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Limiting Factors in the Simultaneous Saccharification and Fermentation Process for Conversion of Cellulosic Biomass to Fuel Ethanol

GEORGE P. PHILIPPIDIS* AND TAMMY K. SMITH

Alternative Fuels Division,
National Renewable Energy Laboratory (NREL),
1617 Cole Boulevard, Golden, CO 80401

ABSTRACT

The cellulosic fraction of biomass feedstocks can be converted to ethanol, a promising alternative fuel, using the simultaneous saccharification and fermentation (SSF) process. This process integrates the enzymatic hydrolysis of cellulose to glucose with the fermentation of glucose to ethanol. Its performance depends on the characteristics of the biomass, the quality of the cellulase enzyme complex, and the behavior of the fermentative organism. This study of cellulose conversion progress in batch mode indicates that during the early stage of SSF, cell growth is the rate-determining step in ethanol production. At later times, however, and for most of the duration of the SSF process, enzymatic hydrolysis becomes the limiting factor. Further experimental probing has shown that cellulose accessibility to the enzyme is the key cause of the decreased rate of cellulose hydrolysis. It is therefore concluded that accessibility needs to be enhanced to improve the productivity of the SSF process.

Index Entries: SSF; biomass conversion; ethanol production; SSF limitations.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Renewed interest has focused recently on producing alternative fuels from cellulosic biomass, which is abundantly available in the United States in the form of energy crops, agricultural and forestry residues, pulp and paper industry waste streams, and municipal solid waste. Fuel ethanol produced from cellulosic biomass has excellent properties for internal combustion engines, and is expected to mitigate global warming and urban air pollution and decrease the dependence of the US economy on foreign energy resources (*1*).

The cellulosic portion of biomass (typically about 40–45% w/w on a dry basis) can be efficiently and economically converted to ethanol using the SSF process (*2,3*). This process combines the enzymatic hydrolysis of polymeric cellulose to monomeric glucose with the fermentative conversion of glucose to ethanol in the same environment. The hydrolytic step is catalyzed by the synergistic action of three types of activities encompassed in an enzyme complex commonly referred to as cellulase (*4*):

1. Endoglucanases, which randomly attack cellulose chains to produce polysaccharides of shorter length;
2. Exoglucanases, which attach to the nonreducing ends of those shorter chains and remove cellobiose moieties; and
3. β-glucosidases, which hydrolyze cellobiose and other oligosaccharides to glucose.

In this work, we will be referring to the combined endo- and exoglucanase action as cellulase to distinguish it from the cellobiolytic action of β-glucosidase.

Although SSF is the front-running option for cellulose conversion, its performance needs to be optimized to minimize the cost of the ethanol produced. A mathematical model that conceptualizes the kinetics of SSF has been developed to design rationally an SSF optimization strategy (*5*). The model takes into consideration:

1. The quality of the cellulosic biomass;
2. The quality of the cellulase and β-glucosidase enzymes that synergistically convert cellulose to the dimer cellobiose and cellobiose to glucose, respectively;
3. The interaction between the enzymes and the substrate; and
4. The interaction between the enzymes and the fermentative microorganism.

Based on this analysis, the properties of the substrate, the enzymes, and the microorganism are considered the key factors in the SSF process that need to be manipulated to enhance its performance. Here, we present the results of an experimental study that explored the role of these

potentially limiting factors during the course of SSF conducted under realistic conditions and aimed at identifying the factor that controls the rate of ethanol production.

MATERIALS AND METHODS

Substrate

Hybrid poplar wood *Populus eugenii* DN34 was milled to particles that passed through a 9-mesh screen (smaller than 2 mm), pretreated with dilute sulfuric acid (0.73% w/w) at 160°C for 10 min, washed with water, and employed as substrate in the study at a concentration of 60 g/L (dry basis). The pretreatment, performed as reported by Torget et al. (*6*), is intended to hydrolyze and thus remove hemicellulose from the wood structure to render the substrate more accessible to enzymatic attack by cellulase. On a dry w/w basis, the pretreated substrate contained 56.4% cellulose, 4.8% hemicellulose, and 38.5% lignin.

Enzyme

Cellulolytic enzyme, synthesized by a *Trichoderma reesei* strain under the commercial name Laminex, was purchased from Genencor (San Francisco, CA). Its cellulase and β-glucosidase volumetric activities were found to be 84 IFPU/mL and 91 IU/mL, respectively, when measured according to the IUPAC methods (*7*). The Laminex enzyme complex was employed in all experiments at a concentration of 25 IFPU/g of cellulose.

SSF

The SSF experiments were performed with 60 g/L of pretreated wood in YP medium (10 g/L yeast extract, 20 g/L peptone) or corn steep liquor (CSL) (10 g/L) of initial pH 5.0 at 38°C in either 1.7-L BioFlo III fermenter vessels (New Brunswick Scientific, New Brunswick, NJ) operated anaerobically or 250-mL flasks equipped with stoppers and carbon dioxide traps to simulate anaerobic conditions. In all cases, agitation was maintained at 150 rpm. Inocula were prepared in the same media supplemented with 50 g/L glucose under aerobic conditions. The fermentative microorganism was *Saccharomyces cerevisiae* D_5A, a strain developed at NREL from bakers' yeast (*8*). A 10% v/v inoculum was used to initiate each SSF run.

Chemical Analysis

During the fermentation runs, samples were periodically removed from the vessels, filtered through 0.45-μm nylon filters, and analyzed by high-performance liquid chromatography (HPLC) for the presence of

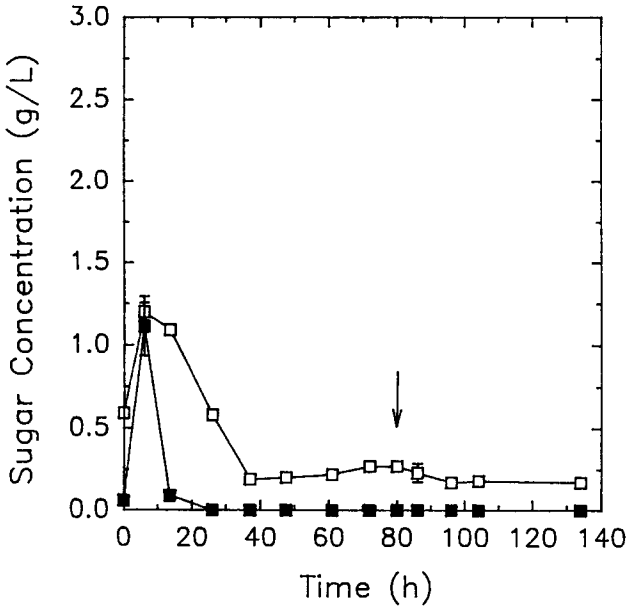

Fig. 1. Time-course of the residual cellobiose (■) and glucose (□) concentrations during the SSF of pretreated wood chips. The arrow indicates the time of spiking.

sugars (cellobiose, other oligosaccharides, and glucose) and metabolites, such as ethanol, organic acids, and glycerol. The HPLC unit 1090LC (Hewlett-Packard, Avondale, PA) was equipped with an organic acids column (HPX-87H, Bio-Rad, Richmond, CA), which was routinely used for measurement of all compounds except for oligosaccharides, which were monitored with a carbohydrate column (HPX-42A, Bio-Rad). The HPLC-based measurements of glucose and ethanol concentration were confirmed using a YSI glucose analyzer (YSI, Yellow Springs, OH) and a gas chromatograph (Model 5890, Hewlett-Packard), respectively.

RESULTS AND DISCUSSION

The SSF runs were performed in shake flasks and fermenters under identical conditions, and the concentrations of sugars and metabolites were monitored (Fig. 1). No differences in the SSF pattern were detected between flasks and vessels. Furthermore, the two employed media, YP and CSL, yielded identical results (data not shown). In all cases, glucose accumulated during the first 6–12 h of the process (Fig. 1). This indicates that the cells could not consume glucose at the rate it was released by the enzymes during the early phase of SSF. A buildup in glucose concentration is expected to have a strong inhibitory effect on the activity of β-glucosidase (*9*), which would cause cellobiose to accumulate, as was indeed

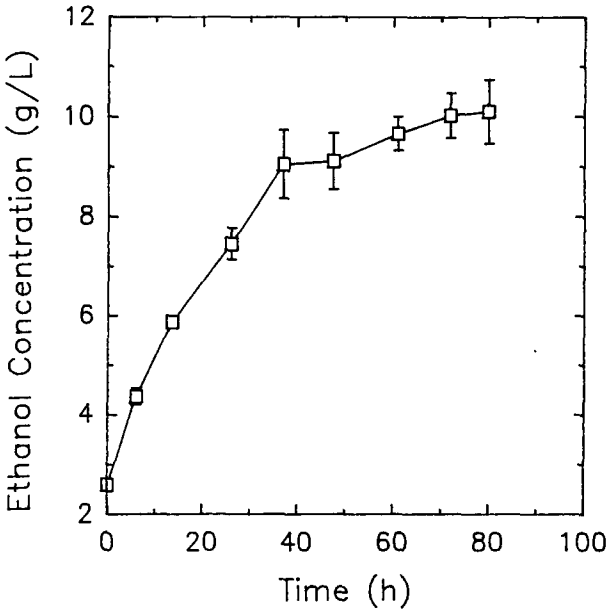

Fig. 2. Time-course of ethanol production during the SSF of pretreated wood chips. The mean values of ten independent runs are depicted. All runs were spiked at 80 h, as described in Table 1.

observed (Fig. 1). Interestingly, other oligosaccharides, such as triose, tetraose, and pentaose (intermediate products of cellulose hydrolysis by the endoglucanase and exoglucanase components of cellulase), did not accumulate. This may be caused by high exoglucanase activity exhibited by cellulase and also by the higher affinity that β-glucosidases reportedly exhibit toward oligosaccharides larger than the dimeric cellobiose (*10*).

As the cell mass progressively increases over time, the microorganism enhances the rate of glucose uptake, bringing the glucose concentration to very low levels, practically at the sensitivity limits of the employed analytical instruments. As a result, β-glucosidase is relieved of sugar inhibition and can continue hydrolyzing cellobiose, leading to a dynamic equilibration between the rates of sugar (glucose and cellobiose) production and consumption. Thus, both saccharide levels remain extremely low for the rest of the SSF process (Fig. 1). This higher cellular activity is verified by the high rate and yield of ethanol production (Fig. 2), which coincided with the disappearance of glucose and cellobiose (Fig. 1). The SSF experimentation was highly reproducible, as indicated by the small error bars of Fig. 2, which summarizes the data of ten runs.

After about 40–60 h of SSF, however, ethanol productivity begins to decline significantly, and by 80 h, it becomes marginal, although only about 65% of the cellulosic content of biomass has been hydrolyzed by that time (Fig. 2). In an effort to elucidate the causes of this cessation, the SSF process was spiked at 80 h in separate, but identical experimental setups with:

Table 1
Effect of Spiking After 80 h of SSF Operation on Residual Ethanol Concentration (the Control Experiment Was Left Undisturbed)

Ethanol concentration, g/L	SSF process spiked with				
	Control	Substrate, 20 g/L	Enzyme, 10 IFPU/g	Cell mass, 1 g/L	Nutrients, YP
Right before spiking	10.95	9.17	9.95	10.28	10.14
6 h after spiking	10.95	10.64	10.00	10.93	10.26
% Change	0.0	16.0	0.5	6.3	1.2

[a]In each case, the mean value of duplicates is presented; the standard deviations did not exceed 6%.

1. 20 g/L of fresh pretreated cellulosic substrate;
2. 10 IFPU of fresh cellulase enzyme/g of estimated residual cellulose;
3. 1 g/L of cell mass on a dry basis; and
4. Fresh YP medium, as a source of fermentation nutrients.

All additions, including a control run that was left undisturbed to serve as reference for comparisons, were tested in duplicate. The concentrations of sugars and metabolites were monitored before and after spiking. Data collected within 2 min before and after the additions were used to correct for changes in the volume of each run caused by the additions. The effect of each addition on ethanol yield 6 h after spiking was averaged for each pair of duplicates and summarized in Table 1; the standard deviations did not exceed 6%. The only addition that yielded results statistically distinguishable from the control runs was that of fresh cellulose, which enhanced ethanol production by 16%. This indicates that the organism and the enzymes retain all or a significant portion of their activity after 80 h of SSF. The problem seems to lie with the residual cellulose, which appears to be inaccessible to cellulase, as verified by the failure of fresh cellulase enzyme to lead to any further ethanol production. Similarly, the provision of fresh cell mass made practically no difference, since no more ethanol was generated from the residual cellulose. Finally, nutrient supplementation showed that nutrient depletion is not an issue even after more than 3 d of SSF.

During the second phase of SSF, when the rate of cellulose hydrolysis slows, the fermentative microorganism seems to be starving. When glucose was exogenously added at 80 h, the cells quickly fermented it to ethanol (data not shown). The present study proves that the cells retain their metabolic capability despite the depletion of glucose, because they are able to ferment the hydrolyzed fresh cellulose. However, further continuation of cell starvation during the SSF process results in utilization of

ethanol as carbon source; sampling allows air to enter the culture environment and enables the microorganism to switch its metabolism toward aerobic uptake of ethanol.

Taking the data of all four spiking experiments and their consistent reproducibility into account, it can be concluded that the progressive decline in ethanol production and yield during the course of the SSF process is caused mainly by a certain fraction of the cellulosic content of biomass, which remains unavailable for hydrolysis by cellulase, irrespective of the concentrations of cellulase and β-glucosidase. The notion often encountered in the literature that incomplete biomass digestion is caused by enzyme deactivation or irreversible binding to lignin and cellulose could not be verified in this study. Rather, substrate accessibility limits the progress of SSF. This conclusion was confirmed with a lignin-free substrate, α-cellulose (Sigma, St. Louis, MO), using the ethanologenic yeast *Brettanomyces custersii* (data not shown).

CONCLUSION

These experiments with real lignocellulosic substrate showed that the conversion of cellulose to ethanol in batch mode demonstrates two regimes: (1) an initial rather short phase, when the fermentation of glucose is the rate-determining step of the overall process because of the initially small number of cells, and (2) a subsequent longer phase that is controlled by the rate of the enzymatic hydrolysis. Optimization of the SSF operation needs to address both problems. By increasing the initial cell concentration, the length of the first regime is expected to be shortened, thus improving the overall ethanol productivity. However, attacking the substrate accessibility problem is much less trivial. To address this issue and improve biomass conversion, work is currently under way to enhance both the efficiency of the pretreatment method and the kinetics of the SSF reactions.

ACKNOWLEDGMENT

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