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Simultaneous Saccharification and Cofermentation of Dilute-Acid Pretreated Yellow Poplar Hardwood to Ethanol Using Xylose-Fermenting *Zymomonas mobilis*

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Abstract

Simultaneous saccharification and cofermentation (SSCF) was carried out at approximately 15% total solids using conditioned dilute-acid pretreated yellow poplar feedstock, an adapted variant of National Renewable Energy Laboratory (NREL) xylose-fermenting *Zymomonas mobilis* and either commercial or NREL-produced cellulase enzyme preparations. In 7 d, at a cellulase loading of 12 filter paper units per gram cellulose (FPU/g), the integrated system produced more than 3% w/v ethanol and achieved 54% conversion of all potentially available biomass sugars (total sugars) entering SSCF. A control SSCF employing Sigmacell cellulose and a commercial cellulase at an enzyme loading of 14 FPU/g achieved 65% conversion of total sugars to ethanol.

Index Entries: Hydrolyzate conditioning; *Zymomonas mobilis*; enzymatic saccharification; ethanol fermentation; SSCF; integrated testing.

Introduction

There are many different technologies for converting biomass to ethanol. Potential process routes include biomass pyrolysis and combustion to produce synthesis gases that can be biologically converted to ethanol *(1)* and hydrolysis (i.e., saccharification) of biomass using concentrated-acid hydrolysis or a combination of dilute-acid hydrolysis and enzymatic saccharification followed by fermentation *(2,3)*. Our team at the National Renewable Energy Laboratory (NREL) is chartered with developing

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economical enzyme-based biomass-to-ethanol process technology utilizing dilute-acid pretreatment and enzymatic saccharification of cellulose. We have chosen the following components for integrated process development: hardwood yellow poplar sawdust feedstock; co-current single stage dilute-acid pretreatment; *Trichoderma reesei*-based cellulase enzyme production; and ethanol production by a glucose- and xylose-fermenting *Zymomonas*. We are developing process technology using these components based on an overall simultaneous saccharification and cofermentation (SSCF) process configuration.

The SSCF Process

Figure 1 shows a simplified schematic representation of the overall design of the SSCF-based process technology being developed. This figure shows the six major unit operations in the process; utilities and waste treatment are not shown. The sawdust biomass feedstock is already of sufficiently reduced particle size that feedstock milling is not required. However, for other feedstocks, the first step in the process would be to mill the biomass to an appropriate size. The biomass is then subjected to dilute-acid pretreatment to hydrolyze (solubilize) the predominantly pentose sugars in the hemicellulose fraction and thereby open up the lignocellulosic structure to make it more susceptible to enzymatic attack *(4)*. Although pretreatment is necessary to release hemicellulosic sugars and increase enzymatic digestibility of cellulose, all pretreatment options available, including dilute-acid pretreatment, create at least some inhibitory compounds such as acetic acid, furfural, hydroxymethyl furfural (HMF), and mixed soluble phenolics that can detrimentally affect microbial metabolism *(5)*. Therefore, the acidic biomass slurry generally must be conditioned after pretreatment to reduce its toxicity and thereby enable the downstream biological processing steps of seed production, cellulase production, and SSCF to effectively occur. Depending on the nature of the feedstock and pretreatment conditions, the extent of conditioning required can vary from simple neutralization to more intensive methods based on a variety of approaches including steam stripping, ion exchange, extraction, and absorption *(5)*.

After conditioning, portions of the xylose-rich hydrolyzate liquors and cellulose-rich lignocellulosic solids are used as substrates for cellulase enzyme production and growth of the fermentative seed culture. The remainder of the conditioned hydrolyzate liquors and solids are transferred to SSCF. In SSCF, cellulase enzymes in the form of unfiltered production broth are added to enzymatically hydrolyze cellulose to glucose and a fermentative microorganism is added to ferment glucose and xylose to ethanol. After SSCF, the ethanol "beer" is recovered and concentrated using conventional distillation technology. The largely inert lignin fraction is carried through the process and, along with the residual cellulose, can be sent to a boiler (or a cogeneration plant) to supply steam (and electricity) for the process.

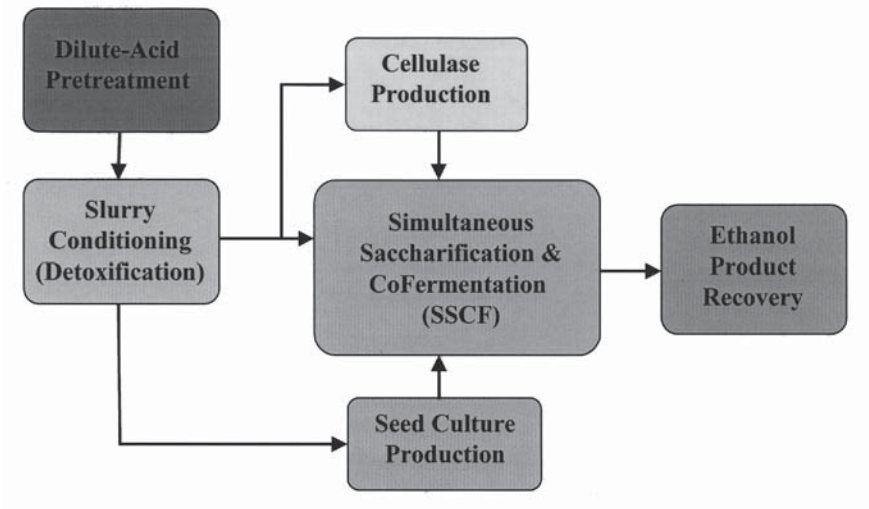

Fig. 1. Simplified schematic of SSCF-based bioethanol production process design.

As Fig. 1 shows, SSCF is at the heart of the overall process. As a result, the economics of an integrated SSCF process benefit from reductions in direct SSCF operating costs and from improvements made in the processing elements that precede it.

Economic Considerations

Lignocellulosic feedstocks ("biomass") such as hardwood sawdust contain (on a dry mass basis) approximately 45% cellulose, 25% hemicellulose, and 25% lignin *(6)*. In general, processes for converting biomass feedstocks into ethanol must achieve high ethanol yields on the biomass substrate to be economically viable because the cost of delivered feedstock accounts for a large percentage of process costs *(7,8)*. Economic analyses of biomass-to-ethanol process costs show that higher ethanol yields and final ethanol concentrations are the most important factors influencing the overall ethanol production cost, followed by increased volumetric productivity *(7–9)*. Substantial capital and operational savings can be achieved by developing advanced process designs that consolidate processing steps, such as the SSCF process configuration in which hexose and pentose sugars are fermented simultaneously ("cofermentation") *(10)*. The SSCF process configuration also reduces process complexity and like traditional simultaneous saccharification and fermentation (SSF) offers the potential to maximize overall ethanol process yields by eliminating end-product inhibition of the cellulase enzymes through continuous fermentation of released glucose to ethanol *(11,12)*. On the other hand, the optimum operating conditions for enzymatic cellulose hydrolysis are generally different than those for mixed sugar fermentation. Thus, one of the challenges to developing efficient SSCF technology is to identify appropriate compromise operating conditions where effective enzymatic saccharification and microbial fermentation can both occur.

Integrated Testing

The performance of integrated production technologies must be tested at the bench and pilot scales to develop and validate operating conditions suitable for economically viable large-scale bioethanol production. However, most published literature documenting experimental efforts to develop biomass-to-ethanol conversion technology, at NREL and at other laboratories, report on demonstrating and improving the performance of individual unit operations in the process (e.g., pretreatment, pentose fermentation, cellulose saccharification/hexose fermentation) rather than on demonstrating and improving the integrated process. Different feedstocks and processing approaches are reported in the available literature *(13,14)*, meaning that previous reports are of limited value for predicting the performance of integrated bioconversion technology based on the use of a specific biomass feedstock.

This paper presents results of bench-scale experiments performed to characterize the baseline performance of an integrated yellow poplar sawdust-to-ethanol SSCF-based bioconversion process (Fig. 1). Performance is reported for integrated SSCFs incorporating cellulase enzymes and fermentation seed culture produced using pretreated yellow poplar feedstock. The purpose of this work was twofold. The first objective was to demonstrate, at a baseline level, successful integration of pretreatment, cellulase production, and cellulose saccharification/biomass sugar fermentation. The second objective was to characterize the effect of cellulase enzyme loading on SSCF process performance.

Materials and Methods

Biomass Pretreatment

Dilute-acid pretreatment of yellow poplar sawdust was performed in NREL's 1 ton/d pilot scale Sunds Hydrolyzer pretreatment reactor *(15)*. Pretreatment was carried out at a temperature of 200°C and a sulfuric-acid concentration of 0.32% (w/w) using a residence time of 4.6 min, as described previously *(16)*. The proximate composition of raw feedstock was 41% cellulose, 28% hemicellulose, and 26% lignin (dry weight basis). Pretreatment resulted in 60% recovery of soluble monomeric xylose in the hydrolyzate liquor and produced pretreated lignocellulosic solids containing 60% cellulose (dry weight basis) with an estimated enzymatic digestibility of 83% *(16)*.

Conditioning Hydrolyzate for Fermentation

Pretreated biomass contains compounds that inhibit subsequent fermentation. Considerable work that is not reported here was performed to develop methods for conditioning acidic yellow poplar hydrolyzate for fermentation. The outcome of this work was to identify liquid-liquid

extraction followed by overliming (LLE-OL) as an effective conditioning method that conserves biomass sugars and enables high ethanol fermentation yields to be achieved. The LLE-OL conditioning procedure used to produce materials for the experiments reported here involved three sequential extractions of the hydrolyzate (liquor or slurry) with methyl tert-butyl ether, followed by heating of the extracted hydrolyzate to remove entrained solvent. The extracted hydrolyzate was then overlimed by adding calcium hydroxide powder to bring the pH up to 10–10.5, holding at 50°C for 30 min, and readjusting the pH to 6.0 using concentrated sulfuric acid *(17)*.

Cellulase

Two cellulase enzyme preparations were used, a commercial preparation from Iogen Corporation (Ottawa, Ontario, Canada) and an NREL-grown preparation produced using the *Trichoderma reesei* strain MTC-a-13 ("MTC"). The MTC enzyme was produced using lactose and washed pretreated yellow poplar solids as the carbon sources, as reported previously *(18)*. To reduce the volume of enzyme preparation added to SSCFs, the more dilute MTC preparation was concentrated approximately fourfold by ultrafiltration. The titers of enzyme preparations were measured as filter paper units per milliliter (FPU/mL) using the standard IUPAC cellulase activity assay *(19,20)*. The concentrated commercial preparation was assayed to have a cellulase enzyme titer of 70 FPU/mL. The cellulose contents of solids (also necessary to calculate the amount of enzyme solution to add to achieve a target cellulase loading in FPU/g cellulose) were also measured according to established standard procedures *(21,22)*.

Microorganism

An adapted variant of recombinant *Zymomonas mobilis* ATCC 39676 (pZB4L) developed through prolonged continuous culture to progressively higher levels of overlimed yellow poplar hydrolyzates was used in all experiments *(23,24)*.

Medium

All seed production and SSCF experiments were carried out using various concentrations of yellow poplar hydrolyzate liquor conditioned either by overliming (OL) or by LLE-OL. Pure cellulose controls were run by replacing solid pretreated lignocellulose with Sigmacell-50, a microcrystalline cellulose preparation (Sigma Chemical, St. Louis, MO). Conditioned hydrolyzates were supplemented with low levels of clarified and filter-sterilized corn steep liquor (CSL) (GPC, International, Muscatine, IA) previously shown to be effective for *Zymomonas* growth and fermentation *(25,26)*. Seed production and SSCFs were carried out by supplementing with 1.5% (v/v) CSL and 1.0% (v/v) CSL, respectively.

Inoculum Cultivation

Frozen stock cultures of the adapted *Zymomonas* strain were revived in sterile RM medium (1.0% (w/v) yeast extract and 0.2% (w/v) KH_2PO_4) containing 2.5% (w/v) xylose and 2.5% (w/v) glucose and tetracycline antibiotic at a level of 10 mg/L. Thereafter, pre-seed and seed cultures were cultivated without antibiotic in 30% (v/v) hydrolyzate conditioned in the same fashion as that used in the SSCF (either LLE-OL or OL). The preseed inoculum culture was incubated overnight at 30°C, then used to inoculate a seed fermentor. Seed cultures were cultivated at 30°C and at pH 6.0 for 20–24 h until culture turbidity at 600 nm reached 2.0–3.0 absorbance units.

SSCF Processing Conditions

SSCFs were carried out batchwise for 7 d in 400 mL working volume (500 mL total volume) Multigen fermentors (New Brunswick Scientific, Edison, NJ) agitated at 150 rpm and maintained at temperatures of 34 ± 1°C. SSCFs using pretreated yellow poplar solids were run at a total solids loading of approx 15% (11.5% insoluble solids and 3.5% soluble sugars). Control SSCFs using pure cellulose (Sigmacell-50) were run at a total solids loading of approx 9.5% (6% insoluble solids and 3.5% soluble sugars). Culture pH was automatically controlled at 5.5 by adding 2 *N* KOH. An appropriate aliquot of sterile, filtered enzyme solution was added, then SSCFs were directly inoculated with seed culture at a loading of 10% (v/v) corresponding to an initial cell concentration of approximately 0.1 g dry cell mass/L.

Analytical

Daily samples were taken for off-line analysis of pH and soluble components. In addition, large samples of the whole slurry were obtained at the initial and final endpoints of the SSCFs for complete compositional analyses to enable carbon mass balances to be performed. High-performance liquid chromatography (HPLC) was used to determine the concentrations of glucose, xylose, and ethanol in sample supernatants over the SSCF time courses. Samples were diluted 1:6 with distilled water before HPLC analysis but were otherwise run as described previously *(26)*. Mixed component concentration verification (CV) standards were periodically run in duplicate to verify calibration accuracy, and analyses were repeated if the reported concentrations of CV standards deviated from their actual values by greater than ± 2.5%. Initial and endpoint whole slurry samples were carefully separated into liquid and solid fractions and gravimetrically analyzed to determine the percent insoluble solids in the unconverted and converted process samples. The liquid and solid fractions of each sample were then analyzed for insoluble carbohydrates, soluble carbohydrates, and fermentation products using established NREL standard procedures *(21,22)*.

Calculations

Ethanol yield on total available sugars present at the beginning of SSCF, also referred to as process yield, or Y_P, is the most important measure of process performance. Metabolic ethanol yield or ethanol yield based on sugars consumed during the process is a useful measure of fermentation strain product selectivity. Previously, we reported process yields calculated as the net (corrected) grams of ethanol produced per grams of glucose and xylose available at the onset of the fermentation *(27)*, assuming 1.1 g of potentially available glucose/g of cellulose (i.e., assuming 100% cellulose digestibility). Here we report process ethanol yields calculated based on all potentially available sugars (monomeric glucose, xylose, mannose, galactose, arabinose, oligomers, and insoluble carbohydrates, including cellulose). The process yield calculation is corrected for ethanol carried over in the inoculum, but not for ethanol produced from sucrose present (as a stabilizer) in the concentrated CPN cellulase enzyme preparation; no sugars were present in the MTC cellulase enzyme preparation. Carbon-balance calculations were performed assuming cell stoichiometric production of carbon dioxide using NREL's standard methodology *(28)*. Cell mass concentrations were estimated from soluble sugar levels using a previously established cell-mass yield of 0.042 g dry cell mass/g substrate *(23)*.

Results

Previous work with xylose-fermenting *Z. mobilis* showed the importance of reducing acetic-acid levels present in pretreated hardwood hydrolyzates to achieve good growth and fermentation *(26)*. Similarly, many workers found biomass hydrolyzates to be highly inhibitory to fermentation unless proper conditioning occurred *(5)*, with OL historically being the most common method used to reduce hydrolyzate toxicity. After considerable investigation, a conditioning method consisting of LLE-OL was developed. This combined conditioning method reduced acetic acid levels and rendered the hydrolyzates much more fermentable than OL alone.

The efficacy of the LLE-OL conditioning process was ultimately tested by carrying out SSCFs on slurries consisting of conditioned hydrolyzates and cellulose. Figure 2 compares soluble substrate and product profiles for SSCFs carried out using pure cellulose (Sigmacell-50) and a commercial cellulase at a loading of 14.0 FPU/g cellulose. The total solids levels in these experiments are approximately 9.5% (6% insoluble cellulose and 3.5% soluble sugars, representing a hydrolyzate liquor dilution of about 50% [v/v]). The plot on the left shows SSCF performance in LLE-OL conditioned hydrolyzate liquor, while the plot on the right shows performance in OL conditioned hydrolyzate liquor. As Fig. 2 shows, the SSCF carried out using LLE-OL conditioning begins with lower levels of acetic acid than are present in the OL hydrolyzate and proceeds well. The concentrations of soluble glucose and xylose fall to undetectable levels within 24 h and 48 h, respectively, with concomitant rapid ethanol production. Ethanol production

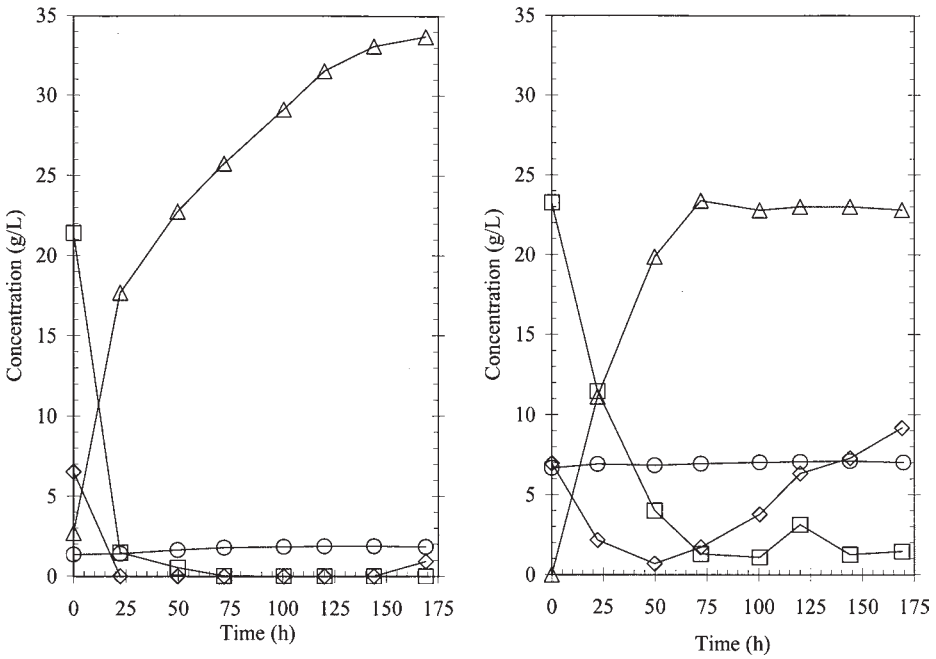

Fig. 2. Influence of conditioning on SSCF performance. Plots show SSCFs of pure cellulose in the presence of hydrolyzate conditioned using either LLE-OL (left) or OL alone (right) using commercial cellulase and adapted *Z. mobilis*. Symbols: (◇), glucose; (□), xylose; (○), acetate; (△), ethanol.

continues once the soluble sugars have been exhausted because of the continuous enzymatic hydrolysis of cellulose to glucose. Ethanol production continues for the entire 7-d duration of the experiment, reaching a final concentration above 33 g/L. As illustrated by Fig. 2, much poorer SSCF performance is observed using hydrolyzate conditioned by OL alone that contains higher levels of acetic acid. Glucose and xylose are not consumed to low levels until 48 h and 72 h, respectively, with correspondingly slower ethanol production. More importantly, the fermentation stalls after soluble glucose and xylose are consumed and the ethanol concentration remains constant at approximately 23 g/L from 72 h onward. Enzymatic cellulose hydrolysis continues after the fermentation stalls, causing the glucose concentration to increase after 48 h.

Based on these results, which confirmed the efficacy of LLE-OL conditioning, four SSCFs were carried out using LLE-OL conditioned pretreated yellow poplar substrate ("whole slurry"). These SSCFs were run at a total solids level of approximately 15% (w/w) (approximately 11.5% insoluble solids and 3.5% soluble sugars, representing a hydrolyzate slurry dilution of about 50% [v/v]). To assess the effect of cellulase enzyme loading, experiments were carried out at enzyme loadings ranging from 3.5 FPU/g cellulose to 12.0 FPU/g cellulose using a cellulase enzyme preparation produced at NREL using lactose and pretreated yellow poplar solids. Fig-

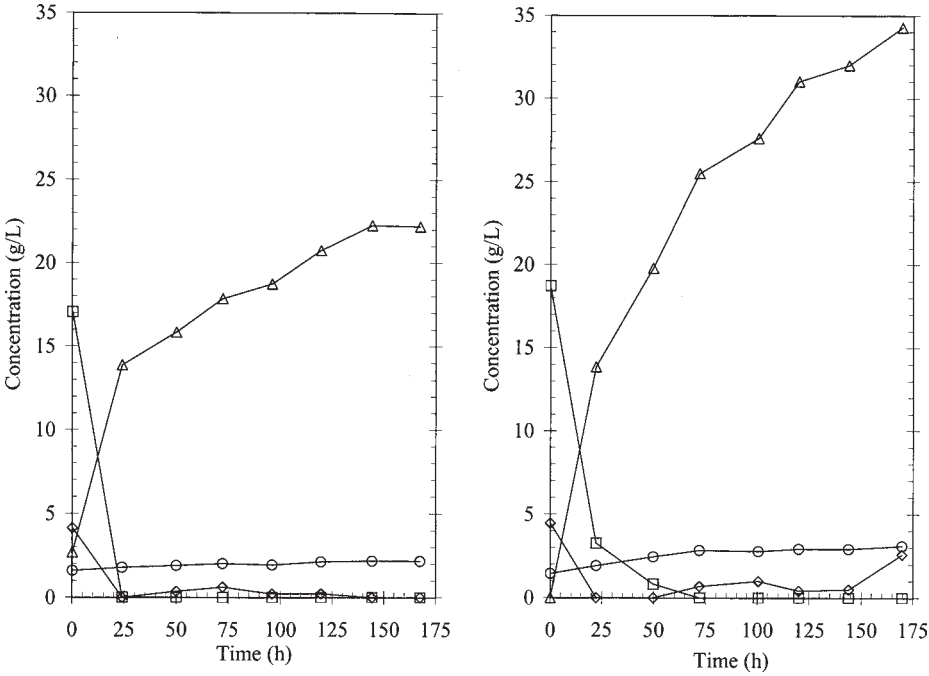

Fig. 3. Integrated SSCF performance achieved on LLE-OL conditioned pretreated yellow poplar whole slurry using adapted *Z. mobilis* and NREL-grown cellulase at enzyme loadings of 5.9 FPU/g cellulose (left) and 12.0 FPU/g cellulose (right). Symbols: (◇), glucose; (□), xylose; (○), acetate; (△), ethanol.

ure 3 shows profiles for SSCFs carried out at cellulase enzyme loadings of 5.9 FPU/g cellulose (left) and 12.0 FPU/g cellulose (right). As this figure illustrates, rapid consumption of glucose is observed at both enzyme loadings, with glucose reaching essentially undetectable levels within 24 h. However, xylose consumption is somewhat faster at the lower enzyme loading. Xylose concentration reaches zero within 24 h using an enzyme loading of 5.9 FPU/g cellulose but does not fall to zero until 72 h using the higher enzyme loading of 12.0 FPU/g cellulose. Despite slower xylose consumption, much better ethanol production is observed in the SSCF carried out at the higher enzyme loading, probably because of more rapid conversion of cellulose to glucose. Ethanol concentration reaches approximately 22 g/L at 168 h in the SSCF carried out at an enzyme loading of 5.9 FPU/g cellulose, whereas it reaches 34 g/L in the same time using the higher enzyme loading of 12.0 FPU/g cellulose.

Figure 4 shows ethanol production profiles achieved in all four SSCFs carried out using conditioned yellow poplar whole slurry, i.e., at cellulase enzyme loadings of 3.5, 5.9, 8.9, and 12.0 FPU/g cellulose. For comparison, a profile is also shown for the control SSCF carried out using pure cellulose and a commercial cellulose preparation (at a loading of 14.0 FPU/g cellulose; *see* Fig. 2). All of the profiles shown in Fig. 4 are similar at 24 h, the point where ethanol has predominantly been produced by fermentation of

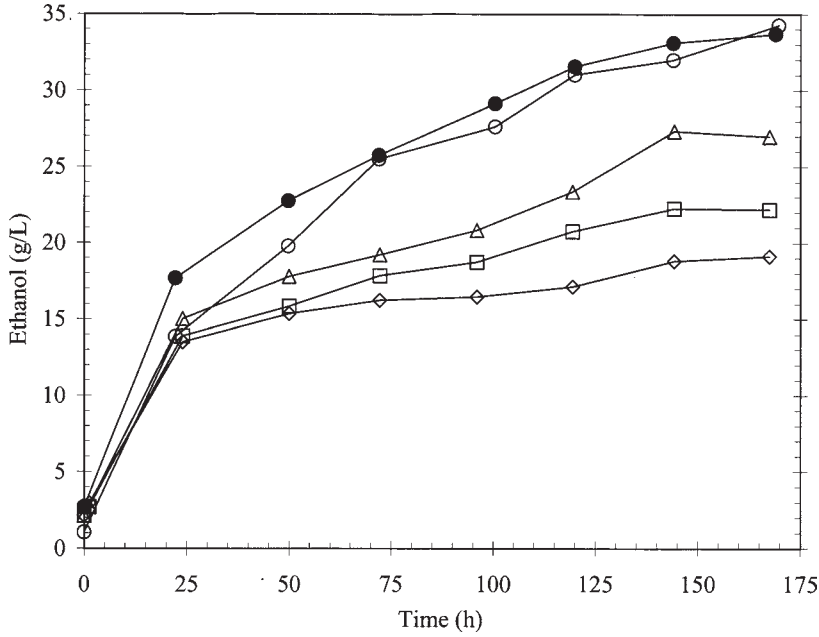

Fig. 4. SSCF ethanol production profiles achieved at different enzyme loadings using LLE-OL conditioned whole slurry. The open symbols depict performance at cellulase enzyme loadings of 3.5 (◇), 5.9 (□), 8.9 (△) and 12.0 (○) FPU/g cellulose, respectively, using an NREL-produced cellulase enzyme. The solid symbol (●) shows comparative performance on purified cellulose and conditioned hydrolyzate using a commercial cellulase preparation at a loading of 14.0 FPU/g cellulose.

soluble sugars rather than from glucose released by enzymatic hydrolysis of cellulose. After 24 h, ethanol production rates are constrained by the rate of cellulose hydrolysis and decrease markedly. Overall, Fig. 4 illustrates that, as expected, final 7-d SSCF ethanol production levels increase using higher loadings of cellulase enzyme.

Table 1 summarizes performance levels achieved in the integrated SSCF experiments; values achieved using purified cellulose and the commercial enzyme preparation are shown for comparison. Figure 5 shows how ethanol concentration, average volumetric productivity, and overall process ethanol yield vary with enzyme loading for the four fully integrated experiments carried out using NREL-grown cellulase enzyme and LLE-OL conditioned pretreatment whole slurry. As Fig. 5 and Table 1 show, net ethanol concentrations and average volumetric ethanol productivities in the integrated SSCFs vary in an approximately linear fashion with enzyme loading, increasing from a low of 17.6 g/L and 0.11 g/L-h, respectively, achieved at an enzyme loading of 3.5 FPU/g cellulose to a high of 32.2 g/L and 0.19 g/L-h achieved at an enzyme loading of 12.0 FPU/g cellulose. As Fig. 4 and Table 1 indicate, the control SSCF carried out using the commercial enzyme preparation at a loading of 14.0 FPU/g cellulose performed quite similarly to the integrated SSCF carried out at the highest

Table 1
Integrated SSCF Performance Results

Solids type	Liquor type	Enzyme source	Enzyme loading (FPU/g)	Ethanol produced (g/L)	Average volumetric productivity (g/L-h)	Ethanol metabolic yield (% theor.)	Ethanol process yield (% theor.)	Carbon balance closure (%)
SC[a]	OL	Iogen	14.0	21.8	0.13	111%	44%	114%
SC	LLE-OL	Iogen	14.0	31.9	0.19	96%	65%	105%
PYP[b]	LLE-OL	NREL	3.5	17.6	0.11	93%	28%	113%
PYP	LLE-OL	NREL	5.9	21.1	0.13	74%	34%	101%
PYP	LLE-OL	NREL	8.9	25.3	0.15	84%	41%	109%
PYP	LLE-OL	NREL	12.0	32.2	0.19	76%	54%	97%

[a]Sigmacell-50.
[b]Pretreated yellow poplar.

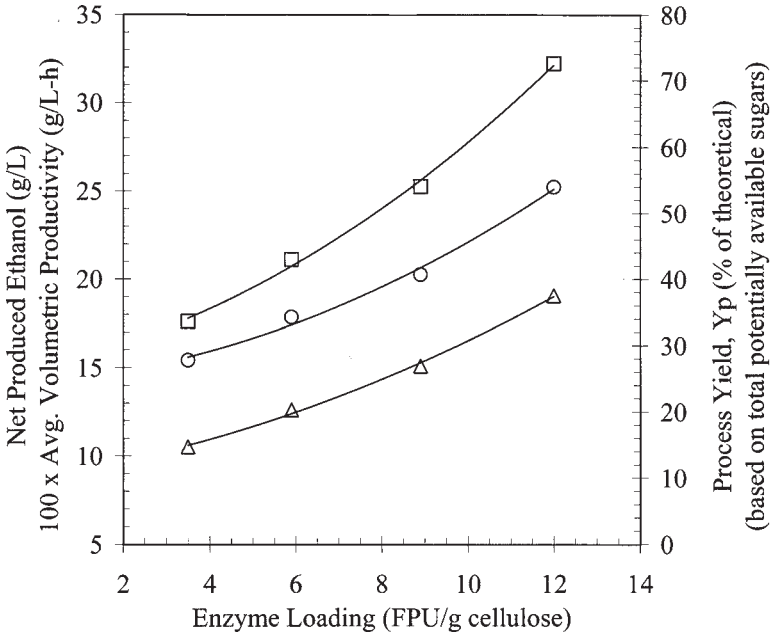

Fig. 5. Net ethanol production (□), average volumetric productivity (△), and process yield (○) achieved in integrated SSCF as a function of cellulase enzyme loading.

enzyme loading (12.0 FPU/g cellulose), producing 31.9 g/L ethanol (net) and achieving an overall average ethanol volumetric productivity of 0.19 g/L-h.

Table 1 shows that metabolic ethanol yields (those based on consumed glucose and xylose) do not show a discernible trend with enzyme loading, varying in the range of 74–93% of theoretical. Process ethanol yields (those based on the total amount of potentially available sugars [total sugars] charged to the system in the form of cellulose, oligomers, and all hexose and pentose biomass sugars) increase modestly with enzyme loading, rising from a low of 28% of theoretical at an enzyme loading of 3.5 FPU/g cellulose to a high of 54% of theoretical at an enzyme loading of 12.0 FPU/g cellulose. In comparison, the control SSCF carried out using pure cellulose and a commercial enzyme preparation at a loading of 14.0 FPU/g cellulose achieved a process yield of 65%. Overall, as Table 1 shows, the quality of the integrated SSCF performance data is relatively high, with carbon mass balances closing to 106.3% ± 6.8% for all six experiments.

Discussion

We previously reported that NREL's metabolically-engineered xylose-fermenting *Zymomonas mobilis* bacterium performs well in a SSCF process configuration *(27)*. Using 6.0% (w/v) pure cellulose (Sigmacell-50) and 3.5% (w/v) reagent-grade xylose and commercial cellulase at a loading of 25 FPU/g cellulose, 5-d SSCF process yields of above 75% of theoretical

were achieved assuming a 5-d cellulose digestibility of Sigmacell-50 of 90% *(29)*. The performance of integrated SSCFs achieved in the experiments reported here is clearly below that previously achieved using pure substrates. This reflects the fact that both enzymatic hydrolysis and sugar fermentation are detrimentally affected by inhibitory components in real pretreated lignocellulosic feedstocks. Nonetheless, this demonstration of integrated processing shows that pretreatment and saccharification/fermentation steps, and to a lesser extent the enzyme production step, can be effectively linked, provided the hydrolyzate is effectively conditioned. In addition, the overall process yield achieved in the control SSCF carried out using LLE-OL conditioned hydrolyzate liquor and pure cellulose increases from 65% to approximately 70% of theoretical, assuming 90% digestibility of Sigmacell-50. This level of performance, although still well below the levels required for economically viable ethanol production from nonzero cost lignocellulosic feedstocks, is not that far below what was achieved in nonoptimized SSCF carried out using pure substrates at modestly lower total solids loading, but using an enzyme loading more than twofold higher. Thus, the baseline integrated SSCF performance demonstration results reported here are promising and indicate that efforts to further improve SSCF-based bioethanol production are warranted.

The overall performance results presented in Table 1 and in Fig. 5 show that all three key SSCF performance measures—process yield, average volumetric productivity, and final ethanol concentration—increase with increasing enzyme loading. This finding suggests that higher process yields will be achieved when SSCFs are carried out at cellulase enzyme loading above 12 FPU/g cellulose. Based on previous work, higher SSCF process yields also are expected when whole broth cellulase enzyme preparations are used rather than clarified preparations *(30)*. Additional data need to be obtained at higher (and perhaps lower) enzyme loadings to determine whether the relationships between the critical process performance parameters and enzyme loading exhibit statistically significant curvature. Additional data are also necessary to determine the impact of using whole broth cellulase enzyme preparations and to determine whether overall SSCF performance plateaus with respect to enzyme loading at higher cellulase loadings. Rough extrapolation of enzyme loading performance data suggests that integrated SSCF process yields above 70% of theoretical based on total sugars should be achievable using cellulase enzyme loadings of 18 FPU/g cellulose or higher.

Conditioning

The results shown in Fig. 2 clearly suggest that conditioning methods beyond overliming alone will be required for our adapted xylose-fermenting *Zymomonas* to effectively ferment hardwood hydrolyzates. These types of hydrolyzates contain higher levels of acetic acid than those produced from other biomass feedstocks because hemicellulose in hardwood is acetylated to a higher degree than in herbaceous crops and agricultural residues

(31). To achieve economically viable rates of ethanol production, the high levels of acetic acid that occur, particularly if pretreatment and SSCF processing are carried out at high total solids levels, must be reduced or the tolerance of the fermentative microorganism for acetic acid must be increased. Although LLE-OL conditioning may not prove to be the best method for detoxifying hardwood hydrolyzate, the results shown here clearly demonstrate that this method is effective and deserving of further consideration.

Considerable work not reported on here was conducted to develop an effective conditioning process and LLE-OL was only one of several promising methods that were identified. A reproducible shake-flask fermentation test, developed by modifying a previously described test tube-based toxicity assay *(17)* to incorporate larger volumes, better mixing, and periodic manual pH adjustment, was used to evaluate the relative toxicity of sawdust hydrolyzates conditioned by a variety of different methodologies. Ultimately, this bioassay was used to identify several conditioning methods, including LLE-OL, that could be used to detoxify hydrolyzates sufficiently to enable good fermentation performance to be achieved. Although the economic viability of LLE-OL conditioning clearly needs to be determined, the process has the positive attributes of being scalable and being able to be conducted directly on the whole slurry, i.e., pretreated solids and hydrolyzate; no solid-liquid separation is required to extract and overlime the hydrolyzate slurry. Moreover, the used extraction solvent can be back-extracted with alkali and water and then reused.

Cellulase Production

The cost of cellulase production and the quantity of cellulase enzyme required to achieve high cellulose conversion yields are important cost factors in this process. Provided good enzyme production performance can be achieved, it is economically attractive to produce cellulase enzymes using process-derived feedstocks as substrates rather than more expensive soluble carbon sources such as lactose and corn syrups that are believed to be currently favored for cellulase production *(32)*. In the work reported here, NREL-produced cellulase preparations were employed to better integrate the SSCF process; commercial (purchased) cellulase preparations were used in all our previous SSCF work. Washed pretreated yellow poplar solids and soluble lactose were used to produce the cellulase enzyme. Current work focuses on demonstrating enzyme production from pretreated feedstock without using lactose, but this work had not been initiated at the time the integrated SSCF testing was performed *(33)*.

It is generally hypothesized that the cellulase enzymes produced by a *T. reesei* culture exposed to a particular cellulosic feedstock during growth and induction of enzyme biosynthesis will be more effective at saccharifying this feedstock than a cellulase complex produced using a different substrate, i.e., the amounts and ratios of the various components in the cellulase enzyme complex will be produced to optimize saccharification of the par-

ticular feedstock type being used for enzyme production. Recent results provide support for this hypothesis. MTC cellulase enzyme complex produced on pretreated yellow poplar solids recently has been shown to be more effective at saccharifying pretreated yellow poplar solids than a commercial cellulase preparation grown using soluble lactose *(18)*. This finding suggests that the enzyme loading required for effective saccharification of pretreated biomass may be less than previously estimated if the enzyme is produced using the same pretreated biomass.

Conclusions

Baseline performance of an integrated SSCF process for converting hardwood sawdust to ethanol was demonstrated and the effect of cellulase enzyme loading on SSCF performance was characterized. SSCFs of pretreated yellow poplar sawdust were carried out using pretreated whole slurry conditioned (detoxified) by LLE-OL. Experiments were performed at approximately 11.5% insoluble solids (15% total solids) and contained cellulose and soluble biomass sugar levels representative of 50% (v/v) pretreatment slurries. Performance results, although below economically viable production levels, demonstrate that integrated SSCF processing can be accomplished and provide a baseline against which future process improvements can be measured. Results of integrated SSCF testing carried out at enzyme loadings of 3.5–12.0 FPU/g cellulose show that all major process performance measures increase with enzyme loading. Process ethanol yields increase from a low of 28% of theoretical at an enzyme loading of 3.5 FPU/g cellulose to a high of 54% of theoretical at an enzyme loading of 12.0 FPU/g cellulose. Extrapolation of the data for performance as a function of enzyme loading suggests that even without optimizing the process, SSCF process yields above 70% of theoretical based on total sugars should be achievable using cellulase enzyme loadings of 18 FPU/g cellulose.

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