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# Process Improvement in Acetone-Butanol Production from Hardwood by Simultaneous Saccharification and Extractive Fermentation

MINISH M. SHAH AND Y. Y. LEE*

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

## ABSTRACT

The acetone-butanol production by simultaneous saccharification and extractive fermentation (SSEF) was investigated. In the SSEF employing cellulase enzymes and *Clostridium acetobutylicum*, both glucan and xylan fractions of pretreated aspen are concurrently converted into acetone and butanol. Continuous removal of the fermentation products from the bioreactor by extraction was an important factor that allowed long-term fed-batch operation. The use of membrane extraction prevented the problems of phase separation and extractant loss. Increase in substrate feeding as well as reduction of nutrient supply was found to be beneficial in suppressing the acid production, thereby improving the solvent yield. Because of prolonged low growth conditions prevalent in the fed-batch operation, the butanol-to-acetone ratio in the product was significantly higher at 2.6–2.8 compared to the typical value of two.

**Index Entries:** Acetone-butanol; hardwood; *Clostridium acetobutylicum*; simultaneous saccharification and fermentation; extraction.

## INTRODUCTION

Simultaneous saccharification and fermentation (SSF) is a viable process option applicable for direct bioconversion of cellulosic materials into fuels and chemicals (*1*). The SSF reduces the end-product inhibition on

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

cellulase enzyme, and eliminates the need for two separate reactors for enzymatic hydrolysis and fermentation (*1*). When the SSF process is applied for the production of acetone-butanol from pretreated hardwood, the bioconversion capacity and the final product concentration are severely limited because of the inhibition of butanol on *Clostridium acetobutylicum* (*2,3*). To alleviate this problem, we have developed an integrated process comprised of SSF and product separation by extraction (*4*). This integrated process was termed simultaneous saccharification and extractive fermentation (SSEF). In the SSEF process, both cellulose and hemicellulose fractions of pretreated hardwood were hydrolyzed into glucose and xylose, respectively, by cellulase enzymes. These sugars were then concurrently converted into acetone, butanol, and ethanol (*4*). Because of on-line product removal in the SSEF process, the culture could be kept viable over a long period of time, and a fed-batch operation became feasible (*4*).

In normal SSEF process, the enzymatic hydrolysis of cellulose is the rate-limiting step. The microbial process thus proceeds under glucose limitation. Because of its multibranched fermentative pathway, *C. acetobutylicum* produces various products in different proportions depending on carbon and electron fluxes through the cells. Under glucose limitation, the production of acetic and butyric acids increases at the expense of acetone and butanol (*5–7*). In our previous investigation, it was evident that the acid production increases toward the end of a fed-batch operation (*4*). Typically, a solvent yield of 28% (w/w) was obtained in the SSEF. When extractive fermentation of glucose was carried out at a high-glucose supply rate, a solvent yield of 34–35% (w/w) was attainable (*8*). In order to achieve higher solvent yield in the SSEF process, the production of acids must be suppressed so that most of the substrate is channeled into the solvent production.

This investigation was undertaken to improve the SSEF process previously developed in our laboratory, especially the yield of solvents. We were primarily interested in the substrate feeding rate (or the sugar supply rate), which may influence the bioenergetics of the cells, and the supply rate of nonglucose nutrients, which may affect the physiological status of the cells.

## MATERIALS AND METHODS

### Microorganism and Medium

*Clostridium acetobutylicum* ATCC 824, was used throughout this research. The growth medium contained the following components in 1 L of distilled water: $KH_2PO_4$ 0.75 g, $K_2HPO_4$ 0.75 g, $MgSO_4 \cdot H_2O$ 0.4 g, $MnSO_4 \cdot H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, L-cysteine 0.5 g, yeast extract 5 g, asparagine $\cdot H_2O$ 2 g, $(NH_4)_2SO_4$ 2 g. Each SSEF experiment was initiated using the above nutrient medium supplemented with pretreated hardwood at 50

g/L. The nutrient medium at six times the above concentration was added to the bioreactor intermittently during a fed-batch operation. Pretreated hardwood was also added intermittently to the bioreactor. The supply of nutrients relative to the supply of sugars during a fed-batch operation was measured on the basis of a nutrient-to-sugar ratio used in the batch fermentation. For this purpose, the nutrient fraction ($F_n$) was defined as follows:

$$F_n = (N/S \text{ in SSEF} / N/S \text{ is a typical batch fermentation}) \quad (1)$$

where $N/S$ is the ratio of nutrients to sugars. For example, $F_n = 0.5$ indicates that the ratio of the overall supply of nutrients to the overall supply of sugars during the entire fed-batch run was 50% of the ratio normally employed in the batch fermentation of glucose. In a typical batch fermentation, $N/S$ was total amount of nutrients in the above list divided by 60 g glucose.

## Substrates and Enzyme

Aspen chips of 0.16–0.64 mm nominal size were pretreated with 20% aqueous monoethanolamine (MEA) solution at 186°C for 3 h (2). The pretreated aspen contained 66.5% (w/w) glucan and 24.5% (w/w) xylan. Cellulase enzyme (85 IFPU/mL), a product of Genencor, Inc. (San Francisco, CA) was used for saccharification of polysaccharides. This enzyme exhibited cellulase and xylanase activities.

## Simultaneous Saccharification and Extractive Fermentation

The details of the apparatus and operating procedure have been previously described (4). Briefly, simultaneous saccharification and fermentation was initiated by adding the cellulase enzyme and *C. acetobutylicum* to the bioreactor. When butanol level in the broth reached 6–8 g/L, the extraction was begun by circulating oleyl alcohol through a silicone membrane tubing. The pretreated chips (dried and sterilized) and cellulase enzyme were added to the bioreactor intermittently at 10–14-h intervals. The dried chips were fed into the bioreactor to keep the liquid volume essentially constant. Concentrated growth medium was also added to the bioreactor at 24-h intervals. Samples were taken to keep the working volume in the bioreactor at constant level. If it is assumed that the feeding and bleeding were done in a continuous manner, the dilution rate would be a meager 0.0005 $h^{-1}$.

## Analytical Methods

Fermentation products and sugars were analyzed as described previously by GC and HPLC, respectively (4,9). To determine the concentration of cells, the sample was first centrifuged at 300 rpm to settle wood

Table 1
Effect of Substrate Feeding Rate
and Nutrient Supply on Yield of Products in Runs 1-3[a]

| Run # | $F_n$ | Average substrate feeding rate g/L/h | Solvent yield | Acid yield |
|---|---|---|---|---|
| 1 | 1.0 | 0.32 | 23.7 | 9.9 |
| 2 | 0.5 | 0.32 | 27.5 | 5.9 |
| 3 | 1.0 | 0.47 | 28.2 | 5.1 |

[a]Yield—g product formed/100 g sugars consumed.

fibers. The supernatant was then diluted with water by a factor of 250, and the turbidity was measured in Nephelometric Turbidity Units (NTUs) on a turbidimeter (Hach model 2100A). The following correlation between cell concentration and turbidity was obtained from the fermentation run using glucose:

$$\text{Cell concentration (g/L)} = \text{Turbidity (NTU)} \times 0.85 \quad (2)$$

## RESULTS AND DISCUSSION

Initially, three SSEF runs were carried out at different substrate feeding rates and nutrient-to-sugar ratios. The substrate feeding interval was 30–50 h, and overall enzyme loading was 16 IFPU/g substrate. Table 1 summarizes the product yield data for these runs. In run #1, the average substrate feeding rate was 0.32 g/L/h, and $F_n$ was 1.0. The low rate of sugar supply was believed to be the reason for the high acid contents in the product. *C. acetobutylicum* is known to produce acids when the supply of sugars is low (*5–7*). In run #2, the average substrate feeding rate was 0.32 g/L/h, and $F_n$ was reduced to 0.5. In this run, the acid yield was significantly reduced to 5.9% (w/w), and the solvent yield increased to 27.5% (w/w). An ensuing run (#3) was made in which $F_n$ was kept at 1, and the substrate feeding rate was increased to 0.47 g/L/h. The solvent yield in this run improved to 28.2% (w/w), whereas the acid yield decreased to 5.1% (w/w). These results confirmed that the substrate feeding rate and nutrient-to-sugar ratio were key factors influencing the yield of products. The data reaffirmed a previous finding by Fond et al. that the rate of sugar uptake by cells (or catabolic flux) has a strong influence on the metabolism of cells and, hence, distribution of products (*6*). Maintaining a sufficient rate of hydrolysis in the SSEF is therefore important not only in keeping the process viable, but also in achieving high solvent yield.

Table 2
Product Yields in SSEF (Runs 4–6)[a]

| | Run 4 | Run 5 | Run 6 |
|---|---|---|---|
| $F_n$ | 0.5 | 0.35 | 0.35 |
| Average substrate feeding rate (g/L/h) | 0.65 | 0.65 | 0.95 |
| Butanol | 21.9 | 22.9 | 23.3 |
| Acetone | 8.2 | 8.1 | 8.9 |
| Ethanol | 1.4 | 1.5 | 1.7 |
| Total solvents | 31.5 | 32.5 | 33.9 |
| Butyric acid | 2.3 | 1.6 | 0.9 |
| Acetic acid | 2.4 | 1.7 | 1.4 |
| Total acids | 4.7 | 3.3 | 2.3 |
| Butanol/acetone ratio | 2.67 | 2.83 | 2.6 |
| Butanol/ethanol ratio | 16.1 | 15.2 | 13.6 |

[a]Yield—g product formed/100 g sugars consumed.

With the enzyme loading normally applied to the SSEF, the rate of enzymatic hydrolysis is slower than the rate at which cells can consume sugars. In runs 1–3, the acid yield was higher than or equal to 5% (w/w). As a result, the solvent yield was no higher than 28% (w/w). Low solvent yields have been reported for the fermentation carried out under a glucose-limited condition (*5,6*). In continuous fermentation using glucose, solvent yield as high as 34% (w/w) was obtainable, but only with sufficient sugar supply (*8*). For the SSEF process to be effective, an inherent disadvantage of sugar limitation must be overcome.

In the subsequent runs (#4–6), the substrate feeding interval was reduced to 10–14 h, and overall enzyme loading was 14 IFPU/g substrate. In addition, the overall substrate feeding rate was increased, and the nutrient-to-sugar ratio was reduced. Table 2 summarizes the product yield data for these runs. Run #4 was carried out at a substrate feeding rate of 0.65 g/L/h and $F_n$ value of 0.5. Figure 1 shows the concentration profiles of cells, sugars, and products. The cell concentration reached 9 g/L in the first 200 h. Glucose and cellobiose were not detected for most of the run. Acetic acid concentration increased in the latter phase of the run, reaching a final value of 4 g/L. Butyric acid concentration remained between 1.5 and 2 g/L. The overall solvent yield in run #4 (Table 2) was significantly higher than that of run #3 (Table 1). The improved solvent yield in run #4 can be attributed to reduction in the nutrient supply and increase in substrate feeding rate (Tables 1 and 2).

Run #5 was carried out with a substrate feeding rate of 0.65 g/L/h, and $F_n$ value of 0.35. The concentration profiles are given in Fig. 2. A notable

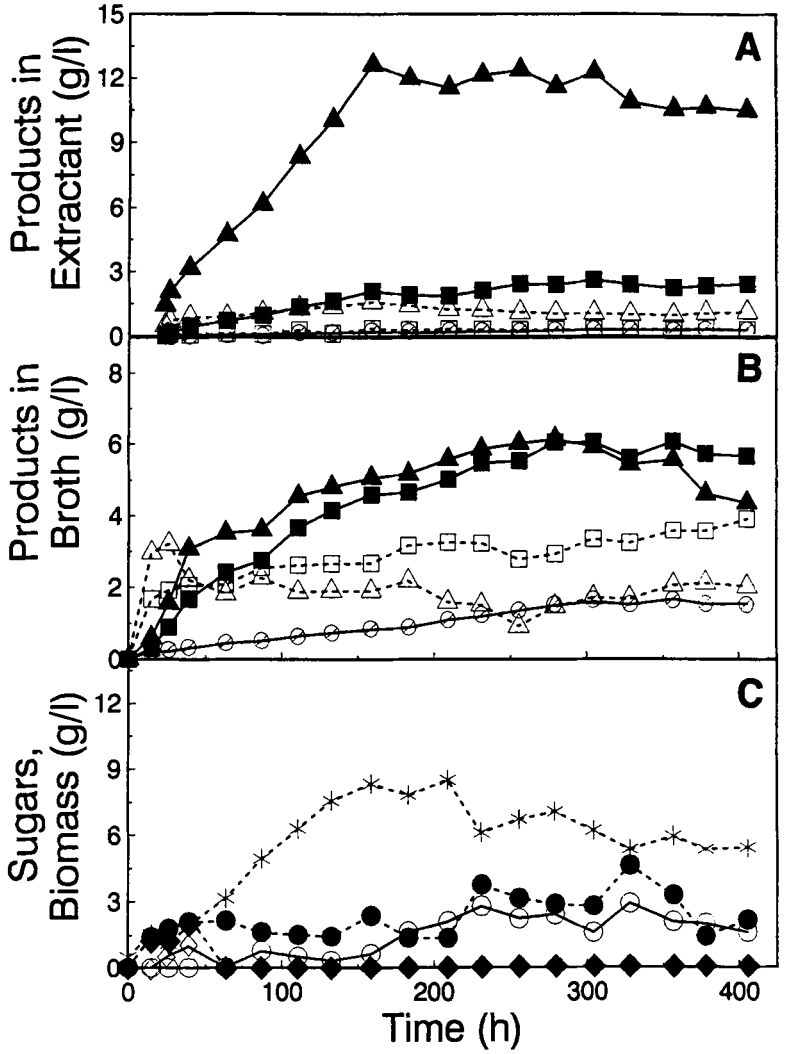


Fig. 1. Time-course of fed-batch SSEF of pretreated hardwood (Run #4); average substrate feeding rate 0.65 g/L/h, cellulase 14 IFPU/g substrate, $F_n$ = 0.5. Legends for panels A and B: —∅— ethanol; —■— acetone; —▲— butanol; --□-- acetic acid; --△-- butyric acid. Legends for panel C:--◆-- cellobiose;—◇— glucose; --●-- xylobiose; —○— xylose; --*-- biomass.

change observed in this experiment was that the increase in the cell concentration was much slower compared to that in run #4 (Figs. 1C and 2C). In the first 200 h of the run, cell concentration reached 6.5 g/L, which was 27% lower than the value attained in run #4. Lower cell growth in this run seems to be the result of lower nutrient supply compared to that in run #4 and increased butanol inhibition compared to that in run #4 because of higher concentration of butanol in run #5. The concentrations of xylose and xylobiose in this run were quite low, not exceeding 3 g/L for the first

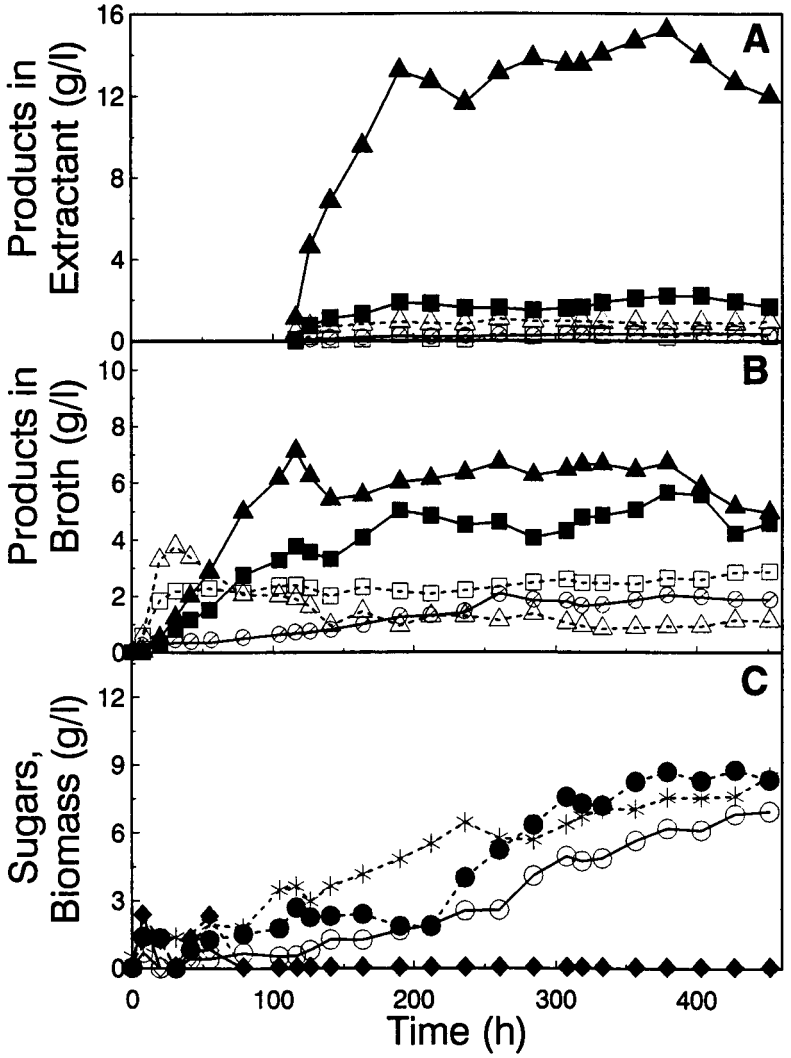


Fig. 2. Time-course of fed-batch SSEF of pretreated hardwood (Run #5); average substrate feeding rate 0.65 g/L/h, cellulase 14 IFPU/g substrate, $F_n$ = 0.35. Legends same as in Fig. 1.

200 h of operation, and then gradually increased thereafter. In this experiment, the butanol concentration in the broth remained below 7 g/L, and the acetic acid below 3 g/L (Fig. 2B). The acetic acid concentration increased more slowly (Fig. 2B) than in the previous run (Fig. 1B). The butyric acid concentration also remained at a low level. The yield of acids in run #5 was 30% lower than that in run #4 (Table 2). The low acid production seems to be related with lower cell production since acid production occurs mostly during exponential growth phase of the cells. Consequently, the solvent yield was improved to 32.5% (w/w).

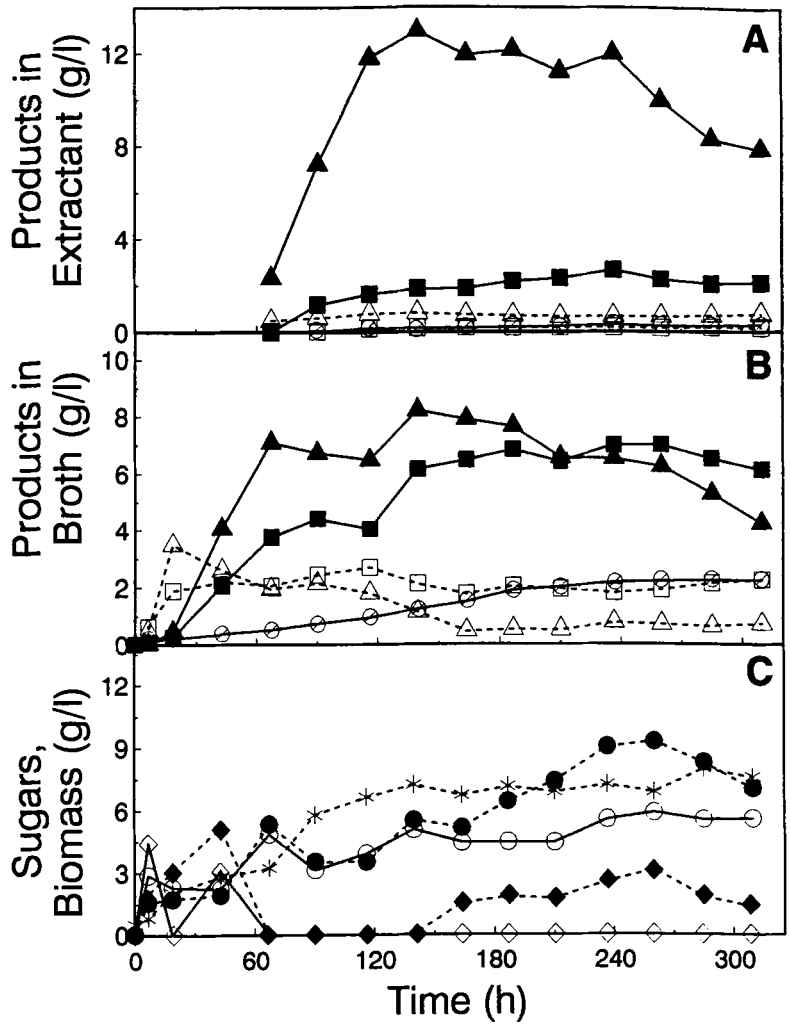


Fig. 3. Time-course of fed-batch SSEF of pretreated hardwood (Run #6); average substrate feeding rate 0.95 g/L/h, cellulase 14 IFPU/g substrate, $F_n$ = 0.35. Legends same as in Fig. 1.

In run #6, the $F_n$ value was kept at the same level (0.35) as in run #5, whereas the substrate feeding rate was increased to 0.95 g/L/h. The concentration profiles for this run are shown in Fig. 3. Most noticeable in this run was a significant reduction in the acid production. The acetic acid concentration in the bioreactor did not exceed 2 g/L (Fig. 3B), which was the lowest value observed in all SSEF runs. The concentration of butyric acid in the bioreactor was also decreased to 0.5–0.7 g/L. The terminal acid yield decreased to 2.3% (w/w), which was 30% lower than that in run #5. The overall solvent yield for run #6, in turn, increased to 33.9% (w/w) (Table 2). The solvent productivity as well as the substrate utilization rate were also higher in run #6 than in run #5.

These results collectively indicate that the substrate feeding rate is an acute factor influencing acid production and solvent yield. We offer the following explanation for this correlation. With low sugar supply, the cells may face a situation where ATP produced by the glycolysis pathway is insufficient to meet their energy needs. The cells then promote acid production to provide extra ATP. On the other hand, with ample sugar supply, the cells generate sufficient ATP by glycolysis without resorting to the production of acetic or butyric acid.

A decrease in the nutrient supply was also found to influence the solvent yield. It has been reported that the solvent yield is improved under phosphate limitation (*10*) or ammonium limitation (*11*). The reasoning here is quite simple. With a limited supply of nutrients, cell growth is suppressed, and more substrate is channeled into product formation. In addition, since acids are produced mostly during the growth phase, reduction of cell growth would decrease acid production, thus making more sugars available for solvent production.

In fact, both these parameters have similar impact on the productivity of the microbial cells. When cell concentration is kept constant and substrate feeding rate is increased, the specific sugar supply rate increases. Similarly, when substrate feeding rate is kept constant and nutrient supply is decreased, cell concentration decreases; hence, the specific sugar supply rate increases. Thus, both increase in substrate feeding rate and decrease in nutrient-to-sugar ratio increased cell-specific sugar supply rate. The increase in solvent yield is, therefore, attributed to the increase in cell-specific sugar supply rate.

## Consumption of Glucan and Xylan in SSEF

Glucose was not detectable in the broth for the most part of all the SSEF runs, which indicates that the culture was under glucose limitation. In the absence of glucose, the metabolism of nonglucose sugars was triggered, thus enabling the *C. acetobutylicum* cells to utilize cellobiose, xylose, and xylobiose concurrently along with glucose (Figs. 1–3). If the cells were subjected to a mixture of glucose and xylose, they will preferentially consume glucose over xylose. In the SSEF process, however, such diauxic consumption was avoided because of glucose-limited condition. This was a unique and beneficial feature of the SSEF process.

Table 3 provides data on carbohydrate hydrolysis and consumption in the SSEF process. The extent of hydrolysis of both glucan and xylan was very high at 94–96% (Table 3). These values were somewhat lower than the value of 99–100% obtained in our previous batch SSF experiments (*3*). The difference may be the result of the physical characteristics of the substrate. In batch SSF runs, the substrate was added to the bioreactor in the wet form (*3*), whereas in the SSEF runs, oven-dried chips were used. The dried chips became solubilized into the broth rather slowly, some of

Table 3
Extent of Carbohydrate Hydrolysis and Consumption in SSEF (Runs 4–6)

| | | Run 4 | Run 5 | Run 6 |
|---|---|---|---|---|
| % Hydrolyzed | Glucan | 94.6 | 93.7 | 95.7 |
| | Xylan | 95.3 | 95.8 | 97.7 |
| % Consumed | Glucan | 100.0 | 99.9 | 99.0 |
| | Xylan | 92.9 | 76.4 | 80.7 |
| % Overall utilized | Glucan | 94.6 | 93.6 | 94.7 |
| | Xylan | 88.5 | 73.2 | 78.8 |

them floating at the top. It seems that glucan and xylan in the floating chips could not be hydrolyzed by cellulase.

The efficiency of *C. acetobutylicum* in utilizing soluble sugars of glucan approached the theoretical maximum (Table 3). The microorganism was able to consume all of the glucose and cellobiose available. Only in run #6 was some cellobiose left unused in the bioreactor. This may be because of a high substrate feeding rate applied in that particular run. The microorganism also consumed the majority of sugars derived from xylan. In run #4, the microorganism was able to consume 93% of the available xylose, resulting in an overall xylan utilization of 88.5% (Table 3). However, the xylan consumption was somewhat lower at 76 and 80% in runs 5 and 6, respectively. An overall utilization of xylan in runs 5 and 6 was 73 and 79%, respectively.

From observation of the concentration profiles of xylose, xylobiose, butanol, and acetic acid, in runs 4–6, it appears that the utilization of xylose and xylobiose is affected by acetic acid and butanol present in the bioreactor as well as the sugar supply rate. In run #4, for the first 200 h of operation, the butanol concentration was lower than 6 g/L and xylose, and xylobiose, concentration remained below 3 g/L. The levels of xylose and xylobiose were higher at 3–6 g/L in a subsequent period between 200 and 350 h (Fig. 1C). During that period, the butanol level in the bioreactor was also higher at about 6 g/L (Fig. 1B). Moreover, the acetic acid, a potential inhibitor to *C. acetobutylicum* (*12*), also started increasing in that time span. Butanol and acetic acid appears to inhibit the uptake of xylose at concentrations lower than those for the glucose uptake. Butanol has been shown to inhibit the xylose uptake completely at 8 g/L (*13*).

In run #5, the xylose and xylobiose levels remained below 3 g/L for the first 200 h of operation (Fig. 2C). Thus, the xylose uptake was quite high during the first half of the experiment. However, in the latter phase of the run, both xylose and xylobiose began accumulating in the bioreactor (Fig. 2C). Final concentrations of these sugars were 6–9 g/L, and butanol concentration was between 6 and 7 g/L in the latter phase of run #5 (Fig.

2). Similarly, it is evident from the concentration profiles in run #6 (Fig. 3) that whenever butanol concentration was increased, the concentration of xylose and xylobiose also increased. The accumulation of xylose and xylobiose of 6–9 g/L in run #6 is most likely linked to butanol in the broth that was high enough to inhibit the xylose uptake. The results of runs 4, 5, and 6 indicate that the inhibition threshold for xylose uptake is 6 g butanol/L.

The results of these runs also suggest that the xylose uptake is affected by the sugar supply rate. When the rate of glucose available to microbial cells is lower than the rate at which they can metabolize it, they are able to consume xylose to satisfy their metabolic need. The cells, however, will stop consuming xylose when all of their metabolic needs are satisfied by glucose alone. In run #4, the cells grew rapidly, attaining high cell concentration and creating a high capacity to consume sugars. Therefore, concentrations of all the sugars remained low. In run #5, however, the cell growth was rather gradual, eventually reaching a moderately high level of cell concentration. Therefore, the capacity of the culture to consume sugars fell to a much lower level than that in run #4. Because cells will preferentially consume all the glucose available to them, their capacity to consume nonglucose sugars might have been lower in run #5 than in run #4. In run #6, the rate of substrate feeding was increased by 50% over that in run #5. This apparently satisfied the metabolic demand of the cells from glucose alone as evidenced by accumulation of cellobiose, xylose, and xylobiose toward the end of run #6 (Fig. 3C). These results are in accordance with the findings of Fond et al. (*6*).

## Butanol-to-Acetone Ratio

The data on butanol-to-acetone ratio in runs 4–6 (Table 2) draw a notable point. This ratio was between 2.6 and 2.8, which is substantially higher than a typical value of two found in batch fermentation. Since butanol commands a higher market price than acetone, this would be an important economic factor. The increase in a butanol-to-acetone ratio seems to be related with prolonged stationary phase of the fed-batch operation. We have observed that this ratio steadily increases with progression of the fed-batch operation. The following is our reasoning for this phenomenon. The hydrogenase enzyme activity in *C. acetobutylicum* cells is suppressed during the stationary phase (*14*). Consequently, hydrogen production is decreased, and more reducing energy in the form of NADH is produced. The production of butanol would then be the only way for the cells to regenerate NAD. The ethanol production can also regenerate NAD. Ethanol yield, however, did not increase (Table 2), probably because of a limited activity of the ethanol-producing enzymes in *C. acetobutylicum*. Thus, cells maintained in a stationary phase enhanced butanol production.

High butanol production may also be linked to continuous removal of butanol from the broth by extraction (*9,15*). The removal of butanol negates

any inhibitory effect on the butanol-producing enzymes. However, a reduction in hydrogenase activity must also take place in order to make sufficient reducing energy available for butanol formation. The following evidence suggests that the latter constitutes a more plausible explanation for high butanol-to-acetone ratio. The butanol concentration in run #6 was considerably higher than that in run #4. However, butanol-to-acetone ratios in these two runs were very close. If reduced inhibition on butanol-producing enzymes was a main factor for higher butanol production, then butanol-to-acetone ratio in run #6 should have been significantly lower. Literature information is abundant that the hydrogenase enzyme plays a major role in controlling the butanol-to-acetone ratio. An inhibition of the hydrogenase activity by carbon monoxide resulted in an increase in the butanol-to-acetone ratio (*16*). Junelles et al. were able to increase the butanol-to-acetone ratio under iron limitation (*17*). Because hydrogenase and acetoacetate decarboxylase (involved in the acetone formation) enzymes contain iron, its limitation reduces the synthesis of these enzymes, and thus hydrogen and acetone production.

The SSEF, being a multistep biological/physical process, undergoes a complicated and dynamic change in various process variables and parameters. They include supply of solid substrate, supply and consumption of soluble sugar substrates, sugar accumulation, product accumulation/removal, and biomass accumulation. Our focused attention on the specific substrate supply rate reveals that it is one of the most important operational parameters that is directly linked to product yield, efficiency in sugar utilization, and distribution of products.

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