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Sequential saccharification of corn fiber by the brown rot fungus, *Gloeophyllum trabeum*, and ethanol production by *Saccharomyces cerevisiae*

by

Mary Lynn Rasmussen

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Civil Engineering (Environmental Engineering)

Program of Study Committee:
Johannes van Leeuwen, Co-major Professor
Anthony Pometto III, Co-major Professor
Samir Kumar Khanal, Co-major Professor

Iowa State University

Ames, Iowa

2006

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This is to certify that the master's thesis of

Mary Lynn Rasmussen

has met the thesis requirements of Iowa State University

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CHAPTER 1. GENERAL INTRODUCTION

1. Introduction

Corn processing by wet and dry milling for ethanol fermentation produce large quantities of the low-value, lignocellulosic co-products – corn fiber and distillers' dried grains (DDG). Saccharification of the cellulose in corn fiber, for instance, could liberate simple sugars for subsequent yeast fermentation to produce more ethanol. The hemicellulose in corn fiber is another possible source of sugars for ethanol production, provided the strain of yeast or bacteria employed is able to ferment five-carbon sugars to ethanol. Various approaches, including concentrated and dilute acid hydrolysis and enzymatic hydrolysis, have been investigated to find an economical method of converting lignocellulose to fermentable sugars (U.S. DOE EERE, 2006). Enzymes may be produced separately and added, or released *in situ* by microbes growing on the lignocellulosic substrate. Pretreatment of the fibrous feedstock by physical or chemical means, such as milling, acidic or alkaline pre-hydrolysis, steam explosion and ammonia fiber explosion, is particularly important for the efficient conversion of lignocellulose to sugars via enzymatic hydrolysis.

An alternate approach to the addition of enzymes is to utilize eukaryotic microorganisms, such as wood rot fungi, directly for the bioconversion of cellulose and hemicellulose to simple sugars. White rot and brown rot fungi are among the most widely known agents of decay for woody biomass (Dix and Webster, 1995). Brown rot fungi, such as *Gloeophyllum trabeum*, rapidly metabolize the hemicellulose and cellulose in wood, leaving behind a chemically-modified lignin residue (Goodell, 2003). The enzymatic and non-enzymatic mechanisms used by *G. trabeum* to degrade wood could be employed for the bioconversion of other fibrous substrates, such as corn fiber, switchgrass, and corn stover to fermentable sugars. Fermentation of wet and dry corn milling co-products by brown rot fungi may be possible under submerged or solid-state conditions. The research presented in this thesis includes submerged-culture and solid-state fermentations of corn fiber by *G. trabeum* to produce fermentable sugars, with subsequent ethanol production by *Saccharomyces cerevisiae*.

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2. Thesis organization

The thesis is organized into two main parts, a literature review and a paper prepared for submission to the journal *Bioresource Technology*. The review discusses the processing of corn kernels to ethanol, and the potential methods of hydrolyzing the fibrous co-products of wet and dry corn milling to simple sugars, followed by fermentation to ethanol. The ecology and decay mechanisms of the brown rot fungus, *G. trabeum*, as well as the characteristics of solid-state fermentation, are also presented as background. The research presented in the paper for journal submission was conducted using both submerged-culture and solid-state fermentation of corn fiber by *G. trabeum* to produce fermentable sugars. I wrote the paper (Chapter 3) and was principally involved in the data collection, analysis, and interpretation, in collaboration with the major professors and a fellow graduate student, Prachand Shrestha.

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CHAPTER 2. LITERATURE REVIEW

This chapter first provides a review of the processing steps for production of ethanol from corn kernels. The various hydrolysis methods for conversion of lignocellulose to simple sugars are then discussed. Background on the brown rot fungus, *Gloeophyllum trabeum*, solid-state fermentation, and the microorganisms used for ethanol fermentation are also presented to justify the research methods described in Chapter 3. The approach used in this research included solid-state fermentation of corn fiber by *G. trabeum* to produce fermentable sugars, followed by ethanol production by *Saccharomyces cerevisiae*.

1. Corn kernel composition

Corn kernels are composed of four parts, the endosperm, pericarp, germ and tip cap (Fig. 1). The endosperm provides energy to the germinating seed in the forms of starch and protein, and contributes about 82% of the kernel's dry weight (NCGA, 2005). The pericarp, or hull, is the outer cover that protects nutrients inside the kernel by resisting water and deterring insects and microorganisms (about 5% of the dry weight) (Butzen and Hobbs, 2002). The only living part of a kernel is the germ, which contains the genetic information, enzymes, vitamins and minerals that enable growth into a corn plant. Corn oil accounts for about 25% of the germ. Water and nutrients are transferred to the kernel through the tip cap, the point of attachment to the cob.

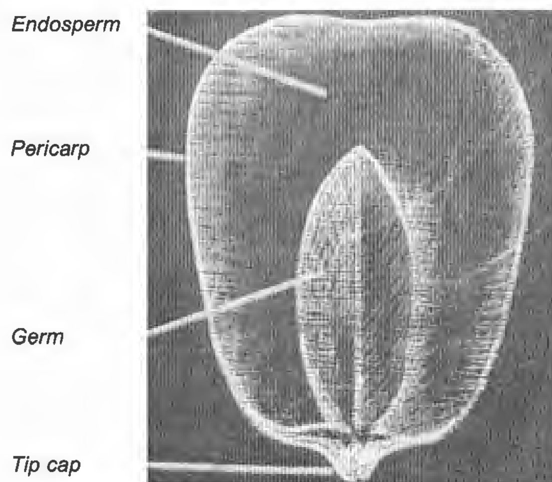

Fig. 1. Basic parts of corn kernel, including endosperm, pericarp, germ and tip cap (NCGA, 2005).

2. Corn wet-milling processes

There are two processes for the production of ethanol from corn kernels, wet milling and dry milling (Brown, 2003). The two methods differ mainly in the initial treatment of the corn kernels and the by-products. A corn wet-milling plant resembles a petroleum refinery in concept, by generating numerous products from corn, including corn syrup, high fructose corn syrup, corn oil, food and industrial starches, beverage and industrial alcohol, carbon dioxide, corn gluten feed, corn gluten meal, and glucose (Abbas et al., 2004). One of the major fermentation products is ethanol.

The first step of the wet-milling process is to clean the corn and convey it to steep tanks, as indicated in Fig. 2 (Brown, 2003). Steeping in a dilute solution of sulfur dioxide (1000-2000 ppm) at 120-140°F for 24 to 40 hours (Butzen and Hobbs, 2002) causes the corn to swell and soften, and breaks down the starch-protein matrix (Abbas et al., 2004; Brown, 2003). The resulting steep water contains some protein and is a potential source of nitrogen and vitamins. After steeping, the wet kernels are coarsely milled and screened to separate the germ from the other components. The germ is further processed by drying and pressing or adding solvents to extract the oil (Brown, 2003). The endosperm and hull (pericarp) are ground and divided into the fine particles of starch and gluten, and the coarser fiber. Centrifugation is used to separate the gluten and starch. The gluten by-product is sold primarily as corn gluten meal (60% protein) for poultry feed. The fiber is rinsed to recover residual starch, and then dried and mixed with the other by-products, including oil cake, corn steep liquor and stillage. The mixture is dried and pelletized for sale as corn gluten feed (21% protein) for dairy cattle. The typical yields for a wet-milling plant per bushel of corn processed are as follows: 2.5-2.6 gal ethanol, 1.7 lb corn oil, 3 lb corn gluten meal, 13 lb corn gluten feed, and 7.7 lb carbon dioxide. The demand for corn gluten feed is not expected to increase significantly. Therefore, as wet-milling processes expand, additional uses of the corn fiber will be required to prevent an oversupply of corn gluten feed.

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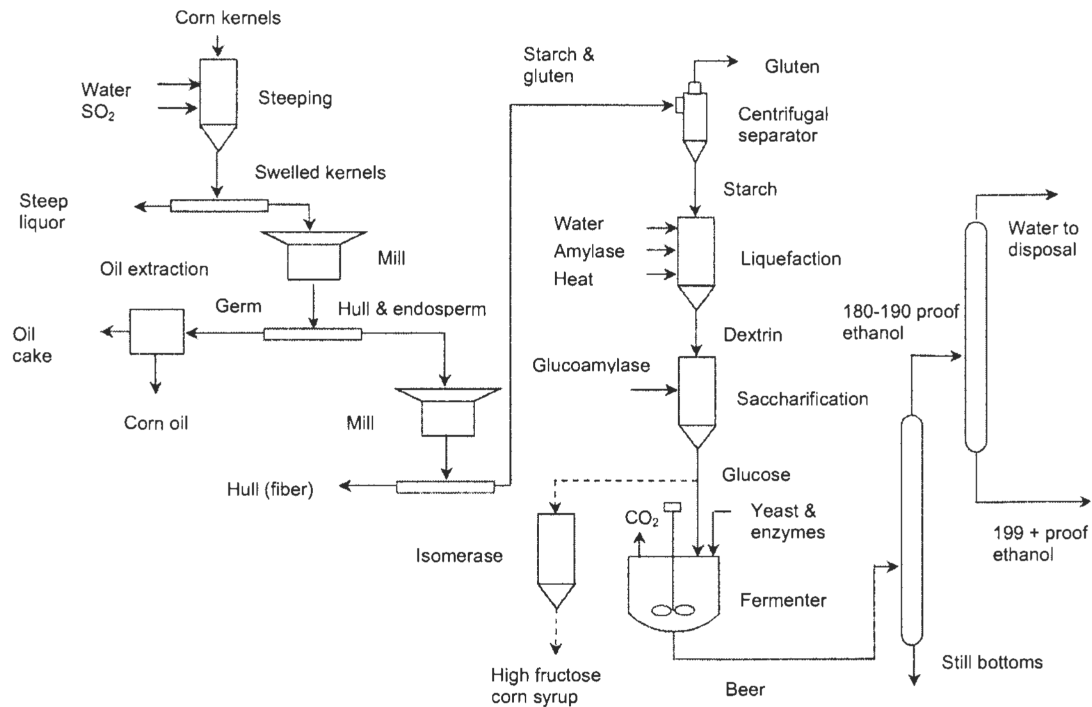

Fig. 2. General process schematic for wet corn milling (Brown, 2003).

Corn fiber is an abundant and convenient feedstock, which requires no additional harvesting or transportation since it is already at the processing facility. The approximate composition of corn fiber is 17% starch, 35% hemicellulose, 18% cellulose, 11% protein, 3% oil, 6% ash, 5% galactan, 1% mannan, and 4% other materials (Abbas et al., 2004). The principal constituents of the hemicellulose are xylose (55%) and arabinose (36%). Their chemical structures are shown in Fig. 3. Fractionation of corn fiber using combinations of enzymatic and thermochemical hydrolysis can be applied to obtain various value-added products (Abbas et al., 2004).

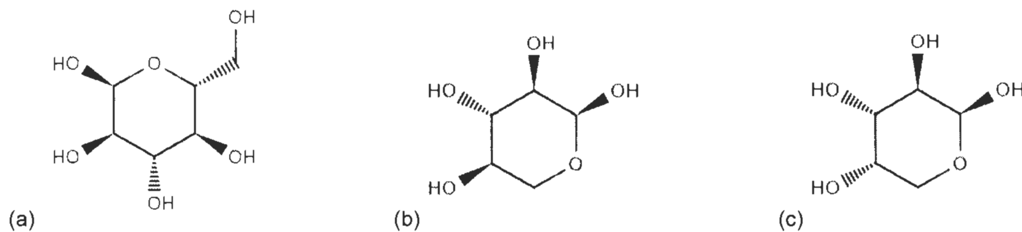

Fig. 3. Chemical structures of (a) α-D-glucose, (b) α-D-xylose, and (c) α-L-arabinose (BMRB, 2006).

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3. Corn dry-milling processes

Dry milling of corn in the food and fuel industries also produces a fibrous co-product, by a combination of simple grinding and screening procedures to produce flour, meal and grits (Brown, 2003). The first step is to clean and then temper the corn to a moisture content of 15-20%. The kernel is split to germ, endosperm, and hull by grinding with roller mills or crushers and separation by gravity. The oil in the germ (18-25%) is extracted by solvents. The starch in the endosperm can be further ground and sifted to the different particle sizes of flour, cornmeal, and grits. The typical distribution of products from the kernel by weight is: 75% flour, cornmeal or grits (starch), 15% oil, and 11% fibrous feed. The starch can either be used as food or feedstock for ethanol fermentation.

Separation of corn into the various food products is not required for ethanol production by dry milling (Brown, 2003). Corn kernels are first ground in a roller mill into coarse flour, referred to as meal in the industry, and then slurried with water to form a mash (Fig. 4). The enzymes α-amylase and glucoamylase are added to the mixture during cooking (Butzen et al., 2003). The first step, liquefaction, uses steam and α-amylase to break down the starch molecule. Glucoamylase is added at a lower temperature in the next step, saccharification, to produce fermentable sugars (i.e. mainly glucose). The mash is then cooled and transferred to the fermenters. The yeast in the fermenters convert the glucose to ethanol and carbon dioxide. After fermentation, the beer and fibrous residue (whole stillage) are separated; the beer is transferred to a series of distillation columns to concentrate the ethanol (199+ proof). The whole stillage is centrifuged to separate the coarse grain from the solubles. The solubles are concentrated to about 30% solids by evaporation, and are known as condensed distillers solubles (CDS) or syrup. The syrup is mixed and dried with the fibrous and other unfermented residues to produce distillers' dried grains with solubles (DDGS).

DDGS retain most of the nutrient value of the corn, including protein (25%), minerals, fats, and fiber, and are sold as cattle feed (Brown, 2003). The protein content of DDGS is significantly higher than in corn fiber alone (11% protein). The typical yields per bushel corn processed by dry milling are about 2.5-2.6 gal ethanol, 17-18 lb DDGS, and 16-17 lb CO_2. The carbon dioxide produced during fermentation may be considered a co-product, by capturing and selling it to the

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carbonated beverage industry or to dry ice manufacturers. The profitability of corn dry milling, as with wet-milling plants, depends on the sale of the ethanol and the co-products. Ethanol dry mills produced approximately 9 million metric tons of DDGS in 2005 (RFA, 2005).

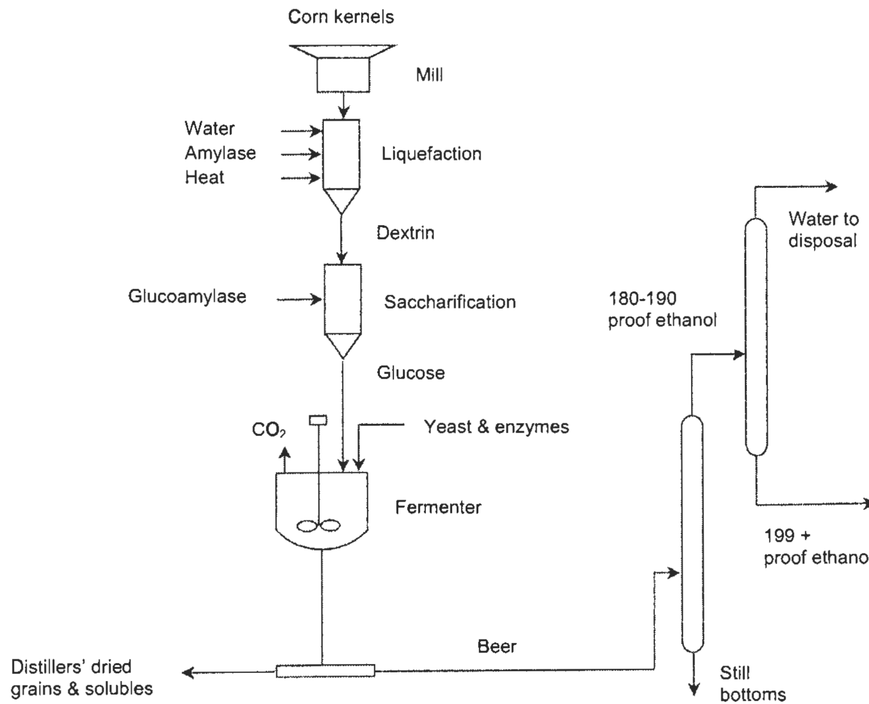

Fig. 4. General process schematic of dry corn milling (Brown, 2003).

4. Hydrolysis of lignocellulose

The profitability of corn wet- and dry-milling plants currently relies on the sale of the fibrous residues as cattle feed. Value-adding, for instance by using this lignocellulosic biomass as feedstock for more ethanol production, is becoming more critical as the demand for ethanol and number of ethanol plants increase. Having a market for the co-product and using lignocellulose, particularly corn fiber which is already at the processing facility, are also important to maintaining or increasing the energy return of ethanol production (Hammerschlag, 1990). The energy return on investment, r_E, as discussed by Hammerschlag (1990), is the ratio of energy in a liter of ethanol to the nonrenewable energy used in manufacturing it (i.e. MJ/MJ). A value of r_E greater than 1 implies some renewable energy return on the fossil fuel investment.

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The values of r_E from six studies on the current corn ethanol technology were calculated by Hammerschlag (1990), and ranged from 1.29 to 1.65, with the exception of a study by Pimentel and Patzek (2005)(r_E = 0.84). Since the gasoline displaced by ethanol has upstream energy inputs, the r_E value of gasoline, 0.76, is also less than 1. Therefore, even with a low value of r_E = 0.84, ethanol still seems to improve fossil fuel consumption on an energy basis when used in place of gasoline.

Almost all ethanol plants manufacture one or more co-products in addition to ethanol, by utilizing the non-starch content of the kernel (i.e. corn fiber). These co-products are often considered as providing an "energy credit" against the gross energy input. In other words, the net energy input used to calculate r_E equals the gross energy input minus the co-product energy input. This means that co-products, such as corn fiber sold as corn gluten feed or used to produce more ethanol, are important to the energy return of ethanol. Furthermore, the three cellulosic ethanol studies showed significantly higher returns of renewable energy on nonrenewable energy investments, with r_E values ranging from 4.40 to 6.61 (Hammerschlag, 1990).

Fig. 5 outlines the common approaches for the hydrolysis of lignocellulose and fermentation to ethanol (Galbe and Zacchi, 2002). Three common methods used to hydrolyze the cellulose and hemicellulose in plant cell walls are concentrated acid, dilute acid and enzymatic hydrolysis. Dilute acid treatment is adequate for releasing sugars from hemicellulose; however, the options for cellulose hydrolysis require more severe acidic conditions, or pretreatment (i.e. steam/ammonia explosion) and the addition of enzymes. The advantages, disadvantages and commercial development are discussed in detail in the next sections. The major concerns, as indicated in the figure, are the recovery of acid during concentrated acid hydrolysis, and the pretreatment of lignocellulose, production of enzymes, and prevention of end-product inhibition (by using simultaneous saccharification and fermentation) during enzymatic hydrolysis.

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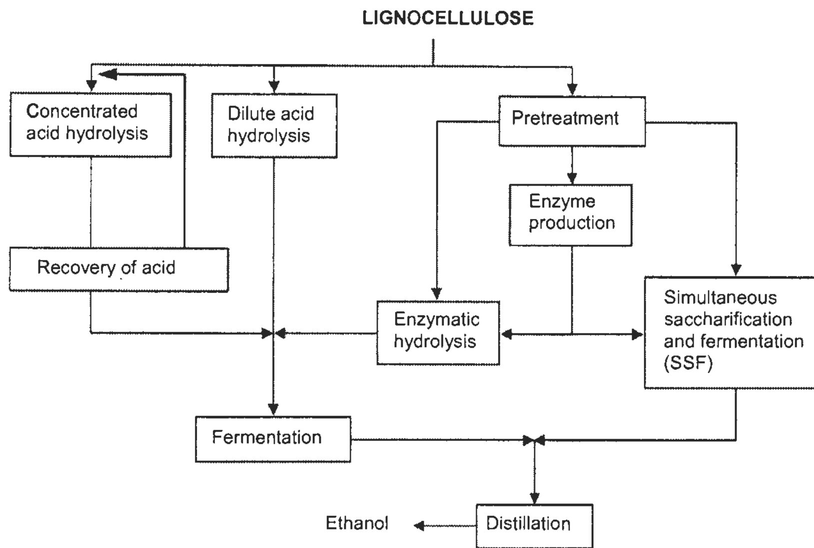

Fig. 5. Production of ethanol from lignocellulose (Galbe and Zacchi, 2002).

Acid hydrolysis of corn fiber converts the starch and hemicellulose to oligosaccharides (Abbas et al., 2004). Cellulose is also hydrolyzed provided the conditions are adequately severe. In general, the hydrolysis of corn fiber proceeds in the order of starch, hemicellulose branches, hemicellulose xylose backbone, and then cellulose as the treatment severity increases. The current research frequently involves a mild acid treatment followed by enzymatic hydrolysis of the remaining oligosaccharides or polysaccharides. Using this approach, starch and hemicellulose are first hydrolyzed to oligosaccharides, and then to monosaccharides. Hydrolysis of the starch yields glucose, and hydrolysis of the hemicellulose yields xylose, arabinose, galactose, and mannose. Acid hydrolysis of lignocellulose may also produce acetic acid from the hemicellulose polymer and degrade a fraction of the monosaccharides to hydroxymethylfurfural (HMF) and furfural (Fig. 6). These products are reported to inhibit yeast during fermentation of corn fiber hydrolysates to ethanol.

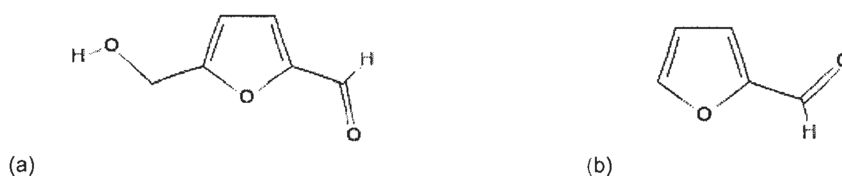

Fig. 6. Chemical structures of (a) hydroxymethylfurfural and (b) furfural (ChemIndustry.com, 2005).

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One acid hydrolysis approach that produces a low concentration of inhibitory degradation products is to subject the corn to an initial thermochemical step, in which the residual SO_2 in the corn fiber from steeping acts to hydrolyze the polysaccharides (Abbas et al., 2004). The residual fiber produced contains primarily cellulose (depending on the severity of the acid treatment) and protein. The volume reduction of the hydrolyzed fiber leads to a higher protein content in the corn gluten feed, which is another method of value-adding since the feed value is based on protein content.

5. Concentrated acid hydrolysis

The process of using concentrated acid for lignocellulose treatment has a long history. As early as 1883, the hydrolysis of cellulose in cotton using concentrated sulfuric acid and dilution with warm water was reported in literature (Harris, 1949). Concentrated acid hydrolysis is a fairly simple process that is attractive for the near theoretical yields of sugar released during the dilution step (U.S. DOE EERE, 2006). The basis of the process involves decrystallization of cellulose followed by dilute acid hydrolysis to produce glucose. The concentrated acid disrupts the hydrogen bonds between cellulose chains, making them more readily hydrolyzed. The subsequent dilution with water at modest temperatures hydrolyzes the amorphous cellulose to glucose. Following neutralization, the fermentable sugars released from the lignocellulose can be fermented to ethanol.

A concentrated acid process patented by Arkenol (Farone and Cuzens, 1996) starts by drying the biomass to 10% moisture and then adding 70–77% concentrated sulfuric acid (Fig. 7). The acid to cellulose and hemicellulose ratio in this step is 1.25:1 with temperatures of less than 50°C. Simple sugars are released by diluting the acid to 20-30% and heating at 100°C for an hour. The solution from this reactor is pressed to separate the residual solids from the acid/sugar stream; the solids are then subjected to a second hydrolysis step. The acid and sugar are separated using a chromatographic column, a crucial improvement in the process. A triple effect evaporator is used to concentrate the acid for reuse. Arkenol claims that at least 98% of the sugar is recovered in the acid/sugar separation column, and not more than 3% of the acid is lost.

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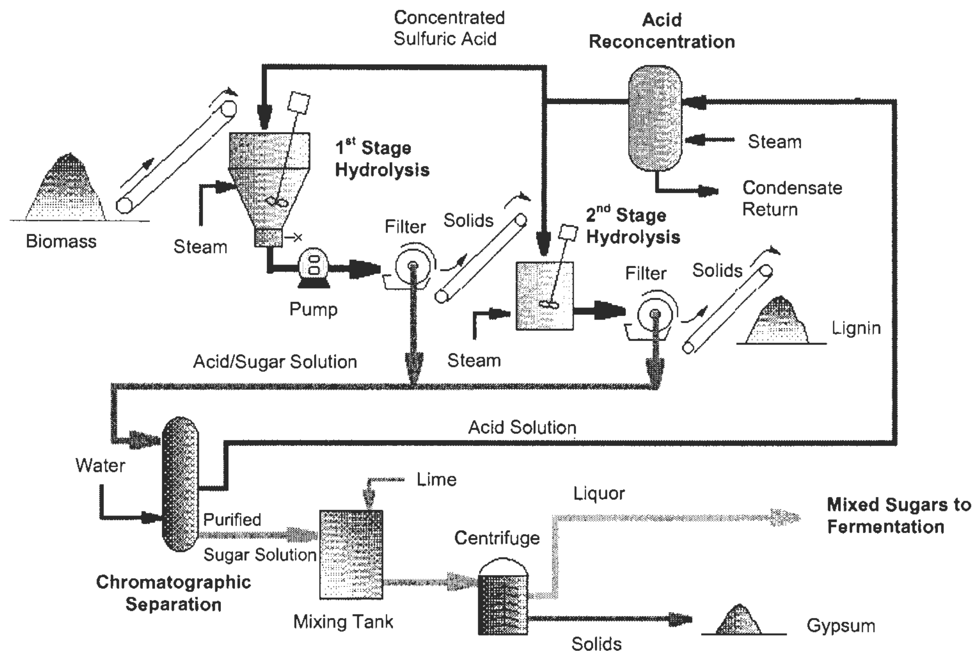

Fig. 7. Diagram of conversion of lignocellulose to simple sugars using Arkenol's concentrated acid hydrolysis process (Arkenol, Inc., 1999).

The recovery and reuse of acid are critical operations for the economic success of ethanol produced using a concentrated acid process. Sulfuric and hydrochloric acid have both been considered for commercial applications; however, sulfuric acid is more attractive as it is less corrosive and less expensive (Brown, 2003). Addition of limestone for neutralization also poses a concern, since a large quantity of gypsum (calcium sulfate) is generated as a waste stream; 1 lb of gypsum (40% moisture) is produced by Arkenol's process per gallon of ethanol (Arkenol, Inc., 1999).

5.1. Commercial development and status

A two-stage process for production of sugars from corncobs was proposed in 1918 by researchers at the U.S. Department of Agriculture (USDA) (LaForge and Hudson, 1918). This process introduced dilute acid pretreatment of lignocellulose to prevent the formation of fermentation inhibitors by removing the hemicellulose first, followed by concentrated acid hydrolysis (75%) of the cellulose. The traditional yeasts used for production of ethanol are unable to ferment the five-carbon sugars released from the hemicellulose.

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The former Soviet Union and Japan were able to commercialize the concentrated sulfuric acid process successfully during times of national crisis (Wenzl, 1970). However, the large volumes of acid required generally prohibit the economic competitiveness of ethanol production by concentrated acid hydrolysis. Improvements in the separation of sugar and recovery of acid may lead to commercialization of the process in situations where the use of biomass can alleviate waste disposal problems. Two companies in the United States, Arkenol and Masada, are currently working with DOE and NREL to commercialize this technology (U.S. DOE EERE, 2006). Arkenol is working with DOE on a commercial facility that will use rice straw as feedstock for ethanol production. New regulations restrict the practice of open field burning of rice straw, which creates a disposal problem. The economic success of the facility is based on the availability of this cheap feedstock. Masada plans to operate a municipal solid waste (MSW)-to-ethanol plant in Middletown, NY. The facility will use technology based on a concentrated sulfuric acid process to convert the lignocellulosic fraction of MSW into ethanol. This project takes advantage of the relatively high tipping fees for collection and disposal of MSW in New York.

6. Dilute acid hydrolysis

Dilute acid hydrolysis of lignocellulose is the oldest technology for the conversion of biomass to ethanol (U.S. DOE EERE, 2006). The hydrolysis of biomass using dilute acid (≤1% acid solution) has evolved into a system of two stages to maximize sugar yields and to account for the difference between hemicellulose and cellulose. The first stage involves milder acidic and heat conditions to hydrolyze the hemicellulose, while the second stage is optimized to hydrolyze the more resistant cellulose. The product stream recovered from the two stages is neutralized, and the simple sugars are fermented to ethanol. Residual cellulose and lignin in the waste solids can be used as boiler fuel for electricity or steam production. The amount of acid required is significantly lower than used in concentrated acid hydrolysis of lignocellulose (Brown, 2003). The process is operated at higher temperatures of 100-160°C for hemicellulose and 180-220°C for cellulose to accelerate the hydrolytic conversion; however, the yields of simple sugars are greatly reduced to 55-60%. Oligosaccharides released from lignocellulose decompose at higher temperatures to products such as furfural and

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acetic acid, which inhibit the fermentation of sugar to ethanol. Pretreatment to separate the hydrolysis of hemicellulose and recovery of pentoses, as depicted in Fig. 8, is one option for reducing the amount of inhibitory degradation products in subsequent acid hydrolysis. Fermentation of five-carbon sugars in addition to the conventional fermentable sugars (six-carbon or disaccharides) is critical to the economic success of ethanol production from lignocellulosic feedstocks.

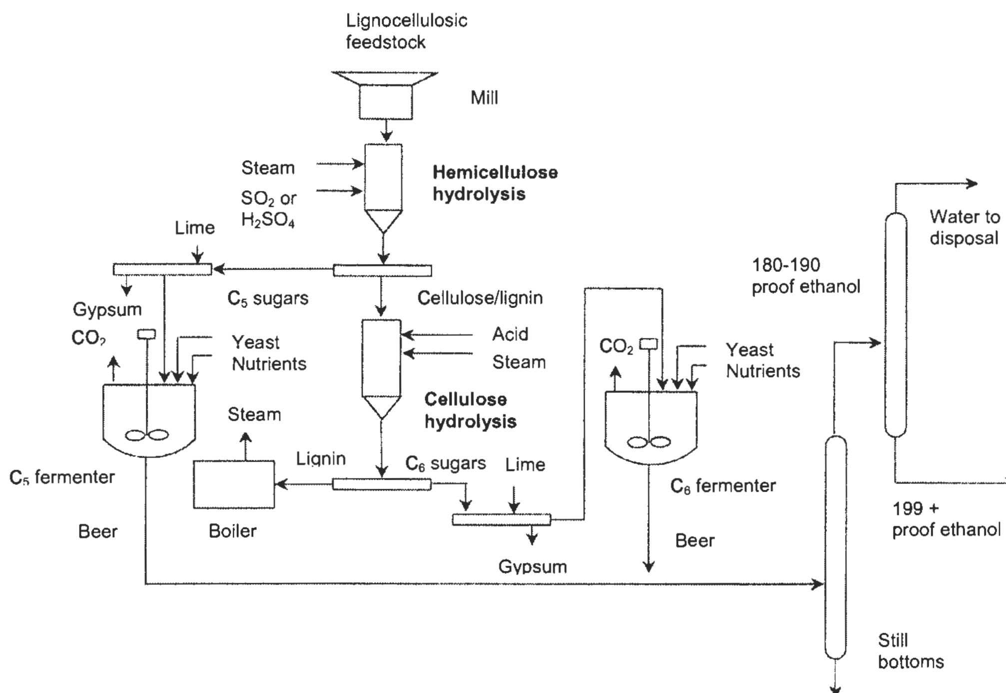

Fig. 8. General process schematic for dilute acid hydrolysis of lignocellulose (Brown, 2003).

6.1. Commercial development and status

The first attempt to commercialize the production of ethanol from wood was in Germany in 1898 (U.S. DOE EERE, 2006). The process used dilute acid to hydrolyze the cellulose in wood to glucose, which was then fermented to produce about 8 L of ethanol per 100 kg of wood waste (18 gal per ton). The optimized industrial process had yields of around 50 gal per ton of biomass in Germany, and lead to the development of two commercial plants operating in the southeast U.S. during World War I. The plants utilized a single stage of dilute sulfuric acid hydrolysis, known as the American Process. The throughput of this process was significantly higher than the German process,

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but yields were halved (25 gallons of ethanol per ton). Shortly after the end of World War I, a decline in lumber production forced the plants to close.

The Scholler Process is an improved percolation method developed in Germany in 1932 (Faith, 1945). The process involves pumping a dilute solution of sulfuric acid through a bed of wood chips. The USDA's Forest Products lab enhanced the productivity and yield of the Scholler Process, with a modified version known as the Madison Wood Sugar Process (Harris and Beglinger, 1946). The dilute acid hydrolysis percolation design developed in 1952, after further refining by the TVA pilot plant studies, is still one of the simplest methods of sugar production from biomass.

Petroleum shortages in the 1970s renewed interest in dilute-acid hydrolysis technology (U.S. DOE EERE, 2006). Most researchers recognized by 1985 that percolation systems had achieved their maximum potential. The relatively high glucose yields of around 70% were produced in highly dilute streams. Studies shifted to alternate designs, including plug flow reactors and progressing batch systems, which resemble countercurrent operation. Plug flow systems in the lab and the pilot plant attained glucose yields of around 50%, close to the theoretical limits for continuous reactor systems. NREL recently reported results for a dilute acid hydrolysis of softwoods under the following conditions: stage 1 – 0.7% sulfuric acid, 190°C, and a 3-minute residence time, and stage 2 – 0.4% sulfuric acid, 215°C, and a 3-minute residence time. These lab-scale tests achieved yields of 89% for mannose, 82% for galactose and 50% for glucose.

Over the last 50 years, Germany, Japan, and Russia have operated dilute acid hydrolysis percolation facilities intermittently (U.S. DOE EERE, 2006). The dilute acid process has significant industrial experience; however, the percolation designs tend not to be economically competitive. Dilute acid hydrolysis requires stainless steel equipment and gypsum waste disposal. The conversion of lignocellulose to hexoses and pentoses by this method costs about $0.20 per gal ethanol (Tengerdy and Szakacs, 2003).

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7. Enzymatic hydrolysis

The first application of cellulase enzymes for hydrolysis of wood was used to replace the acid hydrolysis of cellulose, in a configuration referred to as separate hydrolysis and fermentation (Fig. 9) (U.S. DOE EERE, 2006). The challenges of enzymatic hydrolysis include high enzyme concentration requirements and costs, low conversion rates and end-product inhibition. A major breakthrough in 2004 reduced the cost of cellulase enzymes dramatically, from $5 per gal of ethanol in 2001 to 10–18 cents per gal (Patel-Predd, 2006). The companies Novozymes and Genencor, with support from the National Renewable Energy Laboratory (NREL), modified microbes genetically to produce large quantities of efficient cellulase enzymes. End-product inhibition results from the enzyme ß-glucosidase ceasing to hydrolyze cellobiose in the presence of glucose. The resulting build-up of cellobiose inhibits further cellulose degradation. The introduction of simultaneous saccharification and fermentation was an important modification to the enzymatic hydrolysis process, and is patented by Gulf Oil Company and the University of Arkansas (Gauss et al., 1976; Huff and Yata, 1976). In this process, reactors combine cellulase and fermenting yeasts, so that as sugars are produced they are converted to ethanol. This system reduces the number of reactors and solves the enzymatic problem of end-product inhibition. The process of simultaneous saccharification and fermentation was improved recently to include the fermentation of sugars from hemicellulose and cellulose in the same reactor, known as simultaneous saccharification and cofermentation (illustrated in Fig. 10).

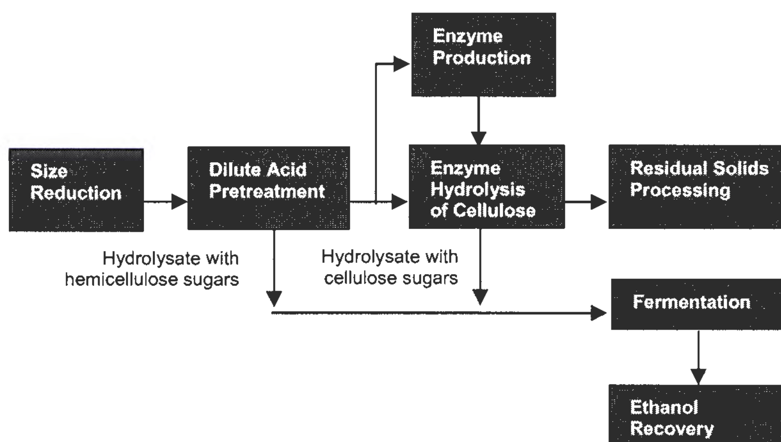

Fig. 9. The enzymatic hydrolysis process configured as separate hydrolysis and fermentation (U.S. DOE EERE, 2006).

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The separate hydrolysis and fermentation process (Fig. 9) uses separate reactors for hydrolysis of hemicellulose (dilute acid pretreatment), enzymatic hydrolysis of cellulose and fermentation (Brown, 2003). The advantages of this approach include the ability to operate each process at the optimal temperature and pH (50°C and pH 4 - 5 for enzymatic hydrolysis and 20-32°C for fermentation) and to avoid undesirable interactions. The disadvantage is the inhibition of ß-glucosidase by the end-product, glucose. To achieve reasonable yields, lower solids loadings are required, resulting in lower sugar and ethanol concentrations; the cost of fermentation and distillation are thus higher.

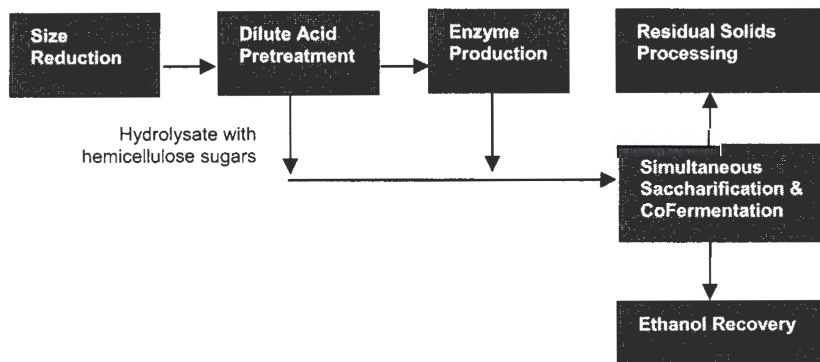

Fig. 10. The enzymatic hydrolysis process configured as simultaneous saccharification and cofermentation (U.S. DOE EERE, 2006).

End-product inhibition is avoided by combining cellulose hydrolysis and ethanol fermentation in a single reactor. This simultaneous saccharification and fermentation process is demonstrated in Fig. 11. The first step is to mill the feedstock and to hydrolyze the hemicellulose, which requires milder acidic conditions than cellulose hydrolysis (Brown, 2003). Complete hemicellulose removal is achieved by treatment with 1% sulfuric acid at 140°C for 30 min or at 160°C for 5 to 10 min. A better alternative is the addition of sulfur dioxide gas at 2-3% to moist biomass at 150°C for 20 min. Sulfur dioxide is converted to sulfuric acid within the biomass pores, and is less corrosive to the equipment. After neutralizing with lime, cellulase and hemicellulase enzymes, yeast and nutrients are added. The enzymatic hydrolysis of cellulose requires a system of enzymes known collectively as cellulase, which can be produced on site or purchased. The cellulose is degraded to glucose, the rate limiting step, and the hemicellulose to hexoses and pentoses (primarily xylose and arabinose). The optimal

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operating conditions, 38°C and pH 5.0 (Philippidis and Hatzis, 1997), are chosen to accommodate the cellulase activity and ethanol fermentation by yeast.

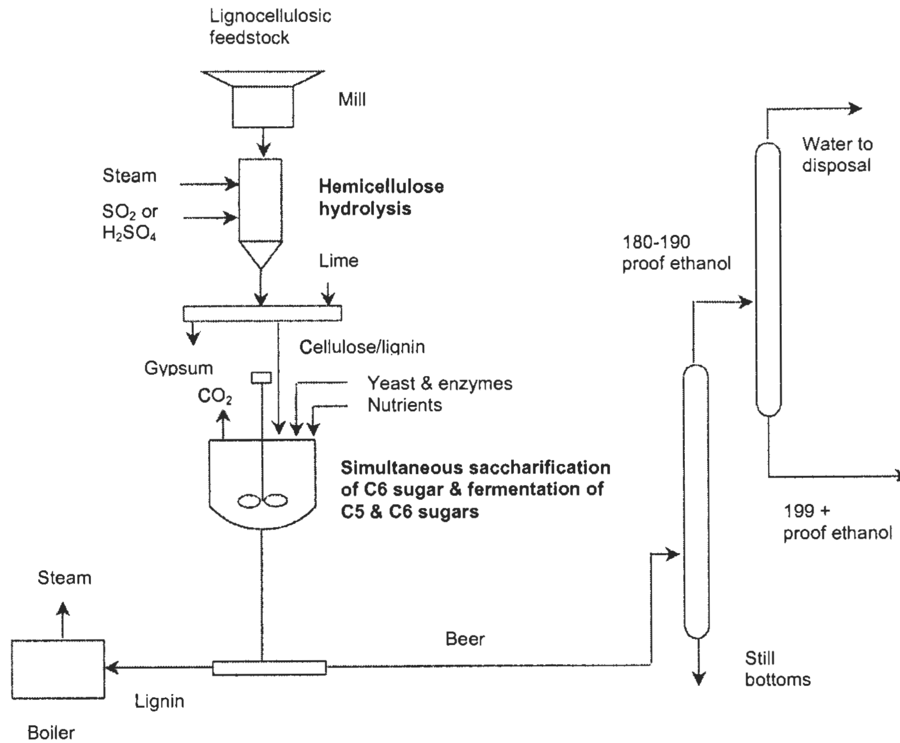

Fig. 11. General process schematic for enzymatic hydrolysis of lignocellulose (Brown, 2003).

7.1. Commercial development and status

The use of enzymes for the production of ethanol from biomass is a relatively new concept (U.S. DOE EERE, 2006). Basic research on the biological causes of cellulose degradation began during World War II when the U.S. Army struggled with deterioration of military clothing and equipment in the jungles of the South Pacific. The formation of the U.S. Army Natick Laboratories and the screening of thousands of samples collected from the jungle lead to the identification of *Trichoderma viride* (renamed *Trichoderma reesei*). *Trichoderma*, a green-spored, phialidic anamorph, is among the most important fungi in forest soils (Kendrick, 1992); it has contributed significantly to the development of cellulase enzymes for commercial use. Cellulases are produced by a variety of fungi and bacteria (Brown, 2003). Some of the fungal producers include *T. reesei*, *T. lignorum*, *T. koningii*, *Penicillium* spp., *Fusarium* spp., and *Aspergilllus* spp.

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Research on cellulase enzymes today focuses on increasing their hydrolytic capabilities (U.S. DOE EERE, 2006). The preparations of extracellular enzyme made from *T. reesei* in the mid-1960s increased scientific and commercial interest in cellulases. Genetic enhancement of *T. reesei* created mutant strains with 20 times the productivity of the original strains by 1979. Cellulases produced from submerged-culture fungal fermentations have been commercially available for nearly 20 years. One highly profitable cellulase market is in the textile industry, where cellulase enzymes are used to replace pumice stones in the production of "stone-washed" jeans.

Compared to other enzymes, our understanding of cellulase is still primitive (U.S. DOE EERE, 2006). There is considerable complexity involved in systems where soluble enzymes act on insoluble substrates, such as cellulase on cellulose. Furthermore, the cellulase enzyme is actually a synergistic system of enzymes that work together to degrade native cellulose. The three major groups of cellulase enzymes are endoglucanases, exoglucanases, and ß-glucosidases. The endoglucanases act on soluble and insoluble glucose chains. The exoglucanases include glucanhydrolases, which liberate glucose monomers from the end of the cellulose chain, and cellobiohydrolases, which liberate cellobiose (glucose dimers) from the end of the cellulose chain. The ß-glucosidases release D-glucose from cellobiose dimers and soluble cellodextrins. Though our understanding of the modes of action of cellulase has improved, there is still much to learn in order to develop enzymes with increased activities.

8. Pretreatment

Pretreatment of biomass for size reduction by thermal and chemical means is necessary to increase the access of enzymes to the cellulose. A focus of the U.S. Department of Energy Biomass Program is to reduce the cost of ethanol production from biomass by improving the pretreatment technology for lignocellulosic feedstocks (U.S. DOE EERE, 2006). Pretreatment is one of the most expensive steps, accounting for about 33% of the processing costs for conversion of lignocellulose to fermentable sugars (Brown, 2003). It usually involves a mild thermochemical treatment to hydrolyze hemicellulose and possibly solubilize some lignin. This treatment releases sugars and disrupts the protective sheath around cellulose, making it more vulnerable to enzymatic hydrolysis.

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A variety of different pretreatment approaches have been investigated over the past 25 years. The pretreatment methods include biological, alkaline, steam explosion, pre-hydrolysis, ammonia fiber explosion and treatment with organic solvents (Brown, 2003). The goal of pretreatment processes is to produce a solid biomass residue that is more amenable to enzymatic hydrolysis than native biomass. Many pretreatment methods, including dilute acid and steam/pressurized hot water, use the approach of hydrolyzing a significant amount of the hemicellulose in biomass and then recovering the soluble monosaccharides and oligosaccharides. The alkaline-based methods, however, are more effective at solubilizing the lignin while leaving behind much of the hemicellulose in an insoluble, polymeric form. Most pretreatment approaches do not hydrolyze significant amounts of the cellulose in biomass; rather, they enable more efficient enzymatic hydrolysis of the cellulose by degrading the surrounding hemicellulose and lignin and by modifying the cellulose structure.

8.1. Milling

Size reduction, and the resulting increase in surface area, is an important component of lignocellulose pretreatment (Brown, 2003). Hammer mills are used for grinding the particles to sizes that pass through 3 mm screen openings. Alternative methods are usually employed for further size reduction, as the energy requirements for milling increase substantially with decreasing particle size.

8.2. Organic solvents

Organic solvents, usually lower alcohols such as ethanol (Diaz et al., 2004), are used for the selective removal of lignin from biomass (Brown, 2003). They can be added to the acidic or alkaline solutions of pulping or pretreatment processes.

8.3. Alkali metal hydroxides

Pretreatment of biomass with alkali metal hydroxides, such as sodium hydroxide, disrupts the lignin-hemicellulose bonds, dissolves the lignin and hemicellulose, and results in cellulose swelling at high concentrations (5-20%) (Brown, 2003). The disadvantages include the loss of sugar from the breakdown of hemicellulose, and the large quantities of chemicals required to neutralize the acidic carboxylic groups in biomass. The kraft pulping process is an example of commercial use of hot sodium hydroxide to dissolve and break down lignin and hemicellulose in the pulp and paper

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industries. In the kraft process, biomass is steamed and then added to a digester with a hot mixture of sodium hydroxide and sodium sulfide, known as white liquor. The end-products are, among other things, soluble lignin (kraft lignin), polysaccharides, and carboxylic acids from the degradation of hemicellulose.

8.4. Steam and ammonia fiber explosion

The steam explosion process relies on the diffusion of steam into biomass pores; subsequent rapid decompression results in explosive expansion of the steam (Brown, 2003). This expansion increase the surface area by breaking the biomass into fiber strands. The mechanical effect on biomass was initially studied at relatively higher temperatures (220-270°C) for only 40-90 seconds. However, the chemical changes that occur prior to rapid decompression may be more important; large pores are created by the removal of hemicellulose and condensation of lignin, which enables the cellulases to reach the cellulose fibers. Comparable removal of hemicellulose is also possible at lower temperatures (190-210°C) with longer residence times (3-15 min). The addition of liquid ammonia (NH_3) to the steam explosion pretreatment of agricultural wastes has sugar yields of 80-90% of theoretical (Brown, 2003). The process employs ammonia loadings of 1.0-2.5 kg per kg dry mass at pressures exceeding 12 atm and ambient temperatures, and provides time for the ammonia to penetrate the lignocellulose, from several minutes to an hour.

8.5. Pressurized hot wash

A variation of dilute acid hydrolysis of hemicellulose includes a high temperature separation and washing of the pretreated solids (Nagle et al., 2002). The addition of a pressurized hot water step is thought to prevent re-precipitation onto the solids of the lignin and xylan solubilized under pretreatment conditions. The presence of lignin negatively affects the subsequent enzymatic hydrolysis of the pretreated solids. The pressurized hot wash is employed immediately after pretreatment by first pressing the pretreated biomass to separate liquid hydrolyzate from the solids. Hot water is then added, while still under pressure, to maintain a temperature of 130°C and to wash away any solubilized lignin or xylose from the cellulose. A continuous-flow percolation reactor system was used for initial research with dilute sulfuric acid (~0.1% w/v) and a hardwood feedstock. The

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process yielded greater than 95% recovery of the xylose oligomers and monomers; the pretreated solids were also significantly more reactive during enzymatic hydrolysis. A hybrid process, including batch or high-solids plug-flow pretreatment followed by a hot separation and washing of the pretreated solids, is under investigation to minimize required liquid volumes.

8.6. Biological

The effectiveness of pretreatment is based in part on the degradation of lignin, which exposes hemicellulose and cellulose to enzymatic action (Brown, 2003). The removal of lignin also decreases enzyme requirements since cellulases tend to adsorb to lignin. Biological pretreatment relies on the enzymes produced by lignin-degrading microorganisms. This method faces the challenge of potentially long reaction times, in days or weeks rather than hours. These microbes also produce cellulases and hemicellulases, and consume some of the sugars released. This loss reduces the potential sugar yields for subsequent ethanol production.

White-rot fungi are among the best degraders of lignin, and are able to completely degrade lignin to carbon dioxide and water (ten Have and Teunissen, 2001). However, white-rot fungi do not utilize lignin as their sole source of energy and carbon. Rather the breakdown of lignin enables the fungi to access the actual energy and carbon sources, hemicellulose and cellulose. The lignin-degrading system of white-rot fungi consists of extracellular enzymes, such as peroxidases and H_2O_2-generating oxidases, and low molecular weight cofactors. The enzyme lignin peroxidase (LiP), excreted by white-rot fungi, is capable of oxidizing and cleaving lignin and lignin-related compounds. Hydrogen peroxide is a co-substrate for this enzyme. In addition to LiP, the oxidative enzyme manganese peroxide (MnP) is involved in lignin degradation. MnP oxidizes Mn^{2+} to the oxidant Mn^{3+}, which acts as a mediator for the oxidation of various phenolic compounds. Laccase, a phenol oxidase, is an oxidative enzyme that was identified long before LiP. However, laccase alone is not potent enough to oxidize the nonphenolic components of lignin. The enzymes released by white rot fungi could therefore be employed as pretreatment for lignin degradation. Production and utilization of enzymes directly, by using the feedstock as a growth media for the fungus, is another approach.

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9. Brown rot fungi

Culturing a brown rot fungus, such as *Gloeophyllum trabeum*, directly on lignocellulosic feedstock offers a potential alternative for hydrolysis of the cellulose and hemicellulose without pretreatment to degrade the lignin. Brown rot fungi are among the most important agents of wood decay in forest ecosystems. In the decay process, brown rot fungi rapidly metabolize the hemicellulose and cellulose in wood, leaving behind a chemically-modified lignin residue (Goodell, 2003). The degraded wood loses strength, and typically appears cubical, crumbly and brown after advanced decay. Brown rot fungi employ both enzymatic and non-enzymatic mechanisms to degrade lignocellulose. Though unable to produce lignin-degrading enzymes, they are able to modify and slowly remove lignin from wood. Research suggests that brown rot fungi produce non-enzymatic, low molecular weight agents, which initiate the depolymerization of wood cell walls. Reports were recently published on the ability of brown rot fungi, such as *Gloeophyllum* species, to use non-enzymatic mechanisms, such as production of hydroxyl radicals by Fenton's reaction, to degrade pollutants, including benzaldehyde, chlorophenols, and poly(ethylene) oxides (Kamada et al., 2002; Fahr et al., 1999; Kerem et al., 1998). Background on the morphology and ecology of brown rot basidiomycetes, such as *G. trabeum*, as well as the mechanisms of lignocellulose decay are needed to assess the importance and potential benefits of brown rot fungi.

9.1. Taxonomy and morphology

Gloeophyllum trabeum is a bracket fungus that belongs to the Kingdom Eumycota, Phylum Basidiomycota, Class Holobasidiomycetes, Order Aphyllophorales and Family Polyporaceae. Most brown rot fungi are members of Polyporaceae. *G. trabeum* forms leathery fruiting bodies when fresh, 0.2-0.8 cm thick and 1-8 cm wide (Overholts, 1953; Gilbertson, 1974; Cockcroft, 1981). The basidiocarps present on wood are annual and sessile. The upper surface is velvety with colors varying from gray to cinnamon-brown. As basidiocarps age, the colors darken and the velvety texture becomes smoother. The lower pore surface is tan-colored and varies from lamellate to daedaloid with spore tubes 1-3 mm in width. Mature basidiospores are hyaline, cylindrical (7-10 x 2.5-4 μm) and smooth. Both oidia and chlamydospores have been reported as asexual reproductive structures

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in this species. The hyphal system is dimitic with generative hyphae supplemented by skeletal hyphae, which gives the basidiomata a tough texture (Fig. 12). The generative hyphae are hyaline, branching, thin-walled, 2.5-4 μm in diameter, and septate with clamp connections. The skeletal hyphae are cinnamon-brown, thick-walled, 3-6 μm in diameter, and aseptate with no clamp connections.

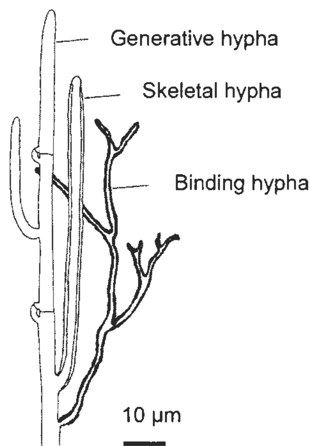

Fig. 12. Diagram of trimitic hyphae (Kendrick, 1992).

9.2. Ecology

Brown rot fungi, though common in nature, are relatively few in number, comprising less than 10% of all wood decay basidiomycetes (Dix and Webster, 1995). Fungal species that cause brown rot preferentially attack softwood, and therefore grow mainly on living and dead trees in conifer forests of the northern hemisphere. They are among the most important saprophytes involved in the degradation of dead wood and nutrient cycling in coniferous ecosystems. *G. trabeum* is an example of a brown rot fungus that grows on the surface of dead trees in temperate North American forests. It is an exception among the brown rot fungi as it is usually found on deciduous trees and only occasionally on coniferous trees.

9.3. Importance to humans and environment

Brown rot is the most common and most destructive type of decay of wood products in the northern hemisphere. Brown rot fungi account for as much as 80% of the destructive decay observed

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in wooden structures (Goodell, 2003). An estimated equivalent of 10% of the timber cut in the United States decays in service each year. Since brown rot fungi preferentially degrade softwood timber, used for the majority of construction wood in the U.S., they are responsible for much of this destruction.

Brown rot fungi are of potential interest in biotechnological applications such as bioremediation. Researchers have investigated using them to degrade aromatic pollutants (Goodell, 2003). The capacity of some brown rot fungi, such as *Gloeophyllum* species, to degrade aromatic compounds is already well documented. *G. trabeum*, for instance, mineralizes benzaldehyde and chlorophenols. Benzaldehyde and its metabolic intermediates were effectively degraded by the brown rot basidiomycetes *Tyromyces palustris* and *G. trabeum* in research by Kamada et al. (2002). This research demonstrated that brown rot fungi are capable of metabolizing certain aromatic compounds to CO_2 and H_2O, even though they are unable to degrade lignin. In research by Fahr et al. (1999), wheat straw cultures of the brown rot fungi *Gloeophyllum striatum* and *G. trabeum* degraded 2,4-dichlorophenol and pentachlorophenol. Degradation of chlorophenols are important as they are toxic and, in some cases, highly persistent priority pollutants of aqueous systems, soils, and waste materials.

Research by Kerem et al. (1998) further suggests that brown rot fungi may be useful for pollutant bioremediation by producing one-electron oxidants. *G. trabeum* is unusual in its ability to degrade an aliphatic polyether via extracellular oxidation. Even white rot fungi, which are able to oxidize lignin and many other organic chemicals, are unable to depolymerize poly(ethylene) oxide (PEO) significantly. PEOs resist biodegradation and are widespread, persistent environmental contaminants.

9.4. Characteristics and mechanisms of brown rot decay

Brown rot is a form of fungal decay of woody biomass caused by basidiomycetes. The main feature of brown rot decay is the rapid, extensive depolymerization of the hemicellulose and cellulose (Eriksson et al., 1990). Fungal attack on wood generally starts by hyphae penetrating through a pit into the narrow central cavity of a cell, after which it grows attached to the inner, lignified S3 layer of

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the secondary wall (Fig. 13 and 14). Unlike hyphae of soft rot and white rot fungi, which lie within cavities dissolved in the cellulosic S2 layer and channels eroded in the tracheid wall, respectively, hyphae of brown rot fungi lie on the inner surface of the tracheid wall without eroding a cavity (Fig. 15). Microscopic examination of wood after decay shows generalized thinning of the cell walls, without localization of decay around the hyphae. The S2 layer is degraded rapidly while the S3 layer resists attack.

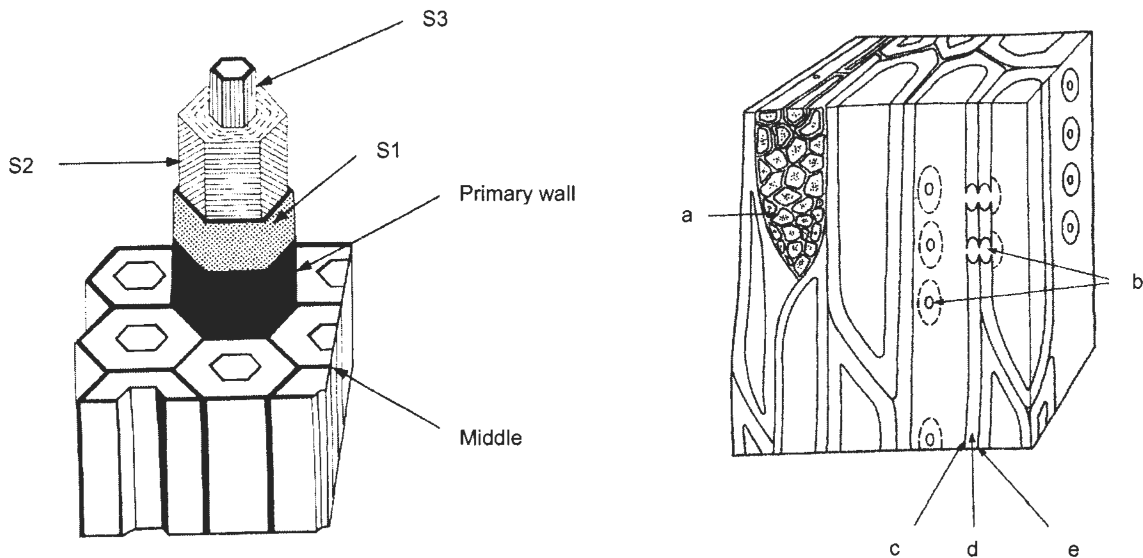

Fig. 13 and 14. The cell wall layers of a mature tracheid on left (Dix and Webster, 1995). Diagram of cellular structure of wood on right: (a) medullary ray, consisting of cells which are alive in living trees, (b) pits, (c) S3 wall, abundant in lignin with cellulose framework, (d) S2 wall, mainly cellulosic, and (e) S1 wall, lignified (Carlile et al., 2001).

The overall effect is that the wood loses strength and shrinks, developing longitudinal and transverse cracks (Carlile et al., 2001). When the cracks eventually join, the wood breaks into dark brown cubical crumbs. Although some degradation of lignin is apparent, the residual substrate after advanced degradation is composed primarily of lignin. Hyphae are surprisingly sparse in the decayed wood. It is hypothesized that older parts of the mycelium are enzymatically digested, allowing valuable materials such as organic nitrogen to be reused by growing hyphae.

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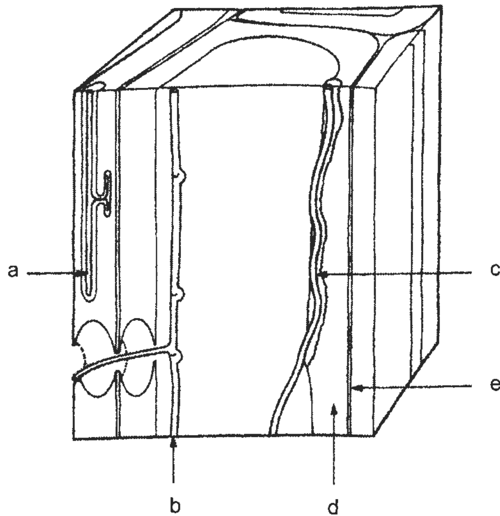

Fig. 15. Diagram of wood cell walls showing typical modes of attack of hyphae producing different types of decay: hypha of (a) soft rot fungus, (b) brown rot fungus, and (c) white rot fungus; (d) the S2 wall of a tracheid and (e) the more heavily lignified middle lamella (Carlile et al., 2001).

9.4.1. Lignin degradation

Circumvention of the lignin barrier is required in order to gain access to the cellulose and hemicellulose of woody biomass. Lignin is a three-dimensional polymer formed from phenylpropanoid subunits joined randomly (Fig. 16). White rot fungi degrade lignin by secreting peroxidases and hydrogen peroxide, H_2O_2. Brown rot fungi are able to alter the lignin via hydroxylation and demethylation reactions, which result in a loss of strength in the woody biomass. According to Goodell (2003), residual lignin in wood degraded by brown rot fungi is hydroxylated and demethylated, with some oxidation of the propanyl side chain. No oxidative enzymes such as laccase or peroxidases were detected in protein extracts from liquid culture or solid-state fermentation using the brown rot fungus, *G. trabeum* (Varela et al., 2003). However, the hypothesis that lignin is slowly metabolized by most brown rot fungi has recently been recognized and supported with evidence. Researchers have reported oxidative removal of lignin by brown rot fungi as high as 25% in some cases (Goodell, 2003). It is thought that some brown rot fungi produce low molecular weight lignin degrading agents and potentially even lignin degrading enzymes, which are capable of at least localized activity. A diffusible, non-enzymatic system that oxidizes lignin must also exist to account

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for the chemical changes that lignin is subjected to during brown rot decay. The unique ability of brown rot fungi to circumvent the lignin with only minor modification is still somewhat a mystery.

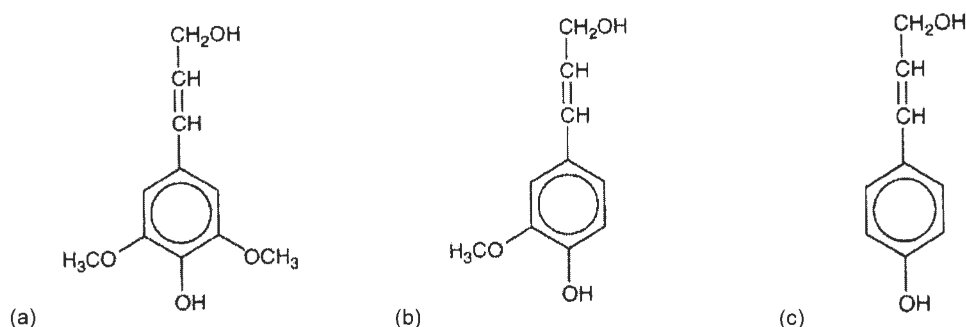

Fig. 16. Molecular structures of the three most common phenylpropanoid subunits of lignin: (a) sinapyl alcohol, (b) coniferyl alcohol, and (c) coumaryl alcohol (Carlile et al., 2001).

9.4.2. Cellulose and hemicellulose degradation

Hemicellulose degradation generally occurs in the early stages of cell wall degradation; the branched and relatively low molecular weight polysaccharides of hemicellulose are more accessible to chemical attack than crystalline cellulose. Both hemicellulose and cellulose are depolymerized to soluble oligosaccharides or monosaccharides, and diffused through the fungal matrix (hyphal sheath) to the wood cell lumen (Goodell, 2003). Fungal extracellular enzymes then act on the remaining complex sugars to produce simple sugars, which are absorbed through the fungal cell membrane and metabolized.

In the past, degradation of holocellulose to simple sugars was considered a solely enzymatic process. Brown rot fungi, such as *G. trabeum*, express a variety of wood-degrading enzymes including endoglucanases, β-glucosidases, and hemicellulases, and typically lack cellobiohydrolase and other exoglucanases (Eriksson et al., 1990). Under laboratory conditions, *G. trabeum* has been shown to produce a β-glucosidase (Herr et al., 1978), a xylanase (Ritschkoff et al., 1994), and two endoglucanases (Mansfield et al., 1998). Therefore, brown rot fungi do not seem to degrade crystalline cellulose by synergistic action between endo- and exoglucanases (Fig. 17). Some brown rot fungi produce endoglucanases that have mixed functions (Goodell, 2003). Glucanases have been isolated that act in a classical endo-manner of cleaving cellulose to shorter chains, but also

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demonstrate exo-activity by decreasing chain length through endwise hydrolysis. *G. trabeum* releases a processive endoglucanase during the hydrolysis of crystalline cellulose in order to cleave cellulose internally and also to liberate oligosaccharides (Cohen et al., 2005). The processive cellulases tend to be larger, and likely act on the crystalline cellulose later in the decay process, after the porosity of the wood has increased. The cellulases and hemicellulases produced by G. trabeum are not repressed by glucose (Varela et al., 2003).

The absence of exoglucanases also suggests that the fungi have a non-enzymatic mechanism for cellulose degradation. This finding is supported by the observation that degradation of wood by brown rot fungi is not localized near the hyphae (Xu and Goodell, 2001). Degradation reagents are capable of diffusing through the wood cell wall. The pore size in native wood cell walls does not permit penetration of compounds the size of known extracellular, cellulolytic enzymes. Thus, at least the initial stages of cellulose degradation by brown rot fungi include a non-enzymatic process as well. After the initial stages of cell wall degradation, the enzymatic and non-enzymatic systems produced by brown rot fungi are thought to act together in further decay.

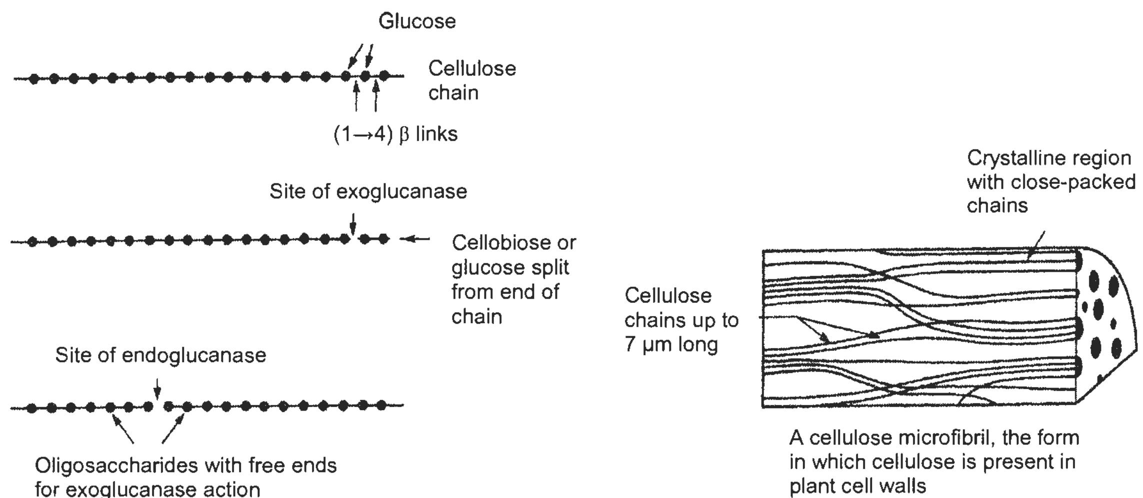

Fig. 17. The modes of action of different cellulase enzymes on a cellulose chain. Endoglucanases hydrolyze internal bonds in the chain. Exoglucanases remove monomers or dimers from the ends of the cellulose molecule (Carlile et al., 2001).

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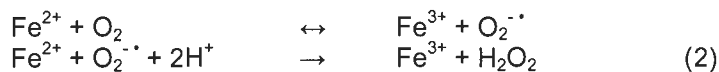
The hydroxyl radical generated by Fenton's reagent is one proposed non-enzymatic agent in wood decay by brown rot fungi (Varela et al., 2003). The Fenton reaction consists of a ferrous ion and hydrogen peroxide reacting as follows: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + {-OH}$. The hydroxyl radicals formed disrupt the structure of crystalline cellulose enabling cellulase enzymes to act. Several mechanisms have been proposed to explain how the fungal cultures generate Fe^{2+} and H_2O_2. One hypothesis is that traces of ferrous iron in wood react with hydrogen peroxide to generate transient free radicals (Carlile et al., 2001).

Recent isolation of extracellular biochelators (siderophores) from culture filtrates of *G. trabeum* suggests an alternative mechanism for the production of ferrous ions and hydrogen peroxide. Wang and Gao (2003) identified a peptide of low molecular weight in liquid cultures of *G. trabeum*. This biochelator produced by *G. trabeum* (termed Gt chelator or Gt factor) has a high affinity for binding and reducing iron. It may therefore mediate reactions with iron in the initial stages of cellulose degradation by brown rot fungi. Researchers suggest that the Gt chelator is able to oxidize cellulose, or a breakdown product of cellulose, and to transfer the electrons to ferric ions, Fe^{3+} (Equation 1). In the presence of molecular oxygen, the ferrous ion, Fe^{2+}, reduces O_2 to $O_2^{-\bullet}$, which then reacts to form H_2O_2 (Equation 2). The Fenton reaction of hydrogen peroxide and ferrous ions creates hydroxyl radicals (Equation 3), which are highly oxidative. Hydroxyl radicals disrupt inter- and intra-hydrogen bonds in cellulose and cause cleavage of the polysaccharide chain. The resulting depolymerization of cellulose chains produces more reducing and non-reducing ends, which makes cellulose accessible for further degradation.

$$Fe^{3+} + \text{Gt factor} \rightarrow Fe^{2+} \quad (1)$$



$$Fe^{2+} + O_2 \leftrightarrow Fe^{3+} + O_2^{-\bullet}$$
$$Fe^{2+} + O_2^{-\bullet} + 2H^+ \rightarrow Fe^{3+} + H_2O_2 \quad (2)$$

$$H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + HO^{-} + Fe^{3+} \quad (3)$$

Equations 1-3. Equations involving the Gt factor (1), formation of H_2O_2 (2) and Fenton reaction (3) (Wang and Gao, 2003).

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9.5. Conclusions on brown rot fungi

Brown rot fungi are important to the environment and humans in many ways, both positive and negative depending on the viewpoint. They are often considered as harmful organisms that cause serious losses of wood and wood products. Their primary importance, however, is in the continuous functioning of the carbon cycle that enables life to exist on earth (Gilbertson, 1974). Wood decay organisms play a natural and essential role in maintaining productive forests. The decay of dead trees prevents the continuous accumulation of large amounts of woody biomass on the ground, and returns organic material and mineral nutrients to the soil. In addition to their natural roles in the environment, brown rot fungi may become important agents in bioremediation of aromatic pollutants. Research suggests that the non-enzymatic mechanisms of brown rot fungi to modify and slowly degrade lignin are useful for the degradation of certain aromatic compounds. Further studies on brown rot fungi may lead to a better understanding of the degradation mechanisms for lignocellulose, and methods of using them in beneficial ways, including the *in situ* hydrolysis of lignocellulose to fermentable sugars.

10. Solid-state fermentation

Solid-state fermentation (SSF) is an important alternative to submerged-culture fermentation in the bioconversion of plant biomass. SSF is particularly suited to microorganisms, such as wood rot fungi, which are adapted to growth on solid substrates in nature (Tengerdy and Szakacs, 2003). Filamentous fungi grow differently on wood than in liquid media, necessitating different conditions, cellular structures, enzymes, and metabolites. Nutrient availability, for instance, is more limited in natural solid substrates than in liquid cultures. Therefore, fungi develop more efficient enzyme systems for degradation; the enzymes produced may lead to more efficient hydrolysis of the substrate in a bioreactor. Lignocellulose is a potential SSF feedstock for the production of biofuels, enzymes and other biochemical products.

10.1. Growth on lignocellulose

Filamentous fungi, unlike bacteria and yeast, are able to utilize substrate-bound water for growth in the absence of free water (Tengerdy and Szakacs, 2003). The growth is limited on

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lignocellulosic substrates, however, by steric hindrance and substrate accessibility. The fungus grows by adhering to the substrate, colonizing via spores or hyphae, and then spreading from particle to particle by branching. The packing density, or volume of mycelial population over the volume available for fungal growth (v/v), provides an indication of space utilization. Theoretically, tightly-packed cylindrical hyphae may achieve a packing density of 0.9 (v/v). However, within finely milled lignocellulose feedstock, such as wheat straw, the packing density is only 0.05 to 0.07 (v/v). This spatial limitation affects the substrate conversion, which rarely exceeds 30 to 40% using SSF.

10.2. Enzyme production

Commercial enzymes such as cellulase and xylanase are currently produced by submerged fermentation primarily. The high production costs, however, impede economical applications of the lignocellulolytic enzymes. SSF is an alternative that is expected to offer an enzyme complex of higher hydrolytic efficiency, particularly when prepared with host-specific fungi in single or mixed cultures (Tengerdy and Szakacs, 2003). Enzyme-assisted ensiling of sweet sorghum provides an example of SSF process efficiency (Fig. 18).

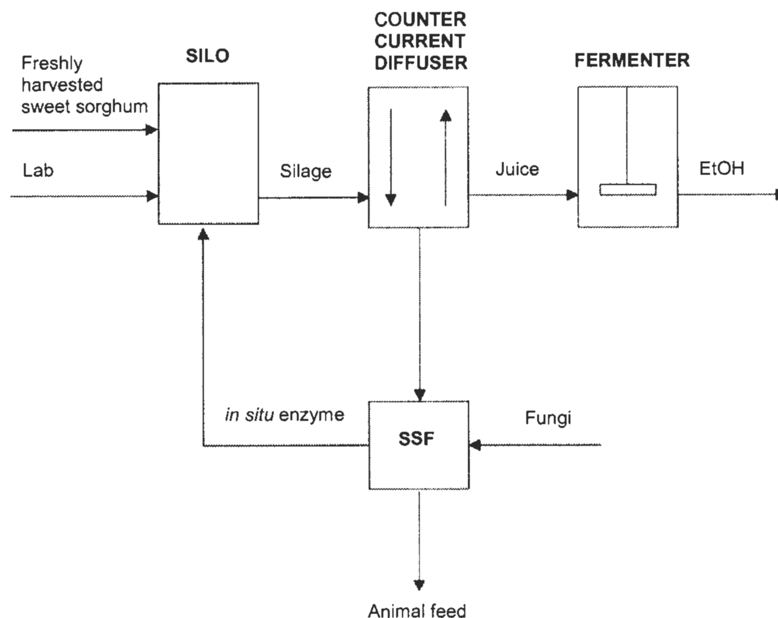

Fig. 18. Process schematic of solid-state fermentation of sweet sorghum pulp for production of enzymes by *Gliocladium* sp. (Tengerdy and Szakacs, 2003).

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Enzymes were produced *in situ* using an 8-d SSF with *Gliocladium* sp. on 2-3% of the fermented pulp, and then added to the freshly harvested sweet sorghum during ensiling (Tengerdy et al., 1996). The enzymes improved sugar yields by converting cellulose to glucose, and increasing the permeability of the cell wall for sugar extraction. The protein in fungal biomass and enzymes also enriched the animal feed. The cost of the SSF enzymes was estimated as $0.12 per MT sweet sorghum, compared to $9.50 per MT sweet sorghum for commercial enzymes.

10.3. Advantages and challenges of SSF

Typical SSF applications use static, non-aseptic conditions with lower energy and equipment costs, and less water and aeration requirements, compared to submerged-culture fermentations (Table 1) (Raimbault, 1998).

Table 1
Comparison between liquid and solid state fermentations (Raimbault, 1998)

FACTOR	Liquid State Fermentation	Solid State Fermentation
Substrates	Soluble substrates (sugars)	Insoluble substrates (starch, cellulose, lignin)
Aseptic conditions	Heat sterilization, aseptic control	Vapor treatment, non-sterile conditions
Water	High volumes of water consumed, effluents discarded	Limited consumption of water, no effluent
Metabolic Heating	Easy control of temperature	Low heat transfer capacity
Aeration (0_2)	Limitation of soluble oxygen. High level of air required	Easy aeration and high surface exchange air/substrate
pH control	Easy pH control	Buffered solid substrates
Mechanical agitation	Good homogenization	Static conditions preferred
Scale up	Industrial equipments available	Need for engineering & new design equipment
Inoculation	Easy inoculation, continuous process	Spore inoculation, batch process
Contamination	Risk of contamination for bacteria	Risk of contamination for low rate growth fungi
Energy	High energy consuming	Low energy consuming
Volume of Equipment	High volumes, high cost technology	Low volumes, low equipment costs
Effluent & pollution	High volumes of polluting effluents	No effluents, less pollution
Concentration/Product	30-80 g/l	100-300 g/l

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SSF systems also tend to produce a more concentrated product stream, which results in considerable energy savings during product recovery, particularly by distillation. The challenges of SSF include less control over the operating variables, including temperature, moisture and pH, and more difficulty in scale-up and continuous operation. One of the major obstacles in full-scale SSF applications is the limited knowledge related to the design and operation of large-scale bioreactors (Ashley et al., 1999), such as rotating drum (Kalogeris et al., 2003), packed bed and gas-solid fluidized bed (Mitchell et al., 2000). Despite the obstacles, SSF offers a number of advantages over submerged fermentation processes, and is currently underutilized for the production of commercially-valuable products (Kalogeris et al., 2003).

11. Ethanol fermentation

The simple sugars liberated from lignocellulosic feedstock, via acid or enzymatic hydrolysis, are available for fermentation to ethanol. The conventional microbes used for the fermentation of sugars to ethanol efficiently convert hexoses to ethanol and carbon dioxide (Brown, 2003). The sugar and starch crops traditionally used as feedstock yield six-carbon sugars, which are fermented to ethanol by common baker's yeast, *Saccharomyces cerevisiae*. Hydrolysis of a lignocellulosic feedstock, such as corn fiber, releases both hexoses and pentoses. The hemicellulosic fraction yields pentoses, particularly xylose and arabinose, which are not fermentable to ethanol by the microbes traditionally used for ethanol production. A variety of wild-type bacteria and fungi can ferment both five- and six-carbon sugars to ethanol; however, the maximum yields are significantly lower compared to *S. cerevisiae*. These wild-type microbes include: the yeast species *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus*, filamentous fungi within the genera *Fusarium*, *Rhizopus* and *Paecilomyces*, and the thermophilic bacteria *Clostridium thermohydrosulfuricum, C. thermosaccharolyticum* and *C. thermocellum*. The maximum yields for ethanol production from lignocellulose is 50 g/L for wild-type yeast, as compared to 130-150 g/L for the hexose-fermenting *S. cerevisiae* (Brown, 2003; Devantier et al., 2005).

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Various approaches, including host selection and modification, and metabolic and genetic engineering, have been employed to improve the utilization of sugars released by the hydrolysis of lignocellulose. Genetic engineering of yeast using recombinant DNA techniques incorporates the desired genes on plasmids or by direct chromosomal integration (Kendrick, 1992). This method has created new strains of microbes that are able to ferment both hexoses and pentoses to ethanol. Recombinant strains of the yeast *C. shehatae* and *P. stipitis* are able to ferment D-xylose, and recombinant *S. cerevisiae* ferments D-xylose and L-arabinose (Becker and Boles, 2003). Strains of bacteria including *Zymomonas mobilis*, *Escherichia coli*, *Klebsiella oxytoca*, and *Bacillus stearothermophilus* have also been engineered to ferment D-xylose and L-arabinose to ethanol (Abbas et al., 2003; Deanda et al., 1996; Ingram et al., 1987).

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CHAPTER 3. SEQUENTIAL SACCHARIFICATION OF CORN FIBER BY THE BROWN ROT FUNGUS, *GLOEOPHYLLUM TRABEUM*, AND ETHANOL PRODUCTION BY *SACCHAROMYCES CEREVISIAE*

A paper prepared for submission to *Bioresource Technology*

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Abstract

Hydrolysis of the corn wet-milling co-product – corn fiber to simple sugars by the brown rot fungus, *Gloeophyllum trabeum*, was studied in both suspended-culture and solid-state fermentations. The suspended-culture experiments were not effective for harvesting the sugars released from the corn fiber by fungal hydrolytic enzymes. Similar reducing sugar contents with and without *G. trabuem* inoculum, despite losses in the cultured fiber biomass, suggest that reducing sugars released by the extracellular enzymes were consumed by the fungus. Whereas, solid-state fermentation with *G. trabeum* for three days followed by buffered anaerobic incubation converted up to about 11% (108 mg/g initial fiber) of the corn fiber into harvestable reducing sugars. With the addition of the commercial yeast *Saccharomyces cerevisiae*, sugars released by *G. trabeum* were fermented to a maximum yield of 4.0 g ethanol/100 g fiber. This is also the first report, to our knowledge, of the fungus *G. trabeum* producing ethanol (up to 3.4 g/100 g fiber) under anaerobic or stressed conditions.

Keywords: Ethanol; Corn fiber; Lignocellulose biomass; Brown rot fungus; Solid-state fermentation; Suspended-culture fermentation; Saccharification

1. Introduction

Ethanol production by corn wet-milling plants produces large quantities of the low-value, lignocellulosic co-product – corn fiber. During the wet-milling operation, the hull of the corn kernel (pericarp) is first separated and washed to recover residual starch, and then dried and combined with other by-products including oil cake, corn steep liquor and stillage (Butzen and Hobbs, 2002). The mixture is dried, pelletized and currently sold as corn gluten feed (21% protein) for dairy cattle. Wet

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mills produced nearly 3 million tons of corn gluten feed in 2005 (CRA, 2006). The typical yield is 13 lb corn gluten feed per bushel of corn processed (Brown, 2003), and the demand for it is not expected to increase significantly. Therefore, as wet-milling processes expand, additional uses of the corn fiber will be required to prevent an oversupply of animal feed. One approach for value-adding is to utilize the corn fiber as feedstock for production of more ethanol. Saccharification of the cellulose could liberate simple sugars for subsequent yeast fermentation to ethanol. The hemicellulose is another possible source of sugars for ethanol production, provided the strain of yeast or bacteria added is able to ferment five-carbon sugars to ethanol. The approximate composition of corn fiber is 17% starch, 35% hemicellulose, 18% cellulose, 11% protein, 3% oil, 6% ash, 5% galactan, 1% mannan, and 4% other materials (Abbas et al., 2004).

Various approaches, including concentrated and dilute acid hydrolysis and enzymatic hydrolysis, have been investigated to find an economical method of converting lignocellulose to fermentable sugars (U.S. DOE EERE, 2006). Significant obstacles hinder the commercialization of these processes. Concentrated acid hydrolysis is appealing for the near theoretical yields of sugars released; however, the chemical expense, acid recovery and corrosiveness, and gypsum waste steam impede economical, full-scale development. Though dilute acid hydrolysis requires less acid, it is still not considered economically competitive due to the similar expenses of chemicals, corrosion-resistant equipment, and gypsum waste disposal, and the potential production of fermentation inhibitors at the higher temperatures.

Enzymatic hydrolysis is considered the most economically promising approach (U.S. DOE EERE, 2006). The lignocellulolytic enzymes may be produced separately and added, or released *in situ* by microbes growing on the fibrous substrate. The current challenges include pretreatment of the feedstock and fermentation of mixed sugars (pentoses and hexoses). The direct bioconversion of cellulose and hemicellulose to simple sugars by microorganisms cultured on the feedstock is advantageous by avoiding the need to purchase cellulase enzymes. White rot and brown rot fungi have great potential for hydrolyzing corn fiber, as they are among the most important agents of decay for woody biomass (Dix and Webster, 1995). Brown rot fungi, such as *Gloeophyllum trabeum*, rapidly

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metabolize the hemicellulose and cellulose in wood, leaving behind a chemically-modified lignin residue (Goodell, 2003). The enzymatic and non-enzymatic mechanisms used by *G. trabeum* to degrade wood could therefore be employed for the bioconversion of other fibrous substrates, such as corn fiber, to fermentable sugars. This research was conducted using both submerged-culture and solid-state fermentation (SSF) of corn fiber by *G. trabeum* to determine the potential for release of simple sugars. Buffered anaerobic conditions were created following solid-state fermentation by *G. trabeum* to restrict the consumption of sugars by the fungus, and to enhance cellulose and hemicellulose saccharification by the fungal hydrolytic enzymes produced during SSF. The yeast *Saccharomyces cerevisiae* was also added under the anaerobic conditions to enhance ethanol production from the fermentable sugars (hexoses).

2. Methods

2.1. Microorganisms

Gloeophyllum trabeum and *Saccharomyces cerevisiae* were obtained from the American Type Culture Collection (ATCC 11539 and 24859, respectively, Rockville, MD, USA). Stock cultures of *G. trabeum* were maintained on potato dextrose yeast agar (Difco, Sparks, MD, USA). The mycelia were stored as freeze-dried and frozen cultures at 4ºC and -80ºC, respectively. *S. cerevisiae* cells were freeze-dried in skim milk (1:1 ratio of cells to 20% skim milk) and stored at 4°C. The contents of one serum vial with ~5.6×10^8 cells was used as inoculum for ethanol fermentation.

2.2. Corn fiber

Corn fiber for submerged-culture and solid-state fermentation experiments was obtained from the coarse fiber (pericarp) stream at an ADM wet-milling plant (Decatur, IL, USA). The corn fiber was cleaned, steeped, milled, and passed over screens and through a press. The fiber samples were dried in a convection oven at 200°C and 0% humidity for 7 h at the ADM facility. The fiber was stored in bags at 4°C, and dried for 4 d at 80°C prior to use in the experiments. The composition of the corn fiber is 45.3% hemicellulose, 16.4% cellulose, 1.3% lignin, 37.0% cell solubles (11.0% of the fiber is protein) and 0.03% ash, determined based on duplicate samples using the Neutral and Acid

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Detergent Fiber procedures (ANKOM Technology, 2005). The moisture content of fiber after drying at the ADM facility was 6.8%.

2.3. Inoculum preparation

G. trabeum inoculum was prepared by thawing 2 ml of frozen mycelia and transferring aseptically to 1 L of yeast mold (YM) broth (Difco), sterilized at 121°C for 20 min in a 2-L Erlenmeyer flask. The culture broth was then incubated aerobically by shaking at 30°C and 150 rpm for 5 to 7 days, until a dense growth of mycelia was observed in the broth. The basal salts solution used for rinsing and inoculation consisted of the following in 1 L of water: 0.250 g KH_2PO_4, 0.063 g $MgSO_4$, 0.013 g $CaCl_2$, and 1.25 ml trace elements solution (Kirk et al., 1978). The trace elements solution contained the following in 1 L of water: 1.500 g nitrilotriacetic acid, 3.000 g $MgSO_4 \cdot 7H_2O$, 0.500 g $MnSO_4 \cdot H_2O$, 1.000 g NaCl, 0.100 g $FeSO_4 \cdot 7H_2O$, 0.181 g $CoSO_4 \cdot 7H_2O$, 0.082 g $CaCl_2 \cdot 2H_2O$, 0.100 g $ZnSO_4$, 0.010 g $CuSO_4 \cdot 5H_2O$, 0.010 g $Al_2(SO_4)_3 \cdot 2H_2O$, 0.010 g H_3BO_3, and 0.010 g $NaMoO_4$. The basal salts solution (1 L) and one 1-L centrifuge bottle were sterilized by autoclaving at 121ºC for 20 min. The mycelia were rinsed by transferring the inoculated YM broth to the centrifuge bottle, centrifuging for 20 min at 7,277 x *g*, and decanting the supernatant. An equivalent quantity of sterile basal salts solution was then added, and the procedure was repeated. The centrifugation step was skipped in later experiments since the small mycelial spheres settled well. Sterile basal salts solution was added to the rinsed mycelia to a final volume of 1 L for use as inoculum in suspended-culture and solid-state fermentation experiments.

2.4. Inoculation, incubation and harvesting for suspended-culture experiments

Suspended-culture fermentation with *G. trabeum* was conducted in a slurry at about 5% total solids (corn fiber). The corn fiber (5 g) was sterilized by autoclaving in a 250-ml flask at 121°C for 75 min with blue autoclave wrap over the flask mouth. The slurry was then prepared aseptically by adding 100 ml of basal salts solution and 10 ml of rinsed, gravity-dense fungal mycelia using a peristaltic pump and sterilized tubing. Similar control flasks were prepared with the corn fiber and basal salts solution, but without fungal inoculum. Once inoculated, the flasks were covered with

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sterile blue autoclave wrap and aluminum foil to prevent loss due to evaporation (Fig. 19), and incubated with shaking at 30°C and 150 rpm.

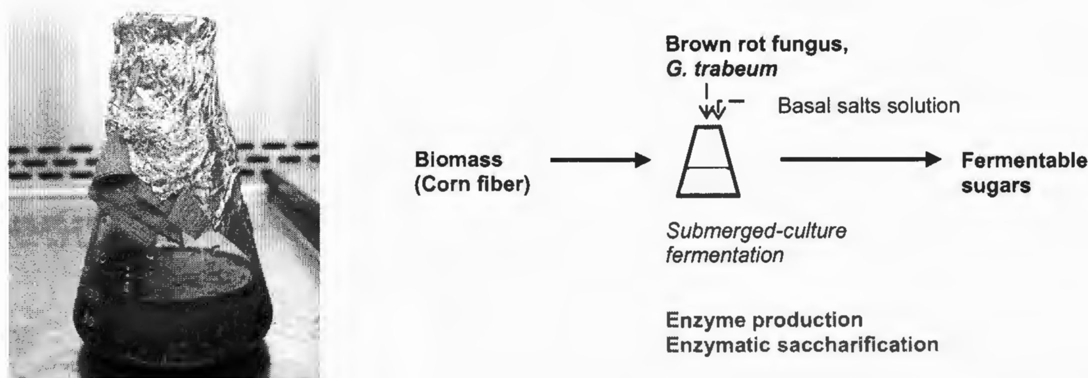

Fig. 19. Photo of a slurry of basal salts solution, *G. trabeum* inoculum, and corn fiber in a 250-ml flask during a suspended-culture experiment (on left). Lab-scale process schematic for submerged-culture fermentation (on right).

Culture and control flasks were harvested and analyzed on a weekly basis for weight loss and reducing sugar content. The flasks were harvested by placing in a steamer cabinet (steaming) at ~100°C for 1 h and then pouring the contents of each flask into a pre-weighed centrifuge bottle (250 ml). After centrifuging at 15,344 x *g* for 20 min, the supernatant was filtered (Whatman #54 filter paper) following the procedure outlined in Pometto III and Crawford (1986) and analyzed for reducing sugar content.

2.5. Inoculation and incubation for solid-state experiments

Solid-state fermentations (SSF) of corn fiber by *G. trabeum* was performed by adding 25 g of corn fiber and 5 ml deionized water to 1-L polypropylene bottles, including five marbles. The procedure was modified from a method by Pometto III and Crawford (1986). The bottles and fiber were sterilized by autoclaving with the caps loosely fitted for 1 h at 121°C. The autoclave was vented at fast exhaust for 3 min to drive steam out of the bottle. Steam created within the bottle helped to ensure sterilization of the substrate. The bottle mouths were then covered with blue wrap and autoclaved for 15 additional min at 121°C. The fiber was inoculated with rinsed fungal mycelia in basal salts solution (75 ml) using a peristaltic pump and sterilized tubing (Fig. 20a). The bottles were rolled to coat the fiber on the inside, and incubated on the bottle's side at 30°C (Fig. 20b). Similar

control bottles were prepared with the basal salts solution, but without the fungus. To maintain high humidity in the incubator and to reduce moisture loss in the SSF bottles, air was continually bubbled through water in troughs at the bottom of the incubator.

Fig. 20. Examples of (a) inoculation and (b) incubation setups for SSF experiments.

2.6. Harvesting for solid-state experiments (without addition of S. cerevisiae)

Sugar was harvested after different lengths (days) of SSF from a set of control and culture bottles by adding 300 ml of deionized water. The bottles were then placed in a steamer cabinet (steamed) for 1 h, and the contents transferred to pre-weighed centrifuge bottles (500 ml). The liquid was collected by centrifugation at 17,696 x *g* for 20 min and filtration (Whatman #54 filter paper). The reducing and total sugar contents of the filtrate was measured colorimetrically.

Sugar was also harvested from a set of control and culture bottles after adding 300 ml of acetate buffer (pH 4.8) to the bottles, which were then capped tightly and placed in a water bath at 37°C. The acetate buffer added per bottle contained 0.533 g acetic acid and 1.732 g sodium acetate. This procedure created static conditions, which are referred to as anaerobic conditions in this paper due to some fermentation of ethanol by *G. trabeum* (discussed in section 3.5). These conditions limited the fungal consumption of sugars while using a buffer to enhance cellulose and hemicellulose saccharification by fungal hydrolytic enzymes produced during SSF. Samples were taken on a 24-h basis. After 48 h of anaerobic conditions, the bottles were placed in the steamer cabinet for 1 h, and the liquid was collected by centrifugation (17,696 x *g* for 20 min) and filtration (Whatman #54 filter paper) for reducing and total sugar analyses.

2.7. Ethanol production following solid-state fermentation

SSF by *G. trabeum* in bottles coated on the inside with 25 g corn fiber in 75 ml basal salts solution was first incubated for 2 and 3 d at 30°C, followed by the aseptic addition of 300 ml water with glucose-free yeast nutrients in one set of bottles (Kunduru and Pometto III, 1996). The nutrients per bottle included 1.80 g yeast extract (Ardamine Z, Red Star BioProducts, Juneau, WI, USA), 0.07 g $CaCl_2 \cdot 2H_2O$, 0.45 g KH_2PO_4, 1.20 g $(NH_4)_2SO_4$, and 0.30 g $MgSO_4 \cdot 7H_2O$. One vial of freeze-dried yeast inoculum (~5.6×10^8 cells) was added per bottle to a subset of the bottles. This procedure created static (anaerobic) conditions to reduce the fungal consumption of sugars and enable fermentable sugar bioconversion to ethanol (Fig. 21). Bottles were capped loosely to allow gas exchange without contamination and incubated anaerobically without shaking for 6 and 12 d at 30°C. Similar control bottles were prepared with yeast nutrient solution, and with or without yeast inoculum.

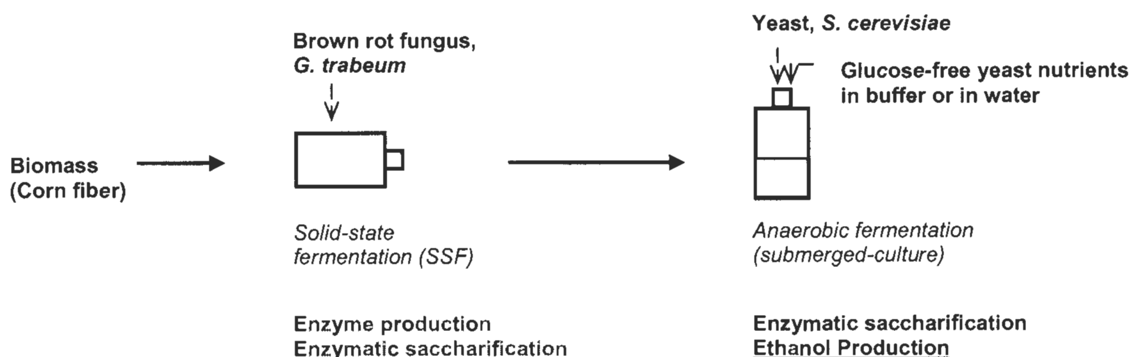

Fig. 21. Lab-scale process schematic for sequential solid-state fermentation of corn fiber by *G. trabeum* and ethanol production by *S. cerevisiae.*

Sterile acetate buffer was added to one set of controls with and without culture *(G. trabeum)* to determine the potential levels of reducing sugar under more optimal pH and temperature conditions for the extracellular cellulases secreted by *G. trabeum*. No yeast was added to this set of bottles. Sterile acetate buffer with glucose-free yeast nutrients was added to another set of controls with and without culture *(G. trabeum)*; yeast was added to some of these bottles. This set was used to assess the effect of acetate buffer on ethanol production by *G. trabeum* in the presence and absence of yeast nutrients.

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Samples were taken aseptically every 24 h and filtrates were analyzed for total and reducing sugar and ethanol contents.

2.8. Filtrate analyses

The quantity of free reducing and total sugars in the filtrates from suspended-culture or solid-state experiments was determined by using a modified Somogyi-Nelson carbohydrate assay (Antai and Crawford, 1981) and the phenol-sulfuric method (Wood and Kellogg, 1988), respectively.

The ethanol content of samples was measured by using a Waters High Pressure Liquid Chromatograph (Millipore Corporation, Milford, MA, USA) equipped with a Waters Model 401 refractive index detector, column heater, autosampler and computer controller (Kunduru and Pometto III, 1996). The ethanol and other sample constituents were separated on a Bio-Rad Aminex HPX-8711 column (300 x 7.8 mm) (Bio-Rad Chemical Division, Richmond, CA, USA) using a mobile phase of 0.012 N sulfuric acid with a flow rate of 0.8 ml/min, an injection volume of 20 µl, and a column temperature of 65°C. Results were cross-checked by using a Multidimensional Gas Chromatography-Mass Spectrometry-Olfactory system (GC-MS-O) (Microanalytics, Round Rock, TX, USA), as described by Cai et al. (2006). The system incorporates GC-O with conventional GC-MS (Agilent 6890N GC/5973 MS, Wilmington, DE, USA) and a flame ionization detector (FID). The non-polar precolumn was 12 m x 0.53 mm with a film thickness of 1 µm of 5% phenyl methylpolysiloxane stationary phase (SGE BP5), and was operated at a constant pressure of 8.5 psi. The polar analytical column was a fused silica capillary column (30 m x 0.53 mm) coated with a 1-µm film of polyethylene glycol (WAX, SGE BP20); the column pressure was held constant at 5.8 psi. Both columns were operated in series with system automation and data acquisition software (MultiTrax™ V. 6.00 and AromaTrax™ V. 6.61, Microanalytics, and Chemstation™, Agilent). The run parameters were as follows: injector, 260°C; FID, 280°C; column, 40°C initial, 3 min hold, 7°C/min, 220°C final, 10 min hold; carrier gas, helium. The range of the mass-to-charge ratio (*m/z*) was set at 33 to 280. Spectra were collected at 6 scans/s, and the electron multiplier voltage was set to 1200 V.

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2.9. Weight loss calculations

Weight (biomass) loss was calculated as the difference between the initial quantity of fiber (dried 4 d at 80°C) added before sterilization/inoculation and the weight of dry fiber residue from the filter paper and centrifuge bottle after harvesting. The filter paper containing fiber residue and the centrifuge bottles with the remaining fiber were dried in an oven at 80°C for two and four days, respectively, and equilibrated in a desiccator before weighing. The percent loss was then determined by dividing the weight loss by the initial fiber weight and multiplying by 100%. The fungal mass on the inoculated fiber was included in residual biomass measurements; therefore, the actual fiber loss in the experiments was larger than the weight loss values reported.

3. Results and Discussion

3.1. Effect of suspended-culture fermentation on reducing sugar production and biomass loss

Three suspended-culture experiments for the fermentation of corn fiber by *G. trabeum* were performed, with some variations from the procedure described in sections 2.3 and 2.4. The first two experiments were conducted at 37°C for inoculum preparation in YM broth and fermentation of the corn fiber slurry with *G. trabeum*.

The results of the first and second suspended-culture experiments are presented in Fig. 22. Flasks were harvested on a weekly basis starting from the day of inoculation (week 0). The average reducing sugar content of harvested slurry cultures (*G. trabeum*) fluctuated between 11 and 17 mg/g initial fiber over the three-week fermentation period (Fig. 22a). The initial decrease in reducing sugars after the first week of fermentation (17 to 11 mg/g fiber) could be attributed to fungal consumption of available reducing sugars from the fiber or residual YM broth in the inoculum. The slight increase in reducing sugar concentrations from weeks 1 to 2 may result from the release of more reducing sugars by the extracellular enzymes than are consumed by the fungus. For controls, the sugars harvested varied between 13 and 14 mg reducing sugars/g initial fiber. The reducing sugar contents of culture and control flasks were similar overall.

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The slurry cultures with *G. trabeum* demonstrated an increasing trend in weight loss from an average of 14% of initial fiber on the day of inoculation (week 0) to 34% on week 2 (Fig. 22b). The reduction in biomass seemed to reach a plateau after week 2, with an average loss of 35% on week 3. The weight loss in the controls varied on average from 14 to 16%. The similar reducing sugar values for the harvested control and culture flasks, despite the loss in cultured biomass, suggests that reducing sugars released by the fungal extracellular enzymes were consumed by the fungus. Saccharification of the corn fiber to oligosaccharides may have also contributed to biomass loss, which was not accounted for in reducing sugar measurements.

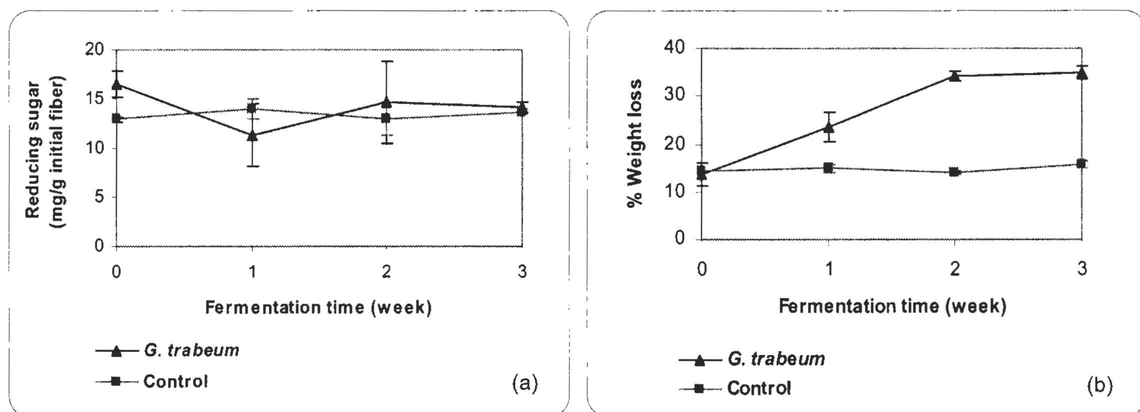

Fig. 22. (a) Reducing sugar content and (b) weight loss in slurries with 5 g corn fiber, 10 ml *G. trabeum* inoculum, and 100 ml nutrient solution (containing in 1 L water: 0.25 g KH_2PO_4, 0.063 g $MgSO_4$, 0.013 g $CaCl_2$, 0.92 g ammonium tartrate, 0.75 g asparagine and 1.25 ml trace elements solution). No fungal inoculum was added to the controls. Corn fiber slurries were prepared in 250-ml flasks with blue autoclave wrap and aluminum foil as cover, and incubated at 37°C with shaking at 150 rpm. The flasks were harvested by steaming for 1 h and filtration. (n=2)

The third suspended-culture experiment differed from the first two experiments in the incubation temperature of 30°C and basal salts solution for inoculum preparation and fermentation of the fiber slurry with *G. trabeum* (Fig. 23). This experiment was conducted to check the effects of lowering the temperature from 37 to 30°C and excluding nitrogen sources (ammonium tartrate and asparagine) from the inoculation solution, prior to starting solid-state experiments. Nitrogen availability affects the production of hydrolytic enzymes and oxidoreductase activity by *G. trabeum* (Varela et al., 2003). The SSF experiments had the same incubation temperature and basal salts in the rinsing and inoculation solution as used in this experiment.

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The harvested slurry cultures with *G. trabeum* in the third experiment demonstrated an increase in reducing sugars from 14 to 20 mg/g initial fiber from week 0 to week 1 (Fig. 23a), and then a decrease to 16 mg/g initial fiber on weeks 2 and 3. These results are similar to the previous experiments, which averaged between 11 and 17 mg reducing sugar/g initial fiber in the culture flasks. The initial increase may indicate enhanced enzyme activity during the first week of fermentation as compared to the previous experiments, in which the reducing sugars decreased from the week 0 to week 1 (Fig. 22a). The reducing sugar levels in the harvested controls were fairly constant with values ranging from 14 to 16 mg/g initial fiber.

The reduction in biomass for cultured fiber in the third experiment followed a similar trend to the first two experiments, with an increase from 13% on week 0 to 30 and 35% on weeks 2 and 3, respectively (Fig. 23b). The weight losses in the controls were somewhat lower than the previous experiments (14 to 16% loss), with values varying between 10 to 13% over the three-week fermentation. Since the reducing sugar and weight loss results for the three experiments were comparable, lowering the temperature (from 37 to 30°C) and the absence of nitrogen sources in the rinsing/inoculation solution did not seem to adversely affect the suspended-culture fermentation of corn fiber with *G. trabeum*.

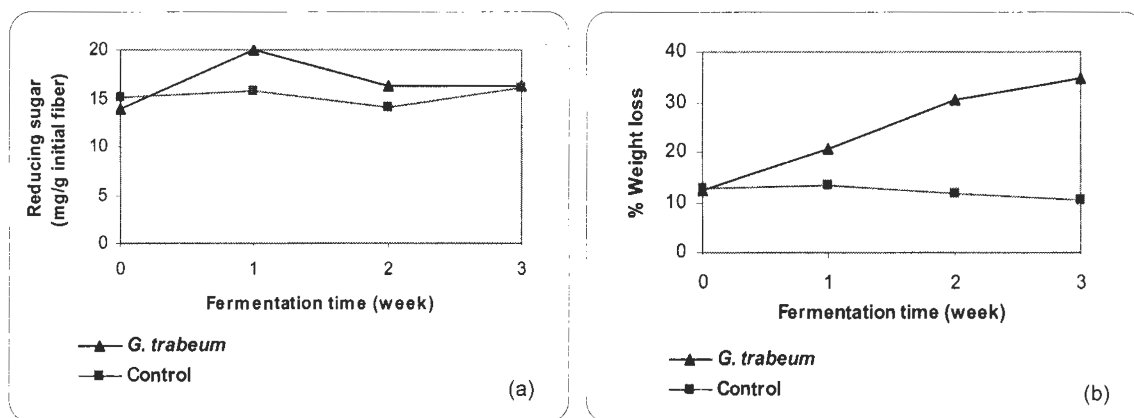

Fig. 23. (a) Reducing sugar content and (b) weight loss in slurries with 5 g corn fiber, 10 ml *G. trabeum* inoculum, and 100 ml basal salts solution (containing in 1 L water: 0.25 g KH_2PO_4, 0.063 g $MgSO_4$, 0.013 g $CaCl_2$, and 1.25 ml trace elements solution). No fungal inoculum was added to the controls. Corn fiber slurries were prepared in 250-ml flasks with blue autoclave wrap and aluminum foil as cover, and incubated at 30°C with shaking at 150 rpm. The flasks were harvested by steaming for 1 h and filtration. (n=1)

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3.2 Effect of sequential solid-state fermentation and anaerobic conditions (without addition of S. cerevisiae) on reducing sugar content and biomass loss

Six SSF experiments were conducted using *G. trabeum* and corn fiber. The first experiment was exploratory work to determine an appropriate length and sampling frequency for subsequent SSF experiments. The second, third and fourth experiments included buffered anaerobic incubation following SSF to enable the enzymes released by *G. trabeum* to hydrolyze cellulose and oligosaccharides to simple sugars with minimal or no fungal consumption. The fifth and sixth experiments (as discussed in sections 3.4 and 3.5) evaluated the sequential SSF of corn fiber by *G. trabeum* and anaerobic fermentation by *S. cerevisiae* of the fermentable sugars released.

The SSF of corn fiber in the first experiment was conducted over three weeks, with bottles harvested on a weekly basis. Significant growth of *G. trabeum* was observed as a mat on the surface of the corn fiber by the fifth day of SSF. The reducing sugar content of filtrates from the bottles fluctuated between 10 and 14 mg/g initial fiber for the cultures, and were slightly lower for the controls, at an average of 9 mg/g initial fiber (Fig. 24a). The biomass loss increased significantly from 11 to 35% of initial fiber by week 1 in the culture bottles, and then seemed to plateau, indicating minimal fungal activity from weeks 1 to 3 (Fig. 24b). The harvested control bottles had weight losses of 11% on average.

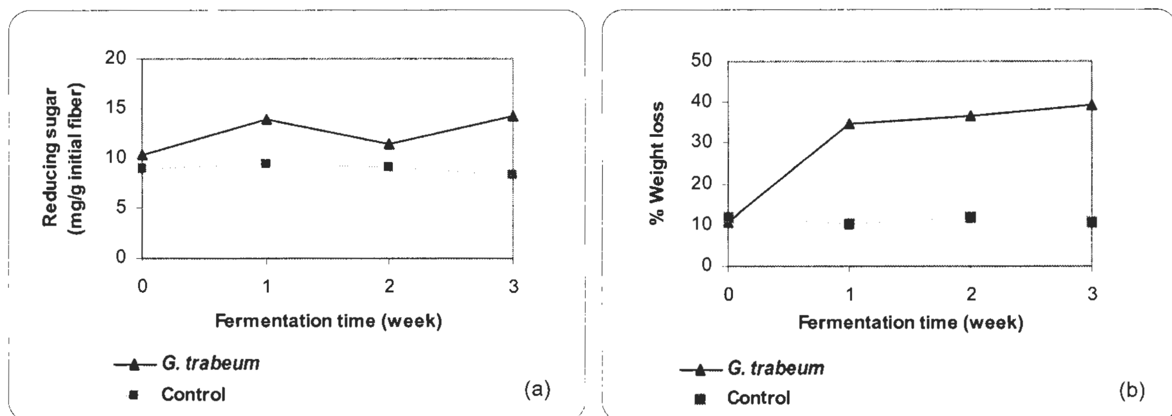

Fig. 24. (a) Reducing sugar content and (b) weight loss in SSF bottles. SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. No fungal inoculum was added to the controls. The bottles were harvested by adding 300 ml deionized water, steaming for 1 h and filtration. (n=1)

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Based on the preliminary results from the first experiment, the length of SSF was shortened in the next two experiments to less than two weeks with more frequent sampling. One set of SSF control and culture bottles was harvested by adding 300 ml deionized water, steaming for 1 h and filtration. Non-sterile acetate buffer (300 ml), pH 4.7 to 4.9, was added to another set of control and culture bottles after SSF, which were then capped tightly and incubated anaerobically for 48 h at 37°C (without shaking). The bottles were steamed for 1 h, and the liquid was collected by filtration.

The harvested reducing sugar content of culture bottles (*G. trabeum*), without buffered anaerobic post-treatment, reached an average peak of 18 mg/g initial fiber after 7 d of SSF (Fig. 25). The additional treatment of buffering and incubating anaerobically before steaming increased the reducing sugar content of culture bottles to as high as 72 mg/g initial fiber on day 3, and to 48 and 43 mg/g initial fiber in the 5-d and 7-d bottles, respectively. The lower levels of reducing sugars in SSF culture bottles with buffered anaerobic post-treatment harvested after day 7 in the second experiment (Fig. 25a) and day 3 in the third experiment (Fig. 25b) may indicate a reduction in the enzyme activity or the quantity of enzymes due to secretion of proteolytic enzymes by *G. trabeum*. The quantity of reducing sugar in control bottles harvested after SSF alone was 8 mg/g initial fiber, and was somewhat lower following anaerobic post-treatment (6 mg/g initial fiber). The lower reducing sugar contents in controls with anaerobic post-treatment may indicate some contamination since the buffer added and sampling were non-sterile in these experiments.

SSF with *G. trabeum* followed by addition of acetate buffer and anaerobic incubation converted up to 7.2% of the biomass into harvestable reducing sugars, with the remainder converted presumably to nonfermentable sugars (oligosaccharides, disaccharides), carbon dioxide, water, and additional fungal biomass. The significant increase in reducing sugars after post-culture buffering and anaerobic incubation indicates that better harvesting methodology, in addition to steaming, could prevent consumption of sugars by the fungus. Furthermore, the phenol-sulfuric assay (total sugars) of the filtrates shows that substantially more sugars are present than reducing sugars alone. The trend is similar for control and culture bottles indicating that steaming releases oligosaccharides into solution. For instance, the fiber cultured by *G. trabeum* for 7 d, then buffered for 48 h and steamed,

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had a total sugar content of 110 mg/g initial fiber (Fig. 25c). The corresponding reducing sugar content was 55 mg/g initial fiber (Fig. 25a). It may therefore be possible to have a larger fraction of the fiber mass loss converted into fermentable sugar under optimal conditions.

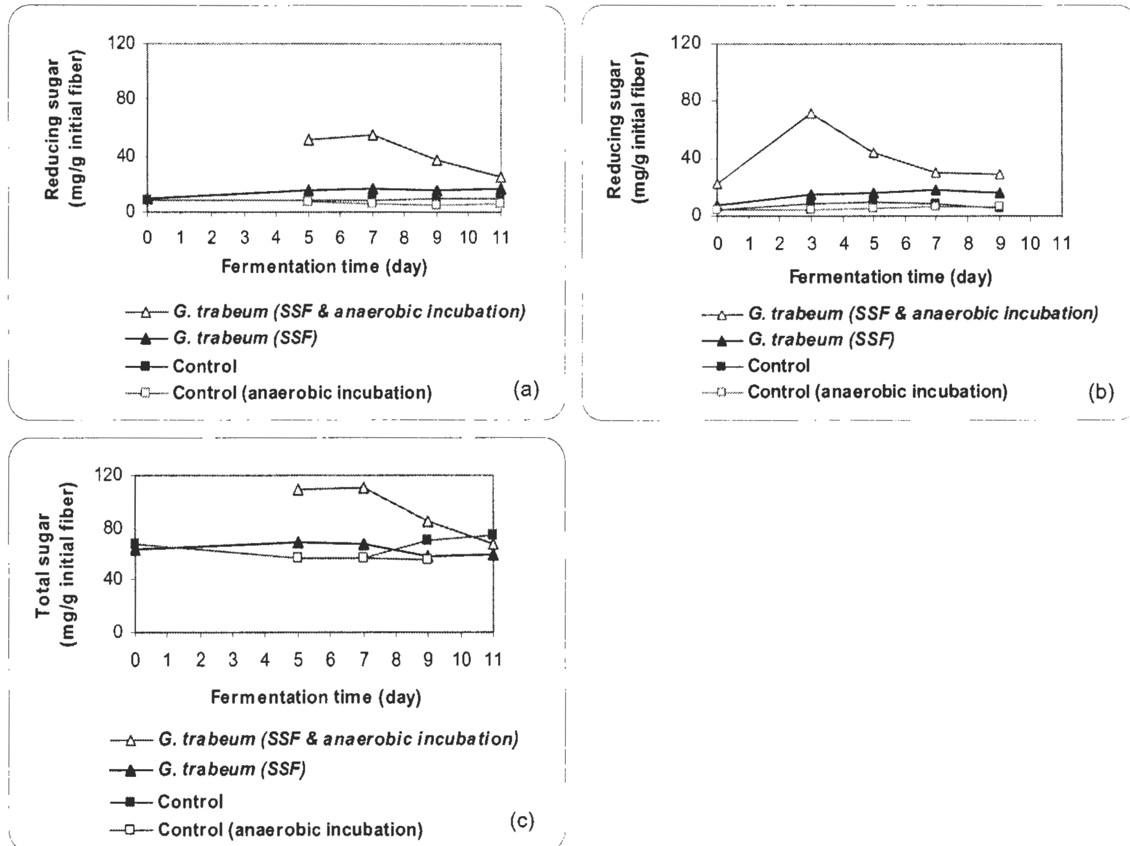

Fig. 25. Reducing sugar content in bottles from the (a) second and (b) third SSF experiments (n=2), and the (c) total sugar content from the second experiment (n=1). SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. No fungal inoculum was added to the controls. One set of control and culture bottles was harvested by adding 300 ml deionized water, steaming for 1 h and filtration. Non-sterile acetate buffer (300 ml), (a) pH 4.7 and (b) pH 4.9, was added to another set of SSF control and culture bottles, which were incubated anaerobically for 48 h at 37°C without shaking. This set of bottles was then harvested by steaming for 1 h and filtration.

SSF of corn fiber with *G. trabeum*, and steaming for 1 h with 300 ml deionized water, reduced the biomass by an average of 26 and 36% of initial fiber in the 5-d and 9-d bottles, respectively (Fig. 26). The set of harvested culture bottles with buffered anaerobic post-treatment had higher biomass

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losses of 33% by day 5 and 40% by day 9. The results of Fig. 25 and 26 confirm cellulase activity. Biomass losses in the controls, with and without buffered anaerobic post-treatment, averaged 10%.

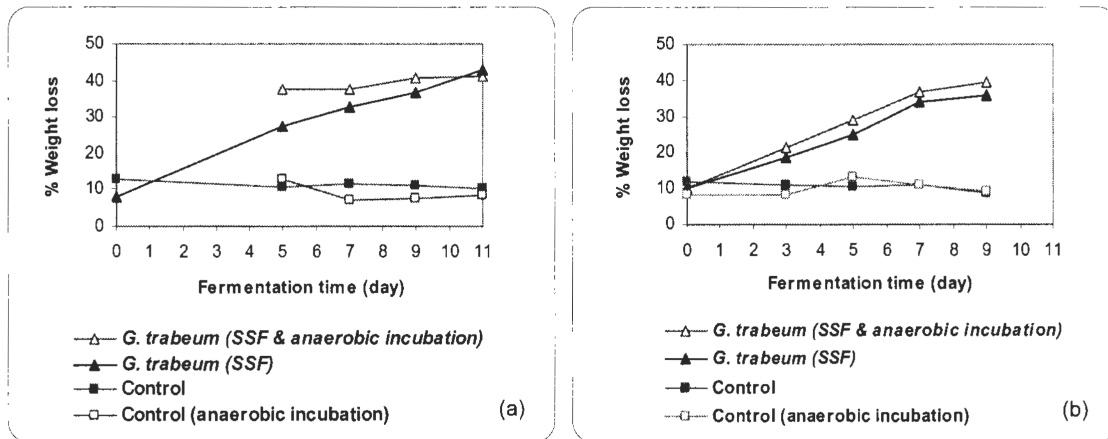

Fig. 26. Weight loss in SSF bottles from two experiments, (a) and (b), as a percent of initial corn fiber added. SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. No fungal inoculum was added to the controls. One set of SSF control and culture bottles was harvested by adding 300 ml deionized water, steaming for 1 h and filtration. Non-sterile acetate buffer (300 ml), (a) pH 4.7 and (b) pH 4.9, was added to another set of SSF control and culture bottles, which were incubated anaerobically for 48 h at 37°C without shaking. This set of bottles was then harvested by steaming for 1 h and filtration. (n=2)

The fourth SSF experiment differed mainly in the basal salts solution used for rinsing and inoculation, which included an inorganic nitrogen source (Fig. 27). The solution for the first three SSF experiments included 0.25 g/L KH_2PO_4, 0.063 mg/L $MgSO_4$, 0.013 g/L $CaCl_2$, and 1.25 ml trace elements solution (as described in section 2.3); no nitrogen source was added. This experiment was conducted to check if available nitrogen or basal salts concentrations in the inoculum were limiting enzyme production.

As in the previous experiments, the 3-d SSF culture bottle with the additional treatment of buffering and incubating anaerobically (37°C for 48 h) exhibited the highest quantity of reducing sugar, 50 mg/g initial fiber in this experiment (Fig. 27a). The culture bottles had lower reducing sugar levels, with 39 and 29 mg/g initial fiber on days 5 and 7, respectively, as compared to 48 and 43 mg/g initial fiber in the previous SSF experiments. SSF of corn fiber with *G. trabeum*, after buffered anaerobic post-treatment, reduced the biomass by 20% in 3 d and 33% in 5 d (Fig. 27b). The

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corresponding losses in the preceding experiments were comparable (21 and 33%). The reducing sugar content and biomass loss in the control bottles from this experiment, with and without anaerobic buffering, were slightly lower than in previous SSF experiments, with 5 mg reducing sugars/g initial fiber and 6% weight reduction, respectively.

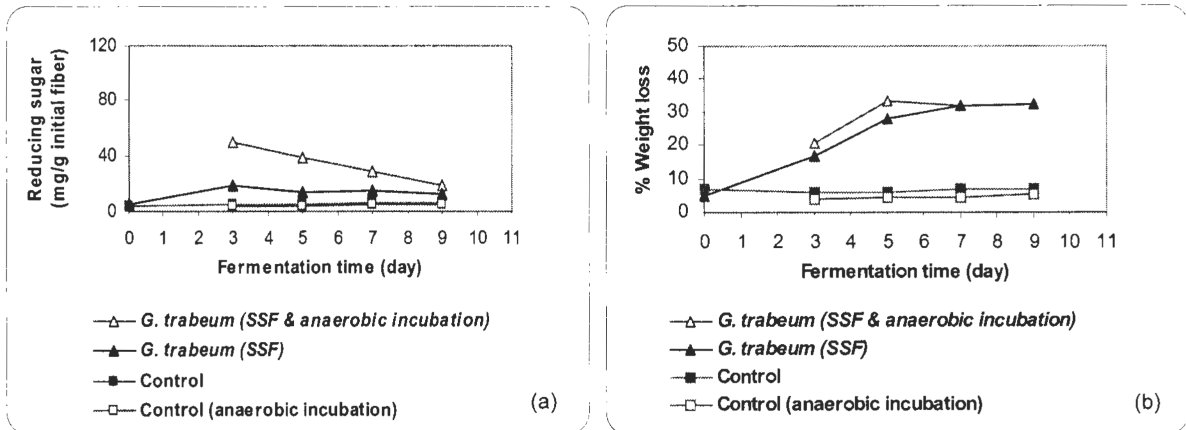

Fig. 27. (a) Reducing sugar and (b) weight loss in bottles from the fourth SSF experiment. The basal salts solution used for inoculation contained in 1 L water: 1.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g $(NH_4)_2SO_4$, 0.1 g $CaCl_2$, and 1.25 ml trace elements solution (Wang and Gao, 2003). SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. No fungal inoculum was added to the controls. One set of control and culture bottles was harvested by adding 300 ml deionized water, steaming for 1 h and filtration. Non-sterile acetate buffer (300 ml), pH 4.8, was added to another set of SSF control and culture bottles, which were incubated anaerobically for 48 h at 37 °C without shaking. This set of bottles was then harvested by steaming for 1 h and filtration. (n=1)

SSF with *G. trabeum* converted up to 5.0% of the biomass into harvestable reducing sugars in the fourth experiment, as compared to a maximum of 7.2% conversion in the previous SSF experiments. The added nitrogen source and higher basal salts concentration in the fourth experiment did not improve the harvestable reducing sugar yields; therefore, the composition of the basal salts solution from the first three SSF experiments (given in section 2.3) was used in subsequent fermentations. The length of anaerobic incubation was also extended in the sequential SSF and anaerobic fermentation experiments because the reducing sugar levels in the 3-d SSF culture samples with buffered anaerobic incubation did not reach a plateau in 48 h, as indicated in Fig. 28.

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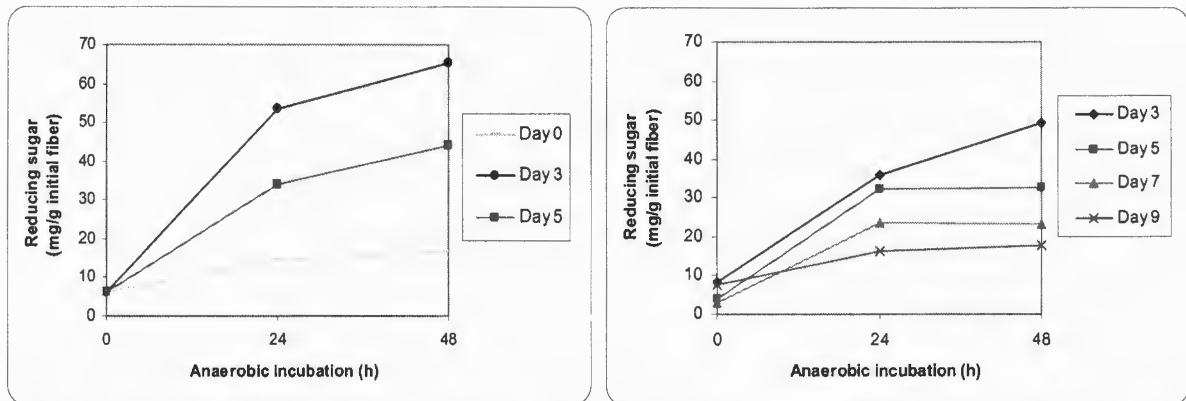

Fig. 28. Reducing sugar content in samples from 0, 3, 5, 7 and 9-d SSF bottles during the buffered anaerobic incubation step of two experiments. SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. Non-sterile acetate buffer (300 ml), pH 4.8, was added to this set of SSF culture bottles, which were incubated anaerobically for 48 h at 37°C without shaking. (n=2)

3.3. Visual observations of fungal growth during solid-state fermentation with G. trabeum

Visual observations of the inside of SSF bottles indicated that some growth was evident after three days of fermentation of the corn fiber by *G. trabeum*, with short hyphae extending from the hyphal spheres used as inoculum (Fig. 29a). Significant growth of *G. trabeum* was observed as a mat on the surface of the corn fiber on day 5 (Fig. 29b) and day 9 (Fig. 29c) after inoculation. Visual observations were important for determining the length of SSF before anaerobic fermentation in the experiments for ethanol production.

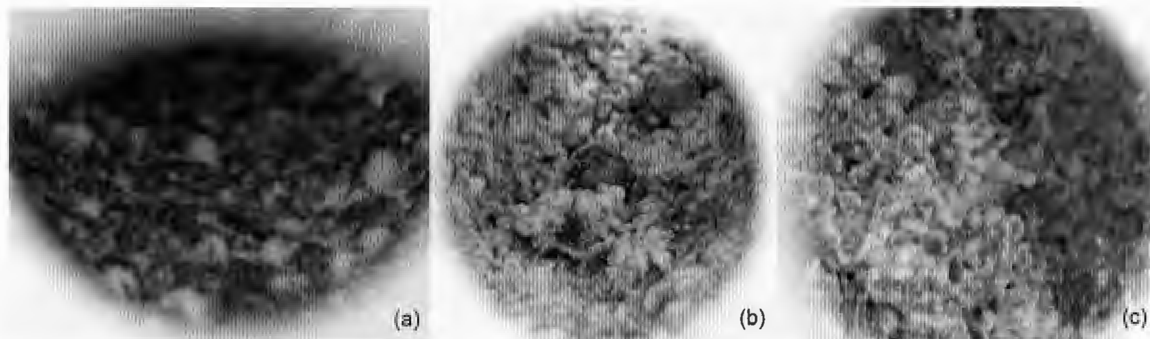

Fig. 29. Views in SSF bottles on (a) day 3, (b) day 5, and (c) day 9.

3.4. Effect of sequential solid-state fermentation by G. trabeum and anaerobic fermentation with S. cerevisiae on reducing sugar content

Based on the reducing sugar results from experiments with SSF followed by buffered anaerobic incubation, a 3-day SSF with *G. trabeum* was selected for subsequent fermentations. In previous experiments, bottles were harvested only after three or more days of SSF. Therefore, anaerobic fermentation was also performed on SSF bottles two days after inoculation with *G. trabeum* to determine if two or three days is preferable for reducing sugar and ethanol production. Only 5 to 10 hyphal spheres with extending mycelia were evident after two days of SSF, significantly fewer hyphae than were present in the bottle after three days (Fig. 29a). Two sequential SSF and anaerobic fermentation experiments were performed with three sets of bottles each. The sets differed based on the solution – acetate buffer, glucose-free nutrient solution, or acetate buffer containing glucose-free nutrients – added prior to anaerobic fermentation by *S. cerevisiae*. The sampling period in the second experiment was extended until the daily ethanol contents of samples from the bottles under anaerobic conditions had reached a plateau (i.e., from 144 h to 288 h).

3.4.1. SSF, addition of acetate buffer, and anaerobic conditions

Acetate buffer was added to one set of control and culture bottles after two and three days of SSF. This set of bottles was used as a control to determine the levels of reducing sugars achievable under more optimal pH and temperature conditions for the enzymatic hydrolysis in solution. Two buffers with different pH values, 4.6 and 4.8, were used to check the effect of pH on the results. Samples were taken on a 24-h basis and analyzed for reducing sugar and ethanol content. No yeast was added to this set of bottles.

The trends for the reducing sugar contents in the 2-d and 3-d culture bottles differed in the first experiment, but were similar in the second experiment (Fig. 30a and 30b). The reducing sugar content of the 2-d culture bottle increased almost linearly in the first experiment over the 144-h sampling period to a maximum value of 52 mg/g initial fiber. However, in the second experiment, the reducing sugar trend in the 2-d culture bottle increased significantly to 91 mg/g initial fiber at 96 h, and then seemed to plateau. The 3-d culture bottles had increasing reducing sugar contents, with an

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average of 71 mg/g initial fiber after 48 h of incubation. As shown in Fig. 30a, the 3-d culture bottle containing buffer of pH 4.6 had considerably more reducing sugars than the buffer of pH 4.8, with 84 mg/g initial fiber at 48 h and 108 mg/g initial fiber at 144 h (Fig. 30a). Therefore, maintaining an optimal pH appears to be very important for the hydrolysis of corn fiber to reducing sugars. The 2-d and 3-d control bottles demonstrated relatively steady concentrations of reducing sugars, averaging 6 mg/g initial fiber over the 6 to 12-d sampling period.

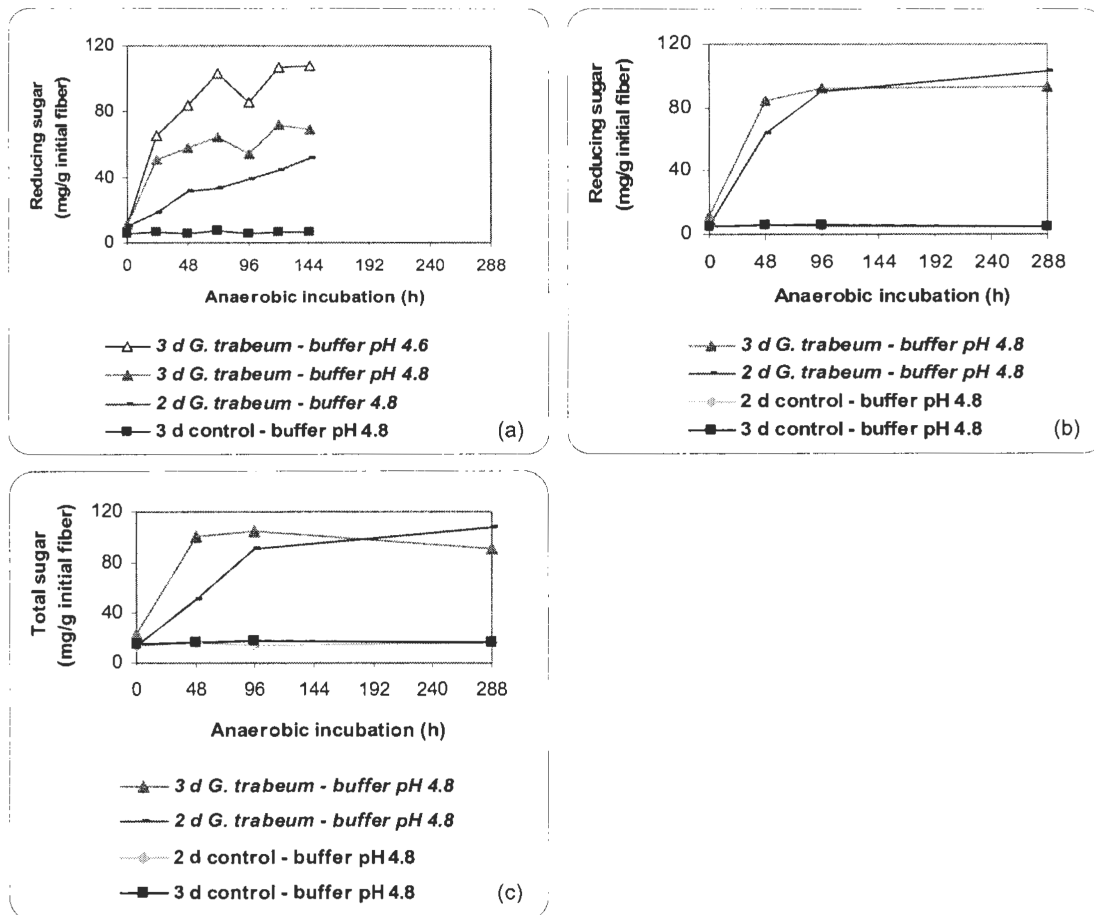

Fig. 30. Reducing sugar content in bottles from the (a) first and (b) second SSF experiments (n=2) with anaerobic fermentation, and (c) total sugar content from the second experiment (n=1). SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. No fungal inoculum was added to the controls. Acetate buffer (300 ml) was added to this set of SSF bottles, which were incubated anaerobically for 48 h at 37°C without shaking (no yeast added).

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The total and reducing sugar values followed similar trends in the second experiment (Fig. 30b and 30c); the 2-d and 3-d total sugar levels in the controls were on average 11 mg/g initial fiber higher than the reducing sugar content. The total sugar content for 3-d culture bottles was about 14 mg/g initial fiber higher than the reducing sugar, whereas the total and reducing sugar levels were the same, on average, for the 2-d culture bottle. This suggests that a higher proportion of the total sugars released in the 2-d culture bottles were reducing sugars, as compared to the 3-d culture bottles. Overall, more total and reducing sugars were present in the 3-d culture bottles up to 144 h.

3.4.2. SSF, addition of yeast nutrients, and anaerobic fermentation

The second set of bottles included control and culture bottles from the SSF step, and the addition of 300 ml glucose-free yeast nutrients (procedure and contents provided in section 2.7). A subset of these bottles were inoculated with yeast (~5.6×10^8 *S. cerevisiae* cells per bottle).

The reducing sugar levels in the 2-d and 3-d culture bottles with yeast nutrients and yeast were relatively steady, averaging 11 mg/g initial fiber and 15 mg/g initial fiber, respectively (Fig. 31a and 31b). The relatively steady levels of reducing sugars in bottles containing yeast suggest that the remaining reducing sugars were nonfermentable. The 2-d culture sample without yeast, used in the second experiment only, demonstrated an increase in reducing sugar values from 6 to 40 mg/g initial fiber during the 288-h sampling period without reaching a plateau. The 3-d culture sample without yeast seemed to reach a plateau after 48 h with an average reducing sugar value of 40 mg/g initial fiber. These reducing sugar values were lower than those obtained with the addition of acetate buffer, in part because ethanol was produced under anaerobic conditions in SSF culture bottles even without the addition of yeast (as reported in the section 3.5). The reducing sugar contents of SSF control samples under anaerobic conditions were similar with and without the addition of yeast, averaging 6 mg/g initial fiber (Fig. 32a and 32b).

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The difference between total and reducing sugar levels (Fig. 31b, 31c, 32b and 32c) was slightly higher for the culture and control bottles with yeast versus those without yeast; the average difference was 16 and 14 mg/g initial fiber for the SSF bottles with and without the addition of yeast, respectively.

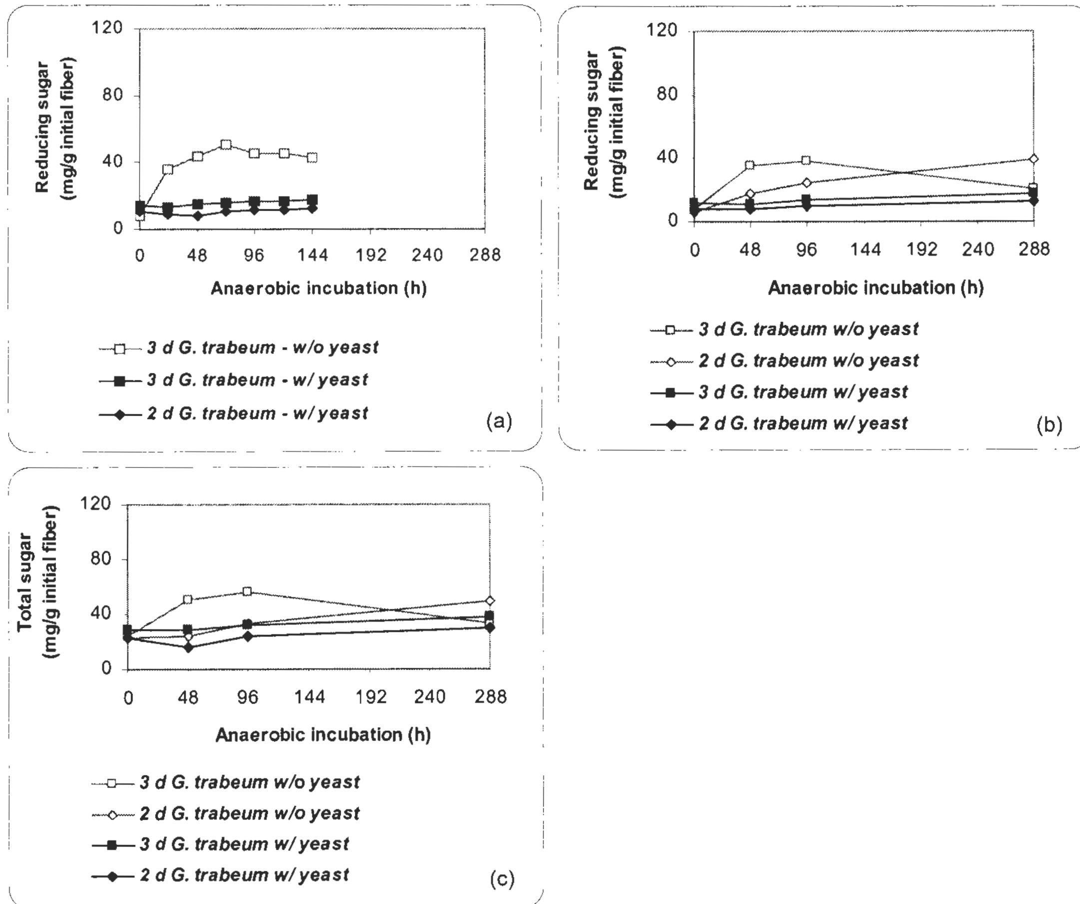

Fig. 31. Reducing sugar content in SSF bottles cultured with *G. trabeum* during the (a) first and (b) second SSF and anaerobic fermentation experiments (n=2), and (c) total sugar content from the second experiment (n=1). SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and was incubated at 30°C without shaking. Glucose-free yeast nutrient solution (300 ml) and one vial of yeast ($5.6x10^8$ cells) were added to this set of bottles, which were incubated anaerobically for 48 h at 37°C without shaking; a subset of the bottles had no yeast added.

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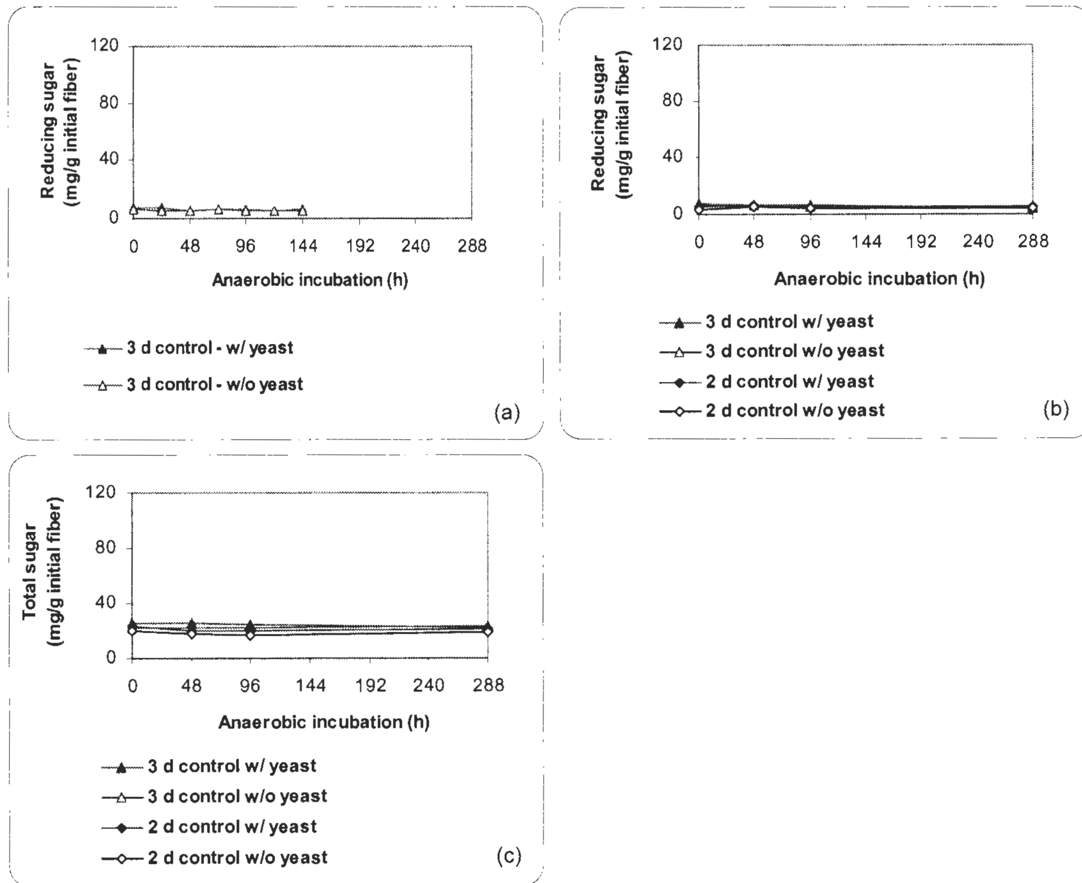

Fig. 32. Reducing sugar content in control bottles (*no G. trabeum*) from SSF in the (a) first and (b) second experiments (n=2) with SSF and anaerobic fermentation, and (c) total sugar content from the second experiment (n=1). SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. No fungal inoculum was added to the controls. Glucose-free yeast nutrient solution (300 ml) and one vial of yeast (5.6×10^8 cells) were added to this set of bottles, which were incubated anaerobically for 48 h at 37°C without shaking; a subset of the bottles had no yeast added.

3.4.3. SSF, addition of acetate buffer with yeast nutrients, and anaerobic fermentation

A third set of bottles included SSF control and culture bottles and the addition of acetate buffer containing glucose-free yeast nutrients. This set was added in the second experiment to determine whether the absence of yeast nutrients prevented ethanol production by *G. trabeum* in bottles with acetate buffer and without yeast. The resulting trends were similar to the bottles with yeast nutrients in water. The 2-d culture bottle without yeast demonstrated an increasing trend in reducing sugar contents and did not reach a plateau in 288 h; the maximum was 83 mg reducing sugars/g initial fiber (Fig. 33a). The 3-d culture bottle without yeast began to reach a plateau at 48 h

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with a reducing sugar content of 75 mg/g initial fiber. The 2-d and 3-d culture samples with yeast averaged 13 and 19 mg/g initial fiber, with a slight increase from 0 to 96 h. The relatively steady levels in culture samples with yeast may indicate that the remaining reducing sugars were nonfermentable. The control bottles with and without yeast were fairly steady with an average value of 6 mg/g initial fiber. The total sugar contents (Fig. 33b) for the SSF bottles with and without yeast were higher than the reducing sugars by 18 mg/g initial fiber for the controls, 17 mg/g initial fiber for the 3-d cultures, and 9 mg/g initial fiber for the 2-d cultures.

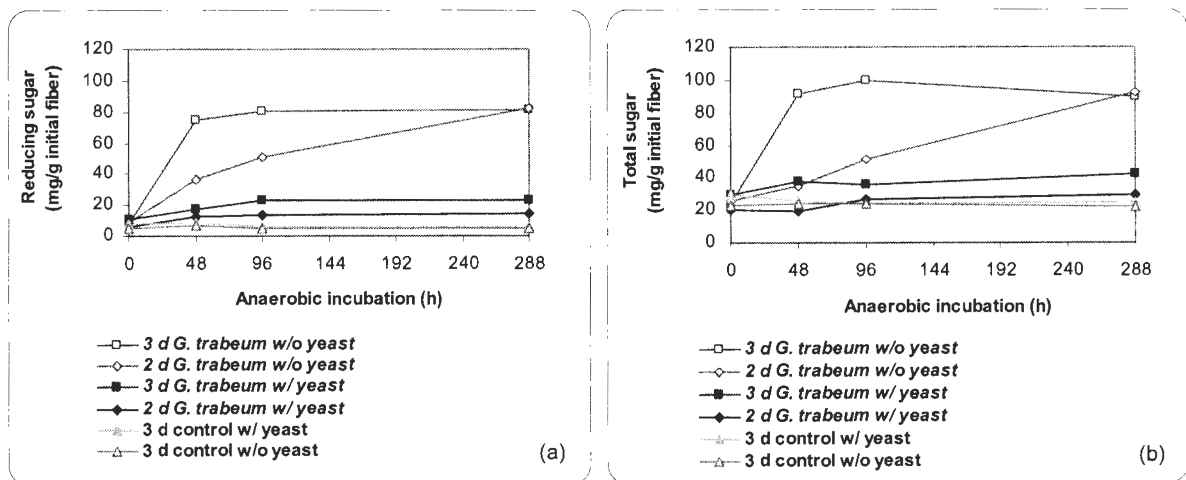

Fig. 33. (a) Reducing sugar and (b) total sugar contents in SSF control and culture bottles during the second SSF experiment with anaerobic fermentation. SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. No fungal inoculum was added to the controls. Acetate buffer containing glucose-free yeast nutrients (300 ml) and one vial of yeast ($5.6x10^8$ cells) were added to this set of bottles, which were incubated anaerobically for 48 h at 37°C without shaking; a subset of the bottles had no yeast added. (n=1)

3.5. Effect of sequential solid-state fermentation by G. trabeum and anaerobic fermentation with S. cerevisiae on ethanol production

Ethanol was detected in the SSF culture bottles containing glucose-free yeast nutrients, with and without yeast (Fig. 34). The 3-d culture bottle with yeast was the first to plateau at about 72 h with an average conversion of 2.8% of the initial fiber to ethanol. The 2-d culture with yeast had ethanol values exceeding the 3-d culture samples with yeast after 96 h; it seemed to plateau around 192 h at 3.5% of initial fiber (3.7% at 288 h). The 2-d and 3-d culture samples with yeast from 144 h

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were checked by GC-MS-O, and had higher conversion values of 6 and 5% of initial fiber to ethanol, respectively. The 3-d culture bottle without yeast had varying trends between the two experiments. In the first experiment, it demonstrated a somewhat linear increase in ethanol with a conversion of about 2% of initial fiber after 144 h, as measured by both HPLC and GC-MS-O (Fig. 34a). The trend was similar, though somewhat lower, to the 2-d culture sample with yeast in the second experiment (Fig. 34b); the 192-h sample had a 3.2% bioconversion of corn fiber to ethanol (3.4% at 288 h). The ethanol in the 2-d culture sample without yeast, only used in the second experiment, seemed to increase slowly and almost linearly over the 288-h period to 1.5% of initial fiber.

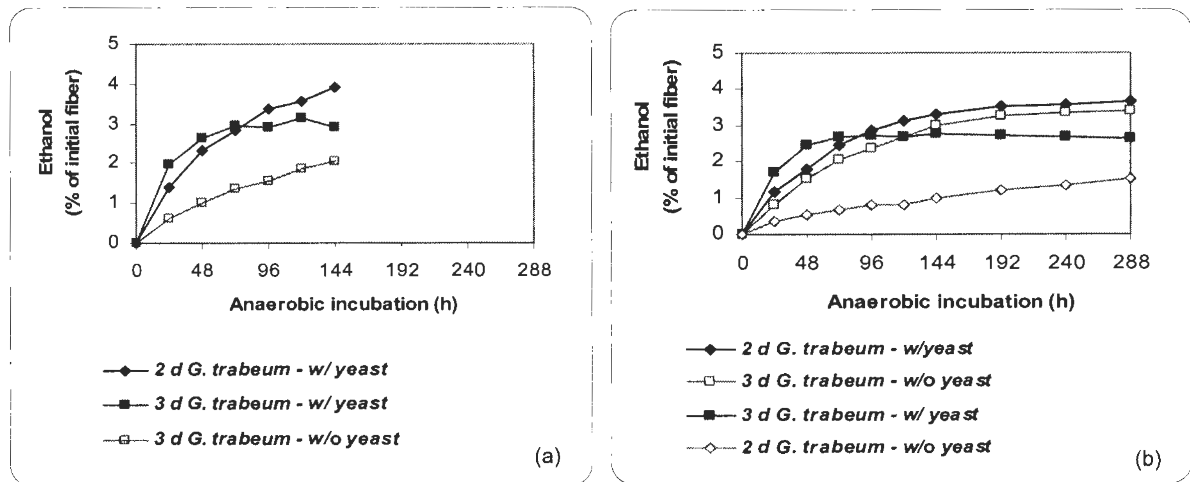

Fig. 34. Ethanol production during anaerobic fermentation of SSF culture bottles in the (a) first and (b) second SSF and anaerobic fermentation experiments. SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. Glucose-free yeast nutrient solution (300 ml) and one vial of yeast (5.6×10^8 cells) were added to this set of bottles, which were incubated anaerobically for 48 h at 37°C without shaking; a subset of the bottles had no yeast added. (n=2)

The production of ethanol in bottles without yeast added suggests that *G. trabeum* is capable of producing ethanol during anaerobic fermentation or stressed conditions (up to 3.4% conversion of initial fiber to ethanol). The photo in Fig. 35 shows the bubbles produced by the yeast during anaerobic fermentation and floating fiber in 3-d SSF bottles with and without yeast.

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Fig. 35. Photo of 3-d *G. trabeum* SSF bottles with and without *S. cerevisiae* after 48 h of anaerobic incubation. The bottle on the left had no yeast; the bottle on the right had yeast. Fiber was floating in both; however, bubbles (within floating fiber) were visible in the bottle with yeast only.

No ethanol was detected in most samples with only acetate buffer added, as expected. A small quantity of ethanol (0.2% conversion of initial fiber) was detected in 48-h and 120-h samples from the 3-d SSF culture bottle with acetate buffer (without yeast) in the second experiment. The culture bottles with acetate buffer, glucose-free yeast nutrients and yeast produced ethanol, with trends similar to the bottles with water instead of buffer. The set of bottles with yeast nutrients and acetate buffer, however, had no ethanol production in the 2-d culture sample without yeast, and the 3-d culture sample without yeast produced significantly less ethanol around 0.3% of initial fiber (Fig. 36). The 3-d culture sample with yeast reached a plateau at 72 h with an ethanol content of 3.4% of initial fiber (somewhat higher than the similar sample with yeast nutrients in water, 2.8%). The 2-d culture sample with yeast seemed to reach a plateau around 240 h, with a conversion of 4.0% by 288 h; this was the highest conversion of corn fiber to ethanol obtained in the research to date.

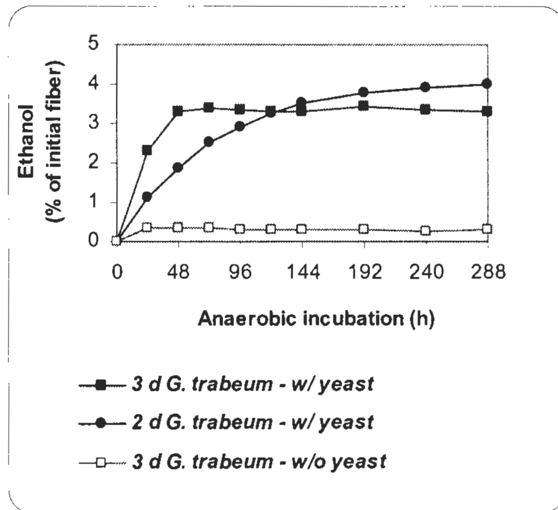

Fig. 36. Ethanol production during anaerobic fermentation of SSF culture bottles in the second SSF and anaerobic fermentation experiment. SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. No fungal inoculum was added to the controls. Acetate buffer containing glucose-free yeast nutrients (300 ml) and one vial of yeast ($5.6x10^8$ cells) were added to this set of bottles, which were incubated anaerobically for 48 h at 37°C without shaking; a subset of the bottles had no yeast added. (n=1)

The activity of the yeast was checked by adding ~$5.6x10^8$ yeast cells aseptically to a 1-L polypropylene bottle with 300 ml of yeast nutrients and glucose (1.2 g); no corn fiber was added. The bottle was capped loosely and incubated at 30°C. The ethanol content reached a plateau in 24 h, with an increase from 0 g on the day of inoculation to 0.53 g ethanol. No ethanol was detected when the yeast inoculum was added to the glucose-free yeast nutrients using a similar procedure.

3.6. Estimation of the reducing sugar production during sequential solid-state fermentation and anaerobic fermentation

The maximum theoretical yield of 0.51 g ethanol from 1 g glucose was used to calculate the fermentable sugars (Brown, 2003). This is a conservative conversion factor for estimating the quantity of fermentable sugars released by the extracellular enzymes, since typically 5-12% of the carbohydrate is converted to cell biomass. The plot of total (fermentable and nonfermentable) reducing sugars indicates similar trends and values for 3-d SSF culture bottles (*G. trabeum*), with and without fermentation by yeast (Fig. 37). These trends suggests that the fermentation of more sugars by yeast does not increase the release of reducing sugars, as compared to the SSF culture bottle

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with *G. trabeum* and without yeast. Without the yeast, however, not all the fermentable sugars were fermented to ethanol. The total reducing sugar trend for 2-d SSF with *G. trabeum* and fermentation by yeast was similar to the 3-d samples but did not reach a plateau (Fig. 37).

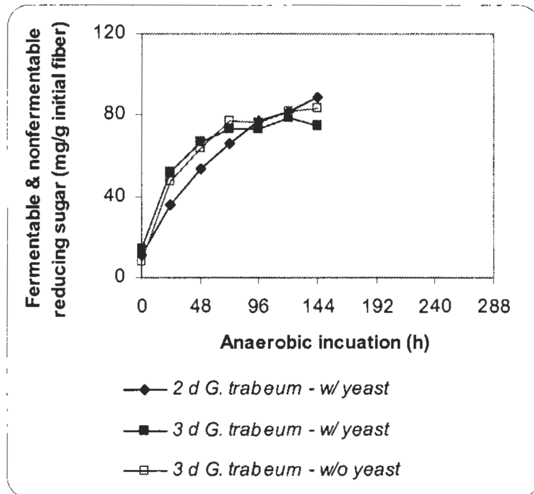

Fig. 37. The estimated total reducing sugar content (fermentable and nonfermentable) in SSF bottles cultured with *G. trabeum* during the first experiment, after considering the quantity of sugars converted to ethanol. SSF was conducted in 1-L polypropylene bottles with 25 g corn fiber, 75 ml *G. trabeum* inoculum, and blue autoclave wrap as cover, and incubated at 30°C without shaking. No fungal inoculum was added to the controls. Glucose-free yeast nutrient solution (300 ml) and one vial of yeast (~5.6×10^8 cells) were added to this set of bottles, which were incubated anaerobically for 48 h at 37°C without shaking; a subset of the bottles had no yeast added. (n=1)

3.7. Effect of sequential solid-state fermentation and anaerobic fermentation on culture broth pH

The pH measurement was taken for each culture broth during the anaerobic fermentation step in the second SSF experiment to determine if the pH was correlated with a plateau in ethanol production. The initial pH of the acetate buffer before addition to one set of bottles was 4.8. The pH values for SSF control and culture samples with acetate buffer during anaerobic conditions (without yeast) fluctuated between 4.6 and 4.8.

The pH of SSF culture samples with glucose-free yeast nutrients (initial pH of 5.3) added, with and without yeast, varied between pH 4.1 and 4.4. Samples from the control bottles with yeast nutrients and without yeast had pH values of 4.0 to 4.1. The pH of samples from control bottles with yeast nutrients and yeast had the only increasing pH values, from about 4.0 to 4.5 (Fig. 38). The pH values of control and culture bottles with acetate buffer containing yeast nutrients varied the least,

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between 4.6 and 4.7. The pH results indicate that ethanol production did not reach a plateau due to a change in pH, since the pH of samples from bottles fermenting to ethanol were relatively steady over the fermentation period. However, as indicated in Table 2, the higher conversions of corn fiber to ethanol by yeast seem to correlate with the higher pH values and temperatures (samples with acetate buffer and yeast nutrients added) for the 48-h SSF culture samples.

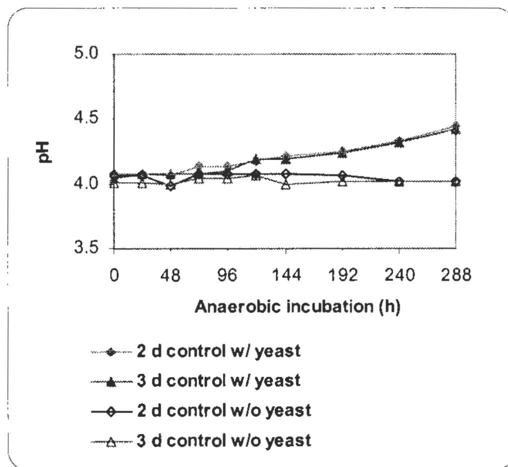

Fig. 38. The pH of samples from control bottles during the SSF step and with glucose-free yeast nutrients added before anaerobic incubation with and without yeast. (n=1)

Table 2
Summary of results for the 48-h samples from 2-d and 3-d SSF cultures

2 d SSF with G. trabeum* (48 h sample)	Yeast	Reducing sugar (mg/g initial fiber)	Ethanol (% of initial fiber)	Temp. (°C)	pH (0 h,	48 h)
Acetate buffer	No	48	0	37	4.7	4.6
Glucose-free yeast nutrients	No	17	0.5	30	4.2	4.1
	Yes	8	1.4	30	4.3	4.3
Acetate buffer containing glucose-free nutrients	No	37	0	37	4.7	4.6
	Yes	13	1.9	37	4.7	4.7

3 d SSF with *G. trabeum* (48 h sample)*	Yeast	Reducing sugar (mg/g initial fiber)	Ethanol (% of initial fiber)	Temp. (°C)	pH (0 h,	48 h)
Acetate buffer	No	71	0.1	37	4.8	4.7
Glucose-free yeast nutrients	No	40	1.2	30	4.3	4.2
	Yes	13	2.6	30	4.4	4.3
Acetate buffer containing glucose-free nutrients	No	74	0.3	37	4.8	4.7
	Yes	18	3.3	37	4.7	4.7

*Results of two experiments (n=2), except for acetate buffer containing glucose-free nutrients and pH measurements (n=1).

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4. Conclusions

The suspended-culture experiments conducted in this study for the fermentation of corn fiber by *G. trabeum* were not effective for harvesting the simple sugars released by hydrolytic enzymes. The harvested control and culture samples had similar reducing sugar contents, despite the loss in cultured biomass of 35% after three weeks of fermentation; this trend indicates that reducing sugars released by the fungal extracellular enzymes were consumed by the fungus. Saccharification of the corn fiber to oligosaccharides also contributed to biomass loss.

Solid-state fermentation with *G. trabeum* for three days, followed by the addition of acetate buffer (pH 4.6) and anaerobic incubation at 37°C, converted up to 11% of the corn fiber into harvestable reducing sugars; the remainder was converted to nonfermentable sugars (oligosaccharides, disaccharides), carbon dioxide, water, and additional fungal biomass. Maintaining an optimal pH was found to be important for the hydrolysis of corn fiber to reducing sugars. The percent of the corn fiber hydrolyzed to reducing sugar was lower (7% conversion) with the addition of acetate buffer of pH 4.8 rather than pH 4.6.

The sugars released by *G. trabeum* were fermented to yield up to 4.0 g ethanol/100 g fiber with the addition of *S. cerevisiae*. The results of this study also indicated that *G. trabeum* was able to produce ethanol under anaerobic or stressed conditions, with glucose-free yeast nutrients as supplement (as high as 3.4 g ethanol/100 g fiber); this observation is a new finding for *G. trabeum* to the our knowledge. The simultaneous release of sugars by enzymes produced during SSF with *G. trabeum* and production of ethanol by *S. cerevisiae* can be improved by optimizing the anaerobic culture conditions, such as pH, moisture and temperature, and the quantity of fungal inoculum. Another opportunity for increasing ethanol yield is to utilize a strain of yeast or bacteria capable of fermenting both pentoses and hexoses simultaneously. The sequential SSF and buffered anaerobic fermentation procedure developed in this research can be used to screen different fungal strains with potentially higher cellulolytic efficiencies under these culture conditions and other lignocellulosic substrates. Further research is required to design pilot- and full-scale processes where large quantities of corn fiber are subjected to fungal saccharification and fermentation to ethanol.

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CHAPTER 4. GENERAL CONCLUSIONS

The cellulose and hemicellulose in corn fiber offers an abundant, readily-available feedstock for the production of more ethanol. Significant obstacles have hindered the commercialization of lignocellulose to fermentable sugars via concentrated and dilute acid and enzymatic hydrolysis (U.S. DOE EERE, 2006). Concentrated acid hydrolysis is appealing for the near theoretical yields of sugars released; however, chemical expenses, acid recovery and corrosiveness, and gypsum waste steam impede economical, full-scale development. Though dilute acid hydrolysis requires less acid, it is still not considered economically competitive due to similar expenses of chemicals, corrosion-resistant equipment, and gypsum waste disposal, and the potential production of fermentation inhibitors at the higher temperatures.

Enzymatic hydrolysis is considered the most economically promising approach (U.S. DOE EERE, 2006). The current challenges include the high concentration requirements and cost of cellulase enzymes, pretreatment of feedstock, and fermentation of both pentoses and hexoses. The alternative to purchasing enzymes is to culture microbes on the lignocellulosic feedstock directly, under submerged or solid-state conditions. The enzymatic and non-enzymatic systems of microorganisms, such as brown rot fungi, can therefore be utilized *in situ* without the need for more expensive substrates.

In this research, conducted using solid-state fermentation with *G. trabeum* for three days followed by buffered anaerobic incubation, a maximum of about 11% (108 mg/g initial fiber) of the corn fiber was converted into harvestable reducing sugars. Enzymes released by *G. trabeum* during SSF hydrolyzed corn fiber to simple sugars, which were fermented to produce up to 4 g ethanol/100 g fiber with the addition of *Saccharomyces cerevisiae*. The results of this research also demonstrated that *G. trabeum* produced ethanol under anaerobic or stressed conditions. Development of *in situ* enzyme production by *G. trabeum* encountered challenges, particularly the fungal consumption of the simple sugars released. However, the approach developed, including sequential SSF and anaerobic fermentation, has the potential to become a relatively inexpensive method of using corn fiber, a co-product of corn wet milling, as a feedstock for more ethanol production.

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