Biofuels

Methods and Protocols

Edited by

Jonathan R. Mielenz

Oak Ridge National Laboratory, Oak Ridge, TN, USA
Preface

This editor believes the public finally understands that bioenergy should be a critical new component of our world’s future energy supply. While this new enlightenment might have come from bioenergy’s environmentally friendly attributes and its renewable and sustainable nature, the reality is that the primary driver has been both the high cost of energy, led recently by petroleum products, and the unique climatic changes such as local weather events and reports of melting of ancient glaciers. I believe that much of the public understands the primary cause of these changes is the growing demand for fossil fuels for heating and transportation needs driven by both population increases and dramatic economic development around the globe. This increased demand, as well as global politics, has impacted the costs for these energy products. At the same time, increased use of fossil fuels has been liberating unprecedented levels of CO₂ and other greenhouse gases (GHGs) that must be impacting the global climate. As a result, the world is looking for alternative solutions. Renewable energy is one of the leading options as long as it is pursued in a sustainable fashion. Of course, renewable energy has numerous sources, including wind, solar, geothermal, ocean currents/tides, and bio-based energy. The latter category of renewable energy is centered primarily on use of green plant matter, i.e., biomass, with limited use of animal fats and greases. Biomass in its many forms is the focus of this book.

The increased interest worldwide has many indicators, including governmental support from every (inhabited) continent for developing new bio-based sources of energy, increased interest in the academic and industrial sectors, and the concrete development of bio-based energy resources, led by food-crop-based corn/wheat ethanol as well as soy biodiesel in the United States. Unfortunately, the aforementioned increasing cost of energy has impacted global food supplies raising (invalid and valid) concerns about food versus fuel balances. Recognizing this, most recent interest in bio-based energy has been aimed at biomass-derived energy supplies. Universities and research organizations are building new departments and institutes aimed at bioenergy and biomass development throughout the world. For example, the U.S. Department of Energy is supporting three Bioenergy Research Centers aimed at developing fundamental science and technology needed to supply renewable bio-based energy for the United States. Similarly, a number of multinational oil companies have announced joint research relationships with academic and government laboratories to accelerate development of bioenergy solutions.

This dramatic growth in interest of biomass-based energy R&D has drawn a growing number of both experienced and young researchers to this exploding field. Fortunately, research on how to provide biomass in a sustainable manner and then convert it into usable forms of energy is not new. For example, this editor is a co-chair for an international meeting that specializes in showcasing the latest in biomass R&D, which just held the 31th annual Symposium on Biotechnology for Fuels and Chemicals in San Francisco. Attendance by both academic and industry researchers at this highly technical meeting has grown by over 200% the last three years. This increased interest is very beneficial to the growth of this new industry. Still, the technology for growing, handling, characterizing, and converting biomass to products is just now beginning to be taught in leading universities.
Preface

Around the world. As a result, this methods volume aims to help fill in the gap regarding basic methods for the biomass bioenergy technology and its application. This authoritative volume provides the reader with 18 chapters that cover a very broad range of topics on biomass supply, characterization, and conversion techniques regarding basic methods.

Production of the raw material for bioenergy, which is biomass, is a relatively new agronomic endeavor whose foundation comes from the food, feed, natural fibers, and pulping industries. Supplying a biomass crop to a bioconversion facility (i.e., a biorefinery) has many details that are well documented in the first chapter on biomass supply and logistics. Critical to both the agronomic sustainability and the cost of production are decisions regarding tilling, harvesting, baling, storing, and transporting the biomass, and the first chapter by Sokhansanj and Hess addresses these issues in great detail. Closely associated with this chapter are two chapters on the field production of two of the leading new biomass feedstock in the United States, native switchgrass by Parrish and Fike, and Miscanthus agronomic practices in Illinois by Pyter et al. Directly associated with this work is a discussion of methods for genetic modification of switchgrass. In Chapter 4, authors from the Noble Foundation describe switchgrass transformation and genetic manipulations with details likely applicable to closely related plant species.

Plants have evolved to survive the assaults of the environment (climate, intra-annual cycles, pests, etc.) by building highly resistant structures with polymeric building blocks of carbohydrates (cellulose and hemicellulose) with extremely complex polyphenolic lignin, often thought as the “glue” for plant carbohydrates. To initiate the deconstruction of plant matter, a number of methods have been developed to begin the breakdown processes which are collectively called pretreatment. This pretreatment step is a key process to permit efficient conversion of lignocellulosic feedstock to ethanol or other fermentation products. There are five chapters provided by leading laboratories from universities across the United States that describe the details of most of the recognized methods for pretreating biomass materials. These methods range from alkaline, mildly acidic, to strongly acidic attack of the biomass structure. Balan et al. and Lee et al. describe similar alkaline pretreatment processes in separate chapters. Sierra et al. document a quite different alkaline lime pretreatment in Chapter 9, while Kim et al. describe a method based on liquid hot water. Yang and Wyman outline details of dilute acid pretreatment in a couple modes. These different pretreatment processes accomplish varying degrees of deconstruction and depending upon details of the process, feedstock type, and accuracy of controls of the process (chemicals, time, and temperature), these pretreatment processes will generate a variety of unwanted breakdown products that are inhibitory to any subsequent microbial fermentation to varying degrees. The chapter by Chambliss addresses identification of these inhibitory compounds as the first step to improving biomass conversion conditions to improve the volumetric fermentation yield.

The world is blessed with a plethora of different types of biomass materials, and the utilization of these many kinds of plant matter requires detailed knowledge of their composition both as the raw material and during any conversion process. Three chapters address important aspects of this vital composition determination. There is a long history of methods for analysis of biomass, and Hames has outlined important details for these methods, such as sampling, drying, and wet chemical analysis, which are often overlooked or otherwise not described in the literature. Complementary to this are important aspects of characterization and use of biomass lignin provided by Compere and Griffith. Finally, as work expands regarding identification of improved biomass cultivars, automated methods are increasingly important. Sykes et al. describe an emerging method for mass spectrometry for rapid analysis of biomass.
Given the complexity of the many types of biomass, biochemical conversion of plant material to fuels or chemicals after pretreatment remains a complex challenge. Cellulose is the predominant component of biomass, and significant progress has been made in development of assays of cellulases (cellulose hydrolyzing enzymes). These are discussed in the chapter by Zhang et al. that provides important methods for the many complex cellulase assays. Different cellulases are numerous in nature, and identification of the best enzymes needed for cellulose and hemicellulose fermentation is described by Dow. This chapter is complementary to a chapter by Peterson et al. that describes the classic simultaneous saccharification and fermentation (SSF) process, which includes the impact and benefit of genetically engineering microorganisms. New ethanol-producing microbes permit SSF with a co-fermentation process (i.e., SSCF) that is able to ferment the variety of hexose and pentose sugars found in biomass hydrolysates to ethanol or other products. Conversion of carbohydrates in biomass to chemicals such as ethanol is an anaerobic process, and Strobel outlines the critical details needed for manipulating and using strict anaerobic bacteria for fermentation, as these microorganisms are much more difficult to handle in an oxygen-free environment compared to yeast, E. coli, and Zymomonas. Lastly, biodiesel is a growing option to complement ethanol used in transportation vehicles. Biodiesel feedstock is largely plant oils such as soybean and canola, with minor contributions from animal fats. It is vital to convert these fatty biomass feedstock with methods that yield a blendable biodiesel fuel that is not detrimental to engines and vehicles. In the last chapter, Van Gerpen outlines methods of production of biodiesel that produce blendable fuel that should meet required fuel standards possibly overlooked by the rush to production of this “home grown” fuel.

Overall, the intention of this volume of Methods in Molecular Biology is to provide the growing cadre of new researchers endeavoring to develop new sources of bioenergy from biomass with a full range of important topics including biomass production and delivery to a biorefinery, as well as many details needed for biochemical and biotechnological conversion of plant matter to fuels and chemicals. This editor believes this new bio-based industry is vital to dramatically reduce the world’s use of nonrenewable fossil fuels (petroleum/natural gas/coal) that have limited resources and elevated costs, and contribute to GHG accumulation. Time is an important issue, as we cannot wait for nuclear energy expansion or future hydrogen economy development by 2030–2050. We are currently subjected to a worldwide expanding energy demand coupled with a finite supply of fossil fuels and their associated fluctuating costs. I believe this volume on biofuels methods will contribute to the development and optimization of technology needed for the production of significant quantities of biomass fuels over the next few years.

Oak Ridge, TN

Jonathan R. Mielenz
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Contributors

Venkatesh Balan • Department of Chemical Engineering and Materials Science, Michigan State University, Lansing, MI, USA
Bryan Bal • Department of Chemical Engineering and Materials Science, Michigan State University, Lansing, MI, USA
Christopher Becker • Department of Chemistry & Biochemistry, Baylor University, Waco, TX, USA
Sarah K. Brandon • Microbiology Department, University of Georgia, Athens, GA, USA
C. Kevin Chambliss • Department of Chemistry & Biochemistry, Baylor University, Waco, TX, USA
Shishir P.S. Chundawat • Department of Chemical Engineering and Materials Science, Michigan State University, Lansing, MI, USA
Alicia L. Compere • Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA
Bruce E. Dale • Department of Chemical Engineering and Materials Science, Michigan State University, Lansing, MI, USA
Mark Davis • National Renewable Energy Laboratory, Golden, CO, USA
Emily DeCrescenzo-Henriksen • Microbiology Department, University of Georgia, Athens, GA, USA
Bruce Dien • National Center for Agricultural Utilization Research, ARS-USDA, Peoria, IL, USA
Frank Dohleman • Department of Plant Biology, University of Illinois, Urbana-Champaign, IL, USA
Joy Doran-Peterson • Microbiology Department, University of Georgia, Athens, GA, USA
Nancy Dowe • National Renewable Energy Laboratory, Golden, CO, USA
John H. Fike • Crop and Soil Environmental Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
Yaxin Ge • The Samuel Roberts Noble Foundation, Ardmore, OK, USA
Cesar Benigno Granda • Department of Chemical Engineering, Texas A&M University, College Station, TX, USA
William L. Griffith • Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA
Rajesh Gupta • Department of Chemical Engineering, Auburn University, Auburn, AL, USA
Bonnie R. Hames • Ceres, Inc., Thousand Oaks, CA, USA
Emily Heaton • Department of Agronomy, Iowa State University, Ames, IA, USA
Rick Hendrickson • Laboratory of Renewable Resources Engineering, Purdue University, West Lafayette, IN, USA
Contributors

J. Richard Hess • Biofuels and Renewable Energy Department, Idaho National Laboratory, Idaho Falls, ID, USA
Mark Thomas Holtzapple • Department of Chemical Engineering, Texas A&M University, College Station, TX, USA
Jiong Hong • Biological Systems Engineering Department, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
Lonnie O. Ingram • Department of Microbiology & Cell Science, University of Florida, Gainesville, FL, USA
Amruta Jangid • Microbiology Department, University of Georgia, Athens, GA, USA
Tae Hyun Kim • Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA, USA
Youngmi Kim • Department of Agricultural and Biological Engineering, and Laboratory of Renewable Resources Engineering, Purdue University, West Lafayette, IN, USA
Matias Kirst • School of Forest Resources and Conservation, University of Florida, Gainesville, FL, USA
Michael R. Ladisch • Department of Agricultural and Biological Engineering, and Laboratory of Renewable Resources Engineering, Purdue University, West Lafayette, IN, USA
Y.Y. Lee • Department of Chemical Engineering, Auburn University, Auburn, AL, USA
Stephen Long • Department of Crop Sciences and Department of Natural Resources and Environmental Sciences, University of Illinois, Urbana-Champaign, IL, USA
Derek Marshall • Department of Chemical Engineering and Materials Science, Michigan State University, Lansing, MI, USA
Nathan S. Mosier • Department of Agricultural and Biological Engineering, and Laboratory of Renewable Resources Engineering, Purdue University, West Lafayette, IN, USA
Evandro Novaes • School of Forest Resources and Conservation, University of Florida, Gainesville, FL, USA
David J. Parrish • Crop and Soil Environmental Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
Gary Peter • School of Forest Resources and Conservation, University of Florida, Gainesville, FL, USA
Richard Pytcer • Department of Crop Sciences, University of Illinois, Urbana-Champaign, IL, USA
Lekh N. Sharma • Department of Chemistry & Biochemistry, Baylor University, Waco, TX, USA
Rocio Sierra • Department of Chemical Engineering, Texas A&M University, College Station, TX, USA
Shahabaddine Sokhansanj • Environmental Science Division, Oak Ridge National Laboratory, Oak Ridge, TN, USA
Herbert J. Strobel • Department of Animal and Food Sciences, University of Kentucky, Lexington, KY, USA
Robert Sykes • National Renewable Energy Laboratory, Golden, CO, USA
Contributors

Jon Van Gerpen • Department of Biological and Agricultural Engineering, University of Idaho, Moscow, ID, USA
Tom Voigt • Department of Natural Resources and Environmental Sciences, University of Illinois, Urbana-Champaign, IL, USA
Zeng-Yu Wang • The Samuel Roberts Noble Foundation, Ardmore, OK, USA
Charles E. Wyman • Chemical and Environmental Engineering Department, University of California, Riverside, CA, USA
Yajun Xi • Northwest A and F University, Yangling, Shaanxi, China
Bin Yang • Center for Environmental Research and Technology, Bourns College of Engineering, University of California, Riverside, CA, USA
Xinhao Ye • Biological Systems Engineering Department, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
Matthew Young • National Renewable Energy Laboratory, Golden, CO, USA
Y.-H. Percival Zhang • Biological Systems Engineering Department, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
Chapter 1

Biomass Supply Logistics and Infrastructure

Shahabaddine Sokhansanj and J. Richard Hess

Summary

Feedstock supply system encompasses numerous unit operations necessary to move lignocellulosic feedstock from the place where it is produced (in the field or on the stump) to the start of the conversion process (reactor throat) of the biorefinery. These unit operations, which include collection, storage, pre-processing, handling, and transportation, represent one of the largest technical and logistics challenges to the emerging lignocellulosic biorefining industry. This chapter briefly reviews the methods of estimating the quantities of biomass, followed by harvesting and collection processes based on current practices on handling wet and dry forage materials. Storage and queuing are used to deal with seasonal harvest times, variable yields, and delivery schedules. Preprocessing can be as simple as grinding and formatting the biomass for increased bulk density or improved conversion efficiency, or it can be as complex as improving feedstock quality through fractionation, tissue separation, drying, blending, and densification. Handling and transportation consists of using a variety of transport equipment (truck, train, ship) for moving the biomass from one point to another. The chapter also provides typical cost figures for harvest and processing of biomass.

Key words: Feedstock, Biomass, Bioenergy, Logistics, Supply chain, Equipment, Storage, Transport, Pelletizing

1. Introduction

Biomass feedstock supply logistics includes all the activities and operations of removing the biomass from the location of production and inserting it into the conversion process. The unit operations of a feedstock supply system include harvesting and collection, storage, transportation, and preprocessing (Fig. 1). Moisture content and the form of biomass (the size of particles and packaging) will often dictate the sequence of operations, as shown in Fig. 1. Dry biomass (i.e., straw, stover, dry grasses, dry
wood chips) is characterized as having a moisture content of less than 15–20%, which is sufficiently low for aerobically stable storage to reduce the chance of spoilage or combustion. Wet biomass (i.e., moist straw, forage, wet wood chips) is aerobically unstable, resulting from a moisture content greater than 20%, and requires either drying prior to storage in a dry storage system or storage as silage. For silage, the wet material is chopped and pressed to minimize oxygen to enhance preservation. For granulation (pelletization), biomass is dried and ground to small pieces prior to briquetting or pelleting. Biomass bales are often dry and stored directly. New research is underway to develop ways of storing bales in a wet form (1).

It is important to note that a feedstock supply system may not just be a harvesting, custom baling, or transportation operation. It is the collective combination of all unit operations from the field to the point of entry to the first stage of chemical treatment in a conversion plant. This integrated system approach creates opportunities to rearrange and modify unit operations for feedstock cost reductions and feedstock value-added improvements.
to both the biorefinery and production system. Central to the integrated feedstock supply system is value-added biomass pre-processing (i.e., size reduction, moisture adjustment, cleaning, fractionation, and densification). Preprocessing may occur at any single point or at multiple points throughout the feedstock supply system.

The primary objective of the integrated feedstock supply system is to reduce the supply logistics portion of the final fuel or production cost of the chemical product. This objective can be achieved in three ways: (1) select and develop technologies that reduce the cost of individual unit operations, (2) select the machinery and sequence of operations that minimize cost, and (3) deliver a higher value biomass feedstock product that improves conversion efficiencies and final product yields. Cost and quality improvements are most effectively achieved when one considers all the unit operations required for moving feedstock from the field to the entry point of the conversion reactor as an integrated system.

2. Objective

This chapter reviews the most common unit operations of biomass feedstock supply systems for biorefineries. Specifically, the review considers the performance characteristics of a unit operation that have the most influence on cost and quality of the feedstock. Examples are provided for a suite of equipment options that can lead to a minimum cost in supplying feedstock to biorefineries.

3. Estimating Biomass Quantity

Biomass production includes all operations involved in raising the crop to the point of harvest. The crop may be grown for a primary product, such as grain, and produce enough residue so that a secondary biomass product can be harvested for bioenergy. Production operations may also include altering crop rotation strategies by establishing biomass energy crops as the primary product, such as miscanthus or switchgrass, and implementing alternate tillage and agronomic practices to make more of the grain crop residue available as a bioenergy product. The integrated feedstock supply system interfaces with crop production at the point when and where biomass is ready for harvest.
Currently, crop residue yield is not measured directly, and grain yield data is the only information available to estimate residue production. The residue yield \((\text{t ha}^{-1})\) is estimated from the biomass-to-grain ratio (Harvest Index [HI]) and may range from 0.5 to almost 2 \((2)\). Table 1 lists typical crop yields and mass ratios of residue to crop. For cotton, the mass ratio of residue to lint is 3. The large variation in HI in published data has been attributed to crop species, tillage, residue management, nitrogen application, climate, and the plant maturity stage at harvest \((3)\).

Pordesimo et al. \((4)\) measured the aboveground fractions of corn (\textit{Zea mays L.}) before and after harvest on test plots in Tennessee. The HI reached a maximum value of 0.86 on day 104 after planting, when the grain moisture content was 41% wet basis (wb). The HI decreased to 0.68 by day 143, when the grain moisture content was 13% wb. Seecharan et al. \((5)\) reported an HI range of 0.83–1.33 for dry-land wheat, depending on the soil type, and up to 1.7 for irrigated wheat. For estimating straw availability in Idaho, Patterson et al. \((6)\) reported HI values ranging from 1.33 to 1.88 for winter wheat and 1.17–1.67 for spring wheat. The low values came from dry-land wheat and the high values from irrigated wheat. Nelson \((7)\) used an HI of 1.7 for winter wheat and 1.3 for spring wheat. Boyden et al. \((8)\) measured

### Table 1

**Typical crop and residue yields based on an average residue to crop ratio**

<table>
<thead>
<tr>
<th>Crop</th>
<th>Typical crop yield (dry t ha(^{-1}))</th>
<th>Ratio of residue to product</th>
<th>Biomass yield (dry t ha(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>11.0</td>
<td>1.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Sorghum</td>
<td>4.3</td>
<td>1.0</td>
<td>4.3</td>
</tr>
<tr>
<td>Barley</td>
<td>3.8</td>
<td>1.5</td>
<td>5.8</td>
</tr>
<tr>
<td>Oats</td>
<td>2.2</td>
<td>2.1</td>
<td>4.7</td>
</tr>
<tr>
<td>Winter wheat</td>
<td>3.6</td>
<td>1.7</td>
<td>6.1</td>
</tr>
<tr>
<td>Spring wheat</td>
<td>2.7</td>
<td>1.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Soybeans</td>
<td>2.9</td>
<td>2.0</td>
<td>5.8</td>
</tr>
<tr>
<td>Rice</td>
<td>8.8</td>
<td>1.5</td>
<td>13.0</td>
</tr>
<tr>
<td>Cotton lint</td>
<td>0.9</td>
<td>3.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Others</td>
<td>2.9</td>
<td>1.0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Source: Ref. \((2)\)
straw-to-grain ratio from spring wheat in Saskatchewan, Canada, as ranging from 1.0 to 1.5 depending on the location.

Most of the reported ratios of biomass to grain do not represent the manual removal and fractionation of the entire aboveground biomass (straw and grain). Rather, the reported HI or biomass-to-grain ratio represents the performance of the collection equipment. Stumborg et al. (9) showed the biomass-to-grain ratio was strongly related to the combine settings, especially the height of the cut. For rice, 40% of biomass was in internodes, 43% in leaves, 4% in nodes, and 3% in panicles (10). For corn stover, the bottom 1–4 stalk sections comprised 66% of the total dry mass (11). Similar types of data relating the mass of grain to different sections of the stalk are not yet available for small grains such as wheat and barley.

### 3.2. Biomass Fractions

The aboveground portion of a plant consists of various mass fractions of stems, leaves, fruit, and related parts. Pordesimo et al. (12) conducted a series of field experiments in Knoxville, Tennessee, to measure the mass fraction and moisture content of the corn grain, stalk, leaf, cob, and husk during a harvest season. Aboveground biomass distribution for two corn cultivars (Pioneer 32K61 and 32K64 Bt) was measured in standing plants from approximately 1 week before physiological maturity of the grain until 4 weeks after grain harvest. Physiological maturity of the grain was considered to be 40% moisture content (mc) wb. The dry matter yield peaked when the stover fractions were harvested roughly 118 days after planting. At that time, the grain had a moisture content of 30.6% wb, and the grain, stalk, leaf, cob, and husk accounted for 13.0, 7.9, 3.3, 2.4, and 2.0 t ha⁻¹, respectively. Distribution of aboveground dry matter was 45.9% grain, 27.5% stalk, 11.4% leaf, 8.2% cob, and 7.0% husk.

As the drying of the plant components progressed, the loss of biomass due to weathering (wind and rainfall) increased. Leaf blades, tassels, tops of the stalks, and husk leaves disintegrated. By the end of the study period, 212 days after planting, the leaves had lost 74% of their maximum recorded dry matter. Dry matter losses for husk and stalk fractions were lower than for the leaf fraction but were still substantial at 54% and 38% of their original mass, respectively. Dry matter losses in the cob and grain fractions at 18% and 13%, respectively, were minimal, most likely representing deterioration of the dry matter by respiration and/or microbial action.

**Figure 2** shows the drying rate of various parts of the plant. All the plant fractions lost moisture gradually except the leaf, which dried very rapidly after grain physiological maturity around kernel moisture content of about 35% wb. The stalks retained the highest moisture content throughout the study period. When the grain moisture was 18.3% wb, the stalk, cob, husk, and leaf moisture
contents were 53.8%, 30.4%, 13.9%, 11.5%, respectively. From 180 days to 212 days after planting (not shown on Fig. 2), there was virtually no change in moisture content for any of the standing corn plant components. By 180 days after planting, the grain, cob, stalk, leaf, and husk had final moisture contents of 13.1%, 12.5%, 11.6%, 10.5%, and 10.4%, respectively.

Not all of the crop residue produced can be removed because varying amounts of surface residue are required for erosion control, depending upon soil texture and field slope. Coarse textured (sandy) soils require relatively large quantities of residue for control of wind erosion. The amount of surface residue required to control water erosion increases with field slope (13). Campbell and Coxworth (14) recommended retaining an average of 1,300 kg ha\(^{-1}\) of crop residues on all soils for soil erosion purposes. Kline (15) recommended that 30–50% of the straw residues be left to effectively protect the soil from wind and water erosion. Lindstrom et al. (16) used 50–75% of straw residues to protect soil from wind and water erosion. The United States Department of Agriculture (USDA) Natural Resource Conservation Service (NRCS) requires that 30% of the field be covered in the spring. For corn, 1,625 kg ha\(^{-1}\) of chopped stover retained in the field fulfills the requirement (17). For spring wheat, the conservation amount is 812 kg ha\(^{-1}\), and for winter wheat, 1,159 kg ha\(^{-1}\) (17).

**3.3. Harvestable Biomass**

![Graph showing the drying rate of fractions of the stover in the field.](image)

Fig. 2. Drying rate of fractions of the stover in the field.
4. Harvest Operations

Harvesting involves cutting the crop and performing one or a few preprocessing steps. These preprocessing steps can be as simple as conditioning (e.g., crimping or shredding the biomass) and dropping the biomass into a windrow for drying and later collection, or as complex as threshing and separating the biomass (i.e., separating grain from residue or select residue fractions). As defined here, harvesting does not include collection, though harvesting and collection operations are often combined into a single-pass operation.

In the context of biomass supply logistics, harvesting is defined as cutting or severing a plant from its standing condition, preparing it for removal from the field, and transporting and storing it either next to the farm or at a larger, more centralized, storage area. Figure 3 depicts various forms of a biomass after a grain crop is harvested. The biomass can be chopped, baled, or formed directly into large stacks (loaf). The selection of a particular biomass format depends upon availability of equipment, type of biomass, and logistics. The objective is to increase the bulk density of biomass to a level to minimize the cost of storage and transport. Table 2 lists typical values for the bulk density of biomass in several forms. The bulk density of pelletized biomass is almost one order of magnitude larger than the bulk density of loose chopped biomass. Figure 3 also shows various transportation equipment used to move the biomass to the side of the farm.

Fig. 3. Options for biomass collection.
4.1. Mowing and Conditioning

*Mowing* is the process of cutting the plant. The term *conditioning* describes operations that speed up the drying of the cut plant. Mowing may be integrated with conditioning, as in a mower-conditioner, where the cut material passes through two or more conditioning rolls to bruise the plant stem. A bruised stem dries in half the time it takes to field-dry conventionally mowed forage (18, 19). To this end, recent conditioners produce various degrees of bruising (sometimes called maceration or super conditioning). Figure 4 from Hunt (18) compares the drying of mowed alfalfa over a 24-h period starting from noon. Crimping increases the speed of drying but even more is the effect of crushing. However, the graph shows that the more severed bruised (crushed) plant tends to pick up more moisture as well. This shows that super-conditioning or crushing may not be suitable for rainy regions.

The field speed of a mower-conditioner is almost the same as a conventional mower: about 6–8 km h⁻¹; however, the power requirement of the mower-conditioner can be twice as much as a conventional mower, depending on the degree of conditioning (19). When field-drying, other complementary operations may be needed to ensure that the mowed/conditioned biomass dries quickly and uniformly in the windrow. Evaporation from a freshly cut windrow occurs primarily from the windrow surface. The swath can be disturbed to expedite drying and allow the crop to dry uniformly. *Raking, tedding,* and *swath inversion* are major operations in order to hasten drying. These operations also have problems, including the loss of valuable parts of the plant such as seed or leaf.

Most modern corn grain combines cut and take only the top portion of the plant, which includes the corn ear. The lower portion that is comprised of the stalk is often left standing. Most of the standing stalks are anchored firmly to the ground and an extra

<table>
<thead>
<tr>
<th>Form of biomass</th>
<th>Shape and size characteristics</th>
<th>Density (kg m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chopped biomass</td>
<td>20–40 mm long</td>
<td>60–80</td>
</tr>
<tr>
<td>Ground particles</td>
<td>1.5 mm loose fill</td>
<td>120</td>
</tr>
<tr>
<td>Ground particles</td>
<td>1.5 mm pack fill with tapping¹</td>
<td>200</td>
</tr>
<tr>
<td>Briquettes</td>
<td>32 mm diameter × 25 mm thick</td>
<td>350</td>
</tr>
<tr>
<td>Cubes</td>
<td>33 mm × 33 mm cross section</td>
<td>400</td>
</tr>
<tr>
<td>Pellets</td>
<td>6.24 mm diameter</td>
<td>500–700</td>
</tr>
</tbody>
</table>

¹Biomass is spread into the container while tapping the container
The operational step of shredding is often needed (20). The shredded material is evenly dispersed across the field for drying, and then has to be raked into windrows for baling. Some operators may forego raking and instead set up the shredder to discharge the fractured and broken stalks directly into a windrow, the trade-off being a possible increase in field drying time.

Collection and packaging of biomass follows the mowing operation. Figure 3 shows that removal of biomass from a field can be accomplished in one of three formats: bale, chop, or loaf. A forage harvester picks up a cut crop from the swath, chops the crop to pieces 25 to 50 mm and blows the pieces into a wagon. The forage harvester can be self-propelled or pulled by a tractor and operated with the tractor’s power takeoff (PTO). Self-propelled forage harvesters are for larger acreages and are usually used for high-temperature dehydrating operations or by custom operators. The field moisture content of the chopped forage could vary from 80%, for the standing crop, to between 10 and 15%, for the crop lying in the field. To make silage, the moisture content of the forage should be between 40% and 60%. Various forage cutting heads are available for standing field crops (i.e., alfalfa, grass) windrowed field crops, and row crops such as sorghum and corn grain or corn stover.

The capacity of the forage harvester varies depending on the crop harvested. The Prairie Agricultural Machinery Institute (21) tested the performance of forage harvesters in terms of specific capacity (t/kWh), which is defined as the mass of forage chopped per unit of energy supplied to the harvester. Figure 5 shows specific capacities for a typical forage harvester for three
green crops for silage: alfalfa, corn, and barley. The specific capacity of barley is double the specific capacity of alfalfa. Corn has a slightly higher specific capacity than alfalfa. Forage refinement (uniformity in length of cut) can be achieved either by adjusting the rotary cutter bars to a shorter cut-length setting or by installing a recutter screen. Reducing the cut length produces smaller biomass particles at the expense of machine capacity and increased chopping energy per ton, especially in barley. Based on a 10-mm cut setting, Hoechstein (21) reported chopping energy requirements of 5 kWh/dry t for cutting alfalfa. For corn silage the energy requirement was slightly less (4.5 kWh/dry t) and for barley silage it was around 2 kWh/dry t.

The chopped biomass is transported out of the field using forage wagons or trucks. For wet biomass, a filled forage wagon is moved to the side of the farm. The contents are unloaded either into a bunker or into a silage bag. The material is compacted to promote fermentation and produce silage (22).

### 4.3. Harvesting Biomass in Bale form

The most common method of collecting and removing biomass from a field at present is by baling. **Bailers** bundle and bind biomass in round or rectangular packages, or **bales**. Most round balers produce bales that weigh in the range of 250–1,087 kg and vary in size from 760 mm (diameter) × 1,000 mm (wide) to 1,900 mm (diameter) × 1,570 mm (wide). The upper weight range is generally a maximum for silage bales. Round bales of the size 1500 m × 1,800 m are popular on most U.S. farms (23).

The power requirement of a round baler increases as a bale is made. **Figure 6** shows the power measured for two styles of round balers: a variable-chamber baler and a fixed-chamber baler.
In this figure, the power requirement of the variable-chamber baler increases initially during the formation of the bale. Once the initial core is made, the power requirement stays fairly constant and increases only slightly at the end of the bales’ formation. Shinners and Binversie (24) tested the productivity of baling for round bales of switchgrass in Wisconsin. The baler productivity ranged from 13.5 Mg h⁻¹ for twine wrap to 17.7 Mg h⁻¹ for the net wrap. Twine wrapping required four times longer wrapping time than net wrapping. The density of bales (1.57 m wide by 1.6 m diameter) averaged 163 kg dry m⁻³.

The power requirement of the fixed-chamber baler, on the other hand, increases continuously as the bale is made. According to Srivastava et al. (25) the PTO power requirement depends on the bale size and may range from 12 to 55 kW. The drawbar power (pulling power) ranges from 2.5 to 10.5 kW but the requirement can increase to 50 kW in soft hilly fields.

Large rectangular bales may have a cross section of 1.2 m × 1.2 m and length up to 2.4 m. Smaller rectangular bales with a cross section of 0.9 m × 1.2 m are also popular for biomass. There are two advantages to these smaller bales: (1) these bales use the available space on a truck more efficiently thereby allowing 33% more bales to be loaded on each truck; and (2) these bales can be compressed to a higher bulk density. The net effect of using rectangular bales is that trucks can be loaded to meet the maximum road load limit. The bales range in mass from 500 kg to more than 900 kg each depending on the type and moisture content of the biomass.

Fig. 6. Power consumption by two styles of round balers.
Balers are currently powered from the pulling tractor. The rotary parts are powered mechanically through PTO, while machine adjustments and bale unloading are performed through hydraulic connections. Figure 7 shows the instantaneous power requirements of a large square baler. In this figure, the peaks are due to the plunger action. The average power requirement is lower than the maximum instantaneous power. For the tractor pulling and powering this particular baler, the recommended power is 83 kW. Average power demand for the draw bar for this baler is estimated at 20 kW. The power used to pull these balers is more than 140 kW.

**4.4. Harvesting Biomass in Stack form**

*Loose Biomass Stack Wagon* is an alternate method of collecting biomass in a large package, which is an older forage handling technology. When biomass is dry, a loose biomass stack wagon picks up the cut biomass from the windrow and blows it into a container. The dimensions of the container are about 2.4 m wide, up to 6 m long, and 3.6 m high (26, 27). The roof of the container is mechanically lowered to apply pressure to the collected biomass in order to increase its bulk density. During this compaction cycle, the stack wagon stops collecting material. The frequent stops during biomass collection reduce the efficiency of the stack wagon.

Once filled, the stack wagon transports the biomass to a storage area and unloads the stack. The top of the stack retains the dome profile of the stack wagon roof and easily sheds water. Loose forage stack wagons were originally developed to provide a means of on-farm livestock feeding of hay. The loose stacks proved to be awkward to transport and their use was mostly discontinued in the 1970s, when the new large balers became popular. However, it appears the concept of large loose stacks has merit for a low-cost biomass collection system (28, 29).

**4.5. Biomass Collection Costs**

To arrive at typical collection costs, the Integrated Biomass Supply Analysis and Logistics (IBSAL) model (30, 31) was used to calculate cost and energy inputs for the supply chains of biomass. Table 3
Table 3
Cost, energy, and emission components for each unit operation in biomass collection

<table>
<thead>
<tr>
<th>Collection options</th>
<th>Collection cost ($/t)</th>
<th>Energy consumption (GJ/t)</th>
<th>Carbon emission (kg/t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square bales</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mow</td>
<td>4.02</td>
<td>0.053</td>
<td>4.1</td>
</tr>
<tr>
<td>Rake</td>
<td>1.94</td>
<td>0.030</td>
<td>2.4</td>
</tr>
<tr>
<td>Bale (large squares)</td>
<td>9.66</td>
<td>0.133</td>
<td>10.4</td>
</tr>
<tr>
<td>Roadsiding and stacking</td>
<td>4.54</td>
<td>0.083</td>
<td>6.5</td>
</tr>
<tr>
<td>Tarping</td>
<td>1.56</td>
<td>0.012</td>
<td>0.9</td>
</tr>
<tr>
<td>Overall</td>
<td>23.72</td>
<td>0.339</td>
<td>26.5</td>
</tr>
<tr>
<td>Loafing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mow</td>
<td>4.02</td>
<td>0.053</td>
<td>4.1</td>
</tr>
<tr>
<td>Raking</td>
<td>1.84</td>
<td>0.029</td>
<td>2.2</td>
</tr>
<tr>
<td>Loafing</td>
<td>13.15</td>
<td>0.227</td>
<td>17.8</td>
</tr>
<tr>
<td>Overall</td>
<td>19.69</td>
<td>0.319</td>
<td>24.9</td>
</tr>
<tr>
<td>Chopping dry – piling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mow</td>
<td>4.02</td>
<td>0.053</td>
<td>4.1</td>
</tr>
<tr>
<td>Rake</td>
<td>1.94</td>
<td>0.030</td>
<td>2.4</td>
</tr>
<tr>
<td>Harvest</td>
<td>22.14</td>
<td>0.398</td>
<td>31.1</td>
</tr>
<tr>
<td>Pile</td>
<td>1.74</td>
<td>0.013</td>
<td>1.0</td>
</tr>
<tr>
<td>Overall</td>
<td>35.71</td>
<td>0.592</td>
<td>46.3</td>
</tr>
<tr>
<td>Chopping moist – ensiling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harvest</td>
<td>22.48</td>
<td>0.399</td>
<td>8.5</td>
</tr>
<tr>
<td>Ensiling</td>
<td>12.58</td>
<td>0.071</td>
<td>1.5</td>
</tr>
<tr>
<td>Overall</td>
<td>35.12</td>
<td>0.470</td>
<td>10.0</td>
</tr>
</tbody>
</table>

summarizes the typical cost of collecting biomass in various forms. Square baling cost is the highest at $23.72/t followed by loose stacking at $19.21/t. The low collection cost using loose biomass stack wagon is due to its reduced number of operations and
the size of the loaf. The higher cost for dry chopping and piling ($35.17/t) and for ensiling ($35.75/t) is due to the higher cost of the forage chopper. Mowing and raking operations are eliminated in silaging operation, but the extra cost of pit and packing the silage offsets the lower cost of harvest. The input data for the silage system also include the cost of silage pit at $4,757.00 per year.

Table 3 also lists energy inputs for the collection options. The energy inputs range from 0.319 GJ/dry t for loose stacking to 0.590 for the dry chop system. The energy inputs are dependent on the size of power used to operate the equipment. Forage choppers require large amounts of power – more than 200 kW. Using 16 GJ/dry t as the energy content of dry switchgrass, the energy input to the system ranges from roughly 2% for loafing to less than 4% for dry chopping. The energy expenditure for silaging is slightly less than for dry chopping.

5. Biomass Storage

Because harvest times and yields vary, biomass must be stored so that it can be supplied to the biorefinery regularly throughout the year. Storing biomass in a dry form and in conventional formats (bales) is easier than storing high-moisture biomass. Major considerations for dry storage systems include overall gross shrinkage (dry matter loss), biomass material degradation leading to mass without yield (biological shrinkage), and quality changes. The key factors for controlling biological changes are to maintain less than 15% moisture content as the material enters storage and to protect it from moisture throughout the storage period. The cost of measures taken to protect a biomass during storage needs to balance against the value of the ultimate sugar yield. Storage format, stack configuration, and protective barriers can all be used to reduce sugar-yield losses in storage. Additionally, dry storage system designs need to be cognizant of fire risks. In relation to these risks, distributed on-farm storage will be the system design discussed herein.

5.1. Dry Matter Loss

Biomass loses mass during various phases of harvest and during storage. For a crop residue such as corn stover, these losses may start from the time when the leaves begin to dry. In general, dry mass losses can be divided into two categories: (1) those that occur during machine operation; and (2) those that result while waiting for the next operation. Biomass losses during a machine operation are mainly physical (i.e., losses due to physical disintegration of the biomass to a degree that it cannot be collected
Machine-induced losses depend upon moisture content of the plant at the time of harvest, yield, physical features of the field, design features of the machine, and prevailing weather conditions (wind, rain, and snow). Chemical losses are due to breakdown of structural and nonstructural carbohydrates. These breakdowns can be biotic, such as mold and respiration. Abiotic breakdowns are oxidative reactions, including a degree of pyrolysis.

Shinners and Binversie (24) conducted tests on storing stover indoors and outdoors in Wisconsin. They used a variety of outside storage configurations, including on the ground and on pallets. Table 4 is an extract of their data and shows the range of moisture content and storage configurations. Indoor storage had the least dry matter loss, ranging from 2.2 to 4.9% for moisture content ranging from 3.6 to 19.2%. The bales stored outside gained substantial moisture content, especially those setting directly on the ground. The dry matter loss in the bales stored outside was also high, reaching 38.5% in some cases.

Rider et al. (32) classified different portions of a round bale according to susceptibility of each portion to deterioration. Figure 8 depicts these divisions of a round bale. Assuming a uniform bale density, the outer 15 cm of a round bale accounts for more than 22% of the total mass of the bale. As the round bale settles, approximately 30% of its surface contacts the ground. A substantial amount of moisture can be absorbed through this contact area, resulting in spoilage as far as 30 cm into the bale. If the weather affects the outer 15 cm of the round bale that is not in contact with the ground, plus an additional 15 cm at the bottom, as much as 42% of the total bale volume can be affected.

### Table 4

Moisture content ranges and dry matter losses in stored stover over a period of 9 months

<table>
<thead>
<tr>
<th>Storage style</th>
<th>Moisture content (% wb)</th>
<th>Dry matter loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square bales indoor</td>
<td>13.2–19.3</td>
<td>1.1–4.8</td>
</tr>
<tr>
<td>Round bales indoor</td>
<td>13.6–19.2</td>
<td>2.2–4.9</td>
</tr>
<tr>
<td>Round bales outside net wrap outside on pallets</td>
<td>23.5–47.9</td>
<td>7.0–8.2</td>
</tr>
<tr>
<td>Round bales outside twine outside on pallets</td>
<td>30.9–55.4</td>
<td>11.0–36.1</td>
</tr>
<tr>
<td>Round bales net wrap outside on ground</td>
<td>30.3–53.3</td>
<td>10.7–14.7</td>
</tr>
<tr>
<td>Round bales twine outside on ground</td>
<td>36.4–59.1</td>
<td>14.3–38.5</td>
</tr>
</tbody>
</table>

Source: Ref. (24)
Similar to round bales, dry matter losses for square bales depend upon the method of storage. Square bales stack easier than round bales and when covered properly (under the tarp or under a shed) maintain their original shape better than round bales. Uncovered square bales when exposed to elements (rain and snow) readily absorb moisture, which may cause spoilage. The stacks of square bales are tighter and more prone to heating because of lack of airflow and natural drying. Table 5 lists results

### Table 5
Partial results of storage stability of baled hay

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of stacks monitored</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Moisture content, %</td>
<td>11.5</td>
<td>14.4</td>
<td>17.7</td>
<td>16.8</td>
</tr>
<tr>
<td>Density, kg m⁻³</td>
<td>111</td>
<td>173</td>
<td>87</td>
<td>188</td>
</tr>
<tr>
<td>Dry matter loss, %</td>
<td>2.1</td>
<td>2.5</td>
<td>3.1</td>
<td>3.0</td>
</tr>
<tr>
<td>Maximum temperature, °C</td>
<td>26</td>
<td>25</td>
<td>26</td>
<td>37</td>
</tr>
<tr>
<td>Average temperature, °C</td>
<td>17</td>
<td>18</td>
<td>21</td>
<td>25</td>
</tr>
</tbody>
</table>

Source: Ref. (33)
of storage studies by Buckmaster et al. (33). The data show that the dry matter loss increases with moisture content and temperature. In stacks that had a moisture content of more than 26%, the temperature increased to 47°C.

6. Preprocessing

The biorefinery requires that biomass be delivered in a form that will potentially yield the maximum conversion products. Among desirable specifications is cleanliness; the biomass, should be free of dirt, stones, synthetic fibers, and oil. The biomass also needs to have a uniform moisture content and particle-size distribution for ease of handling, which can be accomplished by granulating. Densely granulated biomass requires much less space than a bulky, fibrous biomass and can flow more easily. Further physical and chemical specifications will become important as conversion technologies advance. Biomass can be engineered to address the requirements for both the biorefinery and the handling and transportation systems. Figure 9 shows various preprocessing operations for pretreating and modifying biomass in preparation for transportation, long-term storage, and downstream processing.

6.1. Densification Processes

Loose, dried forage is light-weight and needs to be densified for ease of handling and transportation. Table 2 lists some bulk dimensions and specific densities of packaged and granulated biomass. The densified forms of forage include pellets, cubes, briquettes, and compacted small and large bales. Pellets are pressed and hardened ground biomass.

Fig. 9. Unit operations involved in preprocessing.
Figure 10 shows a schematic flow diagram of a biomass preprocessing plant. The process consists of five main operational steps: (1) receiving and feeding chopped feedstock into the mouth of a three-pass drum dryer; (2) drying of chopped biomass; (3) fine grinding of dried biomass; (4) conditioning and pressing material into pellets; (5) cooling and storing.

If the incoming biomass from field is already in chopped form, then no shredding is required prior to drying. If the biomass is in the form of bales, these bales have to be shredded into smaller pieces for drying. In addition to general-purpose tub grinders, specialized grinders for grinding bales in an industrial scale have been developed. Warren and Baerg (Baerg 2007) manufactures a horizontal grinder specifically designed for large squares and round bales. The unit shreds a bale using two rotary cutters while minimizing dust generation. The cutters can be of hammer mill style or knife style. The capacity may range from 10 to 60 t h⁻¹ depending on the screen size (from 3 to 150 mm hole size) and a power use from 150 to 300 kW.

The dryer is typically 4.3 m in diameter and 12 m long. The chopped material dries at temperatures of 200–900°C for approximately 3 min. The dryer temperature is automatically controlled by the degree of pre-wilting of the chopped biomass. The moisture content of the input material may range from 45 to 75%, or higher. The final moisture content of dried biomass ranges from 7 to 9%. The typical capacity of the dryer is 5–10 t of dry material per hour.

The dehydrated, chopped biomass is milled to pass through a 2.8-mm-diameter screen by severe beating and cutting within a hammer mill. The average grind size is about 0.35 mm. The ground material is separated from air in a meal cyclone. The ground meal is then treated with superheated steam if required. Saw mill residues do not require steam conditioning because of
their contents of natural resins which act as binder. The moisture content of the mix increases to 12% if steam-conditioned. The heated, moist meal is then extruded through a series of ring dies ranging in diameter from 4.7 to 15.6 mm. The pellets lose about 1.5–2% of moisture in the cooler, where they are cooled to within 5°C of the ambient temperature. The cooled pellets are conveyed to storage areas using mechanical or pneumatic conveying systems. In some processing plants, pellets are passed over a screen (hole size about 3–4 mm) to remove small particles and then weighed before storing.

Typical pelleting plant capacities vary from 10,000 to 50,000 t of pellets a year with the production cost varying with the scale of operation. Table 6 presents the costs for a specific pellet system with a capacity of 6 t/h yielding an annual production of 45,000 t (34). The plant operates 24 h for 310 days annually (annual utilization period 85%). Table 6 summarizes the cost of pellet production including variable costs using the system. For the base case, wood shavings at 10% (wb) moisture content was considered as a burner fuel with a fuel cost of $40/t delivered to the pelleting plant. Cost of wood shavings is considerably high because of the high demand for animal bedding materials and as a fuel for the pulp mills. The capital and operating cost of

### Table 6

<table>
<thead>
<tr>
<th>Pellet process operations</th>
<th>Capital cost ($/t)</th>
<th>Operating cost ($/t)</th>
<th>Total cost ($/t)</th>
<th>Energy use (GJ/t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drying operation</td>
<td>2.46</td>
<td>7.84</td>
<td>10.30</td>
<td>0.350</td>
</tr>
<tr>
<td>Hammer mill</td>
<td>0.25</td>
<td>0.70</td>
<td>0.95</td>
<td>0.100</td>
</tr>
<tr>
<td>Pellet mill</td>
<td>1.43</td>
<td>1.88</td>
<td>3.31</td>
<td>0.268</td>
</tr>
<tr>
<td>Pellet cooler</td>
<td>0.13</td>
<td>0.21</td>
<td>0.34</td>
<td>0.013</td>
</tr>
<tr>
<td>Screening</td>
<td>0.11</td>
<td>0.05</td>
<td>0.16</td>
<td>0.006</td>
</tr>
<tr>
<td>Packing</td>
<td>0.56</td>
<td>1.37</td>
<td>1.93</td>
<td>0.006</td>
</tr>
<tr>
<td>Pellet Storage</td>
<td>0.07</td>
<td>0.01</td>
<td>0.08</td>
<td>0.026</td>
</tr>
<tr>
<td>Miscellaneous equipment</td>
<td>0.42</td>
<td>0.33</td>
<td>0.76</td>
<td>0.052</td>
</tr>
<tr>
<td>Personnel cost</td>
<td>0.00</td>
<td>12.74</td>
<td>12.74</td>
<td>–</td>
</tr>
<tr>
<td>Land use and building</td>
<td>0.21</td>
<td>0.05</td>
<td>0.26</td>
<td>–</td>
</tr>
<tr>
<td>Total cost*</td>
<td>5.64</td>
<td>25.18</td>
<td>30.83</td>
<td>0.821</td>
</tr>
<tr>
<td></td>
<td>3.18</td>
<td>17.34</td>
<td>20.53</td>
<td>0.471</td>
</tr>
</tbody>
</table>

*First row of total cost includes drying. Second row of total cost does not include drying.
producing biomass pellets are $5.64 and $25.18/t of pellet production, respectively. The cost of producing pellets ($30.83/t) may be further reduced if the plant capacity is increased. Sokhansanj and Turhollow (35) calculated a cost for cubing of corn stover at $26.17/t using corn stover as source of heat in the biomass dryer.

Table 6 lists energy inputs to produce pellets. A value of 0.821 GJ/t is calculated for the entire process. This is roughly 5% of the 16 GJ energy content in a ton of dry switchgrass. The most energy-consuming operation is the dryer (assumed drying from 50% to 10% moisture content), which constitutes more than 40% of the entire energy used for pelleting. Next in the list is the pelleting process followed by the grinder.

7. Handling and Transportation

Biomass is handled and transported numerous times throughout the feedstock supply chain. As part of the collection operation, the biomass is transported from the field to a field side or to centralized on-farm storage. From the on-farm storage, the biomass is transported to the biorefinery, or to a centralized preprocessing facility and then conveyed/transported to the biorefinery (Fig. 11). Various methods of handling and transportation are used within each operation, and the methods of transport depend upon the biomass format at the time of handling. The primary concern with such operations is the minimization of the length.

Fig. 11. Logistics of biomass supply from farm to biorefinery.
of storage, the number of handling operations, and the distance for transport.

The traditional way of handling biomass transport cost is to consider a constant cost component and a variable cost component. For truck transport, the constant cost component is the cost of loading and unloading. The variable cost component is the “per kilometer and per ton” cost of trucking, accounting for fuel, depreciation, maintenance, and labor. The constant cost in case of rail transport includes the capital cost of rail siding, rail cars, and equipment for loading and unloading biomass. The variable cost includes the charges of the rail company, which include capital recovery and maintenance for track and engines and fuel and operating costs. **Table 7** summarizes the cost of transporting biomass using three modes of transport: truck, rail, and pipeline. The cost equation for pipeline is developed on the basis of the data of Kumar et al. (36).

**Figure 12** compares the cost of transporting biomass using three modes of transport. For pipeline the annual capacity is assumed 1 million dry tons. In this model, the transport cost in $/t for truck and rail does not change with capacity (in real situation, the size of contracts with transport companies affect the prices). Pipeline has the steepest cost curve because of the increased capital cost with distance.

Truck and rail costs intersect at about 110 km for the cost figures used in this analysis. It should be mentioned that the cost structures for rail are much more complicated than what is given in this analysis. In cases where a multimode transport is required, the cost structures will be a combination of two or three of these modes. At this point, we would like to caution against overgeneralization of the equations in **Table 7** and graphs in **Fig. 12**. The cost of trucking, rail, and even pipeline much depends upon available infrastructure, custom rates, road travel regulations, and size of contracts.

### Table 7

<table>
<thead>
<tr>
<th>Transport mode</th>
<th>Cost ($/t)</th>
<th>Energy consumption (MJ/t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Truck</td>
<td>5.70 + 0.1367 $L$</td>
<td>1.3 $L$</td>
</tr>
<tr>
<td>Rail</td>
<td>17.10 + 0.0277 $L$</td>
<td>0.68 $L$</td>
</tr>
<tr>
<td>Pipeline</td>
<td>2.67$L^{-0.87} + 0.37LQ^{-0.44}$</td>
<td>$160.2Q^{-0.87} + 22.2 LQ^{-0.44}$</td>
</tr>
</tbody>
</table>

$L$: distance (km); $Q$: annual supply (million dry t)

*The cost and energy values for pipeline are in $ and in MJ*
The major bottleneck in transporting biomass is when it is loaded onto or unloaded from transport vehicles. For example, the loading of a 36-bale truck may take 30–40 min, which is roughly the same amount of time it takes to unload the truck. Altering the format of the biomass to a bulk flowable form (e.g., grinding) can greatly improve handling efficiencies, but the cost to reformat the biomass and final bulk densities must also be considered.

8. Discussion

The current dry, bale-based feedstock supply system relies on the bale as the basic biomass handling package and, as such, these supply systems are designed to produce and handle bales throughout. Biomass preprocessing of bales then occurs at or near the biorefining facility just prior to the conversion processes. For dry biomass, this bale-based system is the primary method of handling biomass today. The design and purpose of bales has been formatted for feeding livestock. Because this is the feedstock assembly system through which biomass is made available today, this system serves not only as the baseline against which to measure future improvements, but it is likely to be the system that will be used, at least in part, by some of the first biorefineries. While bale-based feedstock supply systems are generally considered to be too costly (30), availability of equipment, proven operation, and established costs make bales the format of choice for pilot and precommercial biorefinery demonstration facilities today.
There can be several reasons for combining the harvesting and collection operations, including single-pass efficiencies, field traffic reductions, collection of biomass material that is too fine to return to the ground for later collection, and reduction of incidental dirt and rock pickup by eliminating the windrow pickup collection step. These reasons are driven by requirements that need further quantification as to the benefit and value in a dry system. Therefore, for the dry feedstock assembly system, collection starts with windrowed biomass that is field-dried to a moisture content of less than 15%. Depending on the biomass type and collection/storage system used, biomass moistures in the 10–12% range may be required. The windrowed biomass is then collected into a form that allows it to be removed from the field and stored. Subsequent feedstock assembly preprocessing, transportation, and handling options will allow or constrain the type of collection systems that can be used. Therefore, while collection is under the farmer’s control, how the agribusiness will or can receive the biomass will dictate the use of bale, bale type, or non-bale-collection technologies. Collected biomass in bale or non-bale form is then moved to the field side for distributed on-farm storage. Depending on the collection system used, collecting and transporting biomass to the field side can be two separate operations or combined.

By rearranging the elements of a feedstock supply chain, the functional purpose of each operation can be optimized. For example, a primary purpose of preprocessing is to convert the biomass into a bulk flowable product that can be handled in bins, hoppers, and conveyors within the biorefinery. By moving the preprocessing operation forward in the feedstock assembly system from the conversion facility to the point of field-side storage, non-bale-collection systems and bulk material handling and transportation equipment can be used. Furthermore, this rearranged feedstock supply system does not preclude the use of bales (square, round, or otherwise) or most any other collection format that could be chosen. Moving preprocessing forward in the feedstock assembly greatly increases the flexibility and adaptability of the feedstock assembly system, and allows for implementing advanced biomass densification options that can further improve downstream supply system efficiencies.

When considering biomass densification options, there are a number of densification strategies that are less costly and have lower energy consumption than a baling system. It is possible to move the grinding operation to the field and grind to a bulk density as high as 128 kg/m³. This change in the process sequence would reduce the cost of transporting loose stover and give almost the same density as a bale without the baling cost. Costs might be lowered by as much as $10/t. Integrating a pad or depot-based pelleting facility operating 300 days instead of
240 days/year will reduce densification costs and greatly enhance handling, storage, and transportation efficiencies. Achieving a higher density cube and higher pellet mill throughput, as with alfalfa, would also contribute to lowering costs. Other additional opportunities to reduce costs would include having multiple feedstock that are available as a fresh supply for as much as 180–240 days of the year, (i.e., eliminate/reduce storage costs). While the first biorefineries will likely base their respective feedstock supply systems on forage and/or forest logging systems, advancements in biomass densification, size reduction, and moisture management will result in high-capacity feedstock supply systems which will operated at higher efficiencies and lower costs for supplying the next generation of lignocellulosic biorefineries.

References

Chapter 2

Selecting, Establishing, and Managing Switchgrass (Panicum virgatum) for Biofuels

David J. Parrish and John H. Fike

Summary

Switchgrass is being widely considered as a feedstock for biofuel production. Much remains to be learned about ideal feedstock characteristics, but switchgrass offers many advantages already and can perhaps be manipulated to offer more. When planning to grow switchgrass, select a cultivar that is well adapted to the location – generally a lowland cultivar for the southern United States and an upland cultivar at higher latitudes. Plant non-dormant seed after soils are well warmed, preferably with no-till methods and always with good weed control. Except for weeds, few pests appear to be widespread; but disease and insect pests could become more important as acreages increase. Fertilization requirements are relatively low, with 50 kg N/ha/year being a good “generic” recommendation where a single harvest is taken after plants have senesced; more will be needed if biomass is harvested while still green. Switchgrass should be harvested no more than twice per year and may generally be expected to produce 12 to ≥20 mg/ha/year across its usual range of distribution. A single harvest may provide for maximum sustainable yields – especially if the harvest is taken after tops die back at the end of the season. Several harvesting technologies are available, but the preferred technology may depend on logistics and economics associated with the local processing point, or biorefinery.

Key words: Switchgrass, Panicum virgatum, Energy crops, Crop management, Fertilization, Harvesting, Logistics

1. Introduction

In agriculture, we disperse plants across the landscape to serve as solar collectors and biochemical factories. Those crops use the sun’s energy to form complex, energy-rich materials – especially carbohydrates – that can serve as food, feed, fiber, and fuel. In some crops, major portions of the carbohydrates are stored in
seeds or grains as starch, which can be easily hydrolyzed and then fermented. That process is the basis for activities dating back to antiquity, as well as for the currently burgeoning ethanol-from-corn (Zea mays) industry.

In this chapter, we focus on a crop that produces little in the way of starchy grains but whose biomass contains an abundance of other carbohydrate polymers: cellulose and hemicellulose, which can also be hydrolyzed to simple sugars. When used for biofuel purposes, such crops are often categorized as “cellulosic.” They are also sometimes described as “lignocellulosic” because of significant concentrations of lignin in their biomass. Lignin – a mixture of complex, heterogeneous polymers of aromatic moieties – is often considered a recalcitrant, complicating, anti-quality factor in a biofuel feedstock; but it can contribute favorably to overall energy throughput in some conversion processes (1).

After corn, the herbaceous (and cellulosic) species that has garnered the most attention as an energy crop in the United States is switchgrass (Panicum virgatum) (2, 3). (In this context, we consider ethanol-from-corn an interim measure at best; growing corn for ethanol production on land not suitable for corn production, which is a natural sequitur of rising corn prices, is not sustainable (4).) Switchgrass (hereafter noted as SG) gained biofuel preeminence after Oak Ridge National Laboratory (ORNL), funded by the U.S. Department of Energy (DOE), studied it for a decade and a half in an era when little other research was being done on energy crops. Subcontractors with ORNL screened numerous non-woody, cellulosic species in the 1980s before ORNL adopted SG as a “model species” for another 10 years of agronomic, breeding, and biotechnology studies (5). More recently, the U.S. Department of Agriculture (USDA) has become a prime mover in SG-for-bioenergy work (6).

Switchgrass, a perennial species that was common historically on tall-grass prairies, occurs naturally over most of the eastern two-thirds of the United States, in Central America, and in southern Canada (7). It is a diverse species, occurring in two distinct forms, or “cytotypes” – upland and lowland. Cultivars and populations of the lowland cytotype all appear to be tetraploid, while the much larger group of upland cytotypes is mostly octaploid. Upland variants are generally shorter (1.5–2.5 m) and finer stemmed than lowland types (≥3 m) and are typically of more northern origin, i.e., they have developed or evolved at higher latitudes. Both types can be deeply rooted (≥2 m) in favorable soils.

The ecology of SG is tied closely to grazing animals, fire, and competition for resources in a tall-grass prairie ecosystem. This knowledge can be used to advantage in the establishment and management of the species as an energy crop (3). In the following sections, we emphasize how the biology of the species can inform decisions about using it as an energy crop.
2. Materials

2.1. Cultivar Selection and Species Improvement

In this section, we will discuss plant materials and cultivar selection from a grower’s viewpoint; but first we will look at SG from a molecular biologist’s perspective. How might SG be manipulated genetically to make it more suitable as an energy crop?

Much of the effort in SG improvement as an energy crop has focused on developing more productive lines with lower levels of lignin and ash. Genetic engineering is now being employed to these and other ends. Incorporating genes from other species may improve establishment, convey drought or heat tolerance, increase efficiency of nutrient acquisition and use, increase selected herbicide resistance, and reduce anti-quality factors. In addition to improving such agronomic and quality traits, molecular biology is likely to be used to alter the chemistry and enzymology of feedstock in order to produce specialty chemicals and improve feedstock degradability for “one-step” processing (see below).

Currently, the entry point for many cellulosic crops, to include SG, into the energy system is via combustion and conversion to electricity – typically co-fired with coal. In such systems, high levels of alkali metals and silica in the feedstock can produce slag that fouls boilers (8) and reduces heat transfer efficiency – a problem that is compounded when the feedstock is contaminated with soil. Similarly, burning biomass with higher levels of N or S would be more likely to contribute to NOx or SOx emissions, although this is not considered a large problem for biomass power (9). Still, selecting or transforming cultivars to have lower concentrations of the offending agents may be a reasonable goal for breeding or molecular biology efforts.

While some biomass is used for direct combustion and generation of electricity, much of current U.S. research effort is focused on producing liquid fuels, particularly ethanol. For microbial or enzyme-based biomass-to-ethanol systems, the first step in the conversion process is often called “pretreatment” (1). During pretreatment, polysaccharides are liberated from lignin, and the structure of crystalline cellulose is partially disrupted. The pretreatment step alters the physical and chemical properties of lignocellulose and makes the cellulose and hemicellulose more susceptible to microbial/enzymatic degradation – considered the key technological limitation for creating an economically viable ethanol-based economy built on lignocellulose conversion. Thus, cultivar improvement currently is geared in some cases to developing lines that have greater concentrations of carbohydrates that can be readily hydrolyzed for subsequent fermentation. Selection is for carbohydrate, but also against lignin. Lignin in this case is...
an anti-quality component in biomass, because it encrusts and bonds with the fibrous polysaccharide fractions \((10)\). This both increases the energy requirement for particle reduction during pretreatment and acts as a physical or chemical barrier to subsequent enzymatic hydrolysis.

One of the interesting aspects of the bioenergy revolution – one that has implications for molecular biologists – is that, as of this writing, no industrywide standards for lignocellulosic conversion technologies have yet emerged \((1)\). Several methodologies appear to be “in the pipeline,” but no technology seems to have yet attained commercial success, let alone primacy; and this technological murkiness has implications for SG improvement efforts. For example, although lignin has generally been viewed as an anti-quality factor, it is in fact energy rich. It could contribute in a positive way in thermochemical conversion technologies such as the Fischer–Tropsch reaction, which produces alkanes, and in other pyrolytic processes that produce “bio-oils” \((1)\). Besides these potentially positive bioenergy values, lignin has potential for use in chemistries of greater value than liquid fuels \((11, 12)\). Thus, conversion technologies that could fractionate herbaceous biomass and yield economically significant quantities of lignin might create additional income streams for a biorefinery; and this could put pressure on selection for lignin. But diametrically opposed goals, e.g., manipulating the plant to produce more lignin or less lignin, are not necessarily so incongruent for metabolomic and genomic applications. A good understanding of the metabolic fluxes associated with and the genetic control of lignin synthesis could potentially be used to manipulate lignin levels and types in either direction \((e.g., 13)\).

2.2. Cultivar Selection for Switchgrass Plantings

When considering cultivar selection from a grower’s standpoint, recall that SG plants are divided into two broad forms, or cytotypes – upland and lowland. As a generalization, lowland forms have greater production potential, are taller and thicker stemmed, and are indigenous to lower latitudes than upland forms. Given their greater potential productivity, it might be supposed that lowland forms would be the cultivars of choice, but that is not automatically the case.

Switchgrass behaves as a determinate plant, producing a flush of reproductive tillers only after a period of vegetative (leaf) growth. The amount of biomass produced is partially determined by how soon (or late) the transition to reproductive development occurs. Flowering is triggered when days shorten to a specific length; the requisite day length is specific to the cultivar and the region in which it developed. This relationship can be used to advantage in production scenarios. In the Northern Hemisphere, moving southern-adapted varieties northward can delay flowering, prolong vegetative growth, and boost yield. However, this
strategy has limits, because southern-adapted lines often lack the hardiness needed to survive in colder climates.

Because most lowland cultivars are of southern origin, (e.g., “Alamo,” the most common lowland cultivar, originated in Texas), they are not well adapted to the northern Great Plains or southern Canada. Even “Kanlow,” a well-known northern lowland cultivar, should not be planted too far north of its Kansas provenance. Most of the named cultivars of SG are upland cytotypes, and many of them are derived from northern populations. If planting SG in areas north of the U.S. Corn Belt, one should choose an upland cultivar. Furthermore, the suggested rule of thumb is that a cultivar should not be planted more than 500 km (14) or more than one USDA hardiness zone (15) north of its region of origin.

It should have been noted earlier perhaps, but we want to make clear before leaving this topic, that few commercial, named SG cultivars have been developed specifically for biomass or biofuel production. We anticipate that regionally adapted lines developed specifically for bioenergy purposes will be appearing.

3. Methods

3.1. Establishment

Switchgrass can often be grown in places and sites that would not favor other, more domesticated crops. It is rather remarkably well adapted to many soil types – from sandy to clayey (but not the heaviest clays) and from only moderately drained to well-drained. Low soil pH would seldom be limiting. To maintain productivity, it needs at least 50 cm of rain per year; and it can benefit from 75 cm or more.

Switchgrass has a perhaps undeserved reputation for being difficult or slow to establish. Climatic factors can militate against success, and inattention to details can result in poor performance; but difficulties encountered in SG establishment often stem from overlooking matters related to seed dormancy (3). Freshly harvested SG seeds can be highly dormant – often germinating at 5% or less. Over time, this dormancy disappears as the seeds “after-ripen”. Accordingly, seeds that are ≥2 years old are often more desirable than those from a more recent crop. Some seed producers are now stratifying seeds (3 or 4 weeks exposure to cool, wet conditions) to break the dormancy more quickly, which can greatly increase the seeds’ ability to germinate when planted at an optimum date. Farmers can do this also with relatively small batches (40–50 kg) of seed and a standard refrigerator (16). It is sometimes suggested that SG be planted in winter or early spring to allow the seeds to stratify in situ, a strategy that allows the dormant seeds to
eventually germinate; but this can lead to problems with weeds that germinate before or with the SG (see Note 1).

No single method or approach for establishing SG will work in all situations. However, attention to several key factors can increase the likelihood of success.

- Plant after the soil is well warmed (>20°C); this runs contrary to standard practice in many places; plantings as late as midsummer can be successful if moisture is favorable.
- Use seeds that are highly germinable, i.e., not dormant.
- Seeding rates of 2 kg/ha can be adequate with highly germinable seed lots.
- Planting rates must go up to compensate (≥10 kg/ha) for dormant seeds or earlier plantings.
- Plant 0.5–2 cm deep (can be deeper in coarser soils).
- Incorporation and good seed-to-soil contact are crucial.
- No-till or conventional methods can be used, but always use a planter that places seeds accurately.
- Provide minimal fertilization (no N) at planting to minimize weed competition (see below).
- Control weeds pre and post planting (see below).

Weeds can be a major impediment to SG establishment. Good weed control at the time of planting is crucial – whether with herbicides in no-till applications or by tillage combined with chemicals in conventional plantings. We favor no-till planting and two applications of glyphosate – one 4 or 5 weeks before planting and another at the time of planting. If legal and safe, burn the killed vegetation before planting. This minimizes difficulties encountered with dead vegetation “hair pinning” into the slit of the no-till drill and interfering with seed-to-soil contact. In an optimal scenario, planting non-dormant seeds into a well-warmed soil will produce seedlings that can outcompete many weeds that might coemerge with them. Perennials that escape weed control efforts can be problematic, but broadleaf species in particular can often be easily defeated with post-emergence herbicides (see more in section on pest control).

Planting failures due to weed infestations may be more apparent than real. Some experienced producers tolerate heavy weed infestations in the first year, perhaps only mowing at a height slightly above the SG seedlings to minimize the effects of over-topping. In subsequent years, the growth habit and phenology of the now-established SG plants often help them to outcompete many weeds. A good rule of thumb appears to be that, if there are at least 20 SG plants per square meter, the planting can succeed (16, 17). Initially weedy SG stands can become monocultures with good management and persistence.
The conventional wisdom is to avoid N fertilization of SG during the first year to minimize the potential for providing some advantage to weeds. If weeds are not a problem, SG seedlings might benefit from a more aggressive fertilization program. We do not recommend high rates in any event, since we suspect mycorrhizal and other symbiotic associations may minimize the need for fertilizer supplements.

### 3.2. Fertilization

Despite conflicting reports on responses of SG to fertilizer applications – especially with N and when multiple harvests are involved – some general recommendations for managing the fertility of SG as an energy crop are beginning to emerge. As with any crop, the overall goal is to replace nutrients that are removed in the harvests, i.e., to avoid “mining” the soil and to build or maintain fertility. If one knows how much N, P, K, etc. are being hauled to the biorefinery, steps can be taken to replace them. While this simple approach works well for most nutrients, management of N is a bit more complex. We shall discuss N first and in more detail – partially because its management is more involved but also because N is frequently the nutrient limiting yield most.

#### 3.2.1. Nitrogen Fertility and Fertilization

Actively growing SG has relatively low N concentrations in its biomass compared to many crops: generally ≤2%. Toward the end of the season, the stems and leaves of SG undergo senescence during which N-containing materials are mobilized out of the shoots and into belowground parts (3). Thus, by the time the tops die and might be harvested, N levels can be ~0.5% in standing biomass (Table 1; 18). At this point, a harvest would remove only 5 kg N/mg of biomass collected.

We will discuss harvest strategies in more detail below, and we will make a biological case for harvesting SG only once per year – after the tops have senesced and died; but, from a nutrient-use standpoint alone, a single harvest of senesced biomass seems propitious. Harvesting dead matter will remove far fewer nutrients (Table 1) and, in addition to requiring less N inputs, this management practice could have the concomitant benefit of generating a smaller post-process waste stream. That being said, there are reasons why one might want to harvest midsummer (see below). If this is done, however, one will be removing biomass that is 1–2% N; and even a modest harvest of 5–7 mg/ha will remove 50–140 kg N/ha. In such management scenarios, additional N should be applied after the midsummer harvest.

Interestingly, there have been several reports of two-harvest management (a midseason harvest plus an end-of-season harvest) where ≥200 kg N/ha/year were removed in the biomass over multiple years, yet ≤100 kg N/ha/year was being applied (see Table 1 of ref. 3). It appears SG is not only quite thrifty in its use of N but it is also able to tap soil N reserves that are not available.
to many other crops. We would not suppose that those reserves can be tapped indefinitely, however. The N that is removed must eventually be replaced, or the soil’s N pool will become depleted, and SG yields will suffer.

Confusion about SG N requirements has resulted from reported responses to N ranging from essentially nil to ≥400 kg/ha/year (3, 19). The inconsistent responses to N are likely due to soils that are capable of mineralizing significant N and to differing cutting managements. While SG can be highly productive in some soils with limited fertilizer additions, the literature suggests that positive responses to N are likely on soils of low inherent fertility and poor nutrient holding capacity – especially when N is applied in combination with P.

Ideally, recommendations for N management would be based on site-specific factors such as site history, precipitation, and crop removal rates. Failing such information, in the early stages of optimizing a SG-for-biofuel management system, we recommend fertilizing with 50 kg N ha/harvest/year. The cost of N and the potential environmental costs of its overuse argue for judicious N use in energy cropping systems. In addition, high N application rates can promote lodging and reduce stand density in some situations. We note that, in some parts of the United States, almost half of the 50 kg N/ha/year needed for a one-harvest system falls out of the sky as NOx.

Table 1
Tissue nutrient levels and nutrient amounts removed in the biomass of Alamo switchgrass harvested either once or twice per year

<table>
<thead>
<tr>
<th>Variable</th>
<th>Nutrient</th>
<th>One cutting November</th>
<th>Two cuttings June</th>
<th>November</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue nutrient concentration</td>
<td>N</td>
<td>0.48</td>
<td>0.97</td>
<td>0.65</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.10</td>
<td>0.18</td>
<td>0.12</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>0.56</td>
<td>1.27</td>
<td>0.65</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Ca</td>
<td>0.32</td>
<td>0.28</td>
<td>0.39</td>
<td>–</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nutrient removed in the biomass</th>
<th>Nutrient</th>
<th>One cutting November</th>
<th>Two cuttings June</th>
<th>November</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>66.2</td>
<td>66.6</td>
<td>63.3</td>
<td>129.9</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>12.6</td>
<td>11.7</td>
<td>10.6</td>
<td>22.2</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>78.0</td>
<td>86.1</td>
<td>65.8</td>
<td>151.9</td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>44.9</td>
<td>30.4</td>
<td>36.8</td>
<td>67.2</td>
<td></td>
</tr>
</tbody>
</table>

Each data point is the average of eight southeastern sites (four reps per site) in 1997 through 1999 (unpublished data of Parrish and Wolf)
Switchgrass responses to P, K, and lime are variable and limited. Typically, production systems will need only to maintain soil P and K at moderate levels in order to support high productivity in single-harvest systems; and a soil with pH $\geq 5$ should require no liming. While SG actively recycles N, cycling of other mineral elements appears to be more passive. Delaying harvest of senesced material allows for weathering of dead biomass, with consequent mineral leaching (20, 21). Thus, the P, K, and other nutrients removed in the biomass (Table 1) may be even lower where post-senescence harvests are delayed. Again, the producer’s goal should be to replace nutrients that are hauled away in the biomass. This can be done with commercial fertilizers or perhaps by returning materials from the processing waste stream (22).

Although weeds can be a source of great consternation at planting, they are less likely to be a cause for concern in well-managed SG stands post establishment. Well-managed stands will typically be too competitive for resources – water, nutrients, and light – to give weeds a niche. Weed recruits may become established if gaps develop in the stand or if stand management allows sufficient light to reach the soil surface during the growing season. Untimely fertilizer applications may also support the invasion of stands by cool-season species, especially in regions with warmer winters.

Post-establishment weed control remedies for cool-season weeds are available. Applications of auxenicals (2,4-d, dicamba, and picloram) are effective for broadleaf weeds. Systemic, broad-spectrum herbicides such as glyphosate – though lethal to SG – can be applied in fall on post-senescent stands but would likely have better application in spring, prior to bud break, for control of cool-season invaders.

SG has few insect pests of economic importance. While grasshoppers (Saltatoria) have been observed to feed on SG, pressure from these pests will vary by site, year, and cultivar. Other insect pests such as yellow sugarcane aphid (Sipha flav) and thrips (Thysanoptera) may feed on SG, but it is not a preferred host. While new insect pests may arise – particularly if and when SG plantings become more extensive – these pressures are not a major cause for concern in established stands at this point.

Disease pressures, while not particularly heavy at present, may become a greater issue for a budding SG-for-biomass industry when plantings become extensive. Ruts (Puccinia spp.) can reduce plant yield, with resistance varying among cultivars. The greater resistance of certain lines again points to the need for site-appropriate cultivar selection as well as breeding efforts. Fungal pathogens such as sharp eyespot (caused by Rhizoctonia cerealis) and helminthosporium spot blotch (caused by Bipolaris
sorokiniana)) and a viral disease caused by Panicum mosaic virus have also been observed in SG. A SG smut (caused by Tilletia maclagani) has been observed in the Midwest (23), and a very close relationship was observed between percent tiller infection and overall yield reduction. There is some concern that this could become a major limitation to production in some areas.

The goal of any producer is to maximize and sustain economic yield. A combination of relatively low N inputs, when compared to most crops, and a single harvest taken at the end of the season appears to be an optimal, sustainable strategy for managing SG for biofuel purposes. Note: See arguments for a single harvest in the section above on fertilization, and read on to find additional reasons why one post-senescence harvest is likely to maximize sustainable yield.

Taking two harvests within a season appears to provide minimal or no yield advantage compared to a single cutting (24, 25). However, a single, end-of-the-season harvest may not fit the needs of all producers and processors. Taking two harvests per season might help maintain a continuous feedstock supply for a biorefinery; and it could also benefit contract harvesters, who often would prefer to keep their equipment running. The economic merit of making two harvests must necessarily consider not only the costs associated with the extra harvest but also the consequences of a midseason harvest on feedstock quality and fertilizer demands.

If taken, midseason cuttings should occur near the time the SG would be producing its seedheads. This will vary with cultivar and locale but would generally occur in June or July. The cutting height for a mid-season harvest should be 15–20 cm to allow regrowth to occur from axillary buds on the stems; and a second application of N (50 kg/ha) after the midsummer harvest will help replace the N that has been removed in the harvested biomass.

If a single harvest is to be taken each year, it is best delayed not just until biomass accumulation has ceased, which will be in August or September, but until after shoots have senesced and died, which may not be till November or December. This timing will forego some biomass yield in the short term (for the first year or two), but it will provide for highest sustainable yields in the long term. Yields decline by 10–20% if harvest is delayed from August to November. These losses in standing biomass reflect a season-ending strategy of plants in which they translocate N-rich materials and carbohydrates from senescing shoots into below-ground organs (3). The shift results in a loss of harvestable biomass within the season; however, it is in the stand’s and grower’s best interest for the grower to absorb this loss, because a growing body of literature suggests that early autumn harvests that
maximize SG yield in the short term will hurt yield and persistence in the long term (see 3 and references therein).

For a management strategy in which the energy crop is collected after all end-of-the-season processes have occurred in the standing biomass, harvests may come after several frosts – not just “the first hard freeze.” SG can retain green tissues after several < 0°C nights in the fall. Full drydown of the standing biomass may not occur till well into November or even December at mid-latitudes in the United States. Leaving the SG standing increases the opportunity to leach mineral components from the biomass, which could have positive consequences on soil nutrient status as well as on feedstock quality. Cutting height for a single end-of-the-season harvest might ideally be as close to the ground as feasible with the machinery available – while avoiding contamination of the harvested biomass with soil (See notes for one caveat.).

Providing a continuous supply of feedstock to biorefineries is a crucial issue for an emerging biorefinery industry. Logistical requirements for harvesting, handling, storing, and transporting low-density feedstock must be addressed if practical, commercially feasible biomass-to-bioenergy systems are to be developed (4, 26).

In a dedicated energy cropping system based on a perennial grass such as SG, there are several options for harvest and handling, including chopping with direct haul, chopping with module handling, or cutting and baling (4). Each of these harvest/transport systems has certain advantages. Chopping provides size reduction and converts the biomass into a flowable material, but chopper-based systems will need some form of collection vehicle or vessel to receive the material to keep the harvester moving through the field. Linking module technology with chopper-based harvest may improve the feasibility of in-field chopping. This may present an advantage at the process facility – the material is to a degree already preprocessed. However, module system economics must surmount greater requirements for labor (creating the modules) and storage (27).

Cutting and baling provides certain advantages over chopping, because it breaks the link between the harvest and in-field hauling operations, i.e., allows bales to be left in the field for the time being. Moreover, round bales of coarse feedstock such as SG can shed water, reducing or eliminating the need for storage. However, bale-based systems must address the logistical and cost constraints associated with collecting and moving the material from field to facility (4) (see Note 2).

Because biomass has a low density, shipping costs will play a large role in the economic feasibility of the production system. One way to reduce this cost is to pack more on the truck or train – typically by pelleting or cubing. While these technologies can
significantly increase the bulk density of biomass, they require careful control of moisture content and have a relatively high energy input and cost to make the pellets or cubes, which also affects the economics (28, 29).

While most research on dedicated bioenergy systems to date has focused on agronomics (what and how much can be grown) and on processing technologies (what to do with the material), neither of these concerns is of any merit if the millions of available tons of biomass cannot be readily collected and moved to facilities for conversion. These are major constraints for a biomass-to-bioenergy system, and their resolution is awaited (see Note 3).

4. Notes

1. In areas where winters are mild enough so that winter-annual weeds can become problematic, e.g., southern United States, one strategy to control such weeds involves taking a harvest in mid- to late summer and allowing some regrowth of the SG to accumulate. That regrowth will suppress weeds that would otherwise encroach into an area with only stubble. The overwintering biomass can either be harvested or burned in early spring, just before the SG initiates new growth. (The species clearly can tolerate periodic burnings. That is part of its ecology.)

2. Anecdotal reports suggest that leaving a stubble of 6–10 cm, which would be typical for making hay with many crops, can lead to greatly increased rates of tire failure on harvesting equipment. Cutting at a somewhat greater height (~15 cm) and/or using heavier duty tires might be desirable in large-scale operations.

3. Biofuel production from a dedicated energy crop, such as SG, faces a number of interesting uncertainties, many of which are related to economics. From a farmer’s standpoint, yields and prices received will clearly be large drivers in the economics of energy cropping and therefore how much land might be devoted to it. But, because biomass’ hauling costs are high relative to its feedstock value (30), biomass yields and the availability of nearby land for biomass production will also be key drivers in determining processing facilities’ location and size (31, 32). Alternatively, increasing a biorefinery’s yield per unit of feedstock input can reduce the land base required to grow the biomass and, accordingly, the hauling costs (4). Put in a slightly different way, land base issues and associated transport considerations will have a strong impact on decisions to site biorefineries. Because of important economies of scale, facilities
are likely to be located in areas where land is productive and a significant portion of the land would be used to grow feedstock. Land areas with low production potential – or with little likelihood many landowners will convert to energy-crop production – will be unsuitable because of attendant higher transport costs. This transportation constraint works against mega facilities and likely will act to limit facility size even in locations with ample productive land. However, optimum size remains to be defined and will depend on a calculus that must take into account land productivity, land availability, and the yield on substrate at the facility. We wish to note here only that potentially independent resolutions of genetic, agronomic, and technologic problems associated with SG (or any energy crop) must eventually be accompanied by simultaneous resolutions of economic (and even sociologic) issues about where biorefineries will be sited and who will grow the crop. These clearly are not trivial concerns.

References


Chapter 3

Agronomic Experiences with Miscanthus x giganteus in Illinois, USA

Richard Pyter, Emily Heaton, Frank Dohleman, Tom Voigt, and Stephen Long

Summary

Since 2002, researchers at the University of Illinois, Urbana-Champaign, Illinois, have been studying the perennial warm-season grass Miscanthus x giganteus (M. × g.) to determine its potential as a biomass feedstock. M. × g. originated in Japan and is a hybrid believed to have M. sinensis and M. sacchariflorus as its parents. Until recently, it was used as a landscape plant in the United States, but it is now the subject of research interest because of its potentially great biomass production. In central Illinois, M. × g. begins growth in April, typically reaches 2 m by the end of May, and is normally greater than 3 m by the end of September. The grass is sterile and propagated asexually using plantlets produced in tissue culture or by rhizome divisions. Following field planting, it generally takes at least three growing seasons to become fully established and reach optimal biomass production. In central Illinois, the senesced stems are harvested from early December through early March and can potentially be treated to produce lignocellulosic ethanol. In University of Illinois, research started in 2002. M. × g. produced an annual average of 22.0 t/ha in northern Illinois, 34.7 t/ha in central Illinois, and 35.4 t/ha in southern Illinois per year in 2004, 2005, and 2006.

Key words: Miscanthus x giganteus, Giant Miscanthus, M. floridulus, M. sacchariflorus, M. sinensis, Rhizome, Vegetative propagation, Biofuel, Renewable energy

1. Introduction

Since 2002, researchers at the University of Illinois, Urbana-Champaign, Illinois, U.S.A., have been studying the perennial warm-season grass Miscanthus x giganteus (Greef & Deuter ex Hodkinson & Renvoize; hereafter referred to as Giant Miscanthus or M. × g.) to determine its potential as a biofuel crop. Results
from most of these studies have been positive (1) and encouraged continued research. \( M. \times g. \) is well adapted to the growing conditions in Illinois, and biomass yields in experimental plots have been greater than other potential herbaceous biomass feedstock. Moreover, there has been no evidence of yield-reducing insect pests or diseases, and there is a great amount of interest about it in the agricultural community.

This is not a new grass to the University of Illinois. Since 1988, horticultural researchers have grown \( M. \times g. \) to identify and study its potential landscape uses. European interest in the grass for landscapes began after it and other \emph{Miscanthus} species were collected in Yokohama, Japan, in 1935, and taken to Denmark. Cultivated by nurseryman Karl Foerster, it was spread through Europe (2). Planted as a landscape ornamental, \emph{Miscanthus} has become naturalized in Europe (2).

More recently, the high productivity of Giant Miscanthus, combined with low need for annual agronomic inputs such as fertilizers and pesticides, its broad adaptation to temperate growing environments, and a growing demand for renewable sources of energy has made it a candidate for study as an energy feedstock (3). In fact, a scan of the scientific literature shows that the majority of publications written since 1990 about \( M. \times g. \) grown as a biomass feedstock have originated in Europe.

In this Chapter, we will review appropriate European \( M. \times g. \) literature and share University of Illinois agronomic experiences. The University of Illinois research presented herein can be viewed as a starting point. There is currently significant applied and basic biofuel research being planned and conducted in the United States, so findings from this new \( M. \times g. \) research will likely alter what is written here.

2. Materials

2.1. Plant Material: \emph{What is Miscanthus} \( \times \) giganteus?

The genus \emph{Miscanthus} (Poaceae Family) comprises a group of approximately 14 grass species (4). While a few species originate in Africa, the majority of species occur from southeastern Asia to China and Japan and into Polynesia (2). There can be frequent naturally occurring hybridization within the genus (2).

One such hybrid, \( M. \times g. \), is believed to have \emph{M. sinensis} and \emph{M. sacchariflorus} as its parents (4, 5, 6, 7). Cultivars of \emph{M. sinensis} have been widely planted in the United States and Europe as landscape ornamentals. For example, three of the older and widely available types widely planted in U.S. landscapes are \( M. s. \) “Gracillimus” (Maiden grass), \( M. s. \) “Strictus” (Porcupine grass), and \( M. s. \) “Zebrinus” (Zebra grass) (8). While there is large morphological
variability within *M. sinensis*, most types are clump formers. *M. sacchariflorus* (Silver Banner Grass or Amur Silver Grass) has also been grown as a landscape ornamental (8). It produces strong rhizomes and has a spreading habit. The cross of *M. sinensis* (a diploid species) and *M. sacchariflorus* (a tetraploid species) has produced a triploid, *M. × g.*, which is sterile and does not produce viable seed. (Note 1)

In central Illinois (latitude 40.12N, longitude 88.2W), Giant Miscanthus begins growth in late April, and growth is completed with senescence occurring following a killing frost, usually in mid- to late- October. Shoots develop each spring from the buds on scaly rhizomes. Established plants typically reach nearly 2 m by the end of May and are often more than 3 m at the end of a typical growing season. Flowering occurs in most years, and in central Illinois, it begins in late September or early October. Because it is a triploid, no viable seeds are produced.

Lower canopy leaves drop as the growing season progresses, leaving “bare legs” characteristic of bamboo-type grasses. As below-freezing temperatures occur in autumn, the dark green foliage at the top of the canopy turns tan and drops, becoming part of the leaf litter on the ground. The stems, and sometimes leaves and sterile flowers occurring at their apex, remain. The stems are the commercially important portion of *M. × g.*, resemble bamboo canes, and are harvested between early December and early March. An established stand of Giant Miscanthus produces 50–110 shoots per square meter per year, and commercially desirable stems are usually 1.2–2.0 cm in diameter and more than 2.9 m long.

A positive aspect of *M. × g.* is its perennial life cycle. One of the major arguments against biofuels is the relatively low energy-in versus energy-out ratio. This is largely due to the high energy inputs such annual tillage, planting, and fertilizer application that go into the agronomic production of annual grain crops such as corn. Since *M. × g.* is a perennial crop which has shown little response to fertilizer application, these agronomic inputs are largely reduced, making *M. × g.* an ideal candidate for bioenergy production.

1. Water

As with many agricultural crops, *M. × g.* yield increases as the water available to the crop increases (1, 9). Yields in European trials, when plotted against April through September precipitation, ranged from 20 t/ha at 300 mm to 36 t/ha at 700 mm. Beale et al., (9) showed that for each gram of dry biomass produced, 9.5 kg of water is transpired. Therefore each ton per hectare of yield requires 11 mm of precipitation at the minimum. Based on this analysis, if annual precipitation was 600 mm, the maximum potential yield would be 54 t/ha, assuming that all of the precipitation is
available to the crop. Also, Beale et al. (9) determined that this water-use efficiency would be lower in regions with high crop-atmosphere water vapor deficits, and higher in regions with low crop-atmosphere water vapor deficits.

These analyses suggest that given the high summer rains and humidity in the eastern half of the United States, high potential yields should be obtainable. The eastern corn belt appears to be particularly favorable because of its deep water retaining soils.

2. Temperature
A *M. × g.* biomass yield of 20 t/ha requires approximately 1,000 growing degree days (°Cd), assuming a basal temperature for growth of 10°C (1). However, there is inadequate information from high-temperature areas to establish whether yield would continue to increase linearly with increase in °Cd, but analysis of the temperature response of leaf photosynthesis suggests an optimum of 30°C (10, 11). Lines with higher temperature optima may be needed for warmer climates.

*M. × g.* is among the most cold-tolerant C₄ species known, allowing much higher productivities than corn and sorghum in cool climates (12). It is able to develop leaves that are photosynthetically functional at 8°C, (11, 13), and established stands at the University of Illinois in Urbana, planted over 15 years ago, have survived repeated winters with periods below −20°C during the winter without loss (authors’ observations). Autumn frosts appear to be necessary to induce senescence and drydown. Because senesced stems are harvested in the late fall and winter, areas lacking regular late autumn frosts may be unsuited to *M. × g.* (Note 2)

3. Soils
Yields in Europe showed no apparent relationship to soil type, given adequate moisture. However, establishment is faster on more fertile soils, such that ceiling yields may be obtained within 2–3 years on fertile soils but require 3–5 years on poor soils. Trials in Illinois have produced similar results, with establishment being slowest at the two least fertile of the seven field sites on which we have established trials.

Remarkably, there was no significant effect of nitrogen fertilization on *M. × g.* yields in European trials (1). Beale and Long (14) found that harvested *M. × g.* contained 5.0 [N], 0.6 [P], and 12.0 [K] g/kg. In trials in Illinois, in contrast to those in Europe, the N content of the harvested biomass was only 1.2 [N] g/kg, suggesting that within *M. × g.*, there may be genetic differences in nutrient use efficiency (15). Although only 25 kg [N]/ha was applied during establishment followed by no subsequent additions, the crop in year 4 was found to contain 500 kg [N]/ha when measured prior to senescence. This suggests that the crop is remarkably effective at capturing and recycling N (15).
3. Methods

3.1. Propagating Miscanthus × giganteus

1. European Propagation

Because M. × g. is sterile, it is propagated vegetatively using micro-propagation of plantlets and macro-propagation of rhizomes. Typically, plants produced from rhizome division have proven to be more resistant to winter losses than plantlets (16). However, poor over-wintering of the rhizomes after the first growing season was a problem in northern Europe (17).

Rhizome division can take place after the end of the growing season in the fall or before the growing season in late winter/spring (16). Venturi et al. (18) reported successful propagation using 2-year-old field-grown stock plants that produced rhizomes of 40–100 g. Conventional methods of rotary tilling and harvesting can be used to split the mother plants. Adapted potato harvesters and planters have been used to effectively harvest and plant the rhizomes (19). Using this method, a 30- to 40-fold increase in plants was achieved over a period of 2–3 years (20).

2. Illinois Propagation Experiences

Our M. × g. was planted at the Landscape Horticulture Research Center (Urbana, Illinois) in the late 1980s as part of an ornamental grass demonstration and were obtained from the Chicago Botanic Garden (Glencoe, Illinois). Since we began our biomass studies in 2002, we have been using these plants for both tissue culture and rhizome propagation material (Note 3).

3. Tissue Culture Propagation

In Illinois, we have conducted several studies over the past 3 years in both field and greenhouse settings. First, a University of Illinois laboratory has successfully reproduced the grass in tissue culture (21). Ninety grass plants produced in tissue culture were field-planted in 2006 with greater than 60% survival over the first winter. Additional tests will be conducted to determine survival rates of tissue-cultured plants in locations with milder winter conditions.

4. Greenhouse and field Propagation

The majority of the University of Illinois M. × g. plantings (between 6 and 8 ha at the time of this report) have been established using rhizome divisions from mother plants aged 1, 2, or 3 years old. We have produced new plants using rhizomes in both the greenhouse and by direct field planting.

4a. Propagation of M. × g. in the greenhouse

Select quality rhizomes of approximately 25 g that are approximately 0.6–1.2 cm in diameter and buff colored with multiple nodes. They appear scaly and can be branched or un-branched at planting. Plant into either soil-less container mixes such as Premier Pro-Mix (Premier Horticulture, Inc., Quakertown, Pennsylvania)
or Sunshine Mix (Sun Gro Horticulture, Bellevue, Washington) or pasteurized mixes containing various combinations and ratios of native soil, sand, perlite, sphagnum peat moss, and/or vermiculite; Plant the rhizomes into square 12.5 × 12.5 cm pots; irrigate as necessary to maintain active growth, and fertilize biweekly using Peters 20–20–20 water-soluble greenhouse fertilizer (J.R. Peters, Allentown, PA); provide 12 h per day of supplemental lighting; divide greenhouse-produced plants approximately every 28–56 days.

4b. Field Propagation of \( M. \times g. \) stock

Plant in late April to early June into soil that has been rotary-tilled to a depth of 15 cm; select 1-, 2-, or 3-year-old rhizomes of approximately 50 g that are approximately 10–15 cm long; plant the rhizomes approximately 10 cm deep in rows 60 cm apart with 60 cm between rhizomes within the rows if the rhizomes are to be harvested after one growing season and increase the spacing between rows to 90 cm if the rhizomes are to be harvested after two growing seasons; rhizomes can be planted into holes dug by hand or into furrows produced by a mechanical vegetable or nursery transplanter; the soil should be firmed around the rhizomes using a roller to ensure soil-to-rhizome contact and reduce water loss from the soil; irrigate and remove weeds by hand or by mechanical tillage as necessary to ensure establishment and maximum \( M. \times g. \) growth; with irrigation, in Drummer silty clay loam soils, 1-year clumps typically yield 7–10 harvestable rhizomes and 2-year clumps 25 or more rhizomes in central Illinois. We have obtained 75–80 rhizomes from 3-year plants, but the clumps become too large to easily manage.

Harvesting, cleaning, and dividing large quantities of field-produced rhizomes have proven to be major tasks. Until recently, rhizome clumps were dug by hand or by front-end loaders. We have constructed cleaning tables using expanded sheet metal placed over metal livestock water troughs. The rhizome clumps were placed on the expanded sheet metal, and hand-held power washers were used to wash soil from the clumps. Hand pruners were used to divide the rhizome masses into roughly 50-g segments for replanting. Using this labor-intensive method, it required approximately 400 man hours to prepare rhizomes for planting on two hectares at a rate of 10,300 rhizomes per hectare.

Our future goals include mechanization of this process for commercialization. We will be evaluating several methods with proven success in Europe. In one method, the rhizome clumps will be disked or rotary-tilled prior to harvest with a potato harvester. This method will hopefully reduce the time required to harvest and divide the propagules.
3.2. Planting and Growing Miscanthus × giganteus

1. European Planting and Growing Research

While there is great potential for high biomass yields in Illinois (1), the main problem of growing M. × g. is the high cost of establishment (3, 17). A major reason that establishment costs are high is that the plant is sterile and therefore requires vegetative establishment (16). Another cost is for the labor required during the second season to replant rhizomes into areas where the initial planting did not survive. And finally, a third establishment cost is for the weed control required during the planting season, and sometimes during the second growing season.

The factors found to affect establishment in Europe were rhizome size, planting depth, and storage prior to planting. In a German study (19), successful establishment required rhizome pieces of 200 mm length be planted at a soil depth of 200 mm. Good establishment rates were recorded when rhizomes of similar size were planted at a depth of 100 mm, but winter survival rates were low. Jorgensen (22) wrote that in Denmark, rhizomes were divided into groups of smaller than 100 mm and larger than 100 mm. These rhizomes were hand-planted at a soil depth of 50–100 mm. Rhizome size had a significant effect, with 34% of the smaller size surviving and 82% of the larger size surviving the first winter. In this study, rhizome-planting depth did not have a significant effect on winter survival. Moreover, rhizomes that had been in storage prior to planting had lower winter survival than those planted directly after harvest.

Huisman and Kortleve (23) reported emergence rates of 70–95% from rhizome pieces planted immediately after field harvest and emergence rates of 50–60% from rhizome pieces which were stored before planting. In a Dutch study, rhizomes weighing greater than 50 g which were planted a short time after harvesting resulted in successful establishment with emergence rates of 91–98%. A study lead by ADAS Consulting Ltd., experimented with storage conditions of M. × g. rhizomes and found that storage at 3°C showed the greatest viability. The study also showed that storage under ambient conditions without wetting was the least viable treatment (20).

During the establishment phase, M. × g. is relatively slow growing and has little ability to compete with weeds (19). The slow initial growth may be due to the perennial nature of the plant and the need to develop a rhizome system (24). Weed control during the establishment phase is especially critical for M. × g. because the planting process causes soil disturbance which promotes seed growth and low planting densities which in turn creates large open spaces for weeds to grow (19). After proper establishment, weed competition is suppressed initially by the leaf litter on the ground in the spring and during the growing season by the closed canopy which reduces the amount of light reaching the understory (19).
Previous research investigating the tolerance of \( M. \times g. \) to any herbicides is minimal. The possibility of mechanical weed control has been suggested (19), but this is energy intensive and costly. Chemical weed control will be critical to maintain this crop’s potential as a biofuel. Glyphosate and paraquat have been used successfully to control emerged weeds before \( M. \times g. \) sends up shoots in the spring (19).

2. Illinois Planting and Growing Research
In Illinois, we studied whether rhizomes harvested in the autumn or winter could be held in cold storage for spring planting. Pyter (25) found that \( M. \times g. \) rhizomes will tolerate storage at 4°C for up to 4 months with little effect on period to break dormancy. Survival was less than 100% only when rhizomes had a mass of less than 25 g. For field production, survival of 50–60 g rhizomes that had been in storage for up to 4 months should be high (25). Rhizome storage gives growers another planting window which accommodates situations when planting following rhizome harvest is not immediately possible. In future research, the maximum period of storage for rhizomes needs to be determined in order to give growers the largest possible planting window.

Planting depth of \( M. \times g. \) seems to be important in terms of consistent yield. No significant differences were found between planting depths of 5, 10, 15, and 20 cm in a 2005 University of Illinois study (25), but variation was highest when planted at 5 cm. Repeated in 2006 under a shortened growing season, a planting depth of 10 cm yielded the highest and offered the least variation (25). Research suggests that a \( M. \times g. \) planting depth between 10 and 15 cm provides a buffer from fluxes in temperature and moisture availability. A planting depth greater than 15 cm appears to decrease biomass yield which may be attributed to delayed emergence and a shortened growing season (25).

3. Field Planting and Growing Experiences in Central Illinois
To grow \( M. \times g. \) in the field using rhizomes, we have selected rhizomes that are firm, not shriveled, without apparent disease or harvest damage and relatively free of soil, weigh approximately 50 g, and measure approximately 10–15 cm long; rotary-tilléed the site as soon as soils were workable in spring to prepare for planting; planted the rhizomes using a single-row vegetable transplanter approximately 10 cm deep and 1 m apart within the row; planted rows approximately 1 m apart to equal a planting density of approximately 10,000–12,000 rhizomes per hectare. We have found it unnecessary to apply fertilizers to new planting given the high native fertility of central Illinois soils. We also applied herbicides to control weeds. Experimentally, we have found that \( M. \times g. \) is tolerant of applications of the pre-emergence herbicides pendimethalin, pendimethalin/atrazine, S-metolachlor, and S-metolachlor/atrazine and the post-emergence herbicides
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2,4-D ester and dicamba at rates typically used to control weeds in corn (25).

Approximately 60–70% of the rhizomes typically sprouted during the planting summer and gaps are replanted in subsequent growing seasons. Without fertilization, at the end of the initial growing season the plants are approximately 1–2 m tall with multiple tillers. Thereafter, plants normally develop a closed cover by the end of the second growing season and were more than 3 m tall by the end of the third growing season. (Note 4) Presently, we are conducting research designed to fine-tune herbicide application rates and timing, determine tolerances to additional herbicides, and identify methods of eradicating *M. × g*.

3.3. Harvesting

*Miscanthus × giganteus*

Harvesting does not commence until the crop has fully senesced because earlier harvesting would result in high nutrient removal and plant biomass with high moisture content. There is a trade-off between the quantity and the quality of harvested biomass in relation to harvest time (26). Winter losses of dead and decaying leaves and upper stem parts can cause yield reductions from 30 to 50% dry matter (27). Harvesting can be carried out using existing farm machinery, thereby avoiding costly investment in specialized equipment. Corn silage choppers appear to be the most suitable forms of machinery that are currently available. However, bailers following mowers are also commonly used (26).

In central Illinois, we have harvested stems at various times from early December through early March using a two-step process. First, a hay mower-conditioner drops the stems. This step is followed by a hay baler that produces bales that are approximately 85 cm wide, 90 cm deep, and 1.83 m long. In 2006, an early December harvest yielded bales that weighed approximately 318 kg.

Plot yields in 2004, 2005, and 2006 at three Illinois sites have varied depending on the crop age and the weather during the growing season. In replicated studies of unfertilized *M. × g.* planted in 2002 using small potted plants, the average yields over the 2004, 2005, and 2006 growing seasons were 22.0 t/ha in Shabbona, Illinois (latitude 41.85N; soil is a fine-silty, mixed, superactive, mesic Typic Endoaquolls; formed from loess or silty material and the underlying till; slope of 0%), 34.7 t/ha in Urbana, Illinois (latitude 40.12N; soil is a fine-silty, mixed, mesic Typic Endoaquoll; very deep and formed from loess and silt parent material deposited on the till and outwash plains; slope of 0%), and 35.4 t/ha in Simpson, Illinois (latitude 37.45; soil is a fine-silty, mixed, active, mesic Oxyaquic Fragiudalfs; formed in loess and underlying weathered stone; moderately permeable above the fragipan and very slowly permeable in the fragipan; slope of 0–2%) (15, 28). A separate demonstration plot in Urbana,
Illinois, yielded approximately 31.6 t/ha of dry Giant Miscanthus biomass in 2006 at the end of the third growing season (28).

3.4. Conclusions

Nothing in our 5 years of research has dissuaded us from continuing to work with \( M. \times g \). As observed in Europe, University of Illinois researchers have found that the productivity of \( M. \times g \), its broad adaptation to Illinois growing environments, and our increasing interest in biomass feedstocks make it an ideal candidate for expanded study.

In the future, our basic research will continue to improve understanding of \( M. \times g \) growth and physiology, as well as to develop additional genotypes with adaptations to varied growing environments. In addition, we also plan to conduct applied research that will improve methods of propagating, planting, and harvesting in order to increase the commercial attractiveness of the grass.

4. Notes

1. There appears to be naming and taxonomic confusion within the U.S. nursery industry concerning \( M. \times g \). In the landscape plant trade, it is sometimes offered as Miscanthus \( \times giganteus \), \( M. \) sinensis “Giganteus” or \( M. \) sacchariflorus, or, quite commonly as \( M. \) floridulus(8). According to Darke (8), \( M. \) floridulus has rarely been grown for landscape applications in the United States, and grasses that are sold as \( M. \) floridulus are, in reality, often Miscanthus \( \times giganteus \).

2. \( M. \times g \) is able to withstand high winds and ice storms. In central Illinois, wind gusts greater than 54 kph are common. There have been no occurrences of lodged stems in either our landscape or in biofuel plantings. In addition, winter storms have covered stems with ice and bent stems nearly to the ground. As temperatures rise and the ice melts, the stems routinely spring back into vertical orientation.

3. At the time of writing, large amounts of Giant Miscanthus plants or rhizomes are not readily available in the United States. Giant Miscanthus can be sometimes obtained from some landscape plant nurseries.

4. We have also used greenhouse-produced potted plants in field plantings. Our planting steps are similar to those using rhizomes with two exceptions. First, prior to field planting, the potted grasses are removed from the greenhouse in mid-April to mid-May and the pots are placed in a protected outdoor area for approximately 1 week to harden. After hardening, the same
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planting rate as with rhizomes (approximately 10,000–12,000 plants per hectare) should be used, and the potted grasses are planted at the same depth as it was growing in the pots. The second difference is that since the plants have leaves that are transpiring, the plants are irrigated following planting and until establishment.

Acknowledgments

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Chapter 4

Genetic Transformation of Switchgrass

Yajun Xi, Yaxin Ge, and Zeng-Yu Wang

Summary

Switchgrass (Panicum virgatum L.) is a highly productive warm-season C4 species that is being developed into a dedicated biofuel crop. This chapter describes a protocol that allows the generation of transgenic switchgrass plants by Agrobacterium tumefaciens-mediated transformation. Embryogenic calluses induced from caryopses or inflorescences were used as explants for inoculation with A. tumefaciens strain EHA105. Hygromycin phosphotransferase gene (hph) was used as the selectable marker and hygromycin was used as the selection agent. Calluses resistant to hygromycin were obtained after 5–6 weeks of selection. Soil-grown switchgrass plants were regenerated about 6 months after callus induction and Agrobacterium-mediated transformation.

Key words: Agrobacterium, Biofuel crop, Genetic transformation, Panicum virgatum, Switchgrass, Transgenic plant

1. Introduction

Switchgrass (Panicum virgatum L.), a perennial C4 warm-season grass native throughout North America, has been identified by the U.S. Department of Energy as the main species of emphasis for development into an herbaceous biomass fuel crop (1, 2). Switchgrass has many characteristics which make it suitable for a biomass energy crop, including high productivity, low nutrient (N, P, K) requirement, long stand longevity, excellent disease and pest resistance, high water use efficiency, good soil restoring properties, adaptation to marginal soils, and low cost of production. Switchgrass contains abundant sugars in the form of cellulose and hemicellulose, which can be converted to ethanol...
by hydrolysis and subsequent fermentation. A cellulosic ethanol industry based on warm-season grasses also offers significant potential for CO$_2$ reduction because switchgrass has a high energy output/input ratio.

Switchgrass is a wind-pollinated polyploid monocot species with a high degree of self-incompatibility. This makes conventional selection and breeding schemes complex, especially for traits with low heritability. Genetic improvement is one of the most effective ways to increase productivity and to reduce recalcitrance to saccharification of switchgrass. The fast-paced advancement of cellular and molecular biology offers novel genetic engineering techniques to accelerate or complement conventional breeding efforts. Transgenic approaches offer the opportunity to generate unique genetic variation that is either absent or has very low heritability (3). Like many other monocot species, switchgrass is considered recalcitrant for genetic transformation. To date, there are only two reports on switchgrass transformation, one based on particle bombardment (4) and the other based on Agrobacterium infection (5). Both reports used the phosphinothricin acetyltransferase (bar) gene as selectable marker and bialaphos as selection agent (4, 5). Although not universally accepted, it has been reported that Agrobacterium-mediated transformation tends to result in lower copy number, fewer rearrangements, and an improved stability of expression over generations than the free DNA delivery methods (5–7).

The protocol described in this chapter is based on our recent work in switchgrass transformation. We used embryogenic calluses as explants and hygromycin as selection agent. The use of highly embryogenic calluses is one of the key factors affecting successful generation of transgenic plants. The whole process of obtaining transgenic switchgrass plants takes about 6 months.

## 2. Materials

### 2.1. Plant Material

This comprises seeds and greenhouse-grown plants of the switchgrass cultivar Alamo.

### 2.2. Agrobacterium tumefaciens Strain and Selectable Marker

The *Agrobacterium tumefaciens* strain EHA105 was used in combination with the binary vectors pCAMBIA 1301 and pCAMBIA 1305.2. The pCAMBIA 1301 carries a hygromycin phosphotransferase gene (*hph*) and a β-glucuronidase gene (*gusA* from *E. coli*); the pCAMBIA 1305.2 carries an *hph* and a β-glucuronidase gene (*GUSPlus* from *Staphylococcus* sp.). All genes are under the
control of CaMV 35S promoter (www.cambia.org). Hygromycin was used as selection agent.

### 2.3. Culture Media for Agrobacterium tumefaciens


### 2.4. Tissue Culture

1. Calcium hypochlorite: Prepare fresh 3% (w/v) calcium hypochlorite solution in a glass bottle; add a few drops of Tween-80.

2. 2,4-Dichlorophenoxy-acetic acid (2,4-D, 1 mg/ml, Phyto-Technology Laboratories, Shawnee Mission, KS).

3. 6-Benzylaminopurine solution (BAP, 1 mg/ml, PhytoTechnology Laboratories).

4. M5 medium: MS medium (PhytoTechnology Laboratories) supplemented with 5 mg/l 2,4-D, 3% (w/v) sucrose, and solidified with 0.75% (w/v) Agar (Sigma, St. Louis, MO). Adjust pH to 5.8 and autoclave.

5. SM5 medium: M5 medium supplement with 0.15 mg/l BAP.

6. MSB medium: MS medium (PhytoTechnology Laboratories) supplemented with 3 mg/l BAP, 3% (w/v) sucrose, and solidified with 0.75% (w/v) Agar (Sigma). Adjust pH to 5.8 and autoclave.

7. M1 medium: MS medium (PhytoTechnology Laboratories) supplemented with 1.5 mg/l 2,4-D, 3% (w/v) sucrose. Adjust pH to 5.8 and autoclave.

8. Acetosyringone (ACROS Organics, Morris Plains, NJ): Prepare fresh 100 mM acetosyringone (3’,5’-dimethoxy-4’-hydroxyacetophenone) by dissolving acetosyringone in dimethylsulfoxide (DMSO).

9. Hygromycin B (50 mg/ml, PhytoTechnology Laboratories. Avg. activity 1,000 units/mg).

10. Cefotaxime (Agri-Bio, North Miami, FL): Prepare 250 mg/ml stock in double-distilled H₂O (ddH₂O), filter-sterilize and store at ~20°C.

11. M1 selection medium: MS medium (PhytoTechnology Laboratories) supplemented with 1.5 mg/l 2,4-D, 3% (w/v) sucrose, and solidified with 0.75% (w/v) Agar. Adjust pH to 5.8, autoclave, and then add 75 mg/l hygromycin and 250 mg/l cefotaxime.
12. Kinetin: 1 mg/ml stock (PhytoTechnology Laboratories).
13. MSK medium: MS medium (PhytoTechnology Laboratories) supplemented with 0.2 mg/l kinetin, 3% (w/v) sucrose, and solidified with 0.75% (w/v) agar. Adjust pH to 5.8, autoclave, and add 250 mg/l cefotaxime.
14. MSO medium: half-strength MS medium (PhytoTechnology Laboratories) solidified with 0.75% (w/v) agar. Adjust pH to 5.8 and autoclave.
15. Sterile distilled water.
16. 70% ethanol.
17. Sterile filter paper (7-cm diameter).
18. Autoclaved glass flask (125 ml).
19. Sterile 6.6-cm-diameter (190 ml) plastic culture vessels (Greiner Bio-One, Longwood, FL).
20. Sterile 5.0-cm-diameter (175 ml) plastic culture vessels (Greiner Bio-One, Longwood, FL).
22. Forceps, scalpel, and blades.
23. Drummond Pipet-Aid and sterile disposable pipettes.
24. Magnetic stirrer and stir bars.
25. Spectrophotometer.
27. A rotary shaker.
29. A swing rotor centrifuge.

3. Methods

3.1. Sterilization and Callus Induction

Calluses induction from caryopses (see Note 1):
1. Immerse seeds in 3% calcium hypochlorite solution in a glass bottle. Put a magnetic stir bar in the bottle and place the bottle on a magnetic stirrer. Agitate for 2.5 h.
2. Rinse the seeds three times with sterile distilled water and leave the seeds overnight at 4°C.
3. Sterilize the seeds again for 1 h in 3% calcium hypochlorite solution the next day, and rinse the seeds three times in sterile distilled water.
4. Place about 50 caryopses/seeds per 9-cm culture dish containing M5 medium. Keep dishes, sealed with Parafilm, in the dark at 24°C.
5. Calluses formed within 8–12 weeks are used for Agrobacterium-mediated transformation.

Calluses induction from inflorescences (see Note 1):
1. Collect top nodes from E4–E5 stage (8) plants in the greenhouse.
2. Peel off the outer leaves and wipe the nodes with 70% EtOH.
3. Split the nodal segments longitudinally and place on MSB medium with the cut surface down.
4. Inflorescences can be induced after 15–20 days on MSB medium. Cut the inflorescences into 0.5–1.0 cm segments and place them on SM5 medium. Calluses induced after 6–8 weeks can be used for transformation.

3.2. Agrobacterium Preparation

1. Streak A. tumefaciens from a glycerol stock onto LB agar plate with antibiotic selection appropriate for the vector used. Incubate at 28°C for 2 days.
2. Transfer a single colony from the plate into a 15-ml sterile tube containing 5 ml LB medium with antibiotic selection appropriate for the vector used. Incubate the cultures on a shaker/incubator at 250 rpm at 28°C for about 8–10 h.
3. Transfer 1 ml of the Agrobacterium cultures into a flask containing 50 ml LB medium with antibiotic selection appropriate for the vector used. Incubate the cultures on a shaker/incubator at 250 rpm at 28°C overnight, until the cultures have reached an OD₆₀₀ of about 1.0. Add 50 µl 100 mM acetosyringone (AS) to the bacterium cultures 1–2 h before use.

3.3. Inoculation of Explants and Cocultivation

1. Centrifuge the Agrobacterium cultures at 2,400 × g for 15 min.
2. Pour off supernatant, resuspend the pellet with M1 medium to 50 ml, adjust OD₆₀₀ to 0.5, and add 50 µl of 100 mM acetosyringone. The Agrobacteria are now ready for transformation.
3. Transfer switchgrass calluses into 6.6-cm culture vessels and break up the calluses into small pieces.
4. Add Agrobacterium suspensions to the culture vessels and immerse the callus pieces.
5. Place the culture vessels in a polycarbonate desiccator and draw vacuum (62 cmHg) for 10 min.
6. Release vacuum and incubate the callus pieces and Agrobacteria for 20–25 min with gentle shaking on a rotary shaker.

7. Remove the bacteria after the incubation, transfer the infected callus pieces onto M1 wetted filter papers, and place them in empty culture dishes in the dark at 24°C for cocultivation (see Note 2).

3.4. Selection and Plant Regeneration

1. After 2–3 days of cocultivation, transfer the filter papers supporting the infected callus pieces onto M1 selection medium.

2. After 1 week, transfer the infected callus pieces to new M1 selection medium. Resistant calluses normally become visible after 3–4 weeks of selection.

3. When the resistant calluses become large enough (normally after 4–6 weeks on M1), transfer them onto regeneration medium MSK.

4. Keep the regenerating cultures at 24°C in fluorescent light (40 µE/m²/s) at a photoperiod of 16 h in the growth chamber for 4–6 weeks.

5. Transfer the regenerated shoots to 5.0-cm culture vessels containing MSO medium.

3.5. Greenhouse Care and Seed Harvest

1. Transfer well-rooted plantlets to 3.5-in. pots filled with Metro Mix 350 soil (see Note 3) and grow them under greenhouse conditions (390 µE/m²/s, 16 h day/8 h night at 24°C). Plants can be grown on Ebb-Flo benches and watered once a day with fertilized water containing 50 ppm N (Peters Professional 20–10–20 General Purpose is used as the water-soluble fertilizer).

2. Transfer the established plants to 12-in. (5-gallon) pots filled with Metro Mix 350 soil and grow them under greenhouse conditions (390 µE/m²/s, 16 h day/8 h night at 24°C).

3. Plants normally flower in 2–3 months. Because switchgrass is an outcrossing species, crosses need to be made between independent plants (e.g., transgenic and non-transgenic plants) in order to obtain seeds. For cross pollination, emasculate recipient inflorescence and then bag them together with two panicles from the pollen donor plant; supply water to the donor panicles from a 50-ml conical tube fixed to a bamboo stake. Seeds can be harvested 1 month after cross pollination.
4. Notes

1. Calluses induced from either caryopses or inflorescences can be used for transformation.
2. The amount of callus pieces on each filter paper was equivalent to approximately 20 original intact calluses.
3. Before transfer to soil, rinse the roots with water or remove excessive medium with a damp paper towel.

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References

Chapter 5

Lignocellulosic Biomass Pretreatment Using AFEX

Venkatesh Balan, Bryan Bals, Shishir P.S. Chundawat, Derek Marshall, and Bruce E. Dale

Summary

Although cellulose is the most abundant organic molecule, its susceptibility to hydrolysis is restricted due to the rigid lignin and hemicellulose protection surrounding the cellulose microfibrils. Therefore, an effective pretreatment is necessary to liberate the cellulose from the lignin–hemicellulose seal and also reduce cellulosic crystallinity. Some of the available pretreatment techniques include acid hydrolysis, steam explosion, ammonia fiber expansion (AFEX), alkaline wet oxidation, and hot water pretreatment. Besides reducing lignocellulosic recalcitrance, an ideal pretreatment must also minimize formation of degradation products that inhibit subsequent hydrolysis and fermentation. AFEX is an important pretreatment technology that utilizes both physical (high temperature and pressure) and chemical (ammonia) processes to achieve effective pretreatment. Besides increasing the surface accessibility for hydrolysis, AFEX promotes cellulose decrystallization and partial hemicellulose depolymerization and reduces the lignin recalcitrance in the treated biomass. Theoretical glucose yield upon optimal enzymatic hydrolysis on AFEX-treated corn stover is approximately 98%. Furthermore, AFEX offers several unique advantages over other pretreatments, which include near complete recovery of the pretreatment chemical (ammonia), nutrient addition for microbial growth through the remaining ammonia on pretreated biomass, and not requiring a washing step during the process which facilitates high solid loading hydrolysis. This chapter provides a detailed practical procedure to perform AFEX, design the reactor, determine the mass balances, and conduct the process safely.

Key words: AFEX, Biomass, Pretreatment, Hydrolysis, Fermentation

1. Introduction

The growing United States appetite for liquid transportation fuels, together with growth in demand from China, India, and the rest of the world, has pushed petroleum prices to new highs. The United States uses over 20 million barrels of petroleum per
day, of which 58% is imported. Prices rose to almost $70 per barrel (bbl) in August 2005. It is estimated that the crude oil price will remain well above $60 per bbl through 2012, presumably rising after that. Fermentation production of ethanol (bioethanol) is one of the low-cost, consumer friendly ways to reduce gasoline consumption and net carbon dioxide emissions from automobiles (1). Bioethanol is a clean fuel that can be used in today’s cars by blending with current petroleum products such as gasoline. Because bioethanol is produced from renewable feedstocks, it does not contribute to a net carbon dioxide increase in the atmosphere (1). Thus, a considerable amount of research is underway to produce ethanol from lignocellulosic biomass to supplement corn-based bioethanol already in production (2, 3).

Pretreatment is an important process by which cellulose is made more accessible to the enzymes that convert the complex carbohydrate polymers into fermentable sugars, as shown in Fig. 1. The ultimate goal is to break apart the lignin polymers and dis-

![Fig. 1. Process flow diagram showing how lignocellulosic feedstocks are converted to ethanol and byproducts. Processing steps are presented in gray scale while inputs and outputs are presented in white.](image)
rupt the crystalline structure of cellulose. Many of the present-day biomass pretreatments (ethanol organosolv pretreatment, dilute acid pretreatment, ammonia recycle percolation) fractionate the various biomass components (lignin, hemicellulose, and cellulose) into separate process streams (4). The removal of lignin and/or hemicellulose can substantially reduce the recalcitrance of biomass to enzymatic hydrolysis (5).

Ammonia fiber expansion (AFEX) pretreatment (6) is a novel alkaline pretreatment process that creates a physicochemical alteration in the lignocellulosic ultra and macro structure. An advantage of AFEX is that the ammonia used during the process can be recovered and reused. Also, the downstream processing is less complex compared to other pretreatment processes. Studies have shown that AFEX pretreatment increases enzymatic digestibility several fold over untreated lignocellulosic biomass (7). AFEX pretreatment results in the decrystallization of cellulose (8), partial depolymerization of hemicellulose, removal of acetyl groups (9) predominantly on hemicellulose, cleavage of lignin–carbohydrate complex (LCC) linkages, lignin C–O–C bond cleavage, increase in accessible surface area due to structural disruption, and increased wettability of the treated biomass (10). The AFEX process demonstrates attractive economics compared to several leading pretreatment technologies based on a recent economic model (11) for bioethanol from corn stover. This chapter provides a brief overview of the AFEX process summarizing current mechanistic understanding, key experimental variables, sample preparation for AFEX, the method of pretreatment, construction of AFEX experimental equipment, and data analysis and mass balance calculations.

2. Materials

Corn stover was provided by the National Renewable Energy Laboratory (NREL, Golden, CO). Distiller’s dry grains plus solubles (DDGS) was obtained from Big River Resources, LLC (West Burlington, IA). Switchgrass was provided by Dr. David Bransby at Auburn University (Auburn, AL), and sugar cane bagasse was obtained from Biocel, Brazil. Spezyme CP (cellulase) was provided by Genencor International, Inc. (Rochester, NY). Novo 188 (β-glucosidase, was purchased from Sigma (Cat. No. C6150). All other reagents and chemicals, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO).
The biomass samples were milled to the appropriate size (100–2,000 μm). The milled biomass with appropriate moisture (kg water/kg dry biomass) was transferred to a high-pressure Parr reactor and anhydrous ammonia (1 kg of ammonia/kg of dry biomass) was slowly charged to the vessel. The temperature was raised using a heating mantle to the desired level and maintained for 5 min residence time at that temperature before explosively relieving the pressure. The instantaneous drop of pressure in the vessel caused the ammonia to vaporize, causing an explosive decompression of the biomass and considerable fiber disruption. The pretreated material was allowed to stand under a hood overnight to remove the residual ammonia and stored in a freezer until further use. The ammonia left after the pretreatment process can be recovered, recycled, and reused (12). A pictorial representation of the AFEX reactor is shown in Fig. 2.

The enzyme loading in the saccharification step also determines the rate and extent of polysaccharide hydrolysis. The pretreatment and saccharification steps are intimately coupled. The effects of a poor pretreatment can be overcome, to some extent, by higher enzyme loadings. Similarly, an effective pretreatment can reduce the required enzyme loading substantially. AFEX does indeed significantly reduce the required enzyme loadings. We tested the effects of reducing the enzyme loading on the hydrolysis of AFEX-treated corn stover. These results indicate that reducing the enzyme loadings fourfold (from 60 FPU to 15 FPU/g of glucan) decreases the glucan conversion only by 4% and xylan conversion by 5%. A further cut to 7 FPU/g of glucan results in 13% and 21% reduction in glucan and xylan conversion, respectively. For screening purposes, we fixed the cellulase loading at 15 FPU and 64 pNPGU of β-glucosidase/g of glucan and looked at the performance of both glucan and xylan conversion under various pretreatment conditions. The details about the hydrolysis procedure are given under the mass balance section. Once the desired pretreatment conditions are determined, we further study the role of different accessory enzymes such as xylanases, pectinases, feruloyl esterases, and other surface-active additives.

The key variables during the AFEX process are treatment time \( t \), temperature \( T \), ammonia-to-biomass ratio, and moisture content. An optimum combination of these variables is dependent on the recalcitrant nature of the lignocellulosic biomass. In order to determine the best AFEX conditions for any given biomass, the parameters are varied individually. For example, in the case of corn stover, we first fixed the moisture content and ammo-
nia-to-biomass ratio as 60% and 1:1, respectively, while varying the temperature from 60 to 110°C to establish 90°C as the best pretreatment temperature. In the second set of experiments, the temperature and ammonia-to-biomass ratio was fixed as 90°C and 1:1, respectively, while varying the moisture content from 20 to 80%. In the third set of experiments, the temperature and moisture content were fixed as 90°C and 60%, and the ammonia-to-biomass ratio was varied from 0.5:1 to 2:1. All of these runs were done at a constant holding time of 5 min. Based on these experiments, the best AFEX conditions were found to be 90°C, 1:1 ammonia-to-biomass ratio and 60% moisture content for this particular feedstock, i.e., corn stover. Similar AFEX pretreatment screening was done for other biomass like DDGS (13), switchgrass (12), and bagasse (cane sugar waste). The hydrolysis results both for untreated and AFEX treated sample using 15 FPU of
cellulase (Genencor) at 50°C are shown in Fig. 3. The sugar concentration in the hydrolyzate was measured by the high-performance liquid chromatography (HPLC) (14). It is important to mention that each biomass is a unique complex based on how the cellulose, hemicellulose, and lignin are embedded together. Hence, the pretreatment conditions and ammonia loading vary for different feedstocks. We have shown glucan and xylan conversion for 15 FPU of cellulase and 64 pNPGU of β-glucosidase loading. However, higher conversions were seen when xylanase supplementation was included with cellulase (data not shown).

3.1.4. Mechanistic Understanding of AFEX

Thermochemical pretreatments (i.e., AFEX and steam explosion) used to pretreat biomass result in a physicochemical alteration of the lignocellulosic ultra and macro structure, which greatly enhances biological susceptibility. However, few efforts have been directed to gain a fundamental mechanistic understanding of AFEX. Most chemical pretreatments have been found to improve digestibility by reducing the physical (i.e., lignin and hemicellulose surrounding cellulosic micro fibrils) and chemical (i.e., LCC interactions, hemicellulose acetylation) barriers to accessing cellulose and hemicellulosic polysaccharides. During the AFEX process, ammonia penetrates the biomass and exothermically reacts with water present in the biomass forming ammonium hydroxide. The hydroxide ions catalyze several thermochemical reactions within the lignocellulosic ultra structure. These ammonia-solubilized compounds (i.e., lignin and hemicellulose-derived products) are thought to be extracted from within the ultra structure and re-deposited on the exterior cell walls, thereby improving physical and chemical accessibility of cellulose to several hydrolase enzymes (14).
During the pretreatment process, several alkaline catalyzed degradation products are formed, some of which are biologically inhibitory. Microbial inhibitors such as furans, phenolics, and other organic acids have been found in dilute acid-treated lignocellulosic hydrolyzates, which severely inhibit fermentation (15). The AFEX process also generates some quantities of short-chain oligosaccharides, organic acids (i.e., lactic, acetic, succinic), aromatic phenolic acids, and aldehydes due to base-catalyzed cleavage of lignin-carbohydrate polymers in corn stover (unpublished data). Detailed compositional analysis of AFEX degradation products is currently under progress.

Visualizing the macromolecular changes in the plant cell wall upon AFEX pretreatment could greatly enhance the mechanistic understanding of the process. Scanning electron microscopy-based imaging of dilute acid pretreated corn stover has shown enlarged cell wall pits and pores after acid pretreatment compared to untreated stover (16). Thus, naturally occurring sites such as pits and pores are preferentially degraded during acid pretreatment, aiding in further transport of chemicals and enzymes (17). AFEX pretreatment is found to increase defibrillation of fibrous biomass, as observed under SEM. However, there has been little work done that elaborates on changes in the biomass surface after AFEX treatment. Electron spectroscopy for chemical analysis (ESCA) is an analytical tool used to quantify the atomic composition of biomass surfaces (18). The oxygen-to-carbon (O/C) ratio is a good indicator of the relative amounts of oxygen- and carbon-rich species on the sample surface. After the AFEX process, there is a significant drop in the O/C ratio, indicating deposition of carbon-rich species (i.e., aromatic acids and phenolics, oligosaccharides, organic acids, cell-wall extractives) on the biomass surface (14). Further detailed microscopic analysis using laser confocal fluorescence microscopy and atomic force microscopy indicates deposition of lignin and hemicellulosic degradation products on the cell-wall ultra structure after AFEX (unpublished data).

Since ammonia is used during AFEX, the pretreatment equipment must be constructed using materials that are not reactive to ammonia. A good material to use in building AFEX equipment is #316 stainless steel. All pressure and temperature gauges as well as transducers must be constructed of stainless steel and/or other ammonia–compatible material to prevent corrosion in the presence of highly alkaline ammonia–water mixtures. It is also important to check the specifications of every part used in the reactor for its compatibility with liquid and gaseous ammonia. For example, polytetrafluoroethylene (PFTE) is a good material to use for seals on AFEX reactor parts.
3.2.2. Temperature and Pressure

Since the AFEX reaction is carried out at elevated temperatures close to ammonia’s critical temperature (405.4 K) \(^{(19)}\) and the pressure of the ammonia gas can go as high as its critical pressure (113 bar) \(^{(19)}\), care must be taken in choosing reaction vessels, piping, and transducers. There are two main pressure vessels used in any AFEX reaction: the reaction vessel and the charging vessel (Fig. 2).

3.2.3. AFEX Reaction Vessel

This pressure vessel will hold the feedstock as well as the water–ammonia mixture required for the reaction. Since biomass is not very dense, a sizeable reaction volume is needed for a relatively small amount of feedstock. For example, 200 g dry weight of “loose” milled corn stover will occupy approximately 2 l volume. Of course, most biomass can be pressed down to higher densities, but it is not known how the density of the feedstock affects pretreatment. The reactor volume occupied is shared by the feedstock, water in the feedstock, and the added ammonia. The biomass generally soaks up all of the water added under usual AFEX conditions. The ammonia-water mixture equilibrates in the vapor–liquid phase based on the thermodynamic state of the system \(^{(20, 21)}\). Excess ammonia vaporizes and fills the remainder of the vessel as high-pressure ammonia gas.

3.2.4. AFEX Charging Vessel

This stainless steel pressure vessel is used to deliver a precise amount of ammonia to the reaction vessel. This vessel is weighed to ensure that the correct amount of ammonia is delivered. When choosing charging vessels, ensure that the vessel does not have a “seamed” construction. Some pressure containers, sometimes called “lecture” bottles, are unsuitable for AFEX work. For safety considerations, the charging vessel must not be filled with a volume of liquid ammonia that exceeds 50% of the charging vessel volume at room temperature. For example, a 50 g dry weight feedstock could require up to standard 500 ml charging cylinder, because with 3:1 loading the ammonia required is 150 g. Since the approximate density of liquid ammonia at room temperature and 140 psi is 0.6 g/cm\(^3\), 150 g of ammonia will require approximately 250 ml. When this charging cylinder is heated, the internal pressure increases rapidly and can cause under-rated pressure vessels to burst. Using the 50% rule, the charging vessel will be always safe to handle when heated slightly before charging into the reaction vessel. There will be some amount of residual ammonia left in the charging vessel after ammonia is added into the reaction vessel. This amount of ammonia gas retained will depend upon the temperature and volume of the charging vessel.

3.2.5. Reactor Headspace Volume

Another consideration when choosing reactor volume is “headspace”. This is the unoccupied reactor volume that will be taken up by the ammonia–water vapor. The vapor and liquid phase of the ammonia-water mix pretreat the biomass with varying
effectiveness. It is desirable to have as little headspace as possible. Unfortunately, the less dense the feedstock, the more headspace it will inherently produce. When calculating headspace, use 1.1 g/cm³ as the approximate biomass density. Also, add in water content at its respective density, and the remainder will be the reactor volume available for ammonia to occupy. The water in the system will absorb a considerable amount of this ammonia. The ammonia will dissolve in water forming ammonium hydroxide. Using ammonia vapor tables (20), one can estimate the amount of liquid ammonia available for pretreatment and the amount of ammonia gas in the headspace.

### 3.2.6. Heat Transfer Issues

The use of a cylindrical reaction vessel presents heat transfer issues during reactor scale-up (from 10 to 300 ml). As the volume of the reactor increases, a greater heating mantle temperature must be used to heat the reaction to the desired temperature within a reasonable amount of time. When this mantle temperature is too high, the biomass at the reactor walls will char rapidly. The use of a volumetrically smaller reaction vessel can help prevent the feedstock from charring and reduce the overall pretreatment time.

Another consideration for heat transfer is that the amount of ammonia loaded will affect the amount of time and energy it takes to heat the mixture. Larger amounts of ammonia tend to create more ammonia vapor, which will pressurize the container. Also since various AFEX conditions have different energy requirements to heat to target temperature, it follows that for a given temperature reservoir (heating mantle) the different mixtures will heat at different rates.

### 3.2.7. Vacuum Requirement During AFEX

It is important to apply partial vacuum to the sealed reaction vessel with biomass to remove air before charging the vessel with liquid ammonia. This helps in discharging the ammonia in the slightly heated charging vessel into the reaction vessel, as well as removing oxygen from the system that may promote undesirable alkaline-induced oxidation degradation reactions.

### 3.3. Conclusions

Alkaline pretreatment using ammonia offers several advantages compared to other pretreatments. In AFEX, it is possible to achieve better conversions at lower temperature severity. No separation of a liquid stream during the AFEX process occurs, and the catalyst (namely ammonia) can be recovered, recycled, and reused. The AFEX process is greatly influenced by several key factors such as mixing, lower residence time, heat transfer, lower ammonia loadings, and the amount of ammonia in the gaseous and liquid phase. By reducing the reactor volume from 300 to 22 ml, the heat transfer issues are addressed to some extent. In order to make AFEX a commercially viable process, several of the above-mentioned issues need to be addressed.
4. Notes

4.1. Mass Balance

In order to evaluate the effectiveness of a pretreatment process, a careful mass balance must be performed around the entire process between the pretreatment and subsequent hydrolysis. Various pretreatments and process parameters may result in the solubilization and removal of portions of certain biomass extractives. Such a loss must be accounted for; or else, comparisons between different treatments will not have a consistent basis. Thus, the final evaluation, whether it be sugar or ethanol yields or an economic or environmental metric, must be given per-unit dry, untreated biomass. The overall mass balance should exhibit approximately 100% closure. Details of the mass balance are given in Fig. 4.

4.1.1. Precision in Wording

In addition, precise wording is needed in order to make the results of a pretreatment clear. It is possible to report results on either a conversion or yield basis. Percent conversion refers to the amount of glucan in the hydrolysis reactor that is broken down into glucose, whereas percent yield refers to the total glucose obtained compared to the theoretical maximum. This difference becomes important if either glucose-containing oligomers or monomers such as sucrose are present or if a portion of the cellulose is lost prior to hydrolysis whether by removal or by degradation during pretreatment. For example, a harsh pretreatment may allow the glucan content to become easily digestible, leading to a subsequent high conversion during hydrolysis, but it may also degrade large amounts of the cellulose to unfermentable byproducts.

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**Fig. 4.** Overall mass balance chart based on which the glucan and xylan conversions are calculated.
In such a case, the high conversion of the remaining cellulose is misleading, as the overall yield may still be low. Since all comparisons should be made on a per-unit dry, untreated biomass basis, percent yield should always be reported. Percent conversion of cellulose or hemicellulose may give useful information in some cases, but should not be used without percent yield as well.

Other similar terms that need to be clear are cellulose and glucan composition. Glucan refers to all polymers of glucose, including both cellulose and starch. When determining percent yield, the glucan content should be used rather than cellulose content, as both may be broken down. Furthermore, although they are not polymers, if any free glucose, such as that in sucrose, is present in the biomass, it should also be counted as glucan, as it too would be included in the final glucose concentration. The cellulase enzymes, however, are added on the basis of the cellulose content. If there is a significant amount of non-cellulose glucan in the biomass, these two terms must be distinguished in order to avoid confusion. Another source of possible confusion is in labeling the moisture content of the biomass. It is necessary to always be clear whether the moisture content is determined on a dry-weight (gram water per gram dry biomass) or total weight (gram water per gram wet biomass) basis (Table 1).

<table>
<thead>
<tr>
<th>DBM</th>
<th>Water</th>
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DBM dry biomass; DWB dry weight basis; TWB total weight basis
DWB moisture = 100 \times \text{water/DBM}
TWB moisture = 100 \times \text{water/(water + DBM)}
AFEX is a complicated chemical pretreatment that involves numerous chemical reactions. Ammonia and water may be added to the biomass during cleavage reactions. Likewise, smaller molecules that are cleaved may vaporize either during the process or while the biomass is drying after AFEX. In addition, if using high moisture content, portions of the biomass will solubilize and water may spray out during the explosion. Thus, it is necessary to carefully weigh the solid biomass before and after AFEX to ensure a proper mass balance. If a high moisture content is used in the pretreatment, the liquid that escapes during blasting must be blasted into a container and its solid contents analyzed. If no liquid escapes, then in most cases the final weight of the biomass will be approximately the same as or slightly higher than the initial weight. Because ammonia does not degrade cellulose, the total cellulose present after AFEX can be assumed to be equal to the amount prior to pretreatment. A nitrogen balance may also be performed to determine the amount of ammonia that is irreversibly bound to the biomass.

Intermediate steps may be performed prior to hydrolysis and after AFEX in order to extract specific components as co-products or as a further pretreatment. If so, such a step will likely be a wet step, and thus have a separate liquid stream that may include biomass compounds but will not be present during hydrolysis. Thus, an accurate mass balance during solid–liquid separation is necessary. Separation can be performed through centrifugation or filtration, although centrifugation is preferred as little moisture will be lost on the filter. The volume of the extract must be recorded, and the solids should be washed with excess water to remove residual soluble compounds and centrifuged again. Both the original extract and the rinse water must be analyzed and summed together to obtain the final concentration of the desired end product. In addition, the sugar concentration in these streams must also be determined, as they may still be included in the total sugar yield. The solid biomass recovered must be weighed and analyzed for moisture content in order to determine how much of the biomass has solubilized during the process. Cellulose content may be analyzed on the remaining solids, or may be calculated assuming no loss occurred if the intermediate step does not degrade cellulose. The overall mass change between AFEX and any intermediate steps must be known prior to hydrolysis in order to ensure that the biomass loaded can be traced back to a specific known amount of untreated biomass.

The procedure for hydrolysis is based on the NREL protocol, although it is expanded to include larger volumes and higher solid loadings (NREL 1996). The solid loading, reported as weight of biomass per total weight of the mixture, can be recorded as either percent solids or percent cellulose. The biomass is added
first, taking into account its moisture content, and most of the water is added to allow mixing. Citrate buffer (1M, pH 4.5) is used to bring the final buffer concentration to 0.05 M and 1 M citric acid is added to further reduce the pH to 4.8 if necessary. Cycloheximide and tetracycline are added at 30 and 40 mg/l, respectively, to control fungal/yeast and bacterial growth. Cellulases and other enzymes are added proportional to the cellulose content in the biomass as per the NREL method as described in Subheading 3.1.2. As the final concentrations of the buffer, enzymes, and antibiotics are all small, their specific gravity can be assumed to be 1 and thus added volumetrically. A fed-batch approach for the biomass and enzymes may be used in order to increase the final solid loading or yield. In this case, the pH must be checked after each addition to ensure that it is correctly buffered, and excess citric acid may be required to reduce the pH back to 4.8. The hydrolysis is performed at 50°C with shaking at 200 rpm. As water is consumed during the process, one cannot rely on the initial volume of the reactor when analyzing sugar concentrations. Thus, solid–liquid separation is required, as detailed above, and the final volume of the liquid portion measured in order to determine the final sugar yields.

For screening purpose, experiments are done in the 15-ml scale and 1% cellulose loading in scintillation vials. Samples are collected at 72 and 168 h. The collected sample is transferred to a sealed centrifuge tube, heated to denature the enzymes, and then filtered through a 0.2-µm nylon syringe filters. The filtered samples are frozen in HPLC vials for subsequent analysis. For sugar analysis, an HPLC system is used which consists of a system with refractive index detector and an Aminex HPX-87P carbohydrate analysis column (Biorad, Hercules, CA) or equivalent equipped with a deashing guard cartridge (BioRad). Degassed HPLC-grade water is used as the mobile phase at 0.6 ml/min at a column temperature of 85°C. The injection volume is 10 ml with a run time of 20 min. Mixed sugar standards are used for quantification of cellobiose and monosaccharides (glucose, xylose, galactose, arabinose, and mannose) in the samples. Because the biomass loading is low, the initial volume is used when quantifying results.

4.1.4. Equations

\[
\text{Glucan to Glucose} = \frac{100 \times (\text{kg G in} \# + \text{kg G in} \#)}{\text{(kg glucan in} \# \times 1.11)}
\]  
(1)

\[
\text{Xylan to Xylose} = \frac{100 \times (\text{kg X in} \# + \text{kg X in} \#)}{\text{(kg xylan in} \# \times 1.136)}
\]  
(2)
These are the basic percent yield equation where the circled numbers refer to the process steps in Fig. 4.

**Mass balance around unit operation:**

$$\frac{g_{\text{Dry}O_{BM}}}{100 g_{\text{Dry}I_{BM}}} = \frac{(a)g_{O_{BM}}}{100 g_{O_{BM}}} - \frac{(b)g_{\text{Dry}O_{BM}}}{100 g_{O_{BM}}} - \frac{(c)g_{U_{BM}}}{100 g_{I_{BM}}} + \frac{(d)g_{\text{Dry}I_{BM}}}{100 g_{I_{BM}}} \quad (3)$$

**Overall mass balance:**

$$\frac{g_{\text{Dry}C_{BM}}}{100 g_{\text{Dry}U_{BM}}} = \frac{(e)g_{\text{Dry}A_{BM}}}{100 g_{\text{Dry}U_{BM}}} - \frac{(f)g_{\text{Dry}B_{BM}}}{100 g_{\text{Dry}A_{BM}}} - \frac{(g)g_{\text{Dry}C_{BM}}}{100 g_{\text{Dry}B_{BM}}} \quad (4)$$

Note that Eq.4 can be expanded or contracted to the total amount of unit operations present.

**Ammonia balance:**

$$\frac{g_{\text{NH}_{\text{bound}}}}{100 g_{\text{Dry}U_{BM}}} = \frac{17 g_{\text{NH}_{1}}}{14 g_{N}} \left( \begin{array}{c} \frac{(b)g_{N}}{100 g_{A_{BM}}} - \frac{100 g_{ABM}}{100 g_{A_{BM}}} \\ \frac{(j)g_{\text{Dry}A_{BM}}}{100 g_{\text{Dry}U_{BM}}} \\ \frac{(k)g_{N}}{100 g_{U_{BM}}} - \frac{100 g_{UBM}}{100 g_{U_{BM}}} \end{array} \right) \quad (5)$$

Note that this equation assumes that no biomass nitrogen was lost due to evaporation or in a liquid stream. If an aqueous stream is present, it will be necessary to measure its nitrogen content as well.

**Enzyme addition during hydrolysis:**

$$g_{\text{enzyme}} = \frac{(m)g_{\text{Dry}BM}}{100 g_{\text{Dry}U_{BM}}} - \frac{(n)g_{\text{Dry}BM}}{100 g_{\text{Dry}U_{BM}}} - \frac{(o)g_{\text{cellulose}}}{100 g_{\text{Dry}U_{BM}}} \quad (6)$$

$$\frac{(p)g_{\text{FPU}}}{g_{\text{cellulose}}} \cdot \frac{g_{\text{enzyme}}}{(q)g_{\text{FPU}}}$$

**Glucose recovery:**

$$\frac{g_{\text{glucose}}}{100 g_{\text{Dry}U_{BM}}} = \left( \frac{(r)g_{\text{glucose}}}{L} \cdot \frac{(s)L}{(r)g_{\text{glucose}}} + \frac{(t)L}{(u)L} \right) \cdot \frac{(m)g_{\text{Dry}BM}}{100 g_{\text{Dry}U_{BM}}} \quad (7)$$
Note that this equation can be expanded to include glucose recovered in aqueous streams prior to AFEX. If so, simply apply Eq. 7 to these streams as well, and sum the results to obtain the final glucose concentration.

100% Theoretical glucose:

\[
\frac{\mathcal{G}_{\text{glucose}}}{100 \, \text{g} \text{Dry} \mathcal{U}_{\text{BM}}} = \left( \frac{(o) \mathcal{G}_{\text{cellulose}}}{100 \, \text{g} \text{Dry} \mathcal{U}_{\text{BM}}} \cdot \frac{180 \mathcal{G}_{\text{glucose}}}{162 \mathcal{G}_{\text{cellulose}}} \right)
\]

\[
+ \left( \frac{(v) \mathcal{G}_{\text{starch}}}{100 \, \text{g} \text{Dry} \mathcal{U}_{\text{BM}}} \cdot \frac{180 \mathcal{G}_{\text{glucose}}}{162 \mathcal{G}_{\text{starch}}} \right)
\]

\[
+ \left( \frac{(w) \mathcal{G}_{\text{sucrose}}}{100 \, \text{g} \text{Dry} \mathcal{U}_{\text{BM}}} \cdot \frac{180 \mathcal{G}_{\text{glucose}}}{342 \mathcal{G}_{\text{sucrose}}} \right)
\]

Note that other forms of glucose may be present as well. If so, further terms may be added along with the respective ratio of glucose monomers to the polymer present.

**Glucose yield:**

\[
\% \, \text{yield} = \left( \frac{x \mathcal{G}_{\text{gluc cos e}}}{100 \, \text{g} \mathcal{DUBM}} \right) \times 100\%
\]

Note that Eqs. 7–9 can be repeated for xylose or arabinose recovery.

- \( \mathcal{D} \mathcal{B} \mathcal{O}_{\text{BM}} \): Dry biomass exiting unit operation
- \( \mathcal{D} \mathcal{B} \mathcal{I}_{\text{BM}} \): Dry biomass entering unit operation
- \( \mathcal{D} \mathcal{B} \mathcal{U}_{\text{BM}} \): Dry, untreated biomass
- \( \mathcal{A}_{\text{BM}}, \mathcal{B}_{\text{BM}}, \text{etc.} \): Biomass exiting the first, second, etc. unit operation

(a) Weight of wet biomass exiting operation, measured
(b) Moisture content (total weight basis) of biomass exiting operation, measured
(c) Weight of biomass entering operation, measured
(d) Moisture content (total weight basis) of biomass entering operation, measured
(e) Dry weight of biomass exiting first unit operation (AFEX), calculated from Eq. 3
(f) Dry weight of biomass exiting second unit operation (washing, extraction, etc.), calculated from Eq. 3
(g) Dry weight of biomass exiting third unit operation (hydrolysis), calculated from Eq. 1
(h) Nitrogen content of AFEX treated biomass, measured
(i) Moisture content (total weight basis) of AFEX treated biomass, measured
(j) Dry weight of biomass exiting AFEX, calculated from Eq. 3
(k) Nitrogen content of untreated biomass, measured
(l) Moisture content (total weight basis) of untreated biomass
(m) Amount of dry treated biomass added to hydrolysis reactor, determined by experiment
(n) Overall mass balance to this point in the process, calculated from Eq. 4
(o) Cellulose content in dry untreated biomass, measured
(p) Activity of enzymes desired, determined by the experiment
(q) Activity of enzyme, measured
(r) Concentration of hydrolysate, measured
(s) Volume of hydrolysate, measured
(t) Concentration of glucose in rinse water, measured
(u) Volume of rinse water, measured
(v) Starch content in dry, untreated biomass, measured
(w) Sucrose content in dry, untreated biomass, measured
(x) Total glucose recovered, calculated from Eq. 7
(y) Total theoretical glucose, calculated from Eq. 8

References


Chapter 6

Pretreatment of Biomass by Aqueous Ammonia for Bioethanol Production

Tae Hyun Kim, Rajesh Gupta, and Y.Y. Lee

Summary

The methods of pretreatment of lignocellulosic biomass using aqueous ammonia are described. The main effect of ammonia treatment of biomass is delignification without significantly affecting the carbohydrate contents. It is a very effective pretreatment method especially for substrates that have low lignin contents such as agricultural residues and herbaceous feedstock. The ammonia-based pretreatment is well suited for simultaneous saccharification and co-fermentation (SSCF) because the treated biomass retains cellulose as well as hemicellulose. It has been demonstrated that overall ethanol yield above 75% of the theoretical maximum on the basis of total carbohydrate is achievable from corn stover pretreated with aqueous ammonia by way of SSCF. There are two different types of pretreatment methods based on aqueous ammonia: (1) high severity, low contact time process (ammonia recycle percolation; ARP), (2) low severity, high treatment time process (soaking in aqueous ammonia; SAA). Both of these methods are described and discussed for their features and effectiveness.

Keywords: Aqueous ammonia, ARP, SAA, Pretreatment, Cellulase, Xylanase

1. Introduction

1.1. Ammonia as a Pretreatment Reagent

Aqueous ammonia has many desirable characteristics as a pretreatment reagent. It is a proven delignification reagent. With ammonia treatment, the lignin content of the biomass can be lowered to a desired level (1–5). Delignification opens up the biomass structure, making the biomass amenable for enzymatic hydrolysis. A low lignin level in biomass also increases the efficiency of enzyme action because it reduces irreversible binding of enzymes to lignin (6–11). As is the case with most alkaline treatments, ammonia treatment does not cause
significant loss of carbohydrates. Ammonia is chemically reactive enough to cause significant morphological changes in the biomass other than delignification. Use of ammonia brings about not only the pretreatment effects but also fractionation of biomass, especially separation of lignin from biomass (12). This is an important attribute of ammonia pretreatment, as it offers flexibility in utilizing biomass. The lignin generated in this process is sulfur- and sodium-free unlike those generated from pulping processes. It is generally of higher quality and therefore commands higher byproduct credit. It is quite conceivable that the uncontaminated lignin can be marketed as additives in fuel and polymers, as adhesives, and as an asphalt extender (13–16).

High volatility of ammonia in comparison to water makes it easy to separate from aqueous mixtures. Flash evaporation is sufficient to remove most of the ammonia from the mixture, making the unbound ammonia easily recoverable and recyclable, a key feature in the ammonia-based pretreatment scheme.

Ammonia is inexpensive to the extent that it is used for synthesis of N-fertilizer (urea). Ammonia, although generally considered toxic as a vapor, is not an acute health hazard. It is also one of the most heavily used industrial commodity chemicals. To this point, there is no evidence of harmful byproduct formation from interaction with lignocellulosic biomass at elevated temperatures, and any residual nitrogen will act as a nitrogen supplement to the bioconversion microorganism, such as yeast or bacteria.

With proper use of ammonia treatment, one can achieve selective removal of lignin from biomass, leaving the carbohydrates intact (4–5). The fact that the hemicellulose is retained after pretreatment is a positive factor in a pretreatment process. It is well known that commercial cellulase enzymes exhibit considerable xylanase activity as well as cellulase activity. Therefore hydrolysis of cellulose and hemicellulose occurs simultaneously by the action of those cellulases. Certain microbial processes are designed to utilize both hexose and pentose including simultaneous saccharification and co-fermentation (SSCF) for ethanol or butanol production, or simultaneous fermentation and isomerization of xylose (SFIX). If xylose processing is a separate one, then additional acid hydrolysis of hemicellulose would be required. When xylose stream is generated during the pretreatment process, it is usually contaminated with extraneous components of biomass known to be inhibitory to enzymes and toxic to microorganisms (17–22). Overall, it is believed that the pretreatment designed to retain hemicellulose offers more advantages than the potential problems it generates.

1.2. Hemicellulose Retention
There are potential problems associated with the ammonia-based pretreatment processes. The most important one has to do with consumption of ammonia due to interaction with lignin and neutralization by acetates and other buffering components known to exist in biomass. The nature of reactions involving ammonia and lignocellulosic biomass is quite complex. However, most of the ammonia input to the process is recovered and reused. In general, ammonia equivalent to 2–5% of dry biomass is irreversibly consumed during the pretreatment process. From a process viewpoint, there is a concern as to the high-pressure condition that may develop due to the highly volatile nature of ammonia. However, within the range of expected reaction conditions of 60–180°C and 5–15% NH₃, the upper limit of the pressure is about 30 kg/cm² or 450 psi. It is somewhat higher than a normal pulp mill digester pressure, but certainly within a manageable range. Ammonia is mildly corrosive, but far less corrosive than sulfuric acid at high temperature. Overall, it does not present a major technical problem to design and operate a pretreatment process of this nature.

Two different types of pretreatment methods based on aqueous ammonia are described in this chapter:

1. High severity, low contact time process (ammonia recycle percolation; ARP),
2. Low severity, high contact time process (soaking in aqueous ammonia; SAA).

The main technical features of ARP are the following:

1. Aqueous ammonia (ammonium hydroxide) is used as the pretreatment reagent;
2. High-severity treatment condition is used to limit the reaction time within 20 min;
3. A packed-bed flow-through type of percolation reactor is employed and operated under a recirculation mode;
4. Ammonia is continuously regenerated and recycled during the process. The concept of this percolation process applies well to a continuous pretreatment reactor design. The distinctive feature of the percolation process in comparison to a straight batch process is that the process stream is continuously fed and withdrawn from the reactor. This process design offers a unique advantage in that the lignin and other extraneous components are cleanly separated from the biomass structure. This prevents recondensation of lignin in the biomass. It also eliminates the need for washing the pretreated biomass which will have a significant bearing in the operation cost, especially in the ammonia regeneration step.

SAA is a batch process applied under low-severity condition. Because of lower severity, longer treatment time is required. At a typical condition of 15% NH₃ and 60°C, which gives the system pressure of near 1 atm, a reaction time of several hours is required to achieve an acceptable level of pretreatment effects. In order to
attain an acceptable level of delignification and to prevent lignin recondensation, liquid-to-solid ratio of 4 or higher is normally required in the SAA process. Because of low process energy and low equipment cost, the overall processing cost of SAA is substantially lower than that of ARP. On the other hand, SAA as a pretreatment process has limited application. It works best with feedstock having a low lignin content, especially agricultural residues of annual plants such as corn stover, sugarcane bagasse, and wheat straw.

In addition to two basic modes of ammonia pretreatment described here, the process can be easily modified employing additional reagent, i.e., hydrogen peroxide (23, 24), or applying a two-stage operation (hot water followed by aqueous ammonia (12)).

Pretreatments using aqueous ammonia have been investigated against a number of different feedstock: agricultural residues (corn stover/cobs, rice hulls straw, wheat hulls straw, and sugarcane bagasse), energy crops (switchgrass and hybrid yellow poplars), and cellulosic waste (pulp mill sludge). Aqueous ammonia pretreatment of corn stover and hybrid poplar has recently been studied in our laboratory. Ammonia treatment of corn stover proved to be highly effective in delignification and in improving the digestibility of biomass by cellulase enzymes, whereas the treatments of hybrid poplar, which contained much higher amount of lignin, yielded poorer results. After ARP treatment, glucan digestibility of corn stover increased from 13% to 90% with cellulase loading of 15 FPU/g glucan. Retention of the hemicellulose portion of the biomass carbohydrates is a desirable feature since the resulting sugars can contribute to the ethanol yield when using an appropriate co-fermentation microorganism (25–27). Hybrid poplar showed much higher retention of hemicellulose than corn stover after ammonia treatment, and glucan digestibility of hybrid poplar by commercial cellulases was much lower than with corn stover. It is known residual xylan in the treated biomass is a factor hindering accessibility of the cellulases to cellulose (6, 11). External xylanase may be supplemented to improve hemicellulose hydrolysis during the enzymatic hydrolysis. We have shown that xylanase addition indeed improved the total carbohydrate digestibility of ARP- or SAA-treated hybrid poplar significantly (28, 29). When fermentation of ammonia-treated corn stover and hybrid poplar was carried out with the SSCF process, ethanol yield near 80% of the maximum theoretical yield was obtained as a result of simultaneous consumption of xylose and glucose by recombinant Escherichia coli strain ATCC 55124® (KO11) (4, 5, 26).

1.5. Expected Outcome

Pretreatments using aqueous ammonia have been investigated against a number of different feedstock: agricultural residues (corn stover/cobs, rice hulls straw, wheat hulls straw, and sugarcane bagasse), energy crops (switchgrass and hybrid yellow poplars), and cellulosic waste (pulp mill sludge). Aqueous ammonia pretreatment of corn stover and hybrid poplar has recently been studied in our laboratory. Ammonia treatment of corn stover proved to be highly effective in delignification and in improving the digestibility of biomass by cellulase enzymes, whereas the treatments of hybrid poplar, which contained much higher amount of lignin, yielded poorer results. After ARP treatment, glucan digestibility of corn stover increased from 13% to 90% with cellulase loading of 15 FPU/g glucan. Retention of the hemicellulose portion of the biomass carbohydrates is a desirable feature since the resulting sugars can contribute to the ethanol yield when using an appropriate co-fermentation microorganism (25–27). Hybrid poplar showed much higher retention of hemicellulose than corn stover after ammonia treatment, and glucan digestibility of hybrid poplar by commercial cellulases was much lower than with corn stover. It is known residual xylan in the treated biomass is a factor hindering accessibility of the cellulases to cellulose (6, 11). External xylanase may be supplemented to improve hemicellulose hydrolysis during the enzymatic hydrolysis. We have shown that xylanase addition indeed improved the total carbohydrate digestibility of ARP- or SAA-treated hybrid poplar significantly (28, 29). When fermentation of ammonia-treated corn stover and hybrid poplar was carried out with the SSCF process, ethanol yield near 80% of the maximum theoretical yield was obtained as a result of simultaneous consumption of xylose and glucose by recombinant Escherichia coli strain ATCC 55124® (KO11) (4, 5, 26).
2. Materials

2.1. Pretreatment

Pretreatments using aqueous ammonia have been investigated for a number of different feedstock in our laboratory. Examples of corn stover and hybrid poplar are presented as representative feedstock in this section. Composition of the corn stover and hybrid poplar used are shown in Table 1. Air-dried and ground corn stover was supplied by the National Renewable Energy Laboratory (NREL, Golden, CO). The corn stover was screened to a nominal size of 9–35 mesh. The moisture content of corn stover was approximately 5%, and it was stored at 4°C. Hybrid poplar chips (1/4 in.) were supplied by NREL, which contained approximately 50% moisture, and were stored at −20°C (see Note 1).

Ammonium hydroxide (29–30 wt%) (Sigma-Aldrich) diluted to desired concentration was used for use in the pretreatment process.

For the ARP process, a tubular reactor made out of SS-316 (0.9 in. ID × 10 in. length) was used (Fig. 2). In the SAA process, screw-capped laboratory glass bottles were used for low-temperature (below 60°C) operation. For runs above 60°C, stainless steel reactors (1.375 in. ID × 6 in. length) were used.

2.2. Analysis and Evaluation

2.2.1. Compositional Analysis

Sulfuric acid (Cat. No. A298–212) was from Fisher Scientific, Pittsburgh, PA. For HPLC analysis of biomass carbohydrates, 72% sulfuric acid was prepared by diluting the purchased acid with deionized (DI) water by adjusting the density to 1.6389 using a hydrometer (Fisher, 11–520D).

Table 1
Composition of feedstock

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<th>Components</th>
<th>Corn stover [wt%]</th>
<th>Hybrid poplar [wt%]</th>
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</thead>
<tbody>
<tr>
<td>Glucan</td>
<td>36.1</td>
<td>45.1</td>
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<tr>
<td>Xylan</td>
<td>21.4</td>
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<td>Mannan</td>
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<tr>
<td>Lignin</td>
<td>17.2</td>
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</tr>
<tr>
<td>Ash</td>
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</tr>
<tr>
<td>Acetyl group</td>
<td>3.2</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Note: All data in the table are based on the oven-dry biomass weight
2.2.2. Enzymatic Digestibility

The enzymes used for determination of digestibility were cellulase (Spezyme-CP, Genencor, Palo Alto, CA), xylanase (Multifect Genencor, Palo Alto, CA), and β-glucosidase (Novozyme-188, Sigma-C-6105, Sigma Chemical Co, St. Louis, MO). Although the commercial cellulases have a certain level of xylanase activity, additional xylanase was used as a supplementary enzyme in some experiments. The protein content of Multifect xylanase was 42 mg/ml. The activity of β-glucosidase was determined to be 750 CBU/ml. These enzymes were diluted with 0.05 M citrate buffer solution to a desired enzyme loading (see Note 2). Enzyme loadings of 15 FPU/g glucan (cellulase) and 30 CBU/g glucan (β-glucosidase) were used in enzymatic hydrolysis experiments to evaluate the digestibility of pretreated biomass. In experiments where xylanase supplementation was used, xylanase loading of 31.5 mg/g glucan was applied. Citrate buffer was used in the enzymatic hydrolysis to maintain the pH at 4.8. Tetracycline (Fisher, ICN10301225) and cycloheximide (Fisher, AC35742–0050) solutions were prepared according to NREL LAP-009 (30) and used as antiseptic in enzymatic hydrolysis tests. α-Cellulose (Sigma, C-8200) and Avicel PH-101 (Sigma, 11365) were used as reference substrates in enzymatic hydrolysis tests.

2.2.3. SSCF Test

Recombinant Escherichia coli KO11 ATCC®55124 (American Type Culture Collection, Rockville, MD) was used for the SSCF tests. LB medium (Sigma, L-3152) containing 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/l chloroamphenicol was used for the growth of E. coli KO11.

3. Methods

3.1. Pretreatment

3.1.1. Aqueous Ammonia Pretreatment at High Severity (ARP)

3.1.1.1. Operation of ARP Reactor

The ARP laboratory experimental setup consists of a stock solution reservoir, pump, temperature-programmable oven, SS-316 column reactor (see Figures 1 and 2), and a receiving tank (see Note 3) which also served as a backpressure vessel. High-pressure metering piston pump was used to pump aqueous ammonia into the reactor system.

1. Pack dry biomass sample into the reactor (see Fig.1 and Note 4).
2. Prepare the ammonium hydroxide solution to the desired concentration (see Note 5).
3. Before the reactor installation, run the liquid through the pump to ensure that there is no void volume or bubbles trapped in the tubes.
4. Place the reactor in the heating chamber and connect the tubing.
5. Nitrogen ($N_2$) backpressure is applied to the reactor system (see Note 6).

6. Preheat the convection oven until the reactor temperature reaches to the desired temperature.

7. Keep the valves closed during preheating.

8. When the reactor temperature reaches the desired temperature, start the feed pump and set this as the zero point of reaction time.
9. During the course of the reaction, check the process conditions (flow rate, temperatures, and system pressure) for potential malfunction and take the needed corrective action.

10. When the reaction is completed, let the oven to cool down to room temperature and switch the chemical reagent from ammonium hydroxide to DI water.

11. Flush the treated biomass sample with DI water (see Note 7).

12. After the reactor cools down, release the pressure by opening the valve in the receiving tank.

13. Open the reactor and collect the treated biomass in the reactor (see Note 8).

14. Wash the collected biomass by vacuum filtration (see Note 9).

15. Transfer all washed solid sample to a clean container and weigh the total treated biomass.

16. The wet solids discharged from the reactor are separated into three portions. One is dried at 105°C in an oven for measurement of weight loss. Another is dried at 45°C in the oven and subjected to composition analysis. The third is stored in the refrigerator and then used for the enzymatic digestibility and fermentation test after composition analysis (see Notes 10–12).

17. Measure the moisture content and calculate the weight of solids remaining.

3.1.2. Aqueous Ammonia Pretreatment at Low Severity (SAA)

1. Prepare the reactor (see Note 13).

2. Prepare the ammonium hydroxide to the desired concentration.

3. Preheat the oven to the desired temperature.

4. Load dry biomass sample into the reactor.

5. Add the prepared ammonium hydroxide into the reactor (see Note 5).

6. Place the reactor in a heated oven.

7. After completion of the reaction, remove the reactor from the oven and quench in ice water.

8. Collect the residual biomass in the reactor (see Note 8).

9. Wash the collected biomass by vacuum filtration (see Note 9).

10. Transfer the washed solid sample to a clean container.

11. Weigh the total wet solid.

12. The wet solids discharged from the reactor are separated into three portions. One is dried at 105°C in the oven for measurement of weight loss. Another is dried at 45°C in the oven and subjected to composition analysis. The third
is stored in a refrigerator and then used for the enzymatic digestibility and fermentation test after composition analysis (see Note 10 and 11).

13. Measure the moisture content and calculate the solid remaining.

1. Use the 45°C oven-dried biomass sample for the solid composition analysis (see Subheadings 3.1.1, step 16 and 3.1.2, step 12).

2. All the analyses are done following the NREL LAP procedure (31) and complying with its quality control standard.

3.2. Analysis and Evaluation (See Note 13)

3.2.1. Compositional Analysis

3.2.1.1. Solid Composition Analysis

3.2.1.2. Liquid Sample Analysis

3.2.2. Enzymatic Digestibility

3.2.3. SSCF Test

1. Take a wet, pretreated biomass sample containing 1.0 g of glucan for enzymatic digestibility tests. The glucan content of the sample is initially determined as glucose by solid composition analysis (see Note 11).

2. The digestibility tests are done following the NREL LAP procedure (30).

1. Collect the liquid samples (liquor in the reactor + collected in the receiving tank + water used for washing the treated solids) and measure the total volume.

2. Centrifuge or filter the liquid.

3. Transfer 100 ml of liquid sample into the beaker.

4. Evaporate ammonia by heating the beaker in a water bath at 60–70°C in a chemical hood.

5. Measure the residual volume after evaporation.

6. Analyze the carbohydrates in the liquid by NREL LAP (32).

1. Take a wet biomass sample containing 3.0 g of glucan (see Note 11 and 14).

2. Prepare the LB (Luria-Bertani) medium.

3. Prepare the microbial inoculum (E. coli KO11) as described in the NREL LAP-008 (33).

4. Use sterilized DI water for makeup liquid.

5. The SSCF test is done without external pH control (see Note 15).

6. The ethanol yield in SSCF test is calculated as follows:

   \[
   \text{Ethanol yield}\% = \left( \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial Sugar (g) in reactor \times 0.511}} \right) \times 100
   \]

   Sugar is interpreted as glucose plus xylose in the SSCF test.
4 Notes

1. Moisture content of feedstock changes during storage. The moisture level of biomass feedstock should be measured before each experiment.

2. Enzyme dilution is a very sensitive factor causing experimental error. Quite often, it becomes a major source of experimental error in the enzymatic hydrolysis tests and SSCF. High accuracy is required in this step, especially with thick commercial enzyme preparations.

3. It is recommended that liquid receiving tank should have at least 10 times the volume of the reactor used in the ARP.

4. Flanged stainless steel reactor is recommended since the reaction takes place at high temperature and pressure (Fig. 2). The weight of packed biomass should be determined before the reaction. There should be no void space in the reactor. If small amounts of biomass is to be used, fill the reactor void with an inert material such as perforated Teflon rod.

5. The calculation of ammonia solution is as follows:

\[
V_{\text{NH}_4\text{OH/water}} = \frac{C_{\text{NH}_4\text{OH}}^T}{(C_{\text{NH}_4\text{OH}} - C_{\text{NH}_4\text{OH}}^T) \times S.G._{\text{NH}_4\text{OH}}}
\]

Repeatability in the pretreatment step strongly depends upon the concentration of reagent used. Ammonia is highly volatile and evaporation of ammonia from the solution would lower the effective concentration of the solution. Proper care needs to be taken while preparing and storing the 15% ammonium hydroxide solution in order to avoid its evaporation. The container of the ammonium hydroxide solution should be checked for any leakage.

6. Nitrogen backpressure should be applied to prevent flash evaporation of ammonia. The backpressure is adjusted according to the concentration of ammonia and reaction temperature in order to prevent ammonia from evaporation. In case of 15 wt% of ammonia and 170°C, 2.0 MPa of backpressure is applied.

7. At the completion of the run, the reactor is pumped with DI water to remove the residual sugar and ammonia trapped in the treated biomass. It is more efficient to wash the biomass sample in the reactor than to wash the sample by filtration.

8. Use DI water and tray to collect the treated solids from the reactor.
9. For filtration, a fluted cellulose filter of coarse to medium pore size is recommended.

10. The solid remaining after reaction is calculated as follows.

\[
S.R. = \frac{(W_{Total} - W_{Jar}) \times (1 - \% \text{Moisture} / 100)}{W_{Untreated}} \times 100
\]

Where,

- S.R. = Solid remaining after pretreatment [%]
- \(W_{Total}\) = Weight of wet pretreated biomass + jar
- \(W_{Jar}\) = Weight of jar
- \(W_{Untreated}\) = Weight of untreated biomass, dried
- \%Moisture = Moisture content of wet pretreated biomass

11. All pretreated biomass should not be dried or frozen prior to the enzymatic digestibility or fermentation test. Irreversible pore collapse or unknown structural changes can occur in the microstructure of the biomass, affecting enzymatic reaction with the biomass. The wet samples for the digestibility and fermentation test must be kept in a tightly sealed container to maintain constant moisture level.

12. For reactions at temperatures higher than 60°C (with 15 wt% aqueous ammonia), use of a pressure vessel is recommended. For reaction below 60°C, autoclavable glass laboratory bottle can be used.

13. For ARP and SAA, the solid analysis and evaluation procedures are identical.

14. Sample and flask preparation, shaking, incubating conditions, and analysis procedures are almost identical to the SSF procedure of NREL LAP (33). SSCF employs the Recombinant \(E. coli\) ATCC® 55124 (KO11).

15. The initial pH of the fermentation is 7.0, and it gradually decreases to the terminal pH of 5.0–5.5. Growing and test conditions for KO11 are anaerobic, 37°C, 150 rpm (1.9 cm diameter orbit shaker), and pH 7.0.

References


Chapter 7

Liquid Hot Water Pretreatment of Cellulosic Biomass

Youngmi Kim, Rick Hendrickson, Nathan S. Mosier, and Michael R. Ladisch

Summary

Lignocellulosic biomass is an abundant and renewable resource for fuel ethanol production. However, the lignocellulose is recalcitrant to enzymatic hydrolysis because of its structural complexity. Controlled-pH liquid hot water (LHW) pretreatment of cellulosic feedstock improves its enzymatic digestibility by removing hemicellulose and making the cellulose more accessible to cellulase enzymes. The removed hemicellulose is solubilized in the liquid phase of the pretreated feedstock as oligosaccharides. Formation of monomeric sugars during the LHW pretreatment is minimal. The LHW pretreatment is carried out by cooking the feedstock in process water at temperatures between 160 and 190°C and at a pH of 4–7. No additional chemicals are needed. This chapter presents the detailed procedure of the LHW pretreatment of lignocellulosic biomass.

Key words: Cellulosic biomass, Pretreatment, Liquid hot water, Ethanol

1. Introduction

The use of ethanol-blended gasoline is forecast to soar in the next few decades, due to government-mandated increase in renewable fuels (1,2). Unlike petroleum, biofuels such as bioethanol and biodiesel are derived from domestic, sustainable, and renewable sources. Advantages of using biofuels include reducing petroleum imports, boosting the rural economy, improving air quality, generating less greenhouse gas emission than non-renewable fuels, and recycling CO₂ from the atmosphere.

To meet the escalating demand for the biofuel, fuel ethanol production from lignocellulosic biomass such as corn stover, wood, and switchgrass is essential. Most of the current ethanol
plants produce ethanol from corn starch via dry or wet milling processes. Grain-based ethanol has limited benefits in terms of total oil displacement and greenhouse gas emissions, owing to the substantial fossil fuel inputs required to grow the grains and convert them to fuel ethanol. Therefore, future directions must move toward conversion of more abundant lignocellulosic material, to meet the increasing demand of fuel ethanol. However, producing fuel ethanol from lignocellulosic feedstock has been challenging owing to the recalcitrant nature of this material. More particularly, ethanol production from lignocellulosic biomass requires a high operating cost to make biomass readily fermentable to ethanol. As a result, additional processing steps are needed to improve hydrolysis efficiency and yield of fermentable sugars from lignocellulosic material.

Pretreatment is a key step of the cellulosic ethanol production. Yields of fermentable sugars from untreated native lignocellulosic materials are low because of the highly packed cellulose structure and the presence of hemicellulose and lignin in the cell wall structure which shields cellulose from enzymatic hydrolysis. To improve ethanol yield and lower ethanol cost, maximal utilization of sugars from lignocellulosics is essential. Pretreatment of lignocellulosics is required to improve hydrolysis efficiency of cellulose by removing hemicellulose and lignin, loosening cellulose structure, and increasing porosity of materials so that they may be accessed by hydrolytic catalysts. As a result, lower amounts of enzyme are required to convert cellulose to glucose, thereby reducing the cost of enzymes in the process.

Among the various pretreatment methods, hydrothermolysis using steam or water has shown to be effective in removing and solubilizing hemicellulose, thus improving the subsequent hydrolysis efficiency. Liquid hot water pretreatment (LHW) of lignocellulosic biomass at a controlled pH effectively dissolves hemicelluloses and some of the lignin while minimizing the formation of monosaccharides and further degradation of sugars to toxic substances during the pretreatment step. Previous studies have shown that the LHW pretreatment of corn fiber at 160°C for 20 min resulted in 50% dissolution of the initial material. Additionally, the loss of dissolved carbohydrates to degradation products was less than 1%.

The pH during the LHW pretreatment is restricted to 4-7 to minimize formation of monomeric sugars and sugar degradation products. Continuous pH monitoring of the pretreatment and addition of a base, such as potassium hydroxide, can help maintain the pH within the specific range of 4-7 during the pretreatment. Previous studies have found that optimum LHW pretreatment temperature for most of lignocellulosic materials ranges between 160-200°C. At 200°C, the pH of pure water itself is nearly 5.0. The pH of LHW
pretreated lignocellulosic biomass is generally within a range of 4-5 without any addition of base or buffer since the biomass itself is often self-buffering (9, 12, 17).

Figures 1 and 2 show the effect of LHW pretreatment on the hydrolysis of corn stover. Without pretreatment, about 15% of cellulose was converted to glucose after 72 h of hydrolysis at the same
enzyme loading (10). Figure 1 demonstrates the effect of cellulase enzyme loadings on the yields of sugars from the hydrolysis of pretreated and washed corn stover at 2% solids loading, while Fig. 2 compares the yields between unwashed and washed pretreated corn stover as well as hydrolysis of pretreated and washed corn stover at low (2%) and high (15%) solids loadings between low and high solids loadings hydrolysis. It should be noted that the corn stover substrates for Figs. 1 and 2 were different, and these differences could add to the variability of results. Substrate variability should be considered when using this method, with biological replicates (i.e., samples of different lots and sources of the cellulotic material) to account for this variability. At the same pretreatment and hydrolysis conditions (pretreatment at 190°C, 15 min, hot water wash upon pretreatment, 2% dry solids loading, and 15 FPU cellulase/g glucan), the corn stover used in Fig. 1 produced higher glucose and xylose than the one used in Fig. 2. About 90% of cellulose and 80% of xylose was converted to its corresponding monomeric sugars by 15 FPU/g glucan cellulase in Fig. 1, while the yields were 86% and 70% in Fig. 2.

The effect of washing the pretreated corn stover is shown in Fig. 2. Without removing the liquid fraction of the pretreated corn stover and subsequent washing of the remaining solids, the glucose yield was only 50%, while it was 86% with filtration and hot water washing of the pretreated corn stover. The direct causes of this phenomenon are still unclear, although it is possible that dissolved phenolic compounds, acetic acid, and hemicellulose-derived sugars may have inhibitory effects on the cellulase enzymes, resulting in a low sugar yield if present (17).

Another important factor that can affect the digestibility of the LHW pretreated material is percent dry solids loading for the hydrolysis. At 15% dry solids loading, only 66% of the total glucan was converted to glucose, which is 20% lower than the yield at 2% solids loading, even though the enzyme/glucan ratio is held constant. A proportionately greater amount of enzyme is added when the solids loading (i.e., concentration of lignocellulose substrate) is increased. Mass transfer and diffusion limitation in the hydrolyzate due to the high level of the dry solids as well as the localized high concentrations of glucose that may exert inhibitory effects on the cellulase enzyme could explain the observations, although further investigation is needed to verify the impact on inhibition and/or mass transfer. These factors are of considerable importance given the goal of reducing enzyme usage, and therefore cost for glucose production.

The purpose of this chapter is to present detailed procedures for laboratory-scale LHW pretreatment of lignocellulosic biomass that was used to produce these results. By following the procedure given in this chapter, a researcher should be able to obtain consistent and reproducible samples.
2. Materials

1. Cellulosic biomass: Biomass for LHW pretreatment can be either wet or dry. If wet (>15% moisture or higher), the material should be stored in a freezer until used. For short-term use, the substrate may be stored in a refrigerator up to a week. The initial substrate can be used as it is or ground to small particles. If the substrate is wet, it has to be dried prior to milling. The drying step is done at 45–50°C to prevent and minimize collapsing of pores in the initial material.

2. Pretreatment reactor: A batch reactor tube is fabricated from 316 stainless steel tubes having a length of 4.5 in., an outside diameter of 1.0 in., wall thickness of 0.065 in., and a measured internal volume of 45 ml (see Note 1). A pair of 1-in. OD Swagelok tube end fittings (Swagelok, Solon, OH) are attached to each end of the reactor tube (see Note 2–4). The standard working volume is 33.7 ml to give about 25% of headspace for liquid expansion during the pretreatment.

3. Pretreatment medium: Pretreatment medium for LHW pretreatment is water. Distilled water is generally used for laboratory-scale experiments.

4. Other equipment: A fluidized sand bath that can hold a 5-in.-long reactor tube is needed. An example of a fluidized sand bath for this purpose is the Techne SBS-4 fluidized sand bath (Cole-Parmer, Vernon Hills, IL). A metal wire (about 15–20 in. long) is used to hold the tube in position inside the sand bath. A bucket of cold tap water and ice is needed to quench the reactor tube after pretreatment.

Prior to pretreatment, the cellulosic biomass has to be measured for moisture content. Moisture is measured by using either a drying oven at 105°C overnight or an infrared moisture analyzer. Measurement of moisture is needed to calculate the amount of water to be mixed with the substrate at a specific percent dry solids loading.

The pretreatment reactor tube has 33.7 ml of working volume. The amount of biomass and water to be loaded is calculated first. It is generally assumed that the biomass has a density of 1 g/ml. Total volume of the biomass and water to be loaded into the reactor tube is 33.7 ml. The dry solids loading can be either on weight/weight or weight/volume basis. The percentage of dry
solids loading (weight of dry solids/liquid volume) per tube is calculated as follows:

\[
\% \text{ dry solids loading (wt/vol)} = \frac{W_B \cdot (1 - M_B)}{W_B \cdot M_B + V_W} \times 100
\]

\(M_B\) = Moisture content of the biomass  
\(V_W\) = Volume of water to be added (ml)  
\(W_B\) = Weight of biomass (g)

First, the tube is loaded with the measured amount of biomass and water. The cap is tightly closed using a wrench. The reactor tube should be left for at least 2 h at room temperature to provide sufficient time for full soaking of the biomass.

### 3.3. Pretreatment

The apparatus for the LHW pretreatment is shown in Fig. 3. During the experiments, a researcher should wear proper laboratory safety equipment (*see Note 5*). Optimum conditions for the pretreatment should be determined beforehand by carrying out a series of runs with different pretreatment temperatures, times, and solids loadings. A sand bath is set at the previously determined pretreatment temperature. First, a metal wire is wound around both the end caps of the reactor tube which is packed with the biomass inside. The metal wire holds the tube in position inside the sand bath and functions as a grip to enable easy insertion and removal of the reactor tube. The batch reactor tube is placed into the sand bath and left for the optimal time period. The reactor tube should be positioned near the thermocouple inside the sand bath (*see Note 6*).
Fig. 4. Heat-up profiles for the reactor tube (1 in. OD × 4.5 in. length) containing corn stover at three different percent solids loadings with a target temperature of 190°C. The corn stover was ground to 40 mesh. Deionized H₂O was used as the pretreatment medium.

The duration of the pretreatment should include a heat-up time for the tube itself. The heat-up time required for a reactor to reach the target temperature is determined by a thermowell with a thermocouple inserted in the tube (see Note 7). The heat-up time should be measured prior to the pretreatment of biomass so that the total duration of the reaction is known. If the optimum pretreatment time is 20 min and the heat-up time is 4 min at the optimum temperature, the total duration of pretreatment in the sand bath is 24 min. **Figure 4** shows heat-up profiles for the 1-in.-OD reactor tube with a target temperature of 190°C, which is given here as an example. Depending on the biomass utilized and concentration of dry solids loading in the reactor, the heat-up time can vary significantly. The heat-up time needs to be measured separately for each condition and cellulosic biomass used.

An additional sand bath may be used to preheat the sample prior to submersing the tube in the main sand bath to carry out pretreatment at the final temperature. In this approach, a sand bath is set to a temperature 20°C above the target temperature. The reactor tube is first immersed in the bath at the higher temperature to partially heat up at a faster rate due to the greater driving force (difference in temperature). After a short time, the reactor tube is quickly transferred to the sand bath at the target temperature. Hence, a faster warm-up time
can be achieved. Many materials undergo little chemical change at temperatures up to 110°C when held at this temperature for less than 15 min.

Make sure that the tube did not leak during the pretreatment (see Note 8). After pretreatment, the tube is quenched by submerging it in water at room temperature for about 1 min and then in ice water until the tube is completely cooled down (see Note 9). The whole contents of the tube (both solids and liquid fractions) can be directly hydrolyzed with the addition of enzymes. The pretreated biomass can be filtered and washed with hot deionized (DI) water (80–90°C) prior to the enzymatic hydrolysis (see Note 10). After each experiment, the tubes are cleaned in an ultrasonic bath with soap to remove all depositions on the tube wall and inside of the end fittings.

4. Notes

1. The reactor tube can be constructed in a smaller scale than suggested above, if the amount of available feedstock is limited.

2. The Swagelok tube-end fittings for the reactor tube should be carefully placed as described by the manufacturer. If the end fittings are not correctly placed, the liquid inside the tube may leak during the pretreatment.

3. It is very important to keep the parts (tube, ferrules, caps) matched with each other. We advise marking the two end fittings differently so that each end fitting is placed back to its corresponding end where it was first screwed in when the tube has to be recapped. Each tube and its two end fittings also need to be inscribed with the same characters so that they are matched every time they are used. This is to prevent leaking of the tube caused by the mismatching of tubes and end fittings.

4. Chance of leaking can be greatly reduced by opening and closing one side of the end fittings when you reuse the reactor tube. The other end remains screwed in place at all times.

5. Wear lab coat, safety goggles, and heat-protective gloves to prevent injury.

6. The tube should be positioned at the same level as the thermocouple of the sand bath. If the tube is too close to the surface, in other words, inserted too shallow, the temperature of the reactor will be lower than the target temperature due to heat loss from the blowing sand on the surface of the sand bath.
7. The tube is loaded with the cellulosic material at a certain percentage of solids loading. One of the end fittings of the tube is replaced with a 1-in.-OD Swagelok end-fitting fitted to a thermocouple which is connected to a handheld thermometer. The tube is submerged in a sand bath to measure the heat-up time. Heat-up time is defined as the duration of time necessary for the material inside the tube to reach 97% of the target temperature.

8. If the tube leaks, you should be able to see wet clusters of sand around the rim between the tube and end fitting. Do not use the pretreated material for further experiments if there has been leakage of the tube during the pretreatment.

9. Upon pretreatment, the tube has to be quenched in a bucket of water at room temperature first, before it is further cooled down in ice water. A sudden temperature change can cause thermal contraction of the tube and, consequently, tube deformation.

10. If you intend to wash the pretreated biomass with hot water before the enzyme hydrolysis, skip the quenching step in ice water. Once the tube is cooled down in water at room temperature, you can open the tube and start the hot-water washing step. Hot-water washing of the pretreated biomass is done by filtering the pretreated biomass from one tube through a Whatman No. 1 filter paper, followed by washing the solids with 200–300 ml of hot DI water (80–90°C). Only the solids recovered from the filtration and hot water washing is further hydrolyzed by cellulase enzymes.

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References


Chapter 8

Dilute Acid and Autohydrolysis Pretreatment

Bin Yang and Charles E. Wyman

Summary

Exposure of cellulosic biomass to temperatures of about 120–210°C can remove most of the hemicellulose and produce cellulose-rich solids from which high glucose yields are possible with cellulase enzymes. Furthermore, the use of dilute sulfuric acid in this pretreatment operation can increase recovery of hemicellulose sugars substantially to about 85–95% of the maximum possible versus only about 65% if no acid is employed. The use of small-diameter tubes makes it possible to employ high solids concentrations similar to those preferred for commercial operations, with rapid heat-up, good temperature control, and accurate closure of material balances. Mixed reactors can be employed to pretreat larger amounts of biomass than possible in such small-diameter tubes, but solids concentrations are limited to about 15% or less to provide uniform temperatures. Pretreatment of large amounts of biomass at high solids concentrations is best carried out using direct steam injection and rapid pressure release, but closure of material balances in such “steam gun” devices is more difficult. Although flow of water alone or containing dilute acid is not practical commercially, such flow-through configurations provide valuable insight into biomass deconstruction kinetics not possible in the batch tubes, mixed reactors, or steam gun systems.

Key words: Dilute acid, Autohydrolysis, Pretreatment, Reactor, Lignocellulosic biomass, Batch, Flowthrough

1. Introduction

Pretreatment refers to the disruption of the naturally recalcitrant structure of lignocellulosic biomass to make cellulose and hemicellulose susceptible to an enzymatic hydrolysis step for generation of fermentable sugars. Over the years, various biological, chemical, and physical pretreatment technologies have been explored as ways to increase sugar yields, and several chemical technologies
show great promise (1). Judging the suitability of pretreatment options must take into account their impact on other steps with respect to such features as the sugar release patterns and solid concentrations for each pretreatment to ensure compatibility with the overall process, feedstock, enzymes, and organisms to be used.

Autohydrolysis occurs when biomass is pretreated with just steam and has been favored because of its long history of development and substantial industrial experience, which includes the use of large-scale equipment such as the batch Masonite gun used in the fiber board industry and the continuous screw fed STAKE II reactor (2–4). However, hemicellulose sugar yields from autohydrolysis are limited to less than about 65% of the maximum possible, while adding dilute sulfuric and other acids can recover up to about 90% of the theoretical maximum. As early as 1898, sulfuric acid was employed to catalyze the hydrolysis of cellulose and hemicellulose in biomass to release sugars, although the costs were too high owing to the high concentration of the acid used and the low sugar yields (5). Further developments lowered the concentration of sulfuric acid required, but a two-step thermochemical process was employed to accommodate the different temperature histories needed to obtain high yields of sugars from both cellulose and hemicellulose (6). More recently, biological catalysis was substituted for the second thermochemical step to enhance glucose yields from cellulose (7), but a pretreatment step was required, with removal of most of the hemicellulose prior to enzymatic hydrolysis of the cellulose in the solid residue being one of the first approaches. Not only did dilute acid achieve higher hemicellulose sugar yields than pretreatment with just water or steam in a batch or co-current flow mode, but it also produced a much higher ratio of monomeric to oligomeric sugars in the liquid (7–16).

Pumping water or dilute sulfuric acid through the solids in a flow-through configuration produced better performance than dilute acid in a batch mode as measured by higher hemicellulose recovery, higher lignin removal, higher glucose recovery from cellulose, and less inhibitors than the conventional system (17–24). However, high water and energy consumption and the difficulty in equipment development impede commercial applications of this method. Thus, the flow-through approach primarily has value for providing time release data that can enhance our understanding of hemicellulose hydrolysis, improve the technical foundation for biomass pretreatment, and lead to innovative, advanced pretreatment technologies. In this chapter, our focus is on the description of laboratory-scale equipment and methods that have been employed successfully for autohydrolysis and dilute acid pretreatments in batch and flow-through operation modes.
2. Materials

1. Batch tubular reactors are 0.5 in OD × 0.035 in. wall thickness stainless steel or Hastelloy C276 tubing (Maine Valve and Fitting Co., Bangor, ME). Reactors are heated by a 4-kW fluidized sand bath (model SBL-2D, Techne Co., Princeton, NJ) and monitored with a thermocouple probe (Omega CASS-18U-12, Omega Engineering Co., Stamford, CT).

2. The mixed batch reactor testing requires a 1-l cylindrical reactor made of Carpenter-20 or Hastelloy C (Parr Instruments, Moline, IL), which is rotated with an adjustable speed DC motor drive (A1750HC, Parr Instruments, Moline, IL).

3. The flow-through reactor parts can be purchased from Maine Valve and Fitting Co., Bangor, ME, or other supply houses to build a small tubular reactor (½ in. ID × 1.84 in. length) with an internal volume of 3.6 ml and a larger tubular reactor (½ in. ID × 6 in. length) with an internal volume of 14.3 ml. Flow-through reactions are monitored with a K-type thermocouple (Extech Instruments, 421501) and a pressure gauge (pressure range 0–1,500 psi, Cole-Parmer Instrument Co., Vernon Hill, IL) and a backpressure regulator (Maine Valve and Fitting Co., Bangor, ME). Temperature is monitored with a 0.25 in. stainless steel thermocouple (Omega CASS-18U-12, Omega Engineering Co., Stamford, CT).

3. Methods

3.1. Tubular Batch Pretreatment Reactors

Tubular pretreatments are generally carried out over a temperature range of approximately 140–180°C when dilute sulfuric acid is used, or from approximately 170 to 220°C when just water is employed. One key for batch pretreatment in tubular reactors is to ensure that the temperature is as uniform as possible across the tube diameter, and thermal analyses have shown that a tube diameter of less than 0.5 in. can meet this requirement (25, 26). Furthermore, because it is difficult to add biomass solids to very small diameter tubes, batch tubular reactors are often made from 0.5 in. OD × 0.035 in. wall thickness stainless steel or Hastelloy C276 tubing for water-only or dilute acid treatment, respectively. As shown in Fig. 1, the tubing is cut to 6 in. or other lengths compatible with the heating system and fitted with Swagelok couplings and removable threaded end caps. Although the total volume is about 14.3 ml, only about 6 ml of wet biomass solids is added to each to provide room for thermal expansion during
heat-up. If the end caps are made from stainless steel to keep costs low, Teflon plugs must be installed at both ends of the tubing to protect them from the acid \((24, 27)\). A second key for small batch tubular reactors is the provision to rapidly heat up and cool down the reactor contents; the extended heat-up times for conventional electrical heaters and prolonged cool down with air do not generally meet this requirement. However, heated fluidized sand baths provide high heat transfer rates with safe fluid, and quenching by submerging in ice water is effective for rapid cool down.

For pretreatment with just water, biomass of the desired moisture content is just loaded into the tubes. For dilute acid systems, the biomass is first soaked overnight in a large volume of water of the targeted dilute acid concentration. Then, excess water is squeezed out until the desired moisture level is obtained prior to adding to the reaction tubes. A thermocouple probe is then inserted \(\frac{3}{16}\) in. and 2 in. deep in the center of the reactor to monitor the pretreatment temperature over the course of the reaction. Based on thermal modeling, a three-bath heat-up procedure \((28)\) is used to minimize the effect of thermal transients in batch tubular reactors. First, the reactor is preheated to 100°C in boiling water for 2 min, and it is then immediately transferred to a sand bath held at 20°C above the target reaction temperature for 1 min. Next, the reactor is moved to a second sand bath controlled at the target reaction temperature at a reaction time defined as zero. After the target time is passed, the reactor is quickly transferred to an ice water bath and held there for 5 min.
to quench the reaction. The tube reactor is taken out from the ice water and dried. The end caps and plugs are then removed, and the pretreated substrate is pushed out with a piston-like metal rod and then filtered to separate the liquid from the solids for analysis (28, 27). Batch tube pretreatments are run at least in duplicate so that the solids and liquid from one of the tubes can be used for analysis of sugar recovery and composition changes of the solid substrate during pretreatment and the pretreated solids from the other tube are used to determine the effect of pretreatment on cellulose digestibility (Note 1).

Pretreatment of much larger amounts of biomass requires that the contents be mixed to maintain uniform temperatures. For example, a 1-l cylindrical reactor made of Carpenter-20 or Hastelloy C can be employed with a 3.5-in.-diameter helical impeller on a two-piece shaft driven by an adjustable speed DC motor drive (29). As shown in Fig. 2, the agitator motor is attached to the reactor, and the combined systems suspended from a chain hoist mounted to a wall crane. Biomass is loaded into the reactor after being presoaked in the target concentration of dilute sulfuric acid solution at 5% solids (w/w) at room temperature for at least 4 h. A K-type thermocouple and a 1/8-in.

Fig. 2. Mixed Parr reactor pretreatment and fluidized sand bath system.
stainless steel thermocouple probe are inserted through a port in the vessel head to monitor the reactor temperature. The reactor is then closed per manufacturer recommendations, and the impeller is set to the target agitation speed. The reactor is lowered into a 320°C sand bath to the bottom of the reactor head flange to rapidly heat up the biomass to the target temperature in about 2 min. As the target temperature is approached, the reactor is raised out of the sand bath until the bottom is about 1–2 cm above the sand surface. The position of the reactor is then adjusted manually to maintain the temperature within ±2°C of the target temperature. After completion of the target reaction time, the vessel is hoisted out of the sand bath and lowered into a 19-l poly bucket filled with water at room temperature to quench the reaction. The pretreated contents are filtered through a Buchner funnel to separate the liquid from the solids. Room-temperature deionized water is then used to wash the solid cake until the filtrate pH is above 6 (typically about 1 l water is needed). The solid and liquid fractions are then analyzed (Fig. 2) (Note 2).

3.3. Flow–Through Tubular Reactors

As discussed above, flow of water through a solid bed provides interesting information about the release patterns of hemicellulose, lignin, cellulose, and other components in the biomass (24, 30). Two small reactor designs have been employed to keep the cost of the high-pressure pump reasonable: a small tubular reactor (½ in. ID × 1.84 in. length) and a larger tubular reactor (½ in. ID × 6 in. length) with an internal volume of 14.3 ml. However, larger units can be constructed if the higher pressure drop can be handled safely and the pump capacity is sufficient. The reactors can be made from Hastelloy C276 tubing for use with dilute acid or from 316 stainless steel for use with just water. Two gasket filters (316 stainless steel, average pore size 5 µm) cover both ends of the reactors. A 316 stainless steel tube (0.25 in. OD × 0.35 in. wall thickness × 50 in. length) is used to preheat water to the desired pretreatment temperature before entering the reactor. Another 316 stainless steel tube (0.25 in. OD × 0.35 in. wall thickness × 50 in. length) is attached to the reactor outlet to cool the effluent.

The flow-through system is illustrated in Fig. 3. A high-pressure pump capable of a flow rate ranging from 0 to 40 ml/min is connected to the flow-through reactor to deliver water. A pressure gauge and a backpressure regulator are used to monitor the pressure to the reactor. A 0.25-in. stainless steel thermocouple monitors the temperature at the outlet of the reactor. Biomass substrate is loaded into the flow-through reactor, which is then connected to the inlet and outlet tubing. Water alone or containing dilute sulfuric acid at room temperature is pumped through the reactor for a few minutes to purge out air until the pressure in the reactor increases to the set pressure of 350–400 psig. Flow is then stopped to completely wet the reactor contents, and the reactor
and preheating coil are submerged in a 4-kW fluidized sand bath at 100°C for 2 min. Next, the reactor and the preheating coil are quickly moved to a second sand bath set at the target reaction temperature, and liquid is pumped through the device at a set flow rate until the end of desired reaction time. The time of transfer to the second sand bath is defined as zero. Upon reaching the desired reaction time, the reactor and preheating coil are transferred to an ice water bath to quench the reaction. The liquid hydrolyzate leaving the reactor is collected for analysis and measurement of its pH. The contents left in the reactor at the completion of the run are transferred to a filter and washed with a sufficient amount of water for its pH to be greater than 6.

Samples of the liquid hydrolyzate are dried at 60°C in a vacuum dryer until weight loss ceased, to determine the solids content of the liquid fraction. The sugars in the liquid hydrolyzate are analyzed using an HPLC equipped with a refractive index detector. The collected solid residue in the reactor is dried at 105°C, weighed, and then used for analysis of Klason lignin, cellulose, hemicellulose, and other fractions. The amount of lignin removal is estimated on the basis of the difference in the Klason lignin content between that in the original biomass substrate and the pretreated solids residue (Note 3).

### 3.4. Steam-Heated Reactors

Solids concentrations of about 50% by weight are important to application to commercial conditions, but maintaining uniform temperature throughout the course of pretreatment is critical for meaningful interpretation of the data. Tubular reactors can handle high solids successfully, but tube diameters needs to be less than about ½ in. to provide uniform temperatures, thereby limiting the amount of biomass than can be readily pretreated. Larger amounts of material can be produced in mixed reactors, but the solids content must be less than about 10% by weight so mixing is adequate to ensure uniform temperatures. One of the best ways found to process larger amounts of biomass...
at high solids concentrations while controlling temperatures is through direct steam heating. Steam heating can provide very rapid heat-up to reaction temperatures of typically between 140 and 260°C (37–260 psig), and the temperature can be controlled well by appropriate selection of the steam pressure. Furthermore, rapid release of the pressure at the completion of the run almost instantly drops the temperature to 100°C, terminating the reaction. The term “steam gun,” as frequently used to describe this approach, arises from the rapid pressure release when the contents are discharged into a receiver vessel. Residence times can span a range from less than 1 min to 4 h or more.

A number of steam gun reactors have been used to conduct larger batch steam explosion or steam expansion pretreatment with catalysts (e.g., dilute acid, SO₂) or without them (31–34). We recently installed the steam gun, shown in Fig. 4, in our

Fig. 4. Steam gun pretreatment reactor system.
laboratory powered by an electric boiler that is rated at 75 kW and can generate up to 800 psig. The steam gun is a cylinder of 4 in. nominal inside diameter and 24 in. length and is constructed of Hastelloy C to handle dilute sulfuric acid if needed. The bottom portion of the unit tapers down gradually to a ¾ in. discharge line so that steam will be distributed evenly throughout the biomass and dead spaces will not be formed in corners at the bottom. A discharge valve is attached to the bottom as close to the reactor body as possible to minimize accumulation of biomass in the dead space above the valve and can be actuated remotely by an electrical device. A ½-in. steam injection line enters the side of the discharge line above and as close to the discharge valve as possible to minimize the dead volume between the steam entrance position and the top of the valve. The vessel is jacketed along its entire length including the bottom of the reactor so that steam can be applied to the internal vessel walls through the jacket, which minimizes heat losses that result in excessive condensation and dilution of the sugars released. The outside of the vessel is heavily insulated to minimize heat losses, but is removable for maintenance. A valve is attached to the port on the top of the steam gun to allow biomass to be readily added to the vessel, and a thermocouple is run through the top into the middle of the interior. One of the bigger challenges with the steam gun is accurate material balance closure because of the loss of steam during discharge, the hang up of some biomass inside the vessel, and difficulties in weighing the discharged material. However, we developed an approach using a drum ring to hold a heavy-gauge polyethylene bag to a drum top attached below the bottom valve to collect material upon discharge and facilitate rapidly closing material balances.

The steam gun above can process about 3–5 lb of dry biomass at a time. Prior to pretreatment, the feedstock is preweighed in batches of 200–350 g dry in plastic containers or bags, and presoaked/preimpregnated with just water or with a catalyst solution (e.g., dilute acid or SO₂) overnight. The steam boiler is turned on following standard procedures, the pressure is set at the target pressure for steam explosion, and the insulated and heat-traced vessel is preheated with steam in the jacket for 2–4 min. to ensure that it is at the target temperature. The preimpregnated feedstock is then loaded into the reactor from the top, and the reactor is sealed. Fresh steam is introduced into the bottom of the vessel, raising the inside temperature to the target value in about 15 seconds. The boiler pressure is usually set at about 10–15 psi above the operating pressure for the steam gun but below the maximum pressure of the relief valve. The steam inlet valve is closed about 10 seconds before the end of the operating time,
the discharge valve at the bottom of the steam gun is opened, and the contents are blown into the collecting bag to rapidly bring the temperature to below 100°C and quench the reaction. The discharge valve is opened at least twice to ensure that the vessel is fully depressurized and empty. The reaction time is defined to be from the moment steam is introduced into the reactor until the contents are discharged. The pretreated material is collected, filtered, and washed with water, and the hydrolyzate is sampled for further analysis. The solids are stored at −20°C (Note 4).

3.5. Conclusions

Dilute acid pretreatment is one of the leading pretreatment techniques because of the high yields possible in the coupled operations of pretreatment and enzymatic hydrolysis, while autohydrolysis is often favored because of its long use and ease of application. However, the ability to design these systems in conjunction with enzymatic hydrolysis a priori is limited, and detailed material balance data is vital for the assessment and commercialization of this technique.

4. Notes

1. The small-batch tube reactors provide a low-cost, lab-scale option to optimize pretreatment conditions at high solids loading (up to 30%), and several reactor simulation models have been developed on the basis of mass balance results from tube reactors (35, 25).

2. A mixed reactor can handle larger amounts of material than tube reactors, but handling high solids concentrations (>10%) is challenging for this type of device (29).

3. We have successfully employed a flow-through reactor configuration to provide valuable data on the kinetics of biomass deconstruction (36, 37) and to test the applicability of rate laws. However, although high sugar yields and high lignin removal can be achieved with flow-through systems (31, 24), no one has yet found a way to employ them commercially.

4. The steam gun reactor can be operated at the high solids concentrations desired commercially, process large amounts of biomass, heat up biomass rapidly, control temperature well, and cool the products quickly. However, these systems are more expensive and present bigger challenges to closing material balances as accurately.
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Chapter 9

Lime Pretreatment

Rocio Sierra, Cesar Benigno Granda, and Mark T. Holtzapple

Summary

Lime pretreatment has proven to be a useful method for selectively reducing the lignin content of lignocellulosic biomass without significant loss in carbohydrates, thus realizing an important increase in biodigestibility. In lime pretreatment, the biomass is pretreated with calcium hydroxide and water under different conditions of temperature and pressure. It can be accomplished in one of three fashions: (1) short-term pretreatment that lasts up to 6 h, requires temperatures of 100–160°C, and can be applied with or without oxygen (pressure ~200 psig); (2) long-term pretreatment taking up to 8 weeks, requiring only 55–65°C, and capable of running with or without air (atmospheric pressure); and (3) simple pretreatment requiring 1 h in boiling water, without air or oxygen. Nonoxidative conditions are effective at low lignin contents (below ~18% lignin), whereas oxidative conditions are required for high lignin contents (above ~18% lignin).

Key words: Lime, Pretreatment, Biomass, Lignocellulose, Oxidative, Alternative fuels

1. Introduction

Because of its high carbohydrate content and abundance, lignocellulosic biomass is an excellent biochemical feedstock; however, it resists biodegradation for the following reasons: high lignin content, high crystallinity, high degree of polymerization, low surface area/pore volume, and presence of acetyl groups on hemicellulose (1–5). To overcome this recalcitrance, pretreatment is required.

Currently available pretreatment methods are biological, chemical (cellulose solvents, acids, or bases), or physical (mechanical size reduction, comminution, steam explosion, vibratory ball milling, compression milling, and hydrothermalysis). Lime pretreatment is a chemical method, and like other alkaline treatments (e.g., paper...
pulping), the major effect is lignin removal (6–11). In addition, acetyl groups are removed, which enhances digestibility (5) and removes an important inhibitory component from ethanol fermentations. Lime pretreatment offers three important advantages over other pretreatments: (1) it is not severe, preserving most of the cellulose and hemicellulose; (2) it is inexpensive, allowing its application in the production of nonrenewable fuels and chemicals; and (3) it is safe to handle (12, 13).

Lime pretreatment involves mixing the biomass with calcium hydroxide (or calcium oxide) and water at temperatures between 25 and 200°C, with a pretreatment time ranging from hours to weeks. Under nonoxidative conditions, lime pretreatment removes approximately a third of the lignin. A higher lignin removal may be accomplished in oxidative conditions using oxygen or oxygen-containing gases at pressures ranging from atmospheric to 300 psig.

In lime pretreatment, the effects of temperature, time, and pressure are interdependent. Gentle conditions (temperatures from 25 to 65°C, and atmospheric pressure) require substantially more time than more severe conditions (i.e., temperatures from 100 to 200°C and pressures from 100 to 300 psig). The initial lignin content of the biomass determines the conditions of pretreatment, which are as follows: for woody biomass (lignin greater than ~23%), severe conditions (e.g., 150°C, 200 psig oxygen, 6 h) are required; for herbaceous biomass (lignin less than ~18%), less severe conditions (e.g., 100°C, 1 h, no oxygen or 55°C, 4 weeks, no oxygen) are sufficient; for biomasses with lignin contents between ~18 and ~23%, moderately severe conditions (e.g., 65°C, 4 weeks, air at atmospheric pressure) are required. In any case, if after pretreatment the lignin content of the biomass is ~12% or less, a significant increase in digestibility is expected (5, 10). Accordingly, lime pretreatment has been divided into three categories: long-term pretreatment (1–8 weeks), short-term pretreatment (1–24 h), and simple lime pretreatment (1 h in boiling water).

Although lime pretreatment is fairly selective at removing lignin, a variety of carbohydrate degradation reactions occur. Some products of carbohydrate degradation under oxidative conditions are glucoisosaccharinic and xylosaccharinic acids. At more severe conditions (higher temperature, alkali concentration, and pressure), glycolic and lactic acids are also formed (14–18).

It has been experimentally observed that carbohydrates are better preserved during short-term lime pretreatment. It is also observed that cellulose is more stable than hemicellulose. Possible explanations follow: (1) The crystalline nature of cellulose protects it from degradation whereas the amorphous nature of hemicellulose makes it more susceptible; (2) Hemicellulose is covalently linked to lignin, so when lignin is degraded it takes
hemicellulose with it. Some literature sources suggest that carbon dioxide resulting from carbohydrate degradation reacts with calcium hydroxide to form calcium carbonate protective layers which prevent further carbohydrate degradation (10).

Alkaline delignification occurs in three different simultaneous phases: initial, bulk, and residual (19, 20). Each phase has been shown to have first-order kinetics. Consequently, the model for delignification is expressed as follows:

$$W_L = a_1 e^{-k_1t} + a_2 e^{-k_2t} + a_3 e^{-k_3t}$$

where $W_L$ is the fraction of the residual lignin (gram of lignin remaining/gram of lignin in raw biomass); $a_1$ is the maximum fraction of lignin fragments released in the initial stage; $a_2$ is the maximum fraction of lignin fragments released in the bulk stage; $a_3$ is the maximum fraction of lignin fragments released in the residual stage; and $k_1$, $k_2$, $k_3$ are the reaction rate constants for the initial, bulk, and residual delignification stages, respectively.

The parameters in this equation have been estimated for long-term pretreatment using corn stover (21) and bagasse (10).

As a result of lignin removal and carbohydrate degradation during pretreatment, the biomass composition changes. Figure 1 shows the compositional analysis of poplar wood during long-term pretreatment. A rapid decrease of the lignin content is observed with good preservation of glucan and xylan (i.e., carbohydrates) up to the sixth week of pretreatment. After that, the lignin content continues to decrease but with more significant carbohydrate degradation. In the case of short-term pretreatment, the lignin content decreases proportionally with pretreatment time and pressure in the reactor (Fig. 2). Regardless of the pretreatment method, the lowest lignin content does not necessarily result in
the highest overall yield of glucan (i.e., glucan recovered after pretreatment and enzymatic hydrolysis). Aggressive conditions that remove lignin also degrade carbohydrates; thus, for any given biomass, experimentation is required to obtain the right balance between lignin removal and carbohydrate preservation. An important result of short-term lime pretreatment of poplar wood is glucan recovery of about 93% obtained for 6-h pretreatment at 200 psig and 150°C (Fig. 3).

Fig. 2. Lignin remaining after oxidative short-term lime pretreatment of poplar wood at 150°C.

Fig. 3. Improvement in digestibility of poplar wood obtained for short-term lime pretreatment. Reactor initially pressurized with oxygen at 200 psig. Hydrolysis conditions: 15 FPU cellulose/g dry biomass.
2. Materials

2.1. Reactants

1. Calcium hydroxide, reagent American Chemical Society (ACS) Calcium hydroxide should be stored in a well-closed container to avoid contact with carbon dioxide in air. Alternatively, calcium oxide may be used.

2. Oxygen-containing gases (compressed air or pressurized oxygen).


2.2. Apparatus

1. Chipper, grinder, chopper, shredder, or the like.

2. Reaction chamber.

3. Tubing for oxygen or air supply.

3. Method

3.1. Oxidative Long-Term Pretreatment

The procedure below describes the use of oxidative conditions. In the case of nonoxidative conditions, omit the steps related to air.

1. Reduce particle size of the biomass to be pretreated (in some cases, this step may be omitted; see Notes 1 and 2).

2. Determine the biomass moisture content after grinding.

3. Determine the desired loading of calcium hydroxide. In general, the longer the pretreatment and/or the acidic content of the biomass, the more calcium hydroxide is required. Usually, the lime consumption is 0.1–0.2 g Ca(OH)₂/g dry biomass (see Note 3).

4. Determine the desired loading of water. It must be provided in sufficient quantity to make the mixture slurry-like. Roughly 10 g water/g dry biomass is the minimum, but this quantity must be adjusted in case of very dry and hygroscopic biomass (see Note 4).

5. Provide appropriate conditioning to the air used in the pretreatment. This is accomplished by scrubbing carbon dioxide from air and saturating it at the pretreatment temperature. To scrub the air of carbon dioxide, pass it through a lime–water slurry in a bottle. To saturate the air, pass it through a column of water that is at the pretreatment temperature (see Notes 5 and 6).

6. Select the appropriate reaction chamber. Any metal, glass, or plastic vessel that can stand high alkalinity and mild temperature for a long period of time can be used as a reaction chamber for long-term lime pretreatment (see Notes 7 and 8).
7. Provide a system for supplying air, for example, use a rigid hose (1/4-in. inner diameter) with the opening located at the bottom of the reactor. A diffusion stone may be used to ensure best distribution of air in the reactor (see Note 9).

8. Confirm that the reactor is at the pretreatment temperature (the best results have been observed for temperature ranging between 55 and 65°C) and the air supply is operating properly.

9. Thoroughly mix the appropriate amounts of calcium hydroxide, water, and biomass, making a homogeneous mixture.

10. Check the pH of the mixture. If the pH is not 11 or higher, add more lime in proportion to the amount of dry biomass being pretreated.

11. Transfer the mixture into the reaction chamber and ensure the air is flowing properly.

12. Check the pretreatment apparatus and pH on a daily basis. The pH must be maintained at 11 or more during pretreatment. Refill water and add more lime if necessary. The pH in the water–lime slurry bottle for CO₂ scrubbing must also be ~11. If it is not, more lime must be added.

13. To determine when to stop the pretreatment, it is advisable to measure the lignin content of the biomass. If it is 12% or less, the pretreatment may be stopped. The estimated time of pretreatment ranges between 1 and 2 months. When the pretreatment time has elapsed, terminate it by cooling down and transferring the contents of the reaction chamber into a separate container (see Notes 10 and 11).

3.2. Oxidative Short-Term Pretreatment

Figure 4 shows a schematic representation of oxidative long-term pretreatment as it would be practiced in industry. The procedure below describes the use of oxidative conditions. In the case of non-oxidative conditions, oxygen can be replaced with nitrogen.

1. Follow steps 1–4 as in long-term pretreatment; however, in this case, it is important to ensure that the volume of the

Fig. 4. Cross sectional view of a pretreatment pile.
mixture takes no more than 50% of the available volume inside the reactor.

2. Use gaseous oxygen. No conditioning of the oxygen is necessary. Use a regulator to control pressure.

3. Short-term pretreatment must be run in a hermetic stainless steel reactor of which the wall thickness is adequate to hold the operating pressure.

4. Select the temperature and pressure of pretreatment, and heat up an oven to the desired temperature. For poplar wood, temperatures between 140 and 160°C and pressures between 150 and 250 psig have given good results.

5. Thoroughly mix the appropriate amounts of calcium hydroxide, water, and biomass making a homogeneous mixture, place it in the reactor, and close it hermetically.

6. The pressure in the reactor can be maintained using tubing that connects the oxygen tank and the reactor. An example of the experimental setup is presented in Fig. 5 (see Note 12).

7. The reactors can be preheated to 100°C for 15 min using boiling water. Then place them in the preheated oven.

8. It is important to maintain good mixing at all times. This can be attained using a shaking mechanism (e.g., a swinging arm to which the reactor is attached, slow rotary motion, etc.)

9. When the pretreatment time has elapsed (usually 6 h), close the oxygen valve, remove the reactor from the oven, and cool it down in cold water. Release the pressure before opening. Transfer the contents to another container (see Note 13).
Herbaceous biomass with low lignin content (less than about 18%) can be pretreated using the following simple method:

1. Add biomass, lime (0.1–0.2 g Ca(OH)$_2$/g dry biomass), and water (5–15 g H$_2$O/g dry biomass) to a beaker or pan.
2. Heat to 100°C with a Bunsen burner or hot plate.
3. Periodically stir by hand with a spatula.
4. Add water as necessary to compensate for evaporation.
5. Terminate by cooling down after 1 h of pretreatment.

After lime pretreatment, it may be desirable to neutralize excess lime. The following alternative methods have proven useful:

1. Using good mixing, add dilute acetic or hydrochloric acid to reach the desired pH. In both cases, the neutralized lime forms a soluble product, which can be removed by washing (see Note 14).
2. Using good mixing, add dilute sulfuric acid to reach the desired pH. The reaction product is calcium sulfate, which is not soluble. The resulting precipitate blocks access to lime, which slows the neutralization process. To achieve complete neutralization, it is necessary to add sulfuric acid over an extended time period (see Note 14).
3. Using good mixing and a diffusing stone, bubble carbon dioxide into the slurry to reach the desired pH. Insoluble calcium carbonate or bicarbonate is produced, depending upon the pH. As described in Option 2 above, the resulting precipitate blocks access to lime, so an extended neutralization period is required.

1. Good results have been observed on sugarcane bagasse obtained from the sugar mill (e.g., bagasse fibers of 1.5 cm or less). However, this does not necessarily apply to other types of biomass. In general, the particle size for herbaceous biomass may be ½ in. or less, whereas for woody biomass it is necessary to reduce the particle size to ½ cm or less.
2. If the biomass is high in moisture content, it might be difficult to grind. In this case, it is necessary to air-dry the biomass before grinding.
3. To determine how much lime is needed, in a preliminary experiment add excess lime (e.g., 0.5 g Ca(OH)$_2$/g dry biomass). After the pretreatment is completed, back-titrate with HCl to determine the excess lime. Once the actual lime consumption is known, future experiments can be
run by eliminating the excess lime and adding only what is required.

4. Water will provide a medium in which the lime can be dispersed and will allow uniform temperature due to its good heat transfer coefficient. Excessive water will have no effect on the pretreatment, but its presence will demand more energy.

5. Scrubbing carbon dioxide from air is necessary to avoid consumption of the lime in an undesirable reaction. Saturating the air at the pretreatment temperature will prevent consumption of water in the reactor by the air.

6. It is highly recommended to bubble air in the reactor to provide oxygen, but as stated in the introduction, some improvement in digestibility has also been observed without air, particularly for low-lignin biomass (e.g., lignin content lower than ~18%).

7. Some advisable reactors for laboratory-scale follow: Erlenmeyer flasks, beakers, centrifuge bottles, or the like. Reactors made of polyvinylchloride (PVC) pipe have also been used in our laboratory.

8. In case of large-scale pretreatment (pilot plant or larger), the mixture of biomass and calcium hydroxide can be formed into a pile of convenient dimensions, located on top of a gravel bed through which air is blown as illustrated in Fig. 2. Liquid is recirculated as it trickles through from the gravel bed to the top of the pile.

9. The flow rate of air may be very low because the oxygen consumption is very small, but implementing a high flow rate can provide extra mixing in the reactor.

10. Lignin contents described herein are based upon NREL standard procedure “Determination of Structural Carbohydrates and Lignin in Biomass (2004)”.

11. Running pretreatment for more than 2 months may lead to a significant degradation of carbohydrates; therefore, if after two months the lignin content has not reached the target of 12% or less, it may be better to try the more severe conditions of short-term pretreatment. Short-term lime pretreatment will degrade lignin more selectively.

12. Alternatively, the reactor can be pressurized with a single charge of oxygen at the beginning of the pretreatment. In this case, the pressure will be dynamic and initially increase because of the formation of steam, and later decline as oxygen is consumed. It is essential that the reactor be hermetically closed; otherwise an undesirable loss in pressure may occur.

13. Pretreatment on herbaceous biomass may take less than 6 h; the time in this case may vary between 2 and 4 h depending on the initial lignin content.
14. Use ~5% acid. If overly concentrated, some acids (e.g., sulfuric) can “burn” the biomass, so add with care. Very dilute acid can be used if the water concentration in the slurry is not important.

References


Chapter 10

Analytical Characterization of Fermentation Inhibitors in Biomass Pretreatment Samples Using Liquid Chromatography, UV-Visible Spectroscopy, and Tandem Mass Spectrometry

Lekh N. Sharma, Christopher Becker, and C. Kevin Chambliss

Summary

A variety of chemicals are produced upon pretreatment of lignocellulosic biomass. Aliphatic acids, aromatic acids, aldehydes, and phenolic compounds are of particular interest due to their presumed inhibitory influence on downstream enzymatic or microbial steps in biomass-to-ethanol conversion. Herein, we describe a series of analytical protocols that collectively enable quantitative monitoring of 40 potential fermentation inhibitors in biomass pretreatment samples. Solid samples are accommodated by first employing pressurized fluid extraction to generate an aqueous “wash stream.” Sample preparation for liquids involves an initial precipitation-filtration step, followed by liquid–liquid extraction and reconstitution of extracts in water. Samples are analyzed using high–performance liquid chromatography (HPLC) in combination with ultraviolet (UV) absorbance and tandem mass spectrometry (MS/MS) detection. A standard addition approach is utilized for quantitation to alleviate complications arising from co-extracted sample matrix.

Key words: Biomass, Analysis, Fermentation inhibitors, Pretreatment, Hydrolysate, Organic acids, Lignocellulose, Degradation products, HPLC, LC–MS, MS/MS

1. Introduction

Minimizing the production and downstream effects of inhibitory compounds has been cited as one of the key roadblocks currently impeding process efficiency in biomass-to-ethanol conversion (1–3). The majority of available process configurations for converting lignocellulosic biomass into ethanol involve three sequential steps: (1) pretreatment, (2) enzyme hydrolysis, and (3) fermentation.
The intended outcomes of pretreatment are dissolution of sugars in monomeric or oligomeric form and improved accessibility of enzymes to carbohydrate oligomers, most notably to cellulose. However, a variety of alternative chemicals also result from pretreatment of biomass, many of which are inhibitory to downstream enzymatic and/or microbial steps in the process (4–8).

Two approaches are recognized for mitigating the deleterious influence of inhibitors. The most studied of the two involves inhibitor removal prior to enzyme hydrolysis. A variety of methods have been investigated for inhibitor removal, including over-liming (9–15), ion exchange (9, 16, 17), treatment with polymeric sorbents (18), treatment with enzymes (9), and treatment with microorganisms (19). An alternative strategy relies on the identification of pretreatment conditions that minimize the production of inhibitors (20). To date, assessments of the effect of each approach have been largely based on empirical observations of improved fermentability relative to a reference condition (21–23). While this approach provides guidance in terms of process optimization, it does not enable one to confirm which constituents of the pretreatment samples are responsible for observed inhibitory effects. Accordingly, the availability of molecular-level compositional information on pretreatment samples is paramount to improved fundamental understanding of biomass pretreatment and downstream inhibition processes.

This chapter describes an analytical method for monitoring 40 lignocellulosic degradation products, representing a variety of confirmed and potential fermentation inhibitors in biomass pretreatment samples. The rationale for selection of target analytes has been presented elsewhere (24). For purposes of the present discussion, it is important to note that these compounds are derived from the three primary biopolymers that make up the composition of lignocellulosic materials (i.e., cellulose, hemicellulose, and lignin), are structurally diverse, and collectively represent the full range of physicochemical properties that may be expected for inhibitory degradation products derived from pretreatment of biomass. The method is applicable to both solid and liquid pretreatment samples, as well as pretreatment liquids that have been treated by one or more technologies noted above that result in inhibitor removal.

The analytical protocol is summarized in Fig. 1. Although the most common sample type resulting from pretreatment of biomass is liquid hydrolysate, some pretreatments (e.g., ammonia fiber expansion; AFEX) result in solid samples. The general approach for analysis of hydrolysates involves an initial precipitation–filtration step, followed by liquid–liquid extraction with methyl tertiary-butyl ether (MTBE) and subsequent high-performance liquid chromatography (HPLC) analysis with dual UV spectroscopic and tandem mass spectrometric (MS/MS)
detection (i.e., HPLC-UV-MS/MS analysis). Solid samples require the additional generation of an aqueous “wash stream” via pressurized fluid extraction prior to precipitation–filtration but are otherwise accommodated using the same approach as that employed for liquids. Although the method is presented with specific reference to 40 target compounds, the approach is also expected to be applicable to alternative analytes, provided that a suitable MS/MS transition and/or UV signature can be identified.

2. Materials

2.1. Chemicals, Reagents, and Miscellaneous Supplies

1. Distilled water, purified and deionized to 18 MΩ with a Barnstead Nanopure Diamond UV water purification system (or equivalent water polishing unit).

2. The following reagent-grade chemicals from commercial vendors: methyl tertiary-butyl ether (MTBE), sulfuric acid, formic acid, and ammonium bicarbonate (NH₄HCO₃).

3. The target analytes listed in Table 2 (excepting internal and surrogate standards) in highest available purity from Sigma-Aldrich (St. Louis, MO).
4. The internal standards benzoic acid-$d_5$ (i.e., benzoic acid with the five aromatic hydrogen atoms replaced by deuterium) and *para*-tertiary-butylphenoxyacetic acid in highest available purity from Alfa Aesar (Ward Hill, MA).

5. The surrogate standard 3,5-dihydroxybenzoic acid in highest available purity from Sigma-Aldrich.

6. HPLC solvent A: 0.025% (v/v) formic acid in water.

7. HPLC solvent B: 90% (v/v) acetonitrile, HPLC far UV grade (Acros, Fair Lawn, NJ) and 10% water, containing 0.025% (v/v) formic acid.

8. Glass-fiber filters for use with 22-ml extraction cells in pressurized fluid extraction (Dionex, Sunnyvale, CA; Part no. 047017).

9. Pall IC Acrodisc hydrophilic polyethersulfone Supor membrane syringe filters: 25 mm diameter; 0.45 μm pore size (VWR Scientific, Fair Lawn, NJ).

### 2.2. HPLC Columns

1. 1 mm reverse phase (RP) C18 OPTI-Guard column (Alltech, Deerfield, IL).

2. 150 mm × 4.6 mm (S 03 μm, 99) YMC Carotenoid column (Waters, Milford, MA).

### 2.3. Equipment

1. TurboVap LV Concentration Workstation (Caliper Life Sciences, Hopkinton, MA, USA) or equivalent device capable of evaporating solvents under a gentle stream of nitrogen gas at controlled temperature.

2. Accelerated solvent extractor ASE-200 (Dionex, Sunnyvale, CA), equipped with 22-ml stainless steel extraction cells.

3. HPLC-UV-MS/MS instrumentation. In the present study, employed instrumentation was obtained from Varian, Inc. (Palo Alto, CA) and consisted of a ProStar model 210 HPLC binary pumping system equipped with a high-pressure mixing chamber. Automated sample injection was facilitated using a model 410 autosampler equipped with a standard six-port valve (50 μl sample loop). This autosampler also provided a thermostated column oven, which housed the specified guard and analytical columns (items 1 and 2, respectively, in Subheading 2.2 above) connected in series. The HPLC system was connected to a ProStar model 335 UV-visible photodiode array (PDA) detector, and the PDA was connected in series to a model 1200L triple-quadrupole mass spectrometer equipped with an atmospheric pressure electrospray ionization (ESI) source. A flow splitter was inserted between the PDA and the mass analyzer to divert 50% of the liquid exiting the PDA detector to waste. The remaining 50% was directed to the mass spectrometer. A schematic depicting the general
instrumental configuration is provided in Fig. 2. Note that instrumentation from alternative vendors may be substituted provided that it offers equivalent capability.

### 3. Methods

#### 3.1. Preparation of Standard Solutions

1. **Internal standard mixture** – Dissolve appropriate masses of benzoic acid-$d_6$ and $p$-t-butylphenoxycetic acid (weighed to the nearest 0.1 mg) in methanol to provide a concentration of 400 and 1,000 µg/ml, respectively, when diluted to volume.

2. **Surrogate standard mixture** – Dissolve an appropriate amount of 3,5-dihydroxybenzoic acid (weighed to the nearest 0.1 mg) in methanol to provide a 400 µg/ml solution when diluted to volume (see Note 1).

3. **Stock mixture (400 mg/l) of target analytes** – Combine appropriate masses of each target analyte listed in Table 2 (weighed to the nearest 0.1 mg) in a glass beaker and dissolve in a minimal volume of methanol. Transfer the mixture to an appropriately sized volumetric flask, and dilute to the mark with methanol.

4. **Stock mixture (100 mg/l) of target analytes** – Pipette 25 ml of 400 mg/l stock mixture into a 50-ml beaker, and evaporate
the solvent under a gentle stream of nitrogen gas. Reconstitute the residue in a minimal volume of water, transfer the aqueous solution to a 100-ml volumetric flask, and dilute to the mark with water.

5. Calibration standards – Prepare a minimum of ten calibration standards spanning the concentration range 0.01–15 mg/l via serial dilution of the 100 mg/l stock mixture with water. Standard concentrations typically prepared in our lab are as follows: 0.01, 0.03, 0.05, 0.07, 0.1, 0.5, 1, 4, 7, 10, and 15 mg/l. Before diluting to a final volume ≥10 ml, acidify each solution by adding one drop of concentrated sulfuric acid. Prior to analysis, carry each calibration standard through the sample preparation protocol described in Subheading 3.3 below (beginning with step 4).

3.2. Analytical Sample Preparation for Solid Samples

1. Place and seat (with gentle tamping) one circular glass-fiber filter into the bottom of a 22-ml stainless steel extraction cell.

2. Fill a tared extraction cell with pretreated biomass and record the weight to the nearest 0.1 mg (see Note 2). Screw the end caps tightly to the cell (see Note 3), and place the cell into the ASE apparatus with the end containing the filter pointed down.

3. Extract the sample using the following instrumental parameters: pressure, 1,500 psi; oven temperature, 70°C; preheat time, 3 min; heat time, 5 min (software default); static time, 10 min; static cycles, 2; flush volume, 150%; purge time, 60 s (see Note 4).

4. Allow the extraction tube and collection vial to cool to room temperature. Once cooled, transfer the liquid extract to a 50-ml volumetric flask and dilute to volume with water (see Note 5).

5. Proceed according to Subheading 3.3 below.

3.3. Analytical Sample Preparation for Liquid Samples

1. Adjust the pH of a 30–50 ml aliquot of liquid hydrolysate (or aqueous “wash stream”) to 7–8 by adding solid NH₄HCO₃ (with stirring), and store the sample at 4°C for 30 min. After this period, a brown precipitate will likely be visible (see Note 6).

2. Transfer the mixture to an appropriately sized plastic syringe, and remove the precipitate by filtration using the filter discs described in Subheading 2.1 above (item 9).

3. Adjust the pH of the filtrate to 1–2 by dropwise addition of concentrated sulfuric acid.

4. Transfer a 5-ml aliquot of acidified, aqueous filtrate to a 50-ml centrifuge tube, and add 25 µl surrogate standard mixture and 45 ml MTBE.

5. Equilibrate the phases for 15 min by gentle end-over-end inversion on a rotating wheel (30 rpm) at 25.0 ± 1°C (see Note 7).
6. Centrifuge the biphasic mixture at $3400 \times g$ rpm for 3 min to ensure complete phase disengagement, and transfer the MTBE phase to one or more glass test tubes, depending on the particular apparatus used for solvent evaporation (see Note 8).

7. Add an additional 45 ml MTBE to the residual aqueous phase from the previous step and repeat steps 5 and 6.

8. Place the test tube(s) containing the combined MTBE extracts into the TurboVap and reduce the volume of MTBE to 1–2 ml under a stream of nitrogen at 55°C.

9. Add 1.5 ml water to the test tube, and evaporate the remaining MTBE under a stream of nitrogen at 55°C.

10. Transfer the aqueous mixture to a 5-ml volumetric flask, add a drop of concentrated sulfuric acid, and dilute to volume with water.

11. Transfer a 1-ml aliquot of the aqueous solution from step 10 above to a labeled 2-ml autosampler vial, add 25 µl of internal standard mixture, cap the vial, and place it in the autosampler tray of HPLC-UV-MS/MS instrumentation (see Note 5).

12. The composition of pretreatment samples is widely variable, and concentrations of target analytes typically span several orders of magnitude in a single sample. Accordingly, it is advised that 10-fold and 100-fold dilutions of the solution from step 10 also be prepared.

13. Transfer the diluted samples to labeled autosampler vials using the procedure described in step 11 above.

3.4. HPLC-UV-MS/MS Analysis

With the exception of quantitation protocols, the discussion presented in this section assumes that the reader is reasonably experienced with HPLC-UV-MS/MS analyses. It is unlikely that an inexperienced analyst would have success in operating the noted equipment on the first try. For this reason, a general description of the procedure employed in our laboratory is presented rather than a stepwise protocol. It is expected that the description will provide a seasoned analyst with all necessary information to facilitate analysis of pretreatment samples using their equipment. Most research and production facilities, academic and industrial alike, maintain personnel who routinely operate analytical instrumentation and facilitate sample analysis for others. Alternatively, a number of private sector laboratories provide analytical services on a contract basis.

In our laboratory, analytes are separated using the noted RP analytical column and the nonlinear solvent gradient defined in Table 1 (see Note 9). Additional chromatographic parameters are as follows: injection volume, 50 µl; column temperature 30°C; flow rate, 750 µl/min. The photodiode array UV-visible detector
Table 1
HPLC gradient elution profile

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile phase composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A solvent(^b)(%)</td>
</tr>
<tr>
<td>0:00</td>
<td>100</td>
</tr>
<tr>
<td>1:40</td>
<td>100</td>
</tr>
<tr>
<td>15:00</td>
<td>93</td>
</tr>
<tr>
<td>20:00</td>
<td>92</td>
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<td>33:00</td>
<td>90</td>
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<td>45:00</td>
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<td>50:00</td>
<td>71</td>
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<tr>
<td>53:00</td>
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<td>60:00</td>
<td>50</td>
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<tr>
<td>63:00</td>
<td>0</td>
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<tr>
<td>80:06</td>
<td>100</td>
</tr>
<tr>
<td>110:00</td>
<td>100</td>
</tr>
</tbody>
</table>

\(^a\)Gradient optimized at a flow rate of 750 µl/min using columns and instrumentation specified in Subheading 2.2 and 2.3. The dwell volume of utilized HPLC instrumentation was 2.6 ml

\(^b\)0.025% (v/v) aqueous formic acid

\(^c\)90:10 mixture of acetonitrile and water, containing 0.025% (v/v) formic acid is set to monitor absorbance over the range 200–400 nm, and analytes are introduced into the mass spectrometer via negative electrospray ionization (−ESI). Representative chromatograms resulting from HPLC-UV-MS/MS analysis of a calibration standard are shown in Fig. 3. It is important to emphasize that the MS/MS data in Fig. 3 depict a total ion chromatogram (TIC), representing the summative MS response. However, the mass spectrometer is operated in multiple reaction monitoring (MRM) mode, and chromatographic run time is divided into eight segments in which only select MS/MS transitions are monitored (see Note 10). Precursor and product ions monitored for each target analyte are provided in Table 2, along with employed collision energies and observed chromatographic retention times. Analysts are strongly encouraged to optimize tandem mass spectrometry settings using their instrumentation prior to analysis (see Note 11). Additional mass spectrometry settings are as follows: nebulizing gas, O\(_2\) at 60 psi; drying gas, N\(_2\) at 22 psi; drying gas temperature, 400°C; needle voltage, 4,500 V; collision gas, Ar at 2.0 mTorr.
1. Construct preliminary calibration plots for each analyte by plotting the observed response factor (area of analyte peak divided by area of internal standard peak) versus analyte concentration for each analyzed calibration standard. Chromatograms corresponding to a single UV wavelength (253 nm for 5-hydroxy-2-methylfurfural (5-HMF) and furfural) or a single MS/MS transition should be utilized in construction of these plots. An alternative approach is required for quantitation of acetic acid (see Note 12).

2. Perform a linear regression (forced through the origin) on calibration data and inspect calibration plots for linearity. Evaluation of correlation coefficients (e.g., $r^2$) or the relative standard deviations of mean response factors may aid assessment of linearity but should not substitute for visual inspection. It is likely that some calibration standards will contain one or more analyte(s) at concentrations that are too low (i.e., not result in a measurable response) or too high (i.e., deviate from linearity) to support linear calibration. Data for these standards may be omitted to improve linearity,
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (min)</th>
<th>Precursor(^a) ion (m/z)</th>
<th>Collision energy (eV)</th>
<th>Product ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Malonic acid</td>
<td>2.9</td>
<td>103</td>
<td>−9.0</td>
<td>59</td>
</tr>
<tr>
<td>2 Lactic acid</td>
<td>3.6</td>
<td>89</td>
<td>−10.0</td>
<td>43</td>
</tr>
<tr>
<td>3 Maleic acid</td>
<td>4.0</td>
<td>115</td>
<td>−7.5</td>
<td>71</td>
</tr>
<tr>
<td>4 Acetic acid(^b)</td>
<td>4.0</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5 cis-Aconitic acid</td>
<td>5.1</td>
<td>173</td>
<td>−8.0</td>
<td>129</td>
</tr>
<tr>
<td>6 Methylmalonic acid</td>
<td>5.4</td>
<td>117</td>
<td>−7.0</td>
<td>73</td>
</tr>
<tr>
<td>7 Succinic acid</td>
<td>6.2</td>
<td>117</td>
<td>−14.0</td>
<td>73</td>
</tr>
<tr>
<td>8 Fumaric acid</td>
<td>6.4</td>
<td>115</td>
<td>−7.5</td>
<td>71</td>
</tr>
<tr>
<td>9 trans-Aconitic acid</td>
<td>8.0</td>
<td>173</td>
<td>−8.0</td>
<td>129</td>
</tr>
<tr>
<td>10 Levulinic acid</td>
<td>11.6</td>
<td>115</td>
<td>−5.0</td>
<td>71</td>
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<tr>
<td>11 Glutamic acid</td>
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<td>131</td>
<td>−11.0</td>
<td>87</td>
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<tr>
<td>12 Itaconic acid</td>
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<td>85</td>
</tr>
<tr>
<td>13 2-Hydroxy-2-methylbutyric acid</td>
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<td>71</td>
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<tr>
<td>14 2-Furoic acid</td>
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<td>15 Gallic acid</td>
<td>15.6</td>
<td>169</td>
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<td>125</td>
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<tr>
<td>16 5-Hydroxy-2-methylfurfural(^b)</td>
<td>15.8</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>17 Furfural(^b)</td>
<td>17.9</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>18 Adipic acid</td>
<td>18.5</td>
<td>145</td>
<td>−12.0</td>
<td>101</td>
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<tr>
<td>19 3,4-Dihydroxybenzoic acid</td>
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<td>153</td>
<td>−13.0</td>
<td>109</td>
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<tr>
<td>20 3,5-Dihydroxybenzoic acid</td>
<td>21.5</td>
<td>153</td>
<td>−13.0</td>
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<td>Surrogate standard</td>
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</tr>
<tr>
<td>21 2,5-Dihydroxybenzoic acid</td>
<td>22.6</td>
<td>153</td>
<td>−18.5</td>
<td>108</td>
</tr>
<tr>
<td>22 3,4-Dihydroxybenzaldehyde</td>
<td>24.1</td>
<td>137</td>
<td>−21.0</td>
<td>108</td>
</tr>
<tr>
<td>23 Salicylic acid</td>
<td>26.5</td>
<td>137</td>
<td>−13.5</td>
<td>93</td>
</tr>
<tr>
<td>24 4-Hydroxybenzaldehyde</td>
<td>31.5</td>
<td>121</td>
<td>−23.5</td>
<td>92</td>
</tr>
<tr>
<td>25 Vanillic acid</td>
<td>34.8</td>
<td>167</td>
<td>−12.0</td>
<td>152</td>
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<tr>
<td>26 Homovanillic acid</td>
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<td>181</td>
<td>−7.0</td>
<td>137</td>
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<tr>
<td>27 4-Hydroxyacetophenone</td>
<td>40.0</td>
<td>135</td>
<td>−23.0</td>
<td>92</td>
</tr>
<tr>
<td>28 Caffeic acid</td>
<td>40.5</td>
<td>179</td>
<td>−14.0</td>
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</table>

(continued)
Table 2 (continued)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (min)</th>
<th>Precursor ion (m/z)</th>
<th>Collision energy (eV)</th>
<th>Product ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29 Syringic acid</td>
<td>40.9</td>
<td>197</td>
<td>−12.0</td>
<td>182</td>
</tr>
<tr>
<td>30 4-Hydroxybenzoic acid</td>
<td>41.2</td>
<td>137</td>
<td>−15.0</td>
<td>93</td>
</tr>
<tr>
<td>31 Vanillin</td>
<td>41.5</td>
<td>151</td>
<td>−12.0</td>
<td>136</td>
</tr>
<tr>
<td>32 Benzoic acid</td>
<td>43.4</td>
<td>121</td>
<td>−11.5</td>
<td>77</td>
</tr>
<tr>
<td>33 Benzoic acid-d₅</td>
<td>42.8</td>
<td>126</td>
<td>−11.5</td>
<td>82</td>
</tr>
</tbody>
</table>

*Internal standard for MS detection*

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (min)</th>
<th>Precursor ion (m/z)</th>
<th>Collision energy (eV)</th>
<th>Product ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34 Syringaldehyde</td>
<td>45.3</td>
<td>181</td>
<td>−12.0</td>
<td>166</td>
</tr>
<tr>
<td>35 4-Hydroxycoumaric acid</td>
<td>45.7</td>
<td>163</td>
<td>−13.0</td>
<td>119</td>
</tr>
<tr>
<td>36 Ferulic acid</td>
<td>48.1</td>
<td>193</td>
<td>−15.0</td>
<td>134</td>
</tr>
<tr>
<td>37 Sinapic acid</td>
<td>48.9</td>
<td>223</td>
<td>−12.5</td>
<td>208</td>
</tr>
<tr>
<td>38 3-Hydroxy-4-methoxycinnamic acid</td>
<td>49.4</td>
<td>193</td>
<td>−15.0</td>
<td>134</td>
</tr>
<tr>
<td>39 4-Hydroxycoumarin</td>
<td>50.9</td>
<td>161</td>
<td>−17.5</td>
<td>117</td>
</tr>
<tr>
<td>40 o-Toluic acid</td>
<td>52.2</td>
<td>135</td>
<td>−10.5</td>
<td>91</td>
</tr>
<tr>
<td>41 p-Toluic acid</td>
<td>54.5</td>
<td>135</td>
<td>−10.5</td>
<td>91</td>
</tr>
<tr>
<td>42 t-Butylphenoxyacetic acid</td>
<td>61.0</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Internal standard for UV monitoring*

*a The precursor ion for all analytes was the molecular ion (i.e., m/z = [M−H]−)*

*b A measurable MS/MS transition was not identified for acetic acid, furfural, or 5-hydroxy-2-methylfurfural (5-HMF). As a result, these compounds were monitored by UV spectroscopy (210 nm for acetic acid and 253 nm for furfural and 5-HMF)*

but arbitrary omission of nonconforming data points occurring between these two extremes is not recommended.

3. Determine the concentrations of detected analytes in pretreatment samples using optimized regression equations from step 2 above. If concentrations returned for undiluted samples exceed the upper calibration limit for a given analyte (i.e., the highest concentration used to define the utilized regression equation), a diluted extract (see step 12 in Subheading 3.3 above) should be used to determine analyte concentration. ESI is prone to interference from co-extracted matrix components. As a result, concentrations determined for analytes monitored by tandem mass spectrometry may be significantly different from actual sample concentrations. Nevertheless, our experience suggests that concentrations determined using this approach
are suitable for estimating spiking levels that support quantitation via the method of standard additions (see Note 13).

4. Based on results from step 3 above, prepare a spiking solution containing appropriate amounts of target analyte(s) to support quantitation via the method of standard additions when 10–100 µl of the solution are added to a 5-ml aliquot of the acidified, aqueous filtrate described in step 4 of Subheading 3.3 above. This solution should be prepared in methanol.

5. Add successive amounts (0–100 µl) of spiking solution to four or more replicate 5-ml aliquots of residual aqueous filtrate from step 3 in Subheading 3.3 above.

6. Prior to analysis, carry each aliquot through the sample preparation protocol described in Subheading 3.3 above (beginning with step 4). Note that preparation of multiple dilutions (described in steps 11–13 of Subheading 3.3) will not be required at this point, as a suitable dilution can be selected based on preliminary evaluation of the undiluted, 10-fold diluted, and 100-fold diluted samples in step 2 above.

7. Determine the concentration of surrogate standard(s) in each analyzed sample, and calculate recoveries using the following equation:

\[
\text{Recovery (\%)} = \frac{C_x}{C_s} \times 100
\]

where \(C_x\) and \(C_s\) represent the observed sample concentration and expected sample concentration (i.e., the surrogate spike concentration), respectively. It is not important that quantitative recovery be observed, but calculated values should generally vary by no more than 20% between samples (see Note 14).

8. Assuming that the condition specified in step 7 above is satisfied, prepare a standard addition calibration plot for each analyte, confirm linearity, and determine unknown analyte concentrations using the approach described in Note 13.

4. Notes

1. The presence of a surrogate standard provides an independent assessment of sample preparation efficiency for each analyzed sample. In analyses subject to rigorous quality assurance monitoring, multiple surrogates are often employed and are typically selected to elute across the full range of chromatographic retention times. Alternative surrogates may also be
added to this mixture if desired. Use of deuterium- or $^{13}$C-labeled compounds is highly recommended to ensure that the surrogate is not a native constituent of samples to be analyzed (or at least is not present at significant concentrations). Our suggestion of unlabeled 3,5-dihydroxybenzoic acid is justified by numerous analyses of pretreatment samples that were derived from dissimilar feedstock (i.e., corn stover or poplar) and divergent pretreatment conditions (dilute sulfuric acid or AFEX with multiple time–temperature combinations). However, it is possible that this compound would not be a suitable surrogate in alternative samples.

2. The sample amount will vary depending on bulk density. Do not pack the sample tightly into the extraction cell, as this can lead to variability in extraction. In our experience, approximately 4 g of AFEX-pretreated biomass is sufficient to fill a 22-ml extraction cell.

3. The ASE-200 maintains constant pressure during extraction by adding liquid to the extraction cell. Accordingly, failure to securely tighten caps can lead to variable backpressures and dissimilar extract volumes. In our experience, a firm twist from an analyst of reasonable strength is sufficient to secure caps. Avoid using pliers or other tools, as these can damage the caps.

4. These instrumental parameters were optimized for AFEX-pretreated corn stover using 22-ml extraction cells. Larger (or smaller) extraction cells may be substituted and instrumental settings may be varied in order to optimize extraction for alternative samples. However, care must be taken to ensure that the volume of liquid flushed through the extraction cell (which is a function of the cell volume, flush volume, and number of static cycles) does not overfill the collection vial. Alternatively, the unit can be programmed to perform one static cycle per collection vial, and extracts corresponding to a single sample can be combined post extraction.

5. These liquid samples may be stored in the dark at −20°C for at least 1 month if continued analysis is not immediately desirable. However, volumetric glassware should never be exposed to extreme temperatures. It is recommended that samples be stored in glass, screw-cap containers.

6. Although the identity of this precipitate has not been confirmed, failure to carry out this step results in a dramatic decrease in HPLC column efficiency after only a few injections.

7. Alternate methods for sample agitation (e.g., shaking, vortexing, etc.) may be substituted. It is also appropriate to carry out extraction at room temperature provided that the laboratory is not subject to large temperature fluctuations. For the interested reader, it is important to note that extraction
efficiencies for most target analytes in Table 2 have been reported and discussed elsewhere (24). However, independent knowledge of extraction efficiency is not required for analyte quantitation when a standard addition procedure is employed as described in Subheading 3.5.

8. Evaporators are typically designed to accommodate test tubes conforming to a particular size range (e.g., 1–35 ml for the TurboVap LV). If the volume of combined extracts exceeds the maximum test tube volume for the available instrument configuration, extract may be split between multiple test tubes and combined as the solvent evaporates. If this approach is required, it is highly recommended that additional solvent be used to rinse the depleted test tube when transferring volumes. It is expected that the 1–2 ml volume specified in step 8 of Subheading 3.3 be confined to a single test tube.

9. A caveat of gradient separations is that they do not typically transfer well from one HPLC system to another due to differences in instrumental dwell volume (i.e., the volume including the gradient mixer and all downstream components between the mixer and the head of the chromatographic column). The primary effect of differing dwell volumes is a shift in analyte retention times when gradient elution is used. In some cases, shifts are also accompanied by changes in resolution. Therefore, it is likely that the gradient program provided in Table 1 will need to be modified for alternative instrumentation. For additional information on dwell volume and its effect on gradient separations, the reader is referred to the text authored by Snyder et al. (25).

10. One of the advantages of mass spectrometry detection is the ability to resolve co-eluting analytes in reconstituted ion chromatograms. An illustrative example is provided in Fig. 4 for select compounds eluting between 39 and 42 min. The total ion current (TIC) trace (Fig. 4a) demonstrates that multiple analytes co-elute over the defined retention time range. However, because a unique MS/MS transition is monitored for each analyte, individual compounds are baseline-resolved in reconstituted ion chromatograms (Fig. 4b–e).

11. Tandem mass spectrometry settings in Table 2 were determined as follows: Independent solutions of each target analyte were prepared in 0.1% aqueous formic acid at a concentration of 10 mg/l and infused individually into the mass spectrometer at a flow rate of 10 µl/min using a syringe pump. Initially, the first quadrupole was scanned from \( m/z \) 40 to \([M + 100]\) (\( M \) = the molar mass of a given analyte). This experiment enabled identification of the most intense precursor ion. Without exception, the most intense precursor for all target analytes was the \([M-H]^-\) ion.
Next, the $[\text{M-H}]^-$ ion was selected in the first quadrupole, and collision energy was varied in the second quadrupole while the third quadrupole was scanned from $m/z$ 40 to $[\text{M} + 20]$. This experiment enabled identification of the most intense product ion and optimal collision energy for each precursor. Similar experiments are supported by most software packages supplied with commercially available mass spectrometry instrumentation.

12. It is important to note that the employed chromatographic conditions result in co-elution of acetic acid and maleic acid (see Table 2). Maleic acid absorbs light over the entire range
of wavelengths that may be employed to monitor acetic acid. As a result, quantitation of acetic acid using a simple regression equation is prohibited. According to Beer’s law, the observed analytical response for these analytes at a given wavelength is mathematically governed by the following equation:

\[ A_{210nm} = b \left( \epsilon_{\text{maleic acid}} \times c_{\text{maleic acid}} + \epsilon_{\text{acetic acid}} \times c_{\text{acetic acid}} \right) \]

where \( A \) is the observed absorbance (mAU), \( b \) is the path length of the flow cell (0.9 cm), \( c \) is the concentration (mol/l), and \( \epsilon \) is the molar extinction coefficient. The extinction coefficient of maleic acid at 210 nm is more than 200 times greater than that of acetic acid (ca. 6,880 l mol\(^{-1}\)cm\(^{-1}\) vs. 34 l mol\(^{-1}\)cm\(^{-1}\)). However, because maleic acid is amenable to quantitation via independent MS/MS monitoring, the concentration of acetic acid may be estimated using the above equation, assuming no other compounds are contributing appreciably to the UV response over the retention time range in which these analytes elute. Depending on the relative concentrations of 5-HMF and gallic acid in pretreatment samples, a similar approach may be required for quantitation of 5-HMF in some cases.

13. The method of standard additions involves adding successive amounts of analyte(s) to replicate aliquots of a sample of unknown concentration. These samples are subsequently analyzed, and the unknown concentration is determined by extrapolating a plot of observed response for the standard addition samples versus spiking level to zero concentration. In practice, a regression equation is established and analyte concentration determined by dividing the observed response for the unspiked sample by the slope (Fig. 5). In order for this analysis to be successful, it is critical that spike concentrations result in sample response factors that fall within the linear range of analyte concentration. Ideally, the observed response factors will also vary by at least a factor of two over the concentration range of added analyte(s). Thus, it is important to approximate analyte concentration(s) and have working knowledge of analyte sensitivity prior to selecting spiking levels. Once linearity has been confirmed for a given pretreatment sample, subsequent analysis of similar samples may be supported by a single-point standard addition approach. In this case, unknown sample concentrations are determined using the following equation:

\[ C_x = C_{\text{add}} \times \frac{RF_x}{(RF_{x+} - RF_x)} \]

where \( C_x \) is the unknown concentration, \( C_{\text{add}} \) is the concentration of analyte added to the sample, \( RF_x \) is the response factor.
RF_{x+} is the response factor observed for analysis of the spiked sample.

14. Acceptable agreement between surrogate recoveries may be greater than or less than 20%, depending on the particular compound employed. In actuality, calculated surrogate recoveries should be control-charted on a continuing basis to more rigorously define analyte-specific quality control criteria.

**Acknowledgments**

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References


Chapter 11

Biomass Compositional Analysis for Energy Applications

Bonnie R. Hames

Summary

In its broadest definition, biomass can be described as all material that was or is a part of a living organism. For renewable energy applications, however, the definition of biomass is usually limited to include only materials that are plant-derived such as agricultural residues (e.g., wheat straw, corn stover) by-products of industrial processes (e.g., sawdust, sugar cane bagasse, pulp residues, distillers grains), or dedicated energy crops (e.g., switchgrass, sorghum, Miscanthus, short-rotation woody crops). This chapter describes analytical methods developed to measure plant components with an emphasis on the measurement of components that are important for biomass conversion. The methods described here can be viewed as a portfolio of analytical methods, with consistent assumptions and compatible sample preparation steps, selected for simplicity, robust application, and the ability to obtain a summative mass closure on most samples that accurately identifies greater than 95% of the mass of a plant biomass sample. The portfolio of methods has been successfully applied to a wide variety of biomass feedstock as well as liquid and solid fractions of both thermochemical pretreatment and enzymatic saccharification (1).

Key words: Biomass analysis, Composition, Carbohydrate, Cellulose, Hemicellulose, Lignin, Extractives, Ash, Rapid analysis, Near infrared

1. Introduction

Because you cannot optimize what you cannot measure, the ability to rapidly and inexpensively obtain an accurate chemical composition of complex biomass feedstock and biomass-derived materials is a key element in enabling the development and commercialization of processes that convert biomass to fuels and valuable chemicals. Biomass process analytical methods also improve our understanding of the economics and environmental impacts of biomass conversion processes by providing values and uncertainties for use in process and life-cycle models.
The analytical methods described in this chapter report data in a form compatible with the Aspen models used by the U.S. Department of Energy, Office of Biomass Programs (2). Because of the heterogeneity, which is an inherent property of biomass, the composition of any feedstock can vary significantly (3). The chemical composition of a biomass feedstock varies as a function of many factors, including plant genetics, growth environment, harvesting method, and storage. Many biomass conversion feedstock are residues of another process. For example, bagasse is a by-product of sugar production from sugarcane. In these situations, the varying efficiency in the original process can impart an additional source of compositional variance in biomass feedstock. All of these sources of compositional variance are difficult, if not impossible, to control. However, the composition of a given feedstock can be measured at any point and that information can be used to adjust process conditions for optimal conversion or steady-state production. The rapid and inexpensive compositional analysis methods described at the end of this chapter are examples of types of new tools that will be needed for the commercialization of processes that convert biomass into fuels and valuable chemicals.

1.1. Background

Plant biomass used as a feedstock for energy applications can take many forms, but all plant materials are comprised of the same basic building blocks, extractives, carbohydrates, lignin, protein, and ash. The relative concentrations of these basic constituents vary, within a plant as a function of tissue type and age, and between plants as a function of genetics and environment (4). As illustrated in Fig. 1, biomass feedstock can vary widely in the number of constituents and the concentration of each constituent. In biomass conversion processes, up to 20 constituents may need to be monitored to characterize the conversion of feedstock into a desired product or products.

1.2. Glossary

Because definitions and assumptions for various biomass methods can vary, a brief description of the terms used in this chapter is given here. These definitions and assumptions should be reviewed for appropriateness when applying the portfolio of analytical methods to any new feedstock or materials. Extractives. The category of extractives in biomass includes all plant materials that are extracellular or not part of the three-dimensional cell wall structure. The definition is behavior-based, in that extractives are all materials that can be easily extracted using water or organic solvents such as ethanol or hexane. Since pretreatment and other conversion processing steps can break down cell-wall components and release soluble products, extractives contents are reported for feedstock samples only.
Carbohydrates, or polysaccharides, in biomass feedstocks fall into four main categories: starch, cellulose, hemicellulose, and pectins. Starch consists of glucose molecules connected by α-glycosidic linkages.

Starch is found mainly in storage organs such as rhizomes, tubers, bulbs, and seeds (5). In this portfolio of methods, the glycosidic bonds in starch are selectively cleaved with amylase enzymes and starch content is estimated from the glucose released following enzymatic treatment.

In stalks, stems, and leaves, carbohydrates are found in the primary and the secondary cell walls (6). The predominant polysaccharide in the primary cell wall is cellulose; the second most abundant is hemicellulose; and the third is pectin.

Cellulose is a polymer of glucose that contains only β-(1,4)-linkages, and it has a highly linear structure that encourages the formation of strong hydrogen bonds between chains of cellulose. The high level of hydrogen bonding among the chains makes it much more difficult to be attacked or depolymerized, either chemically or biologically. In this portfolio of methods, cellulose content is estimated from glucose released after a two-stage acid hydrolysis process.

Hemicelluloses are biopolymers of six- and five-carbon sugars comprised of a main backbone decorated with a wide spectrum
of side-branch substituents. In softwoods, the backbone sugar is mannose with side chains of glucose and galactose. In hardwoods and grasses, the backbone sugar is xylan with side chains of arabinose and glucuronic acid. Un-branched sections of the xylan backbone are often substituted with acetyl esters. In this portfolio of methods, hemicellulose content is estimated from the released of acetic acid, glucuronic acid, and monomeric sugars after a two-stage hydrolysis process. Information about the structure or substitution patterns of hemicellulose is not available.

Pectins are highly branched polysaccharides comprised mainly of galacturonic acid and methyl esters of galacturonic acid. Pectins are most abundant in fruits and in young plant tissues. In this portfolio of methods, pectins are estimated from the release of uronic acids and their methyl esters after a two-stage hydrolysis process.

Lignin, the secondary cell wall produced after the cell has completed growing, also contains the above-mentioned polysaccharides and is strengthened by polymeric lignin covalently cross-linked to hemicellulose. Lignin is a high-energy content biopolymer formed by polymerization and crosslinking of phenylpropanes. In this portfolio of methods, lignin is measured in two parts. The majority of the lignin is measured gravimetrically as the organic material insoluble after hydrolysis of the carbohydrates. For herbaceous materials, the assumption is made that all protein is insoluble, and this weight is subtracted from the acid-insoluble residue before reporting lignin content. In some biomass materials, a portion of the lignin is solubilized during hydrolysis and is quantified using UV (ultraviolet) spectroscopy.

Protein In plant biomass materials, protein has many forms and functions. Proteins in plant cell walls govern cell expansion and cell-wall transport. They are major constituents of photosynthetic organs, enzymes, and hormone systems. In this portfolio of methods, the assumption is made that proteins are the only source of nitrogen in plant tissues. Protein determination requires two determinations: a measurement of the nitrogen content, and an independent method of estimating the relative abundance of amino acids. Protein content is estimated from the nitrogen content and the relative nitrogen content of the most abundant amino acids.

Ash In plant biomass, ash content is synonymous with inorganic compounds. In this portfolio of methods, ash is measured twice, once on the whole biomass and again on the solids remaining after extraction (extractives-free material). Some inorganic salts may be solubilized during the extraction processes. Soluble salts are calculated as the difference in ash contents before and after extraction. Structural ash, which is mainly silica, is the ash content of the extractives-free material.
2. Analysis Methods for Feedstock

2.1. Standard Methods

Standard wet chemical methods for the chemical characterization of biomass feedstock and biomass-derived materials have been validated through the International Energy Agency and are available from the American Society for Testing and Materials (ASTM) (7). In addition, the National Renewable Energy Laboratory (NREL) has developed and validated a collection of standard laboratory analytical procedures specifically for the compositional analysis of biomass including, but going beyond, those of the ASTM (8). These wet chemical methods of analysis are based on the fractionation of the biomass sample and the isolation of purified fractions that can be quantified using conventional analytical instruments. The core methods were developed and validated through the efforts of the International Energy Agency (9). The current methods are derived from two main sources, TAPPI standard methods developed for pulp and paper applications (10) and the Uppsala methods developed for fiber and nutritional analysis (11). These methods are combined in feedstock-specific portfolios containing analysis methods for each of the relevant constituents. The portfolio methods have consistent assumptions and are compatible when used together. Compatible methods minimize opportunities for double-counting constituents; for example, water soluble inorganics could be counted as both ash and extractives. The portfolio of methods described here includes methods for all major constituents of biomass feedstock. In many cases, more than one constituent is measured from the same analytical sample, minimizing sampling errors. In most cases, these portfolios enable the identification and quantification of more than 95% of the dry mass of biomass feedstock and biomass-derived materials.

2.2. Analytical Variance

A complete analysis characterizing greater than 95% of a biomass sample can require up to 30 independent measurements to report the concentration of 10–15 constituents. Each analytical method used to determine the concentration of a biomass constituent is a source of some error or uncertainty. When evaluating the results of biomass compositional analysis, it is important to determine whether or not variance due to sampling and measurement techniques is significant in light of variance from other sources. Table 1 shows the confidence intervals for the portfolio wet chemical methods used to characterize biomass feedstock. The confidence interval of 1.5% for glucan means that when a value of 38% is reported for a biomass sample, there is a 98% probability that the “true” glucan concentration lies between 36.5% and 39.5%. These values were determined in an international round-robin evaluation of four biomass standard reference materials (SRMs) available from the National Institute of Standards Technology (NIST) (9).
In the past, efforts were made to develop universal methods of analysis that could be used for any type of biomass feedstock or biomass-derived material (12). These methods provide instruction for the various procedural steps, but they rarely discuss why a given step is included in the procedure. The assumption is made that all steps in a standard procedure will be followed as written without omission or amendments. When combined in a portfolio for a complete mass analysis, it became obvious that many of the analytical methods were adequate for many samples, but optimal for none. As the biomass conversion industry matures, the need for more accurate and precise methods will increase, as data generated from these methods will be used to evaluate a maturing state of technology for commercialization. In response to this need, newer methods of analysis have been developed in a modular

### Table 1
Confidence intervals for NREL and ASTM standard wet chemical methods

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Method variance</th>
<th>Absolute error; 98% confidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Xylan</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Arabinan</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Mannan</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Galactan</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Uronic acids</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Pectin</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>O-Acyl groups</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Structural inorganics</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Lignin</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Ferulic acid esters</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Extractives</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Phyate</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Cutin</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>
fashion and designed to be incorporated into sample-specific portfolios capable of providing a customized total analysis of any biomass sample. In the portfolio of methods presented here, the individual constituent methods are independently validated relative to standard reference materials. These methods are then validated a second time when the results are combined to reconstruct the entire chemical composition of a biomass sample. Typical mass closures between 96% and 104% confirm that no major components have been overlooked and interferences between methods and double counting of materials are minimized. A summary of portfolio methods for various types of biomass feedstock and biomass-derived materials is shown in Table 2.

Within each portfolio of wet chemical methods, the sequence of application of the various analytical methods is critical, as many methods have been validated with the assumption that all prior steps have been completed. For feedstock, the sequence is sample preparation, then removal of nonstructural materials

| Table 2 | Quick reference guide for the recommended methods for various biomass types and their constituents |
| Methods table | (References for recommended method) |
|---|---|---|---|---|---|
| **Biomass type** | **Constituent** | **Hardwood** | **Softwood** | **Grass** | **Spent grains** | **Process solids** | **Process liquids** |
| Extractives | (8, 17) | (8, 17) | (8, 17) | (17) | N/A | N/A |
| Ash | (7, 8) | (7, 8) | (7, 8) | (7, 8) | (7, 8) | N/A |
| Protein | N/A | N/A | (8, 26, 27) | (8, 26, 27) | (8, 26, 27) | (30) |
| Oil | N/A | N/A | (8) | (8) | N/A | N/A |
| Starch | N/A | N/A | (24) | (24) | (24) | N/A |
| Glucan, xylan, mannan | | | | | | |
| Galactan, arabinan | (7, 23) | (7, 23) | (7, 23) | (7, 23) | (7, 23) | (30) |
| Lignin | (8) | (8) | (8) | (8) | (8) | (30) |
| Ferulates | N/A | N/A | (22) | N/A | (22) | N/A |
| Uronic acids/pectin | (21) | N/A | (21) | N/A | (21) | (30) |
| Acetyl | (23) | N/A | (23) | (23) | (23) | (30) |
| Sample preparation | (7, 8) | (7, 8) | (7, 8) | (7, 8) | (7, 8) | (30) |
| Representative sampling | (7, 8) | (7, 8) | (13) | (7, 8) | (7, 8) | (30) |

Numbers in parenthesis refer to references listed in text
through solvent extraction, followed by hydrolysis of the structural polymers to simple forms for chromatographic or spectroscopic analysis. In the past, many methods for biomass analysis have been “behavior-based,” meaning they define chemical structure based on solubility in certain solvent systems. The newest methods incorporate modern analytical instruments, which allow the various structural components to be measured and tracked on the basis of chemical structure.

Before using biomass analysis methods, analysts are encouraged to review the literature cited here that supports the standard methods. Understanding the science behind the methods helps the analyst understand the ways that the various procedures are connected, the limits within which a step can be altered or modified, and the potential downstream consequences of omitting any given step. With this in mind, the following several paragraphs outline available information concerning the significance, implications, and limitations of each step in a complete characterization of biomass. More details are available in the open literature and the text of the individual published procedures.

2.3.2. Sample Preparation

Sample preparation is an important but often overlooked part of biomass analysis. Biomass sample preparation must take several factors into account. The object of sample preparation is to enable the analysis to be done on a small scale while ensuring that the analysis of the small sample provides valid information about the larger bulk sample. Typically, biomass sample preparation includes drying, milling, sieving, and homogenizing.

Representative Sampling

The natural heterogeneous nature of biomass feedstock samples calls for particular care when subsampling a bulk sample for analytical procedures. Many biomass samples naturally segregate by particle size and density during transportation and storage. Thorough mixing and careful visual inspection is required at all levels of analysis. University extension offices provide detailed protocols for homogenization and sampling of forage samples that can be applied to most biomass types (13).

Drying

Freshly harvested biomass feedstock samples can contain as much as 60% (w/w) water. If the biomass has been degraded in a manner that disrupts the ultrastructure of the plant (chemical treatment or enzymatic digestion), the moisture of the sample could be even higher. Water in a biomass sample, if high enough, can introduce errors during some of the analysis steps by diluting reagents. Moisture in samples can also cause sample fractionation during milling and sieving. Biomass samples with high moisture contents are still vulnerable to biological degradation and may not remain stable and uniform for the duration of a battery of tests. For these reasons, biomass samples are usually dried until the moisture content is less than 10%. Because some of the
constituents are heat-sensitive or volatile, certain protocols must be followed when drying a biomass sample for compositional analysis, to avoid sample loss or degradation. Air-drying is preferred if time, space, and ambient humidity will allow the sample to reach a moisture level below 10%. Drying the samples in a convection oven or vacuum oven where the temperature is not allowed to exceed 45°C is an acceptable alternative. When freezing does not cause collapse of the cellular structure of the biomass, samples can also be safely lyophilized or freeze-dried.

Size reduction is an important step in biomass analysis because standard methods are optimized and validated for materials with a specific particle size. The particle size will affect hydrolysis or digestion rates as well as rates of degradation, and should be specified in any standard procedure. As a general rule, the sample to be analyzed should contain at least 300 particles of the biomass sample in order to be representative of the bulk material. For reasons described earlier for drying protocols, care must be taken during the milling process to avoid heating the biomass sample. This is particularly crucial if the biomass must be reduced to a fine powder. Cryo-mills, where the milling apparatus is submerged in liquid nitrogen or mills are jacketed for the circulation of cooling solutions, are most suitable for milling biomass samples to fine powders. Knife mills such as the standard Thomas-Wiley Mill 4 or equivalent work well for most woody biomass samples (14). Less dense materials such as corn stalks or straws may be easier to feed into a hammer mill. Most knife and hammer mills will retain and reprocess materials until they pass through a screen of a particular particle size, allowing the user to specify the maximum particle size of the processed sample.

If the biomass sample is contaminated with soil, the samples may be washed before further processing. Soils vary considerably in chemical content, and even a few weight percent of extraneous inorganic compounds can neutralize reagents, catalyze side reactions, or affect subsequent analysis in ways that are difficult to observe or control. Washing may be as simple as agitation in clean, room-temperature demineralized water or may require small amounts of mild detergents to be effective. After washing, the sample must be carefully dried before analysis.

Chemical or thermal hydrolysis performs better when the particles are of a uniform size. For this reason, most standard procedures usually specify both a maximum and a minimum particle size. The portfolio methods have been optimized for particle sizes that are greater than 80 mesh and less than 20 mesh. To accomplish this, milled biomass is sieved through a set of standard screens. To prevent errors associated with incomplete hydrolysis, the oversized material is milled again until it passes through the largest (20 mesh) screen. The material that passes through
the larger screen but is retained on the fine mesh screen is of uniform particle size and can be used for compositional analysis. The material that passes through the fine mesh screen may be hydrolyzed too quickly, leaving the hydrolysis products more susceptible to side reactions or degradation before analysis. Since the fine material cannot be reprocessed to increase particle size, this material would ideally be discarded. In some biomass samples, however, sieving to remove fine material chemically fractionates the sample. Fractionation on sieving is often seen in grasses where the friable pith or leaf material may be preferentially removed as fines. As mentioned earlier, the impact of high ash content on the summative analysis cannot be predicted, but poor mass closures are often seen with high ash samples, and mass closure often improves with removal of the –80 mesh fraction (15). Before discarding the fine material, the weight, moisture, and ash content should be determined. The portfolio analysis is performed only on the intermediate, uniformly sized material. The potential impact of discarding the fines fraction should be made considering the dry weight fraction of the fines and the ash content of the fines. When high ash content is causing poor mass closures, the composition of the original sample may best be calculated by assuming that the organic portion (non-ash) of the fines has the same chemical composition as the intermediate fraction that is analyzed.

Making the particle size as uniform as possible also makes it easier for the analyst to select a small but reproducible sample for analysis. When a decision is made not to remove the fines, additional homogenization prior to subsampling may be necessary to select a representative particle size distribution for analysis.

2.3.3. Extractions

Plants store nonstructural materials in their vessels and water transport channels that can interfere with accurate chemical compositional analysis. These materials are commonly known as extractives, which may give the impression that they are a single substance or at least a few closely related substances. The category of extractives is actually a complex mixture of many compounds present in relatively small concentrations (16). Extractable materials include gums, resins, pitch, waxes, sterols, flavinoids, tannins, terpenes, quinones, nonstructural sugars, chlorophyll, and many other minor building block reserves that vary seasonally and by biomass type. Because extractives vary so much in chemical composition, their behavior during the slate of analyses required for a compositional characterization cannot be predicted. For this reason, nonstructural components should always be removed prior to analysis for lignin or carbohydrates. Failure to remove these materials can cause several problems (9). Some of the extractives may be insoluble in acid and will precipitate during hydrolysis, and thus will be falsely counted as lignin.
The presence of some materials will limit access to the carbohydrate polymers, causing incomplete hydrolysis and resulting in low bias in carbohydrate measurements. Some constituent measurements are made on the whole biomass samples, before extraction, such as ash, starch, and nitrogen content used to calculate protein. These measurements may need to be repeated after extraction to determine the concentrations of extractable ash, starch, protein, etc. The contribution of identified extractives should be subtracted from the reported extractives value to avoid “double counting” of any constituent mass, once in their correct constituent category and again as extractives. The solvents and number of extractions required to remove all nonstructural materials will vary with biomass type and constituents present. For example, with grasses a simple water extraction will remove sucrose with minimal degradation. Ethanol and hexanes are used to remove chlorophyll, proteins, fats, and oils. The order of extraction steps can be important. For grains and grain-derived materials, oil is usually defined as hexane-extractible material. In whole plant materials that include grain, water, and ethanol, extraction should be performed prior to hexane extraction to remove materials that could interfere with oil determination (17). Some materials, like wood bark, may require additional extractions with toluene and dichloromethane to remove tars, pitch, and resins (18). The published NREL methods provide instruction for extraction processes using traditional soxhlet extraction apparatus as well as recommended setting for accelerated solvent extraction. The solids remaining after extraction are called extractives-free samples.

Quantifying Extractives

In the current portfolio of methods, sucrose and oil are the only extractives components that are quantified directly. Water extractions are taken to a standard volume, and the concentration of sucrose is measured by HPLC using the standard carbohydrate methods or, more quickly, using a sucrose membrane available for the glucose analyzer sold by YSI Life Sciences (19). Following water and ethanol extraction, an oil measurement can be made by extracting the sample with hexane. All other nonstructural materials are simply weighed together and reported as “other extractives.” For most feedstock, extractives are complex mixtures with most components present in low concentration. For future applications, the portfolio has been specifically designed to allow easy addition of methods for quantifying other compounds reported now as extractives, should interest arise.

2.3.4. Acid Hydrolysis

The biomass analysis portfolio methods for the determination of carbohydrates and lignin in biomass incorporate a two-stage acid hydrolysis to separate individual polymers and hydrolyze them to simple molecules that can be readily analyzed by gravimetric, chromatographic, and spectroscopic techniques. The first stage subjects the extractive-free biomass sample to a concentrated
acid that disrupts the noncovalent interactions between biomass polymers. The portfolio method uses 72% sulfuric at 20°C for 1 h. A critical component of this procedure is the need for regular mechanical disruption of the gels that form as the cellulose fibers swell. Regular stirring of the sample allows contact between the concentrated acid and newly exposed biomass materials. Failure to adequately mix the samples during concentrated acid hydrolysis may result in incomplete hydrolysis of carbohydrate polymers and a resulting low bias in those determinations. A second more dilute stage follows, which is optimized for complete polymer hydrolysis and minimized degradation of monomeric sugars (20). Following concentrated acid hydrolysis, the sample is diluted to adjust the acid concentration to 4%. The dilute solution is then autoclaved at 121°C for 1 h.

Cellulose and Hemicellulose – At the completion of the dilute acid hydrolysis, the liquid phase should contain all carbohydrates in the biomass sample as monomeric sugars. Acetyl groups from hemicellulose will be released as acetic acid. Uronic acids and phenolic esters will also be released into the liquid phase during dilute acid hydrolysis. Some monomeric sugars will be further degraded to furans and lactones. The main degradation products are furfural from five-carbon sugars (arabinose and xylose) and hydroxymethylfurfural (HMF) from six-carbon sugars (glucose, galactose, and mannose). These monomers remain in the liquid phase after hydrolysis and can be quantified using a variety of chromatographic techniques. Both liquid chromatography (LC) and gas chromatography (GC) methods were used during a round-robin test of the core hydrolysis methods, and despite substantial variation in the equipment used, including columns and detectors, the LC methods, which require only sample neutralization, exhibited roughly the same precision and accuracy as GC measurements, which required a complex series of derivatization steps (9). Standard methods for both LC and GC analysis are available through ASTM (7). Internal standards are necessary in the ASTM procedures to minimize errors due to evaporation during hydrolysis. The updated NREL methods have eliminated the need for internal standards in the LC methods by performing the hydrolysis in sealable hydrolysis tubes. Use of these pressure vessels also improves the method precision and accuracy by eliminating the need for quantitative transfer between the two stages of hydrolysis. The LC methods also require the use of both anion and cation de-ashing cartridges, which need to be replaced every 100–300 injections. When selecting chromatography instruments, columns, and conditions, be sure to evaluate some actual biomass hydrolysis samples, since soluble lignin, acetic acid, and some salts can affect chromatography and detection.

Portfolio-compatible methods for measuring hemicellulose substituents such as uronic acids (21), ferulic acid esters (22),
and $O$-acetyl groups (23) should be included in a complete carbohydrate analysis of hardwoods and grasses.

**Starch** – If starch and cellulose are present in the feedstock sample, they will both contribute to the measured glucose concentration after hydrolysis. An independent starch measurement allows the two polymers to be quantified. The NREL portfolio starch analysis method is based largely on the megazyme total starch assay (amyloglucosidase/α-amylase method) (24). In this method, starch is selectively hydrolyzed to glucose using two types of amylase enzymes. Cellulose and hemicellulose are not hydrolyzed by this process. Starch analysis is necessary only when analyzing young plants, or samples that contain root tissues or seeds. In the portfolio methods for feedstock samples, starch analysis is done on the whole biomass sample prior to solvent extractions.

**Loss Factors**

Some degradation of sugars is unavoidable in the two-stage hydrolysis methods. The kinetics of degradation differs for each sugar. Degradation is also concentration dependent. Carbohydrate standards of similar structure and concentration should be run in parallel to assess the magnitude of this degradation and correct the reported values for this loss if necessary. Loss factor standards with a range of concentrations for each sugar are run with each hydrolysis batch to ensure an appropriate measurement of loss for each sugar in unknown samples. Since loss factors are measured with each batch, it is possible to pool this data and minimize errors by applying historical averages instead of individual batch measurements. Loss factors should be reassessed when changing lab equipment – particularly autoclaves – since hydrolysis severity can be affected when heating and cooling rates change.

**Lignin**

Many early lignin determinations were behavioral-based where lignin was defined as the material insoluble in acid after hydrolysis of the carbohydrate fraction. This definition is valid for most wood species, but invalid for many biomass samples, particularly herbaceous materials where ash and protein will condense with the lignin and remain in the insoluble residue. In the biomass analysis portfolio methods, lignin is assumed to partition during the hydrolysis process. The bulk of lignin in biomass feedstock will remain insoluble and can be collected by simple filtration. A portion of the lignin will remain soluble in the dilute acid and will be measured spectroscopically in the liquid phase.

**Acid-Insoluble Lignin**

Following dilute acid hydrolysis, the acid-insoluble residue is collected by filtration. To minimize the need for quantitative transfers, the recommended filtration device is a ceramic crucible that can go directly into an ashing furnace. The acid-insoluble residue is dried, weighed, and then ashed at 575°C to measure the inorganic content of the residue. A nitrogen measurement can be made on the acid-insoluble residue for protein determination (see Subheading 2.3.5). Since both ash
and protein determinations consume the sample, acid hydrolysis must be run in quadruplicate to provide replicate determinations for insoluble ash and protein. In some cases, the assumption can be made that all of the protein in the extractives-free material will condense during hydrolysis. This assumption introduces some error, but eliminates the need for replicate hydrolysis and an additional nitrogen determination.

Acid-insoluble lignin is calculated as the acid-insoluble residue minus acid-insoluble protein and ash. Failure to measure and subtract acid-insoluble ash and protein will result in falsely high mass closures, as portions of protein and ash would be counted twice, once as lignin and once in their respective constituent category.

In the two-stage hydrolysis, a portion of lignin may be released into the hydrolysis liquor. An accurate lignin value includes a measurement of both acid-soluble and acid-insoluble lignin. In the portfolio methods, acid-soluble lignin is measured by UV/Vis spectroscopy. The optimum wavelength for measuring acid-soluble lignin varies with the biomass type. In these determinations, the critical parameters are: accurate dilution of the sample to bring the measured absorbance into the range where detector response is known to be linear with lignin concentration; and selection of an appropriate wavelength and absorptivity. The NREL methods include measurement guidelines for several biomass categories (25).

To estimate crude protein content of biomass or other materials, the nitrogen content of the material is measured by Kjeldahl or combustion methods and multiplied by a conversion factor where: protein (wt/wt%) = nitrogen (wt/wt%) × nitrogen-to-protein conversion factor.

A nitrogen-to-protein conversion factor (N-factor) of 6.25 is commonly used for animal feeds and other materials. The practice of using 6.25 as a N-factor is based on an incorrect assumption that protein in a given material contains 16% nitrogen (100/16 = 6.25) (26). The average nitrogen content of amino acids is 16%, but the amino acids are not present in equal amounts. The correct N-factor for protein found in herbaceous biomass will likely be different from 6.25. Yet, determining a perfectly accurate N-factor for the complex matrices of biomass feedstock and process samples may not be possible. The challenge is then to determine a more appropriate N-factor possible for biomass feedstock and process samples. A strategy based on the consensus in the literature has been incorporated into the portfolio methods. These methods calculate the reasonable N-factor upper and lower limits for a given material. The limits are calculated using data from an amino acid (AA) analysis with multiple hydrolysis times and an accurate total nitrogen analysis substantially similar
to the methods described by Mossé \cite{27}. A spreadsheet designed for easy calculation of N-factors can be found with the portfolio methods on the NREL website.

2.3.6. Ash

In this portfolio of methods, ash is measured twice, once on the whole biomass and again on the solids remaining after extraction (extractives-free material). Ash content is measured gravimetrically as the residue remaining after combustion of the plant material at 575°C. Some inorganic salts may be solubilized during the extraction processes. Soluble salts are calculated as the difference in ash contents before and after extraction. Structural ash, which is mainly silica, is the ash content of the extractives-free material. As described above, the ash remaining in the acid-insoluble residue is also measured in the determination of acid-insoluble lignin.

2.4. Summative Mass Closure

2.4.1. Reconstructing the Composition of the Original Biomass Sample

Following the multistep sequence of the portfolio methods, the constituents’ values can be reported in many forms. It is always important to state the measurement basis for any reported compositional values. Constituent values in solid samples are most commonly reported as percent dry weight on a whole-biomass basis in order to assess the summative mass closure or the total percentage of the sample that has been identified. None of the portfolio methods directly provides the values used for summative analysis. In each step, the dry weight of a constituent is determined and is reported as a percentage of the dry weight of a starting analytical sample. The following list describes the calculations used to convert the measured values into a summative mass closure. Actual equations for these conversions can be found in each portfolio method. Calculation Workbooks designed specifically for summative mass closures of biomass feedstock and biomass-derived materials have been prepared at NREL and are available with the portfolio methods \cite{28}.

**Moisture**: Although moisture (or the inverse, total solids) is determined in every step of the portfolio methods, and these measurements are only used to convert all values to a dry-weight basis. The moisture content of a sample on a dry-weight basis is, by definition, zero.

**Sucrose**: Sucrose concentration in the water extractives is measured as a concentration in a fixed volume of water extractives. The weight of sucrose is determined by multiplying concentration by volume.

**Oil**: Oil or fats in a biomass sample are defined as the hexane solubles. This weight is measured directly after removal of the hexane by evaporation.

**Extractives**: The dry weight of material extracted by each solvent is determined after solvent removal by evaporation. *Total extractives* is the sum of water solubles, ethanol solubles, hexane solubles, etc. When reporting sucrose or oils, their weights
are subtracted from the total extractives and the difference is reported as Extractives (other).

**Extractives-free material**: Structural ash, protein, carbohydrates, and lignin are measured on extractives-free materials. The measured values are converted to a whole-biomass basis using the total extractives measurement and the following equation: whole biomass = total extractives + E-F biomass.

**Ash**: Soluble ash is usually reported as part of the extractives (other). The ash content of the extractives-free material is reported as structural ash after conversion to a whole-biomass basis.

**Protein**: Soluble protein is usually reported as part of the extractives (other). The protein content of extractives-free material is reported as structural protein after conversion to a whole-biomass basis.

**Starch**: The starch content of the extractives-free material is reported as starch after conversion to a whole-biomass basis.

**Carbohydrates**: For most energy applications, individual sugars and side-chain constituents are reported separately for mass closure and tracking. Degradation is estimated from the loss factor standards and added to the values measured by GC or LC. The individual constituent weights are then converted to polymeric or anhydro form. Glucose is converted to glucan; xylose to xylan; and acetic acid to acetyl, etc. This conversion subtracts the weight of water molecules added during polymer hydrolysis. Then constituent concentrations are expressed as a percent dry weight and are converted to a whole-biomass basis for summative mass closure. For some applications, it may be desirable to reconstruct the carbohydrate polymers.

**Cellulose**: For hardwood samples, glucan, and cellulose are assumed to be equivalent. For grass samples, cellulose = glucan – starch. For softwood samples where some glucan comes from the gluco–galacto–mannans, hemicellulose glucan can be estimated as one-third of the mannan content (5). Cellulose can be estimated as total glucan–hemicellulose glucan. The cellulose content is reported after conversion to a whole-biomass basis.

**Hemicellulose**: Hemicellulose content and structure varies with the biomass type. For softwoods hemicellulose = mannan + galactan + hemicellulose glucan. For hardwoods, hemicellulose = xylan + arabinan + acetyl + uronic acids. For grasses, hemicellulose = xylan + arabinan + acetyl + uronic acids + ferulates. The hemicellulose content is reported after conversion to a whole-biomass basis.

**Lignin**: Lignin content is the sum of acid-soluble lignin and acid-insoluble lignin. Lignin content is reported after conversion to a whole-biomass basis.

**Total mass closure**: The summative mass closure of characterized material is the sum of ash, protein, extractives (other), sucrose, oil, lignin, glucan, xylan, mannan, galactan, arabinan, uronic acids, and ferulates. As mentioned earlier, the portfolio of methods is
2.5. Troubleshooting and Quality Control

designed to allow accurate characterization of at least 95% of the dry weight of a biomass feedstock sample.

Each procedure in the portfolio has several levels of quality control to assess method errors and assist in troubleshooting individual measurements. SRMs are available through NIST, with values reported for all major constituents that have been determined using the portfolio methods (29). These materials were selected to represent the categories of materials expected to be used as biomass feedstocks: RM 8492 poplar (hardwood); RM 8493 pine (softwood); RM 8494 wheat straw (agricultural residue); and RM 8491 sugarcane bagasse (process residue). One of these materials should be analyzed as a sample with each batch of unknown samples. Control charting the values obtained in each step from SRMs will allow the calculation of method errors in your laboratory. These method errors can be used to set data quality specifications for both precision and accuracy. Failure to measure an accurate value for any constituent in the SRM is cause for further investigation. Data in batches where errors in determining the constituent concentration on the SRM did not meet data quality specifications should be flagged or rejected.

Chromatographic methods for quantifying acetic acid and/or uronic acids can also be calibrated to monitor carbohydrate degradation products, HMF, and furfural. Unusually high levels of degradation products in the hydrolysis liquor of the SRM are evidence for high severity in the hydrolysis step. Likely causes of excessive degradation are longer hydrolysis times, higher hydrolysis temperatures, changes in the autoclave heating and cooling profile, and acid concentrations that do not meet method specifications. Each of these errors will increase hydrolysis severity and may result in excessive degradation of the accessible sugars. Hemicellulose sugars, especially xylans and arabinans, are the most susceptible to degradation if hydrolysis severity is increased.

Chromatographic methods for the determination carbohydrates can be calibrated to quantify cellobiose. Failure to remove nonstructural materials prior to hydrolysis may result in incomplete hydrolysis of the more recalcitrant carbohydrate polymers, particularly glucans and galactans. The presence of cellobiose in the sample following dilute acid hydrolysis indicates incomplete hydrolysis. Incomplete hydrolysis results in a low bias in carbohydrate values and a high bias in lignin values, since large carbohydrate oligomers are often acid-insoluble.

Failure to regularly mix the sample as specified during the concentrated acid hydrolysis can give simultaneous evidence of excessive degradation of sugars (over-hydrolysis) and cellobiose (incomplete hydrolysis). Failure to mix the sample after dilution to 4% acid can also present the same confusing evidence.
As mentioned earlier, the hydrolysis parameters have been optimized for a −20 mesh/+80 mesh particle size cut. Finer particles may result in excessive degradation products. Hydrolysis may not be complete for larger particle sizes.

Summative mass closure below 95% is rare when each constituent measurement on the SRM meets data quality specifications. Samples with ash contents above 10% may have a low bias in carbohydrate measurements because of the presence of iron or manganese, which may catalyze degradation of sugars to materials that are not measured. Look for evidence of new peaks in the sugar chromatograms. Check for missing constituents. Have all known constituents been measured? Uronic acids and ferulates can account for 3–5% of the mass in hardwoods and grasses.

Summative mass closures above 102% are evidence of double-counting of some materials. Have protein and ash been subtracted from the acid-insoluble residue before reporting acid-insoluble lignin? Are extractives-free measurements used when reporting ash and protein? Using values determined on samples before extraction can result in double counting of soluble ash and protein, once in their respective constituent category and again as extractives. Using total extractives instead of extractives (other) in the summative mass closure calculations will count sucrose and oils twice, once in their respective constituent category and again as extractives.

Whenever possible, each measurement should be made in duplicate and two subsample replicates should be analyzed. Realistic reproducibility metrics can be determined from the SRM control charts. Good moisture measurements are essential to good mass closure since these measurements propagate through any calculation that converts concentration measurement to percent dry weight.

3. Analysis
Methods for Biomass-Derived Materials

The portfolio methods described above for feedstock analysis can be applied without much modification to biomass-derived samples from pretreatment, saccharification, and fermentation.

Extractives are assumed to be released into the liquid phase during pretreatment, and pretreatment solids are considered extractives-free. Some pretreatments may not hydrolyze all carbohydrates to their monomeric substituents. Sugars in the liquid phase following pretreatment and saccharification are analyzed twice, once to measure monomers released and again after a modified 4% hydrolysis stage to measure total sugars. The difference in the two measurements is reported as oligomeric sugars.
Standard methods for measuring carbohydrates, carbohydrate degradation products, and lignin in process samples are available on the NREL website \cite{30}. Protein contents will be elevated in the analysis of fermentation solids due to the inclusion of cell mass from ethanologens or other fermentation organisms. The consistent use of portfolio methods allows the calculation of process mass closures and provides information about the fate of each main feedstock constituent.

4. Rapid Analytical Methods

Standard wet chemical methods, although accurate and robust, were not designed for process applications because they are very expensive (labor intensive) and cannot provide the analysis information in a time frame useful for process control. For example, a complete analysis using standard wet chemical methods costs $1,000–3,000 per sample and the results are typically not available for days, sometimes weeks. In contrast, new methods are being developed that can perform the same analysis for about $20 per sample and provide results in a time frame relevant for process control, meaning that the information can be used to make the process adjustments necessary for steady-state production. One approach to reducing the time and cost of compositional analysis is the development of rapid analysis methods that use multivariate analysis software to extract chemical information from easily obtained spectroscopic data \cite{31}. Rapid analysis methods match the precision and accuracy of their calibration methods, so the savings are obtained without loss of precision or accuracy \cite{32}. New techniques, such as rapid analysis, are needed to provide analytical support for large-scale processes that convert biomass to fuels and chemicals.

In this approach, published compositional data obtained using standard wet chemical methods are used to calibrate rapid and inexpensive spectroscopic techniques, which can then be used for feedstock and process analysis. Rapid analysis is a generic term for methods that couple traditional wet chemical methods of analysis with rapid and inexpensive spectroscopic techniques. The methods described here are often further classified as rapid biomass analysis methods. Although many spectroscopic techniques can be incorporated into rapid analysis methods, for biomass analysis, near infrared (NIR) spectroscopy has several advantages over alternate spectroscopic tools. Techniques have been developed for obtaining quality NIR spectra from bulk samples, minimizing the time and expense of sample preparation. Robust NIR instruments are commercially available for process analysis, process control,
and field applications. Many of the industrial NIR techniques have been developed for use by the chemical, food processing, and agriculture industries and can be applied directly to the characterization of biomass.

Several steps are involved in rapid analysis method development. These include gathering appropriate calibration samples, chemical characterization of the calibration samples, developing spectroscopic methods for the rapid technique, projection-to-latent-structures (PLS) regression, validation of the PLS algorithm, and the development of QA/QC procedures (31).

Rapid analysis methods based on PLS multivariate modeling require calibration based on robust and accurate methods. The first step in developing a new method is to gather appropriate calibration data. Robust methods usually contain at least 100 well-characterized samples. Collecting and characterizing a good calibration set cost about $300,000. This is by far the most expensive and time-consuming step in method development. Calibration samples should have compositions similar to the samples to be analyzed. If possible, the calibration set should include samples that represent all known sources of compositional variance. The range of compositional variability within the calibration samples determines the validated calibration range for each constituent.

Quality spectroscopy is the second essential component of method development. The technique selected must contain information about the chemical composition of each sample. The spectroscopic method is the key to cost reduction and speed of analysis.

In the next step of rapid analysis method development, multivariate analysis is used to identify spectroscopic patterns that correlate with compositional data. In simplified terms, PLS analysis is used to solve hundreds of equations in thousands of variables to obtain a linear equation that predicts compositional information from spectroscopic data. Multivariate analysis is designed for complex systems such as those found in biomass compositional analysis. These powerful mathematical techniques retain the precision and accuracy of the calibration data. It is important to note that PLS analysis can never be more accurate than the methods used to obtain the calibration data (32). For this reason, the best and most accurate wet chemical techniques should be used for method calibrations. Once calibration is complete, compositional analysis becomes as fast and inexpensive as the spectroscopic method.

Figure 2 illustrates the accuracy of a rapid analysis method developed at NREL for the compositional analysis of corn stover by comparing data obtained from the portfolio methods to data obtained using the NIR-based method. The two methods agree within stated analytical errors, demonstrating that the savings in time and cost are obtained without loss of precision or accuracy.
One of the major limitations of rapid analysis methods is that an answer is always provided and the user must determine the validity of the provided data. Robust QA/QC procedures are needed to ensure that the rapid methods are appropriately applied to unknown samples (31). In addition to significant savings in time and money for routine process samples, rapid analysis methods can be used to provide levels of information that were not previously available. For example, feedstock assessment and genetic studies require the screening of hundreds, sometimes thousands, of samples. These studies would have been too costly to pursue without the savings in time and cost provided by rapid analysis methods. For example, approximately 200 biomass samples can be analyzed by a single lab worker in 1 day at a cost of less than $4,000. The ability to accurately analyze hundreds of samples for about $20 each provides a new tool that is being used to assess the compositional variability of different bioenergy feedstocks in the United States as a function of variety, geographical location, harvest time, and collection method. Changes in feedstock composition during storage are also being monitored. With these larger datasets, feedstock composition can be more accurately reported as a range of expected normal values. Variability in feedstock composition can be incorporated into process economic models to evaluate the impact of feedstock quality on biomass conversion processes.

The availability of realistic information concerning sources of variability in commercial biomass conversion processes minimizes investment risk and increases the realistic probability of achieving success in the nation’s goal of energy independence.

![Graph showing predicted vs measured for stover5c eqa](image-url)
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High-Throughput Screening of Plant Cell-Wall Composition Using Pyrolysis Molecular Beam Mass Spectroscopy

Robert Sykes, Matthew Yung, Evandro Novaes, Matias Kirst, Gary Peter, and Mark Davis

Summary

We describe a high-throughput method for estimating cell-wall chemistry traits using analytical pyrolysis. The instrument used to perform the high-throughput cell-wall chemistry analysis consists of a commercially available pyrolysis unit and autosampler coupled to a custom-built molecular beam mass spectrometer. The system is capable of analyzing approximately 42 biomass samples per hour. Lignin content and syringyl to guaiacol (S/G) ratios can be estimated directly from the spectra and differences in cell wall chemistry in large groups of samples can easily be identified using multivariate statistical data analysis methods. The utility of the system is demonstrated on a set of 800 greenhouse-grown poplar trees grown under two contrasting nitrogen treatments. High-throughput analytical pyrolysis was able to determine that the lignin content varied between 13 and 28% and the S/G ratio ranged from 0.5 to 1.5. There was more cell-wall chemistry variation in the plants grown under high nitrogen conditions than trees grown under nitrogen-deficiency conditions. Analytical pyrolysis allows the user to rapidly screen large numbers of samples at low cost, using very little sample material while producing reliable and reproducible results.

Key words: High-throughput screening, Lignin, Molecular beam mass spectrometry, Chemical composition, Cell-wall chemistry

1. Introduction

Analytical pyrolysis (pyrolysis vapors analyzed using mass spectroscopy) has been demonstrated to be a very sensitive and useful technique for analyzing plants and other biomaterials (1). The mass spectra of the pyrolysis vapors provide a chemical fingerprint


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that is useful for classifying and identifying the original material. In classical fingerprinting techniques, little or no interpretation of the mass spectral patterns is attempted, and computer-assisted techniques (chemometrics) are used to identify and classify the samples. Analytical pyrolysis is sensitive to changes in molecular and metabolite levels and cellular structure, and has been successfully used to classify microbes and other unicellular organisms (for example see (2–4)). Pyrolysis combined with gas chromatography and mass spectrometry (PyGCMS) has been used to measure lignin content and determine changes in lignin structure in biomass materials (5–12).

Pyrolysis molecular beam mass spectrometry (pyMBMS) has been used to analyze the chemical composition of many different biomass materials (13–18). The pyMBMS approach has been used to determine the cell chemistry of over 350 loblolly pine samples, and the results of the analysis were used to identify eight quantitative trait loci (QTLs) for cell-wall chemistry (19). Analytical pyrolysis has also been used to determine within-tree variation of lignin content of *Populus* (20). PyMBMS and multivariate data analysis were used to determine that the brown midrib2 (bm2) mutant of maize had reduced levels of di- and trimeric lignin derivatives. Use of this method to examine the cell-wall composition of different plant parts has revealed that the bm2 gene is important for establishing tissue-specific cell-wall composition (21). Here we describe the pyMBMS system that has been successfully used to analyze thousands of biomass samples at the National Renewable Energy Laboratory (NREL). We demonstrate this high-throughput method with the results of a poplar study containing approximately 1,500 wood samples.

In order to use analytical pyrolysis to analyze the vapors arising from the pyrolysis of biomass and make meaningful interpretations, an understanding of the processes occurring during pyrolysis is critical. Key components of the processes involved in cellulose pyrolysis have been reviewed by Evans and Milne (15). Researchers have been able to identify more than 200 unique compounds arising from the pyrolysis of wood via the reaction pathways involved in the pyrolysis of biomass. The compounds released during pyrolysis depend on many factors, including the variety, developmental stage, growing location, and anatomical features. Other factors such as the thermal history during pyrolysis can also account for the large number of compounds that may be present.

The primary pyrolysis pathways for pure cellulose entail sequential depolymerization reactions of cellulose (by either free-radical or heterolytic pathways) to form high yields (60%) of levoglucosan as shown in Scheme 1a. The pyrolysis spectrum of pure cellulose has major peaks at \( m/z \) (mass to charge ratio) = 162.
and \( m/z = 144 \) due to levoglucosan and its ionization fragment, respectively. During cellulose pyrolysis, the majority of contributions to intensities at locations of \( m/z = 57, 60, 73, \) and 98 have also been shown to arise from electron ionization (EI) fragments of levoglucosan. The homogeneity of pure cellulose allows high levoglucosan yields.

Although biomass may have a high cellulose content (~50%), the levoglucosan yields during biomass pyrolysis are low. When small amounts of alkali metals (0.1, wt%) such as sodium and potassium are added to cellulose, the formation of levoglucosan during pyrolysis is inhibited. The resulting products consist of furfural derivatives (\( m/z = 126, 100, 96 \)), low molecular weight carbonyl compounds represented by \( m/z = 43 \), and high molecular weight condensables. Scheme 1b shows the alkali-metal-catalyzed reaction pathway. These two pathways (depolymerization and alkali-metal catalyzed) are also important during the pyrolysis of lignin and carbohydrates such as xylan found in the hemicellulose in biomass.

The pyrolysis spectra of biomass materials contain peaks that can be assigned to the different classes of polymers found within the biomass materials. Spectra of poplar ball-milled lignin, xylan, and cellulose are shown in Fig. 1a–c, respectively.
The peaks labeled in the spectra are peaks that have minimal overlap with other cell-wall components. These unique signature masses also allow us to quickly determine the distribution of lignin, cellulose, and hemicellulose within a biomass sample. For example, the amount of lignin in the cell wall can be estimated from the peaks $m/z = 120, 124, 137, 138, 150, 152, 154, 164, 167, 178, 180, 181, 182, 194, \text{ and } 210$ relative to the carbohydrate peaks $m/z = 114$ (hemicellulose), 98, 126, and 144 (cellulose).

Fig. 1. Representative spectra ($m/z$ 50–450) of (a) poplar ball-milled lignin, (b) xylan, and (c) cellulose. Prominent peaks associated with lignin are listed in Table 1.
Minimal sample preparation is required for analytical pyrolysis experiments. Samples generally consist of ground biomass materials, although leaf punches, matchsticks, and other un-ground materials can be used as long as they can fit within the sample holder. Samples can be extracted using standard procedures to remove low molecular weight materials and other extraneous materials that can interfere with the determination of structural cell-wall components. This can be important when analyzing herbaceous materials that have high extractives contents.

PyMBMS requires small sample amounts to obtain cell-wall chemistry information. The amount of biomass material required depends on the number of replicates desired. The typical biomass sample amount used in our laboratory for pyMBMS is approximately 4 mg/analysis, with all samples run in duplicate (8 mg total sample needed). However, we have successfully analyzed biomass samples as small as 0.1 mg (1/8 in. circular leaf punch) in the past. The pyrolysis system described later in this chapter can be configured to accept several different orifice sizes to maximize the sensitivity of the equipment.

Samples materials such as ground biomass are prepared by placing them into the 80-μl stainless steel sample cups of a commercially available autosampler (Frontier Ltd). The amount of material required varies greatly depending on the sample of interest. Typical biomass samples such as *Eucalyptus*, loblolly pine, or corn stover consist of ~4 mg of milled −20/+80 mesh material. Pure lignin samples and chemical compounds require smaller amounts, as pyrolysis occurs faster and saturation of the instrument can occur with large sample sizes. All samples are randomized throughout the experimental run to eliminate bias due to spectrometer drift.

Due to the high gas flow used in these experiments, a glass fiber filter disc is used to keep the sample material from blowing out of the sample cups. Type A/D glass fiber filter material with no binder is recommended for use in these experiments. After the sample material is placed in the sample cups, the glass fiber filter disc is inserted and pushed firmly into the cups. It is imperative that the glass fiber filter disc completely covers the sample material. If the sample material blows out of the sample cup, the pyrolysis spectra can be affected and transfer lines can become plugged with unreacted biomass materials (see Note 1).

Standard materials should be included in every experimental run. Standard materials serve as an experimental control and provide vital information about the consistency of the mass spectrometer
as well as a means for judging chemical differences present in the samples of interest. The standard materials should be prepared in a similar fashion to the materials being analyzed, and cell-wall chemistry traits should be determined before use. Typically, 10% of the samples in each experimental run are standards with varying chemical composition. An excellent source of standard biomass material is the National Institute of Standards and Technology (http://www.nist.gov). Standard biomass materials include radiata pine (8,493), poplar (8,492), wheat straw (8,494), and sugarcane bagasse (8,491).

The following gases are required during experimental runs:

- Ultrapure helium.
- Nitrogen.

Two compressed gases are used when running pyMBMS experiments. Ultrahigh purity helium from a gas cylinder is used as a carrier gas for pyrolysis vapors. Typical flow rates are 2 l/min through a ruby orifice of 0.012 in. diameter. Nitrogen gas is also used as an ejection gas for the sample cups. The mass spectrometer used in this particular system is capable of scanning \( m/z \) 10–720. However, using nitrogen as an ejection gas causes the range to be limited to \( m/z \) 30–720 to minimize interference from any nitrogen carried over into the mass spectrometer.

In order to incorporate a commercially available autosampler onto the molecular beam mass spectrometry (MBMS) system, several parts were fabricated and modified. A Frontier model PY-2020 iD autosampler used to automatically change samples was interfaced with the molecular beam mass spectrometer using a custom face plate. Swagelok fittings were used to connect the autosampler to the MBMS through a 1/8 in. transfer line to the MBMS face plate. A crystal orifice (Bird Precision) was mounted into a ¼-in. stainless steel tube. A ¼-in. Swagelok male fitting was welded onto the faceplate to allow the ¼ in. orifice tube to be connected to the transfer line. Incorporating the exchangeable crystal orifice into the new faceplate allows the orifice size to be adjusted quickly for different sample sizes. **Figure 2** shows an experimental schematic of the different components.

The Frontier autosampler was originally designed to use a 1/16 in. transfer line. However, the carrier gas flow rate and sample size that are used at the NREL are greater than normal, and we made several modifications to accommodate the higher flow rate and sample size. The transfer line connection after the pyrolysis oven was bored out to 1/8 in. and modified to accept a 1/8-in. Swagelok fitting. This allowed higher carrier gas flow rates with little increase of backpressure inside the pyrolysis tube.
The use of a larger transfer line decreased the chance for clogging due to condensing pyrolysis gases. An in-line glass wool filter was included in the transfer line to prevent small pieces of biomass from clogging the orifice.

### 3. Methods

#### 3.1. Pyrolysis Molecular Beam Mass Spectrometry

A custom-built molecular-beam mass spectrometer using an Extrel Model TQMS C50 mass spectrometer was used for pyrolysis vapor analysis (15, 22). Minor modifications were made to incorporate the autosampler inlet pyrolysis system. Ground biomass samples are introduced into the Frontier autosampler with helium flowing through at 2 l/min (at STP). The autosampler furnace was electronically maintained at 500°C, and the interface was set to 350°C. The 1/4-in. transfer line was wrapped in heat tape and heated to approximately 350°C measured with thermocouples (see Note 1). The total pyrolysis time was 2 min, although the pyrolysis reaction was completed in less than 20 s.

The system does not separate individual components using chromatography but collects the total ion current so that all the chemical information is contained in a single mass spectrum to increase throughput. The pyrolysis mass spectra provide a fingerprint of the cell-wall chemistry that can be used to obtain quantitative chemical information. Mass spectral data analysis comprises the following methods: (1) using multivariate statistical methods...
to select cell wall chemistry phenotypes that significantly differ from cell-wall chemistry of controls; (2) developing partial least squares (PLS) calibrations for well-characterized materials for which traditional wet chemical analyses are available; and (3) estimating changes in concentration or structure of cell-wall components from peak intensities.

### 3.2. Experimental Reproducibility and Quality Assurance

Standards from aspen (*Populus tremuloides*) and a loblolly pine (*Pinus taeda*), known to represent differences in syringyl to guaiacol (S/G) ratio and lignin content, were analyzed along with the samples of interest to measure repeatability within the experiment. We analyzed these internal standards periodically throughout the run (four standards per 48-sample tray) to monitor spectrometer drift and to identify whether other instrumental problems occurred during the analysis. The standard runs in all experiments had excellent reproducibility and showed little to no drift (see Notes 2 and 3). Pooled standard deviations ($s_p$) for the duplicates in each pyMBMS run were calculated using the formula:

$$S_p = \left( \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2 + \ldots + (n_k - 1)s_k^2}{n_1 + n_2 + \ldots + n_k - k} \right)^{1/2}$$  \hspace{1cm} (1)

where $1, 2, \ldots, k$ refer to the different series of measurements, $s_k$ is the standard deviation for each set of measurements, and $n_k$ is the number of measurements in each series (23). Historical data compiled over a 1-year period for lignin content yields a pooled standard deviation of ±1% of the total lignin content based on internal standards.

### 3.3. Data Analysis and Processing

#### 3.3.1. Data Processing

Each MBMS spectrum was averaged and the background was removed using the Merlin Automation Data System version 2.0 software. We then imported the raw data into Microsoft Excel and formatted it for analysis. We used the Unscrambler version 9.7 software program (CAMO A/S, Trondheim, Norway) to normalize the data based on total ion content to eliminate differences due to variation in sample weights. Principal component analysis (PCA) was performed to determine whether the standard samples have different patterns of variation separating them into distinct groups and to provide loading coefficients for the principal components (PCs).

#### 3.3.2 Data Analysis

Data analysis was performed using PCA with the Unscrambler. This statistical method relies on projecting data points on a new set of (orthogonal) axes that are defined in such a way that variation between groups is maximized, while variation within groups is minimized. Johnson and Wichern provide a good reference book on PCA and other multivariate statistical techniques, such as discriminant analysis and PLS (24). PCA can dramatically reduce
the dimensionality of the spectral data. This is achieved by defining a small set (<10) of new variables that are linear combinations of correlated original variables (peak intensity). The data can be visualized in a PCA score plot in which the calculated values for (typically) two PCs are plotted for a group of samples. The basis for the separation of the samples can be determined on the basis of a so-called PC loading, which displays the importance of individual variables that contribute to a given PC. Variables with positive coefficients are positively correlated, and variables that have negative coefficients are negatively correlated. In this manner, PCA is used to identify subtle differences in the MBMS spectra that are difficult to distinguish visually.

The intensities of the major peaks assigned to lignin were summed in order to estimate the lignin contents across the range of samples (Table 1). Lignin peaks with m/z = 120, 124, 137, 138, 150, 152, 154, 164, 167, 178, 180, 181, 182, 194, and 210 were summed and then averaged for the different samples (Table 1). S/G ratios were determined by summing the syringyl peaks at 154, 167, 168, 182, 194, 208, and 210 and dividing by the sum of guaiacol peaks at 124, 137, 138, 150, 164, and 178 (Table 1). Several lignin peaks were omitted in the syringyl or guaiacol summations due to individual peaks having associations with both S and G precursors.

Lignin values estimated using pyMBMS in the study were corrected to approximate Klason lignin values by the following procedure. Klason lignin values were determined for a National Institute of Standards and Technology sample (NIST 8492: *Populus deltoides*) at NREL. Multiple pyMBMS spectra of NIST 8492 were averaged, and lignin was estimated by summing the peaks in the previous paragraph. A correction factor was then determined by dividing the Klason lignin value by the lignin value determined by pyMBMS. We then used this correction factor to correct the remaining samples to values that are comparable to Klason lignin values.

The high-throughput analytical pyrolysis instrument was used to screen samples obtained from large populations of poplar, eucalyptus, and maize samples. Here we describe the data collection and analysis of a large poplar set provided by the Forest Genomics Laboratory from the University of Florida. These poplar trees are the progeny of a cross between a *Populus trichocarpa* x *Populus deltoides* hybrid (clone 52–225) and a *P. deltoides* pure genotype (clone D124). A set of 396 genotypes from this pedigree was clonally replicated six times to accommodate a greenhouse experiment with two nitrogen treatments (0 and 25 mM of NH₄NO₃) and three biological replicates in an incomplete block design. Xylem samples from two biological replicates of this experiment
(~792 samples for each nitrogen treatment) were ground and analyzed with two technical replicates in the pyMBMS at NREL. The ultimate objective of this study is to identify genes regulating carbon partitioning among wood chemicals and allocation among plant organs in poplar. The analytical data provided by the pyMBMS constitutes the carbon partitioning phenotypes that are being used for identification of genomic regions (QTL) associated with phenotypic variability in wood composition. The cell-wall phenotypes determined by the high-throughput analytical pyrolysis method include S/G ratio, lignin content, as well as the individual peak intensities for peaks previously assigned to lignin, cellulose, and hemicelluloses. The wood chemistry phenotypes

Table 1  
Peak and precursor assignments in mass spectra of lignified samples

<table>
<thead>
<tr>
<th>m/z</th>
<th>Assignment</th>
<th>Syringyl (S), para-hydroxy (H), or guaiacyl (G) precursor</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>Phenol</td>
<td>H,S,G</td>
</tr>
<tr>
<td>120</td>
<td>Vinylphenol</td>
<td>H</td>
</tr>
<tr>
<td>124</td>
<td>Guaiacol</td>
<td>G</td>
</tr>
<tr>
<td>137^b</td>
<td>Ethylguaiacol, homovanillin, coniferyl alcohol</td>
<td>G</td>
</tr>
<tr>
<td>138</td>
<td>Methylguaiacol</td>
<td>G</td>
</tr>
<tr>
<td>150</td>
<td>Vinylguaiacol</td>
<td>G</td>
</tr>
<tr>
<td>154</td>
<td>Syringol</td>
<td>S</td>
</tr>
<tr>
<td>164</td>
<td>Allyl- + propenyl guaiacol</td>
<td>G</td>
</tr>
<tr>
<td>167^b</td>
<td>Ethylsyringol, syringylacetone, propiosyringone</td>
<td>S</td>
</tr>
<tr>
<td>168</td>
<td>4-Methyl-2,6-dimethoxyphenol</td>
<td>S</td>
</tr>
<tr>
<td>178</td>
<td>Coniferyl aldehyde</td>
<td>G</td>
</tr>
<tr>
<td>180</td>
<td>Coniferyl alcohol, syringylethane</td>
<td>S, G</td>
</tr>
<tr>
<td>182</td>
<td>Syringaldehyde</td>
<td>S</td>
</tr>
<tr>
<td>194</td>
<td>4-Propenylsyringol</td>
<td>S</td>
</tr>
<tr>
<td>208</td>
<td>Sinapylaldehyde</td>
<td>S</td>
</tr>
<tr>
<td>210</td>
<td>Sinapylalcohol</td>
<td>S</td>
</tr>
</tbody>
</table>

^a Ref. (15)  
b Fragment ion
generated with pyMBMS will be combined with gene expression data and assayed with microarray in a subset of the progeny to aid in identification of candidate genes for the regulation of carbon partitioning.

**Figure 3** shows representative mass spectra of samples that were grown under deficient and luxuriant nitrogen conditions (0 and 25 mM of NH₄NO₃, respectively). The analytical pyrolysis showed that samples receiving no nitrogen treatment (**Fig. 3a**) have a higher lignin content than samples that received the higher nitrogen treatment (**Fig. 3b,c**).

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![Mass spectra](image-url)

**Fig. 3.** Representative spectra (m/z 50–450) of populus samples receiving different nitrogen treatments: (**a**) low nitrogen treatment (**b**) high nitrogen treatment (**c**) high nitrogen treatment. Prominent lignin peaks (see Table 1 for assignments) are listed, indicating that samples receiving the low nitrogen treatment have less lignin than those with high nitrogen treatment.
The analytical pyrolysis experiment also showed that there was more cell-wall chemistry variation in the high-nitrogen-treated samples. Figure 4 shows the result of a PCA of the mass spectra of the poplar samples. The plot depicts PC1 vs. PC2 scores of the pyrolysis vapor mass spectra. PC1 and PC2 explain 64% and 13% of the variation in the mass spectral dataset, respectively. Comparison of PCA scores confirms the high variability in cell-wall chemistry of plants under high nitrogen treatment, as shown by the larger amount of scatter along PC1 when compared to trees grown under nitrogen-deficiency conditions. The PC1 loading plot (Fig. 5) indicates that the samples grown with high nitrogen supply have lower lignin contents than the trees grown with no nitrogen fertilization. The loadings associated with the samples grown under higher nitrogen treatment consist of masses

Fig. 4. Principal component 1 (PC1) vs. principal component 2 (PC2) scatter plot of lignin variability as a function of nitrogen treatment for 1,500 populus trees. Samples with the low nitrogen treatment are denoted by filled circles, and samples with the high nitrogen treatment are denoted by open circles.

Fig. 5. PC1 loadings for plot shown in Fig. 3.
assigned to carbohydrate cell-wall components, whereas the negative loadings associated with the trees grown under nitrogen deficiency consist of masses assigned to lignin (Table 1).

Corrected lignin values using the procedure described in **Subheading 3.3.3** ranged from approximately 13–28% for all the samples analyzed. The S/G ratio measured from the pyrolysis spectra ranged from 0.5 to 1.5 among all the samples. This study demonstrates the ability of pyMBMS to be used as a high-throughput method for screening biomass for chemical composition differences.

### 3.4. Summary

The autosampler pyrolysis system coupled with a molecular beam mass spectrometer has multiple applications for biomass characterization, and facilitates the simplicity of data analysis and the consistency of the data produced. This method allows the user to screen large numbers of samples rapidly and at low cost, using very little sample material while producing reliable and reproducible results.

### 4. Notes

1. The temperature of the transfer line must be carefully monitored. During our original research and design of the high-throughput system, the temperature of the transfer line was maintained at ~400°C. The higher temperature coupled with the longer residence time caused secondary cracking of the high molecular weight ions and resulted in a spectrum that did not consist of primary pyrolysis products. The secondary cracking of the pyrolysis vapors can affect the lignin and S/G estimates by fragmenting into products that are not easily identified as lignin.

2. Ideally, pyMBMS experiments are run on a single day. However, with high-throughput screening, it is not always possible to run an entire experiment in a single day. This can introduce additional variation into an experiment due to day-to-day instrumental drift. Instrumental drift may occur because of the transfer line getting coated with pyrolysis products, the orifice becoming clogged, or the mass spectrometer quadrupole rods becoming fouled with pyrolysis products. It is possible to combine multiple experimental runs for data analysis if there is very little experimental drift. For example, the data presented in this chapter were run over the course of 2 weeks with multiple days of cleaning included.

3. Current experimental conditions allow approximately 1,750 samples (~800 samples in duplicate plus standards) to be run before the equipment needs to be cleaned due to build up of pyrolysis oils. The typical high-throughput experimental
run involves 4 days of running samples and 1 day reserved for cleaning.

Acknowledgments

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References


Chapter 13

Preparation and Analysis of Biomass Lignins

Alicia L. Compere and William L. Griffith

Summary

Lignin, comprised primarily of three randomly polymerized phenylpropenyl monomers, is, arguably, the second most common organic molecule on earth. In current biorefinery applications, lignin is burned, usually in concentrated pulping or hydrolysis liquor, as a source of process steam and both internal and exported electricity. The aromatic content of lignin makes it a potentially attractive feedstock for high-value aromatic chemicals, polymers, and carbon products (graphite, activated carbon, and carbon fiber). Revenue from production of lignin-based chemicals could play a major role in biorefinery profitability if cost-effective methods for lignin separation and purification can be developed. This chapter presents descriptions of methods for assessing and purifying biorefinery lignins so that they can be evaluated for use as feedstock for production of chemical products. Areas covered are: (1) initial evaluations of as-received lignin samples (visual, microscopic, separable organics); (2) analysis of common contaminants (bulk and filterable ash and particulate contaminants in liquid and dry lignin samples); (3) preparation of lignins for experimental use as chemical feedstock (prefiltration, filtration using bench-scale chemical apparatus and larger scale bag filters, one-step lignin precipitation, two-step carbohydrate and lignin precipitation, desalting of dry powdered or precipitated lignin, and lyophilization). These methods have been used successfully at the bench scale to produce the 1–50 kg amounts of wood and grass lignins typically required for bench-scale assessment as chemical feedstocks.

Key words: Lignin, Biomass, Separation, Analysis

1. Introduction

Lignin, cellulose, and hemicellulose, as the three major components of biomass, are the most common organic chemicals on earth. The relatively consistent structure of cellulose makes it possible to use a wide variety of different types of feedstock (for example, seasonal grasses, wood, or stover) for ethanol production.
Most cellulosic biorefinery processes have focused on the utilization of cellulose and hemicellulose as feedstock for fermentable sugars and have relegated lignin to use as a process fuel.

Lignin from pulping streams has long been used as a process chemical or additive (1). However, the variable character of lignin, combined with the difficulty of purification, has limited progress in commercializing processes in which lignin is converted to a chemical feedstock. With the advent of biorefineries, it has been recognized that use of biorefinery lignin as a chemical feedstock could provide significant value. A U.S. Department of Energy (DOE) process economics team has estimated that the projected production of 60 billion gallons of cellulosic ethanol would result in the coproduction of ~225 million tons of biorefinery lignins (2). According to Bozell and coworkers, the resulting biorefinery lignin could be used to produce $11 billion of power (wet combustion of process liquors); $24 billion of alcohols (gasification plus catalytic conversion); $31 billion as mixtures of benzene–toluene–xylene (BTX) aromatics and alcohols, or as carbon fiber; or $35 billion as an optimized product mix of carbon fiber, BTX, and alcohols. The potential value of biorefinery lignins will likely be higher than the values estimated because these authors used conservative estimates based on low-purity lignins.

Although production of syngas will not require extensive purification of lignin, production of aromatics and carbon fiber, like most industrial chemical processes, will require a reasonably clean, consistent, and predictable starting material. The biorefinery industry is at an early stage in which bench-scale development of processes such as chemical conversion of lignins is anticipated. This chapter is a discussion of laboratory-scale methods for purifying biorefinery and wood lignins as chemical feedstock materials. The process begins with initial receipt and assessment of crude lignins in the form of dry powders, pastes, or liquors and covers purification, recovery, assessment, and some analytical methods critical to lignin for chemical feedstock use. At present, lignins are recovered from waste process liquors. If lignin use becomes economically feasible, it may be desirable to recover lignin from other process streams.

Detailed literature references covering lignin biosynthesis, structure, cell integration, and analysis are available. Typical methods include those for evaluating lignin content and structure in non-wood pulps (3), wood (4), cell structure (5), animal fodder (6, 7), or human diet (8–10).

In assessing biorefinery lignins as chemical feedstock, it is important to remember that recovered lignins are process by-products which need to be processed in order to obtain a reasonably consistent, relatively pure chemical process feedstock. Variations in feedstock species, collection or storage times, and hydrolysis methods are expected to produce variations in the
composition, molecular weights, contaminants, and overall processability of biorefinery lignins. Additionally, a number of contaminants are introduced into conventional pulping and biomass lignins by the methods used to recover lignins from liquors. Typically, lignins are precipitated as gels, filtered, possibly rinsed, and dried. A number of different materials (fibers, soil, carbohydrates, waxes, alcohols, resins) are swept out of solution with the lignin precipitate and thereby concentrated in the dry lignin powder. Any nonvolatile salts in the solution from which lignin is precipitated are also concentrated as the lignin dries.

Moderate amounts, typically kilograms of a given lignin, will likely be required to support systematic studies of its suitability for a given use and it may be necessary to process several times as much starting material to obtain a product with the needed purity. The first step is to determine approximately how much starting material will be required for initial studies and which contaminants are likely to interfere with the intended use of the lignin. For carbon fiber preparation and for chemical synthesis to produce aromatics, the contaminants of concern will likely include soil (sand, clay particles), cell-wall fragments and other fibrous materials, carbohydrates, cutin (long-chain waxes, alcohols, and fatty acids), extractives (resins, rosin, or fatty acids), and salts. These are removed to minimize both point defects caused by particulates or low-density areas in the final fiber contaminants (sand, fiber, cell wall fragments) which could plug multifilament fiber spinning dies (11–13). Maintenance of a sharp melting point requires minimization of cutin, extractives, and other materials which affect rheology and phase behavior. For aromatic synthesis, the major concern is minimization of materials which would interfere with catalytic processing and, if used, hydrogenation. Undesired materials will include oxygenated organics which react with hydrogen (carbohydrates, cell-wall fragments, cutin, extractives) and catalysts which might compete with selected process catalysts (clays and silica, possibly salts). It is important to remember that lignin has the potential to be used as a feedstock in many different chemical syntheses and that each type of processing could require assessment and removal of different types of contaminants.

The next step is initial assessment of the as-received lignin, which may be in the form of a liquor, paste, or dry powder. This process is relatively quick and is primarily intended to give an assessment of initial purification requirements for the sample.

The contaminants in lignin can then be assessed using relatively straightforward tests and a scheme for lignin purification developed. Evaluations which will be discussed vary with lignin and intended use but include (1) filterable and bulk contaminants, (2) salt and ash, (3) moisture and volatiles, (4) cutin and extractives, and (5) carbohydrate content. Analytical and purification methods will be discussed for alkaline lignins from wood and
grass. Variations for other types of lignins will be discussed under Subheading 4.

2. Materials

Lignins purified from several commercial and research materials included: (1) Indulin AT dry powdered lignin (Mead–Westvaco), (2) PC-1369 hardwood dry powdered lignin (Mead–Westvaco), (3) Sarkanda dry powdered grass and flax lignins prepared with a no-sulfur-added pulping process (Granit), (4) dry powdered grass and flax lignin (Granit), and (5) various Kraft and NaOH pulping liquors prepared from hardwood and softwood (Weyerhaeuser and North Carolina State University). Materials used in the carbohydrate separation included (1) Kelco Alginate (Merck), (2) Kelco XANFLOOD xanthan gum (Merck), (3) Catiofast polyethyleneimine (BASF), (4) a variable-speed Lightnin mixer, model L5U10F with a propeller-type impeller <100 mm in diameter (VWR Scientific), and (5) stainless steel 20-l blow case (Millipore). Foam control used a combination of Antifoam A (Dow Corning) and P-4000 polypropylene oxide (BASF). Filtration media and equipment included (1) Whatman #2 and VWRbrand 413 papers, Nalgene 2234-0030 or 2234-0050 polyethylene wide-mouth carboys, large Buchner funnels, and digital conductivity meter (VWR Scientific Products); (2) polypropylene filter felt, bag filters (McMaster-Carr); and (3) stand-alone stainless steel bag filter housing (McMaster-Carr). Dried lignins were prepared using a #5006 lyophilizer (Refrigeration for Science).

3. Methods

In most biorefinery streams, by-product lignins are produced in the form of concentrated liquors which are combusted to produce process steam or electricity. Some biorefineries may be able to provide dry powdered lignins for evaluation, while others will send as-processed or concentrated (sometimes almost pasty) liquors. Without filtration, these materials will contain significant amounts of contaminants. Analysis of the lignin starts with assessment of the as-received material and moves through preparation and analysis.

3.1. Initial Evaluation of as-Received Lignin

Contaminants in lignins typically come from the cellulosic material (cell fragments and fibers); growing, harvesting, and handling techniques (sand and soil); and compounds, such as resins, cutin, and suberin, peculiar to different types of plants. In some
cases, contaminants can be derived from unfiltered process water (diatoms) or process chemicals. In order to determine how to remove these contaminants from the lignin, it is important to consider both the pretreatment technologies (feedstock, size reduction, fiber separation) and the overall biorefinery process itself (steam explosion, alkaline pulping).

1. Inspect the lignin sample. Is the lignin a powder or a liquid? Can the material be mixed to provide a representative sample or is it separated into layers or clumped on the wall of the container? Have inorganic materials precipitated at the bottom of the sample?

2. If it is possible to obtain a reasonably representative sample of the lignin, assess it visually and microscopically to get an initial estimate of particulate contaminants and an idea of whether they are large enough to be removed by filtration (see Note 1).

3.1. Visual assessment of lignin, initial sample

3.1.2. Visual and microscopic estimate of particulate contaminants (see Notes 2–4)

1. If the lignin was obtained as a dry powder, mix the powder and take a representative sample.

2. Weigh approximately 100 mg of the sample, place it in a 12 × 100 mm test tube, and add 5 ml of 0.1 N NaOH.

3. Cap the tube and invert several times to wet and dissolve the powdered lignin. If the lignin does not dissolve, see Notes 6 and 7 for recommendations on dissolving lignin.

4. If the lignin was received as a liquid or paste, remove a representative sample and place ~0.5 ml in a 12 × 100 test tube.

5. Add 4.5 ml of 0.1 N NaOH, cap the tube, and invert several times to mix the two solutions.

6. Refrigerate the sample upright for several hours or overnight.

7. Without stirring the sample, look at the tube carefully. Particulates will generally settle into a layer at the bottom of the tube, and high-melting organic materials such as cutin, waxes, resins, and saturated fatty acids will segregate or precipitate on the side of the tube or the top of the liquid, typically as a thin ring or as droplets. Using a long Pasteur pipette, carefully remove a small amount of debris from the bottom of the tube, place a drop on a microscope slide, and examine it in transmitted light at 400×. (As a rule, particulates visible at 400× can be removed using a 1-µm absolute filter.) Examine the specimen to determine whether particulate matter is primarily cell-wall fragments, fiber, sand, soil, or a mixture of different materials (see Notes 5 and 6).

8. If only a small amount of particulate material is apparent, the lignin can be dissolved or diluted and filtered to remove contaminants. If there is a relatively large amount of particulate material, multistep filtration will likely be required.
If the sample contains a separated organic layer or organic droplets, it will be desirable to remove this material prior to further lignin purification. Two types of biorefinery feedstock are known to have significant quantities of separable organics. Grasses, reeds, and waxy plant stems contain cutin, a wax-like material comprised of long-chain fatty acids, alcohols, and waxes. Cutin coprecipitates with lignin and can constitute a significant fraction of recovered lignin. Softwood contains tall (pine) oils, rosins, and other extractives (see Notes 7 for cutin and tall oil removal strategies).

Nuclear magnetic resonance analysis of recovered droplets or organic layers may be of value in determining the structures of constituents. This is useful in planning separations.

The goal of initial evaluation of lignin samples is to compile information about the sample in a short time so that decisions concerning the processing method can be made. If the lignin sample was received as a paste or concentrated liquor, it may be worthwhile to determine whether a small sample can be air-dried (see Note 8.)

1. Stir the lignin paste or liquor.
2. Place a representative sample of 5 g in a clean, preweighed porcelain crucible in air.
3. Loosely cover it with filter paper to prevent dust from settling in the sample and see if it will dry. (This may require several days.)
4. When the sample appears dried, reweigh it.
5. Inspect the sample closely to see if it contains any large biomass or fiber fragments that will need to be removed by prefiltration or prescreening.
6. Dry the sample in a 105°C drying oven for several hours or overnight, cool the watch glass, and reweigh the sample. Based on the initial sample weight, calculate the fraction dry weight in air and at 105°C.
7. If the fraction dry weight is <0.2, it will likely be advantageous to precipitate and dry the liquor or paste for use as a feedstock.

A good understanding of the “removable” contaminants in lignins will be useful in evaluating samples, assessing purification, and designing lignin recovery and purification processes. In most cases, both the bulk (distributed throughout the lignin, can include salts) and filterable (insoluble particulates 1 μm or larger removed by filtration) contaminants in lignins are evaluated. The analytical methods used are rapid, inexpensive, and simple (see Notes 9–12).

Removable contaminants in lignins may include salts (as ash), clay, sand, cell fragments, fiber, carbohydrates, and other materials.
The general method for evaluating contaminants involves weighing a given amount of a representative sample of lignin in a preweighed container (aluminum, ceramic or platinum dish); heating the container to 105°C and holding it for a period of at least 1 h; cooling and reweighing the container and sample. The weight difference provides a measure of materials, such as free water, that evaporate or boil at or below 105°C. The container and sample are then placed in an oven or muffle furnace, heated to 600°C and held for a period of at least 1 h, and again cooled and weighed. The material remaining in the sample is primarily ash. The difference between the weights at 105°C and the weight at 600°C is “volatiles,” which are primarily organic materials such as biologicals (fiber, biological polymers, plant waxes, etc.) and small organic molecules.

There are a number of different ways in which this sort of test can be used to assess contaminants in lignins. Three of the most common are discussed below.

1. A scale reading to 0.1 mg is required for this method.
2. Label, dry, and preweigh aluminum weighing pans (or ceramic dishes) in which to place lignin samples. Prepare ~20% more sample pans than needed for the lignin samples and replicates. The weight of aluminum weighing pans may change because of surface oxidation or volatilization of surface organics. Empty pans are run along with samples to permit correction for weight changes by the pan.
3. Take representative lignin samples and weigh out a consistent amount directly into each preweighed aluminum dish. (For conventional aluminum weighing pans, this is ~0.5–1 g of lignin, or enough to provide an even and thin layer on the bottom of the pan).
4. Place the samples in a prefired aluminum baking dish and put them in a 105°C oven. Leave them at 105°C until the weights of the cooled samples become constant. Although this will vary depending on the preparation, contaminants, and particle size of the lignin, usually at least 2 h will be required and, where possible, the time may be extended to overnight.
5. Cool and weigh the samples and record the weights.
6. Then place the pans and samples back in the aluminum baking pan and put the pan in a cold muffle furnace or oven.
7. Heat the samples in air from room temperature to 600°C and hold them at 600°C for at least 1 h to ash the lignin and other organic materials.
8. At the end of 1 h, open the oven and look at the samples. If black fibers, droplets, or grains are seen in the samples, these are probably due to conversion of lignin to carbon. If this is the
When the samples appear to have fully ashed, cool the pans, reweigh the samples, and record the values.

Calculate the weight changes in samples, adjusting the values for changes in empty weighing pans (see Note 15).

The initial weight loss between room temperature and 105°C is due to evaporation of water, low-boiling volatiles, and some volatile acids and salts. A value on the order of ~10% of the sample weight is expected for commercial dry powdered lignins.

The weight loss between 105°C and 600°C is due primarily to combustion of biopolymers (lignin, carbohydrate polymers, proteins, cellulosic fiber) and volatilization of high-boiling organics (lignin monomers, dimers, resins, rosins, cuticle wax) in the sample.

The materials remaining after firing at 600°C include most inorganic salts; carbonates, oxides, and hydroxides of inorganic cations; minerals (silicophosphates, sand, clay); skeleton and exoskeleton minerals (which may become powdered); and carbon (carbon, carbon fiber).

The final chemical composition of the ash may depend on the firing schedule and time at temperature (for example, some materials form carbon which slowly burns off at 600°C and some multivalent cations form different minerals depending on firing schedule and time held at the final temperature).

Label, dry at 105°C for at least 1 h, and preweigh aluminum weighing pans and 47-mm Gelman A/E glass fiber filters. Use a scale capable of weighing to 0.0001 g for all weights, prepare ~20% more glass fiber filter–weighing pan combinations than needed, and record the weights.

Weigh 0.5–1.0 g taken from a representative sample of each lignin into a small beaker.

Dissolve the lignin in at least 25 ml of 0.1 N NaOH. (More NaOH can be used if the lignin does not dissolve.) A Teflon-coated magnetic stirring bar and a magnetic stirrer are valuable aids in dissolving the lignin sample.

Carefully center a glass fiber filter in a standard filter assembly using flat filter forceps.

Place the top on the filter assembly and place the assembly on a filter flask.

Wet the filter with distilled water and turn the vacuum on.

Pour or pipette the dissolved lignin into the filter assembly.

When all of the liquid has passed through the filter, wash the assembly and filter down with a small amount of distilled
water to ensure that all solids are transferred to the filter and that the NaOH is rinsed out of the filter.

9. Turn the vacuum off, let air into the filter flask, and remove the upper portion of the filter assembly.

10. Using flat filter forceps, carefully remove the filter from the filter assembly and place in the sample pan.

11. Carefully observe the filter holder and the filters in weighing pans. Discard and remake any samples that appear to have suffered handling damage, as even a small amount of damage to a filter can result in a change in weight of 0.0001 g or more.

12. Blanks are prepared using the same procedure as the samples. It is important to pass the same amount of NaOH and distilled water rinse through the filters, as this may appreciably change the weight of the filters.

13. Following the same schedule and procedure as described above for bulk contaminants, dry the samples (pans + filters, placed in an aluminum baking pan) for at least 1 h or overnight at 105°C.

14. Weigh and record the weights of samples and blanks.

15. Then place the pans and samples back in the aluminum baking pan and put that pan in a cold muffle furnace or oven. Heat the samples in air from room temperature to 600°C and hold them at 600°C for at least 1 h to ash the lignin and other organic materials. At the end of 1 h, open the oven and look at the samples. The appearance of black fibers, droplets, or grains in the samples is probably due to conversion of lignin to carbon; close the oven and heat them for a longer period to finish oxidation of the carbon.

16. When the samples appear to have fully ashed, cool the pans, reweigh the samples, and record the values.

17. Calculate the weight changes in samples, adjusting the values for changes in blanks.

18. It may also be desirable to obtain micrographs of as-filtered particulates (see Note 16).

3.3. Preparation of Lignin for Experimental Use

After completion of initial evaluations of the lignin, a decision on further purification, if required, can be made. Depending on the properties needed for the desired use, different preparation methods will be appropriate. The methods discussed below will include prefiltration, filtration, one-step lignin precipitation, two-step carbohydrate and lignin precipitation, desalting of powdered dry lignin, and lyophilization of lignin.

3.3.1. Prefiltration

Particulate contaminants may constitute 10% (or substantially more) of a biomass lignin sample. In many cases, the bulk of particulate material is relatively large – 10 µm or better – fiber
and cell-wall fragments. When this is the case, prefiltration may improve the quality of downstream lignin (see Note 17).

Prefiltration can be easily accomplished using a 1-μm absolute polypropylene filter (see Notes 18 and 19). Depending on the volume to be filtered, (1) a large Buchner funnel fitted with a weighted circle of polypropylene filter felt on a vacuum flask or (2) a bag filter suspended above a bucket or drum can be used. Because the density of polypropylene is lower than that of water, a circle of heavy, nonreactive material, such as a stainless steel or glass, which is slightly smaller than the interior diameter of the Buchner funnel should be placed on top of the polypropylene filter.

1. The filter should be thoroughly wetted in distilled water just prior to use.
2. Pour the dissolved lignin or pulping liquor sample through the filter at a slow rate. Careful control of flow rate is not required for prefiltration, since the goal is removal of most gross particulate contaminants.
3. When all of the pulping liquor or dissolved lignin has passed through the filter, take a representative sample of the liquid and examine a droplet at 400× using transmitted light microscopy (see Note 20).
4. If there are still significant amounts of large particulates, visually check the filter to be sure that it does not have a hole, low-density section, or edge lifted from its support. If appropriate, clean the funnel and replace the filter.
5. Then, filter the pulping liquor or dissolved lignin again.
6. The gross contaminants, along with the filter, can be dried and weighed, rehydrolyzed, or discarded.
7. Circular felt prefilters can be rinsed, air-dried, and reused.

3.3.2. Filtration

This section discusses filtration of pulping liquors and dissolved lignins through both Buchner funnels and layered bag filters attached to a continuous-flow feed system. With reasonable care, a tenfold reduction in particulate contaminants is possible. Although careful selection of filtration media and control of flow rate is required, filtration of pulping liquor and dissolved lignin is very effective in removing particulate contaminants as small as 1 or 2 μm. A number of new products, such as 1-μm absolute polypropylene felt and layered 1-μm absolute bag filters, have greatly improved bench-scale filtration of solutions.

Buchner Funnel

1. Wet with distilled water and layer in a Buchner funnel (1) a medium to slow flow rate paper filter such as Whatman #2 or VWRbrand #413 paper, (2) a polypropylene 1-μm absolute filter felt cut to fit inside the Buchner funnel, (3) a circle of thin polypropylene tissue filter, and (4) a circular weight of a nonreactive material (see Notes 20 and 21).
2. Place the Buchner funnel on a large vacuum flask and connect it to a vacuum line. Use a rubber vacuum flask adapter between the funnel stem and the flask neck to seal vacuum leaks.

3. Rotate the funnel relative to the flask so that the open taper on the tip is pointed away from the vacuum line.

4. Turn the line vacuum on slowly and observe the liquid flow out of the wet filter. If it is too fast (>20 ml/min from a 253-mm-diameter Buchner funnel), turn the vacuum off and remove the filter funnel.

5. Replace the paper layer with a wetted flow paper with a slower flow rate (or two wetted medium flow filter papers on top of each other), replace the filter layers as before, put the weight on top of the filter stack, and again turn the vacuum on slowly.

6. When the flow rate appears to be acceptable, turn off the vacuum line, remove the Buchner funnel and pour off the accumulated water. Then replace the funnel and start the filtration.

7. Initially, open the vacuum line very slightly.

8. Ladle or pour small amounts of the dissolved lignin or pulping liquor over the filter stack, check the flow rate, and observe the filtrate. If it is clear and appears high in refractive index, continue with the separation.

9. If the filtrate appears cloudy, stop the filtration and return the filtered lignin to the feedstock solution.

10. Check the filter stack for leaks, replace or reseat sections if needed, and replace the Buchner on the filter flask.

11. Turn the assembly on at low pressure and restart the filtration.

12. When all of the pulping liquors or dissolved lignin has been filtered, pool it in a large capped bottle.

13. Take a representative sample and evaluate a droplet at 400× using transmitted light microscopy. If particulates appear to be at an acceptably low level, refrigerate the lignin and assess filterable contaminants using the remaining representative sample.

14. If the particulate level in the lignin is not satisfactory, refilter it at a slower rate, check the quality again, and refrigerate the lignin in a capped bottle.

The authors have filtered dissolved lignin and pulping liquors using a layered 1-μm absolute felt bag filter, supported in a free-standing case, with a 20-l blow case. The filter and housing are reasonably inexpensive, scaleable with the assistance of the manufacturer, and come in a range of sizes. The advantage of this approach is that it will filter relatively large amounts of dissolved lignin or pulping liquors (and later, recover lignin precipitate) safely with limited staff time. Tens of gallons per day can be filtered using the size of system the authors use, and it is relatively easy to recover most of the solids collected by the filter. Stainless steel
is the preferred material of construction for both blow cases and filter housing used with alkaline systems such as pulping liquors or dissolved lignins (see Notes 22–24).

1. The filter system is relatively easy to set up. The bag is wetted by folding it, placing it in a bucket, putting a weight on top, and filling the bucket with distilled water.

2. The bag (with an adapter if required by the manufacturer) is placed in the filter housing and the top is bolted on.

3. A tubing or pipeline from the filter to a take up bottle is connected.

4. The blow case is filled, its top is attached, and the blow case output hose is attached to the filter.

5. A weight is attached to the filter output hose and it is placed in a large polyethylene wide-mouth bottle (Nalgene 2234-0030 or 2234-0050).

6. To prevent spills, the filter case and take-up bottle are usually placed in a shallow plastic pan large enough to contain all of the liquid being filtered.

7. The blow case is then connected to an air pressure system and slowly pressurized until very slow liquid flow is observed. (Pressures are typically 2–15 psig.)

8. The system can then be left to complete filtration.

9. When the first 20-l batch is through the filter, take a representative sample of the filtrate and check particulates at 400× using transmitted light microscopy.

10. It may be necessary to refilter the first batch or to adjust the pressure applied to the blow case to get the desired levels of particulate removal.

Other Approaches

If the lignin is relatively low in particulates or if the bulk of particulates have been removed, high-surface area 1-ìm absolute or submicron polypropylene cartridge filters can be used for polishing. These are relatively inexpensive and work well when fed using a blow case system.

3.3.3. One-Step Lignin Precipitation

Generally, lignin can be precipitated from strongly alkaline solutions (pH 10.5) using a mineral acid, such as sulfuric or hydrochloric acid. Although quantitative analytical methods typically precipitate lignin at pH 3 or 4, the authors have found that a better quality lignin with a lower salt concentration can be produced by stopping at pH between 5 and 6. The authors have also incorporated some common fermentation techniques, such as use of antifoam and gradual addition of acid to a solution stirred in a container with a relatively large amount of headspace, into the preparation of lignin. It is also important to remember that reaching chemical equilibrium in lignin solutions is slow and that,
particularly where alkaline solutions have been held for a time, a significant amount of carbonate may have been formed. This leads to the evolution of carbon dioxide gas during pH reduction. If a large amount of gas evolves during lignin precipitation, a solid mass of lignin gel with bubbles may form. This is difficult to predict and can result in a chemical spill or loss of valuable experimental material. This can be prevented by developing a titration curve for the lignin solution, performing the acidification slowly, providing ample containment, and using a small propeller mixer.

The authors have developed two methods for routine lignin precipitation: a one-step process which will precipitate both lignin and dissolved long-chain carbohydrates (described immediately below), and a two-step process in which carbohydrate and lignin are separately precipitated (see Subheading 3.3.4). The goal of both processes is production of a high-quality, readily desalted lignin that can serve as a quality feedstock for other materials.

Before starting a large lignin precipitation, it is advisable to estimate the acid requirements for precipitation using a small sample as described below (see Note 25).

1. Start with a lignin dissolved in a highly alkaline aqueous solution (pH > 10.5). Measure or estimate the volume.
2. Take a representative 100-ml sample and place it in a 400- or 500-ml beaker. Add a stirring bar and a couple of drops of antifoam.
3. Then place the beaker on a magnetic stirrer.
4. Standardize a pH meter and immerse the electrode(s) in the solution.
5. Stir the solution slowly and add 10 ml aliquots of 0.1 N HCl or H$_2$SO$_4$.
6. It is useful to stir the solution for at least 5 min before measuring the pH.
7. Then repeat the process until pH reaches ~5 units.
8. The data can be used to determine how much acid will be required to reduce the pH to 5.5 or 6 units.

1. Although a variety of different batch sizes can be used, one of the simplest methods is to put 10 l of the lignin solution in a wide-mouthed (100 mm) 14- or 20-l polyethylene bottle and place the bottle in a large, deep plastic pan capable of containing any spill or overflow.
2. Place the impeller of a large laboratory mixer in the center of the liquid.
3. Add several drops of antifoam to the top of the lignin. Because foam can develop rapidly in lignin solutions during acidification, place antifoam where it can be easily reached (see Notes 26 and 27).
4. Support a pH electrode and place it in the solution to permit pH monitoring during precipitation.

5. Based on the pH curve obtained earlier for a 100-ml sample, measure the amount of acid required to drop the pH of the lignin solution to 5.5 or 6 units. Dilute the acid threefold by pouring it into slowly mixed distilled water.

6. Support a dropping funnel above the lignin solution and place no more than one-third of the diluted acid in the funnel at a time.

7. Start the stirrer and keep it running at a slow rate. Be sure that the stirrer impeller does not touch the bottom or side of the bottle. Regulate the dropping funnel so that it will take at least 20–30 min to add each third of the diluted acid (see Note 28).

8. Between pH 9.5 and pH 6, lignin precipitation will occur and carbon dioxide will be evolved. This creates a floating solid layer which can trap bubbles and overflow the bottle. It is therefore important to keep a close watch on the bottle during this period and to slow or stop acid addition if it starts to occur.

9. When pH 5.5–6 is reached, turn the dropping funnel off but continue slow stirring for another 5 or 10 min to ensure that dissolved gases are evolved.

10. Then rinse the dropping funnel with distilled water, remove its support, and set it aside to be cleaned and dried. Rinse the pH electrode with distilled water and remove it from the bottle.

11. When gas evolution appears complete, turn the mixer off, remove the stirring paddle, and rinse the paddle off with distilled water.

12. Loosely cap the bottle and let it settle for several hours or overnight. Generally, lignin will collect in a layer at the bottom of the bottle (see Notes 29).

13. Carefully siphon the clear liquid layer from above the lignin.

14. At this point, the lignin precipitate can be desalted or, if high ash content does not interfere with downstream lignin uses, dewatered by filtration.

15. Since a typical lignin layer from a 10-l starting sample is ~3–4 l, a Buchner funnel with a filter stack, as described in Subheading 3.3.2, can be used to remove a substantial amount of water (as well as ash and dissolved organics) from the lignin.

Long chain carbohydrates, such as hemicellulose, dissolved in lignin solutions, pulping liquors, or lignin hydrolysis liquors, coprecipitate with lignin in a one-step precipitation process. Coprecipitated carbohydrates are often the major lignin contaminants and can constitute 10–30% of the total lignin sample. The
bulk of dissolved carbohydrates precipitate at a higher pH than does dissolved lignin, making it possible to remove much of the carbohydrate prior to lignin precipitation.

The first step in this process, which is performed as described in Subheading “Estimating Acid Requirements for Precipitation”, is to estimate acid requirements for precipitation using a small sample. For wood-derived lignin solutions, precipitation of solution carbohydrates, such as hemicellulose, typically occurs by pH 9.8–10. The amount of acid required to reduce pH from 9.5 to 5.5 should be estimated, as this will be used in preparation of carbohydrate-stripped lignin.

1. Prepare clear 1,000 mg/l solutions of two flocculants: a long-chain carbohydrate gum with carboxyl groups (Merck Kelco XANFLOOD xanthan gum or Kelgin alginate) and a polyethyleneimine (BASF Catiofast) (see Notes 30 and 31).

2. Measure out 400 ml of each solution.

3. Using the method and equipment described under batch precipitation in the section above, prepare sufficient diluted sulfuric or hydrochloric acid to reduce the pH of the lignin solution to pH 9.5–9.8.

4. Slowly add the acid to the lignin solution to precipitate the carbohydrate.

5. When pH 9.5–9.8 is reached, slow the stirring down and slowly add ~100 ml aliquots of each flocculent to the bottle.

6. Repeat until the flocculants are completely added. (This should take approximately 5 min.) Continue to stir for 1 or 2 min and stop the stirrer.

7. Remove the stirrer, pH electrode, and dropping funnel, rinse them, and set them aside.

8. Loosely cap the bottle and let it stand several hours or overnight.

9. If there is a layer of clear liquid above the precipitate, remove it carefully by siphoning.

10. Then remove the precipitated carbohydrate by slow low-pressure filtration through a 1-μm absolute polypropylene bag filter. (If the amount of carbohydrate precipitate is low, it may also be removed by filtration through a series of pleated 0.45–1.0-μm cartridge filters.)

11. Collect the filtrate for precipitation of carbohydrate-stripped lignin.

12. If the carbohydrate is desired, remove it from the filter with a spatula, place in a wide-mouth plastic bottle, and refrigerate (see Notes 32 and 33).
1. Using the method and equipment described under batch precipitation, prepare sufficient diluted sulfuric or hydrochloric acid to precipitate the lignin by reducing the solution pH from 9.5 to 5.5 units.

2. Then desalt or lyophilize the lignin precipitate as needed for further processing.

Lignin solutions can form hard-to-remove layers on equipment and clothing if not cleaned properly. Equipment and clothing should be cleaned as soon as possible. If there are difficulties in cleaning, see Notes 34 and 35.

As a result of hydrolysis conditions, many lignins will contain several percent salts. For chemical conversion, salt levels to below one part per thousand are preferred. Soluble salts can be removed from precipitated or dry powdered lignin by leaching with distilled water.

1. For large samples, place 15 kg of powdered dried lignin (or approximately 10 gallons of wet lignin precipitate) in a 30-gallon stainless steel drum.

2. Place a Lightnin mixer on the side of the drum and add distilled water gradually to ~4 in. from the top of the drum while stirring at a slow speed.

3. Continue to stir slowly for at least 10 min to ensure that the lignin is in contact with the distilled water.

4. If the water turns brown, indicating that the lignin is starting to dissolve, adjust the pH to 5.5–6 using hydrochloric or sulfuric acid.

5. Turn the mixer off.

6. Place a UV-absorbing plastic cover over the drum, and let the lignin settle overnight.

7. The following day, carefully siphon the water layer off. The textures of lignin powders and precipitates vary, and it may be necessary to place a 1-μm absolute bag filter in the drum and siphon out of the bag filter (see Note 36).

8. When the water level is close to the top of the lignin layer, remove the siphon, refill the drum with distilled water, and turn the mixer on.

9. If the water turns brown, indicating that the lignin is starting to dissolve, adjust the pH to 5.5–6 with hydrochloric or sulfuric acid.

10. Stir for at least 10 min, turn the mixer off, cover the drum with UV-absorbing plastic, and again let settle over night.

11. Repeat rinsing the lignin every day for roughly a week.
12. At the end of that time, take a representative sample from the lignin in the drum and determine bulk ash content.

13. If it is below one part per thousand, the lignin can be filtered and lyophilized. If not, continue rinsing lignin until acceptable bulk ash content is obtained (see Note 37).

14. When bulk ash content is acceptable, siphon as much water as possible from the lignin.

15. Then set up a large Buchner filter with a wetted medium-to-fast flow paper on a large vacuum flask.

16. Connect it to the vacuum line and turn the vacuum on.

17. Add lignin to the filter until the level of solid lignin is around 1 in. from the top of the filter.

18. When liquid flow through the filter stops, turn the vacuum off.

19. Tap the side of the Buchner funnel several times with a rubber mallet and turn the vacuum on until liquid flow stops (see Note 38).

20. Turn the vacuum off and repeat the rubber mallet taps.

21. Repeat until it is not possible to remove additional liquid from the lignin filter cake.

22. Turn the vacuum off and disconnect the hose.

23. Lay a large piece of plastic or a plastic tray, large enough to hold the lignin cake, on a secure surface (or countertop).

24. Run a wetted large stainless or plastic spatula around the edge of the Buchner funnel and invert the funnel onto the plastic or tray to dislodge the lignin.

25. Remove the filter paper, felt, and any other layers from the lignin and scrape any residual lignin out of the Buchner funnel.

26. Label 4–6-ml (heavy) plastic zipper bags and fill them with cut sections of the lignin filter cake.

27. Try to fill the zipper bags so that, laid horizontally, they form a layer 0.5–1 in. thick.

28. Press air out of the bags, seal them, even out the thickness of the lignin layer, and place them in horizontal layers in a freezer (see Note 39).

29. Repeat until lignin dewatering and packing is completed.

30. Depending on the end use, the frozen lignin can be used as is or can be lyophilized to provide a dry powder.

3.3.6. Lignin Lyophilization

It is difficult to air-dry lignins which contain appreciable amounts of carbohydrates. The authors typically use a large lyophilizer to dry lignin samples. As discussed in Subheading 4, a variety of
different lyophilizers are available, and the drying procedures will vary with the instrument used.

1. Clean the lyophilizer, change the vacuum oil, and wash the freezing chamber the day before a run.
2. The large polypropylene felt filters which decrease the lignin powder sucked into the vacuum oil from each pan are replaced with clean filters (see Notes 40 and 41).
3. Pans are placed on each section and the unit is turned on so that the chamber and vacuum can reach the preferred working ranges by the next morning.
4. For the authors’ lyophilizer, it was found useful to lightly coat the rims of the pans for new samples with silicone vacuum grease.
5. A piece of polypropylene plastic screen that fits loosely at the bottom is placed in each pan to decrease lignin melting.
6. The pans are then covered with plastic wrap and cooled in a freezer for at least 1 h.
7. The frozen ziplock bags of lignin are removed from the freezer, wrapped in terry towels, and struck with a mallet to break the lignin into ~1 in. chunks.
8. The frozen lignin is placed in the frozen prepared pans.
9. Each pan is then individually attached to the freeze dryer and opened to vacuum.
10. During active lyophilization, the pans will typically remain cold enough to see condensation of water.
11. When the bulk of the lignin has been lyophilized, the bottom of the pan will stop condensing water and become dry.
12. After a few additional hours, either determine the temperature of the bottom of the pan or touch it. When it approaches room temperature, the lignin is probably fully dried.
13. Prepare a sieve set (pan, 50 mesh screen, and lid), metal blade spatula, and lignin storage bottle and cap by wiping them with low-residue static control wipes and drying in a 105°C oven.
14. Air tight bottles such as mason jars that minimize water sorption by lignin are preferable for most applications.
15. After cooling, preweigh the sample bottle and cap and write the weight on the bottle.
16. Assemble the sieve set and place it in a plastic pan or on a plastic sheet.
17. Remove individual pans from the freeze dryer.
18. Wipe off the silicone vacuum grease.
19. Remove the polypropylene mesh and empty the pan into the sieve, using the spatula if necessary, cover the sieve, and shake it until the lignin is transferred to the pan. If necessary, cut lignin lumps with the spatula.

20. When all the lignin has been transferred to the sieve set pan, repeat the process with the next freeze dryer pan.

21. When sieving is complete, transfer the lignin to the sample bottle, cap, and weigh (see Notes 42).

4. Notes

1. In most biorefinery streams, by-product lignins are produced in the form of concentrated liquors that are burned to produce process steam or electricity. Some biorefineries may be able to provide dry powdered lignins for evaluation, while others will send in the form of as-processed or concentrated (sometimes almost pasty) liquors. Without filtration, these lignins will contain significant amounts, possibly 10–50%, of contaminants. Analysis of the lignin starts with assessment of the as-received material and moves through preparation of samples for bench-scale process evaluations.

2. Lignins are good absorbers of ultraviolet light and may, as a result, react or polymerize. Daylight or fluorescent room light can be sufficient to present problems. When working with dissolved lignins and lignin gels, it is a good idea to keep containers covered with an ultraviolet-blocking material. The transparent plastic rolls supplied by fabric stores for protection of outdoor furniture from fading by sunlight are inexpensive, easy to work with, and effective. Alternatives include UV-blocking containers (brown glass, for example) and aluminum foil wrappings.

3. In a conventional cellulosic biorefinery, lignins are anticipated to be produced as by-products. Thus, it is likely that lignin and lignin liquors will contain significant amounts of fiber, cell-wall fragments, dirt (sand and clay), and carbohydrates (cellulose and hemicellulose), as well as materials particular to the feedstock (for example, cutin from grasses) and those introduced during fiber separation (acids, bases, alcohols). All these materials need to be removed from lignin if it is to be used as a process feedstock. The goal of the methods discussed below is to enable researchers to produce the 1–100 lb of “clean” lignin needed for a set of process investigations with the minimum expenditure on time, materials, and effort.
4. Upon receipt, a lignin sample should be looked at carefully to determine whether it can, in fact, be used or whether it will have to be purified prior to use. It is possible to learn a great deal about a lignin sample by looking at it with a magnifying glass (sand grains, fiber, cell walls may all be visible) and determining whether it has separated into layers or formed precipitates, and whether a representative sample can be obtained by a simple process, such as mixing.

A bulging lignin sample container could be due to pressure buildup from fermentation of carbohydrate materials or by gases from pulping or hydrolysis chemicals. Such containers can build up enough pressure to pose a significant hazard, and some fermentation microorganisms produce gases which contain significant amounts of flammable methane or hydrogen. If possible, without unduly disturbing the container, freeze it prior to drilling a small gas escape hole. When the container thaws and any pressure is released, you can consider opening the container and evaluating the material.

It would, however, be sensible to determine whether the pressure increase was caused by fermentation or by chemical reaction. First, put a drop of the material on a glass slide and look at it at 400× with a transmitted light microscope. Significant amounts of bacteria and yeasts will likely be visible if gases came from fermentation. If that is the likely cause of the gas buildup, have the next sample sent by refrigerated carrier and minimize the time in transit. Second, check the pH and consider likely chemical reactions that could cause gas evolution, such as chemical or biological acidification of a sample with high levels of carbonates. Third, look at the inside of the container to see whether the container itself reacted with the lignin sample during shipment.

5. The goal of visual and microscopic estimation of particulate contaminants is to determine whether it will likely be possible to remove these materials by filtration. If a layer of settled particulates is clearly visible, the lignin will require prefiltration.

6. If 2% w/v lignin cannot be dissolved in 0.1 N NaOH, these steps can be tried: (1) Increase the concentration of NaOH to 0.2–0.5 N; (2) Warm the sample to 70–100°C by dipping it in boiling water or holding it in a water bath; (3) Evaluate other solvents, such as ethanol–water blends. If none of these methods dissolves the lignin, contact the supplier to discuss the sample you received (Was it lignin?) and to determine whether another sample should be collected and sent.

7. Some samples contain significant amounts of organic materials which separate from solution upon refrigeration. One relatively simple method for removing the bulk of such materials is to simply place a large sample in a covered glass container and
refrigerate it overnight or over a weekend. It is often possible to siphon the lignin solution out of the glass container while retaining the organic material precipitated on the bottle edge. Floating contaminants can typically be separated by low-temperature centrifugation. If there is a lot of separable organic material, it may be necessary to repeat the separation.

If refrigeration and centrifugation do not separate these organics, they may be removed by solvent extraction. Hexane and cyclohexane have been recommended for this purpose, as they typically do not extract lignin.

8. Air-drying is rapid, inexpensive, and relatively easy to scale. It should be tried in case it is feasible. However, most biorefinery lignins are expected to contain sufficient carbohydrate as to preclude air-drying. In wood, hemicellulose retains significant amounts of “bound” water even when the wood appears to be dry. A similar effect has been observed for lignins that contain significant amounts of coprecipitated carbohydrate.

9. Lignins used as feedstock for carbon fiber can be specified as “reagent grade” (>95% pure materials with an ash content <0.1%). To produce specialty chemicals, lignin feedstock will need to meet other purity specifications such as very low carbohydrate content. Most of the contaminants in lignins can be removed by a combination of precipitation and filtration.

10. Analytical filtration and ash evaluations also provide a good prediction of contaminant removal by current bench-scale filtration systems because they use filters that remove the same sizes of particles. This is due to significant recent improvements in bench-scale filtration systems (such as 1-μm absolute felt filter cloth) and to selection of appropriate filtration media (~1-μm glass fiber filters).

11. Bulk contaminants are materials such as salts, sand, cell walls, and dirt that are distributed throughout a lignin sample. Filterable contaminants are particulate materials (sand, cell walls, and dirt) large enough (>1 μm) to be removed from a lignin solution by filtration. Filterable contaminant levels are lower than bulk contaminant levels. Generally, salts that dissolve in lignin solutions are classed as bulk contaminants because they will be retained in lignin precipitates and remain in dried lignin.

12. Preparation of lignins for evaluation as chemical feedstock involves a series of separations, each of which is targeted at the removal of a specific class of contaminants. It is often worth assessing the contaminants in the separated fractions to serve as a basis for assessment and development of new processes. For example, it is useful to know whether a process such as carbohydrate stripping increases or decreases the salt content of a lignin solution.

13. Lignin tends to form carbon fibers and carbon powder if it either contains a high proportion of volatiles or if the
temperature starts at a high level or is raised too rapidly. If a lignin sample forms liquid droplets, rather than volatilizing, during the heating process, it has been observed to (1) boil, forming small masses of carbonized bubbles; (2) form carbon fiber stretching out from the lignin droplet; or (3) form pieces of carbon which are discharged from the weighing pan. Any of these behaviors can change the estimated ash and volatiles in a sample by either removing material from a sample pan or leaving a carbon residual. Carbon pieces, bubbles, and fibers take a several hours, or longer, to oxidize.

When black carbon particles, bubbles, or fibers are visible, the sample firing schedule should be adjusted to give the samples time to vaporize and oxidize, rather than melt. This can often be accomplished by starting the samples in a room temperature, rather than hot, furnace and increasing the time required to raise the firing temperature of the samples to 600°C. If these techniques are not effective, it may be appropriate to decrease the sample size or to ensure that the sample is spread around the bottom of the pan as a thin, even layer prior to heating in a furnace.

14. The changes in the weight of blanks, together with weight variations expected from a balance reading to 0.1 mg, make it sensible to set up samples to have an ash weight of at least 3 or 4 mg. At the same time, it is useful to minimize the weight of lignin to minimize carbon and carbon-fiber formation. It is usually possible to select sample sizes that meet both goals.

15. Blanks are prepared using the same procedure as the samples. It is important to pass the same amount of NaOH and distilled water rinse through the filters, as it may appreciably change the weight of the filters by removing smaller glass fibers or particles and by the leaching of any binders in the filter material. It is also important to handle wet filters carefully to avoid breaking small pieces away.

16. Pass lignin samples through an alkali-compatible membrane filter, such as a polypropylene filter, and dry in room temperature air or a 105°C drying oven. The surface of the filter is usually smooth enough to permit examination by reflected light or scanning electron microscopy. This will provide a good indication of the size, shape, and structure of the particulates and may, additionally, permit collection of photographs or spectroscopic data which can aid in identification of the source of contaminants.

17. In addition to removal of particulate contaminants, a pre filter (or filter) may remove other materials, such as resins and long-chain fatty acids, which adhere to the filter fibers. These materials may decrease the flow rate or completely coat the surface of the prefILTER. If this happens, replace the prefILTER
as needed. (It may be useful to scrape some of the material from the surface of a coated filter in order to determine its composition.) A surface layer, such as a thin layer of polypropylene tissue, may be used to trap some of the materials.

18. There are a number of reasons for using polypropylene filter media. The authors have noted that filtration of lignin solutions resulted in the deposition of thick lignin films on the surface of Teflon and other conventional nonstick filter materials. This slowed or stopped flow through the filters. Polypropylene had the lowest lignin adherence of any common filter material evaluated. Polypropylene has good resistance to acids and bases. Polypropylene filter felts and felt bags are available in a number of different pore sizes and thicknesses. Manufacturers of polypropylene felt bags also manufacture industrial filter cloths from many of the same materials. Their assistance makes it possible to scale separations and estimate equipment sizes and costs. Polypropylene is inexpensive, relatively nontoxic, and easily obtained.

19. Polypropylene filter felts are difficult to cut with common sewing scissors because the fibers are both very strong and very fine. Scalpels, as well as scissors and shears with titanium blades, are effective.

20. Filtration is an art, and the materials and processes used will necessarily vary somewhat among different lignin samples. It is usually good to try a small test sample and check the filtrate for particulate contaminants visually or using transmitted light microscopy. Vary the filter layers and the pressure differential until a satisfactory filtrate is produced. For example, a thin upper fabric layer can be used to permit separation of a solid particulate layer, and different types of filter felt can be stacked to give a graded particulate removal that minimizes pressure drop.

21. A set of filter layers graded so that the upper layer collects coarser particles and lower layers, finer particles, works best. A circle of filter paper will serve as both a felt support and a flow rate control device. If polypropylene or other filter felts and tissues are used, it is important to weigh the edge of the filter stack. A glass ring or large stainless steel circle can be used for this purpose. It is important that the weight holds the filter stack down well enough to prevent leakage around the edge.

22. If a bag filter does not seem to be removing particulate contaminants, examine the seams. The most commonly encountered problem with layered bag filters is leakage along any stitched or poorly fused seam. Welded, bonded, and glued filter layered filter bags have fewer leaks than stitched bags.

23. Blow cases are pressure vessels that can contain and deliver reasonably large amounts of liquid. They can be used with
most filters that have housings which permit tubing fittings or pipe couplings. Blow cases can be pressurized using line air and a regulator. Liquid flow can be controlled using valves. This permits good control of the filtration process with limited staff attention. Because blow cases are designed to empty completely, they also minimize sample loss.

24. It is usually prudent to place freestanding and cartridge filters, with any sample collection bottles, in a plastic or stainless pan large and tall enough to contain any spills. This permits recovery of samples if there is a line or filter leak. Where samples are toxic, at high pH (>10), or a low pH (<2), use of sample containment pans minimizes the chance of a spill. It is also advisable to connect the blow case (or pressure feed tank), the filters, and the take-up bottle with pressure tubing held with compression fittings. When handling high pH, low pH, or toxic samples, appropriate personal protective equipment should be selected and used.

25. A 100 ml sample is usually sufficient for determination of acid requirements. As the lignin gel can be quite thick, a heavy or egg-shaped stirring bar is recommended.

Estimation of acid requirements using a small sample has a number of benefits. (1) It gives a good indication of problems, such as “instantaneous” foaming or the formation of lumpy or thick sections of lignin precipitates; (2) It provides enough time between aliquots of acid to approach a true pH. After lignin precipitation is initiated, the lignin solution will be both viscous and, possibly, lumpy. It may take several minutes to distribute “pockets” of unmixed acid into the solution because lignin can form a thick precipitate layer which prevents mixing around an aliquot of acid; (3) Lignin solutions can contain substantial amounts of carbonates and other materials which form gas on acidification. It may take several minutes for these to form and collect into bubbles large enough to reach the solution surface; (4) Keeping the lignin solution from “over-acidifying” is important because it maintains lignin quality during the drying or lyophilizing process. (Sulfuric acid will concentrate in lignin during drying, resulting in production of a gummy black lignin residue which is not useful as a chemical feedstock.)

26. Foam control and foam breaking are necessary for reasonably safe and efficient lignin precipitation. The authors have used a combination of Dow Corning Antifoam A and BASF P-4000 polypropylene oxide. This pair is commonly used to control foam in fermentations. If this does not work well for a lignin solution, change the proportions of the two materials and, additionally, try each material separately. If foam control is not satisfactory, the Dow Corning website
provides downloadable antifoam selection guides and their technical services may be willing to assemble and provide a test kit containing small amounts of several antifoams. Other manufacturers, such as BASF, also provide information and test materials.

27. The combination of carbon dioxide production and high solids content makes it hard to control foaming in lignin solutions during precipitation. Dow Corning antifoams are polysiloxanes which can also contain finely divided silica. They may contribute to ash in precipitated lignin. As such, the amount should be kept as low as practicable. The BASF antifoams, such as P-4000, are organics which do not leave an ash residue. These two materials are often used together.

In typical applications, antifoams are used at a few milligrams per litre or less and are supplied in concentrations of 25–100%. Initially, two large drops of each foam control compound should be enough for a 10-l sample. However, it is prudent to keep antifoam compounds within reach during lignin solution acidification.

28. If the bottle has areas where little fluid motion is visible, rotate the bottle or reposition the stirring impeller to ensure that all the lignin solution is relatively evenly acidified. Also, decrease the rate at which acid is added to the lignin solution.

29. Precipitated lignin should settle to the bottom of the processing bottle within a few hours. Failure to settle is often due to retention of small bubbles within the lignin precipitate layer. The simplest method for removing bubbles from the lignin mass is to tighten the cap and rock the bottle back and forth several times with some force. Tapping the bottle with a rubber mallet, putting it on a tilt table, or vibrating it may also be effective.

30. Carbohydrate precipitates are loose gels which appear to be <1 \( \mu m \) in diameter. When appropriate flocculants are added, the gels become stiffer and the particle diameter increases. This permits the gels to be removed by low-pressure filtration.

31. For our bench studies, xanthan gum and alginate (also called alginic acid) have, when used with a commercial polyethyleneimine, been effective in strengthening and enlarging carbohydrate gel precipitates. A number of other long-chain carbohydrate gums and long-chain cationic polymer pairs can be used. These are described in detail in a recent patent application (14) which can be viewed on the U.S. Patent Office website. Use of different flocculants can be evaluated using 10-ml samples of lignin solution at pH 9.5 to pH 10. The flocculants can be added with a micropipette, the sample capped and mixed, and flocculation evaluated by transmitted light microscopy.
32. If desired, carbohydrate precipitates can be centrifuged to give a solid concentration of 20% or more.

33. Carbohydrate precipitates will typically include significant amounts, perhaps as high as 50%, of lignin. The lignin may be: (1) chemically bonded to the carbohydrate, (2) dissolved in the water which constitutes most of the carbohydrate gel, or (3) entangled in the carbohydrate chains. If “cleaner” carbohydrate is required, the precipitate can be redissolved with NaOH, reprecipitated, and refiltered.

34. Lignin can dry to form adherent films on equipment surfaces. It is important to clean equipment before lignin hardens. If equipment was not cleaned properly, it may be necessary to clean it with dilute sodium hydroxide or with a cleaner such as Spitfire (Johnson Wax) made to remove organic films. If these approaches do not work, it is possible to fire stainless steel parts in a muffle furnace at 400°C for several hours to remove lignin.

35. Most conventional detergents have little impact on lignin stains. Soaking or washing clothing in trisodium phosphate (from a hardware or paint store) is effective.

36. Polypropylene bag filters tend to float and, additionally, the sides tend to collapse if liquid is being siphoned out through the bag. The easiest method for keeping a polyethylene bag filter open and submerged is to place a coil of stainless steel screen or mesh in the filter. A large stainless steel pipe fitting can also be dropped into the bottom of the filter to keep it open and weight it down. The strap or loop attached to the filter can be attached to a clamp or rod to keep the top of the bag above the liquid surface.

37. Although variation among different types of lignins is expected, there is typically good correlation across a wide range of concentrations (0.01–10%) between the total dissolved solids concentration measured using a conductivity meter and the ash, or salt, concentration in dried lignin. The bulk of lignin gels is comprised of water, and salts in the water are concentrated in the dried lignin.

Conductivity meters are inexpensive and easy to use and give a rapid reading. If salt concentration is a concern, purchase a conductivity meter and record conductivity values at the same time that lignin gel samples are taken for ash content. Once the correlation is established for a given lignin preparation, conductivity can be used to determine when to terminate a desalting process.

38. Lignin gels are thixotrophic and respond to applied shear by water loss. Waves in the surface of the lignin indicate that the shear force from tapping the funnel was effective.
39. Lignin tends to spoil when stored in a refrigerator, and few lignins dry in ambient air. Keeping ziplocks of lignin frozen until they can be lyophilized has been the most effective storage method for the authors’ group.

40. Dried or freeze-dried lignin is a very finely divided powder. As lignin dries, this powder can be sucked into the piping and vacuum pump sections of a lyophilizer. This is a problem because lignin facilitates formation of oil–water emulsions in the vacuum pump and could also remain to contaminate other types of samples.

One of the easier methods for limiting lignin access to the vacuum pump is to place 1-μm absolute filter felt between the lignin and the vacuum intake from the freezing chamber. For the authors’ lyophilizer, this could be accomplished by cutting circles of polypropylene felt to fit the top mount pan holders. The felt, which is soft, was retained with a circle of stiff nonwoven material cut to fit tightly into the top mount. Other lyophilizers will require different placement of filter material. It is important to wash or discard the filter material after every run and to change vacuum pump oil regularly.

41. Frozen lignin remains in good condition for months. If needed, it can be thawed and refrozen to fit the requirements of lyophilizers with trays which freeze samples or lyophilizers which use sample flasks. The major concern is ensuring that the samples remain frozen until the lyophilizer cycle is complete.

42. High moisture content can pose problems when lignin is used as a chemical feedstock. One relatively simple method for minimizing moisture uptake is to place lignin in a moisture-tight container with a clean seal, such as a mason jar. Small containers of lignin can also be stored in dehumidifiers.

References


Chapter 14

Cellulase Assays

Y.H. Percival Zhang, Jiong Hong, and Xinhao Ye

Summary

Cellulose is a heterogeneous polysaccharide, and its enzymatic hydrolysis requires endoglucanase, exoglucanase (cellobiohydrolase), and β-glucosidase to work together. We summarize the most commonly used assays for individual enzymes and cellulase mixture.

Key words: β-Glucosidase, Cellobiase, Cellobiohydrolase, Cellulose, Cellulase assay, Endoglucanase, Sugar assay

1. Introduction

Cellulose, which is the most abundant renewable biological resource, is produced mainly by plant photosynthesis. Cellulose biodegradation mediated by cellulases or cellulolytic microorganisms releases organic carbon in plant, animal, and microbial sediments back to the atmosphere as carbon dioxide and methane. Complete enzymatic crystalline cellulose hydrolysis requires three types of enzymes (endoglucanase, exoglucanase or cellobiohydrolase (CBH), and β-glucosidase) to work together (1–4).

Physical heterogeneity of the cellulosic materials and the complexity of cellulase enzyme systems (synergy and/or competition) on solid enzyme-accessibility-limited substrate surfaces present some challenges for cellulase activity assays (5–8). A number of cellulase activity assays have been summarized (5, 6). In this chapter, we describe the common cellulase activity assays including the total cellulase assays, β-glucosidase assays, endoglucanase assays, and exoglucanase (CBH) assays. This chapter will provide some useful guidance, especially in Subheading 4.
2. Materials

2.1. Total Cellulase Assays

2.1.1. Filter Paper Activity Assay

DNS (3,5-dinitrosalicylic acid) reagent. Dissolve 10.6 g of DNS and 19.8 g of NaOH in 1,416 ml of distilled water. After complete dissolution, add 360 g of Rochelle salts (sodium potassium tartrate), 7.6 ml of melted phenol (at 50°C) (see Note 1), and 8.3 g of sodium metabisulfite, and then mix well. Titrate 3 ml of the DNS reagent using 0.1 M HCl using the phenolphthalein endpoint pH check. It should take 5–6 ml of HCl for a transition from red to colorless. Add NaOH if required (2 g of NaOH added = 1 ml of 0.1 M HCl used for 3 ml of the DNS reagent) (see Note 2).

Citrate buffer (1 M, pH 4.5). Dissolve 210 g of citric acid monohydrate in 750 ml of distilled water, and add 50–60 g solid NaOH until pH is 4.3. Dilute the solution to nearly 1,000 ml and check the pH. If necessary, add NaOH to adjust the pH to 4.5.

Citrate buffer (50 mM, pH 4.8). Dilute 1 M citrate buffer (pH 4.5) by adding 19 times distilled water.

Filter paper strip (50 mg, 1.0 × 6.0 cm). Cut 1.0 × 6.0 cm Whatman No. 1 paper strips with a paper cutter (see Note 3).

Glucose standard stock solution – 10 g/l (see Note 4).

2.1.2. Anaerobic Cellulase Assay Using Avicel

1. Tris–HCl buffer (0.5 M Tris, pH 7.0, 0.1 M CaCl₂, and optional 1.5% NaN₃). Prepare 0.5 l of 1 M Tris–HCl buffer (pH 7.0), dissolve 11.1 g of CaCl₂ and 15 g NaN₃, and add distilled water to make up to 1 l.

2. Dithiothreitol (DTT, 0.5 M). The DTT solution can be stored at 4°C for at least a half year. Less costly cysteine can replace DTT (9).

3. Avicel suspension solution (24.4 g/l). Suspend 20 g of completely dry Avicel (FMC 105 or Sigma Cell 20) in 820 ml of distilled water with a magnetic stirrer.

4. Glucose standard solution – 1 g/l.

5. Phenol aqueous solution (5% w/v). Store at 4°C in darkness.

6. Sulfuric acid ~98% w/w.

2.2. β-Glucosidase Assays

2.2.1. β-Glucosidase Assay Using p-Nitrophenyl-β-D-Glucoside (pNPG)

1. Sodium acetate buffer, 0.1 M, pH 4.8.

2. pNPG (5 mM) in acetate buffer. Dissolve 0.1576 g of pNPG in 100 ml acetate buffer.

3. Glycine buffer (0.4 M) pH 10.8. Dissolve 60 g of glycerin in 1,500 ml of distilled water, add 50% w/v NaOH until the pH is 10.8, and then dilute to 2 l.

4. p-Nitrophenol (pNP; 20 g/l) in acetate buffer (see Note 5).
2.2.2. β-Glucosidase Assay Using Cellobiose

1. Cellobiose (15 mM) in citrate buffer (freshly made substrate solution).
2. Citrate buffer (50 mM, pH 4.8).

2.3. Endoglucanase Assays

2.3.1. Endoglucanase Assay Using Carboxymethyl-cellulose (CMC)/DNS

1. Citrate buffer (50 mM, pH 4.8).
2. CMC (2% w/v) in citrate buffer (above).
3. DNS reagent (above).
4. Glucose standard (2 g/l).

2.3.2. Endoglucanase Assay Using CMC/Bicinchoninic Acid (BCA)

1. Citrate buffer (50 mM, pH 4.8).
2. CMC solution (0.05% w/v) in the citrate buffer.
3. BCA Solution A. Dissolve disodium 2,2'-bicinchoninate (97.1 mg) in a solution of 2.714 g of Na₂CO₃ and 1.21 g of NaHCO₃ with a final volume of 50 ml. Solution A will remain stable for 4 weeks at 4°C in darkness.
4. BCA Solution B. Dissolve CuSO₄·5H₂O (62.4 mg) and L-serine (63.1 mg) in 50 ml of water. Solution B will remain stable for 4 weeks at 4°C in darkness.
5. Working BCA reagent. Mix equal volumes of solution A and B. The reagent is to be made immediately before use.
6. Glucose standard solution (0.9 g/l, 5 mM).

2.3.3. Endoglucanase Assay Using CMC/Viscosity

1. Sodium acetate buffer (50 mM, pH 5.0).
2. CMC solution (0.5% w/v, medium viscosity, degree of substitution of 0.5–0.7) in acetate buffer.

2.3.4. Semiquantitative Endoglucanase Assay Based on Dye Release

1. Congo red solution (1 g/l) prepared by dissolving 100 mg Congo red in 99 ml water and 1% ethanol.
2. NaCl (1 M) solution.
3. Sodium phosphate buffer (0.1 M, pH 6.5).

Microbe-Secreted Endoglucanase Assay on Agar Medium

1. CMC (1% w/v, low viscosity) in 1.5% agar medium. Dissolve CMC before adding agar and autoclave.

Endoglucanase Assay on Agarose Gel

1. CMC (1% w/v, low viscosity) in 0.8% agarose. Dissolve CMC completely before adding agarose.

Endoglucanase Assay on Polyacrylamide Gel

1. CMC (1% w/v) in sodium phosphate buffer whose pH is chosen depending on the specific cellulase.

2.4. Exoglucanase Assays

2.4.1. Exoglucanase Assay Using Avicel

1. Avicel (FMC PH 101 or PH 105 or Sigmacell 20).
2. Sodium acetate buffer (0.1 M, pH 4.8).
3. Phenol (5%) solution.
4. Sulfuric acid, ~98%.
2.4.2. Exoglucanase Assay Using Regenerated Amorphous Cellulose (RAC)

1. Sodium acetate buffer (1 M, pH 4.5).
2. Phenol (5%) solution.
3. Sulfuric acid (~98%).
4. RAC (1% w/v). RAC preparation is given below.

3. Methods

3.1. Total Cellulase Assays

A total cellulase system consists of three enzymatic activities: endoglucanases, exoglucanases, and β-D-glucosidases. Total cellulase activities are always measured using insoluble substrates, including pure cellulosic substrates such as Whatman No. 1 filter paper, cotton linter, microcrystalline cellulose, bacterial cellulose, algal cellulose, as well as cellulose-containing substrates such as dyed cellulose, α-cellulose, and pretreated lignocellulose (2). The two most common assays (filter paper assay and anaerobic cellulase assay) are described here.

3.1.1. Filter Paper Assay (FPA)

FPA is the most common total cellulase activity assay recommended by the International Union of Pure and Applied Chemistry (IUPAC) (6). IUPAC recommends a filter paper activity (FPA) assay that differs from most enzyme assays based on soluble substrate for initial reaction rates. This assay is based on a fixed degree of conversion of substrate, i.e., a fixed amount (2 mg) of glucose (based on reducing sugars measured by the DNS assay) released from 50 mg of filter paper (i.e., both amorphous and crystalline fractions of the substrate are hydrolyzed) within a fixed time (i.e., 60 min). In part due to the solid heterogeneous substrate, reducing sugar yield during hydrolysis is not a linear function of the quantity of cellulase enzyme in the assay mixture. That is, twice the amount of enzyme does not yield two times the reducing sugar within equal time. Total cellulase activity is described in terms of “filter-paper units” (FPU) per milliliter of original (undiluted) enzyme solution. The strengths of this assay are that (1) the substrate is widely available and (2) the substrate is reasonably susceptible to cellulase activity. However, the FPA has long been recognized for its complexity and susceptibility to operator errors (10).

Procedure

1. Place a rolled filter paper strip into each 13 × 100 test tube.
2. Add 1.0 ml of 50 mM citrate buffer (pH 4.8) to the tubes; the paper strip should be submerged in the buffer.
3. Prepare the enzyme dilution series, of which at least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 2.0 mg of glucose (~2.1 mg) and one slightly less than 2.0 mg of glucose (1.9 mg) (see Note 6).
4. Prepare the dilute glucose standards (GSs) as below:
   GS1: 1.0 ml of glucose standard + 4.0 ml buffer = 2 mg/ml
       (1.0 mg/0.5 ml).
   GS2: 1.0 ml of glucose standard + 2.0 ml buffer = 3.3 mg/ml
       (1.65 mg/0.5 ml).
   GS3: 1.0 ml of glucose standard + 1.0 ml buffer = 5 mg/ml
       (2.5 mg/0.5 ml).
   GS4: 1.0 ml of glucose standard + 0.5 ml buffer = 6.7 mg/ml
       (3.35 mg/0.5 ml).
   Add 0.5 ml of GS1–4 solutions to 13 × 100 mm test tubes,
   and add 1.0 ml of 0.050 M citrate buffer.

5. Prepare the blank and controls.
   Reagent blank (RB): 1.5 ml of 50 mM citrate buffer.
   Enzyme controls (EC1–5): 1.0 ml of 50 mM citrate buffer +
   0.5 ml enzyme dilution series whose enzyme concentrations are
   the same as those from E1 to E5 (see Note 7).
   Substrate control (SC): 1.5 ml of 50 mM citrate buffer +
   filter paper strip.

6. Prewarm the enzyme solutions, blank, and controls until
   equilibrium.

7. Add 0.5 ml of the enzyme dilution series to the tubes with
   filter paper substrate (E1–5); add 0.5 ml of the enzyme
   dilution series to the tubes without filter paper substrate
   (EC1–5).

8. Incubate the tubes of E1–5, GSs, RB, EC1–5, and SC in a
   50°C water bath for exactly 60 min.

9. Add 3.0 ml of the DNS reagent to stop the reaction, and mix
   well.

10. Boil all tubes for exactly 5.0 min (see Note 8).

11. Transfer the tubes to an ice-cold water bath.

12. Withdraw ~0.5 ml of the colored solutions into 1.5-ml micro-
    centrifuge tubes and centrifuge at ~10,000 g for 3 min.

13. Add 0.200 ml of the supernatant into 3-ml spectrophotometer
    cuvette tubes, add 2.5 ml of water, and mix well by using a
    pipette or by inversion several times.

14. Measure absorbance at 540 nm, where the absorbance of RB
    is used as the blank.

Calculation

1. Draw a standard sugar curve (sugar along the x-axis vs.
   absorbance at 540 along the y-axis), as shown in Fig. 1.

2. Calculate the delta absorbance of dilute enzyme solutions
   (ΔE1–4) for E1–5 by subtraction of the sum of the absorb-
   ance of EC1–5 and SC.

3. Calculate the real glucose concentrations released by E1–5
   according to a standard sugar curve.
4. Draw the relationship between the real glucose concentrations and their respective enzyme dilution rates (EDRs) (Fig. 1).

5. Link the points less than 2 mg and greater than 2 mg by a line, and identify the EDR by using the point for 2-mg glucose based on the line (Fig. 1).

6. Calculate the FPA of the original concentrated enzyme solution in terms of FPU/ml:

$$FPA = \frac{0.37}{EDR}$$

where 2 mg glucose = $2 \text{ mg} / (0.18 \text{ mg/\mu mol}) \times 0.5 \text{ ml} \times 60 \text{ min} = 0.37 \text{ \mu mol/min/ml}$ (see Notes 9, 10).

3.1.2. Anaerobic Cellulase Assay Using Avicel

Some cellulases or cellulosomes isolated from anaerobic environments need the presence of a reducing agent and some metal ions, such as calcium, to exert maximal hydrolysis ability, for example, the cellulosome from the thermophilic anaerobic bacterium *Clostridium thermocellum* (11). Johnson et al. (11) established a turbidometric method based on the change of 0.6 g/l Avicel (FMC RC-591), which is a blend of microcrystalline cellulose and sodium carboxymethylcellulose, but the results often suffer from large variations. The anaerobic cellulosome assay was modified on the basis of the soluble sugar release during the initial hydrolysis period (12, 13).
1. Add 4.10 ml of the well-suspended Avicel solution into 16 × 125 mm Hungate tubes, and add 0.50 ml of Tris–HCl buffer (each sample needs triplicate tubes).
2. Add the rubber stopper and seal the tubes.
3. Vacuum and flush the headspace gas by ~5 psi (ultra) pure nitrogen at least three times.
4. Add 0.10 ml of 0.5 M DTT solution using a syringe with a 23G needle before enzyme activity assay.
5. Prewarm the tubes in a water bath at 60°C.
6. Prepare the enzyme solution.
7. Add 0.30 ml of the dilute enzyme solution series into the tube using a syringe with a 23G needle.
8. After the first 10 min of adsorption and reaction, withdraw ~0.5 ml of well-suspended sample using a syringe with a 21G needle as the starting point for enzymatic hydrolysis. The larger gauge needle is needed for homogeneous sampling of cellulose slurry.
9. Shake the tubes continuously or manually mix them every 10–15 min.
10. Withdraw another 0.50 ml of well-mixed Avicel suspension every 1 h using a syringe with a 21G needle into the precooled 1.5-ml microcentrifuge tubes or stop the reaction after 1 h by transferring to an ice-cooled water bath.
11. Centrifuge the samples in 1.5-ml microtubes at 13,000 g for 3 min.
12. Measure total soluble sugars in the supernatants by the phenol–sulfuric acid assay.
13. Calculate the net soluble sugar release during the hydrolysis process by subtraction of the sugar at the starting point.
14. Determine enzyme activity at a linear range between sugars released and enzyme concentrations.

β-Glucosidase can cleave β-1,4-glucosidic bonds of soluble substrates, including cellobiose, longer cellooligosaccharides with a DP from 3 to 6, and chromogenic substrates, such as p-nitrophenyl-β-D-glucoside, p-nitrophenyl β-D-1,4-glucopyranoside,
β-naphthyl-β-D-glucopyranoside, 6-bromo-2-naphthyl-β-D-glucopyranoside, and 4-methylumbelliferyl-β-D-glucopyranoside (2). The term “cellobiase” is often misleading because of this key enzyme’s broad substrate specificity.

3.2.1. β-Glucosidase Assay Using pNPG

This pNPG method is an initial reaction rate assay (6).

1. Add 1.0 ml of pNPG solution and 1.8 ml of acetate buffer into test tubes.
2. Equilibrate at 50°C.
3. Prepare the enzyme dilution series.
4. Add 0.2 ml of diluted enzymes into the tubes containing the substrate, and mix well.
5. Enzyme blanks: Add 0.2 ml of diluted enzymes into the tubes containing 2.8 ml of acetate buffer, and mix well; Substrate blank: Add 1.0 ml of pNPG solution and 2.0 ml of acetate buffer into test tubes.
6. Incubate all tubes at 50°C for 15 or 30 min.
7. Add 4.00 ml of glycine buffer to stop the reaction.
8. Measure the absorbance of liberated products of p-nitrophenol at 430 nm based on the substrate blank.
9. Read the net absorbance of the enzyme solutions by subtracting readings of the enzyme blanks.
10. Determine p-nitrophenol release on the basis of the known concentration of p-nitrophenol diluted by glycine at 430 nm.
11. Calculate the enzyme activity on the basis of the linear range between absorbance and enzyme concentrations.

3.2.2. β-Glucosidase Assay Using Cellobiose

The β-glucosidase based on cellobiose assay recommended by IUPAC is based on a fixed amount (1 mg) of glucose formation from cellobiose (6). The glucose concentrations in the cellobiose reaction mixture are determined using at least two different enzyme dilutions. One dilution should release slightly more and one slightly less than 1.0 mg (absolute amount) of glucose in the reaction conditions.

1. Dilute the enzyme solution by citrate buffer in a series. At least two dilutions must be made of each enzyme sample investigated. One dilution should release slightly more and one slightly less than 1.0 mg (absolute amount) of glucose in the reaction conditions (i.e., 0.5 mg glucose released/ml).
2. Add 1 ml of diluted enzyme solution (DES) to the tubes.
3. Equilibrate the enzyme solutions and substrate solutions at 50°C.
4. Add 1.0 ml of substrate solution into the tubes containing the enzyme solution.
5. Incubate at 50°C for exactly 30 min.

6. Immerse the tubes in boiling water for exactly 5.0 min to stop the reaction.

7. Transfer the tubes to a cold water bath.

8. Substrate Blank: A mixture of 1.0 ml of cellobiose solution and 1.0 ml of citrate buffer. Enzyme Blanks: A mixture of 1.0 ml of cellobiose solution and 1.0 ml of DESs. Treat substrate and enzyme blanks identically as the experimental tubes (i.e., equilibrate at 50°C, heat, boil, and cool).

9. Determine glucose release using a commercial glucose oxidase kit (GOD) or a glucose hexose kinase and glucose-6 phosphate dehydrogenase kit (HK/G6PDH) or HPLC.

10. Measure the absorbance of all solutions based on the substrate blank.

Calculation

1. Calculate the delta absorbance of dilute enzyme solutions by subtracting absorbance of the respective enzyme blanks.

2. Calculate the real glucose concentrations released according to a standard glucose curve by the enzyme kit.

3. Link the points less than 1 mg and greater than 1 mg by a line, and determine the EDR by using the point that is supposed to produce 1 mg glucose.

4. Calculate cellobiase solution activity (IU/ml) (see Note 12):

   \[ \text{Cellobiase} = \frac{0.0926}{\text{EDR}} \]

3.2.3. \(\beta\)-Glucosidase Assay Using Cellobiose

\(\beta\)-Glucosidase activity can be measured on the basis of initial reaction rates of cellobiose by combining the methods of Subheading 3.2.1 and 3.2.2. The hydrolysis product – glucose – can be measured by the glucose HK/G6PDH kit (14).

3.3. Endoglucanase Assays

Endo-\(\beta\)-1,4-D-glucanase (EC 3.2.1.4) randomly cleaves accessible intermolecular \(\beta\)-1,4-glucosidic bonds on the surface of cellulose. Because insoluble cellulose has very low accessible fractionation of \(\beta\)-glucosidase bonds to cellulase (3, 8, 15), water-soluble cellulose derivatives such as CMC and hydroxyethylcellulose (HEC) are commonly used for endoglucanase activity assays. The hydrolysis can be determined by measuring the changes in reducing sugars or viscosity or color. Since CMC is an anionic substrate, its properties change with pH. Nonionic substrates such as HEC are recommended sometimes.

3.3.1. Endoglucanase Assay Using CMC/DNS

The IUPAC-recommended endoglucanase (CMCase) assay is a fixed conversion method, which requires 0.5 mg of absolute glucose released under the reaction condition (6). The reducing end concentration is measured by the DNS method.
Procedure

1. Prepare the enzyme dilution series, of which at least two dilutions must be made of each enzyme sample, with one dilution releasing slightly more than 0.5 mg of glucose and one slightly less than 0.5 mg of glucose.

2. Add 0.5 ml of the DESs into test tubes with a volume of at least 25 ml.

3. Equilibrate the enzyme solution and substrate solution at 50°C.

4. Add 0.5 ml of the CMC solution to the test tubes and mix well.

5. Incubate at 50°C for 30 min.

6. Add 3.0 ml of DNS solution and mix well.

7. Boil for exactly 5.0 min in vigorously boiling water.

8. Place the tubes in an ice-cooled water bath to quench the reaction.

9. Add 20 ml of distilled water and seal with parafilm or by a similar method. Mix by inverting the tubes several times.

10. Read the absorbance at 540 nm based on the substrate blank.

11. Prepare the substrate blank (0.5 ml of CMC solution + 0.5 ml of citrate buffer) and the enzyme blanks (0.5 ml of CMC solution + 0.5 ml of dilute enzyme solutions). Treat substrate and enzyme blanks identically as the experimental tubes.

12. Prepare the glucose standards:

- GS1 – 0.125 ml of 2 mg/ml glucose + 0.875 ml of buffer.
- GS2 – 0.250 ml of 2 mg/ml glucose + 0.750 ml of buffer.
- GS3 – 0.330 ml of 2 mg/ml glucose + 0.670 ml of buffer.
- GS4 – 0.500 ml of 2 mg/ml glucose + 0.500 ml of buffer.

13. Calculate the glucose released by the enzyme solutions with deduction of the enzyme blank absorbance based on the glucose standard curve.

14. Draw the relationship between the real glucose concentrations and their respective EDRs.

15. Link the points less than 0.5 mg and greater than 0.5 mg by a line, and identify the EDR by using the point for 0.5 mg glucose.

16. Calculate the CMCase activity of the original concentrated enzyme solution in terms of IU/ml:

\[
\text{CMCase} = \frac{0.185}{EDR}
\]

3.3.2. Endoglucanase Assay Using CMC/BCA

This initial reaction rate enzymatic assay can be conducted at a very low enzyme concentration. The reducing end concentration
is measured by the BCA method, in which the color development reaction is conducted at 75°C in order to avoid β-glucosidic bond cleavage during the color-development process (16).

**Procedure**

1. Dilute the enzyme solution extensively (e.g., 1,000-fold) using the 50 mM citrate buffer and prepare the dilute enzyme solution series.
2. Add 1.8 ml of CMC solution into 13 × 100 mm test tubes.
3. Equilibrate at 50°C water bath.
4. Add 0.2 ml of DES and mix well.
5. Incubate at 50°C for 10 min.
6. Add 2 ml of working BCA reagents and mix well.
7. Incubate at 75°C for 30 min.
8. Read absorbance at 560 nm after subtracting the readings for the enzyme blanks and the substrate blank.
9. Calculate the enzyme activity based on a linear range between reducing end concentrations and enzyme concentrations.

**Substrate blank:** 1.8 ml of CMC solution + 0.2 ml of citrate buffer; enzyme blanks: 1.8 ml of CMC solution + 0.2 ml of dilute enzyme solutions. Treat blanks identically as the experimental samples.

**Glucose standard:** 1 ml of 5 mM glucose diluted by 50 mM citrate buffer by 100-fold to 50 mM glucose standard solution; prepare the sugar standards as below:

- GS1 – 0.4 ml of 50 μM glucose + 1.6 ml of buffer.
- GS2 – 0.8 ml of 50 μM glucose + 1.2 ml of buffer.
- GS3 – 1.2 ml of 50 μM glucose + 0.8 ml of buffer.
- GS4 – 1.6 ml of 50 μM glucose + 0.4 ml of buffer.
- GS5 – 2.0 ml of 50 μM glucose.

**3.3.3. Endoglucanase Assay Using CMC/Viscosity**

This initial reaction rate assay is based on the reduction in specific viscosity of soluble cellulose derivatives such as CMC and HEC (2). Both endoglucanase and exoglucanase can release new reducing sugar ends from soluble substrates. Within a limited degree of hydrolysis, endoglucanase can decrease specific viscosity greatly, and exoglucanase can decrease specific viscosity slowly (7).

**Procedure**

1. Add 6.0 ml of prewarmed CMC solution in a water bath at 30°C into an Ostwald viscometer (water flow time of 15 s at 30°C) (see Note 13).
2. Add 1.0 ml of the prewarmed DES (see Note 14).
3. Determine the flow rates every 5 or 10 min.
4. Calculate specific viscosity ($\eta_{wp}$):

$$\eta_{wp} = \frac{t - t_0}{t_0}$$
where \( t \) is the effluent time of the buffer (s) and \( t_0 \) is the efflux time of the buffer (s).

5. Plot the increasing rate of the reciprocal of the specific viscosity against the enzyme concentration; a linear relation should be obtained.

6. Calculate unit of activity arbitrarily from the linear relationship between enzyme concentration/rate of increase of reciprocal of the viscosity of the CMC solution (see Note 15).

Endoglucanase activity can be detected semiquantitatively on solid supports by staining polysaccharides with various dyes because these dyes are adsorbed only by long chains of polysaccharides. These methods are suitable for monitoring large numbers of samples but differences in enzyme activity levels of less than twofold are difficult to detect by eye. A linear relationship between the halo diameter and the logarithm of endoglucanase activity can be established as \( D = K \times \log(A) + N \), where the \( D \) is the diameter, \( A \) is the enzyme activity, and \( K \) and \( N \) are parameters determined by the standard curve of the known enzyme activity solutions. The activity of unknown samples can be calculated on the basis of the standard curve. Three procedures are described involving in vivo as well as in vitro endoglucanase detection.

1. Inoculate the endoglucanase-secreted microorganisms on the solid CMC medium. The growth time depends on the growth rate of the microorganism and enzyme activity (see Note 16).

2. Stain a 9-cm Petri dish by adding 20 ml of Congo red solution at room temperature for 30 min.

3. Rinse the residual dye on the dish using distilled water.

4. Destain Congo red with \(~20\) ml of 1 M NaCl for 30 min. If the halos are not clear, destain the dish by another \ (~20\) ml of NaCl solution.

5. Detect the clear, weak yellow halos for endoglucanase activity with the red background.

6. Option: In order to increase halo contrast, add \ (~20\) ml of 5% acetate acid or 1 M HCl to the plate at room temperature for 10 min, and pour off. The background of the plate will turn blue.

1. Pour \ (~20\) ml of the melted CMC agarose solution (\ (~50°C) into a 9-cm Petri dish.

2. Drill wells on the solidified agarose gel with a cork borer, and remove the agarose particles in the wells by suction or a pair of tweezers (see Note 17).

3. Add \ (~10–20\) \mu l of the enzyme solution into the holes.
4. Put the plate in the incubator (37°C or desired temperature) for several hours or even overnight.

5. Wash the plate with distilled water.

6. Add 10 ml of the Congo red solution and incubate at room temperature for 30 min.

7. Wash the residual dye on the plate by distilled water.

8. Destain the dye by using 20 ml of 1 M NaCl solution at room temperature for 30 min, and decant the destained solution (see Note 18).

9. Detect the clear yellow halo with the red background.

This method can separate mixed protein components by electrophoresis and then detect endoglucanase activity on polyacrylamide gels (SDS PAGE or native PAGE). If SDS-PAGE is used, cellulose activity must be detected after SDS removal and protein re-naturation.

1. Separate the protein mixtures by native or SDS PAGE.

2. Rinse the gel in distilled water for 5 min.

3. Soak the gel in the sodium phosphate buffer with gentle shaking for 20 min, and repeat the washing procedure three times to remove the SDS.

4. Transfer the gel into the CMC/phosphate buffer for 30 min.

5. Rinse the gel with distilled water.

6. Incubate the gel in 0.1 M sodium phosphate buffer at 40°C for 1 h.

7. Stain the gel with the Congo red solution at room temperature for 30 min.

8. Wash the gel with distilled water, and destain the gel in 1 M NaCl solution at room temperature for 30 min (see Note 19).

3.4. Exoglucanase Assays

Exoglucanase (CBH, EC 3.2.1.91) can release either glucose and/or cellobiose from ends of cellulose chains. *Trichoderma reesei* CBH1 and CBH2 cleave cellobiose units from the reducing end and the non-reducing end of cellulose chains, respectively. In contrast to endoglucanase and β-D-glucosidase, exoglucanases are difficult to measure due to the lack of specific substrates and interference from other cellulase components. Accordingly, exoglucanases have to be assayed in the purified form. The activity of purified exoglucanases is often estimated using Avicel. Avicel is a good substrate for exoglucanase activity assay because of its highest ratio of end/accessibility (3, 7). To some extent, Avicelase is regarded as synonymous with exoglucanase or CBH. In addition, amorphous cellulose can be used for determining of exoglucanase activity.
3.4.1. Exoglucanase Assay Using Avicel

Procedure

1. Suspend 1.25% (w/v) Avicel in acetate buffer (see Note 20).
2. Add 1.6 ml of Avicel suspension solution into the tubes.
3. Dilute a series of enzyme solutions by acetate buffer.
4. Equilibrate the substrate and enzyme solutions in a water bath at 50°C.
5. Add 0.4 ml of the dilute enzyme solutions to the Avicel substrate and mix well.
6. Incubate at 50°C for 2 h.
7. Stop the reaction by submerging the tubes in ice-cooled water bath.
8. Withdraw ~1 ml of hydrolysate into microcentrifuge tubes and centrifuge the sample at 13,000 g for 3 min.
9. Prepare enzyme blanks (0.4 ml of diluted enzymes and 1.6 ml of 0.1 M acetate buffer) and substrate blank (0.4 ml of 0.1 M acetate buffer and 1.6 ml of 1.25% (w/v) Avicel suspension buffer).
10. Determine the total soluble sugars in the supernatant by the Phenol–Sulfuric Acid assay where the absorbance of the substrate blank is used as the blank (see the Phenol-Sulfuric Acid assay in Subheading 3.1.2).
11. Calculate the enzyme activity on the basis of a linear relationship between the total soluble sugar release and enzyme dilution. One unit of exoglucanase activity is defined as the amount of enzyme that releases one micromole of glucose equivalent per minute from Avicel.

3.4.2. Exoglucanase Assay Using RAC

RAC Preparation Procedure (17)

1. Microcrystalline cellulose (0.2 g) is added to a 50-ml centrifuge tube, and 0.6 ml distilled water is added to form a suspended cellulose slurry.
2. Ten milliliters of ice-cold 86.2% H$_3$PO$_4$ is slowly and carefully added to the slurry with vigorous stirring. Before adding the last 2 ml of phosphoric acid, the cellulose suspension solution must be thoroughly mixed. The cellulose mixture turns transparent within several minutes, and should be held for ca. 1 h on ice with occasional stirring.
3. Approximately 40 ml of ice-cold water is added at the rate of approximately 10 ml per addition with vigorous stirring between additions, resulting in a white cloudy precipitate.
4. The precipitated cellulose is centrifuged at ~5,000 g and 4°C for 20 min.
5. The pellet is suspended in about 45 ml ice-cold water followed by centrifugation to remove the supernatant containing phosphoric acid; this step is repeated four times.
6. Approximately 0.5 ml of 2 M Na$_2$CO$_3$ is added to neutralize the residual phosphoric acid, and then 45 ml of ice-cold distilled water is used to suspend the cellulose pellet.

7. After centrifugation, the pellet is suspended in distilled water and centrifuged twice or until the pH reaches 5–7.

8. The carbohydrate concentration of RAC is calibrated by the Phenol-Sulfuric Acid method and diluted to 1%.

9. Addition of 0.2%, w/v sodium azide is optional for extended RAC storage at 4°C (see Note 21).

1. 0.5 ml of 1% (w/v) RAC and 0.05 ml of 1 M citrate buffer plus 0.25 ml water in the tubes.

2. Dilute the enzyme solution series with 50 mM acetate buffer.

3. Equilibrate the tubes containing the enzyme and substrate solutions at 50°C.

4. Add 0.2 ml of the DESs and mix well.

5. Incubate at 50°C for 10–30 min.

6. Place the tubes in an ice-cold water bath.

7. Centrifuge the hydrolysate sample at 10,000 g for 3 min.

8. Prepare enzyme blanks (0.2 ml of the DES, 0.05 ml of 1 M citrate buffer, and 0.75 ml of distilled water) and substrate blanks (0.5 ml of 1% w/v RAC, 0.45 ml of distilled water, and 0.5 ml of 1 M citrate buffer).

9. Measure the total soluble sugar concentration in the supernatants by the Phenol–Sulfuric Acid method and measure the absorbance at 490 nm using the absorbance of the substrate blank as the blank; (see the Phenol–Sulfuric Acid assay in Subheading 3.1.2).

10. A linear relationship between the total soluble sugar release and enzyme dilution is used for calculating the enzyme activity. One unit of exoglucanase activity is defined as the amount of enzyme that releases one micromole of glucose equivalent per minute from Avicel.

### 3.5. Summary

A number of cellulase activity assays have been developed over several decades, but we have presented only the most popular cellulase activity assays here. Heterogeneity of insoluble cellulose, complicated synergy/competition among endoglucanase and exoglucanase, and changes in ratio of enzyme/substrate pose formidable challenges in developing cellulase activity assays (2, 7, 8). Keeping special properties of insoluble substrates (such as limited accessibility to enzyme, degree of polymerization (DP), etc.) in mind (8), there is no clear relationship between the hydrolysis rates obtained on soluble and insoluble substrates,
mainly because of large variations in limited solid substrate accessibility to cellulase (7). A functionally based model has been developed to suggest the complexity among endoglucanase, exoglucanase, their ratio, cellulose accessibility, DP, enzyme concentration, and reaction time (7). The model suggests the challenges in applying the results of total cellulase activity assay measured on one solid substrate to a different solid substrate. Researchers must state clearly all parameters of their assay conditions and resist the temptation to compare their results to those of other researchers using different substrates, experimental conditions, etc. An understanding of enzymatic cellulose hydrolysis mechanisms among substrate characteristics is urgently needed, as well as development of enzyme activities to evaluate cellulase performance on insoluble cellulosic materials, especially for pretreated lignocellulosic materials.

4. Notes

1. Be careful to handle the phenol safely.

2. The DNS reagent can be stored in darkness at 4°C for at least 1 month. It could lose its reducing ability after long storage (18). The freshness of the DNS reagent is often ignored (18).

3. It is important to check each paper strip weight to ensure that the weight variation is less than 1 mg per strip because FPA is subject to the filter paper weight. Handle the paper with forceps or gloved hands.

4. Aliquots of the standard glucose solution can be tightly sealed and stored frozen. The solution should be mixed well after thawing.

5. 4-Methylumbelliferyl-β-glucoside can replace pNPG, which results in an assay with higher sensitivity.

6. Take commercial concentrated cellulase solution as an example. Dilute the enzyme solution 20-fold using 50 mM citrate buffer DES, and then prepare a series of dilutions from E1 to E5 with different dilution rates as below:
   - E1: 0.10 ml of DES + 1.90 ml of citrate buffer (dilute rate = 0.0250).
   - E2: 0.15 ml of DES + 1.85 ml of citrate buffer (dilute rate = 0.0375).
   - E3: 0.20 ml of DES + 1.80 ml of citrate buffer (dilute rate = 0.0500).
E4: 0.30 ml of DES + 1.70 ml of citrate buffer (dilute rate = 0.0750).
E5: 0.35 ml of DES + 1.65 ml of citrate buffer (dilute rate = 0.0850).

7. Commercial enzyme solutions can contain a significant amount of reducing sugars (19).

8. The boiling condition should be severe, and the volume of the boiling water bath should be maintained above the level of the total liquid volume of the test tubes to promote full color development.

9. International Unit (IU) is defined as 1 μmol/min, based on the initial hydrolysis rate, and is different from FPU assay, which is a fixed conversion assay.

10. The β-d-glucosidase level present in the cellulase mixture greatly influences the FPA assay (2, 5, 6, 20) because the DNS readings are strongly influenced by the reducing end ratio of glucose, cellobiose, and longer cellodextrins (16).

11. The Phenol–Sulfuric Acid assay is an extremely exothermic reaction; so be cautious not to spill the liquid.

12. 0.5 mg of glucose produced/ml × 2 ml volume = 1 mg of glucose produced = 5.56 μmol of glucose produced = 2.78 μmol of cellobiose consumed. Since reaction time is 30 min, 0.0926 IU of β-glucosidase can produce 1 mg of glucose from cellobiose within 30 min.

13. A convenient viscometer such as capillary tubing could be used to replace the Ostwald viscometer.

14. Constant temperature during the viscosity measurement is important because viscosity is greatly influenced by temperature change.

15. Exact endoglucanase activity (μmol bond cleavage/min) based on changes in specific viscosity can be calculated through a relationship between the viscosity and the DP of CMC. Viscosity of the substrate is strongly associated with substrate DP, and medium-viscosity CMC is recommended.

16. If CMC inhibits microorganism growth, a second layer of CMC solid medium can be applied to the primary medium containing other carbon sources or nutrients.

17. Strong enzyme activity or the short distance between the wells results in the fused halos, which may be difficult to differentiate or measure.

18. Often, the halo can be observed in 5 min; if the halo is not clear, destain again by adding 20 ml of the NaCl solution. If the band is not clear, destain the gel by using the NaCl solution again.
19. For native PAGE, a one-time soak is enough. Do not use a potassium phosphate buffer because potassium precipitates in the gel.

20. Since Avicel powder could contain approximately 4–8% moisture, weight adjustment is needed.

21. RAC, different from phosphoric acid-swollen cellulose (PASC), is a homogeneous amorphous cellulose that is precipitated from dissolved cellulose. RAC has a constant quality because it is regenerated from homogeneous dissolved cellulose, has easy handling and transferring properties, is a homogeneous substrate, and has high substrate reactivity (17). Take care if azide is used because of both toxicity and the explosive nature of powders.

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References


Chapter 15

Assessing Cellulase Performance on Pretreated Lignocellulosic Biomass Using Saccharification and Fermentation-Based Protocols

Nancy Dowe

Summary

Cellulase enzyme is a key cost component in the production of fuels and chemicals from lignocellulosic biomass. Cellulolytic ability of the enzyme preparation is often measured by activity assays using model substrates such as filter paper. Using lignocellulosic biomass as the substrate to assess enzyme performance has the potential of being more process relevant. We describe two procedures that use washed pretreated cellulosic material to measure the efficacy of cellulase enzymes. First, a saccharification assay that measures glucose yield as a function of the amount of cellulase used in the process. And second, the simultaneous saccharification and fermentation (SSF) assay measures cellulase performance by the amount of ethanol produced from enzymatic hydrolysis of the cellulosic material. You can use both assays to screen cellulases under a variety of substrate types, loadings, and process conditions.

Key words: Cellulases, Lignocellulose, Saccharification, Simultaneous saccharification and fermentation, Pretreatment, Biomass, Ethanol

1. Introduction

Considerable efforts have focused on converting lignocellulosic biomass to a fermentable feedstock for production of fuels and chemicals, especially the production of ethanol (1-3). Typical steps taken to produce ethanol from biomass involve feedstock preparation (cleaning and size reduction), pretreatment, enzymatic hydrolysis, fermentation, and ethanol recovery. An example of a lignocellulosic biomass to fuel ethanol process is one that uses co-current dilute acid pretreatment with enzymatic hydrolysis of the remaining cellulose and cofermentation of the resulting
glucose and xylose to ethanol (4–5). Cellulase enzyme is a key component in this design because of the enzyme’s role in producing fermentable sugars and the contribution to the cost (6–7). Enzyme cost can be reduced with improvements in the production process and specific activity of the enzyme (8–9).

As integrated and cost-effective biomass processes are developed that use cellulolytic enzymes, screening of enzymes for the process is necessary. Filter paper, endoglucanase, and β-glucosidase activity assays are commonly used to measure cellulolytic ability (10). These assays use model substrates such as filter paper, hydroxyethylcellulose, or p-nitrophenyl-β-D-glucoside. However, higher enzyme activity on model substrates may not coincide with hydrolysis efficiency on the pretreated biomass (11). At some point, it becomes necessary to screen enzymes in more process-relevant conditions.

In this chapter, we describe two assays for testing the efficacy of cellulase enzymes using washed pretreated cellulosic material. First, we describe a saccharification assay which measures enzyme performance by the fermentable sugars produced from washed pretreated substrate (glucose yield) as a function of enzyme loading, without fermentation. Since there is no fermentation of the sugars, enzyme performance will be affected by feedback inhibition from glucose and cellobiose, which are the solubilized sugars from cellulose hydrolysis. Second, we describe the simultaneous saccharification and fermentation (SSF) assay which determines the ethanol yield as a function of enzyme loading on a washed pretreated substrate (12). The SSF assay allows you to test enzyme performance without inhibition from the soluble glucose and cellobiose. With both assays, you can screen enzyme preparations on the basis of how much enzyme is needed to achieve a particular ethanol or glucose yield under various substrate loadings and conditions. Glucose and ethanol yield calculations described in this chapter are based on the weight of the contents of the flask being equal to a liquid volume. This is not as problematic when substrate concentrations are low (10%, w/w total solids or less). But with increased substrate loading, the flask contents contain much less liquid relative to the total amount of material in the flask, which complicates the yield calculations. More analytical measurements such as density of the liquid fraction, total and insoluble solids, and weight of substrate loaded are needed to determine yields from sugar and ethanol concentrations (13).

You can expand the assays to include screening on whole slurry, which can provide enzyme performance data under more realistic process conditions. Process conditions such as temperature, amount and types of pretreated biomass, pH, and enzyme mixtures can be varied. The information from these tests could be used in scale-up and in providing enzyme loading information for technoeconomic modeling.
2. Materials

2.1. Enzymes

1. Cellulase enzyme is added to the saccharification or SSF on a milligram of protein per gram of cellulose basis. Protein is measured using Pierce’s bicinchoninic acid (BCA) protein determination assay kit prior to testing enzymes in saccharifications or SSFs. There is a choice of which temperature to run the assay at. We use the enhanced BCA protocol at 60°C.

2. Commercial cellulases may contain salts, low molecular weight (MW) peptides and media components, co-factors, inhibitors, and additives that may interfere with the BCA assay. De-salting the enzyme preparation prior to running the protein assay will eliminate that potential problem. Samples were overloaded into a 2 ml sample loop and subsequently washed from the loop with 1 ml of buffer after sample injection. The proteins within the void volume (either 15 or 20 ml) of the column (Pharmacia HiPrep 26/10, 20 mM acetate buffer, 10 ml/min) were collected, combined, and then assayed for protein concentration.

3. Filter-sterilize all enzyme before use unless you are using a preparation that does not require it be filtered, like a cellulase fermentation broth. Use non-cellulosic based filters.

4. Store enzyme at 4°C in sterile containers for up to a year. Check the protein value before use if stored for 3 months or more.

2.2. Media, Solutions, and Frozen Working Stock Culture of Saccharomyces cerevisiae D5A for Saccharification and SSF

The stock solutions listed below are used for saccharification and SSF. The citrate buffer is the only stock solution used in the saccharification. The yeast extract and peptone solution (10× YP) and citrate buffer are used in the SSF for nutrients and pH control, respectively. The glucose stock and 10× YP stock together make up the inoculum medium and the glycerol stock solution is the cryoprotectant used to freeze back the SSF yeast.

1. Citrate buffer (1.0 M): Dissolve 210 g citric acid monohydrate into 750 ml deionized water. Add 60–70 g sodium hydroxide until pH equals 4.5. Caution should be observed as the solution will become very hot. Let the solution cool to room temperature. Dilute to 1 l and check pH. If necessary add sodium hydroxide until the pH is 4.8. The 1 M stock can be stored in aliquots at −20°C. Before use, filter-sterilize buffer through a 0.45-μm or smaller sterile filter into a sterile container before adding to the saccharification or SSF. When the 1.0 M stock citrate buffer stock is diluted in the saccharification, the pH should be 5.0. When the 1.0 M stock citrate buffer stock is diluted in the SSF to 50 mM, the pH should be 5.2.
2. **10x YP medium:** Dissolve 100 g yeast extract (Difco) and 200 g peptone (Bacto) in about 600–700 ml of deionized water. Add enough deionized water to bring the total volume to 1,000 ml. Filter-sterilize through a 0.45-µm or smaller sterile filter into a sterile container. Store at room temperature up to 3 months.

3. **Glucose stock (500 g/l):** Dissolve 500 g of glucose in about 400 ml of warm deionized water. Add enough deionized water to bring the total volume to 1,000 ml. Filter-sterilize through a 0.45-µm or smaller sterile filter into a sterile container. Store at room temperature up to 3 months.

4. **YPD (5%, w/v glucose) inoculum medium (14):** Using a sterile pipet, add to a sterile baffled shake flask 10 ml of sterile 10x YP medium and 10 ml of 500 g/l glucose stock solution per 1,000 ml. The working volume to flask volume ratio should be 1:5.

5. **Sterile glycerol solution (40%):** Weigh 40 g glycerol into 60 ml of deionized water. Filter-sterilize through a 0.45-µm or smaller filter into a sterile container or autoclave.

6. **Long-term storage of yeasts can be done a number of ways (15).** We have chosen cryogenic storage in a −70°C freezer. Obtain a frozen vial from the master stock of *S. cerevisiae* D5A. Transfer culture into 50 ml of sterile YPD medium in a sterile 250-ml baffled flask. Incubate in a rotary shaker at 38°C until the glucose concentration is around 5 g/l (10–16 h depending on the inoculum size). The pH should be between 4.5 and 5.0 and ethanol between 8 and 10 g/l. Observe the culture under the microscope for contamination. Aseptically mix equal volumes of 40% sterile glycerol solution and inoculum. Dispense 1-ml aliquots into sterile cryovials. Place in a −70°C freezer for up to 1 year.

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### 3. Methods

#### 3.1. Enzyme Testing for the Hydrolysis of Lignocellulosic Biomass: Saccharification Procedure

This procedure measures the glucose yield from pretreated biomass as a function of cellulase enzyme loading. Different enzyme preparations can be compared on the same substrate by determining how much enzyme is needed for a particular glucose yield or the amount of enzyme needed for maximum glucose yield. No microorganism is used.

1. The saccharification method described below applies to substrates where the cellulose content is known (see Note 1).
2. The pretreated biomass is washed to remove the residual sugars and inhibitors. The pretreated biomass is placed in a centrifuge bottle with two weight volumes of water. Mix well. The mixture is centrifuged and the water decanted. The solids are resuspended in another two weight volumes of water, centrifuged, and the water decanted. This is repeated four more times or until the glucose concentration in the wash water is less than 0.05 g/l as measured by a Yellow Springs Instrument (YSI) glucose analyzer (Yellow Springs, OH).

3. A dry-solids measurement is determined by weighing, in triplicate, the washed cellulosic substrate into tared aluminum pans, dried overnight in a 105°C convection oven, and weighed again. The percentage of dry solids is determined by subtracting out the tared weight and dividing the dry solid weight by the wet solid weight (see Notes 2 and 3).

4. Choose a cellulose level, enzyme loading, and total mass for the saccharification. We typically use 6% w/w cellulose level and an enzyme loading of 20 mg protein/g cellulose in 100 g total mass (see Note 4).

5. Calculate how much washed biomass is needed to achieve the targeted cellulose level based on the cellulose content, the percent solids, and total mass. If the total mass is 100 g and the desired cellulose loading is 6%, w/w, 6 g of cellulose is needed. If the biomass is 60% cellulose and the dry solids percent is 25%, 40 g of biomass is needed for 6% cellulose loading.

6. Calculate the amount of cellulase needed from the measured protein concentration, the amount of cellulose present, and the desired enzyme loading (see Note 5). If the desired enzyme loading is 20 mg protein/g cellulose, the amount of cellulose present is 6 g, and the protein concentration is 120 mg/ml, the amount of enzyme needed is 1 ml.

7. Calculate the amount of water and citrate buffer to be added to the biomass and enzyme to achieve the desired total mass and a final citrate buffer concentration of 50 mM. For our example, 5 ml of 1.0 M citrate buffer (pH 4.8) and 54 ml of deionized water will be added to 40 g of biomass and 1 ml of enzyme for 100 g total mass. We assume all liquids have a density of 1.0 g/ml.

8. Add the water and substrate to an autoclavable flask or glass media bottle (Wheaton) with a screw cap. A 1:2.5 ratio of total mass-to-flask volume works well. Record the final weight of the flask. Autoclave at 121°C for 30 min (see Note 6). Cool and re-weigh flask. Determine the amount of water lost in the autoclave step by subtracting the weight after autoclaving from the weight before autoclaving.
9. In a laminar flow hood, aseptically add the citrate buffer, sterile water (water lost from autoclaving), and enzyme mixture. Mix well (see Note 7).

10. Aseptically, take a 3-ml slurry sample at time zero using a sterile 5 ml pipette (see Note 8) with the tip broken off. Store in capped tubes or vials. Place the samples on ice until all the samples of that specific time point have been collected. To ensure no hydrolysis takes place after a sample is taken, boil the sample for 5 min, and then place on ice (see Note 9).

11. Incubate the flasks in a rotary shaker at 0.18 G based on a 19mm diameter circular orbit and 130 rpm (agitation will depend on the working weight and the biomass solids level) and 50°C (see Note 10).

12. Centrifuge samples and filter the supernatant through a 0.45-μm nylon filter into HPLC vials. A small amount of sample can be set aside to measure the pH.

13. Determine the amount of glucose present in each supernatant sample by the YSI glucose analyzer or by HPLC using the Shodex SP0810 carbohydrate column and refractive index detection. The mobile phase is 100% deionized water (isocratic elution mode), the flow rate 0.6 ml/min, and the column temperature 85°C.

14. Appropriate sampling times are 0, 4–8 h, and then daily for 7 days.

15. Calculate the glucose yield as % of the theoretical yield by using the following formula:

\[
\% \text{ Yield} = \frac{[\text{Glucose}] - [\text{Glucose}_0]}{1.111 f[\text{Biomass}]} \times 100\%
\]

where

- \([\text{Glucose}]\) is the residual glucose concentration (g/l);
- \([\text{Glucose}_0]\) is the initial glucose concentration (g/l);
- \([\text{Biomass}]\) is the dry biomass concentration at the beginning of the fermentation (g/l);
- \(f\) is the cellulose fraction in dry biomass (g/g).

16. Comparisons between enzyme preparations can be made by plotting the maximum glucose yield as a function of enzyme loading (Fig. 1). In Fig. 1, a loading of 10.5 mg protein/g cellulose of Enzyme A is needed for 80% glucose yield as compared to Enzyme B loading of 36 mg protein/g cellulose. To achieve 90% glucose yield, 16 mg protein/g cellulose of Enzyme A is needed as compared to >50 mg protein/g cellulose of Enzyme B. This methodology can be applied to measuring the reactivity of different biomass samples to a specific enzyme preparation (see Note 11).
Enzyme evaluations can include SSF testing which integrates enzyme usage into a process such as the production of ethanol from lignocellulosic biomass. This procedure measures the ethanol yield from pretreated biomass as a function of cellulase enzyme loading. When evaluating enzymes, this procedure best applies to washed biomass, which minimizes inhibition to the fermentation microorganisms. This procedure also prevents product (glucose) inhibition to the enzyme because the microorganism ferments the glucose to ethanol.

1. The SSF method described below applies to substrates where the cellulose content is known (see Note 1).

2. The pretreated biomass is washed to remove the residual sugars and inhibitors. The pretreated biomass is placed in a centrifuge bottle with two weight volumes of water. Mix well. The mixture is centrifuged and the water decanted. The solids are resuspended in another two weight volumes of water, centrifuged, and the water decanted. This is repeated four more times or until the glucose concentration in the wash water is less than 0.05 g/l as measured by a YSI glucose analyzer.

3. A dry-solids measurement is made by weighing, in triplicate, the washed cellulosic substrate into tared aluminum pans, dried overnight in a 105°C convection oven and weighed again. The percentage of dry solids is determined by subtracting out the tared weight and dividing the dry solid weight by the wet solid weight (see Notes 2 and 3).
4. Choose a cellulose level, enzyme loading, and total mass for the saccharification. We typically use 6%, w/w cellulose level and an enzyme loading of 20 mg protein/g cellulose in 100 g total mass (see Note 4).

5. Calculate how much washed biomass is needed to achieve the targeted cellulose level based on the cellulose content, the percent solids, and total mass. If the total mass is 100 g and the desired cellulose loading is 6%, w/w, 6 g of cellulose is needed. If the biomass is 60% cellulose and the dry solids percent is 25%, 40 g of biomass is needed for 6% cellulose loading.

6. Calculate the amount of cellulase needed from the measured protein concentration, the amount of cellulose present, and the desired enzyme loading (see Note 5). If the desired enzyme loading is 20 mg protein/g cellulose, the amount of cellulose present is 6 g, and the protein concentration is 120 mg/ml, the amount of enzyme needed is 1 ml.

7. Calculate the amount of water, yeast extract, peptone, and citrate buffer to be added to the biomass and enzyme to achieve the desired total mass: 10 g/l yeast extract, 20 g/l peptone, and a final citrate buffer concentration of 50 mM. Allow 1 ml for the yeast inoculum. For our example, 5 ml of 1.0 M citrate buffer (pH 4.8), 10 ml of 10× yeast extract/peptone stock solution, 1 ml of yeast inoculum, and 43 ml of deionized water will be added to 40 g of biomass and 1 ml of enzyme for 100 g total mass. We assume all liquids have a density of 1.0 g/ml.

8. Add the water and biomass to an autoclavable shake flask equipped with a water trap. We use a glass-blown top attached to a stopper similar in design to what home beer brewers use on their bucket fermentors. A 1:2.5 ratio of total mass-to-flask volume works well. Record the weight of the flask. Autoclave at 121°C for 30 min. Cool and re-weigh flask. Determine the amount of water lost in the autoclave step by subtracting the weight after autoclaving from the weight before autoclaving (see Note 6).

9. Prepare the yeast inoculum medium by autoclaving an empty baffled shake flask with a Morten cap. Use a 1:5 ratio of liquid volume to flask volume. Aseptically, pipette from stock yeast extract/peptone and glucose solutions and sterile deionized water for a 10 g/l yeast extract, 20 g/l peptone, and 50 g/l glucose medium (YPD flask). The amount of inoculum needed will depend on the scale and number of SSF tests.

10. Inoculate the YPD flask with one thawed stock vial of *S. cerevisiae* D5A (see Note 12). Incubate for 10–14 h operating at 38°C and 0.18 G based on a 19 mm diameter circular orbit and 130 rpm.
11. Before transferring the inoculum, check microscopically for contamination and measure the residual glucose and optical density (OD) at 600 nm. The culture should be transferred once the glucose is below 2 g/l. Optimally, there should be some residual glucose to ensure that cells are still in the growth phase.

12. At time of transfer, determine the amount of culture needed to inoculate the SSF flask(s) for a starting OD of 0.5 (see Note 13). For example, if the final OD in the inoculum flask is 8.0, 68.75 ml of inoculum is needed for ten SSF flasks containing 100 g of total mass.

\[
\text{Total vol. from inoc. flask (mL)} = \left( \frac{\text{SSF total mass (g) } \times 0.5}{\text{Inoc. O.D. } \times} \right) \times \left( \text{Number of SSF flasks } + 1 \right)
\]

13. Centrifuge the calculated total volume from the inoculum flask in a tabletop centrifuge to pellet the cells. Decant the supernatant. Resuspend in sterile YP with no carbon and centrifuge again. Resuspend cells in 1 ml of sterile YP with no carbon for every SSF flask (plus one extra). For example, if ten flasks will be inoculated, resuspend cells in 11 ml of sterile YP with no carbon.

14. In a laminar flow hood, aseptically add the 10× yeast extract/peptone, citrate buffer, sterile water (water lost from autoclaving), inoculum, and enzyme mixture. Mix well.

15. Aseptically, take a 3-ml slurry sample at time zero using a sterile 5-ml pipette with the tip broken off. Store in capped tubes or vials. Place the samples on ice until all the samples of that specific time point have been collected.

16. Incubate the flasks in a rotary shaker at 0.18 G based on a 19 mm diameter circular orbit and 130 rpm (agitation will depend on the working weight and the biomass solids level) and 38°C.

17. Centrifuge samples and filter the supernatant through a 0.45-μm nylon filter into capped HPLC vials.

18. Determine the amount of glucose and ethanol present in each supernatant sample by HPLC with the BioRad HPX-87H organic acids column and refractive index detector. We used 0.1M sulfuric acid mobile phase (isocratic elution mode), 65°C column temperature, and 0.6 ml/min flow rate.

19. Appropriate sampling times are 0, 4–8 h, and then daily for 7 days.

20. Calculate the ethanol yield as percent of the theoretical yield by using the following formula:
%Cellulose conversion = \frac{[\text{EtOH}]_f - [\text{EtOH}]_o}{0.51(f[Biomass]1.111)} \times 100%

where

- $[\text{EtOH}]_f$ is the ethanol concentration at the end of the fermentation (g/l);
- $[\text{EtOH}]_o$ is the ethanol concentration at the beginning of the fermentation (g/l);
- $[\text{Biomass}]$ is the dry biomass concentration at the beginning of the fermentation (g/l);
- $f$ is the Cellulose fraction of dry biomass (g/g);
- The number 0.51 is the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast;
- The number 1.111 converts cellulose to equivalent glucose.

21. Comparisons between enzyme preparations can be made by plotting the maximum ethanol yield as a function of enzyme loading (Fig. 2). In Fig. 2, a loading of 20 mg protein/g cellulose of Enzyme A is needed for 90% ethanol yield as compared to 62 mg/g cellulose of Enzyme B (see Note 14). The maximum ethanol yield from Enzyme A was 97% at 54 mg protein/g cellulose, and from Enzyme B 62 mg/g cellulose was needed to achieve a maximum ethanol yield of 90%.

![Fig. 2. Enzyme performance as measured by ethanol yield from SSF.](image-url)
4. Notes

1. A variety of pretreated cellulosic substrates can be used to test the efficacy of the cellulase enzyme. These substrates can be used unwashed. It is recommended that a compositional analysis be completed to determine the cellulose content; however, the enzyme can be added on the basis of total solids if compositional analysis is unavailable.

2. Total solids can be measured using an infrared balance. It is recommended that substrates that have not been washed and contain soluble solids be dried in a 45°C vacuum oven for 72 h to avoid charring the substrate. An accurate solids measurement is necessary for reproducibility and accuracy. It is recommended that a solids measurement be completed at least in duplicate before each test.

3. Do not completely heat-dry biomass and then subject to enzymatic hydrolysis. Once dry, the pores of the biomass may irreversibly collapse, preventing the enzyme from hydrolyzing the cellulose.

4. Higher biomass loadings that more closely mimic production scale can be tested with this procedure. Whole slurry (unwashed pretreated biomass) can also be tested.

5. Filter paper activity per gram of cellulose and milliliter of enzyme per gram of total solids are examples of other methods of loading cellulase in a saccharification or SSF.

6. Autoclaving is not recommended for whole slurry biomass containing solubilized sugars. Antibiotics such as tetracycline (10 mg/l) can be added, or follow aseptic practices by using presterilized glassware, sterile media, and pipettes and work in a laminar flow hood to prevent contamination.

7. It is critical that the enzyme gets mixed in well with the slurry. This is not a problem at low solids with free liquid present, but at high solids mixing can be challenging. When high solids (>20% insoluble solids) are used, we set up a flask (“dummy flask”) that replicates the experimental conditions that is used for obtaining initial data. The rest of the flasks are not sampled until the slurry begins to liquefy.

8. Falcon brand sterile 5-ml pipettes have a scored tip that can be broken off by hand. We break the tips off while the pipettes are still in the sterile wrap.

9. Experiments requiring samples to be taken close together and early in the hydrolysis can be affected by residual enzyme activity. To ensure no hydrolysis takes place after a sample is taken, boil the sample for 5 min and then place on ice.
10. The cellulase enzyme(s) being used should guide the selection of the incubation temperature. Saccharification temperatures typically range from 45 to 54°C. Typically the enzyme manufacturers will recommend a temperature.

11. Other biomass hydrolyzing enzymes can be tested with this method. For example, one can add xylanase and measure xylose yield.

12. A variety of microorganisms besides *S. cerevisiae* D5A can be used to test the enzyme performance in SSF. The appropriate media and growth conditions for the specific organism should be used.

13. Standardizing the inoculum procedure improves accuracy within an experiment and reproducibility between experiments.

14. In SSF, enzyme performance is measured by the product yield. Other products besides ethanol could be used to measure enzyme performance.

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### References


Chapter 16

Basic Laboratory Culture Methods for Anaerobic Bacteria

Herbert J. Strobel

Summary

Oxygen is either limiting or absent in many ecosystems. Anaerobic bacteria are often key players in such environments and these organisms have important roles in geo-elemental cycling, agriculture, and medicine. The metabolic versatility of anaerobes is exploited in a variety of industrial processes including fermented food production, biochemical synthesis, and bioremediation. There has been recent considerable interest in developing and enhancing technologies that employ anaerobes as biocatalysts. The study of anaerobic bacteria requires specialized techniques, and specific methods are described for the culture and manipulation of these microbes.

Key words: Anaerobic bacteria, Batch culture, Continuous culture

1. Introduction

Renewed interest in the production of fuels, chemicals, and other products from biological feedstock has led to a resurgence in the development of industrial processes that employ anaerobic bacteria. Thermophilic anaerobes have garnered particular attention because of the robust enzymatic activities and metabolic pathways possessed by many of these organisms. When compared to mesophilic aerobic processes, fermentations using thermophilic anaerobes have potential benefits including lower risk of contamination, little need to provide reactor cooling, and no oxygen supply requirement. The high level of interest in commercializing technologies that employ anaerobic thermophiles has attracted a diverse group of researchers and engineers, and while many have microbiological experience, it is often limited to work with aerobic organisms. This chapter provides basic protocols for the
laboratory culture of thermophilic anaerobic bacteria. Since the techniques for culturing these organisms are generally similar to those for anaerobes growing at lower temperatures, no distinction between the groups will be made in the description of methods unless warranted for technical reasons. Given the specific requirements for culturing individual bacterial species, readers are urged to review the primary literature that is relevant to the organisms of interest. Also, previous literature has reviewed anaerobic culture methods, and some of this is listed in the References (1–3). Readers are particularly encouraged to carefully review relevant details contained within Hungate’s classic description of anaerobic procedures (1).

Detailed discussion of media composition is beyond the scope of this chapter, and the medium used to support the growth of a specific bacterial strain should either be obtained from previous work or determined empirically. Nevertheless, since anaerobes by their nature are sensitive to oxygen, it is important to emphasize that their culture requires careful attention to excluding oxygen during media and solution preparation, inoculation and transfer, and cell growth. Although there is a spectrum of oxygen sensitivity among bacteria labeled as “anaerobes,” most obligate (“strict”) anaerobic organisms cannot tolerate any oxygen exposure or, at best, only brief exposures to low concentrations. This sensitivity is largely due to a lack of enzymatic systems that detoxify reactive oxygen molecules such as peroxide, superoxide, and various radical species. In this light, equipment and protocols that minimize exposure of the organisms to oxygen will lead to more consistent, reproducible results.

Since some common plastics are gas permeable, most routine culture of anaerobic bacteria is performed using glass vessels. Similarly, tubing that is employed to transfer gas or liquid and stoppers that seal vessels should also be gas-impermeable. Black butyl rubber is commonly used. Alternatives are available, but manufacturers should first be consulted regarding the gas permeability of a particular material.

When preparing medium and culturing organisms, oxygen-free gases (e.g., nitrogen, carbon dioxide, argon, helium, etc.) are often used to sparge solutions and maintain an anaerobic gas phase in vessels. Gases free of oxygen contamination (e.g., “anaerobic” or “oxygen-free” grade) are commercially available, but these are exceedingly expensive for routine laboratory work. Lower quality grades, while more economical, typically contain sufficient amounts of oxygen to inhibit the growth of strict anaerobes, particularly those which are very oxygen-sensitive (e.g., methanogenic bacteria). However, this contamination is conveniently removed by passing the gas over heated copper turnings (approximately 350°C; Fig. 1; see (1) for construction details).
The resultant dark-colored copper oxide can then be subsequently re-oxidized by a flow of hydrogen with release of water vapor in the process (see Note 1).

Although oxygen removal and exclusion are critical elements in culturing strict anaerobes, these steps are not necessarily sufficient to ensure success. In addition to the absence of oxygen, most anaerobes also require an environment that has a relatively low redox potential (typically, several hundred millivolts below that of a standard hydrogen electrode). As Hungate elegantly calculated (1), it is not physically possible to achieve these low potentials by simply removing oxygen from the solution, and a
reducing agent must be added to the growth medium. Although not required for growth, many investigators also include resazurin in the medium as an indicator of redox potential.

The routine laboratory culture of anaerobic bacteria usually occurs in sealed tubes, bottles, or flasks ranging from 5 to 5,000 ml. Depending on the specific experimental objective, batch cultivation can also take place in containers that are open to the atmosphere while continuously sparged with oxygen-free gas. Although cultures can be grown in standard test tubes and bottles with typical rubber stoppers, many laboratories use “Balch” tubes and serum bottles, both of which accept serum bottle-type stoppers that provide a gas-tight seal (3). Aluminum crimp caps are also used to prevent blowout of stoppers during autoclaving or from fermentation gases produced during microbial metabolism. Solutions and inoculations can be conveniently and aseptically added through the stoppers using tuberculin syringes and needles. This system allows investigators to easily manipulate cultures on the bench top without the need for highly specialized and dedicated equipment.

In some situations, dedicated anaerobic chambers (aka “glove boxes”) are employed to grow and manipulate anaerobes. These units isolate the user from the anaerobic environment that is contained within a flexible vinyl bag or rigid metal/plastic box, and manipulations are performed using rubber sleeves and gloves that protrude into the chamber. Airlocks allow introduction of supplies, and metal catalysts (e.g., palladium) remove residual oxygen that invariably enters the chamber. A well-maintained chamber contains less than 2 ppm oxygen (as compared with approximately 2 × 10^5 ppm in air). Although not necessary for the routine culture of most anaerobes, anaerobic chambers can be useful when growing bacteria on solid surfaces (e.g., agar), purifying oxygen-sensitive components from cells or performing other activities that demand exclusion of oxygen during the extensive manipulation of cultures or cells.

The isolation of pure cultures from mixed microbial environments often involves agar plates, but the traditional method of streaking and plating has limited utility when applied to anaerobic organisms. Hungate developed and perfected the “roll tube” technique (1, 4) of isolating strict anaerobes, and this method continues to be used many decades after its introduction. The more recent development of anaerobic chambers and jars provides the option to also use standard Petri plate techniques when working with anaerobes.

The vast majority of microbial culture and experimentation is performed using batch culture where cells typically grow for a period of time in an unrestricted manner. The batch environment is not static;
substrate depletion, metabolic end-product accumulation, and pH shifts are a few of the changes that can occur as the bacteria grow and cell mass increases. The dynamic nature of batch cultures has direct effects on cellular metabolism and consequently limits the interpretation of experimental data and development of biological models. In contrast, continuous culture involves the precise manipulation of medium and substrate inflow rate, usually in a manner that constrains provision of a specific nutrient. This permits direct control of microbial growth rate \((5–8)\). Continuous cultures reach and maintain a steady-state condition where physiological parameters (e.g., metabolite levels, enzyme synthesis rates, end-product production rates, microbial cell mass) are essentially constant and time-independent. For this reason, the continuous culture method is a powerful tool for analyzing bacterial physiology.

At its core, a continuous culture system consists of a growth vessel into which fresh medium is continuously delivered at a known rate. Provision is made for passive (e.g., overflow device) or active (e.g., pump) removal of culture such that a constant volume is maintained in the growth vessel. Control of temperature, agitation, and medium flow is usually part of a basic apparatus. There are various commercial continuous culture systems available, but most investigators either modify these or construct completely customized equipment to suit experimental needs. Automated controls and measurements (e.g., pH, product analysis, cell density) can be incorporated to create sophisticated systems. Although most apparatus are designed to handle liquids, there are also examples based on the delivery of insoluble substrates such as cellulose \((9, 10)\), but these are very specialized and require considerable expertise to operate.

2. Materials

Analytical-grade chemicals should be used, and water should be high purity (e.g., either deionized and distilled or Milli-Q quality). It should be noted that the suppliers listed are not necessarily the only sources of particular items. Additionally, custom-made substitutions are possible for some of the equipment.

2.1. Media Preparation

2.1.1. Equipment

1. Oxygen-free gases. Commercial-grade gases should be passed over oxygen-removing column as outlined in Subheading 1.1.
2. Gas-impermeable tubing (Randolph Austin Co., Austin TX).
4. Sparging implements (Fig. 2):
   (a) Blunt end needles/cannula with female Luer-fittings (Hamilton Co. Reno, NV).
   (b) Observation tubes with male Luer-fittings (Popper and Sons, New Hyde Park, NY).
   (c) gas dispersion tubes.
5. Erlenmeyer flask with custom rubber stopper (Fig. 3).
6. Plastic syringe (30–60 ml as needed) fitted with approximately 30 cm butyl rubber tubing.
7. Serum bottles and stoppers.
8. Autoclave: sufficient to provide 0.3 MPa at 121°C.

2.1.2. Chemicals and Reagents
1. Basal medium. Since most organisms have specific growth requirements, medium composition should be formulated using past literature and experience.
2. Reducing agent (see Note 2).
3. Carbohydrate solution (see Note 3).
4. Buffer solution (see Note 4).

2.2. Anaerobic Transfer of Cultures

2.2.1. Equipment

2.2.2. Chemicals and Reagents
1. Tuberculin syringes and needles: 1–10 ml with 21½G needles.
2. Ethanol: 95% (w/v).
3. Sterile basal medium.
4. Sterile media supplement stock solutions.
2.3. Culture Preservation

2.3.1. Equipment

2.3.2. Chemicals and Reagents

1. Tuberculin syringes and needles: 1–10 ml with 21½G needles.

1. Ethanol: 95% (w/v).
2. Sterile basal medium.
3. Sterile media supplement stock solutions.
4. Glycerol.

Fig. 3. Vessel for media preparation. (a) Gas dispersion tube connected to butyl-rubber tubing. (b) gas vent fitting consisting of 1-ml syringe barrel filled with glass wool. Alternatively, a syringe filter apparatus can be used as a vent.
1. Medium reservoir vessel. An appropriately sized glass vessel fitted with ports for supplement addition, medium outflow, and sparging is required (Fig. 4).

2. Growth vessel. A glass vessel fitted with an overflow device with provisions for temperature control, agitation, and gas sparging is needed (Fig. 4).

3. Effluent collection vessel. A glass vessel is required for collection of culture overflow from the growth vessel (Fig. 4).

4. Peristaltic pump. A pump capable of delivering 5–500 ml/h (see Note 5).

2.4. Batch Culture Growth

See Subheading 2.2.1.

2.5. Continuous Culture Growth

2.5.1. Equipment

1. Medium reservoir vessel. An appropriately sized glass vessel fitted with ports for supplement addition, medium outflow, and sparging is required (Fig. 4).

2. Growth vessel. A glass vessel fitted with an overflow device with provisions for temperature control, agitation, and gas sparging is needed (Fig. 4).

3. Effluent collection vessel. A glass vessel is required for collection of culture overflow from the growth vessel (Fig. 4).

4. Peristaltic pump. A pump capable of delivering 5–500 ml/h (see Note 5).

2.5.2. Chemicals and Reagents

See Subheading 2.1.2.

3. Methods

3.1. Preparation of Media

The following describes the preparation of 1 l medium that is dispensed into individual culture tubes (5–10 ml) or bottles (20–100 ml) that are used for batch growth (see Subheading 3.5 for continuous culture media). The method can be adapted
to prepare larger volumes in vessels that will be directly inoculated instead of being dispensed (see Note 6). It is strongly recommended that oxygen-removing columns be rejuvenated with hydrogen gas prior to medium preparation and other critical procedures that require use of anaerobic gases.

1. Combine basal medium components in a 1-l Erlenmeyer flask and adjust pH as needed.

2. Secure stopper and clamp sparging gas inlet. Do not clamp vent outlet of stopper during autoclaving. Failure to maintain open vent will cause the flask to explode.

3. Degas medium by autoclaving for 10 min at 121°C and 0.3 MPa (see Notes 7 and 8).

4. Sparge medium with oxygen-free gas and cool to room temperature. Exercise extreme caution when sparging hot solutions; introduce gas at slow rate and then gradually increase the rate as needed.

5. Anaerobically add additional medium components as needed (see Subheading 3.1.2).

6. Anaerobically transfer the desired quantity into tubes or bottles (see Note 10):
   (a) While sparging the medium, insert an appropriately sized plastic syringe to which is attached butyl rubber tubing (see Note 11).
   (b) Fill syringe with gas from headspace of media flask. If using a syringe, remove from flask, displace gas, re-enter flask, re-fill with gas, and then displace gas into flask.
   (c) Insert pipette into medium and withdraw required volume.
   (d) Dispense medium into tube or flask that is being flushed with oxygen-free gas.
   (e) Remove gassing needle while simultaneously sealing with stopper.
   (f) Repeat steps 5c–e until all medium is dispensed (see Note 12).
   (g) Crimp seal all tubes or bottles.

7. Sterilize tubes or bottles by autoclaving for 20 min at 121°C and 0.3 MPa.

3.1.2. Media Supplement Preparation

Although it is possible to prepare bulk quantities of a “complete” medium that has all components required for microbial growth, it is usually more convenient, and sometimes even necessary, to prepare a sterilized basal medium instead and then provide supplements (e.g., carbohydrates, reducing agents, buffers, etc.) as needed by adding them as separate solutions. Using this strategy,
the basal medium can be easily “customized” with different types and concentrations of supplements (see Note 13).

1. Add supplement material to empty tube or bottle.

2. Thoroughly flush vessel with oxygen-free gas.

3. Bring an appropriate volume of distilled water or buffer to a vigorous boil in a round-bottom flask for 30 s while sparging with oxygen-free gas.

4. Anaerobically transfer the desired quantity of liquid into tubes or bottles (see step 5 in Subheading 3.1.1) and seal vessel.

5. Sterilize tubes or bottles by autoclaving for 20 min at 121°C and 0.3 MPa (see Note 9).

### 3.2. Anaerobic Transfer of Cultures

The following describes the routine transfer of cultures between sealed tubes or bottles.

1. Sterilize stoppers of all tubes or bottles by applying 95% ethanol and flaming.

2. Aseptically add necessary media supplements (e.g., carbohydrate) to media:
   (a) Insert a sterile needle attached to a 1-ml tuberculin syringe into stock supplement bottle.
   (b) Invert stock solution bottle and withdraw approximately 0.2 ml of solution.
   (c) Withdraw needle from bottle and hold syringe near vertical position.
   (d) Gently tap side of needle to displace any gas bubbles towards needle end of syringe.
   (e) Displace gas from syringe with plunger.
   (f) Reinsert needle into stock bottle, invert, and withdraw desired amount of stock solution.
   (g) Dispense solution into sterile medium tube or bottle.

3. Add the desired amount of inoculum to tube or bottle by performing steps 2a–g using a culture tube instead of a stock bottle.

4. Wipe down work surface with disinfectant.

5. Incubate inoculated tubes or bottles at appropriate temperature in a water bath.

### 3.3. Culture Preservation

The method described does not require specialized equipment or techniques. It is the author’s experience that an effective preservation solution can be designed by inclusion of 20% (v/v) of glycerol to the basal growth medium. Culture viability can often be maintained for 5 years or more when cells are stored in this solution at −70°C or lower. However, it is important to note that
the effectiveness of this approach can vary significantly depending on the specific organism, medium composition, and storage conditions; therefore, it is critical to routinely evaluate the viability of frozen culture stocks. Investigators should also be aware that alternative techniques (e.g., freeze-drying) may be more effective in preserving cell viability.

1. Prepare a basal medium that contains 50% (v/v) glycerol (see steps 1–4 of Subheading 3.1.1).

2. Anaerobically transfer 3 ml of preservation medium into serum vials (see step 5 of Subheading 3.1.1).

3. Sterilize vials by autoclaving for 20 min at 121°C and 0.3 MPa.

4. Allow vials to cool and then transfer 4 ml of a freshly grown culture into vial (see Subheading 3.2).

5. Immediately freeze vials containing culture at −70°C or lower.

6. Revive cultures as needed.
   (a) Thaw preservation vial at room temperature. Do not thaw with warm water.
   (b) As soon as liquid in the vial has thawed, transfer 0.5–1 ml of preserved culture into fresh medium tube containing 10 ml medium and the required substrates and supplements (see Subheading 3.2).
   (c) Immediately re-freeze the preservation vial (see Note 14).

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**3.4. Batch Culture Growth**

The following describes a basic protocol for growing anaerobes in batch cultures ranging from 10 to 1,000 ml. It is presumed that cultures will be grown in sealed vessels (i.e., tubes or bottles). However, it is certainly possible for growth to occur in containers that are open to the atmosphere so long as the medium is either gently sparged with an oxygen-free gas or an anaerobic gas phase is maintained above the liquid.

1. Prepare the basal medium (see Subheading 3.1.1).

2. Add the appropriate supplements to the medium (see Subheading 3.1.2).

3. Using the aseptic and anaerobic technique (see Subheading 3.2), inoculate the medium with 0.5–5% (v/v) of preserved or freshly grown culture.

4. Monitor the growth and metabolic activity using the appropriate and preferred method (e.g., optical density, dry weight, fermentation end products, etc. (see Note 15).

---

**3.5. Continuous Culture Growth**

There are a variety of commercial and custom continuous culture systems available, but description of these and their operation
are far beyond the scope of this chapter. Instead, a generalized protocol is provided that is based on the setup and operation of a relatively simple apparatus (Fig. 4). This system has a growth vessel with a working volume of 350 ml. The preparation of 13 l medium provides sufficient volume to support approximately eight individual samplings that are taken after approximately a 95% turnover of culture volume between samplings. Readers are encouraged to review continuous culture theory and the mathematics associated with it.

1. Prepare approximately 12 l basal medium using the basic protocol (see Subheading 3.1.1) with the following modifications:
   (a) Omit any heat-labile components (e.g., vitamins). Prepare these as separate solutions, by autoclaving or sterile filtering, and aseptically add to sterilized basal medium.
   (b) Sterilize 12 l medium by autoclaving for 90 min at 121°C and 0.3 MPa.
   (c) Cool medium to room temperature.
   (d) Add the heat-labile components and other supplements.
   (e) Add anaerobic, sterile water to bring volume up to 13 l.

2. Sterilize growth vessel, effluent vessel, tubing, connectors, and any other equipment that will come into contact with media and/or culture by autoclaving for 20 min at 121°C and 0.3 MPa.

3. Aseptically connect the medium reservoir and effluent vessel to the growth vessel.

4. Sparge the growth vessel with oxygen-free anaerobic gas and maintain gas flow.

5. Calibrate the pump as described by the manufacturer.

6. Fill the growth vessel with medium, begin agitation, and heat to the desired temperature.

7. Inoculate the growth vessel with 0.5–5% of preserved or freshly grown culture.

8. Allow the culture to grow in batch mode to achieve reasonable cell density.

9. Set the pump to achieve the desired flow rate and begin pumping the medium.

10. Perform measurements and take samples after either set periods of time or turnover.
4. Notes

1. Extreme care must be taken when using hydrogen gas in the proximity of open flames and heated equipment. Hydrogen forms explosive mixtures with air when it accumulates from 4–74% (v/v), and spontaneous ignition can occur even without heating, spark, or flame. It is absolutely critical, when passing hydrogen gas through an oxygen-removing column, that the (1) room be well ventilated to prevent hydrogen accumulation; (2) hydrogen source not be in direct contact with any flames, heated objects, or other potential ignition sources; and (3) residual hydrogen exiting the column be combusted by ignition with a flame.

2. Cysteine is a commonly used reducing agent because it is inexpensive and relatively nontoxic. However, it is oxidized to cystine under basic conditions and this amino acid is toxic to some anaerobes. Therefore, care must be taken with regard to cysteine addition during medium preparation. Some protocols require that cysteine be added as a separate solution after the basal medium is sterilized, but it can often be added directly to the medium mixture prior to sterilization. There is also evidence that photocatalytic interaction with resazurin promotes reduction of the medium by cysteine (11). Cysteine cannot support the growth of some anaerobes since its redox potential is only −170 mV (12). Alternatives include dithiothreitol, titanium salts (e.g., titanium citrate), sodium sulfide, and ferrous sulfide.

3. Carbohydrate stock solutions of 5–20% (w/v) in anaerobic water permit additions of convenient volumes to culture tubes and bottles (e.g., adding 0.2 ml of a 10% carbohydrate stock to a 10-ml medium tube results in final carbohydrate concentration of 2 g/l). Since some carbohydrates are chemically labile, care must be taken in preparing stock solutions (see Note 9).

4. Many anaerobic media utilize a buffering system based on carbon dioxide (in the form of carbonic acid) and a carbonate salt (typically sodium carbonate or sodium bicarbonate). This combination is inexpensive, and a final concentration of 0.4% sodium carbonate equilibrated with carbon dioxide has a pKa of approximately 6.7, which provides a suitable pH for the growth of many organisms. However, other chemicals can serve as buffering components and the reader is encouraged to review relevant literature.

5. Pumping requirements will be based on the working volume of the vessel and the desired dilution rates of the system. Flow rates of 5–500 ml/h will support dilution rates of approximately 0.014–1.4/h.
6. The precise time of sterilization by autoclaving will be determined by the volume of liquid and geometry of the container. Larger volumes will require more lengthy exposures and it is best to empirically determine the appropriate time.

7. As an alternative to removing soluble gases in an autoclave, it is possible to remove oxygen by bringing the solutions to a vigorous boil for at least 30 s. Extended sparging of solutions with oxygen-free gases will also eventually remove oxygen, but this is not usually practical given the lengthy time that is required.

8. If the medium will not be dispensed into tubes or bottles but rather into the vessel (e.g., 1-l flask) directly inoculated with the culture, the solution should be sterilized by autoclaving for an appropriate length of time.

9. Care must be exercised when autoclaving materials (e.g., vitamins, co-factors, etc.) that may be heat-labile. Sterile-filtering and irradiation are alternative means of sterilization. Special care should be taken when dealing with certain carbohydrates (particularly di- and oligo-saccharides; a combination of heat, pressure, and acidic pH can cleave glycosidic bonds). Some of this can be avoided by sparging the solutions with inert gases rather than carbon dioxide (which leads to the formation of carbonic acid).

10. The use of so-called mouth-tube pipettes (1) had been a common practice in transferring anaerobic solutions between vessels, especially when preparing media in Balch tubes. However, it is no longer a recommended practice.

11. Some anaerobes (e.g., some methanogens) are particularly sensitive to the presence of even the smallest traces of oxygen, and routine culture may be possible only if extraordinary precautions and conditions are employed. For instance, it might become necessary to dispense the medium in an anaerobic chamber and/or completely avoid the use of any plastic components (e.g., replacing plastic with glass syringes).

12. There is invariably some intrusion of oxygen when working from a large flask on the bench top and dispensing the medium into tubes and bottles. Resazurin in the medium will indicate such contamination, and the medium will take on a faint shade of purple or pink. In these situations, simply stop the dispensing procedure and let the medium continue sparging with an oxygen-free gas with gentle mixing. The color of the medium will usually revert to its original state within 5–15 min.

13. If supplements are being added to the basal medium prior to sterilization of the medium (i.e., at step 4, Subheading 3.1.1),
there is no need to sterilize the supplement solution. For instance, this is typically the case if a carbonate solution is being used as a buffer system.

14. Repeated thawing of frozen cultures can sometimes significantly reduce viability, and in such cases it is advisable to store and employ multiple single-use vials rather than rely on only a few multiple-use vials.

15. Tuberculin syringes are a convenient way of removing samples from sealed tubes and bottles.

References

Simultaneous Saccharification and Fermentation and Partial Saccharification and Co-Fermentation of Lignocellulosic Biomass for Ethanol Production

Joy Doran-Peterson, Amruta Jangid, Sarah K. Brandon, Emily DeCrescenzo-Henriksen, Bruce Dien, and Lonnie O. Ingram

Summary

Ethanol production by fermentation of lignocellulosic biomass-derived sugars involves a fairly ancient art and an ever-evolving science. Production of ethanol from lignocellulosic biomass is not avant-garde, and wood ethanol plants have been in existence since at least 1915. Most current ethanol production relies on starch- and sugar-based crops as the substrate; however, limitations of these materials and competing value for human and animal feeds is renewing interest in lignocellulose conversion. Herein, we describe methods for both simultaneous saccharification and fermentation (SSF) and a similar but separate process for partial saccharification and cofermentation (PSCF) of lignocellulosic biomass for ethanol production using yeasts or pentose-fermenting engineered bacteria. These methods are applicable for small-scale preliminary evaluations of ethanol production from a variety of biomass sources.

Key words: Saccharification, Fermentation, Lignocellulose, Pretreatment, GC

1. Introduction

Ethyl alcohol has been used by humans since the dawn of history and is thought to be one of the most universally known chemical compounds manufactured (1). Produced by the spontaneous fermentation of sugars, ethanol was used by ancient civilizations that evolved many types of production. Some cultures extracted and concentrated alcohol in crude stills and used it in the manufacture of perfumes, cosmetics, medicinal agents, and beverages. Ethanol found increasing use as a chemical agent, an ingredient, or a raw material for the production of other commodities, as
later civilizations improved upon the purification and distillation processes. With the entry of the United States into World War II, the alcohol requirements for munitions, synthetic rubber, solvents and thinners, and food increased the demand to unprecedented levels (1).

Ethanol may be produced by fermentation using three main groups of feedstock: saccharine-containing materials (molasses, fruit, sugar cane juices, etc.); starchy materials (cereal grains, root crops such as potatoes, etc.); and cellulosic or lignocellulosic materials (wood, waste sulfite liquor from paper pulp mills, agricultural residues such as corn cobs, hulls, stover, etc.). Using lignocellulose for ethanol production is not novel, and considerable effort was invested in converting sawdust and mill waste to ethanol using a dilute sulfuric acid process as early as 1915 (1). Ethanol was produced commercially in the United States by this method until the end of World War I, when molasses became a cheap source of readily available substrate.

Fermentation processes using wood wastes intensified abroad, however, since wood waste was more economically available than molasses or grain. By 1941, 21 foreign plants are reported to have operated on wood wastes. A commercial plant was erected for the development of ethanol from wood wastes in the United States by Defense Plants Corporation and was operational in 1947 (1); however, further development of this technology ceased when wartime scarcity disappeared and the era of inexpensive petrochemical fuels began (2).

Bioconversion of cellulosics was still pursued by the U.S. Army Natick Research and Development Command following WW II, but their interest pertained to protecting cellulosic materials used by the military (e.g., cotton uniforms) from microbial degradation (3). These scientists isolated the dominant organism responsible for decomposition of military clothing, tents, and other equipment in 1943. The organism was the fungus *Trichoderma reesei*, now known as an anamorph of *Hypocrea jecorina* (4). Hereafter, *T. reesei* is referred to as *H. jecorina*. Army researchers further identified an active cellulase complex that was stable and contained all the components needed to hydrolyze native cellulose. A hyper-secreting *H. jecorina* mutant was isolated in 1971, and in 1973 considerable effort focused on process development for production of cellulases and pretreatment options for various feedstock (5). Current commercial processes for producing cellulase are based on modifications of this fungus and its relatives.

In 1974, Gulf Oil Chemicals Company undertook extensive research and development to examine processes that could convert cellulose to chemicals. Their objective was to establish a chemical industry that was based on a renewable resource rather than petroleum (6). The subsequent OPEC oil embargo
emphasized the importance of developing this type of industry for obtaining greater energy independence. In 1976, a method was patented with yeast as the biocatalyst, which prevented glucose accumulation by combining the saccharification and fermentation steps, termed the simultaneous saccharification and fermentation (SSF) process \((7, 8)\). Faster enzyme activity is maintained because the glucose is fermented as soon as it is released by cellulase, thereby minimizing end-product inhibition. The alternative process, i.e., separate hydrolysis followed by fermentation (SHF), generates a sugar stream first, followed by inoculation with the fermenting organism for conversion of the sugars to ethanol. Partial saccharification and cofermentation (PSCF) is a combination of SSF and SHF, whereby the enzymes (usually from fungi) are given a “head start” under optimum conditions \((45–50^\circ C \text{ and } \leq \text{pH 5})\) to liberate some of the available monomeric sugars from the biomass polymers. After a period of a few hours the conditions are altered for the fermenting organism inoculation; the pH is usually raised and temperature often lowered. In this fashion, the fermenting organism rapidly consumes the previously liberated sugars, and the enzymes are still able to work, albeit at reduced efficiency. True SSF requires a lower capital cost, generally produces higher concentrations of ethanol, and reduces risks from contamination because the accumulation of sugars is avoided \((9)\). Selecting appropriate operating conditions of pH and temperature that favor both the enzymes and fermenting organism in an SSF process is crucial for maximizing yield and productivity, although there is some degree of flexibility in the range of both \((10)\).

In the United States, almost all ethanol is produced by fermenting dent corn, which is 60–65% starch; starch is an \(\alpha-1,4\)-linked polymer of glucose that is easily digested by humans or, in the case of ethanol production, by commercial amylases \((11, 12)\). The starch is either converted to glucose by a two-step enzyme process involving liquefaction followed by saccharification, or by a newer one-step process using native starch amylases. In both processes, usually the final release of glucose and fermentation are carried out simultaneously. Fermentation substrates, such as starch, which have competing value for animal and human needs, will be insufficient to meet the increasing demands for fuel ethanol. Therefore, a more plentiful and less expensive source of carbohydrate is needed as the feedstock. Furthermore, lignocellulosic ethanol offers larger reductions in greenhouse gases compared to corn ethanol or petroleum-based fuels \((13)\).

Cellulosic wastes, agricultural residues, and forage and woody crops are significant renewable resources for the production of fermentable sugars \((14–18)\). Ethanol production by fermentation of lignocellulosic biomass-derived sugars involves a fairly ancient art and an ever-evolving science. In general, lignocellulose is treated
to open the plant wall structure and disrupt lignin–hemicellulose complexes (19). Hemicellulose is a heterogeneous branched polymer that yields mostly xylose upon hydrolysis, as well as some arabinose, mannose, glucose, galactose, acetic acid, glucuronic acid, and furfural, depending upon the biomass type. Hydrolysis of the hemicellulose component to yield hexoses and pentoses is relatively easy compared to cellulose hydrolysis; however, efficient cofermentation of hexose and pentose sugars presents a challenge. Conversely, fermentation of the cellulose hydrolysis product, i.e., glucose, is straightforward, while the actual hydrolysis step itself is more difficult (20, 21). In its native form, cellulose is composed largely of crystalline fibers held together by an extensive network of hydrogen bonds. These fibers are embedded in a matrix of hemicellulose and lignin, which serves to further reduce their accessibility to cellulytic enzymes (22, 23). Solvent and mechanical pretreatments increase the accessibility of cellulose to hydrolysis presumably by disrupting the lignin matrix and crystalline structure of the cellulose. Therefore, pretreatment of lignocellulosics improves cellulose conversion (19, 21, 24). Pretreatment methods that have been examined for biomass conversion to ethanol include acid pre-hydrolysis, steam explosion, ammonia fiber expansion (AFEX), alkali treatment, organic solvents, and radiation, as well as numerous others (2, 11, 12, 25, 26). Many of the pretreatment methods, in addition to separating the cellulose, hemicellulose, and lignin, also hydrolyze the hemicellulose to monosaccharides. In contrast, cellulose must be converted to either glucose or cellobiose before fermentation, depending upon the fermenting microorganism’s ability to use dimers of glucose versus the monomeric form. Lignin, the third major component of lignocellulose, is a large phenolic polymer that cannot be fermented to ethanol but when combusted can provide sufficient energy for ethanol recovery (26).

The enzymatic hydrolysis of cellulose is particularly attractive because of its mild reaction conditions, high selectivity, and low impact on the environment (19, 20, 27). The complete hydrolysis of cellulose to glucose requires at least three major classes of enzymes: (1) exoglucanases, which attack nonreducing ends of crystalline cellulose chains; (2) endoglucanases, which degrade amorphous cellulose and may also introduce nicks in crystalline cellulose chains; and (3) β-glucosidase (cellobiase), which completes the process by degrading cellobiose into glucose monomers (20, 28). Deconstruction of intact plant cell walls is much more complex and involves many additional enzymatic activities. For example, enzymes such as phenolic esterases break the bonds between some carbohydrates and lignin and can enhance digestibility (29, 30). If hemicellulose is to be enzymatically digested, an additional suite of activities is required. For this discussion, hemicellulose is hydrolyzed with acid to liberate xylose, and enzymes are used to convert cellulose to glucose.
Microorganisms that degrade cellulose are ubiquitous and abundant in nature. These include fungi, bacteria, and actinomycetes. The ability to produce extracellular cellulolytic enzymes is widespread among fungi, with *Hypocrea jecorina* being one of the most extensively studied. Culture filtrates from this organism contain each of the major cellulolytic enzymes in a number of forms. While a multiplicity of each of these three major components exists, the mode of action of each general group of enzymes can be summarized for fungal enzymes as follows (28):

(a) endoglucanase; (b) β-glucosidase; (c) cellobiohydrolase.

Endoglucanases (EC 3.2.1.4) hydrolyze β-1,4-glycosidic linkages randomly and do not attack cellobiose. Most reports indicate minimal action on crystalline cellulose. Endoglucanases hydrolyze cellohexa- and cellooctaose, phosphoric-acid-swollen cellulose, and substituted celluloses (indicating low specificity). The β-glucosidase enzyme (cellobiase, EC 3.2.1.21) hydrolyzes cellobiose and cello-oligosaccharides to glucose and does not attack cellulose or higher cellooligosaccharides. Cellobiohydrolase (exocellulase, EC 3.2.1.91) splits off cellobiose units from the nonreducing end of the chain, does not attack substituted celluloses, and hydrolyzes cellohexa- and cellooctaose, but not cellobiose. An extensive discussion of plant cell walls and their deconstruction is beyond the scope of this chapter on methods, and the reader is referred to a special edition of the plant journal: *Harnessing Plant Biomass for Biofuels and Biomaterials* (May 2008) and references therein for a more thorough discussion.

This chapter describes methods for dilute acid pretreatment followed by SSF and PSCF of biomass to produce ethanol. The methods encompass bacterial and yeast fermentations in either test tubes or customized bioreactors equipped with automatic pH control. These methods may be modified for a variety of biomass types. Mixed forest residues containing both 5C and 6C sugars were selected to illustrate results obtained via small volume or bioreactor fermentations using either yeast or engineered bacteria as biocatalyst. An overview of the process is presented in Fig. 1.

**2. Materials**

**2.1. General Equipment**

2. pH electrodes (Sensorex S300C, Sensorex Corp., Garden Grove, CA).
3. pH controllers (Jenco 3671, Jenco Instruments Inc., San Diego, CA).
2.2. Chemical Solutions (All of Analytical Grade)

1. 2 M KOH.
2. 2 N HCl.
3. pH buffers.

2.3. Microorganisms

1. *Saccharomyces cerevisiae* D5A (ATCC No. 200062, National Renewable Energy Laboratory, Golden, CO) or other suitable *S. cerevisiae* strain (i.e., NABC BioFerm XR, North American Bioproducts, Norcross, GA).

2. Pentose and hexose fermenting bacterium such as *Escherichia coli* DC863adhC adhR, ATCC 53847 \((31)\).

2.4. Media for Growth of Microorganisms

2.4.1. *Saccharomyces cerevisiae*

Unless otherwise directed, all solutions should be autoclaved for 20 min at 121°C on liquid cycle.

1. 10× YP Broth: 200 g peptone, 100 g yeast extract per liter of (distilled water) \(dH_2O\).
2. Tryptic Soy Agar (TSA) plates: 15 g pancreatic digest of casein, 5 g papaic digest of soybean meal, and 5 g NaCl with 15 g agar per liter of \(dH_2O\).
3. Glucose stock solution (50%, w/v), glucose and dextrose should be filter-sterilized or autoclaved separately from the medium and added to final concentrations indicated below to sterilized YP broth.

4. YP2D broth: 10 g yeast extract, 20 g peptone, 40 g dextrose, per liter of dH2O.

5. YP5D broth: 10 g yeast extract, 20 g peptone, 100 g dextrose, per liter of dH2O.

6. 1× Diluent: 4.25 g NaCl, 0.15 g KH2PO4, 0.3 g Na2HPO4, 0.2 g peptone, 500 ml dH2O.

2.4.2. *Escherichia coli*

1. Modified Luria–Bertani (LB) broth: 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl per liter dH2O.

2. 5× LB broth: 12.5 g in 100 ml dH2O.

3. Glucose stock solution (50%, w/v).

4. 1× Diluent: 4.25 g NaCl, 0.15 g KH2PO4, 0.3 g Na2HPO4, 0.2 g peptone, 500 ml dH2O.

5. Chloramphenicol stock solution: 400 mg chloramphenicol dissolved in 10-ml of 70% ethanol and filter-sterilized through a 0.22-µm filter.

6. LB/Gluc/CAM plate: LB agar plate with the following added after LB agar is cooled from autoclaving: 50% glucose stock solution for a final concentration of 2% w/v glucose, and 40 mg/ml chloramphenicol stock solution for a final concentration of 40 µg/ml.

2.5. *Commercial Enzymes*

1. GC 220 Cellulase (Danisco, Genencor Division, Rochester, NY) or Celluclast 1.5 l (Novozymes, Franklinton, NC).

2. Novo 188 Cellobiase (Novozymes, Franklinton, NC).

3. Pectinase from *Aspergillus niger* (P2736, Sigma Chemicals, St. Louis, MO).

2.6. *Moisture Determination*

1. Convection drying oven able to control heat at 105 ± 3°C.

2. Analytical balance, accurate to 0.1 mg.

3. Desiccator and desiccant.

4. Instead of steps 1–3 above, an automated infrared moisture analyzer may be used.

2.7. *Dilute Acid Pretreatment via Autoclaving and Fermentation of Biomass*

1. Glass screw-top centrifuge tubes (50 ml), heat resistant above 121°C.

2. H2SO4 (1.75%, w/v).

3. Ca(OH)2 (10%, w/v). Ca(OH)2 will not dissolve; solution should be well mixed when using for additions.

4. Citric Acid [HOC(COOH)(CH2COOH)2] (1 M).
2.8. Fermentation of Biomass in Bioreactors

1. Glass bioreactors (250 ml).
2. Pasteur pipettes.
3. Pipette man 200 μl tips.
5. Silicone lubricant.
6. 2× Tryptic soy broth (TSB).
7. 2× LB broth.
8. Aluminum foil.

2.9. Gas Chromatographic Analysis of Ethanol Concentration of Fermentation Samples

1. Gas chromatograph (Shimazdu GC-8A, Columbia, MD, or other equivalent GC) with a flame ionization detector.
2. Gas chromatograph column (J & W Scientific, 0.53 mm ID × 30 m, 3 μm film).
3. N₂, H₂, and compressed air gas cylinders.
4. Isopropanol (2%, v/v) internal standard in a stoppered volumetric flask.
5. Ethanol standards (0.5%, 1%, 2%, 3%, and 4%, v/v) in stoppered volumetric flasks.
6. Sample syringe (Hamilton syringes no. 80300), or autosampler.
7. Centrifugation filters (0.22 μm; Costar 8169, Spin-X centrifuge tube filter).

3. Methods

3.1. Moisture Determination of Biomass to be Fermented

1. Perform the moisture determination in triplicate (see Note 1).
2. Place an aluminum weigh boat into the convection oven at 105 ± 3°C for 4 h. Cool in a desiccator and weigh to the nearest 0.1 mg. Record.
3. Mix sample well, remove a representative sample, and weigh 1–2 g to the nearest 0.1 mg. Add to the predried weigh boat.
4. Place the sample into the convection oven at 105 ± 3°C for a minimum of 4 h. Very wet samples will require overnight drying. Cool the sample in a desiccator and weigh to the nearest 0.1 mg. Record. Determine whether the sample is at constant weight by reheating the sample for 1 h, drying in the desiccator, and reweighing. A 0.1% or less change in the weight is considered a constant weight.
5. To calculate the percent moisture, use the formula (initial weight–final weight)/initial weight) × 100.
6. Alternatively, an infrared moisture balance may be used to determine the moisture content.
3.2. **Biomass Calculations**

1. To determine percent solids of biomass, use: 100 – percent moisture.

2. To determine amount of wet biomass needed per culture to achieve a targeted dry weight, use: \((y \text{ g dry wt})/(\text{percent solids ÷ 100})\), where \(y\) is the total g sample on a dry weight basis required for fermentation.

3.3. **Enzyme Calculations and Preparation**

3.3.1. **To Determine Amount of Commercial Enzymes in U (Units) Per Gram Dry Weight Biomass to Add to Fermentation**

1. Determine total unit (U) of enzyme needed for the total volume of the fermentation and express the enzyme U for the total g dry weight of sample as: \((\text{g dry weight}) \times (\text{U enzyme/g dry weight})\).

2. Determine total volume of commercial enzyme preparation to add to fermentation as: \((\text{total U enzyme calculated above})/ (\text{U/ml of commercial enzyme preparation})\).

3. Filter-sterilize enzymes using a 0.22-µm filter.

3.4. **Microorganism Precultures**

3.4.1. **Saccharomyces cerevisiae**

1. From −80°C freezer stock, streak *S. cerevisiae* D5A onto a tryptic soy agar (TSA) plate; incubate for 2 days at 37°C.

2. Inoculate YP2D broth (5–15 ml) with a single colony from the TSA plate; incubate overnight at 35°C with stirring at 150 rpm (see Note 12).

3. Transfer 1% (v/v) of overnight YP2D culture to YP5D medium; incubate for 24 h at 35°C at 150 rpm.

4. Measure the optical density at 600 nm (OD 600). Prepare the appropriate amount of culture for a final OD 600 = 0.5. Centrifuge the culture, resuspend in 1× diluent, and add to the SSF.

3.4.2. **Escherichia coli**

1. From −80°C freezer stock, streak *E. coli* ATCC 53847 onto an LB plate containing 2% (w/v) glucose and 40 µg/ml chloramphenicol; incubate overnight at 37°C.

2. Inoculate 100 ml of LB broth containing 5% (w/v) glucose and 40 µg/ml chloramphenicol with a single colony from the LB plate; incubate overnight at 37°C without stirring (see Note 12).

3. Measure the OD 550. Prepare the appropriate amount of culture for a final OD 550 = 1. Centrifuge culture, resuspend in 2× LB, and add to the fermentation.

3.4.3. **Determining the Amount of Preculture Needed to Provide an Inoculum of OD 1**

1. Use the following formula: \(C_1V_1 = C_2V_2\), or, substituted, \((OD_z \text{ preculture}) (x \text{ ml}) = (OD_z 1) (y \text{ ml})\), where \(x\) = amount of preculture needed, \(y\) = volume of fermentation, and \(z\) = wavelength.

2. Measure the volume of preculture needed and place in a centrifugation tube or bottle.

3. Centrifuge at 10,000 × *g* for 10 min and remove the supernatant.

4. Resuspend cells in a small volume of 1× diluent YP or LB (depending on fermentation method) and use to inoculate.
3.5. Small-Volume Dilute acid Pretreatment of Biomass by Autoclaving and Fermentation by Saccharomyces cervisiae (Fig. 1)

3.5.1. Dilute Acid Hydrolysis by Autoclaving

1. Measure 1.5 g dry weight of biomass into 50-ml glass screw-top centrifuge tubes.
2. Add 8.5 ml 1.75% (w/v) sulfuric acid (density of concentrated H₂SO₄ = 1.84 g/ml).
3. Autoclave sealed tubes at 121°C for 1 h, and allow tubes to cool to room temperature.
4. Add 1.2 ml Ca(OH)₂, 0.55 ml 1 M citric acid, 1.1 ml 10× YP to each tube and adjust the pH to 5 (see Notes 2 and 3).

3.5.2. Enzyme Saccharification, Inoculation, and Fermentation

1. Prepare enzymes as directed above for an enzyme loading of 5 FPU cellulase/g dry weight biomass and 60 U cellobiase/g dry weight biomass (see Note 7).
2. Add enzymes to pretreated biomass tubes and incubate at 37°C at 150 rpm.
3. Prepare the inoculum as directed above (see Subheading 3.4.1) with 1× diluent.
4. Add the inoculum to pretreated tubes and incubate at 37°C at 150 rpm with the caps on loosely to allow CO₂ ventilation (see Notes 5 and 9).
5. Take two 1-ml samples at 0, 24, 48, and 72 h for analysis of ethanol and reducing sugars; store samples at −20°C (see Notes 10 and 13).

3.6. Small-Volume Dilute Acid Pretreatment of Biomass by Autoclaving and Fermentation by Escherichia coli ATCC 53847 (Fig. 1)

3.6.1. Dilute Acid Hydrolysis by Autoclaving

1. Measure 1.5 g dry weight of biomass into 50-ml glass screw-top centrifuge tubes.
2. Add 8.5 ml 1.75%, w/v sulfuric acid.
3. Autoclave the tubes at 121°C for h and allow the tubes to cool to room temperature.
4. Add 1.2 ml Ca(OH)₂, 0.55 ml 1 M citric acid, 1.1 ml 10× LB to each tube and adjust the pH to 4.5 (see Notes 2 and 3).

3.6.2. Enzyme Saccharification

1. Prepare enzymes as directed above (see Subheading 3.3) for an enzyme loading of 5 FPU cellulase/g dry weight biomass and 60 U cellobiase/g dry weight biomass (see Note 7).
2. Add enzymes to pretreated biomass tubes and incubate at 37°C at 150 rpm for 24 h (see Notes 5 and 9).

3.6.3. Inoculation and Fermentation

1. Add 10 µl of 40 µg/ml chloramphenicol stock to each tube.
2. Prepare the inoculum as directed above (see Subheading 3.4.2) with 1× diluent.
3. Add the inoculum to pretreated and saccharified tubes and incubate at 37°C at 150 rpm with the caps on loosely to allow CO₂ ventilation (see Notes 5 and 9).

4. Take two 1-ml samples at 0, 24, 48, and 72 h for analysis of ethanol and reducing sugars; centrifuge @ 10,000×g, filter supernatant and store samples at −20°C (see Notes 10 and 13).

1. To each bioreactor, add a magnetic stir bar.
2. To each bioreactor cap, grease largest hole for pH electrode, and place Pasteur pipettes and/or pipette manifold tips stuffed with cotton in additional cap holes for ventilation.
3. Place the cap on the bioreactor and cover with a “hat” of aluminum foil.
4. Autoclave at 121°C for 20 min and cool to room temperature.
5. Add 56.7 ml 1.75% (w/v) H₂SO₄ to 10 g dry weight biomass weighed in 250 ml flask. Place a cap on the flask and autoclave separately at 121°C for 1 h. Allow it to cool to room temperature.
6. Transfer the biomass treated with dilute acid in the flask to the autoclaved bioreactor (see Note 4) and insert pH electrode.
7. Add 15 ml 10% (w/v) Ca(OH)₂, 3 ml 1 M citric acid, 20 ml 10× YP to each tube and adjust the pH to 5 (see Notes 2 and 3).
8. Add distilled water to bring the total volume to 200 ml.

Fig. 2. Schematic of the general equipment used in fermentation of biomass in bioreactors using *S. cerevisiae* and *E. coli* ATCC 53847. See Subheadings 2.1, 3.7, and 3.8 for details.
1. Prepare enzymes as directed above (see Subheading 3.3) for an enzyme loading of 5 FPU cellulase/g dry weight biomass and 170 U pectinase/g dry weight biomass (see Note 7).
2. Add enzymes to the pretreated biomass in the bioreactors.
3. Place the bioreactor in a multi-stirrer water bath maintained at 35°C and stir (see Notes 6, 8, 9, and 11).
4. Prepare the inoculum as directed above (see Subheading 3.4.1).
5. Add the inoculum to the above bioreactors with the enzymes to carry out SSF.
6. Take two 1-ml samples at 0, 24, 48, and 72 h to estimate ethanol and reducing sugars centrifuge @ 10,000 × g, filter supernatant and store samples at −20°C (see Notes 10 and 13).

1. To each bioreactor, add a magnetic stir bar.
2. To each bioreactor cap, grease largest hole for pH electrode, and place Pastuer pipettes and/or pipetteman tips stuffed with cotton in additional cap holes for ventilation.
3. Place the cap on the bioreactor and cover with a “hat” of aluminum foil.
4. Autoclave at 121°C for 20 min and cool to room temperature.
5. Add 56.7 ml 1.75% (w/v) H$_2$SO$_4$ to 10 g dry weight biomass weighed in 250 ml flask. Place a cap on the flask and autoclave separately at 121°C for 1 h. Allow it to cool to room temperature.
6. Transfer the biomass treated with dilute acid in the flask to the autoclaved bioreactor (see Note 4) and insert the pH electrode.
7. Add 15 ml 10% (w/v) Ca(OH)$_2$, 3 ml 1 M citric acid, and 40 ml 5× LB to each tube and adjust the pH to 4.5 (see Notes 2 and 3).
8. Add distilled water to bring the total volume to 200 ml.

1. Prepare enzymes as directed above (see Subheading 3.3) for an enzyme loading of 5 FPU cellulase/g dry weight biomass and 170 U pectinase/g dry weight biomass (see Note 7).
2. Add enzymes to the biomass in the bioreactor.
3. Place the bioreactor in a multi-stirrer water bath maintained at 45°C and adjust the pH to 4.5 using either 2 M KOH or 2 N HCl; incubate for 24 h (see Notes 6, 8, 9, and 11).
5. Lower the temperature of the water bath to 35°C and adjust the pH to 5.5.
6. Add 200 μl of 40 μg/ml chloramphenicol stock to each bioreactor.
7. Prepare bacterial inoculum as directed above (see Subheading 3.4.2) and add to the fermentation bioreactor.

8. Take two 1-mL samples at 0, 24, 48, and 72 h to estimate ethanol by GC; store samples at −20°C (see Notes 10 and 13).

1. Open H₂, N₂, and compressed air gas cylinder valves and turn on the GC and the GC integrator.

2. Make sure the GC injector/detector temperature is 250°C and the column temperature is 65°C. The carrier gas (N₂) flow rate should be 0.5 ml/min.

3. Reduce compressed air flow and allow GC to sit for 5 min to accumulate H₂ gas in the column.

4. Ignite the column with a lighter.

5. Return the compressed air flow to the original flow rate and allow the GC to warm up for 15 min.

1. In five individual microcentrifuge tubes, add 50 µl of the 2% (v/v) isopropanol standard solution.

2. Add 50 µl of each standard ethanol solution to the respective tube, mixing well.

1. Inject 1 µl of the 0.5% (v/v) standard solution prepared into the ignited column.

2. Allow ethanol and isopropanol peaks to elute (in that order).

3. Use an integrator or a computer program to record the peak areas for ethanol and isopropanol.

4. Repeat for all standard solutions.

5. Determine the ratio of the ethanol peak to the isopropanol peak for each standard solution: ethanol peak area/isopropanol peak = ratio.

6. Plot ratios (y-axis) versus percent ethanol (x-axis) to calculate the linear equation of the standard curve.

1. For each sample, combine 50 µl of fermentation sample with 50 (−/−) µl of the 2% (v/v) isopropanol standard in a microcentrifuge tube.

2. Mix well.

1. Inject 1-µl of the mixed fermentation sample into the ignited GC column.

2. Press “start” on the integrator and wait for the ethanol and isopropanol peaks to elute.

3. Press “stop” on the integrator and record the ethanol and isopropanol peak areas.
3.9.6. Calculate Ethanol Concentrations

1. For each sample, determine the ratio of the ethanol peak area to the isopropanol peak area (see Subheading 3.9.3, step 5).
2. Using the equation of the line determined in Subheading 3.9.3, step 6, determine the percent ethanol of each sample: \( \frac{(\text{ratio} - (y\text{-intercept}))}{\text{slope}} \).
3. To convert percent ethanol to g/l, multiply percent ethanol by 8 [calculation: \( \frac{x \text{ ml ethanol}}{100 \text{ ml H}_2\text{O}} \times \frac{1,000 \text{ mL}}{1 \text{ L}} \times 0.8 \text{ g/ml} \), where \( x \text{ ml ethanol}/100 \text{ ml H}_2\text{O} \) is percent ethanol and \( 0.8 \text{ g/ml} \) is the density of ethanol] (see Note 15).

4. Notes

1. If the substrate has been frozen, allow it to thaw completely before calculating its percent dry weight. A percent dry weight calculation on a frozen substrate can yield varying results.
2. The volumes of Ca(OH)\(_2\) and citric acid may vary. These are used to adjust the pH of the fermentation to the desired value.
3. It is important to take into account the moisture content of the biomass when determining how much growth medium to add to the fermentation vessel. With high moisture content, it may be necessary to add less of a more concentrated growth medium stock to maintain the correct percent solids but provide the needed nutrients to the fermenting organism.
4. The distilled water that needs to be added to the fermentation can be used to rinse out the remaining biomass in the flask from the dilute acid pretreatment to ensure that all the biomass has been transferred to the bioreactor for fermentation.
5. Mixing issues are common for the small-volume dilute acid-pretreated fermentations. This is most common at the beginning of saccharification and/or the beginning of fermentation, leading to incomplete mixing of substances that are added during the course of fermentation. Mixing problems usually correct themselves as saccharification progresses. However, to ensure proper mixing of added liquids, such as acids or bases, the tubes should be agitated and shaken.
6. Stirring issues are very common at the beginning of saccharification and/or the beginning of fermentation; the biomass is often too viscous to be stirred by the magnetic stir bar. This can lead to incomplete mixing and distribution of the enzymes, inoculum, acids, and bases that are added to the fermentation vessel. Most stirring issues correct themselves once the enzymes are added and sufficient time is allowed.
to begin the breakdown of the biomass. However, if there is insufficient stirring, the fermentation vessel should be agitated and shaken vigorously to ensure complete mixing when substances are added to the fermentation vessel.

7. Commercial enzyme mixtures are concentrated culture supernatants from enzyme-producing fungi. They are not sterile and should never be autoclaved since the heat would denature the enzymes rendering them inactive. All enzyme mixtures should be filter-sterilized and not added to the fermentation vessel until the vessel has been sterilized separately. Enzymes should be added after the pH is adjusted to the desired value so that they are not denatured.

8. If ethanol is used to disinfect pH probes before administering them into the fermentation apparatus, be sure to thoroughly rinse the probes with sterile water to avoid ethanol contamination.

9. During the course of the fermentation, it is critical to maintain the appropriate temperature and pH that is optimal for the specific step in the process. For the saccharification step, enzymes have different rates of catalysis depending on the temperature and pH, so the indicated temperature and pH are crucial for maximized enzyme activity. Also, the fermenting organism requires a certain pH and temperature in which to grow. If the pH or temperature is too high or too low, this could kill the organism. Either of these scenarios with the enzymes or the organism results in poor ethanol yield.

10. When maintaining pH levels throughout the fermentation, do not extract samples from the fermentation apparatus soon after adding any acid or base. The addition of acid or base just before extracting a sample can interfere with the detection of ethanol yields.

11. Ensure that water bath levels are sufficient throughout the entire fermentation. Many of the fermentations are completed at higher temperatures, resulting in faster evaporation rates. This may cause overheating of the heating element.

12. If conducting replicate fermentations, ensure that each fermentation apparatus is inoculated from a separate liquid culture grown from different colonies.

13. When preparing fermentations for frozen storage, they must be centrifuged and filtered. Centrifugation removes large particle size matter (biomass, organisms) that could clog the filter. Filtering ensures that the fermenting organism is removed from the sample, preventing any further conversion
of sugars to ethanol. It is also very important that all samples that are to be analyzed by GC be filtered. This prevents fouling of the column or detector. In addition, it is critical to store samples in tubes that prevent evaporation of ethanol during storage.

14. The GC septum should be changed every time the GC is operated.

15. If antibiotics that have been dissolved in ethanol are added to the fermentation apparatus before inoculation, correct the apparent yields during the fermentation for the ethanol present.

16. These different methodologies may result in differing ethanol yields depending on the biomass source, composition of the biomass, and/or the substrate range of the fermenting organism. Another consideration is that Dilute Acid Hydrolysis (DAH) pretreatment may be ineffective depending on the substrate (production of inhibitory compounds, release of heavy metal, etc.). See Fig. 3 an example of fermenting the same mixed carbohydrate biomass containing 5C and 6C sugars for yeast and bacteria using the small-volume fermentation protocol compared to the bioreactor protocol. These fermentations have not been optimized to increase ethanol yields.

![Fig. 3. Ethanol yields from the fermentation of forest residue biomass by two methods using S. cerevisiae (circles) and E. coli ATCC 53847 (squares) in small-volume DAH and fermentations (dashed lines) and bioreactor DAH and fermentation (solid lines). The 24-h time point corresponds to the beginning of the 24-h saccharification step for the bacteria only (PSCF). Yeast fermentations were true SSF without a preincubation step. Inoculation occurred at time 0 h.](image-url)
Acknowledgments

We would like to thank undergraduate student researchers in the Peterson lab, Brian Gardner, and Sean Suggs for their excellent fermentation help. The authors also acknowledge the assistance of Patricia O’Bryan at the USDA in Peoria for her assistance with the small-volume fermentation procedures. This work was supported, in part, by funding to JDP from the Traditional Industries Program (TIP3) of Georgia.

References


Biodiesel: Small Scale Production and Quality Requirements

Jon Van Gerpen

Summary

Biodiesel is produced by reacting vegetable oils or animal fats with alcohol in the presence of an alkaline catalyst. The resulting methyl esters, which are the biodiesel fuel, are separated from the by-product glycerin, and then washed with water and dehydrated to produce fuel that must meet standardized specifications. Degraded oils containing high levels of free fatty acids can also be converted to biodiesel, but pretreatment with acid-catalyzed esterification is required. The resulting fuel is suitable for use as a neat fuel in diesel engines or blended with conventional diesel fuel.

Key words: Biodiesel, Transesterification, Esterification, Diesel

1. Introduction

Vegetable oils can be used for short periods of time as alternative fuels for diesel engines but experience has shown that extended use leads to injector and cylinder deposits, lubricating oil dilution, and higher emissions (1, 2). Conversion of the oils to methyl esters provides a fuel with properties that are closer to conventional diesel fuel and, if the fuel is produced according to specifications defined by the American Society for Testing and Materials (ASTM), engine operating problems are not typically observed.

Fuel produced by transesterification of triacylglycerides with monohydric alcohols is known as biodiesel and is subject to the requirements of ASTM D 6751 (3). The corresponding standard
in Europe is EN 14214 (4). The fuel is most often produced by reacting a fat or oil with methanol in the presence of an alkali catalyst. While methanol is the most common alcohol, ethanol and other higher alcohols have been used (5, 6). To ensure a complete reaction, 100% excess alcohol is typically used, with the balance recovered after the reaction for reuse.

Transesterification has been known since the start of modern chemistry, but the industrial processes to produce methyl esters from fats and oils were developed in the 1940s to produce higher quality feedstreams for glycerin production (7–9). Current biodiesel producers follow the same steps defined by Bradshaw and Meuly (8, 9), although some refinements in mixing technology have been introduced (10).

**Figure 1** shows a schematic diagram of the major processes that occur in commercial biodiesel production (11). Oil (triacylglycerides), methanol, and catalyst are mixed in a reactor, usually in steady flow, and discharged to a separator where the glycerin by-product is separated from the methyl esters. The reaction is often conducted at 60°C to avoid the need for pressurized reactors, but higher temperatures can significantly increase the reaction rate (12). A small amount of dilute acid is added to the methyl ester stream to neutralize the catalyst and split any soaps that were formed during transesterification. The excess methanol is removed, usually by flash vaporization, and the methyl ester is then washed with warm deionized water. The water washing is frequently done with multiple steps in counterflow to minimize water consumption. Finally, the methyl esters are dried with another flash process to produce biodiesel that meets ASTM D 6751.

The glycerin stream that leaves the separator shown in Fig.1 is also acidulated, although much more acid is required because most of the catalyst and soap goes with the glycerin stream. Acidulation produces a fatty acid phase that can be separated by gravity. This fatty acid stream is typically sold as animal feed but can be esterified and recycled back into the process. The methanol is removed from the glycerin by flash vaporization leaving a crude glycerin product (80–90% glycerin) that is sold to specialized glycerin refiners. The methanol that is recovered from the methyl ester and glycerin streams will frequently contain water, which should be removed with a distillation column before the methanol is reused in the process.

An alternative to water washing is to use a solid adsorbent to remove the soap, catalyst, and other contaminants. One option is to add finely ground magnesium silicate, available commercially as Magnesol, to the biodiesel after the methanol has been removed. The powder absorbs the soap and, after filtering, the fuel is ready for use. This eliminates the need for water treatment and disposal as well as drying the final product. Another alternative is to use a packed bed of ion exchange resin, such as BD10Dry, which can
be used before methanol recovery to remove soap, catalyst, and free glycerin.

The properties of the biodiesel are most strongly influenced by the oil or fat feedstock. Oils and fats are almost entirely composed of triacylglycerides, which consist of three fatty acid chains on a glycerin backbone. The fatty acid chains are mostly C16–C22 in length with varying degrees of saturation. Animal fats and tropical oils are frequently high in saturated fatty acids, and these give a biodiesel fuel that tends to solidify at common operating temperatures but which has an excellent cetane number and oxidative stability. The cetane number is an important quality parameter for diesel fuel that relates to its tendency to ignite readily in the engine. Highly unsaturated oils give fuels with better low-temperature performance but have lower cetane numbers and worse oxidative stability.

Some oils and fats contain sulfur that may render the biodiesel unsuitable for use as an on-highway fuel since the U.S. Environmental Protection Agency limits on-highway diesel fuel to a maximum of 15 ppm of sulfur. Phosphorus is also a concern because, before refining, most oils and fats contain some amount of phospholipids. If these compounds are not removed, and they survive the biodiesel production process, they may poison the catalysts that are used in diesel exhaust aftertreatment devices.
A common contaminant found in triacylglyceride feedstocks is free fatty acids (FFAs). FFAs are formed by splitting fatty acid chains from the triacylglyceride molecules. The formation of FFAs is enhanced by heat, water, and enzymes, and FFA levels in some rendered animal fats can be 30% or higher. When a high FFA feedstock is to be used for biodiesel production, the FFAs will react immediately with the alkali catalyst and produce soap, which may inhibit separation of glycerin. These feedstocks can still be used, but an acid-catalyzed pretreatment is needed before the material can be transesterified (13, 14). The acid catalyst can also be used for the transesterification but it is too slow to be commercially viable (15).

### 2. Materials

1. Oil or fat: The oil or fat should be at least 98% triacylglycerides with less than 150 ppm phosphorus, less than 0.1% water, and less than 0.5% FFAs. Oils and fats with higher levels of FFAs require pretreatment as described in Subheading 3.2.
2. Methanol (see Note 1).
3. Sodium methoxide, 25% in methanol (see Note 2).
4. Sulfuric acid, 96.5%.
5. Hydrochloric acid, conc.
6. Potassium hydroxide, 0.1 N in distilled water.
7. Magnesium silicate – Magnesol (Dallas Group, Jeffersonville, IN).
8. BD10Dry – Ion exchange resin (Rohm and Haas, Philadelphia, PA).

### 3. Methods

#### 3.1. Transesterification of Vegetable Oil

1. Dissolve 10 g of 25% sodium methoxide solution in 100 g of methanol.
2. Weigh out 500 g of canola oil in a 1,000-ml flask and add 80% of the methanol–sodium methoxide solution.
3. Heat the mixture with agitation at 60°C for 1 h with an air condenser.
4. Transfer the mixture to a separatory funnel and let it settle for 30 min.
5. Drain off the lower phase, which consists of crude glycerin.
6. Return the upper phase to the 1,000-ml flask and add the remaining 20% of the methanol–sodium methoxide solution.
7. Heat the mixture with agitation at 60° for 1 h.
8. Transfer the mixture to a separatory funnel and let it settle for 30 min.
9. Drain off the lower phase, if any, which consists of the balance glycerin.
10. The upper phase is the biodiesel, which still contains substantial methanol, soap, catalyst, and some free glycerin, which must be removed.

Feedstock containing more than 0.5% FFA require that special measures be taken to convert them to biodiesel because the FFAs will react with the alkaline catalyst to make soap. For FFA levels up to 4–5%, the usual practice is to add extra catalyst so that the FFAs can be converted to soap while still leaving sufficient catalyst for the reaction. If the feedstock’s FFA level is determined by titration with KOH, and one mole of FFA reacts with one mole of catalyst, the extra amount of catalyst can be determined.

When the FFA level of the feedstock is above 5%, the amount of soap produced inhibits glycerin separation and an alternative method is needed (13). This method, details of which follow, uses sulfuric acid to catalyze the esterification of the FFAs to methyl esters.

1. Measure the FFA level of the feedstock oil by titrating with 0.1 N KOH.
2. Weigh out 200 g of the feedstock oil in a 1,000-ml flask.
3. Calculate the weight of FFA present in the 200 g of oil.
4. Weigh out an amount of methanol equal to 2.3× the weight of FFA present (20:1 molar ratio of methanol to FFA).
5. Weigh out an amount of sulfuric acid equal to 5% of the weight of FFA present (0.15 moles of H₂SO₄ per mole of FFA).
6. Mix the sulfuric acid slowly with the methanol and then add to the oil.
7. Heat the mixture with agitation at 60°C for 1 h with an air condenser.
8. Transfer the mixture to a separatory funnel and let it settle for 60 min.

The lower layer is the pretreated oil, which should have an FFA level of less than 1% and is suitable for the transesterification process described in Subheading 3.1. If necessary, steps 1–8 can be repeated to lower the FFA level further. The upper phase contains
methanol, most of the water produced by the esterification reaction, and the sulfuric acid. In a commercial plant, this material would be neutralized and the methanol would be recycled.

### 3.3. Methanol Recovery

In commercial plants, excess methanol is recovered from the biodiesel (and glycerin) by vaporization. This can be done in the laboratory with a rotary evaporator, or the methanol can be left in the biodiesel for removal by the water-washing step (see Subheading 3.4).

### 3.4. Washing

Biodiesel produced by the steps given in Subheading 3.1 will still contain small amounts of soap, catalyst, free glycerin, and methanol. These should be removed for the fuel to meet the ASTM specifications. The contaminants can be removed from the biodiesel by washing with water as follows.

1. Separate the glycerin phase and use the following procedure on the upper methyl ester phase.
2. Add an equal volume of distilled water at 60°C to the fuel and gently agitate. Combining the two fluids in a separatory funnel and rocking the funnel back and forth works well. Excessive agitation can produce emulsions that are slow to break.
3. Let the methyl ester–water mixture settle for 30 min.
4. Drain off the lower phase (water).
5. Repeat steps 1–4 until the water separates as a clear liquid (usually 3–4 times).
6. Dry the fuel by heating under vacuum in a rotary evaporator or heating to 110°C for 15 min.

### 3.5. Quality Determination

Biodiesel quality is determined by comparison to the requirements of ASTM D 6751 listed in Note 3. The biodiesel industry has established a voluntary producer certification program called BQ-9000 (bq-9000.org). This program documents the processes of certified producers and ensures that proper quality control practices are in place. After producers have demonstrated that the full specification can be met by seven consecutive production lots, they are allowed to reduce their routine testing to a set of critical parameters with only semiannual testing to the full specification. These critical parameters include flash point, water and sediment, cloud point, acid number, free glycerin, total glycerin sulfur, stability, and visual appearance. Visual appearance is not included in ASTM D 6751 but is described in ASTM D 4176 and ensures that the fuel is “clear and bright.”
Table 1
Biodiesel specifications for ASTM D 6751 and EN 14214 (16)

<table>
<thead>
<tr>
<th>Property</th>
<th>Test method</th>
<th>Unit</th>
<th>Limit</th>
<th>Test method</th>
<th>Unit</th>
<th>Limit</th>
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<td>130 min</td>
<td>EN ISO 3679</td>
<td>°C</td>
<td>120 min</td>
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<td>Methanol content</td>
<td>–</td>
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<td>% (m/m)</td>
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<td>EN ISO</td>
<td>mm²/s</td>
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<td>EN ISO 5165</td>
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<td>EN 14105</td>
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<td>EN 14538</td>
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<td>EN 14107</td>
<td>mg/kg</td>
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<tr>
<td>Na and K, combined</td>
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<td>ppm (µg/g)</td>
<td>5 max.</td>
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<td>Oxidation stability</td>
<td>EN 14112</td>
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<td>3 min</td>
<td>EN ISO</td>
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<td>6 min</td>
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<td>360 max.</td>
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*Different specifications for 15 and 500 ppm sulfur*
1. Methanol is used more frequently in the United States to produce biodiesel because of its lower cost. Ethanol can be used, but its higher molecular weight means more mass of ethanol must be used. The larger mass of ethanol is offset by the greater mass of biodiesel produced. Ethanol is also more sensitive to water, so it must be drier than is required for methanol and this can be problematic, especially for ethanol recovery systems because of the azeotrope that ethanol forms with water.

2. The most commonly used catalysts are sodium methoxide (methylate) and sodium and potassium hydroxide. Sodium methoxide is preferred because it is available as a 25% or 30% concentrate in methanol, and so it can be handled and dispensed as a liquid. Further, when a hydroxide catalyst is dissolved in methanol to produce the methoxide ions needed for the reaction, it also provides hydroxyls that compete to form FFAs and soap.

3. In the United States, biodiesel quality is maintained through reference to ASTM standard D 6751 (ASTM 2007). Table 1 shows the specifications for biodiesel provided by ASTM D 6751 along with the corresponding values for the European standard EN 14214.

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