

**INTEGRATED PROCESS FOR MICROBIAL
SOLVENT PRODUCTION FROM WHEY PERMEATE**

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**INTEGRATED PROCESS FOR MICROBIAL
SOLVENT PRODUCTION FROM WHEY PERMEATE**

Final Report

Prepared for

**THE NEW YORK STATE
ENERGY RESEARCH AND DEVELOPMENT AUTHORITY**

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ABSTRACT

Acetone and butanol were historically produced through fermentation of carbohydrate raw materials. Conventional feedstocks such as grain and molasses, and the energy required to recover products by distillation, are too costly for traditional batch fermentation to compete with petrochemical synthesis.

We proposed to evaluate an acetone-butanol-ethanol fermentation of acid whey permeate, a cheap carbohydrate source, using up-to-date bioreactor technology - continuous fermentation with cell recycle - and *in-situ* butanol recovery by gas stripping. *Clostridium acetobutylicum* P262 was the strain chosen, as it assimilates both the lactose and lactic acid in acid whey.

Single-stage continuous culture proved unsuitable for butanol production, since productivity low and cultures degenerated quickly. Two-stage culture improved productivity by a factor of two over batch runs. All continuous cultures showed major oscillations in cell density, substrate concentration and products formed. Under these conditions, cell recycle did not affect productivity in two-stage culture.

Gas stripping with fermentor off-gases recovered a clean condensate of butanol and acetone at 70-90% yield and with purification factors of 14 to 35. Stripping maintained solvent concentrations in the range of 2-4 g/l even at the peak of solventogenesis, eliminating product inhibition. Gas stripping produced a 50% improvement in substrate uptake and a 10-20% improvement in solvent productivity.

Key Words: Acetone, butanol, *Clostridium acetobutylicum* P262, fermentation, continuous culture, bioreactors, gas-stripping.

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SUMMARY

The aim of this research was to develop and evaluate an improved process for production of butanol and acetone from acid whey permeate by *Clostridium acetobutylicum*, which would increase the volumetric productivity and decrease the costs of product recovery. We approached this goal in two ways, combining them at the end of the study. First, we investigated membrane-assisted liquid/liquid extraction and gas stripping as methods for *insitu* recovery of butanol from a continuing fermentation. Second, we compared single-stage continuous culture, cell recycle continuous culture, and two-stage continuous culture fermentation to determine their relative productivity and identify the limitations of each one.

Hollow-fiber membrane aided liquid extraction proved impractical for solvent recovery from our chosen strain, *C. acetobutylicum* P262, because of solvent toxicity problems and the certainty of shear damage to the cells upon repeated recycling through the membrane extractor. On the other hand, gas stripping removes and recovers solvents effectively, is gentle to the cells, and holds the promise of producing a product containing 60 to 80% butanol by weight. Gas stripping held butanol concentration to 0.2 - 0.4% in a batch culture where it typically reaches the toxic levels above 1.5%. Gas stripping improved volumetric productivity by 20% and sugar consumption by 50% in a batch fermentation.

Compared to a standard batch fermentation of whey permeate, single-stage continuous culture raised solvent productivity by 18%, but these cultures invariably stopped producing solvents after 70 to 130 hours. Productivity was approximately doubled in two-stage continuous cultures, though these, too, became unproductive after 140 to 220 hours. In batch and two-stage culture, cell recycle had no effect on solvent productivity, and in single stage culture it lowered productivity.

Work is continuing to combine gas stripping with continuous and fed-batch cultures on whey permeate, to investigate the lactate metabolism of *C. acetobutylicum* P262, and to pretreat acid whey permeate to improve its performance in the acetone-butanol fermentation.

Section 1

INTRODUCTION

BACKGROUND

The acetone-butanol-ethanol (ABE) fermentation, a mainstay of the fermentation industry between 1918 and 1954, has come under intensive study during the past 10 to 15 years because of wide interest in the production of these industrial solvents from renewable resources. The traditional batch fermentation of molasses, which was supplanted by petrochemical processes in the 1950s, is limited by a number of factors, chiefly the high cost of substrate and the toxic or inhibitory effects of the product butanol on the fermentation. Butanol toxicity limits final solvent concentrations to 2.5 to 3% in a batch fermentation. Hence, large reactor volumes are necessary and product recovery is energy-intensive since 30 to 40 units of culture broth must be processed to yield one unit of pure solvents (Spivey, 1978; Gibbs, 1983).

This project addresses three major limitations of the ABE process – high substrate cost, butanol toxicity, and difficult product recovery – to demonstrate a process for fermentation of acid whey permeate to solvents.

Acid whey permeate, a by-product of cottage cheese manufacture, contains around 4% lactose and 0.7% lactic acid. It can be concentrated by a factor of about four for shipping and storage. The best current uses are as animal feed or substrate for single-cell protein production, both very low value uses (Zall, 1983).

Butanol toxicity and solvent recovery may be simplified at the same time through *in-situ* removal of solvents from the fermentation in progress. Several approaches are possible: adsorption of solvents by polymer resin beads, liquid/liquid extraction, perstraction or liquid/liquid extraction through a membrane, gas stripping, and pervaporation or gas stripping through a selective membrane.

Typically a continuous process provides higher productivity than a batch process for a primary product like butanol, the direct chemical result of the cells' energy metabolism. Moreover, variations on continuous culture can further improve productivity. Two-stage continuous culture allows a degree of separation of the phases of cell growth and solvent

production, so that conditions for each phase may be separately optimized. Cell recycle permits the accumulation of high concentrations of cells within a fermentor, and has the potential for significant increases in volumetric productivity if those cells remain in an actively productive state (Afschar and Schaller, 1991; Afschar et al, 1985).

OUTLINE OF WORK PROPOSED AND PERFORMED

The overall goal of this work was to demonstrate a laboratory-scale integrated process for continuous production and recovery of butanol from acid whey permeate. In the original work plan, put forth in 1987, it was assumed that a single-stage continuous culture with recycle and a perstraction system for solvent recovery would prove the best techniques for increasing productivity. Extraction with high-pressure liquefied propane was also thought worthy of consideration. The original workplan included these points:

- Screen solventogenic *Clostridia* and select one best strain
- Build, test, and evaluate a continuous culture/cell recycle system
- Screen organic extractants for a perstraction system
- Build and test a perstraction system for solvent recovery and attach it to the continuous culture/cell recycle system
- Evaluate the resulting integrated process
- Test the liquefied propane extraction system for butanol recovery
- Evaluate the economic potential of byproduct streams from the fermentation
- Analyze the process economics for the integrated system.

As work began it became evident that perstraction and, by extension, liquid propane extraction, would not improve productivity because of the toxicity of the extractants themselves to the micro-organisms. Furthermore, early results showed cell recycle to have no effect on productivity, as cell damage balanced cell concentration in the fermentor. Hence, the workplan was modified to focus on two more promising methods: two-stage continuous culture for the fermentation, and gas stripping for product recovery.

The work actually performed for this contract includes these points:

- Screen solventogenic *Clostridia* and select one best strain
- Build, test, and evaluate a continuous culture/cell recycle system

- Build, test, and evaluate a two-stage continuous culture system
- Build and test a perstraction system for solvent recovery
- Screen organic extractants for a perstraction system
- Build and test a gas stripping system for butanol recovery
- Evaluate gas stripping of a batch whey fermentation.

Our work is continuing to couple the gas stripping to a two-stage continuous fermentation of whey permeate.

Section 2

MATERIALS AND METHODS

MICROBIAL CULTURES

The following microorganisms were used in the strain selection experiments: *Clostridium acetobutylicum* strain ATCC 824; *C. acetobutylicum* strain ATCC 39058, *C. acetobutylicum* strain P262, provided by Professor D.R. Woods (University of Cape Town, South Africa), and *C. beijerinckii* strain LMD 27.6, provided by the Collection of Bacteria, Kluyver Laboratory of Biotechnology, University of Technology, Netherlands. The organism selected for all bioreactor studies was *C. acetobutylicum* strain P262, due to its excellent performance on whey permeate. The P262 strain was stored as spores in spent medium at 4°C (Diez-Gonzalez, 1992).

MEDIA

Whey Permeate Medium

The medium of cultivation was based on acid whey permeate obtained from cottage cheese and provided by Crowley Foods, Inc., Arkport, NY. Its composition was 4.0 g/l of lactose and 0.6 g/l of lactic acid, with a pH of 4.4. The composition of the whey permeate medium was the following: whey permeate, 960.0 ml; distilled water, 37.14 ml; casein hydrolyzate 1.4 g; K₂HPO₄, 0.45 g; KH₂PO₄, 0.45 g; sodium thioglycolate, 0.43 g; MgSO₄·4H₂O, 0.07 g; L-cysteine-HCl, 0.05; MnSO₄·4H₂O, 3.5 mg; FeSO₄·7H₂O, 3.5 mg; resazurine, 1.0 mg; p-aminobenzoic acid, 0.35 mg; thiamine-HCl, 0.35 mg; and biotin, 20 mg. Acid whey permeate was first neutralized with 50% (v/v) NH₄OH solution to a pH of 6.8, then autoclaved at 121°C for 20 min. and cooled down inside an anaerobic glove box (95% N₂ and 5% H₂) (Coy Laboratory Products, Inc.). The whole medium was prepared in the glove box mixing the sterilized whey permeate with the above ingredients as stock solutions. The medium pH was 6.0 (Diez-Gonzalez, 1992).

For gas stripping studies, the whey permeate was cooled under a nitrogen blanket after initial neutralization and autoclaving. Only the clear supernatant was incorporated into the medium. In addition, the medium was supplemented with 20 g/L lactose.

Glucose Medium

The glucose medium was based on the whey permeate medium and was identical to it except for two substitutions: 3 g of ammonium acetate was substituted for the 1.72 g. of casein hydrolysate; and 80.0 g of glucose and distilled water to 1 liter was substituted for the whey permeate and 37 ml distilled water. The media was sterilized by autoclaving at 121 °C for 20 minutes and allowed to cool under nitrogen sparging before mixing with the rest of the media components. The final pH of the media was 6.2-6.4.

INOCULUM PREPARATION

For each new fermentation, 6 to 10 ml of liquid were taken from the spore stock solution and heat-shocked at 75°C for 2 min. From this, 1 ml was used to inoculate 100 ml of one of the media described above in an anaerobic jar. After incubation for 24 h at 34°C, the actively growing culture was then used to inoculate the fermentor at a 5% inoculation level (Diez-Gonzalez, 1992).

FERMENTATION IN SERUM BOTTLES

The strain screening test was conducted in 60 ml of media in 120-ml stoppered serum bottles. An inoculum of 3 ml was introduced into each bottle and the culture checked at 12-hour intervals for cell growth and solvent production. Extractant toxicity was tested by preparing a whey-based fermentation media containing 20, 50, 80, and 100% of the saturation concentration of extractant, then inoculating with an active culture. The culture was observed over the course of a week to determine growth rate and solvent productivity.

SCREENING OF EXTRACTION SOLVENTS

Partition Coefficient

To determine partition coefficients of butanol in water, butanol/water solutions of different butanol concentration were prepared. A 4 ml portion of butanol/water was added to an equal volume of extraction solvent, thoroughly vortex-mixed, and allowed to remain

overnight at 35°C. A sample of the water phase was withdrawn via syringe and injected into the GC or HPLC. Similar experiments were done on spent fermentation broth.

Culture Inhibition

To 60 ml of medium in a stoppered serum bottle was added the appropriate quantity of solvent or solvent/water solution to reach 20, 50, and 100% of saturation. Bottles were well shaken and allowed to stand overnight at 5°C to equilibrate the contents. A 5% inoculum was added and the culture allowed to grow as previously described.

PERSTRACTION

A schematic of the perstraction unit is shown in Figure 2-1 on the next page. It is a locally built replica of the Hoechst-Celanese Liqui-Cel™ Laboratory Liquid Liquid Extraction System. Organic extractant is circulated to the shell side, and aqueous phase to the tube side, of a Hoechst-Celanese Liqui-cel™ hollow fiber liquid/liquid extraction module. Each phase had its own peristaltic pump, flowmeter, and inlet and outlet pressure gauges.

The Liqui-Cel™ extraction module has 0.2 m² membrane surface area of Celgard® X-10 microporous polypropylene hollow fiber membrane with 400 micron i.d. and 0.05 micron pore size. The case and fiber potting material are polypropylene and solvent-resistant epoxy, respectively.

To determine mass transfer coefficients, a feed stream of distilled water or fermentation broth containing 1% butanol was equilibrated in a 35°C water bath, and a reservoir of the extractant, 2-ethyl 1-hexanol, was also equilibrated. The flow of water through the hollow fiber unit was started, and the outlet valve was adjusted to give about 5 psi pressure in the hollow fibers. After the flow of organic phase was established, the tube-side pressure was reduced to 3.2 to 3.5 psi, about 2 psi above the organic side. Aqueous and organic side flow rates were then set and the inlet and outlet valves adjusted to maintain the 2 psi pressure difference. Once flow had stabilized, samples of the aqueous phase were taken at the outlet of the extraction unit for GC or HPLC analysis.

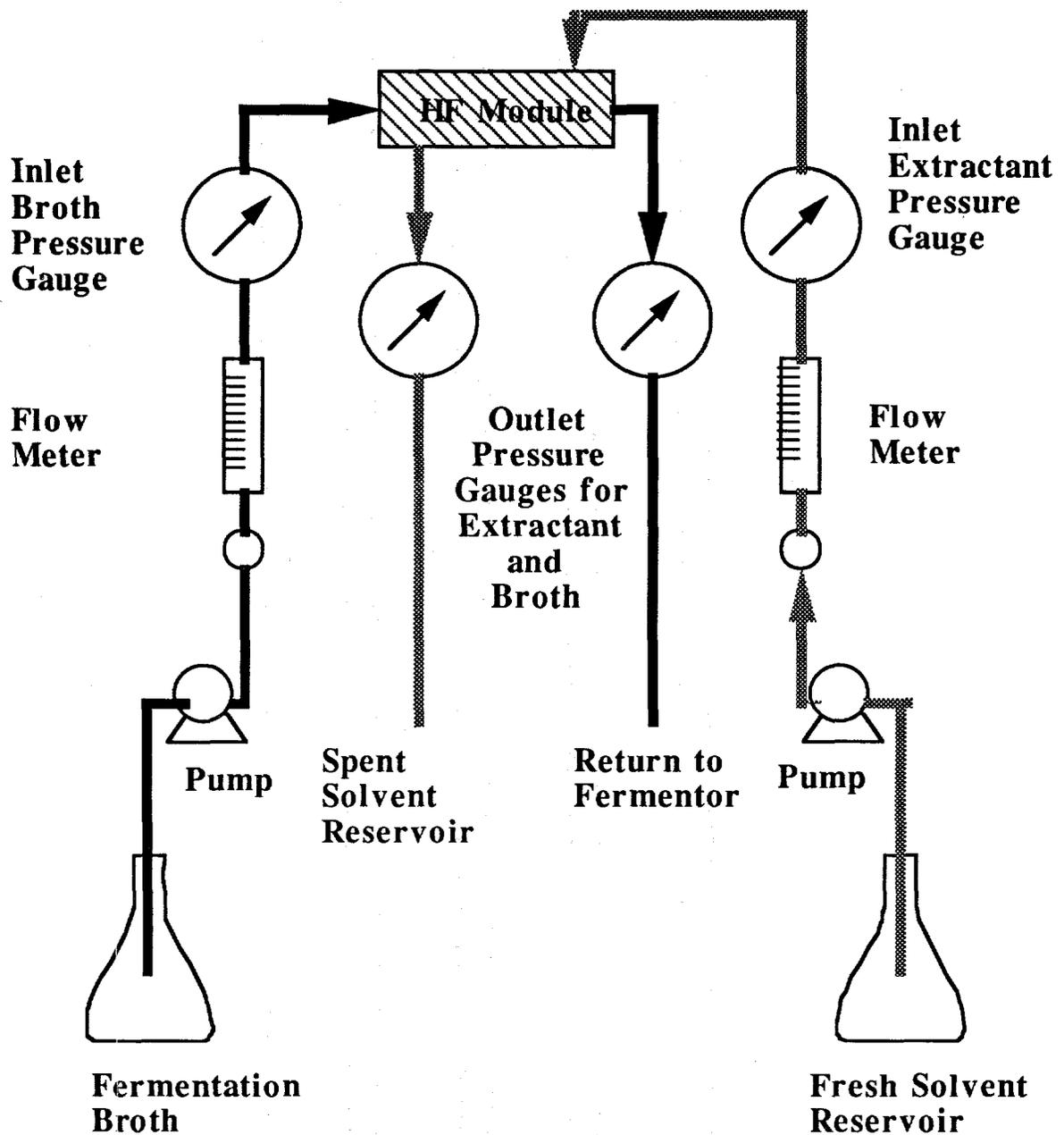


Figure 2-1. Schematic of the Perstraction Unit. Dark Lines: Aqueous Phase; Shaded Lines: Organic phase.

GAS STRIPPING

Equipment

Figure 2-2 shows a schematic of the gas stripping system. The stripper was made of 5 cm i.d. glass pipe, packed with 6 mm diameter ceramic saddles to a depth of 20 cm. It was insulated with 2.5 cm of household pipe insulation to minimize heat loss to the surroundings. A peristaltic pump (Masterflex®, Cole Parmer) pumped whole fermentation broth to the top of the stripper where it was subdivided into four streams to achieve a uniform flow of the broth over the ceramic saddles. Stripped broth was returned to the fermentor by gravity.

The first condenser was a glass shell and tube condenser 4 cm x 30 cm (Ace Glass Co.) It was kept at 0 °C by pumping ethylene-glycol at -10 °C through the shell side of the condenser. Condensate was collected in a 400-ml glass flask below the condenser.

The stripping gases then flowed past a manometer/pressure release valve. This allowed any excess gases produced by the fermentation to vent while maintaining a controlled pressure of around 10 cm water and sterile conditions. An increase in the internal pressure of the system was an easy way to confirm gas production by the fermentation.

The second condenser was a 2 liter capacity thin-walled aluminum cylinder 15 cm in diameter and 30 cm tall with internal baffles. The condenser was kept below -20 °C by immersion in a dry ice/acetone mixture. The condensate was collected as a solid and melted when ice build-up threatened to block the gas passage, or when samples were required for analysis. Dry ice was added to the chilling bath as required during fermentation.

The two 4-liter flasks were used to increase the volume of the gas system. This served as a buffer, decreasing pressure fluctuations due to production or leakage of gases. The gases were recycled with a peristaltic pump (Barnett Co.), with a flow capacity of 10 l/min. Pure nitrogen was used for stripping in the experiments on model broth. In the fermentation work the system was started up on pure nitrogen gas, but any fermentation off-gases were

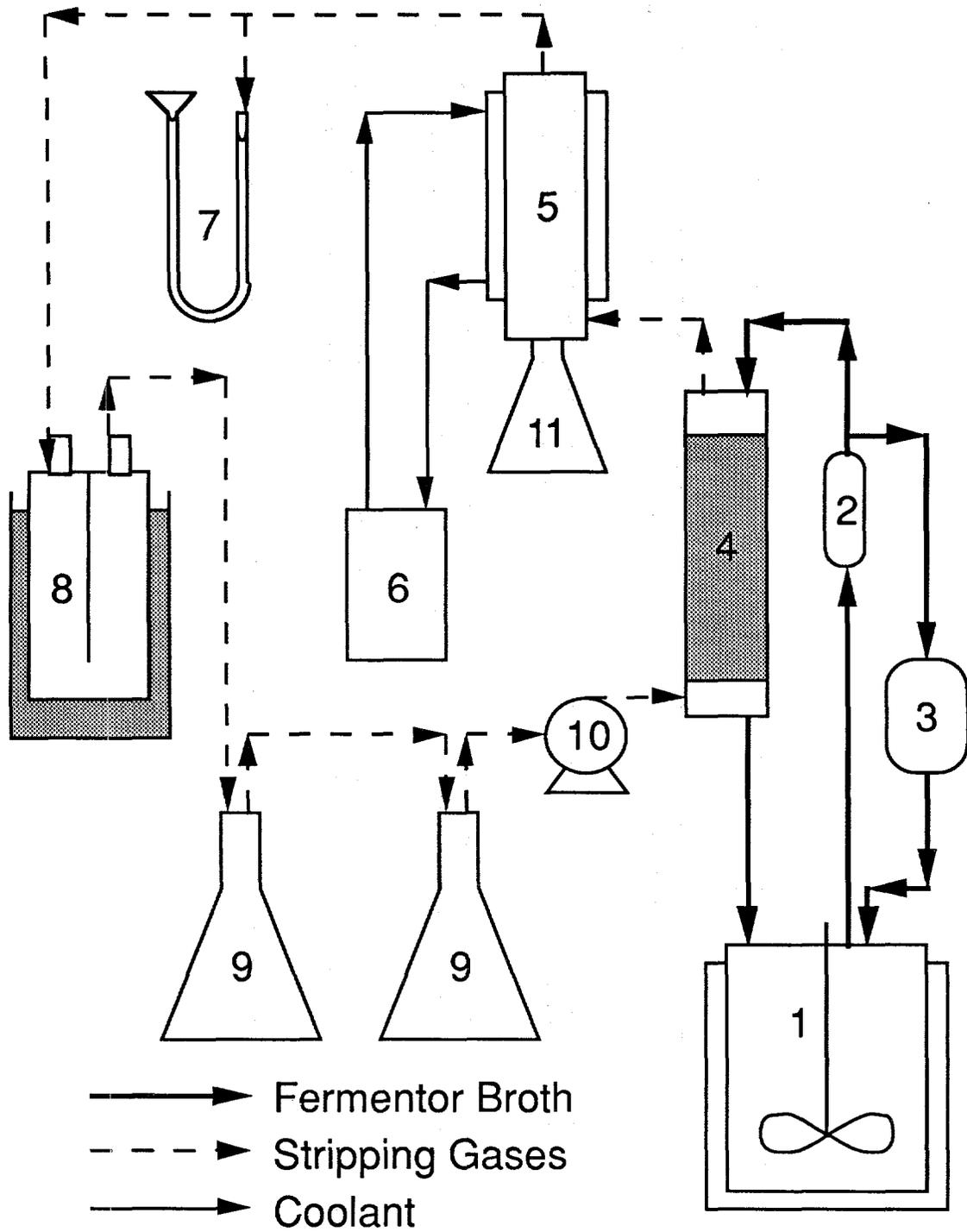


Figure 2-2. Gas Stripping System.

- | | |
|--|--------------------------------|
| 1. Fermentor | 6. Ethylene Glycol chiller |
| 2. pH meter/flow-through cell | 7. Manometer/Excess Gas Vent |
| 3. Spectrophotometer/flow-through cell | 8. Condenser, -20°C |
| 4. Packed Column Stripper | 9. Gas Surge Vessels, 4 L each |
| 5. Condenser, 0°C | 10. Gas Recycle Pump |
| | 11. 0°C Condensate Reservoir |

added to the stripping gases, and the original nitrogen was progressively lost as excess gas vented through the manometer.

Stripping of Model Fermentation Broth

Initial gas stripping work was conducted on a model fermentation broth that contained solvents and acids in concentrations similar to those seen in previous batch cultures. The model broth contained 1000 g distilled water, butanol 20 g, acetone 10 g, ethanol 5 g, lactic acid 5 g, and acetic acid 4 g.

The model broth was heated to 35 °C in the fermenter and pumped through the gas stripper. The stripping gas was nitrogen from a tank, and was passed once through the system and vented. Liquid samples were taken every half hour and analysed. The volume of liquid lost to gas stripping was also determined. Solvent removal rates were determined as well as percent product recovery. Several different condenser systems were tested to determine which were applicable to gas stripping a batch fermentation. A list of these systems can be found in Appendix A.

FERMENTATION IN LABORATORY FERMENTORS

Batch and Continuous Single-Stage Fermentations

A 2.0 liter bioreactor (Biostat-M; B.Braun, Inc.) with 1.0 liter working volume was used for all single-stage studies. The stirrer speed was kept at 80 rpm and the temperature at 35°C. The feed and the spent broth flows were maintained by an 8-channel pump.

The bioreactor was sterilized at 121°C for 20 min with the whey permeate and allowed to cool inside the anaerobic glove box. Other media components were added to prepare the complete starting medium (Diez-Gonzalez, 1992).

The bioreactor was inoculated with an active 24-hour old culture. For batch cultures, samples were taken every 6 to 8 hours. For continuous cultures, the feeding and removal of medium started at 10 to 12 hours, and the reactor was sampled every 6 to 12 hours.

Two Stage Fermentations

For the two-stage experiments, the first stage reactor was a 0.8-liter bioreactor (Bioflo C-30, New Brunswick Scientific Co.) with a 0.4 liter working volume, stirred at 150 rpm, and maintained at 35°C. The preparation of this fermentor was similar to that described above. The Braun bioreactor was used for the second stage. The dilution rates were set to $D_1 = 0.2 \text{ h}^{-1}$ and at $D_2 = 0.09 \text{ h}^{-1}$ for the first and second stage reactors respectively.

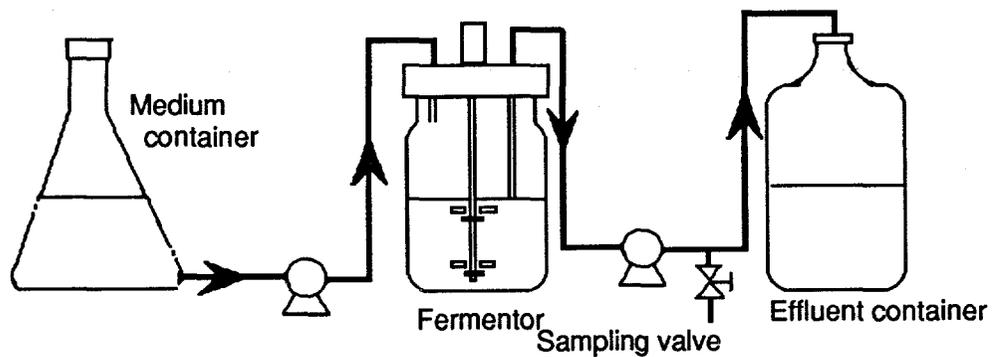
Cell Recycle Fermentations

In the single-stage cell recycle experiments, the cell-free effluent was separated from the fermentation broth in an isotropic hollow fiber microfiltration unit (MiniKros, Microgon, Inc.) with 300 sq cm area, and a 0.2 mm pore size. The unit was sterilized in advance with a detergent wash, rinsed in 75% ethanol, and then rinsed with sterile distilled water.

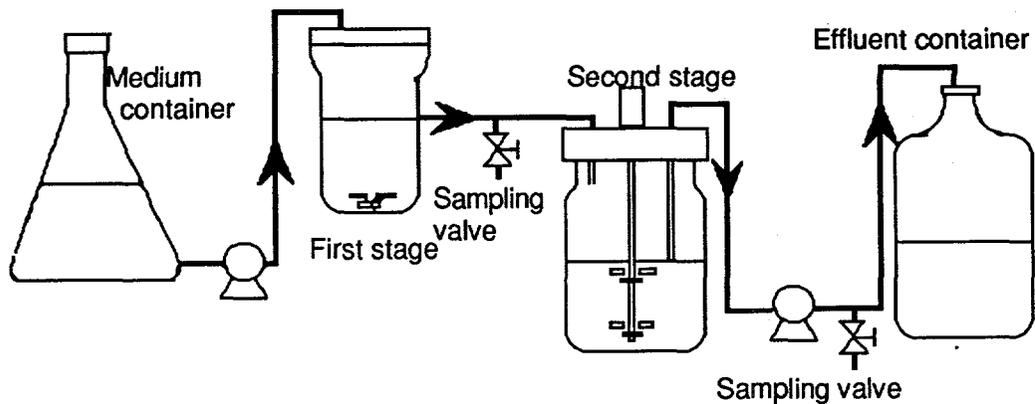
The two-stage cell recycle experiments were carried out using an isotropic hollow fiber microfiltration module (CellFlo, Microgon, Inc.) with 3915-cm² area and 0.2- μm pore size. It was sterilized at 121°C for 30 min before use (Diez-Gonzalez, 1992).

Gas-Stripped Fermentations

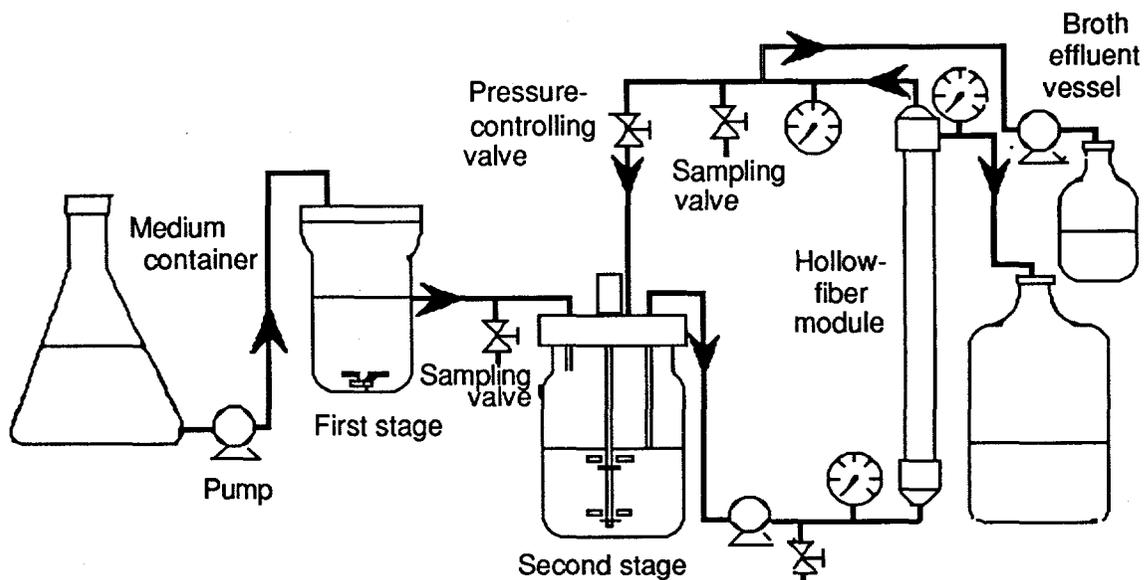
Gas stripping batch cultures were run in the 2-l Braun fermenter as described above. The equipment for gas stripping of glucose based fermentations was sterilized in two different ways. The fermenters, strippers, and 4-l flasks were autoclaved. All other parts, such as the condensers, manometers, and tubing were sanitized with 75% ethanol for 20 min and then rinsed with autoclaved water. After assembly, nitrogen gas was flushed through the entire system for 1 hour to remove any oxygen from the system. For gas stripped fermentations, the fermenter was inoculated with 30 ml of an active 24-hour old culture. The control experiments with no gas stripping were sampled every 4-6 hours and allowed to run until glucose uptake stopped. Gas stripping experiments were allowed to run as normal batch cultures until the solvent break, at which point gas stripping commenced. Samples were taken every 4-6 hours, except for more frequent samples in the first few hours of stripping. Condensate was collected from the condensers at the end of each run.



A) Single-stage continuous culture system



B) Two-stage continuous culture system



C) Two-stage continuous culture with cell-recycle system

Figure 2-3. Continuous Culture Fermentation Systems

ANALYTICAL METHODS

pH and OD-600 Recording

pH measurements were taken with a pH meter (Ingold) on collected samples. In the whey based gas stripping experiments, the pH meter was placed in an external loop and connected to a computer (IBM XT) through an A/D board, and pH measurements were taken automatically every 0.5 hours after a startup period of approximately 5 hours. Cell mass was estimated by optical density of the broth at 600 nm. Samples were diluted if absorbances were above 1.0 units, and an empirical correlation between absorbance and cell dry weight was used to convert the sample absorbances to cell concentration units.

In whey based gas stripping experiments, the optical density was monitored in an external loop with a flow cell having an optical path length of 3 mm. The narrow flow cell provided sufficient liquid velocity to prevent settling of the cells, and also allowed the measured absorbance to remain under 1.0 unit at all times throughout the fermentation. Optical density measurements recorded by the computer every half hour and were converted to cell dry weight data using the correlation described above.

Off-Line Methods

Biomass was estimated by optical density at 600 nm and dry weight analyses. Solvents, lactose, and organic acids were determined by HPLC (Biorad Labs, Inc.) with an Aminex HPX-87H column, a 1750A refractive index detector, and a 1306- ultraviolet light detector. The operation conditions were: eluent, 0.005 mol/l sulfuric acid; flowrate, 0.7 ml/min; column temperature, 40°C. Samples were diluted with eluent, centrifuged, and filtered through 0.45- μ m membrane filter before injection. Pyruvate and valerate were used as internal standards. (Diez-Gonzalez, 1992)

For the extraction work, solvents were analyzed using a Hewlett-Packard Model 5710 gas chromatograph with FID detector, with a 2-m Porosil column and helium as carrier gas. A 0.4 μ l sample was injected. A temperature program of 60°C for 4 min followed by an 8°C temperature rise to 190°C gave good separation and a butanol peak at 15.5 minutes.

Morphological changes in the culture were observed in a phase-contrast microscope (Laborlux S, Leitz GmbH).

Section 3

RESULTS AND DISCUSSION

LIQUID EXTRACTION OF BUTANOL

Culture Inhibition

Shukla and coworkers(1989) reported toxic effects of 2-ethyl-1-hexanol on *C. acetobutylicum* ATCC 824 growing on a glucose-based media. The saturation concentration of 2E1H is 0.66%. At 0.6% 2E1H, Shukla found a 40% decline in glucose uptake and a 25% reduction in butanol formation. In contrast, we found a 50% growth inhibition at only 20% of saturation, and zero growth was observed above 50% of saturation. We conclude that our micro-organism may be more sensitive to the extractant, or that the whey permeate medium may contain factors that sensitize it to the toxic effects of the extractant.

Partition Coefficient

The partition coefficient P is the ratio of the equilibrium concentration of the solute (butanol) in the extractant phase and the aqueous phase: $P = C_e/C_w$, where C_e is the butanol concentration in the extractant phase and C_w in the aqueous phase.

Since C_e cannot be measured directly, it is determined from a material balance on butanol, given the original volumes of extractant and aqueous phase (V_e and V_w , respectively) and the original and final concentration of butanol in the aqueous phase, C_{w0} and C_w . Thus

$$P = (C_{w0}/C_w - 1) * V_w/V_e.$$

A partition coefficient that decreases with C_{w0} is not unusual for a partially miscible system such as butanol/water/2-ethyl-1-hexanol. The average partition coefficient for the 1-2% butanol range was 10.7. The coefficient of 5.8 measured on fermentation broth is close to the 5.9 reported by Shukla and coworkers (1989).

Table 3-1. Partition Coefficient Of Butanol Between Water Or Fermentation Broth And 2-Ethyl-1-Hexanol.

C_{w0}	$C_w, \%$	V_w, ml	V_e, ml	P
1.0% in water	0.072	4	4	12.7
1.5% in water	0.124	4	4	11.1
2.0% in water	0.238	4	4	7.4
1.5% in broth	0.221	4	4	5.8

Mass Transfer Coefficient

A mathematical model for performance of the hollow fiber extractor was developed by analogy to the hemodialyzer model given by Colton and coworkers (1981). Both the Liqui-Cel extractor and the hemodialyzer are hollow fiber mass exchangers run in countercurrent flow. The only difference is that the driving force for solute movement in the Liqui-Cel unit must be modified to account for the partition coefficient P, which is unity in the hemodialyzer.

The model is presented in Appendix B. From it we can define the mass transfer coefficient k_0 for transport of butanol across the hollow fiber membrane, and can also calculate the expected performance of the equipment at any flow rates, partition coefficient, or initial butanol concentration.

In the butanol/water system, aqueous flow rates from 28 to 330 ml/min, and extractant flow rates of 12 to 67 ml/min were tested in various combinations. The overall mass transfer coefficient k_0A ranged from 19 to 103 cm^3/min , with an average value of 51 cm^3/min . The mass transfer coefficient depended on the aqueous flowrate but not on the extractant flow rate. Typically, 30 to 40% of the butanol was removed from the aqueous phase on a single pass, for mass removal rates ranging from 0.1 to 0.8 g/min, with an average of 0.39 g/min or around 23 g/hr under the conditions tested.

In the butanol/fermentation broth system, with broth flow rates of 137 and 200 ml/min, and extractant flow rates of 58 - 120 ml/min, the average overall mass transfer coefficient was 70 cm^3/min , and the average butanol transfer rate was 0.61 g/min or 36.5 g/hr under the conditions tested. The extractant also partitioned into the aqueous phase, building to a final concentration of 0.08 to 0.10%, or 13 to 16% of the saturation concentration. The above

performance is extraordinary but was achieved under impractical conditions. First, the entering extractant stream contained no butanol. For every 1% of butanol in the extractant, the rate would decline by around 20%. Second, the best results were obtained at high water and broth flowrates. In the cell recycle experiments described below, the cells appeared to sustain shear damage at broth flowrates of 10 ml/min. At a broth flowrate of 10 ml/min to the liquid extraction unit, essentially all butanol would be removed from the broth, yielding a transfer rate around 3 g butanol/hr to a butanol-free extractant. At this low flow rate, the broth would also become saturated (0.66%) with extractant; at this concentration, complete inhibition of cell growth and butanol production is likely.

BATCH FERMENTATION OF ACID WHEY PERMEATE

Four solventogenic strains of *Clostridia* were tested to select the best performer on acid whey permeate. In serum bottles, *C. acetobutylicum* strain P262 fermented lactose two times faster and produced three times more butanol than strain ATCC 824, (the type strain), ATCC 39058 or *C. beijerinckii* strain LMD 27.6. These results confirmed previous reports (Yu et al, 1987; Ennis and Maddox, 1985) on the superior ability of P262 to utilize lactose.

In a stirred tank reactor, *C. acetobutylicum* strain P262 grew rapidly on acid whey permeate (Figure 2-2). Both lactose and lactic acid were metabolized simultaneously. The final solvent concentration was 1.14% (0.87% butanol and 0.27% acetone). No ethanol was detected. The final butanol:acetone ratio was 3.5:1. The maximum butanol and solvent production rates were 0.79 g/l-h and 1.11 g/l-h, respectively. The solvent yield was 0.28 g solvents/g lactose, and the overall productivity, 0.32 g/l-h at 35 hours.

P262 fermented a maximum of 5.5% sugar in batch cultures of acid whey permeate supplemented with 2% additional lactose. Under these conditions, the final solvent concentration increased to 1.52% (1.01% butanol, 0.51% acetone), the productivity to 0.46 g/lh. and the solvent yield was 0.27 g solvents/g lactose. These results were similar to reported results for whey, but were relatively low compared to cultures grown on glucose (Bahl et al, 1986). The maximum butanol concentration of typical glucose cultures is 1.9 % and the yield is approximately 0.32 g solvents/g lactose. The relatively high final butyrate concentration detected in cultures fermenting acid whey permeate may explain the slightly low solvent production. The underlying cause for this result might be the cometabolism of lactic acid. Lactic acid consumption caused a pH-control effect in the medium maintaining a relatively high pH that allowed acidogenesis to occur at the same time as solventogenesis.

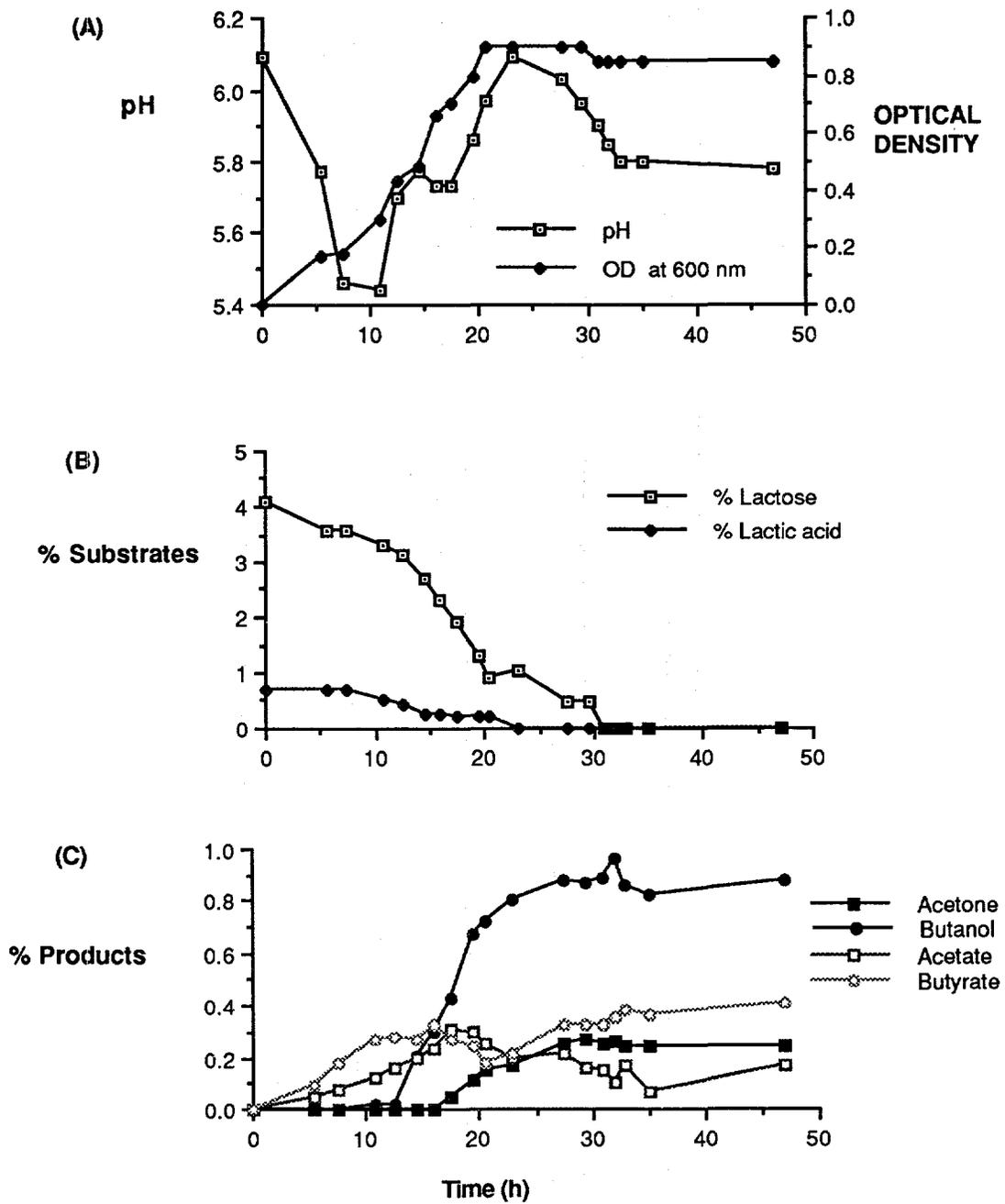


Figure 3-1 Batch Culture of *Clostridium acetobutylicum* strain P262 on acid whey permeate medium.

CONTINUOUS CULTURE OF ACID WHEY PERMEATE

A series of single-stage and two-stage continuous culture experiments were performed to investigate the continuous fermentation of acid whey permeate by P262. A cell-recycle system was also used to determine whether solvent productivity could be increased. A diagram of each of these fermentation systems is shown in Figure 2-3.

Single-stage system

The profile of a typical continuous culture over a period of 10 days is shown in Figure 3-2. The dilution rate (D) was 0.06 h^{-1} . The most noticeable feature of these plots was that no steady-state was obtained. Instead, a series of oscillations characterized the fermentation profiles. The minimum lactose concentration of 0.63% was observed at the same time as the maximum solvent concentration of 1.13% (0.89% butanol) was detected.

Table 3-2. Summary Of The Fermentation Parameters Of Single-Stage Continuous Cultures

EVALUATION PARAMETERS		SINGLE-STAGE CULTURE		
		STANDARD	pH 5.5	CELL RECYCLE
Solvent Productivity (g/l-h)	Mean	0.37	0.07	0.28
	S.D. ¹	0.18	0.07	0.31
	Max. (h) ²	0.69 (143)	0.23 (203)	1.09 (42)
Lactose Uptake Rate (g/l-h)	Mean	1.46	0.75	1.23
	S.D.	0.45	0.31	0.48
	Max. (h)	2.36 (16)	1.14 (203)	2.11 (72)
Yield (g solvents/ g lactose)	Mean	0.26	0.09	0.19
	S.D.	0.11	0.07	0.16
	Max (h)	0.45 (26)	0.24 (108)	0.52 (66)
Butanol:Acetone Ratio	Mean	4.56	---	3.81
	S.D.	1.22	---	0.93
	Max (h)	6.62 (45)	---	5.76 (107)

¹S.D.= standard deviation

²Max (h)= Maximum at (hours)

Table 3-2 summarizes the fermentation parameters of cultures run at $D = 0.06 \text{ h}^{-1}$. The culture at pH 5.5 was undertaken to determine if butanol productivity could be increased by lowering pH, as suggested by Qureshi and Maddox (1991). The culture with cell recycle

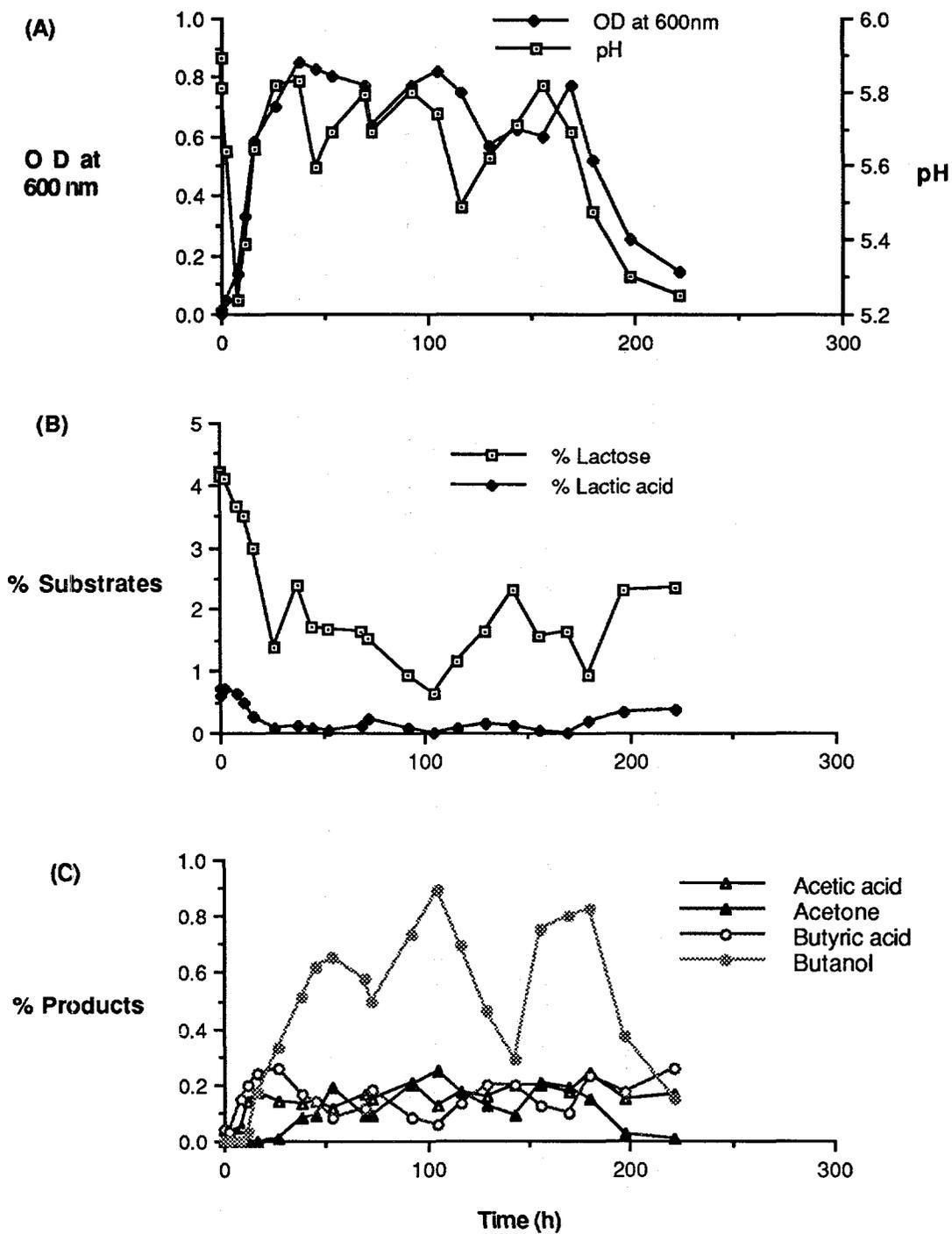


Figure 3-2. Single-Stage Continuous Fermentation of Acid Whey Permeate by Strain P262.

was an attempt to increase butanol productivity by increasing cell density. The overall productivity of all of these cultures appeared to be lower than the batch fermentor because of the metabolic oscillations. However, peak productivities in the standard and cell-recycle cultures were, respectively, 46 and 100% superior to the batch mode. The average solvent yield in all single-stage cultures was lower than the batch culture, 0.32 g solvent/g sugar. The pH 5.5 run performed very poorly, possibly due to the presence of lactic acid in the medium; this was the chief difference between our system and that of Qureshi and Maddox. The use of a microfiltration unit to recycle cells did not improve butanol production.

Two-stage system

A two-stage continuous fermentation system (Figure 3-2) was investigated to improve the stability and butanol productivity of continuous cultures on acid whey permeate. Afschar and Schaller (1991) have claimed that in a two-stage system, acidogenesis and solventogenesis can be separated by running the first fermentor at a greater D than the second reactor. The separation of the fermentation phases could supposedly increase culture stability and, consequently, butanol production in the solventogenic second reactor.

At D values of 0.2 h^{-1} and 0.09 h^{-1} in the first and second reactor, respectively, steadier states were obtained in both vessels compared to the single-stage cultures (Figure 3-3 and 3-4) Oscillations was markedly decreased, though not totally eliminated. The overall fermentation parameters of four two-stage cultures are shown in Table 2-3. Compared to batch and single-stage continuous cultures the overall solvent productivity and the lactose uptake rate increased approximately two-fold and three-fold, respectively. However, the solvent yield and the butanol/acetone ratio remained relatively unchanged. Among two-stage systems, the change in dilution rate and the application of cell-recycle did not have any significant change on the fermentation parameters.

The change of D from 0.06 h^{-1} to 0.09 h^{-1} did not have any effect on the solvent productivity of cultures under standard conditions (mean 0.37 g/lh , maximum 0.70 g/lh), but the solvent yield decreased from 0.255 to $0.115 \text{ g solvent/g lactose}$. This apparently contradictory result is due to an increase in the ratio of acid to solvent production at the faster growth rate.

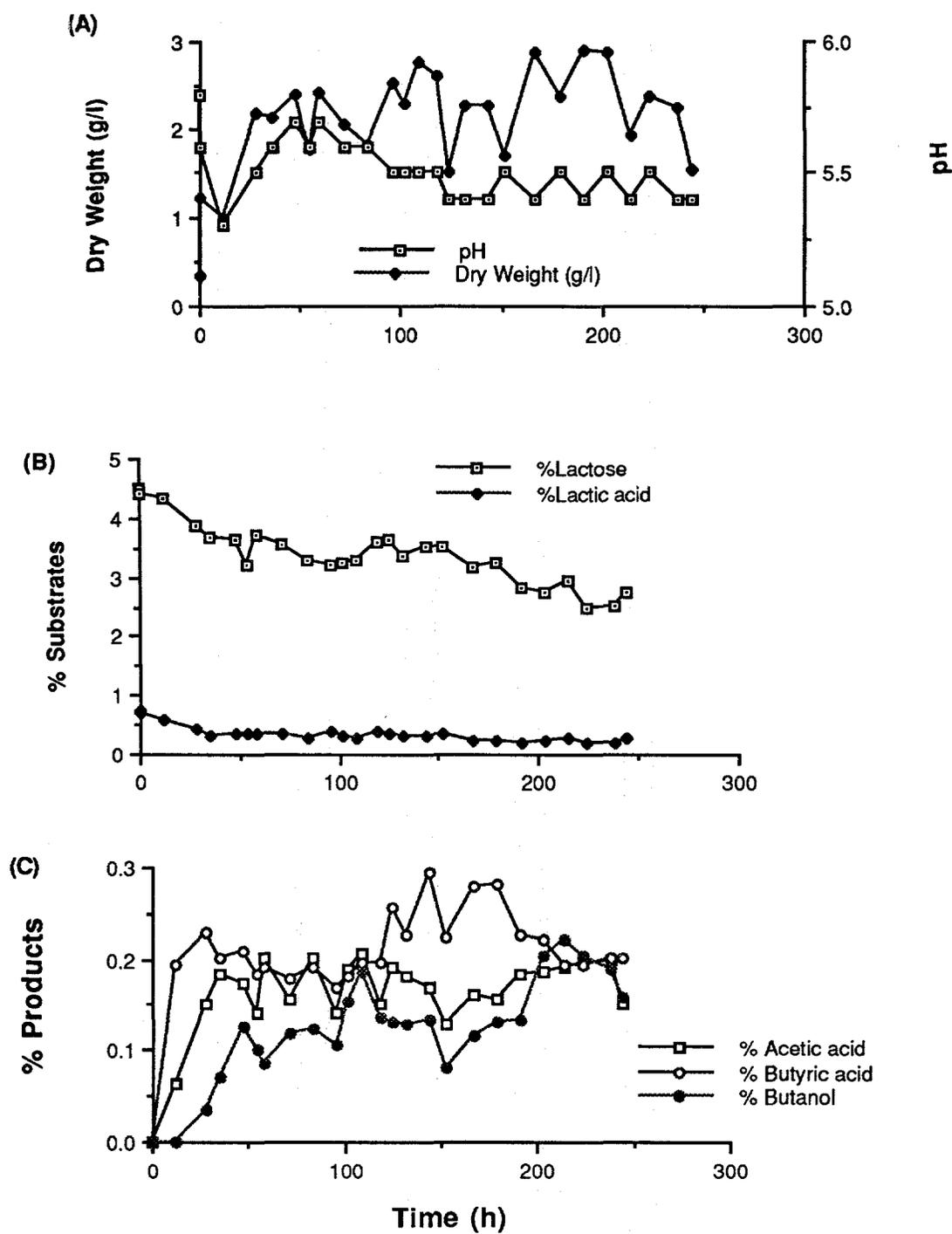


Figure 3-3. Fermentation Profile Of the First Reactor of A Two-Stage Continuous Culture ($D_1 = 0.2 \text{ h}^{-1}$).

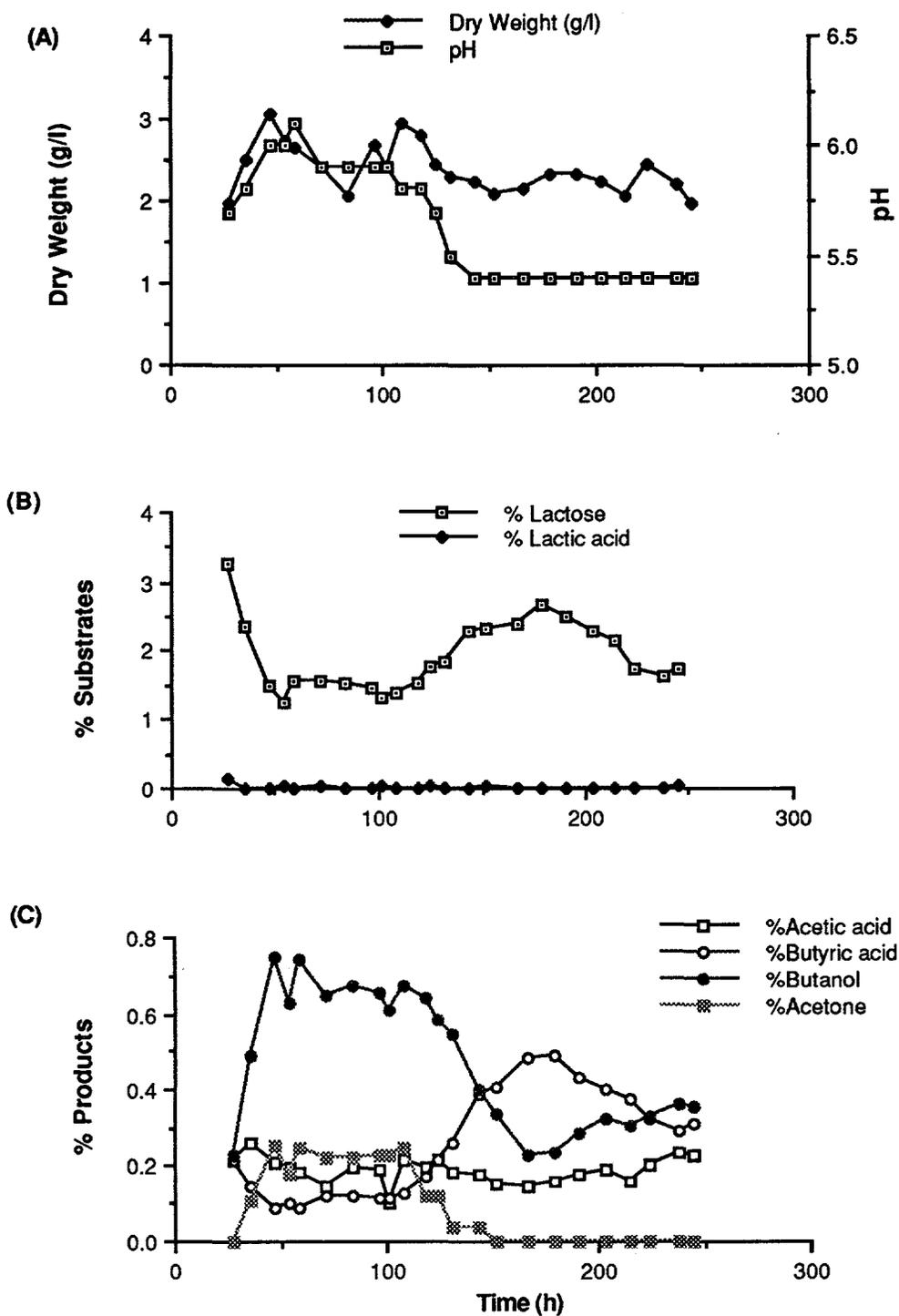


Figure 3-4. Fermentation Profile of the Second Reactor of a Two-Stage Continuous Culture ($D_1 = 0.09 \text{ h}^{-1}$).

Table 3-3. Two-Stage Continuous Cultures: Overall Performance Parameters.

TWO-STAGE CULTURES		OVERALL FERMENTATION PARAMETERS			
		Solvent Productivity (g/l.h)	Lactose Uptake rate (g/l.h)	Yield g solvent/ g lactose	Ratio butanol/ acetone
Low Dilution Rate ⁴	Mean	0.71	4.14	0.16	4.31
	S.D. ¹	0.31	1.26	0.05	2.25
	Max ² (h)	1.10(152)	6.35 (43)	0.24	9.95
High Dilution Rate ⁵	Mean	0.68	3.83	0.2	3.5
	S.D.	0.3	0.91	0.13	0.94
	Max (h)	1.18 (48)	5.89 (224)	0.71	5.29
Total Cell Recycle ⁵	Mean	0.68	3.75	0.17	4.71
	S.D.	0.41	0.61	0.08	1.49
	Max (h)	1.31(66)	4.92 (75)	0.29	7.06
Partial Cell Recycle ⁵ $\partial=0.50$	Mean	0.74	3.53	0.21	3.88
	S.D.	0.36	0.88	0.07	2.76
	Max (h)	1.43(48)	5.45 (48)	0.29	11.5

¹S.D.= standard deviation

²Max (h)= Maximum at (hours); time reported only for solvent productivity and lactose uptake rate

⁴D₁= 0.15 h⁻¹ and D₂= 0.06 h⁻¹

⁵D₁= 0.2 h⁻¹ and D₂= 0.09 h⁻¹

Summary

Clostridium acetobutylicum strain P262 clearly demonstrated its superior ability to ferment acid whey permeate compared to other clostridial species screened. Its ability to ferment lactose and to cometabolize lactic acid represent very suitable substrate preferences for the use of acid whey permeate for solvent production. The ability to utilize lactose may be due to the P262's advantage of having relatively higher levels of intracellular phospho- β -galactosidase and β -galactosidase enzymes to metabolize lactose compared to those of other strains (Yu et al, 1987).

None of the two-stage continuous cultures ever achieved steady state in the second stage. Oscillatory behavior, such as that shown in Figures 3-2 and 3-4, was observed in nearly all continuous runs. Butanol inhibition, substrate starvation, or both effects combined are likely to be responsible for the oscillatory pattern. Similar oscillatory patterns were reported by Clarke et al (1987) and Barbeau et al (1988).

Similar to other reports (Barbeau et al, 1988; Afschar and Schaller, 1991) the two-stage continuous cultivation system achieved higher butanol productivity and more stable operation than the single-stage continuous mode. Overall mean solvent productivity was increased from 0.37 g/lh to 0.71 g/lh and the oscillation's wavelength increased from approximately 70 h to approximately 150 h.

The cell morphology in the second stage of all the two-stage cultures with cell-recycle was characterized by a low concentration of long cells and appearance of significant quantities of cell debris and deformed cells. This suggests that the shear stress caused by the recycling of cells disrupted the longer cells.

In two-stage cultures, the first stage accounted for 25 - 40% of the overall solvent produced. The lactose uptake rates and yields in the first stage were lower than in the second one because most of the lactose was converted to acids instead of solvents. The higher parameters in the second stage were probably due to the re-assimilation of organic acids. In agreement with theoretical predictions, as the cell-recycle ratio in the second stage increased, cell densities increased. The total cell-recycle culture achieved the highest cell concentration reaching 14 g/l dry weight, while the high-dilution rate culture with no cell-recycle attained a maximum of 3.1 g/l dry weight.

The fermentation parameters of the second stage and the overall culture performance evaluation parameters showed no significant differences among these cultures. This indicates that change in the fraction of recycled cells do not cause a sensible effect on butanol production under current conditions. High butanol concentrations affect cells of strain P262 either at high or low populations, therefore the result is similar in all cases. In summary, the overall results of the present work suggests that under oscillatory behavior the mean reactor productivity and culture instability cannot be improved by cell recycle.

GAS STRIPPING

Butanol/Water Model System

Initial studies on a model fermentation broth showed that gas stripping was able to remove solvents from the broth without removing critical product intermediates. A time course of a typical model-broth experiment is shown in Figure 3-5.

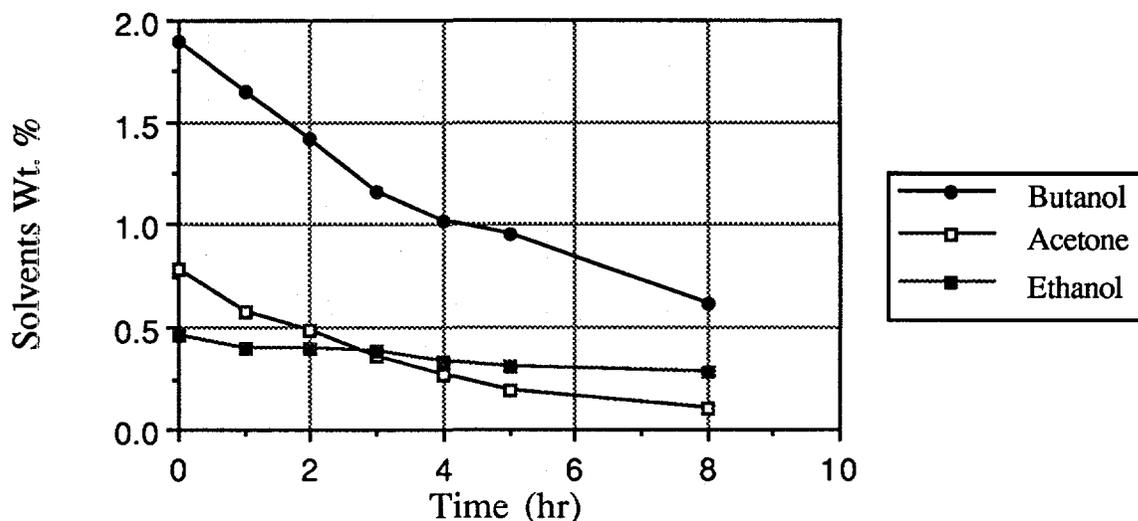


Figure 3-5. Solvent Stripping Rates on a Model Fermentation Broth.

Butanol was removed at a rate of 0.13 g/h-(g/l). Assuming the maximum rate of butanol production of 0.6 g/l-h. and the specified gas stripping rate, an equilibrium is achieved when butanol concentrations reach 0.46%, well below the 1.0% levels that are considered toxic to the bacteria.

Acetone was removed at a rate of 0.22 g/hr-(g/l). At the maximum rate of acetone production of 0.62 g/l-h in a regular fermentation and the specified gas stripping rate, an equilibrium is achieved when acetone concentrations reach 0.28%. Ethanol was removed at a rate of 0.05 g/h-(g/l), much more slowly than either acetone or butanol, but was never produced in any of the fermentations. Fermentation acids, such as lactic and acetic acid, which are reassimilated by the bacteria during solventogenic growth, are not removed by gas stripping.

The model broth systems also tested the ability of the condenser systems to extract the solvents from the vapor stream. Several different condenser systems were tested for their ability to condense the solvent vapors from the gas stream and their feasibility for use in a real fermentation system. They are described in Appendix A. A final condenser system consisting of a glass condenser cooled to 0 °C with ethylene-glycol followed by an aluminum condenser cooled to -20 °C with acetone/dry ice was chosen for the fermentation experiments. Mass balances on this system were able to account for 90% of the butanol and 70% of the acetone removed. Some of the solvent is not recovered because it was held up in the condensers as moisture on the walls of the condensers. This is a one-time loss at the start of the gas stripping run, and is minimized by longer fermentations. Some solvent, especially the more volatile acetone, is lost during sampling and sample preparation for HPLC analysis. This loss is most severe in work with the condensate because of the high solvent concentrations involved.

Using the model broth, at roughly 2% butanol by weight, a two-phase condensate was obtained from the second condenser. The aqueous phase contained approximately 6% butanol by weight, and the organic phase approximately 60% butanol. A two phase condensation would greatly improve the economics of the fermentation by producing a 30-fold purification in a single step.

Fermentation on Glucose Media

A batch glucose fermentation with no gas stripping, shown in Figure 3-6 , produced a total of 10.5 g butanol and 4.0 g acetone in 38 hours, for a total solvent productivity of 0.38 g/l-hr. This fermentation was limited by the solvents produced since there was still plenty of available sugars left at the end of the fermentation. Glucose consumption was limited to 42 g, or 65% of the available glucose. This is not a very efficient use of the available glucose. Maximum rates of butanol and acetone production achieved were 0.6 g/l-hr and 0.62 g/l-hr, respectively. Butanol production started around 15 hours, continuing until the end of the fermentation, and acetone production started at 19 hours and ended at 25 hours.

Gas stripping a glucose-based fermentation is shown in Figure 3-7. It produced 11 g butanol and 5.9 g acetone in 38 hours, for a total solvent productivity of 0.44 g/l-hr. This increase in productivity was brought about by an increase in the solvent producing phase of the fermentation. Glucose consumption increased 1.5 fold from 42 to 63 g, with 84% of all the available sugar consumed in the fermentation.

Although no two-phase condensate was recovered, 70% of the butanol was recovered in the second condenser at a concentration of 14%. This represents a 14-fold increase in the butanol concentration normally available for downstream processing. All of the acetone produced was recovered in the second condenser at a concentration of 14%, which is a 35-fold increase. This increase in solvent concentration should dramatically reduce the cost of purifying the solvents for eventual sale.

During the gas stripping runs, the volume in the reactor slowly declined as water was stripped out of the fermentation along with the solvent vapors. This had the effect of further concentrating the solvents still left in the fermentor as well as the cells and remaining substrate. In a continuous or fed-batch culture, forced evaporation produced the same effect as cell recycle, without the cell damage caused by repeated pumping through the hollow fiber unit.

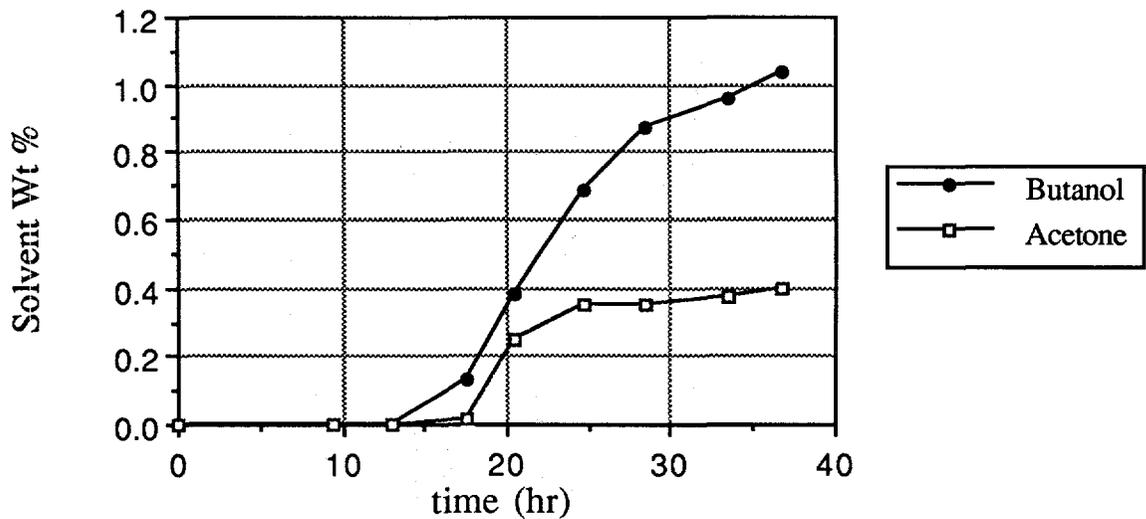


Figure 3-6. Standard Batch Fermentation of Glucose.
A: Substrate Uptake. B: Product Formation.

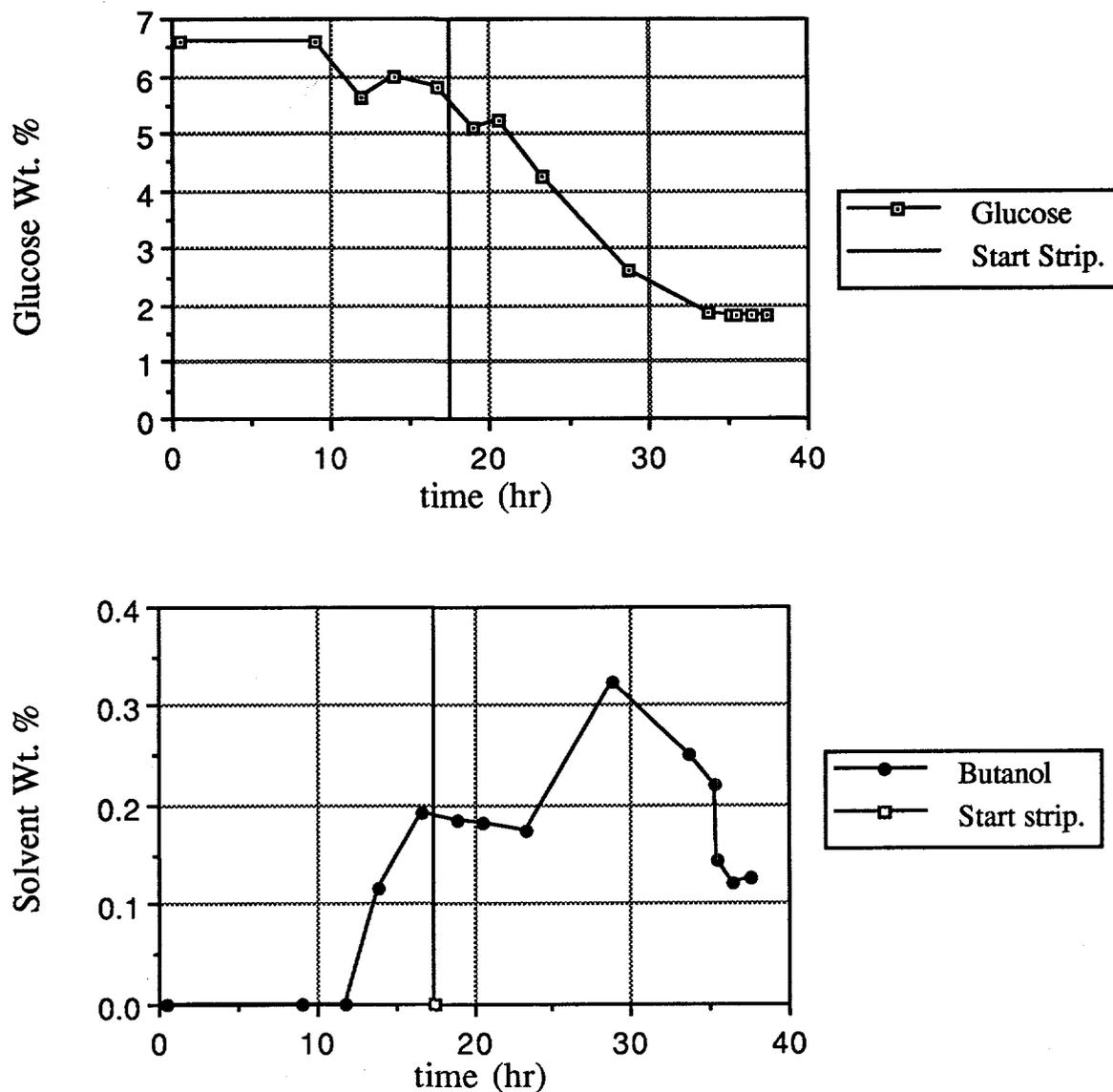


Figure 3-7. Batch Fermentation of Glucose with Gas Stripping. A: Substrate Uptake. B: Product Formation.

Fermentation on Whey Media

Regular batch fermentations were also undertaken on acid whey permeate supplemented with 2% lactose, as shown in Figure 3-8. This medium was chosen to provide more substrate than available in the whey alone. The fermentation profiles of the glucose-based and whey-based fermentations were very similar. In the control fermentation, 10.7 g of butanol and 4 g acetone were produced by the fermentation in 38 hours, giving a total

solvent productivity of 0.39 g/l-hr. Compared with the glucose fermentation, the whey fermentation is slightly slower, with the maximum rates of butanol and acetone production at 0.53 g/l-hr and 0.25 g/l-hr, respectively. The whey fermentation consumed 52 g lactose, or 85% of the available lactose, and a large proportion of the lactic acid.

Gas stripping the whey fermentation, as shown in Figure 3-9, produced 9.7 g butanol and 5.6 g acetone in 38 hours, for a productivity of 0.40 g/l-hr. This very slight increase in productivity is due to a small extension of the solventogenic phase of the fermentation. A total of 57 g of lactose were consumed, or 92% of the available lactose. This represents a 10% improvement over a regular batch fermentation without gas stripping.

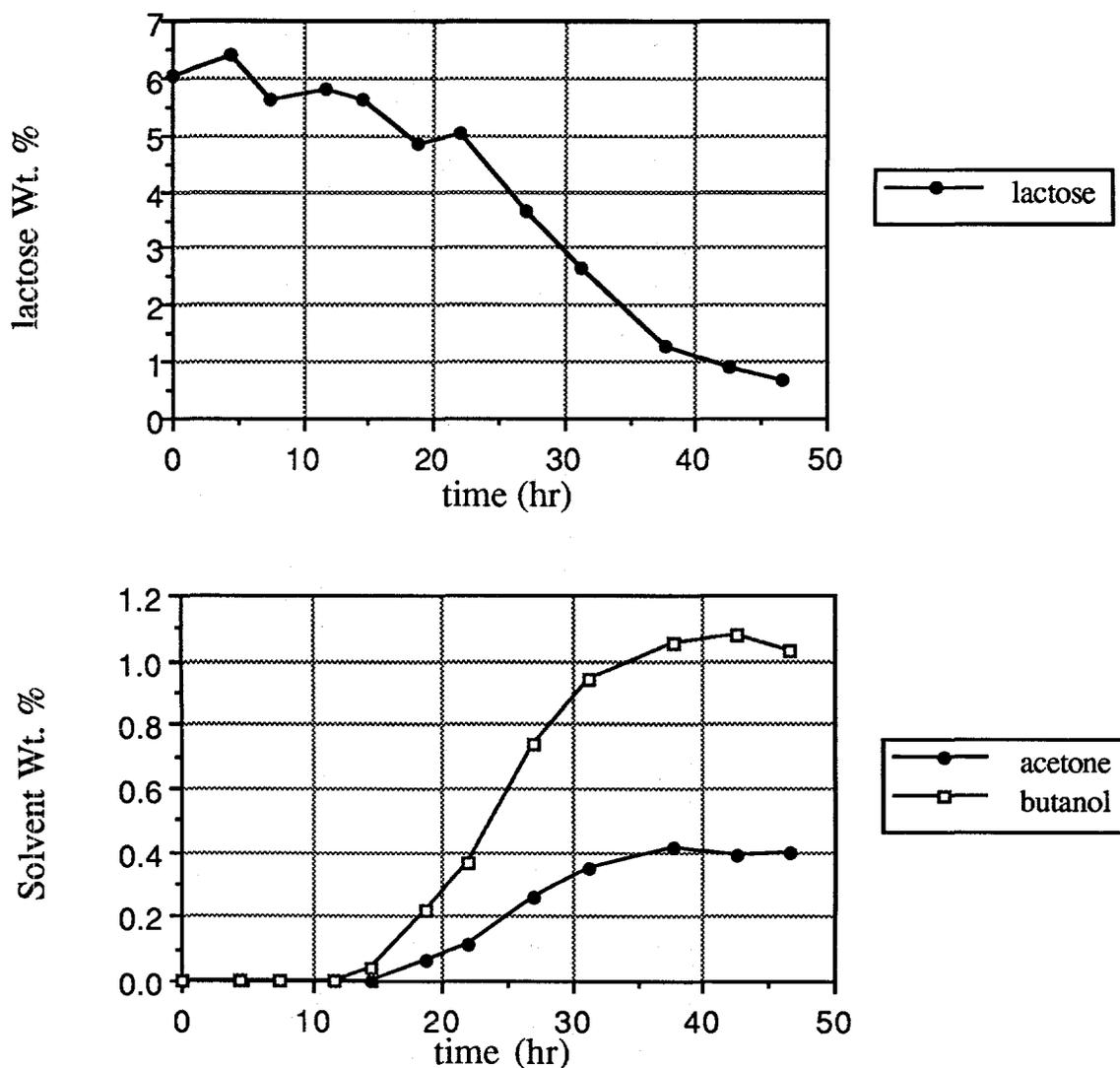


Figure 3-8. Standard Batch Fermentation of Whey Permeate with 2% Added Lactose. A: Substrate Uptake. B: Product Formation.

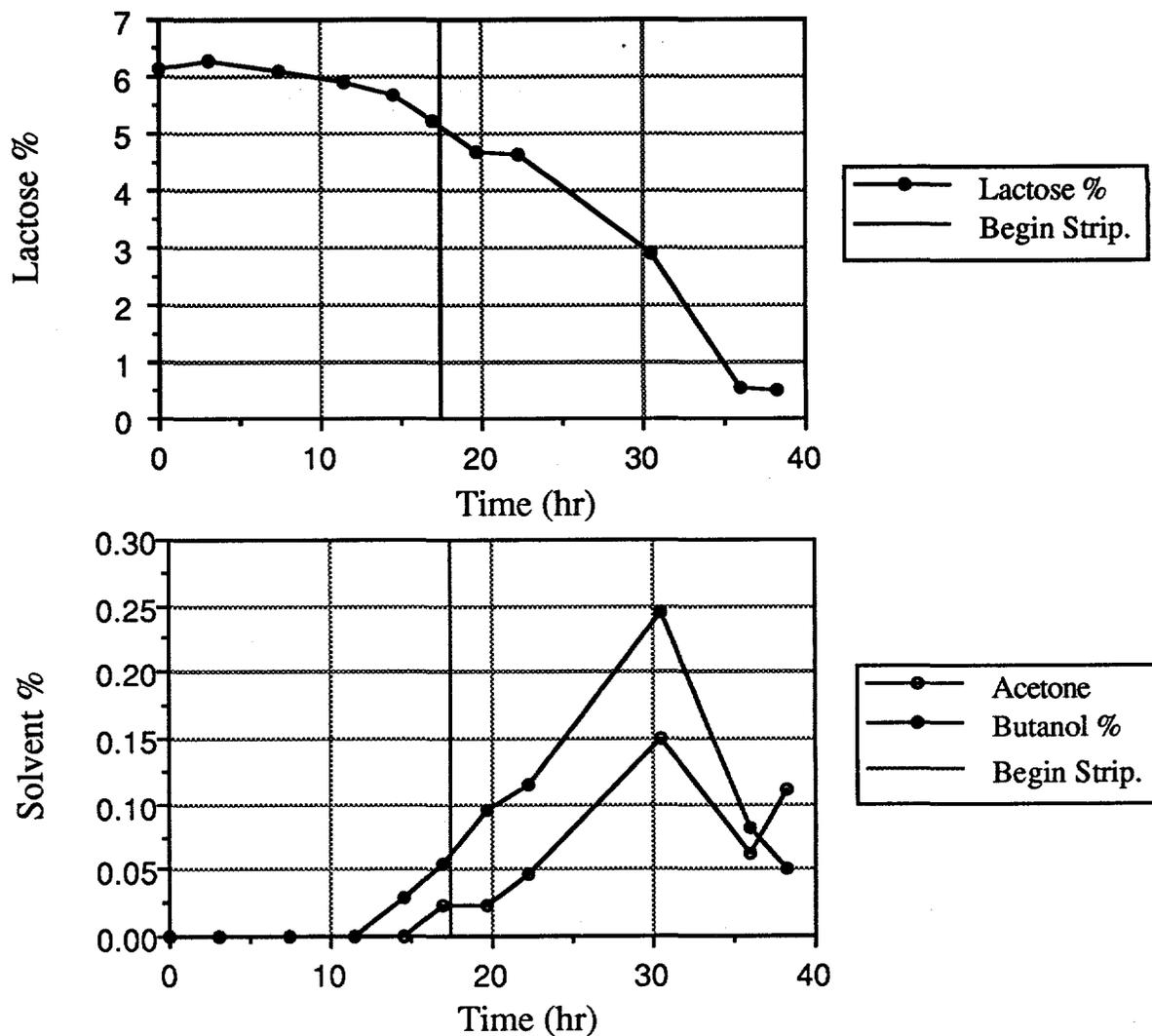


Figure 3-9. Batch Fermentation of Acid Whey Permeate With 2% Added Lactose. Solvent Recovery by Gas Stripping. A: Substrate Uptake; B: Product Formation.

No two-phase condensate was recovered, but 75% of all butanol and 80% of all acetone produced was recovered in the second condenser. Their concentrations were 9% and 5% respectively, again representing large purification factors.

In this experiment, gas stripping principally served as a way to concentrate the products. The productivity increase was small because the control fermentation was near substrate limitation. Gas stripping cannot improve productivity at low initial substrate concentrations since the fermentation terminates from starvation rather than product inhibition. Better performance can be expected from conditions that supply a substrate excess, such as fed-batch fermentation, continuous culture, or a high initial substrate concentration.

Section 4

CONCLUSIONS

Culture screening results showed *C. acetobutylicum* P262 to be the best performer on acid whey permeate. The strain even assimilates lactate as a co-substrate with lactose, taking up 7 g/ of lactic acid in batch and continuous culture. The use of lactate as an additional carbon source may be partly responsible for the higher productivity of P262. From an industrial viewpoint, lactate consumption is additionally valuable since it reduces another source of BOD in the effluent.

Batch and single-stage cultures had approximately the same productivity, approximately 0.31 g/l-h of solvent. Cell recycle did not improve productivity. It is likely that the benefits of higher cell density were offset by the destruction of solvent-producing cells by shear stresses in the pump or hollow fiber microfiltration unit. Two-stage continuous culture approximately doubles the productivity of the system to around 0.7 g/l-hr. This system was not optimized and additional improvements are likely.

Based on our mass transfer studies, hollow-fiber-assisted liquid/liquid extraction is capable of extremely high rates of butanol recovery, on the order of 30 g butanol per hour in our small system. However, solvent toxicity and shear damage of cells make liquid extraction impractical for free-cell fermentation of *C. acetobutylicum* P262. Liquid extraction remains promising for *in-situ* butanol extraction if the problems of cell toxicity and shear damage can be solved. One possibility is an immobilized-cell fermentation, where the non-growing cells are less susceptible to toxicity than the growing cells used in free-cell fermentations.

Gas stripping was shown to recover a clean, clear condensate of butanol and acetone in high yield and with concentration factors of 14 to 35. Gas stripping maintained solvent concentration in the fermentor at 2 to 4 g/l, even at the peak of the solvent production rate. By contrast, the control fermentations accumulated 15 to 19 g/l of solvents and stopped because of product inhibition. Gas stripping produced a 50% improvement in substrate consumption in batch fermentation, and a 10 to 20% improvement in solvent productivity. We believe that the whey permeate fermentation was too close to substrate limitation to show the full benefit of gas stripping, and that even better results will be obtained on an amended whey with 80 to 100 g/l sugars.

Gas stripping completely alleviated solvent toxicity and converted product-limited fermentations to substrate-limited fermentations. The current rates of removal, on the order of 3 g solvent/l-hr, are adequate to maintain a continuous culture fermentation at non-inhibitory butanol levels.

The observed yields, 90% of butanol and 70% of acetone in the model system, were lower than expected because of holdup and losses in the condenser system. Recoveries should improve to near 100% in larger scale systems where condensers are optimized and holdup minimized.

Additional work is proceeding in three areas related to this study: In a direct extension of this work, we are applying gas stripping to continuous solvent production from whey permeate. We are also studying the kinetics and bioenergetics of the lactate metabolism of *C. acetobutylicum* P262. Finally, we are investigating pretreatments for whey permeate substrate to increase its utility in the acetone-butanol-ethanol fermentation.

Section 5

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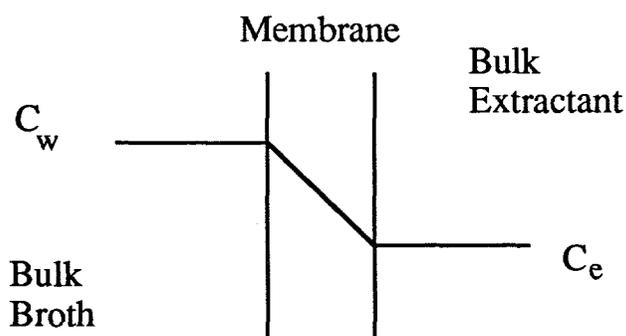
APPENDIX A. CONDENSOR DESIGNS TESTED

<u>Description of Design</u>	<u>Test Results</u>
3 glass/ethylene glycol condensers in series	not cold enough, solvent recovery very poor
2 glass/ethylene glycol condensers followed by two activated carbon adsorbers	Difficult and complicated to extract solvents from adsorbers
2 glass/ethylene-glycol condensers followed by two glass/liquid nitrogen condensers	Too cold, stripping gas (nitrogen) liquefies, shape of condenser allows frost to block gas passage
1 glass/ethylene-glycol condenser followed by one steel/acetone-dry ice condenser	Good recovery of solvents, stripping gas does not liquefy, frost does not block gas passage as badly as above, but steel rusts.
1 glass/ethylene-glycol condenser followed by one aluminum/ acetone-dry ice condenser	No rust, good recovery and gas passage not blocked by frost.

APPENDIX B. MODEL FOR COUNTERCURRENT LIQUID/LIQUID EXTRACTION.

The hollow fiber extractor is a differential, continuous contact apparatus. Unlike conventional liquid/liquid extraction systems, the two liquids do not mix; hence, there are no flooding problems and the aqueous and organic phases may be varied independently. Moreover, the surface area for mass transfer is defined by the available membrane area times the porosity (30%).

Based on the similarity between this system and the hemodialyzer (Colton, 1981), a theoretical model to liquid/liquid extraction may be developed.



Consider a differential length of the extractor. The governing differential equation for diffusive transfer across a differential length of membrane is

$$dM = k_0 (C_w - C_e/P) dA \quad (1)$$

where dM is the moles transferred over differential area dA per unit time, C_e is concentration of solvent in extractant, C_w is concentration of solvent in the fermentor, and P is the partition coefficient. Note that the driving force is affected by the partition coefficient P , unlike hemodialysis where both systems are aqueous and no significant pH gradients are present.

The total mass transfer in the extractor per unit time may be calculated from the inlet and outlet concentrations in each stream; e.g.

$$M = Q_w(C_{wi} - C_{wo}) = \quad (2)$$

$$M = Q_e(C_{ei} - C_{eo}) = \quad (3)$$

Taking the derivatives of (2) and (3) yields

$$dM = -Q_w dC_w = Q_e dC_e \quad \text{or} \quad (4)$$

$$dC_w = -dM/Q_w \quad (5)$$

$$dC_e = dM/Q_e, \quad \text{and } d(C_e/P) = dM/PQ_e \quad (6)$$

Subtracting equation 6a from equation 5 yields,

$$d(C_w - C_e/P) = -dM(1/Q_w - 1/PQ_e) \quad (7)$$

which rearranges to:

$$dM = d(C_w - C_e/P)/(1/Q_w - 1/PQ_e) \quad (8)$$

Combining (1) and (8) we get

$$k_0 (C_w - C_e/P) dA = d(C_w - C_e/P)/(1/Q_w - 1/PQ_e) \quad (9)$$

or

$$d \ln (C_w - C_e/P) = k_0(1/Q_w - 1/PQ_e) dA \quad (10)$$

Integrating over the entire membrane area, and knowing the inlet and outlet concentrations of C_w and C_e , we find

$$\ln \{(C_{w0} - C_{ei}/P)/(C_{wi} - C_{e0}/P)\} = k_0A (1/Q_w - 1/PQ_e). \quad (11)$$

Let us define $x = (C_{w0} - C_{ei}/P)/(C_{wi} - C_{e0}/P)$; then we can solve for the mass transfer coefficient of the entire apparatus,

$$k_0A = \ln x / (1/Q_w - 1/PQ_e). \quad (12)$$