

Enhanced Cofermentation of Glucose and Xylose by Recombinant *Saccharomyces* Yeast Strains in Batch and Continuous Operating Modes

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ABSTRACT

Agricultural residues, such as grain by-products, are rich in the hydrolyzable carbohydrate polymers hemicellulose and cellulose; hence, they represent a readily available source of the fermentable sugars xylose and glucose. The biomass-to-ethanol technology is now a step closer to commercialization because a stable recombinant yeast strain has been developed that can efficiently ferment glucose and xylose simultaneously (coferment) to ethanol. This strain, LNH-ST, is a derivative of *Saccharomyces* yeast strain 1400 that carries the xylose-catabolism encoding genes of *Pichia stipitis* in its chromosome. Continuous pure sugar cofermentation studies with this organism resulted in promising steady-state ethanol yields (70.4% of theoretical based on available sugars) at a residence time of 48 h. Further studies with corn biomass pretreated at the pilot scale confirmed the performance characteristics of the organism in a simultaneous saccharification and cofermentation (SSCF) process: LNH-ST converted 78.4% of the available glucose and 56.1% of the available xylose within 4 d, despite the presence of high levels of

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metabolic inhibitors. These SSCF data were reproducible at the bench scale and verified in a 9000-L pilot scale bioreactor.

Index Entries: Simultaneous saccharification and cofermentation; glucose and xylose fermentation; cellulosic biomass; recombinant *Saccharomyces* sp.; fermentation scale-up.

INTRODUCTION

Agricultural residues represent a readily available source of cellulose and hemicellulose that can be converted to fuel ethanol (1). The recent formation of the SWAN Biomass Company, a partnership between Amoco and Stone and Webster Engineering (2), demonstrates the interest of the private sector in the commercialization of biomass-to-ethanol conversion technology developed by the National Renewable Energy Laboratory (NREL) and Amoco.

Cellulose conversion technology by the simultaneous saccharification and fermentation (SSF) process has made significant progress during the past ten years (3). In contrast, the fermentation of hemicellulose-derived xylose to ethanol remains problematic because of the unavailability of efficient glucose- and xylose-cofermentation organisms (4–6). As a result, the ethanol potential of biomass has not been fully realized and the cost of ethanol remains uncompetitive with gasoline. Fortunately, recent efforts in metabolic engineering have resulted in the development of organisms that can ferment glucose and xylose simultaneously, such as a recombinant *Zymomonas mobilis* (7) and recombinant derivatives of *Saccharomyces* yeasts (8–12).

In 1993, Ho and coworkers first developed recombinant *Saccharomyces* yeasts that could effectively ferment xylose to ethanol. One such yeast is 1400(pLNH33), referred to as LNH33, which was developed by transforming *Saccharomyces* strain 1400, a fusion product of *Saccharomyces diastaticus* and *Saccharomyces uvarum* (13), with the high-copy number yeast—*E. coli* shuttle vector pLNH33 containing the xylose reductase (XR), xylitol dehydrogenase (XD) (both from *Pichia stipitis*), and xylulokinase genes (XK) (from *S. cerevisiae*). These cloned genes were fused to promoters that are neither inhibited by the presence of glucose nor require the presence of xylose for induction (8,10). As a result, LNH33 can ferment xylose to ethanol, as well as efficiently coferment glucose and xylose to ethanol. The xylose-fermenting ability of LNH33 can be maintained by growth on YEP (10 g/L yeast extract and 20 g/L peptone) medium supplemented with xylose (20 g/L) as the sole carbon source. However, like all plasmid-bearing transformants, LNH33 is not stable when successive generations are cultured in nonselective media. This instability could limit the use of LNH33 in a continuous fermentation process.

To overcome this problem, Ho and Chen (12) recently developed an improved recombinant *Saccharomyces* yeast, designated as LNH-ST, which

contains multiple copies of the xylose metabolizing genes, XR-XD-XK, integrated into the chromosome of the host strain 1400. This strain coferments glucose and xylose with improved efficiencies, and is also stable. LNH-ST does not have to be cultured in any special medium to maintain the cloned genes; it can be cultured in nonselective media for an unlimited number of generations and still retain its full capability to ferment xylose to ethanol.

By using the recombinant yeast strains that utilize both glucose and xylose, the process becomes a SSCF. Higher ethanol yields and concentrations, in turn, minimize the risk of contamination in the SSCF bioreactors and further enhance the productivity and lower the cost of the technology. Hence, the current SWAN biomass conversion technology applied to grain by-products consists of three key steps:

1. The pretreatment process, which uses sulfuric acid and heat to hydrolyze hemicellulose to xylose and xylan oligomers and improves cellulose accessibility to enzymatic hydrolysis;
2. The SSCF process, in which cellulose is hydrolyzed to glucose and, at the same time, glucose and xylose are fermented to ethanol by the recombinant yeast; and
3. The downstream separation process, in which ethanol is recovered through distillation and the protein-rich solids are retrieved to be sold as a component of animal feed.

In the present work an evaluation of the cofermentation capabilities of the two recombinant yeast strains, LNH33 and LNH-ST is described, first using mixtures of pure sugars (glucose and xylose) and then pretreated corn biomass. To assist in the scale-up of the process, this study monitored the ethanol productivity of the organisms in both batch and continuous operating modes and compared the SSCF performances at the bench and pilot scales.

MATERIALS AND METHODS

Inoculum Generation

Frozen stock cultures of LNH33 and LNH-ST were prepared in YEPX [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) xylose, pH 5.0], and 20% (w/w) glycerol solution. One-mL aliquots were dispensed into cryopreservation vials and stored at -70°C .

Seed cultures of LNH33 were prepared by inoculating 1 mL of LNH33 frozen stock into 50 mL of 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) xylose, and 1% (w/v) corn steep liquor (CSL) at pH 5.0 in a 250-mL baffled Erlenmeyer flask. Cultures were incubated at 30°C with agitation at 150 rpm. After 24 h of growth, 10% (v/v) was transferred to 2% (w/v) CSL, 1% (w/v) yeast extract, and 2% (w/v) xylose at pH 5.0 for inoculum preparation.

LNH-ST inoculum was also prepared in two stages for the continuous fermentation studies, first by growth in YEPD [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose, pH 5.0] and then in 1% (w/v) CSL, and 2% (w/v) glucose at pH 5.0. For comparative studies between bench-scale and the pilot-scale Process Development Unit (PDU), inoculum was prepared in five stages, to scale-up seed, in the presence of 1% (w/v) CSL and 2% (w/v) glucose at pH 5.0.

Cofermentation of Glucose and Xylose

Pure sugar cofermentation studies, with a mixture of glucose and xylose, were carried out in a two-stage continuous fermentation configuration at 30°C, 150 rpm, and pH 5.0. Two 1.7-L BioFlo III fermentors (New Brunswick Scientific) were employed at a working volume of 1 L per reactor. The first-stage fermentor was started in batch mode with 2% (w/v) CSL, 1% (w/v) yeast extract, 2.4% (w/v) glucose, and 3.4% (w/v) xylose at pH 5.0. Stock solutions of glucose and xylose were filter sterilized separately and added to the fermentor, along with 10% (v/v) inoculum. The first-stage fermentation was operated in batch mode for 24 h, then switched to continuous mode, and the effluent was directed to the second-stage fermentor. Feed for the first stage was prepared in 15-L batches and consisted of the same medium used in the batch phase. The dilution rate of the two fermentors was controlled by the feed rate to the first vessel and was monitored throughout the fermentation. The ethanol and by-product yields and the glucose and xylose conversions were calculated based on the steady-state concentrations. To minimize ethanol evaporation, the condensers on each fermentor were packed with 1-mm glass beads for maximum surface area and equipped with 4°C water circulation. The pH was controlled at 5.0 with the addition of 3M sodium hydroxide. Neither air nor nitrogen were supplied to the fermentors.

Batch Simultaneous Saccharification and Cofermentation (SSCF)

Corn biomass was pretreated in a pilot-scale dilute-acid pretreatment reactor and the pH was adjusted to 5.0 with sodium hydroxide. SSCF batch experiments were performed at 20% (w/v) total solids concentration, 30°C, 150 rpm, and pH 5.0, controlled with the addition of 3M sodium hydroxide. CSL [1% (w/v)] was added as a nutrient source. The SSCF, as well as the continuous pure sugar fermentations, were carried out at 30°C because xylose fermentation is diminished at higher temperatures.

Cellulase enzyme (80 IFPU/mL), supplied by Iogen (Ottawa, Canada), was added to the SSCF at a level of 10 IFPU/g cellulose present in the raw corn biomass. Its activity was measured by the IUPAC methods (14). Glucoamylase (285 IU/mL), from Enzyme Development Corporation (New York, NY), was added at 2 IU/g of starch present in the raw feedstock. Each enzyme preparation was filter-sterilized before addition to the fermentors.

Analysis

Samples were analyzed for ethanol and glucose with the Yellow Springs Instrument (YSI) Model 2700 Biochemistry Analyzer. Hexose and pentose sugars were analyzed by liquid chromatography using a Hewlett Packard 1090 HPLC unit equipped with an HP 1047 IR detector and an HPX87P column operating at 0.60 mL/min and 85°C. Ethanol and metabolic by-products, such as organic acids, glycerol, and xylitol, were quantified by HPLC using an HPX87H column operating at a flow rate of 0.60 mL/min and a temperature of 65°C. SSCF liquor residues were analyzed for oligomeric sugars, and a total compositional analysis was performed on the solids at the initial and final time points of each SSCF study to determine ethanol yields and to close the fermentation carbon balance (15).

RESULTS AND DISCUSSION

Two-Stage Continuous Cofermentation of Glucose and Xylose by LNH33

A two-stage continuous cofermentation was used to examine the ability of LNH33 to simultaneously ferment a mixture of xylose and glucose at levels representative of realistic pretreated corn biomass; and demonstrate its ability to grow in continuous culture under realistic process conditions prior to scale-up and commercialization efforts. The two-stage continuous cofermentation was operated in batch mode for 24 h. During the batch phase, all available glucose, as well as 38.6% of the xylose, was consumed (Fig. 1).

After the 24-h batch period, the fermentation was switched to continuous operation with a feed rate of 0.694 mL/min. This rate produced a 24-h residence time (0.042-h^{-1} dilution rate) in each stage and an overall process residence time of 48 h. After switching the system to continuous operation, the glucose concentration remained undetectable, but the xylose concentration increased during the 265-h duration of the continuous run to 31.2 g/L (Fig. 1), resulting in a xylose conversion of 11.4% in the first stage (Table 1). After the second stage was filled with effluent, the xylose concentration reached a minimum of 6.8 g/L. However, during the continuous phase, the xylose concentration in the second stage increased to 26 g/L (Fig. 1), resulting in a xylose conversion of 16.9%. Hence, the overall xylose conversion was 26.3% at a residence time of 48 h. Even after 11 residence times, the continuous system had not reached a steady state, presumably because of plasmid instability or a decrease in plasmid copy number.

The metabolic ethanol yields (based on the amount of consumed sugar) in the first and second stages were 84.3 and 85.2% of theoretical, respectively. The ethanol process yields (based on available fermentable sugars) were only 40.1 and 14.4%, respectively, because of the limited consumption of xylose, especially in the second stage (Table 1). For the over-

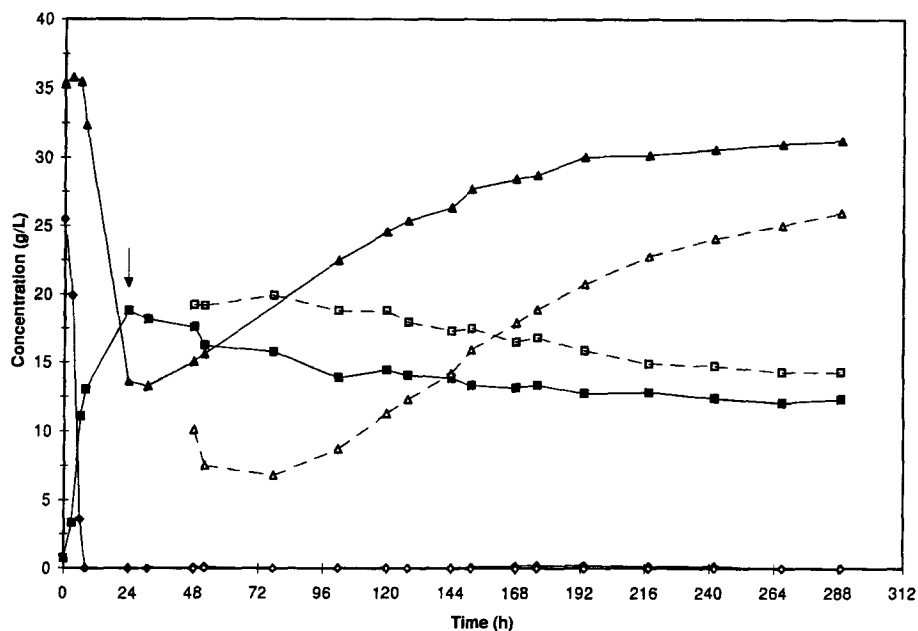


Fig. 1. Time course of the glucose (◆, ◇), xylose (▲, △), and ethanol (■, □) concentrations during the continuous, two-stage pure sugar cofermentation by LNH33. Closed symbols and continuous lines represent the first stage, whereas open symbols and dashed lines represent the second stage. The arrow indicates the switching point from batch to continuous operation.

Table 1
Pure Sugar Two-Stage Continuous Fermentation Performance
of LNH33 and LNH-ST

Strain LNH33			
Stage	1	2	Overall
Residence Time (h)	24	24	48
Glucose Conversion	100.0%	-	100.0%
Xylose Conversion	11.4%	16.9%	26.3%
Ethanol Process Yield (% theoretical)	40.1%	14.4%	47.6%
Ethanol Metabolic Yield (% theoretical)	84.3%	85.2%	84.4%
Strain LNH-ST			
Stage	1	2	Overall
Residence Time (h)	23.3	23.3	46.6
Glucose Conversion:	99.5%	100.0%	100.0%
Xylose Conversion:	58.3%	67.4%	86.4%
Ethanol Process Yield (% theoretical)	58.7%	47.2%	70.4%
Ethanol Metabolic Yield (% theoretical)	78.0%	69.7%	76.5%

Table 2
Product Distribution during Pure Sugar Fermentation and Corn Biomass SSCF
(Expressed as Grams of Product per 100 g of Consumed Glucose and Xylose)

Product	Pure-Sugar Two-Stage		Corn Biomass SSCF by LNH-ST	
	LNH33	LNH-ST	Batch 1	Batch 2
Ethanol	43.14	39.09	41.14	45.00
Cell Mass	7.53	7.04	4.04	3.94
Carbon Dioxide	41.21	37.34	39.35	43.05
Glycerol	3.39	9.37	4.47	5.02
Xylitol	4.94	7.02	2.61	2.75
Succinic Acid	0.00	0.00	0.45	0.55
Total	100.21	99.86	92.05	100.31

all process, the ethanol metabolic yield was 84.4% and the ethanol process yield was 47.6%. The major by-products of the fermentation (expressed per gram of consumed glucose and xylose) were carbon dioxide (0.412 g, calculated on an equimolar basis with respect to produced ethanol), cell mass (0.075 g), xylitol (0.049 g), and glycerol (0.034 g). The overall carbon balance was 100.21% (Table 2).

Because of the poor xylose utilization by LNH33 in the two-stage continuous cofermentation study, strain LNH-ST was used in all subsequent studies.

Two-Stage Continuous Cofermentation of Glucose and Xylose by LNH-ST

The two-stage continuous cofermentation of glucose and xylose with LNH-ST was performed in the same manner as with LNH33, but the residence time was 23.3 h per stage (0.043-h^{-1} dilution rate), resulting in a slightly shorter overall process residence time of 46.6 h compared to 48 h for LNH33. The glucose disappeared within the first 24 h of the batch stage (Fig. 2). During the same period, residual xylose concentration decreased to 7.7 g/L from an original level of 32.1 g/L (76% consumption).

After 24 h of batch operation, the fermentation was switched to continuous mode with a feed rate of 0.716 mL/min. The glucose concentration remained at zero, and the xylose concentration increased from 7.7 g/L to a steady-state value of 13.4 g/L (Fig. 2), which represents a utilization of 58.3% of the feed xylose in the first stage (Table 1). In the second stage, the xylose concentration decreased further to a steady-state value of 4.4 g/L, representing an overall conversion of 86.4%. The overall xylose utilization of LNH-ST was significantly higher than that of LNH33 (86.4% vs 26.3%) under similar experimental conditions (Table 1).

The metabolic ethanol yields in the first and second stages were 78 and 69.7%, respectively, whereas the ethanol process yields were 58.7 and

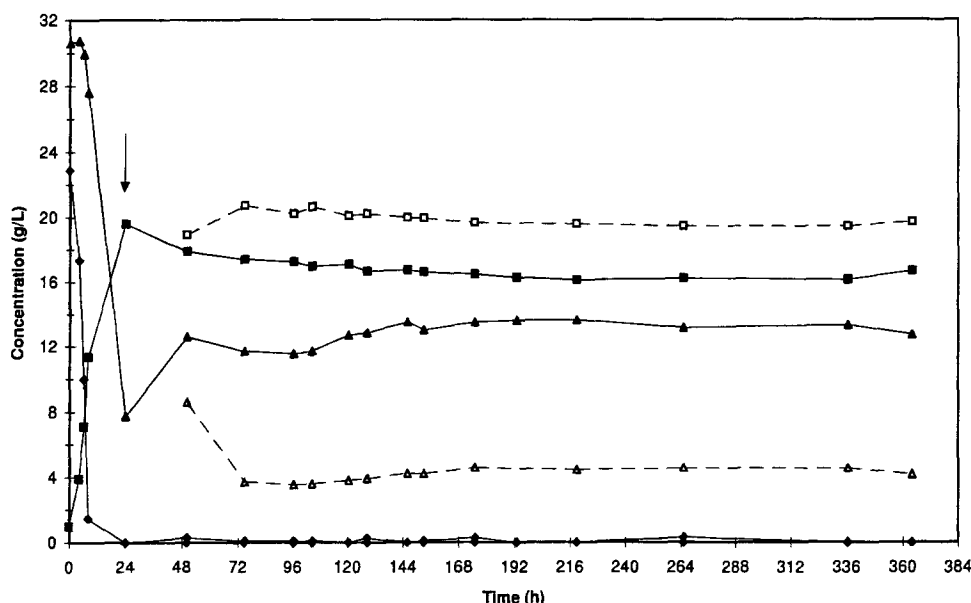


Fig. 2. Time course of the glucose (◆, ◇), xylose (▲, △), and ethanol (■, □) concentrations during the continuous two-stage pure sugar cofermentation by LNH-ST. Closed symbols and continuous lines represent the first stage, whereas open symbols and dashed lines represent the second stage. The arrow indicates the switching point from batch to continuous operation.

47.2%, respectively (Table 1). The lower metabolic yield of LNH-ST (76.5 vs 84.4% of LNH33) was a result of its higher production of glycerol and xylitol. This suggests that LNH33 was slightly more efficient, because it channels 10.5% more carbon into ethanol than LNH-ST. The major by-products (per g of consumed sugar) were carbon dioxide (0.373 g), glycerol (0.094 g), xylitol at (0.070 g), and cell mass (0.070 g) (Table 2). However, from a practical standpoint, LNH33 is a less efficient cofermenter than LNH-ST, because it fermented only 56.4% of the available sugar, whereas LNH-ST fermented 92.0%. The overall mass balance closure was 99.86% (Table 2).

SSCF of Corn Biomass by LNH-ST (Batch 1)

The pure sugar studies provided an indication of the ability of the organisms to use both xylose and glucose during the hydrolysis and fermentation of corn biomass. However, these studies were performed in the absence of metabolic inhibitors, such as acetic acid, lactic acid, hydroxymethyl furfural (HMF), furfural, and lignin-derived phenolics, which abound in biomass hydrolyzates. As the ultimate goal of this research is to examine the feasibility of large-scale conversion of pretreated corn biomass to ethanol, next studied was the ability of LNH-ST to produce ethanol during batch SSCF of pretreated corn biomass.

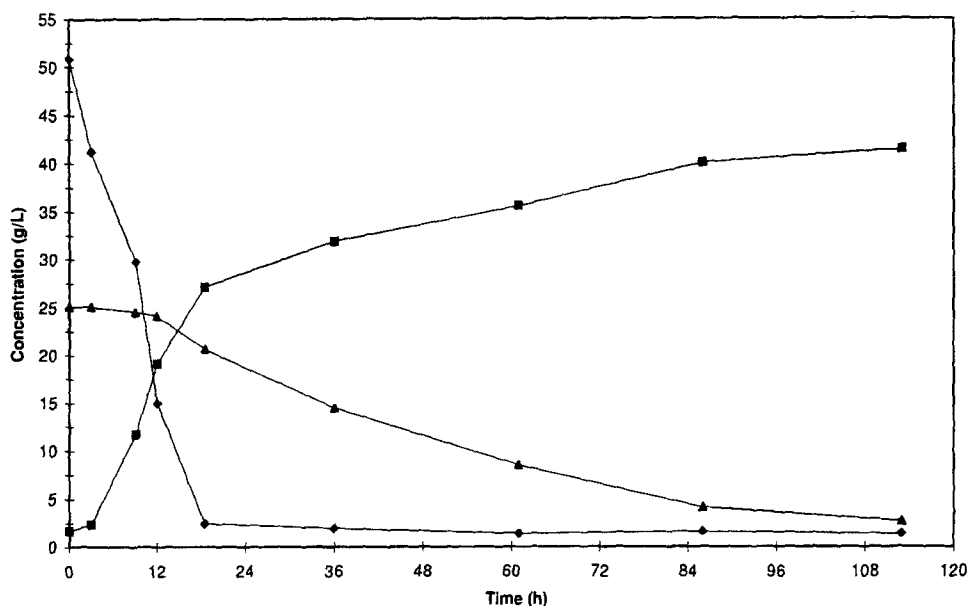


Fig. 3. Time course of the batch SSCF of pretreated com biomass (batch 1) by LNH-ST. The symbols represent the concentrations of glucose (◆), xylose (▲), and ethanol (■).

Acetic acid (2.52 g/L) was the predominant inhibitor present in the pretreated biomass slurry. Other potential inhibitors included furfural (0.18 g/L) and HMF (0.22 g/L), both of which decreased in concentration over the time course of the fermentation. The addition of cellulase was needed for enzymatic hydrolysis of cellulose polymers and oligomers, whereas the addition of glucoamylase ensured the expedient conversion of residual starch to glucose. The pretreatment process liberated 62.3 g/L of soluble glucan (50.1 g/L glucose, 2.7 g/L cellobiose, and 9.5 g/L oligomers) and 39.3 g/L of soluble xylan (28 g/L xylose and 11.3 g/L oligomers) based on 20% total solids.

Within 24 h of inoculation, the available glucose was completely consumed (Fig. 3). Afterward the rate-limiting step to glucan utilization was the liberation of glucose from cellobiose and soluble oligomers in the liquid phase and from cellulose (28.7 g/L) in the solid phase by the catalytic action of the cellulase components endoglucanase, exoglucanase, and β -glucosidase (1). The overall glucan conversion, based on the soluble and insoluble fractions, was 74.1% after 113 h of fermentation (Table 3). The cellulose conversion was 75.4%. Of the 28 g/L monomeric xylose originally available, 26 g/L was consumed, corresponding to a conversion of 92.8% (62.8% conversion of total soluble [monomeric and oligomeric] and insoluble xylan). There was no detectable conversion of soluble oligomeric or insoluble xylan. The ethanol metabolic yield was quite high, 80.5% (Table 3), or 0.41 g/g of consumed sugars.

Table 3
Performance Parameters of Pretreated Corn Biomass Conversion
to Ethanol (SSCF) by LNH-ST

Parameter	Batch 1	Batch 2
Glucose Conversion ¹	74.1%	78.4%
Xylose Conversion ²	62.8%	56.1%
Ethanol Process Yield (% theoretical)	56.9%	63.5%
Ethanol Metabolic Yield (% theoretical)	80.5%	88.1%

¹Based on total glucan as equivalent glucose.

²Based on total xylan expressed as equivalent xylose.

The by-products were similar to those observed in the two-stage continuous cofermentation of pure sugar. As shown in Table 2, glycerol was the major by-product at 0.045 g/g of consumed sugars (glucose and xylose), whereas xylitol was synthesized at 0.026 g/g of consumed sugars. The overall mass balance was closed to 92.1%.

Reproducibility and Scaleability of Corn Biomass SSCF by LNH-ST (Batch 2)

The reliability and scaleability of LNH-ST is of particular importance to the commercialization of the biomass conversion technology, since it dictates the ethanol yield and production rate of ethanol, the desired product. It was, therefore, essential to examine whether the SSCF data could be reproduced and to assess how well the bench-scale performance of LNH-ST compared with that observed at the pilot scale.

Pretreated corn biomass already mixed at 20% solids loading with the appropriate amounts of cellulase and glucoamylase enzymes, CSL, and water (to resemble the batch study) was prepared in NREL's pilot plant and employed in both the bench scale 1.7-L fermentor and the 9000-L PDU fermentor to examine the process scaleability. Again, acetic acid (4.93 g/L) was the predominant inhibitor present in the pretreated biomass slurry, with only minor amounts of furfural (0.33 g/L) and HMF (0.27 g/L). The higher level of acetic acid liberated during the dilute-acid pretreatment step, resulted from slightly more severe conditions used in the PDU pretreatment reactor. The pretreatment generated 63.1 g/L of soluble glucan (51.4 g/L glucose, 5.6 g/L cellobiose, and 6.1 g/L oligomers) and 34.6 g/L of soluble xylan (26.7 g/L xylose and 7.6 g/L oligomers) in the liquid phase, and 26.6 g/L of cellulose and 1.75 g/L of xylan remained in the solid biomass at a 20% solids level.

After 24 h of SSC in the 1.7-L fermentor, most of the available glucose was consumed (Fig. 4). The overall glucan conversion was 78.4% after 167 h of fermentation, similar to the 74.1% observed in the first batch study after 113 h of operation (Table 2). The cellulose conversion was 78.3%. Xylan utilization (56.1%) was slightly lower than that observed in the

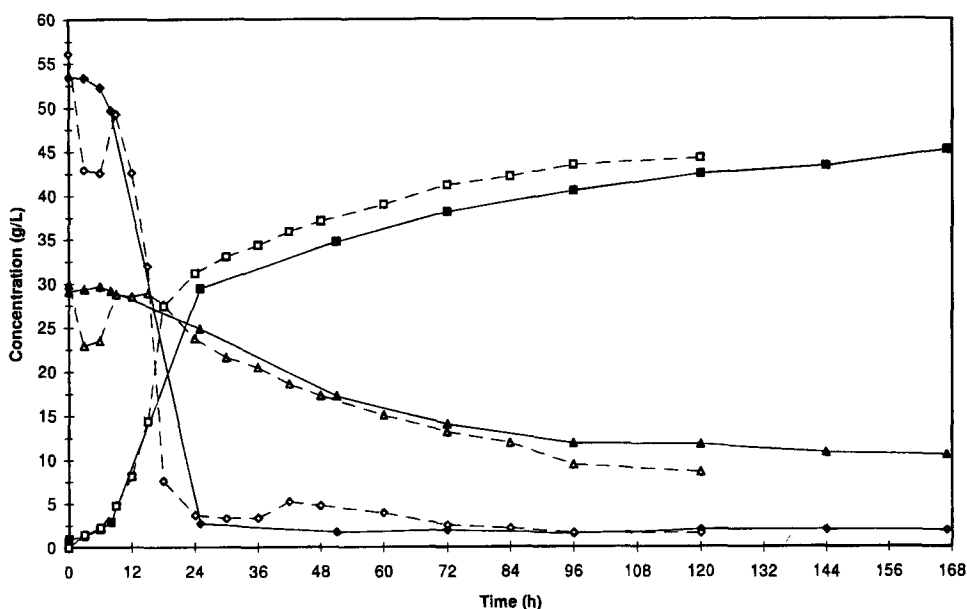


Fig. 4. Comparative study of the bench scale (closed symbols and continuous lines) and pilot scale (open symbols and dashed lines) performance of LNH-ST during the SSCF of pretreated corn biomass (batch 2). The symbols represent the concentrations of glucose (◆, ◇), xylose (▲, △), and ethanol (■, □).

previous study (62.8%). As the severity of pretreatment was the most notable difference between the two batch runs, the lower xylan utilization observed here may be because of the higher acetic acid concentration. The inhibitory effect of acetic acid on xylose utilization and ethanol production has been well documented (16,17). The ethanol process yield was 63.5%, whereas the ethanol metabolic yield was again very high at 88.1% (Table 2) or 0.45 g/g of consumed sugars. By-product formation resembled that in the first study: 5 g/L of glycerol and 2.7 g/L of xylitol. The overall mass balance closure was 100.3%.

The pretreated biomass was also subjected to SSCF in a 9000-L pilot plant fermentor under conditions similar to those at the bench scale. As seen in Fig. 4, the results obtained in the pilot plant run were similar to the bench-scale data. After 24 h of SSCF, the concentrations of glucose, xylose, and ethanol were 2.7 g/L, 24.9 g/L, and 29.4 g/L in the bench-scale fermentor and 3.7 g/L, 23.8 g/L, and 31.1 g/L in the pilot fermentor. At 96 h, the concentrations of these same components were 1.5 g/L, 11.9 g/L, and 40.5 g/L in the bench reactor and 1.7 g/L, 9.4 g/L, and 43.4 g/L in the pilot-scale reactor. Similarly, by-product formation was in close agreement, as indicated by xylitol, which reached 2.4–2.5 g/L in both vessels (data not shown). The good correlation shows that despite the 5300-fold increase in fermentor size, the performance of LNH-ST remained unchanged and very promising.

CONCLUSION

Continuous and batch studies have demonstrated that the chromosome-integrated genes of LNH-ST enable the organism to broaden its substrate range to metabolize xylose in addition to glucose. The observed high levels of glucose and xylose conversion and ethanol yield were consistently reproducible on pure sugars and corn biomass prepared under realistic process conditions. The batch corn biomass SSCF data show that more than 78% of the available glucose and cellulose and more than 56% of the available xylose were fermented by the organism within 4 d. Despite the presence of high levels of acetic acid, a metabolic inhibitor, the overall ethanol process and metabolic yields were 63.5% and 88.1%, respectively, at both the bench and pilot scales. The scaleability and reproducibility of the LNH-ST SSCF performance justify further optimization and scale-up, studies in a continuous pilot plant operation for eventual application of the recombinant cofermenting yeast at the commercial scale.

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