In Situ Extractive Fermentation of Acetone and Butanol

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The productivity of the acetone–butanol fermentation was increased by continuously removing acetone and butanol from the fermentation broth during fed-batch culture. Whole broth containing viable cells of *Clostridium acetobutylicum* was cycled to a Karr reciprocating plate extraction column in which acetone and butanol were extracted into oleyl alcohol flowing counter-currently through the column. By continuously removing these toxic metabolites from the broth, end product inhibition was reduced, and a concentrated feed solution containing 300 g/L glucose was fermented at an overall butanol productivity of 1.0 g/L h, 70% higher than the productivity of normal batch fermentation. The continuous extraction process provides flexible operation and lends itself to process scale-up.

INTRODUCTION

Although many chemicals can be produced by fermentation, the accumulation of toxic or inhibitory metabolites in the fermentation broth often inhibits cell growth and product formation; final product concentrations are low, fermentor productivity is reduced, and wastewater treatment and product separation costs are high. The effects of end product inhibition can be reduced by *in situ* removal of toxic metabolites from fermentation broth. A wide range of *in situ* product recovery methods have been proposed or tested.[1] In the present study liquid–liquid extraction was used to remove inhibitory metabolites from the broth during the acetone–butanol fermentation.

The acetone–butanol fermentation is characterized by strong product inhibition. Butanol, the primary product of the fermentation of sugar or starch by *Clostridium acetobutylicum*, inhibits its further production at concentrations above 10–15 g/L.[2-4] This strong butanol inhibition adversely affects the economics of the acetone–butanol fermentation in three main ways: butanol accumulation in the broth lowers fermentor productivity so that large fermentors are required; butanol inhibition limits the concentration of substrate that can be completely consumed, and thus large volumes of wastewater are produced; and product recovery is expensive due to the low final product concentrations in the fermentor. The economic viability of the acetone–butanol fermentation depends on reducing the effects of butanol inhibition and thus overcoming these problems.

Previous studies have shown that the effects of product inhibition during the acetone–butanol fermentation can be reduced by extractive fermentation.[5-8] For example, batch extractive fermentation, schematically illustrated in Fig. 1b, has been used to reduce butanol inhibition to *C. acetobutylicum*.[5,6] In batch extractive fermentation an immiscible organic solvent is added directly to a batch culture of microorganisms. Inhibitory metabolites dissolve into the solvent as they are produced, and end product inhibition is reduced. By extracting butanol into a mixture of oleyl alcohol and benzyl benzoate, maximum butanol productivity was increased by 60% compared to regular batch fermentation.[6] Attempts to ferment concentrated glucose solutions (above 100 g/L) in batch-extractive fermentation, however, were unsuccessful,[6] probably due to catabolite repression of the cells by high sugar concentrations.[5] Thus, even though batch extractive fermentation increased fermentor productivity, wastewater production was not significantly reduced.
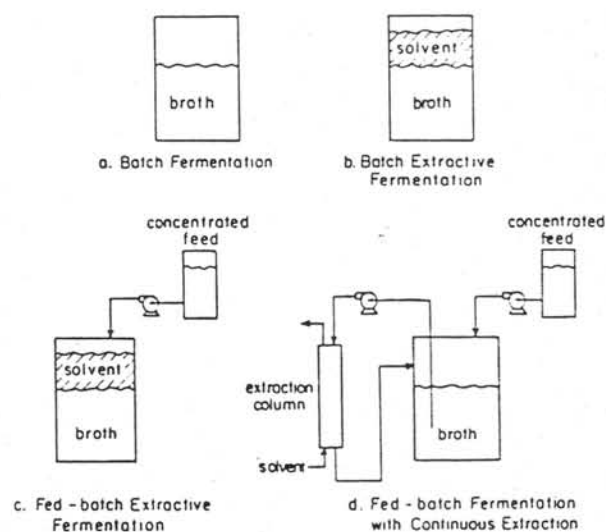

Figure 1. Extractive fermentation process configurations.

Concentrated glucose solutions were fermented in fed-batch extractive fermentation, in which an organic solvent is added to a fed-batch culture (Fig. 1c). Nutrient solutions containing up to 500 g/L glucose were fermented in fed-batch extractive fermentation using oleyl alcohol as the extraction solvent[8] compared to glucose conversions of only 60–80 g/L in regular batch culture. Volumetric productivity was increased from 0.58 g/L h in batch fermentation to 1.5 g/L h in fed-batch extractive fermentation. The final butanol concentration in the oleyl alcohol solvent was about 3 times higher than can be obtained in batch fermentation.[8] Fed-batch extractive fermentation thus reduced the effect of the three main problems of the acetone–buanol fermentation. Inhibitory products, however, eventually accumulate in the fermentation broth as the extraction solvent becomes saturated with products, and the bacteria, as in batch culture, stop growing when inhibitory metabolites reach toxic levels. In addition, the high shear rate generated by the impeller can cause the formation of emulsions. As a result, the agitation rate must be maintained at low levels in fed-batch extractive fermentation. Mass transfer rates will be slow in a large-scale process, and the advantage of extractive fermentation would be lost.

This article describes an extractive fermentation process that overcomes the limits inherent in fed-batch extractive fermentation while retaining its benefits. In the process, shown in Fig. 1d, inhibitory products are continuously extracted from the fermentation broth in an external extraction vessel. Fresh or regenerated solvent is continuously introduced into the extraction column so that the solvent does not become saturated with toxic metabolites. The application of this process to the acetone–butanol fermentation is described in this article, and the benefits of the continuous process for use in large-scale extractive fermentation are discussed.

MATERIALS AND METHODS

Microorganism and Culture Conditions

A strain of *Clostridium acetobutylicum* obtained from the American Type Culture Collection (ATCC 824) was used in this study. Cultures were maintained as previously described.[6]

Fermentation

Cultures were grown on a stable medium described elsewhere[3] in a 15-L Chemap fermentor. Fermentations were started as batch cultures, and fed-batch operation commenced after the concentration of glucose in the medium dropped below 20 g/L. During fed-batch operation concentrated nutrient solution was metered into the fermentor with a peristaltic pump. The concentrated nutrient solution consisted of (in g/L): 2.25 K_2HPO_4; 2.25 KH_2PO_4; 1.55 $MgSO_4(7H_2O)$; 0.019 $FeSO_4(7H_2O)$; 0.015 $MnSO_4(H_2O)$;

3 NaCl; 1.5 asparagine monohydrate; 0.5 cysteine; 30 yeast extract; 300 glucose; and 0.0045 resazurin. The flow of concentrated nutrient solultion was adjusted such that a relatively constant glucose concentration was maintained throughout fermentation. Fermentation conditions were identical to those previously described.[6]

EXPERIMENTAL APPARATUS

Figure 2 shows a schematic diagram of the extractive fermentation apparatus used in this study. Concentrated nutrient solution, maintained under a nitrogen atmosphere in the feed reservoir, was metered into the fermentor as needed. Whole broth, containing viable cells of *C. acetobutylicum*, was pumped into the top of the extraction column while oleyl alcohol, the extraction solvent used in this study, was metered into the bottom of the column. Extracted broth was returned to the fermentor by gravity flow. Solvent collecting in the top of the extraction column was bled into an external settling chamber in order to allow additional time for solvent–broth disengagement. The settler consisted of a vented inclined cylinder with a working volume of about 2 L. Broth collecting in the settler was returned to the fermentor, while solvent accumulating in the settler was fed to a steam stripper. The steam stripper was fashioned from a 45-in.-high and 2-in.-diameter glass column and contained 27 in. of $\frac{3}{8}$-in. Raschig rings. Butanol, acetone, and ethanol were stripped from the oleyl alcohol solvent and collected in the condensate receiver. Regenerated solvent was drained from the bottom of the steam stripper into the solvent reservoir and maintained under a nitrogen atmosphere. Steam condensate collecting in the bottom of the steam stripper was periodically drained into the steam stripper bottom receivers.

A Karr reciprocating plate extraction column was used in this study. In a Karr extraction column perforated plates are connected to a rod running the length of the column. A motor connected to the top of the rod provides agitation in the column by moving the rod and plates rapidly up and down. The agitation rate can be varied by adjusting the frequency and amplitude of plate oscillations. The column used in this study had a diameter of 1 in., total height of 12 ft 8 in., and plate stack height of 8 ft. Stainless steel plates, with about 60% open area, were placed every 2 in. on the rod in the column. The plates were normally agitated at 100–150 min^{-1} with a stroke length of $\frac{7}{8}$ in. The column was maintained at 37°C by blowing warm air through an annulus formed between the column and a plastic cylinder constructed around the column.

The extraction system, including the Karr column, settler, values, and lines, was sterilized in place with live steam. The diaphragm pump used to cycle broth to the extraction column was flushed with a 70:30 wt % ethanol–water solution and then rinsed with sterile water prior to use. Air was removed from the extraction system by flushing it with nitrogen before use.
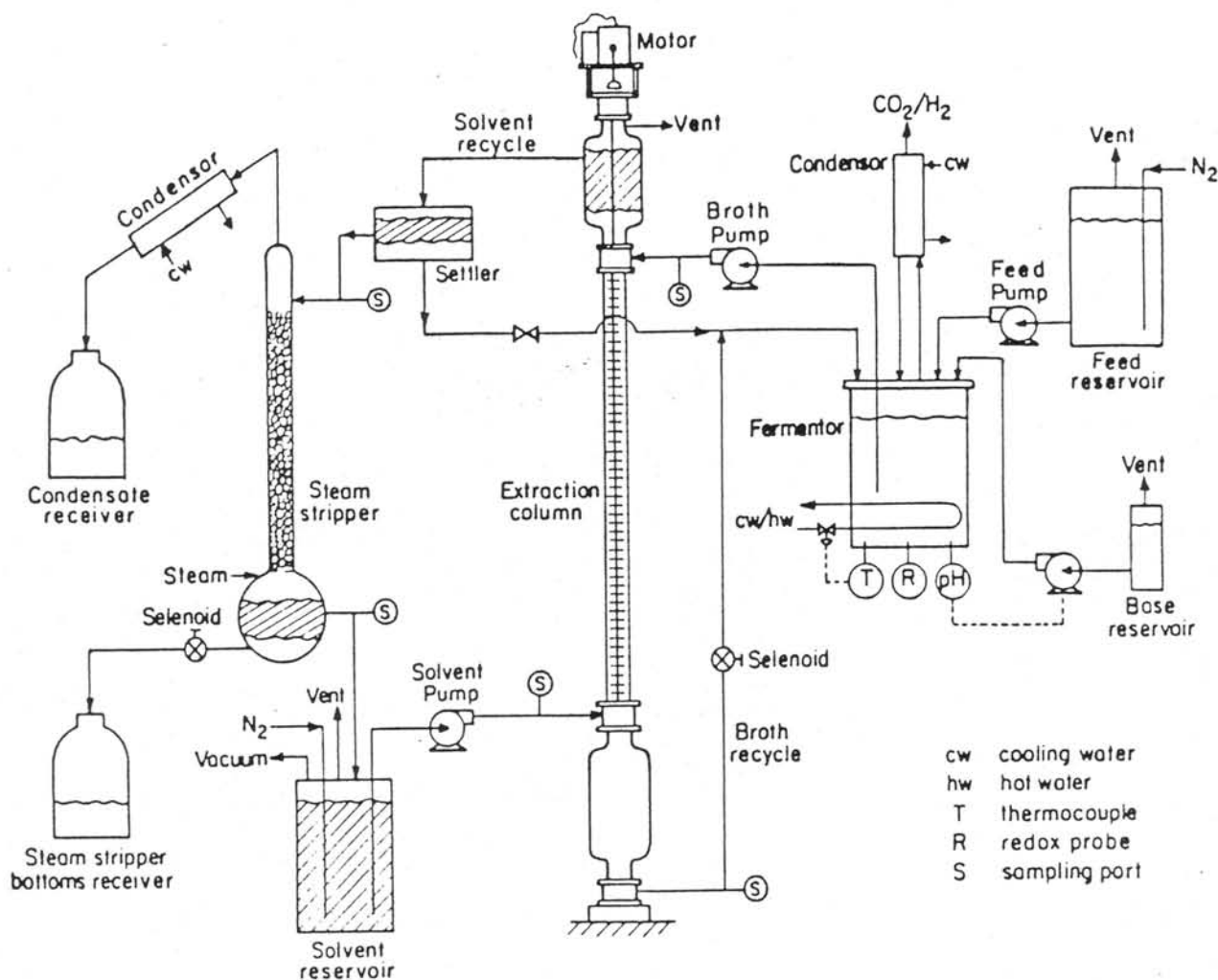
Figure 2. Schematic design of experimental apparatus used for continuous *in situ* extraction of acetone and butanol during fed-batch fermentation.

Analytical Methods

Aqueous Phase Analysis

Aqueous phase samples were periodically removed from the inlet and outlet of the extraction column and from the condensate and steam stripper bottom receivers. Samples containing bacteria were centrifuged and frozen for later analysis. Product concentrations were determined by HPLC as previously described.[6]

Organic Phase Analysis

Samples of organic solvent entering and leaving the extraction column and steam stripper were periodically removed and analyzed using gas chromatography as previously described.[3]

Butanol Production

The amount of butanol produced from time t_1 to time t_2 was estimated from the following equation:

$$B(t_2) - B(t_1) = B_b(t_2)V_b(t_2) - B_b(t_1)V_b(t_1) + B_c(t_2)V_c(t_2)$$
$$- B_c(t_1)V_c(t_1) + B_{sb}(t_2)V_{sb}(t_2) - B_{sb}(t_1)V_{sb}(t_1)$$
$$+ V_s[B_s(t_2) - B_s(t_1)] + \sum_{i=1}^{n} B_{xi}V_{xi}$$

where

t_1 = time 1, h

t_2 = time 2, h

B = total amount of butanol produced, g

B_b = concentration of butanol in fermentor, g/L

V_b = volume of broth, L

B_c = concentration of butanol in steam stripper bottom receiver, g/L

V_c = volume of condensate in receiver, L

B_{sb} = concentration of butanol in steam stripper bottom receiver, g/L

V_{sb} = volume of liquid in steam stripper bottom receiver, L

B_s = butanol concentration in solvent reservoir, g/L

V_s = volume of solvent in reservoir, L

B_{xi} = concentration of butanol in ith sample, g/L

V_{ii} = volume of ith sample, L

n = number of samples taken from t_1 to t_2

The amounts of other products formed by the bacteria were calculated analogously. Volumetric butanol productivity was estimated as previously described.[8]

RESULTS AND DISCUSSION

Extractive fermentation has been shown to increase volumetric productivity and reduce the amount of wastewater produced during the fermentation of glucose to butanol and acetone by *C. acetobutylicum.*[6,8] In those studies, however, extraction solvent was added directly to the fermentor, and as a consequence, agitation in the fermentor had to be maintained at low levels in order to prevent the formation of stable emulsions. In a large-scale extractive fermentation process, a low agitation rate will result in slow mass transfer, and the benefits of extractive fermentation will be lost. Mass transfer limitations and problems of emulsion formation can be reduced by contacting the broth and solvent in an external extraction device.

There are a wide variety of commercially available extractors that can be used to contact solvent and broth during extractive fermentation.[9,10] A Karr reciprocating plate extractor[11] was selected for use in this study. Karr columns have been used in a wide variety of applications, including the extraction of antibiotics from whole broth,[12] penicillin extraction,[13,14] copper extraction,[15] and the recovery of acetic acid, phenol, and other products.[16] It was chosen for use in this study for two reasons. First, shear rate is relatively uniform throughout a Karr column and can be adjusted by varying the reciprocation rate. Solvent droplets can be maintained at a fairly uniform size in the column, and the formation of stable emulsions, always a potential problem when dealing with fermentation broths, is minimized. Second, the Karr column can be scaled from laboratory to production scale in a straightforward manner.[16]

In extractive fermentation viable cells are cycled between the fermentor and extraction column. It is therefore important that the cells remain viable throughout the extraction loop. The feasibility of extractive fermentation was thus tested under realistic operating conditions. Broth from a fed-batch culture of *C. acetobutylicum* was cycled to a Karr column in which butanol and acetone were extracted into oleyl alcohol flowing countercurrently through the extraction column. Figure 3 shows results of an extractive fermentation that was operated for 55 h. The concentrations of acetone and butanol in the oleyl alcohol solvent leaving the extraction column during this experiment are shown in the top section of Fig. 3. Variations in acetone and butanol concentrations in the oleyl alcohol leaving the column reflect changes in the flow rate of oleyl alcohol through the Karr column; product concentrations in the solvent decrease as more oleyl alcohol flows through the column and increase when the oleyl alcohol flow rate is decreased. During the second half of the fermentation, butanol concentration in the oleyl alcohol ranged from 8 to 20 g/L. Figure 3 also shows

the concentrations of products in the aqueous phase during fermentation. The concentrations of acetone and butanol in the broth rose rapidly until the 17th hour, when extractive fermentation was initiated. At this time the concentration of acetone in the aqueous phase leveled off while the concentration of butanol actually decreased. More butanol was removed from the fermentation broth during each pass through the extraction column, as butanol has a higher distribution coefficient than does acetone. The distribution of butanol and acetone between water and oleyl alcohol as a function of concentration are given elsewhere.[6] The concentrations of both butanol and acetone in the broth rose during the later stages of fermentation, indicating that they were produced more rapidly than they were extracted from the broth into the solvent. Nevertheless, butanol concentration was maintained below severely inhibitory levels throughout fermentation. The bottom third of Fig. 3 shows cell and glucose concentrations in the fermentor and the flow rate of glucose into the fermentor during fed-batch operation. By varying the glucose feed rate, residual glucose in the broth was maintained at a fairly constant low level during most of the fed-batch operation.

Figure 4 shows the concentrations of acetone, butanol, and ethanol in the broth entering and leaving the extraction column. The fractional butanol recovery in the extraction column, defined as the difference between the concentration of butanol entering and leaving the column relative to the inlet concentration, was only 15–40% during most of extractive fermentation. The analysis of product recovery, however, is complicated by the fact that bacteria in the broth passing through the extraction column continue to produce solvent products. Under certain conditions the bacteria passing through the extraction column can produce products faster than they are extracted from the broth into the solvent. An analysis of this phenomenon is presented in the literature.[17] Figure 4 shows that acetone and ethanol were in fact sometimes produced faster than they were extracted; the concentrations of acetone and ethanol in the aqueous phase leaving the extraction column were occasionally higher than their concentrations in the broth entering the column. The low recovery of butanol in the extraction column (15–40%) is thus an inherent consequence of cycling viable cells through the extraction column rather than due to poor extractor performance. Fortunately, a high degree of product recovery per pass in the extractor is not necessary since broth is recycled to the fermentor.

Figure 5 shows the total amounts of products produced over the course of extractive fermentation. Butanol, acetone, and ethanol were produced throughout fermentation, showing that the cells remained viable during extractive fermentation. Butyric and acetic acids, on the other hand, were not produced during the second half of fermentation. It is desirable to minimize the production of these acids because their formation consumes substrate that could otherwise be used to produce butanol, ethanol, and acetone. The majority of the acetic and butyric acid in the broth are present as acetate and butyrate salts at the pH of the fermentation. These by-products are thus retained in the aque-

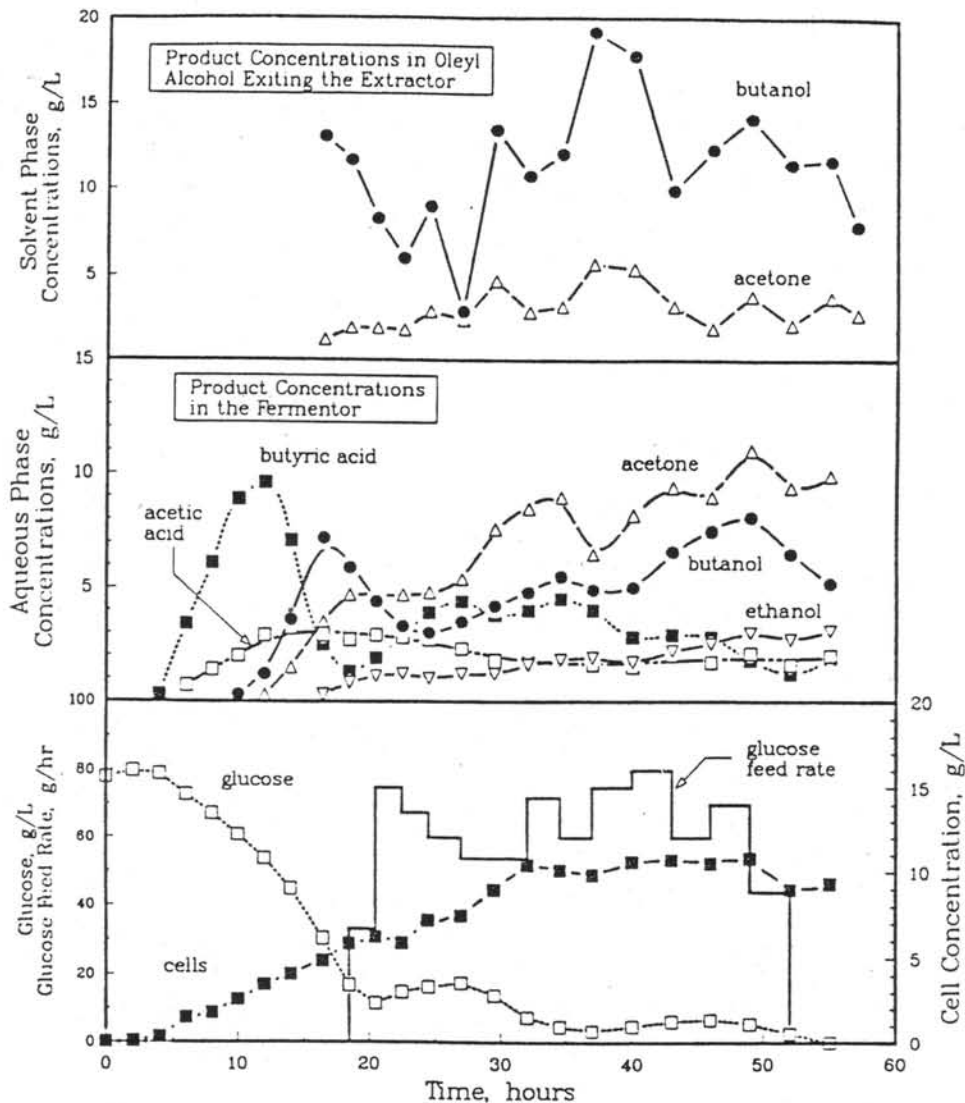**Figure 3.** Product and substrate concentrations during continuous extraction of acetone and butanol from a fed-batch culture of *C. acetobutylicum*. Oleyl alcohol was used as extraction solvent. Concentrated feed solution contained 300 g/L glucose.

ous phase during fermentation since salts are not extracted to any appreciable degree by oleyl alcohol. Acid production is inhibited by the accumulation of these by-products in the broth, and longer fermentation times allow more of these acids to be reassimilated by the bacteria. Extractive fermentation thus helps to minimize the production of by-product acids during the acetone–butanol fermentation.

Table I summarizes results of the continuous *in situ* extraction of acetone and butanol during fed-batch fermentation. At the end of fermentation, which stopped when the glucose feed was depleted, 2730 g glucose were consumed and 525 g butanol were produced. This is equivalent to a glucose consumption of 214 g/L and butanol production of 40 g/L based on final broth volume or about 3 times the amounts that can be obtained in regular batch fermentation. Also, 3 times less wastewater was produced in extractive fermentation compared to regular batch culture.

Table II shows that product yields in extractive fermentation were comparable to those of batch culture. The yields of butanol, acetone, and ethanol were essentially the same in batch or extractive fermentation. The yield of

Table I. Results of fed-batch fermentation with continuous extraction.

Initial broth volume, L	9.7
Average broth volume, L	10.2
Final broth volume, L	13
Total glucose consumed, g	2780
Total butanol produced, g	525
Total acetone produced, g	285
Total ethanol produced, g	77
Total fermentation time, h	55

Table II. Yields of products in batch culture and fed-batch culture with continuous extraction.

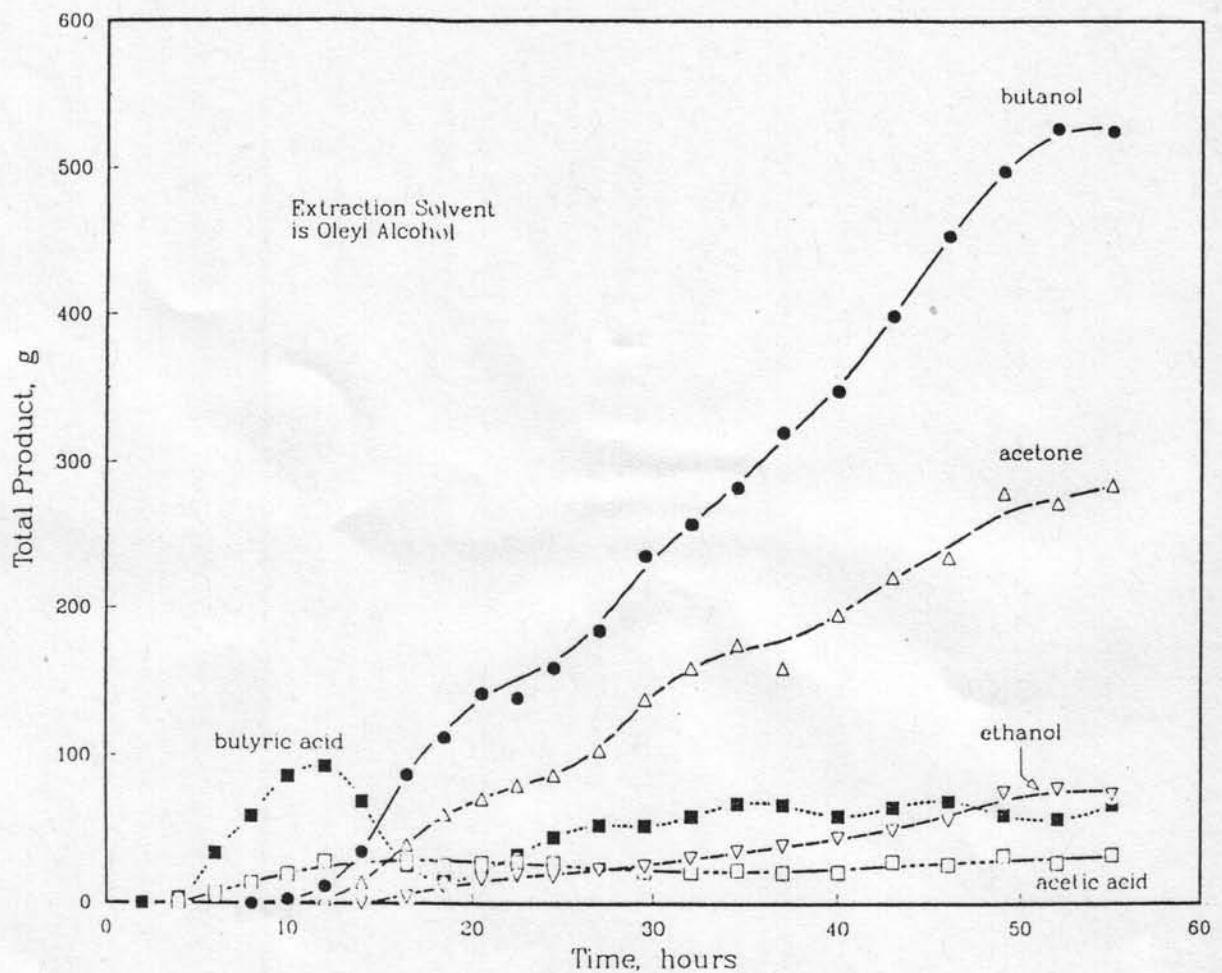| Product	Batch (g product/g glucose)	Fed-batch with continuous extraction (g product/g)
Glucose		
Butanol	0.18	0.19
Acetone	0.10	0.10
Ethanol	0.04	0.03
Acetic acid	0.05	0.01
Butyric acid	0.001	0.02

Figure 4. Concentration of products in aqueous phase entering and leaving extraction column during extractive fermentation.

by-product acetic acid was much lower in extractive fermentation, while the yield of butyric acid was higher than in batch culture. The overall yield of acids during the continuous extraction of products from the acetone–butanol fermentation was about half the yield of regular batch culture.

Because toxic metabolites are removed from the cell environment as they are excreted during extractive fermentation, product inhibition is reduced, and fermentor productivity should be improved. Figure 6 shows the volumetric butanol productivity and glucose consumption rate over the course of extractive fermentation. In regular batch culture, butanol productivity rises rapidly in the early stages of fermenta-

tion, to a maximum productivity of 1.4 g/L h,[6] and then it quickly drops as butanol accumulates in the broth and cell growth is inhibited. Overall butanol productivity in batch culture is about 0.58 g/L h. Butanol productivity during extractive fermentation, on the other hand, was maintained at about 1.2 g/L h for much of the fermentation, indicating *in situ* removal of butanol and acetone effectively reduced product inhibition. Table III shows that the overall volumetric butanol productivity during fed-batch fermentation with continuous extraction was about 70% higher than the butanol productivity of regular batch fermentation. Wastewater volume was also greatly reduced by using extractive fermentation. Cells of *C. acetobutylicum* are totally inhib-

Table III. Comparison of batch culture and extractive fermentation using oleyl alcohol as extraction solvent.

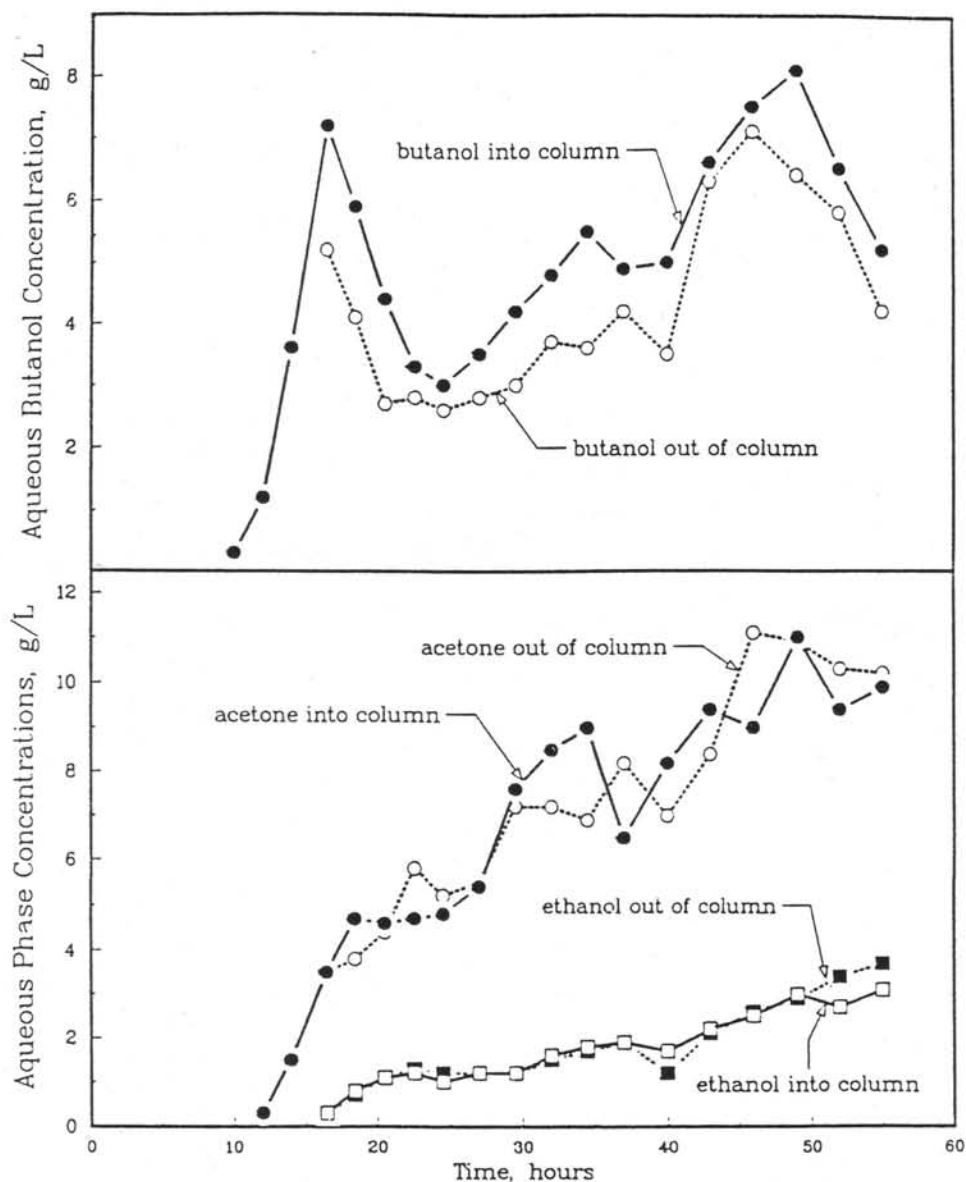| Fermentation	Overall butanol productivity (g/L h)	Concentration of glucose fermented (g/L)	Total glucose fermented* (g/L)
Batch	0.58	81	81
Batch extractive	0.72	98	98
Fed-batch extractive	1.5	333	207
Fed-batch with continuous extraction	1.0	300	214

* Based on final broth volume.

Figure 5. Total amounts of products produced during continuous *in situ* extraction of acetone and butanol from fed-batch culture of *C. acetobutylicum*.

ited by butanol accumulation after only 60–80 g/L glucose are consumed in batch culture. Because butanol is continuously removed in extractive fermentation, an equivalent of 214 g/L glucose was consumed in fed-batch fermentation with continuous extraction, representing a 300% decrease in wastewater volume. Table III also compares results of this study with results of batch and fed-batch extractive fermentation. Overall butanol productivity during fed-batch culture with continuous *in situ* extraction was higher than the productivity of batch extractive fermentation (Fig. 1b). Volumetric productivity in fed-batch extractive fermentation (Fig. 1c), however, was higher than the productivity of the continuous extraction system. There are several possible reasons why the productivity of the continuous extraction process was lower than the productivity of the fed-batch extractive fermentation. First, some bacteria may have been damaged during circulation through the extraction loop; the bacteria had to pass through a diaphragm pump and several valves on each pass through the extraction loop. Also, any

air that entered the extraction system through the many valves present could have inhibited the strictly anaerobic cells of *Clostridium acetobutylicum*.

Clostridium Acetobutylicum

In addition, the broth was not at optimal temperature throughout all of the extraction loop, which may have decreased productivity. Finally, some viable cells were entrained in the solvent and lost viability in the steam stripper. Through better phase separation and temperature control, the effect of these factors can probably be reduced in large-scale extractive fermentation.

Although the overall volumetric butanol productivity of the continuous extraction process was lower than the productivity of fed-batch extractive fermentation, other factors recommend the continuous process for use in large-scale extractive fermentation. Operation of the continuous extraction process is inherently more flexible than is operation of
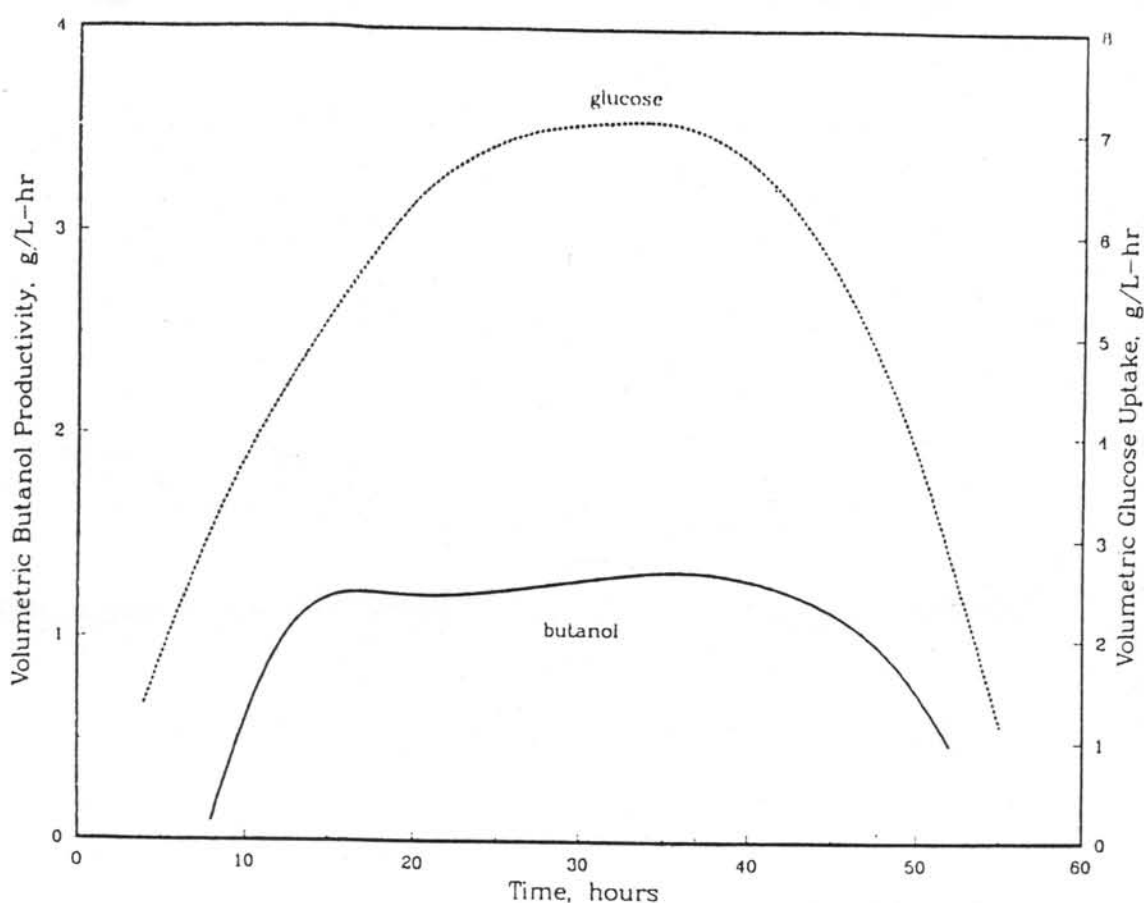
Figure 6. Volumetric butanol productivity and glucose uptake rate during continuous *in situ* extraction of acetone and butanol from fed-batch culture of *C. acetobutylicum*. Curves obtained by differentiating polynomials fit to fermentation results with least-squares routine.

fed-batch extractive fermentation. The concentration of inhibitory butanol in the fermentation broth can be controlled in the continuous extraction process by properly adjusting the relative flow rates of broth and solvent to the extraction column. In fed-batch extractive fermentation, on the other hand, butanol concentration increases throughout fermentation. The ratio of solvent to broth is limited to a factor of about 2 in fed-batch extractive fermentation because of problems with phase inversion and separation.[8] This sets a limit on the length of fermentation since butanol production stops when the solvent "reservoir" becomes saturated with products and inhibitory metabolites accumulate to toxic levels in the broth. In contrast, fermentation can be carried out for long periods in a continuous extraction process: fermentation will continue until salts or inhibitory products that are not extracted into the solvent accumulate to toxic levels in the broth. By adjusting the concentration of salts in the feed and using a solvent that extracts most inhibitory products, fermentation times can be extended to many times those obtainable in batch or fed-batch extractive fermentation.

The continuous extraction system also allows more process flexibility than does fed-batch extractive fermentation. Fermentation and extractive steps can be independently optimized in the continuous extraction process because each step is carried out in a separate vessel. For example, the

agitation in the fermentor can be adjusted to ensure that nutrients and base are well mixed, while the shear rate in the extraction column can be set so that mass transfer rates are high but emulsion formation is avoided. In fed-batch extractive fermentation, on the other hand, fermentation and extraction both take place in the fermentor and cannot be independently optimized. Limits on the level of agitation in order to prevent emulsion formation in fed-batch extractive fermentation will result in slow mass transfer rates in large-scale processes.

CONCLUSIONS

The continuous *in situ* extraction of acetone and butanol from the fed-batch fermentation of *C. acetobutylicum* into oleyl alcohol is feasible. By continuously removing inhibitory products from the broth, a 300-g/L glucose solution was converted to products at a volumetric productivity 70% greater than that obtainable in batch culture. Furthermore, because toxic metabolites were removed from the cell environment throughout fermentation, operation of extractive fermentation could be carried out for long periods. Fermentation was carried out for 55 h in this study compared to only 24 h in batch fermentation.[6] Turnaround time is reduced, and fewer nutrients may be required in the continuous-extraction process since broth is recycled to the fermentor.

Cells remained viable throughout extraction as evidenced by continued gas evolution and product formation inside the extraction loop. The Karr reciprocating plate extraction column performed well during extractive fermentation. Emulsion formation was minimized in the Karr column by varying the reciprocation frequency so as to prevent the formation of very small droplets of oleyl alcohol in the aqueous phase.

The continuous extraction system described in this article can be easily integrated into large-scale extractive fermentation processes. Fermentation and extraction steps are carried out in separate vessels and can therefore be independently operated at optimal conditions. Fermentation products extracted into the organic solvent can be recovered by continuously feeding the loaded solvent into a series of distillation columns or other separation devices. The scale-up of the Karr column to production scale is straightforward,[16] and the scale-up of other equipment in the continuous-extraction process should present no major problems.

Further work is required to optimize the extractive fermentation of acetone and butanol. In the present study an excess of yeast extract and inorganic salts was used. Medium costs can be reduced by minimizing the nutrients used in extractive fermentation. Extractive fermentation should also be carried out using other substrates such as hydrolyzed corn endosperm or starch supplemented with corn steep liquor. The operating conditions of the extraction process should also be optimized. The flow rates of broth and solvent to the extractor, the plate reciprocation frequency in the Karr column, and the degree of product recovery on each pass through the column can be varied. An economic analysis would be helpful in determining optimal operating conditions, minimizing the cost of extractive fermentation.

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