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Nonisothermal Simultaneous Saccharification and Fermentation for Direct Conversion of Lignocellulosic Biomass to Ethanol

ZHANGWEN WU AND Y. Y. LEE*

Department of Chemical Engineering, Auburn University, Auburn, AL 36849

ABSTRACT

The enzymatic reaction in the simultaneous saccharification and fermentation (SSF) is operated at a temperature much lower than its optimum level. This forces the enzyme activity to be far below its potential, consequently raising the enzyme requirement. To alleviate this problem, a nonisothermal simultaneous saccharification and fermentation process (NSSF) was investigated. The NSSF is devised so that saccharification and fermentation occur simultaneously, yet in two separate reactors that are maintained at different temperatures. Lignocellulosic biomass is retained inside a column reactor and hydrolyzed at the optimum temperature for the enzymatic reaction (50°C). The effluent from the column reactor is recirculated through a fermenter, which runs at its optimum temperature (20–30°C). The cellulase enzyme activity is increased by a factor of 2–3 when the hydrolysis temperature is raised from 30 to 50°C. The NSSF process has improved the enzymatic reaction in the SSF to the extent that it reduces the overall enzyme requirement by 30–40%. The effect of temperature on β-glucosidase activity was the most significant among the individual cellulase compounds. Both ethanol yield and productivity in the NSSF are substantially higher than those in the SSF at the enzyme loading of 5 IFPU/g glucan. With 10 IFPU/g glucan, improvement in productivity was more discernible for the NSSF. The terminal yield attainable in 4 d with the SSF was reachable in 40 h with the NSSF.

Index Entries: Simultaneous saccharification and fermentation (SSF); nonisothermal; column reactor; hydrolysis; ethanol; fermentation.

* Author to whom all correspondence and reprint requests should be addressed. E-mail: yylee@eng.auburn.edu

INTRODUCTION

One of the most advanced bioprocesses in fuel ethanol production is the simultaneous saccharification and fermentation (SSF) of cellulosic materials. The SSF is a single-step process in which enzymatic hydrolysis and alcoholic fermentation are carried out in a single vessel. In the SSF, the rate of hydrolysis is much lower than the rate at which the microorganism can consume glucose. The SSF therefore proceeds under glucose-limitation, and the inhibition caused by glucose and cellobiose is eliminated. Consequently, a lower enzyme loading is required *(1–4)*.

The SSF, however, has inherent problems that need to be addressed. The most significant one is the mismatch in optimum temperatures for hydrolysis and fermentation. The saccharification requires temperature of 45–50°C, and the fermentation is most efficient at 20–30°C. Since the two stages are carried out simultaneously, an SSF process is normally operated at a compromised temperature of 35–38°C. This trade-off in the temperature precludes the possibility of achieving maximum enzyme activity and the highest possible fermentation efficiency. This has been a very well-recognized problem. Substantial research effort has indeed been put forward to improve the process. Most of the research work has been focused on the identification and improvement of thermotolerant yeast or bacteria that can produce ethanol at higher temperatures *(5–10)*. This would allow hydrolysis to proceed at higher rates. From these studies, new strains that can withstand temperatures as high as 41°C have been identified. However, results from various studies indicate that thermotolerant microorganisms are less tolerant against ethanol and exhibit low productivity *(11–13)*. Furthermore the temperature range of 38–41°C is still lower than the optimum temperature for cellulases (45–50°C). Research efforts from different angles have also been made. They include changing the temperature profile *(14–15)*, varying the recipe of the enzyme, i.e., supplementing β-glucosidase *(3,16)*, further verifying of the kinetics *(17)*, increasing substrate digestibility by employing novel pretreatment methods *(18–20)*, and developing oligomeric fermenting microorganism *(21,22)*. Each proposed method has its own merits and limitations. None of them, however, has provided a feasible solution for the stated problem.

In this study, a novel bioconversion scheme that is designed to overcome the problem of temperature mismatch is introduced. A nonisothermal bioreactor is introduced to modify the SSF process. The process is named nonisothermal simultaneous saccharification and fermentation (NSSF). The key point of the NSSF is that the hydrolysis and fermentation are carried out in separate zones, yet simultaneously at their respective optimum temperatures. One zone is a column reactor in which biomass is contained and hydrolyzed at elevated temperature, and the other zone is a fermenter. Yeast cells are retained in the fermenter either by using flocculent cells or immobilized cells. A cell-free stream is recirculated

through the column reactor and the fermenter. The focus of this work was on the enzymatic hydrolysis in a packed-column reactor and the overall performance of the NSSF.

MATERIALS AND METHODS

Materials

Switchgrass supplied by the National Renewable Energy Laboratory was used as the main feedstocks. The switchgrass was pretreated with 0.078 wt% sulfuric acid at 175°C for 45 min. On dry basis, the dilute-acid pretreated substrate was analyzed to contain 59% glucan, 5% xylan, and 35% lignin and ash. A commercial α-cellulose from Sigma (St. Louis, MO) (Lot 69F-0373, 93% glucan and 5% xylan) was used as a reference substrate. The cellulase enzyme, Spezyme-CP (Lot No. 41-95034-004) was obtained from Environmental Biotechnologies, Menlo Park, CA. The specific activity of the enzyme as determined by the supplier is: filter paper activity = 64.5 FPU/mL, β-glucosidase activity = 57.6 p-NPGU/mL.

Microorganism and Media

A flocculent yeast cell, *Saccharomyces cerevisiae* (ATCC 26603), was used throughout this study. The stock culture was maintained at 4°C on agar slants containing 20 g glucose, 2 g yeast extract, 3 g malt extract, 5 g peptone, and 15 g agar/L.

SSF Operation

The flocculent yeast was transferred to a growth medium twice. The yeast cells were then grown in a liquid medium containing 2.5 g/L yeast extract, 2.5 g/L malt extract, 4.0 g/L peptone, and 20 g/L glucose at 30°C, pH 5.0, and shaken at 150 rpm. The batch SSFs were run in 250-mL Erlenmeyer flasks containing 100 mL of the fermentation medium, with shaking at 150 rpm and 35°C. The flasks were attached to a water trap to maintain anaerobic condition and to vent CO_2. The fermentation medium contained 1.5 g/L yeast extract, 1.5 g/L malt extract, and 2.5 g/L peptone. Fermentation was initiated with 5% (v/v) inocula. The same media and amount of inoculum were also applied to the NSSFs. The ethanol yield is quantified as percent of the theoretical yield. The theoretical ethanol yield is defined as 0.51 (=92/180) g ethanol/g glucose.

NSSF Design and Operation

The NSSF system consists of a fermenter, a cell settler, a heat exchanger, and a column reactor for enzymatic hydrolysis (Fig. 1). The hydrolysis column reactor is made of glass, with an internal volume of 395 mL (1.24 in. id × 14 in. L). The column was jacketed and maintained at 50°C.

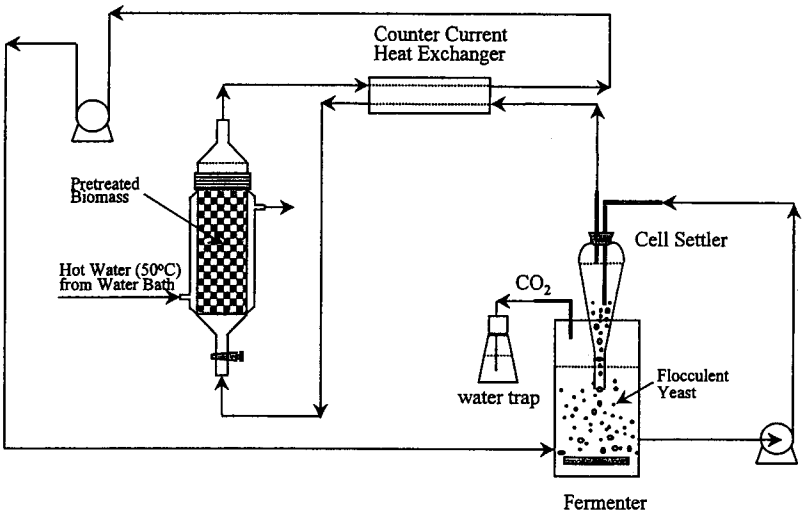

Fig. 1. Schematic diagram of NSSF process.

The cell is a steeply tapered glass tube settler (internal volume 90 mL), which resembles an inverted cone. Fermentation broth is pumped into the cell settler by a peristaltic pump in which flocculent yeast is naturally settled down and returns to the fermenter. A heat exchanger is inserted into the recirculation stream. The heat exchange occurs between the output stream from the cell setter (cold stream) and the output stream from the column reactor (hot stream). The cell-free supernatant containing cellulase enzymes is sucked into the bottom of the hydrolysis column. Lignocellulosic materials are hydrolyzed as the stream slowly passes through the column. The formed glucose is brought back to the fermenter, where it is consumed to produce ethanol. The glucose concentration is kept at a very low level in the entire system. The overall NSSF process scheme therefore retains the same feature as the SSF in eliminating the product inhibition.

Digestibility Test

Enzymatic hydrolysis was performed in 250 mL glass bottles at 50°C, pH 4.8 (0.05 *M* sodium citrate buffer). It was agitated at 150 rpm on a shaker incubator. The enzymatic digestibility is defined as (total amount of glucose released) × 0.9/total glucan. A dehydration factor of 0.9 is used to convert the glucose to glucan.

Analytical Methods

Sugars and fermentation products were analyzed by HPLC using Bio-Rad (Hercules, CA) Aminex HPX-87H column. The HPLC was operated with refractive index detector under conditions of: 0.005 *M* sulfuric acid as eluent, 0.6 mL/min, and 65°C.

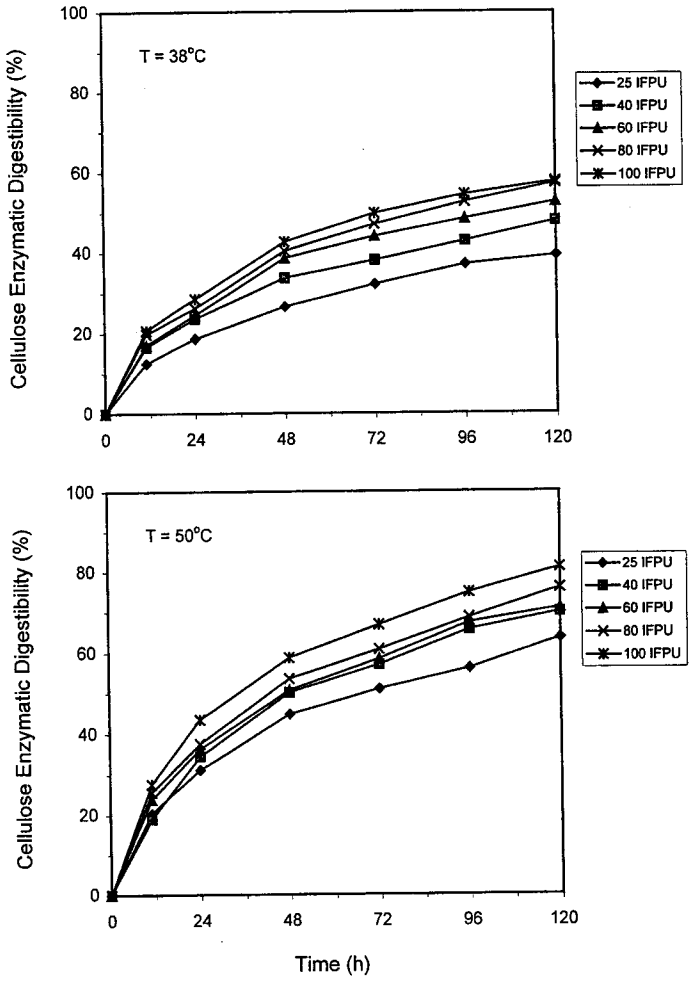

Fig. 2. Enzymatic hydrolysis of cellulose at 38°C and 50°C at various enzyme loadings. The enzyme loading is based on per gram glucan. Hydrolysis condition: 3% (w/v) cellulose, pH 4.8.

RESULTS AND DISCUSSION

Effect of Temperature on Enzymatic Hydrolysis

The effect of temperature on enzymatic hydrolysis was investigated under a typical SSF cellulose loading of 3% (w/v). Two levels of temperature were selected, 38°C and 50°C, representing the optima for the cellulases and the yeast. The effect of enzyme loading on the digestibility was tested within the range of 25–100 IFPU/g glucan (Fig. 2). At 38°C, the digestibility has increased with the enzyme loading. The maximum observed digestibility at 38°C is about 58%, occurring at 120 h, with 100 IFPU/g glucan. At 50°C, the digestibility is consistently higher than those of 38°C by 20–25% for all levels of enzyme loading. For example, at

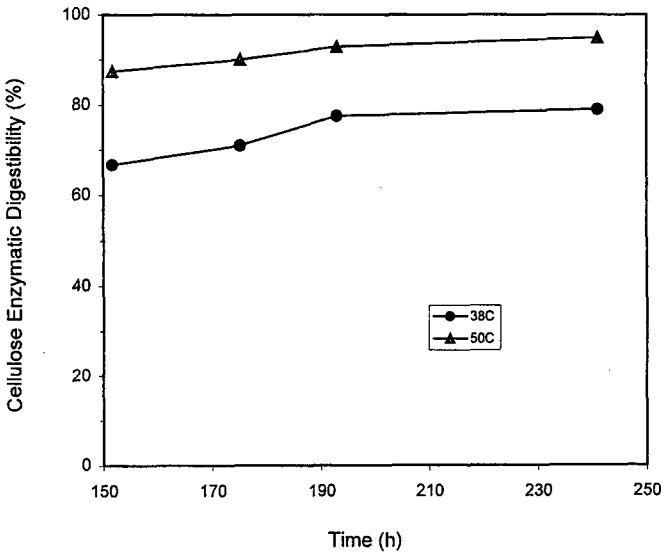

Fig. 3. Cellulose terminal enzymatic digestibility at 38°C and 50°C. Hydrolysis condition: 25 IFPU/g glucan, 3% (w/v) cellulose, pH 4.8.

120 h point, with 100 IFPU/g glucan, the yield reached 80%, a 22% increase over that of 38°C. These results indicate that reaction temperature is a prime factor determining the enzyme loading for a given conversion level. Taking a numeric example in these runs, to achieve 60% conversion at 50°C, a little less than 20 IFPU/g cellulose is needed. To achieve the same at 38°C, 100 IFPU/g cellulose is needed.

Figure 3 shows the effect of temperature on the terminal digestibility of cellulose at an enzyme loading of 25 IFPU/g glucan. At 10 d, about 90% of cellulose has been hydrolyzed at 50°C, at 38°C the digestibility is 10% lower. These results reaffirm that the temperature is an important factor controling the terminal yield of glucose as well.

From a kinetic standpoint, the initial hydrolysis rate may be a better parameter in the study of the temperature effect on the enzyme activity. The enzymatic hydrolysis was carried out under a straight batch mode at pH 4.8 using filter paper (Whatman [Clifton, NJ] No. 1) as the substrate. The initial hydrolysis rate was measured by glucose production in the first 3 h. The results in Table 1 show that the initial hydrolysis rates increase with temperature and with the enzyme loading. The initial hydrolysis rate was shown to increase by a factor of 2–3 when the temperature was raised from 30°C to 50°C. The temperature of 50°C was judged to be near the optimum for Spezyme because it was found that, at 55°C and 60°C, both hydrolysis rate and digestibility rapidly decrease.

Performance of Enzymatic Hydrolysis in a Packed-Column Reactor

The performance of the enzymatic hydrolysis in a column reactor in a recirculation mode is a key element in the NSSF process. There is no

Table 1
Effect of Temperature on Initial Hydrolysis Rate at Various Enzyme Loadings[a]

	Enyme loading (IFPU/g glucan)			
Hydrolysis temperature (°C)	5	15	25	60
30	0.25	0.51	0.85	1.36
38	0.25	0.56	0.93	1.56
45	0.70	1.39	1.56	2.03
50	0.79	1.56	1.66	2.10

[a] Hydrolysis: 1 (w/v)% glucan, pH 4.8, and Whatman No. 1 filter paper as substrate. The initial rate was defined as the average released rate of glucose and cellobiose during the first 90 min; Unit: (g/L/h).

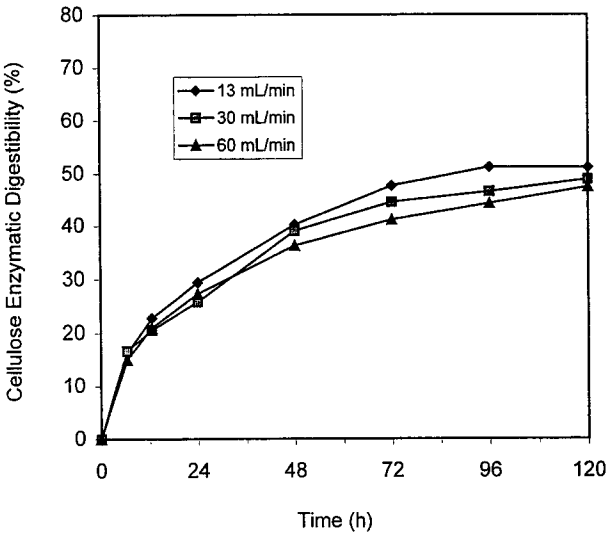

Fig. 4. Effect of recirculating liquid flow rate on cellulose enzymatic hydrolysis. Hydrolysis condition; 25 IFPU/g glucan, 50°C, pH 4.8, 3% (w/v) cellulose.

evidence in the literature that the enzymatic hydrolysis has ever been performed in a packed-column reactor in a recirculation mode. The enzymatic hydrolysis of biomass in a packed column can be affected not only by the temperature, enzyme loading, and substrate concentration, but also by the fluid dynamic conditions in the bed.

Similar to the function of agitation in the enzymatic batch hydrolysis, a certain level of liquid flow rate in the column reactor is required to facilitate the mass transfer. The effect of liquid recirculating rate on the hydrolysis was investigated over the range of 13–60 mL/min. As shown in Fig. 4, there was little difference in digestibility among the three different flow rates applied. The mass transfer or hydrodynamic characteristics appears to be consistent over this flow rate range. Also to be noted that the

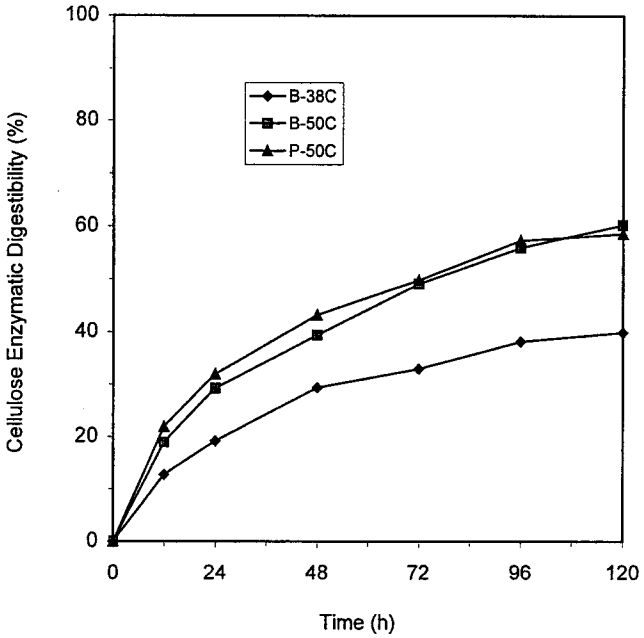

Fig. 5. Comparison enzymatic hydrolysis of cellulose in a packed-column reactor and in a straight batch process. Hydrolysis condition: 25 IFPU/g glucan, 3% (w/v) cellulose, and pH 4.8. B-38C: enzymatic hydrolysis at 38°C in a batch process. B-50C: enzymatic hydrolysis at 50°C in a batch process. P-50C: enzymatic hydrolysis at 50°C in a packed-column reactor.

recirculation needs to be applied in such a way that the liquid is sucked out of the column reactor instead of pumping the liquid through the column reactor to avoid plugging.

The hydrolysis in a packed column was first carried out using α-cellulose as the substrate. A straight batch hydrolysis at 38°C and 50°C were included as references. Average of the results from five runs was shown in Fig. 5. As seen in the figure, the enzymatic hydrolysis of cellulose at 50°C in both the packed-column reactor and the batch process showed little difference in digestibility. However, the effect of temperature was more discernible in these runs.

The performance of hydrolysis in the packed column was further tested using dilute-acid-treated hybrid poplar as the substrate. The hybrid poplar was pretreated with 0.75 wt% sulfuric acid in two stages (150°C for 10 min and 185°C for 10 min) in a percolation reactor. On dry basis, the pretreated hybrid poplar contained 64% cellulose and 33% lignin. The enzymatic hydrolysis was performed with a solid concentration of 1.55% (w/v), equivalent to 1% glucan, 50°C, pH 4.8, and an enzyme loading of 60 IFPU/g glucan. The results of hydrolysis (Fig. 6) are similar for the two modes of operation. The enzymatic hydrolysis in the column reactor has shown a higher initial rate. However, the hydrolysis in the batch mode

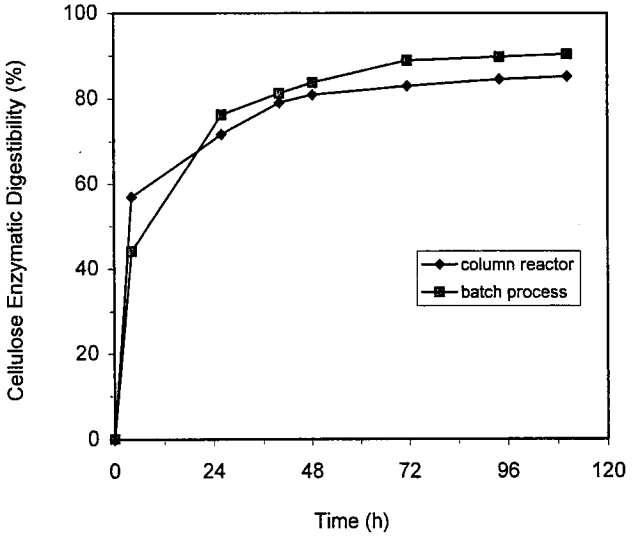

Fig. 6. Comparison of enzymatic hydrolysis of dilute-acid-treated poplar in a packed-column reactor and in a straight batch process. Pretreatment condition: 0.75 wt% sulfuric acid, 150°C, 10 min, and 180°C, 10 min, in a percolation reactor. Hydrolysis condition: 25 IFPU/g glucan, 50°C, pH 4.8, and 1% (w/v) glucan.

regains the activity at the latter phase, eventually yielding higher overall conversion at the end of the run.

Nonisothermal Simultaneous Saccharification and Fermentation

The NSSF runs have been made with enzyme loading of 5, 10, and 25 IFPU/glucan, using dilute-acid-pretreated switchgrass as the substrate. The SSF runs were carried out simultaneously as a reference under the same conditions, except the temperature. In the broth, cellobiose, xylose, and glucose were identifiable. A small amount of glycerol was also identified along with ethanol. At each level of enzyme loading, glucose was accumulated during the first 10–20 h in both process (Fig. 7). In the initial phase of the process, yeast could not consume glucose at the rate it was released by the enzyme. An exception is in the case of the SSF at 5 IFPU/g glucan enzyme loading, in which no glucose was accumulated during this period. An enzyme loading of 5 IFPU/g glucan is obviously too low oversupply glucose even in the initial phase of the SSF. At about 20 h, as the yeast cell mass increases, the glucose concentration in both SSF and NSSF declined to near zero level. From this point on, the process is limited by enzymatic hydrolysis, not by the microbial action. Xylan was also hydrolyzed into xylose. More xylan was converted in the NSSF than in the SSF process.

The advantage of the NSSF is evident in the cellobiose profile. At the enzyme loading of 25 IFPU/g glucan, the profiles are similar, the maximum

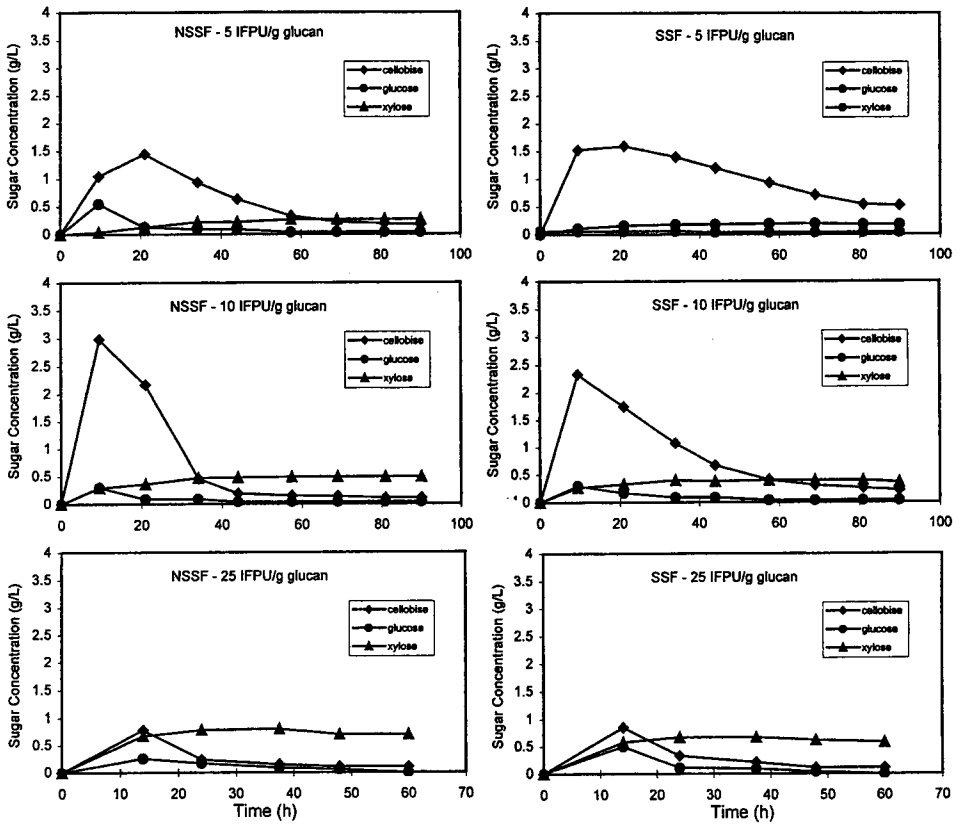

Fig. 7. Concentration profiles of sugars during NSSF and SSF of dilute-acid-pretreated switchgrass. Pretreatment condition: 0.074 wt% sulfuric acid, 170°C, and 45 min, in a batch reactor.

cellobiose concentration being less than 1 g/L for both processes. This indicates that 25 IFPU/g glucan is sufficient for both cases. With 10 and 5 IFPU/g glucan, cellobiose accumulates for both cases, the maximum cellobiose concentration reaching 3 g/L. For the NSSF process, the cellobiose concentration reached its peak value in 10–20 h, and then declined quickly to a much lower level (<0.2 g/L) at 45 h. For the SSF process, however, the cellobiose concentration profile declined gradually, then remained at a relatively high concentration for the rest of the process. Accumulation of cellobiose occurs because of insufficient β-glucosidase activity. Cellobiose is a strong inhibitor to the cellulase enzymes. It can also disrupt the synergism among the individual components of cellulase enzymes. Maintaining a low cellobiose concentration is therefore extremely important to the SSF process. Reduction of the cellobiose concentration in the SSF process can be achieved by increasing the enzyme loading or supplementing β-glucosidase. Either method, however, is uneconomical (*24*). The obvious benefit of the NSSF is that it enhances the overall enzyme activity, a merit of

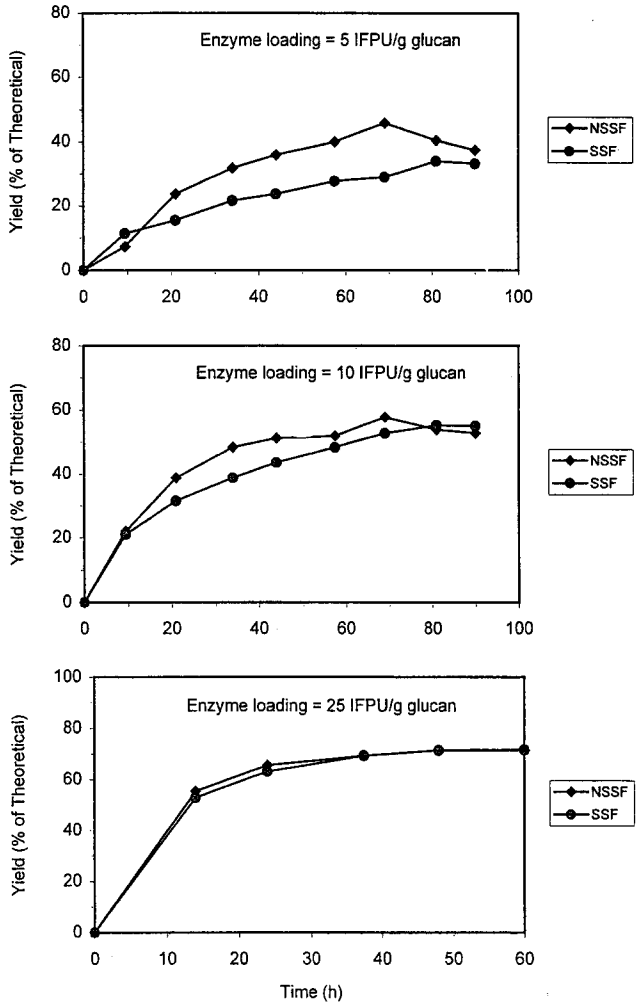

Fig. 8. Ethanol yields of dilute-acid-pretreated switchgrass at different time in NSSF and SSF. Pretreatment condition: 0.074 wt% sulfuric acid, 170°C, and 45 min, in a batch reactor.

temperature adjustment. It is also meaningful that it raises the activity of β-glucosidase to the extent that the supplementation of it is unnecessary, even at low enzyme loading.

An advantage of the NSSF over the conventional SSF is also found in the yield of ethanol (Fig. 8). At the enzyme loading of 5 IFPU/g glucan, both ethanol yield and productivity of the NSSF process were substantially higher than those of the SSF process. The difference of ethanol yield between the NSSF and SSF process varies with time. It reached to the maximum of 17% at 67 h. Unfortunately, after that point the ethanol yield in the NSSF process started to decline. It is believed that the microorganism under extremely glucose-limiting condition utilizes ethanol as a carbon

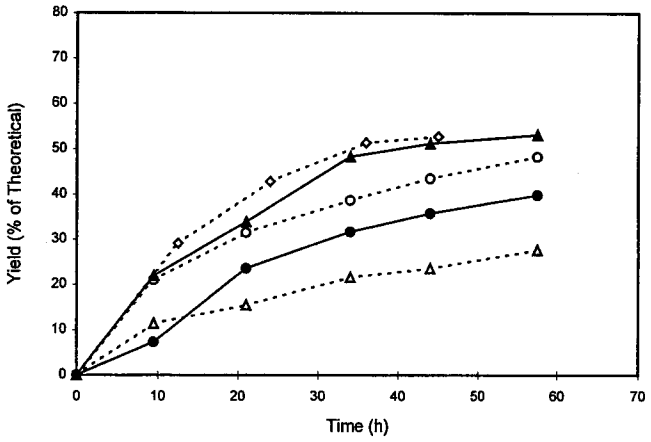

Fig. 9. Comparison of ethanol yields of dilute-acid-pretreated switchgrass in NSSF and SSF.

source. Similar phenomena were also reported by Philippidis and Smith (*25*). In the author's experience, this behavior is observed when the microorganism is under glucose limitation, and when air enters into the culture environment during the sampling process. Under this particular situation, the microorganism seems to switch its metabolism toward aerobic uptake of ethanol. Without the accidental metabolic pathway change, the difference in ethanol yield between the NSSF and SSF processes has been much higher. We also speculate that the microorganism may utilize CO_2 as a carbon source, along with ethanol. This comes from the observation that there is a back siphoning of liquid from the sealing trap into the fermenter that coincides with the microorganism starting to consume ethanol.

When the enzyme loading was increased to 10 IFPU/g glucan, the difference in ethanol yield between the SSF and the NSSF became insignificant. However, the productivity was much higher for the NSSF. For example, it took about 40 h for the NSSF to reach its terminal yield; it took the SSF process 4 d to reach the same conversion. The consumption of ethanol in the NSSF process also occurred after 70 h fermentation with the enzyme loading of 10 IFPU/g glucan. As the enzyme loading increased to 25 IFPU/g glucan, the advantage of the NSSF diminished.

The enzymatic hydrolysis is a limiting step in both SSF and NSSF process. The NSSF processes substantially increase the enzyme efficiency and consequently the productivity and product yield. To achieve a same-level conversion of lignocellulosic biomass, less enzyme is required in the NSSF than in the SSF. The evidence is shown in Fig. 9. The NSSF at an enzyme loading of 10 IFPU/g glucan achieves the ethanol yield achievable by the SSF with 15 IFPU/g glucan. Similarly, a 5 IFPU/g glucan enzyme loading in the NSSF exhibits enzyme activity equivalent to 8 IFPU/g glucan enzyme loading in the SSF. In general, the NSSF process reduces the en-

zyme requirement by 30–40%. The increase in the initial hydrolysis rate is far greater (2–3×) when the reaction temperature is increased from 30 to 50°C. The data collectively support the original claim of the NSSF that the enzyme efficiency and the productivity could significantly improved. Occasional difficulty was encountered in operation of the NSSF using powder cellulose as the substrate. The fine particles have a tendency of forming dense slurry cake, eventually causing channeling or clogging. Special care needs to be taken to cope with this problem in the design and operation of a NSSF reactor, or a recirculation packed-bed column reactor, in general.

CONCLUSION

The focus of this work was placed on the validation of two main items: the enzymatic hydrolysis in a column reactor under a recirculation mode, and the advantage of the nonisothermal SSF process. The data on enzymatic hydrolysis of lignocellulosic substrates prove that the performance of column hydrolysis is essentially the same as the stirred-batch process. The initial enzymatic hydrolysis rate is increased by a factor of 2–3 when the hydrolysis temperature is raised from 30 to 50°C. The optimum temperature for enzymatic hydrolysis of cellulose is about 50°C. Further increase in temperature beyond 50°C resulted in decrease of glucose yield.

The NSSF reactor system proposed in this work can be operated under a stable condition. Experimental results on the NSSF prove that it is a feasible engineering solution to a well-known problem associated with the SSF; the mismatch of temperatures for hydrolysis and fermentation. The NSSF process improves the enzymatic reaction in the SSF. It reduces the enzyme requirement by 30–40%. The increase in the β-glucosidase activities in the NSSF is particularly meaningful, in that it negates the cellobiose inhibition.

REFERENCES

1. Abe, S. and Takagi, M. (1991), *Biotechnol. Bioeng.* **37,** 93–96.
2. Grohmann, K. (1993), in *Bioconversion of Forest and Agricultural Plant Residues*, Saddler, J. N., ed., CAB International, Wallingford, pp. 183–210.
3. Spindler, D. D., Wyman, C. E., and Grohmann, K. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 773–786.
4. Hinman, N. D., Schell, D. J., Riley, C. J., Bergeron, P. W., and Walter, P. J. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 639–649.
5. Ballesteros, I., Ballesteros, M., Cabanas, A., Carrasco, J., Martic, C., Negro, M. J., Saez, F., and Saez, R. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 307–315.
6. Ballesteros, I., Oliva, J. M., Ballesteros, M., and Carrasco, J. (1993), *Appl. Biochem. Biotechnol.* **39/40,** 201–211.
7. Spindler, D. D., Wyman, C. E., Mohagheghi, A., and Grohmann, K. (1988), *Appl. Biochem. Biotechnol.* **17,** 279–293.
8. Spindler, D. D., Wyman, C. E., and Grohmann, K. (1989), *Biotechnol. Bioeng.* **34,** 189–195.
9. Barron, N., Marchant, R., McHale, L., and McHale, A. P. (1995), *Appl. Microbiol. Biotechnol.* **43,** 518–520.

10. Ward, C., Nolan, A. M., O'Hanlon, K., McAree, T., Barron, N., McHale, L., and McHale, A. P. (1995), *Appl. Microbiol. Biotechnol.* **43,** 408–411.
11. Viikari, V. L., Nybergh, P., and Linko, M. (1980), In *Advances in Biotechnology*, vol. 2, Moo-Yooung M., ed., Pergamon, New York, 137–142.
12. Spindler, D. D. and Emert, G. H. (1986), *Biotechnol. Bioeng.* **28,** 115–118.
13. Saddler, J. N., Mes-Hartree, M., Yu, E. K. C., and Brownell, H. H. (1983), *Biotechnol. Bioeng. Symp.* **13,** 225–238.
14. Oh, K. K., Kim, T. Y., Jeong, Y. S., and Hong, S. I. (1996), in *Renewable Energy*, vol. 2, Sayigh, A. A. A., ed., Pergamon, New York, 962–970.
15. Huang, S. Y. Chen, C. J. (1988), *J. Fer. Technol.* **66,** 509–516.
16. Spindler, D. D., Wyman, C. E., Grohmann, K, and Mohagheghi A. (1989), *Appl. Biochem. Biotechnol.* **20/21,** 529–540.
17. Philippidis, G. P., Spindler, D. D., and Wyman, C. E. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 543–556.
18. Shah, M. M., Song, S. K., Lee, Y. Y., and Torget, R. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 99–109.
19. Torget, R., Hatzis, C., Hayward, T. K., Hsu, T.-A., and Philippidis, G. P. (1996), *Appl. Biochem. Biotechnol.* **58/59,** 85–101.
20. Wu, Z. and Lee, Y. Y. (1997), *Appl. Biochem. Biotechnol.* **63/65,** 21–34.
21. Mamma, D., Koullas, D., Fountoukids, G., Kekos, D., Macris, B. J., and Koukios, E. (1995), AIChE Annual Meeting, Miami Beech, FL.
22. Katzen, R. and Fowler, D. E. (1994), *Appl. Biochem. Biotechnol.* **45/46,** 697–707.
23. Wright, J. D., Power, A. J., and Douglas, L. J. (1987), *Biotechnol. Bioeng. Symp.* **17,** 285–302.
24. Hinman, N. D., Schell, D. J., Riley, C., J., Bergeron, P. W., and Walter, P. J. (1992), *Appl. Biochem. Biotechnol.* **34/35,** 639–649.
25. Philippidis, G. P. and Smith, T. K. (1995), *Appl. Biochem. Biotechnol.* **51/52,** 117–124.
26. Iyer, P. V., Wu, Z., Kim, S. B., and Lee, Y. Y. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 121–132.