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UPTAKE OF ORGANIC ACIDS BY *Clostridium acetobutylicum* B18 UNDER CONTROLLED pH AND REDUCED BUTANOL INHIBITION

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Abstract—Uptake of organic acids by *Clostridium acetobutylicum* B18 was studied at controlled pH and under reduced butanol inhibition conditions. A pervaporative membrane module was placed in the fermentor to remove butanol from the fermentation broth. Uptake of added butyric acid followed zero order kinetics at pH 4.75 and first order kinetics at pH 5.75. At pH 5.25 the kinetic order shifted from zero to first order as the butyric acid was taken up. At the point of order shift undissociated butyric acid (UBA) concentration was approximately 0.5 g/L. Unlike butyric acid, uptake of acetic acid followed first order kinetics regardless of pH. The difference in acid uptake kinetics could be explained by the combined effect of acid diffusion across the cell membrane and intracellular enzymatic reaction. The acid concentration for kinetic order shift seemed to be dependent upon pH and the kind of the acid used. Glucose was consumed simultaneously with added acids. Both butyric and acetic acids were taken up simultaneously but the rate was faster for butyric acid. Added butyric acid was completely assimilated whereas acetic acid uptake was incomplete.

Key words: *Organic Acids, Clostridium acetobutylicum, Controlled-pH Pervaporative Membrane, Uptake Kinetics*

INTRODUCTION

Butanol has the potential for becoming an important motor fuel additive because it has some better physical and chemical properties compared to currently popular ethanol. Butanol has a higher heat value and lower water solubility than ethanol. Butanol can be produced by fermentation using a variety of low cost carbon sources. However, butanol fermentation produces organic acids (acetic acid and butyric acid) which are not useful for motor fuels. Organic acid formation reduces butanol yield because it diverts some of the carbon flow through the cell's catabolism. Organic acid production cannot be avoided because butanol-producing bacteria obtain their metabolic energy during organic acid formation, but if organic acid production can be reduced butanol production may be increased.

Organic acid concentrations increase as acids are produced during the initial phases of butanol fermentation, and decrease as the cells produce solvents. Organic acid concentrations decrease because the acids are recycled and converted to solvents. Organic acids added to fermentors were also converted to solvents [Fond et al., 1985; Gottschal and Morris, 1981; Hüsemann and Papoutsakis, 1988; Martin et al., 1983].

A butanol producing strain, *Clostridium acetobutylicum* B18, produced more butanol and less organic acid compared with other strains during batch fermentation [Park et al., 1993; Geng and Park, 1993]. This strain was used in an extractive fermentation process utilizing a solvent-selective separation technique (pervaporation through silicone membrane tubing). Cells increased their glucose consumption rate when butanol concentration in the medium was maintained at non-toxic levels. Organic acids did not accumulate in the medium even though a large amount of glucose was fermented during fed-batch operation [Geng and Park, 1994]. The strain efficiently recycled organic acids it produced, in particular, butyric acid. The lack of organic acid accumulation confirmed that *Clostridium acetobutylicum* B18 has an efficient acid recycling system.

In this paper we determined the kinetics of added organic acid recycling by *C. acetobutylicum* B18. This study was done at controlled pH levels because acid uptake rate was expected to be affected by the degree of acid dissociation. The acids were added after the cells recycled the acids they produced themselves. Pervaporation was used to remove butanol during fermentation to minimize butanol inhibition on cell activity.

MATERIALS AND METHODS

1. Bacterium and Media

Clostridium acetobutylicum B18, a mutant strain isolated from *C. acetobutylicum* NRRL B643, was grown in a yeast extract medium (YEM) [Roger and Palosaari, 1987] modified by adding vitamins (mg/L): p-amino benzoic acid 1.0, biotin 0.02 and thiamine-hydrochloric acid 1.0. The strength of this nutrient is expressed as "one unit of modified-YEM" in the rest of the paper. The inoculum was prepared in this modified-YEM with 2 (w/v) % glucose, and the plating medium was made of inoculum medium plus 1.5% agar. The medium was autoclaved at 121℃ for 20 min. Dissolved oxygen in the medium was removed during autoclaving. The medium was maintained in an anaerobic condition by supplying nitrogen gas to the fermentor head space during cooling. The addition of nitrogen gas was continued until sufficient fermentation gas was produced. A decrease in redox potential was noticed by the color change of resazurine from pink to colorless.

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2. Inoculum Preparation

Inoculum was prepared at 35℃ in an anaerobic chamber (Forma Scientific, Inc., Marietta, Ohio, U.S.A.). First, well-sporulated cells were streaked on an agar plate and incubated for 24 hours. Then, a single colony from the plate was inoculated into a test tube with 10 mL inoculum medium, and incubated for 15 hours. Finally, 5 mL of the test tube culture was transferred into a serum bottle containing 50 mL of modified-YEM medium, and incubated for 5 hours. About 25 mL of the serum bottle culture was inoculated into the fermentor.

3. Fed-Batch Fermentation with Organic Acid Uptake

Fed-batch experiments were performed by feeding glucose and other nutrients. Organic acids were added to the fermentor to determine acid uptake kinetics. A pervaporation module was used to remove butanol from the fermentation broth.

The module was made with eight 3.5 m silicone tubing sections. The total tubing length was 28 m and the surface area based on outside diameter was 0.17 m^2. The silicone tubing had an outside diameter of 1.95 mm and 240 μm-thick walls (Scientific Products, McGaw Park, IL, U.S.A.). The silicone tubing was weaved through holes on two flat autoclavable plastic holders horizontally mounted in a fermentor (Bio Flo I, New Brunswick Scientific Co. Inc., Edison, NJ, U.S.A.). The module used in this study is shown in our previous publication [Geng and Park, 1994]. Fermentor working volume was one liter excluding silicone tubings and plastic holder volumes. Air was supplied at 8 L/min through the lumen side of the tubings using an air pump (Gast DDA-P104-AA, MFG Corp., Benton Harbor, MI, U.S.A.).

The medium pH was controlled at 4.75, 5.25, 5.75 and 6.25 using 4 N NaOH and 4 N HCl. A pH controller (Cole Parmer, 5652-00, Chicago, U.S.A.) and a pH probe (Ingold, 465-35-90-K9 Wilmington, MA, U.S.A.) were calibrated at pH 4.1 and 7.0. Fermentor temperature was maintained at 32℃.

Initially, fermentation was started with 60 g of glucose and 1 unit of modified-YEM in one liter medium volume. Additional glucose and other nutrients were supplemented to make an overall nutrient mixture of 40 g of glucose per 1 unit of modified-YEM. Glucose concentration and the total volume of the supplements were 300 g/L and 200 mL for pH 4.75 and pH 5.75, 200 g/L and 300 mL for pH 5.25 and 333 g/L and 300 mL for pH 6.5. The strength of modified-YEM in the supplements were 2 units for pH 4.75 and 3 units for pH 5.25 and pH 6.5. The supplements were added stepwise at the moments shown in Fig. 1. Including the initial 60 g of glucose and 1 unit of modified-YEM a total of 120 g of glucose and 3 units of modified-YEM were added to the fermentor for pH 4.75, 5.25 and 5.75. For pH 6.25, a total of 160 g of glucose and 4 units of modified-YEM were added to the fermentor including the initial 60 g of glucose and 1 unit of modified-YEM. Nutrient and glucose supplements were maintained anaerobic by nitrogen sparging, and were added to the fermentor after off-line glucose analysis. Glucose concentration was maintained at above 15 g/L during uptake experiments of the added acids because sufficient glucose was necessary for the complete recycling of butyric acid [Park et al., 1993].

Kinetics of organic acid uptake was studied at controlled pH and under reduced butanol inhibition by using pervaporation. Butanol removal was necessary to avoid possible complications in data interpretation due to butanol inhibition on cell growth and fermentation activity. Butyric acid was added after cells completely consumed butyric acid produced by themselves. The addition time was 38, 30.3 and 21.6 hrs and the added amount was 2.17, 3.39 and 2.89 g/L for pH 4.75, 5.25 and 5.75, respectively (Fig. 1). Acetic acid was added after butyric acid. The addition time was 58.5, 37 and 28.2 hrs and the added amount was 2.84, 3.80 and 3.49 g/L for pH 4.75, 5.25 and 5.75, respectively (Fig. 1). At each addition organic acids were premixed with NaOH to minimize pH decrease. Slow agitation (50 rpm) was applied during pH control and the acid uptake periods. Samples were taken every 5 to 15 minutes for butyric acid uptake experiments. Sample intervals were 15 to 30 min for acetic acid uptake experiments.

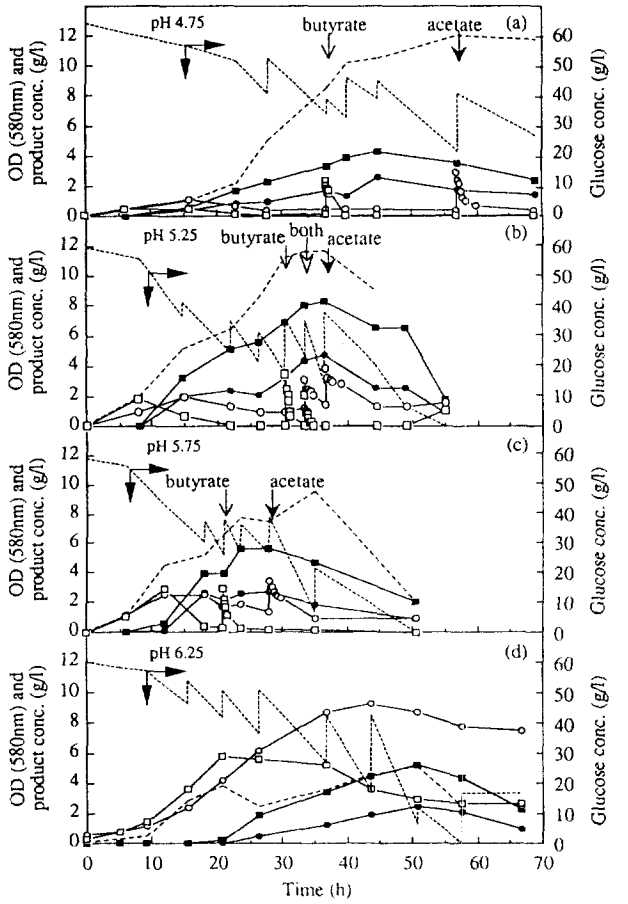

Fig. 1. Profiles of cell mass ---, glucose ···, acetone —●—, butanol —■—, acetate —○—, and butyrate —□—, concentrations during fed-batch fermentation under controlled pH and reduced butanol inhibition.

Acid uptake from a mixture of butyric acid and acetic acid was studied at pH 5.25 (Fig. 1b). Experimental procedures were similar to those described for individual acid uptake experiments. Acid mixture (2 g/L of butyric acid and 3 g/L of acetic acid) was added at 33.5 hrs when the fermentation reached a stationary phase.

In other work a mixture of acetic and butyric acids (1.75 g/L each) was added at the beginning of the fermentation at pH 5.25 to see the effect of added acids on the onset of solvent production. This amount of supplemented acids was slightly higher than that produced by strain B18 during normal batch fermentation at the onset of solvent production [Geng and Park, 1993].

4. Determination of Fermentation Products and Cell Mass

Glucose concentration was determined by using a sugar analyzer (YSI Model 27, YSI Co., Yellow Spring, OH, U.S.A.). Solvent

and organic acid concentrations were determined after the pH of the sample was decreased below 1.8 by adding 4 N HCl by using a gas chromatograph (HP5730A, Hewlett Packard Co., Palo Alto, CA, U.S.A.) equipped with a Flame Ionization Detector and Auto Linear Temperature Programmer. Temperature was programmed with an initial temperature 120℃, final temperature 180℃ and at a rate of 8℃/min. The column was packed with 6.6% Carbowax 2.0 M 80/120 Carbopack BAW (Supelco, Inc., Bellefonte, PA, U.S.A.). Nitrogen was the carrier gas. The optical density of the culture was determined at 580 nm with a spectrophotometer (Spectronic 20D, Milton Roy Co., U.S.A.) using 10-mm path length cuvette and converted to dry cell density using the following relationship determined by experiment: O.D. 1.0=0.4 g dry cell/L.

Optical density was not a true representation of cell growth because some cells were entrapped between silicone tubings. Final cell mass was determined by dry cell weight measurement after fermentation was over. The cell mass was washed twice with distilled water and oven-dried at 70℃ for 24 hrs.

Undissociated organic acid concentrations were calculated using pK values of 4.82 and 4.75 for butyric and acetic acids, respectively.

RESULTS AND DISCUSSION

The profiles of cell, glucose and product concentrations during fed-batch fermentation with pervaporation at pH 4.75, 5.25, 5.75 and 6.25 are shown in Fig. 1. Maximum butanol concentrations during each fed-batch fermentation were between 4 to 8 g/L which were less than 15 g/L for fermentation without pervaporation. Butyric acid and acetic acid uptake experiments were performed at pH 4.75, 5.25 and 5.75. At pH 6.25 cells produced mostly acids and acid addition experiments were not performed.

1. Butyric Acid Uptake

Added butyric acid was exhausted in less than two hours regardless of pH. Uptake kinetics of added butyric acid was dependent upon pH (Fig. 2). At pH 4.75 butyric acid concentration decreased linearly with time (Fig. 2a) and the data were best fitted by zero order kinetics (Fig. 2d). At pH 5.75 butyric acid concentration decreased exponentially with time (Fig. 2c) and the data were best fitted by first order kinetics (Fig. 2f). The rate constants were determined using the following kinetic expressions and undissociated butyric acid (UBA) concentrations. The kinetic constants are listed in Table 1. For zero order kinetics,

$$C - C_o = k_o t \quad (1)$$

For first order kinetics,

$$\ln C - \ln C_o = k_1 t \quad (2)$$

Eqs. (1) and (2) were obtained by integrating the following differential equation.

$$-\frac{dC}{dt} = kC^n \quad (3)$$

where C is the undissociated acid concentration, t is time, k is the rate constant, and n is equal to zero for zero order kinetics and equal to one for first order kinetics, respectively.

At pH 5.25 the butyric acid concentration profile showed a transition (Fig. 2b) and the data were best fitted by the following zero-to-first order shift kinetics.

$$-\frac{dC}{dt} = \frac{k_1 C}{1 + k_2 C} \quad (4)$$

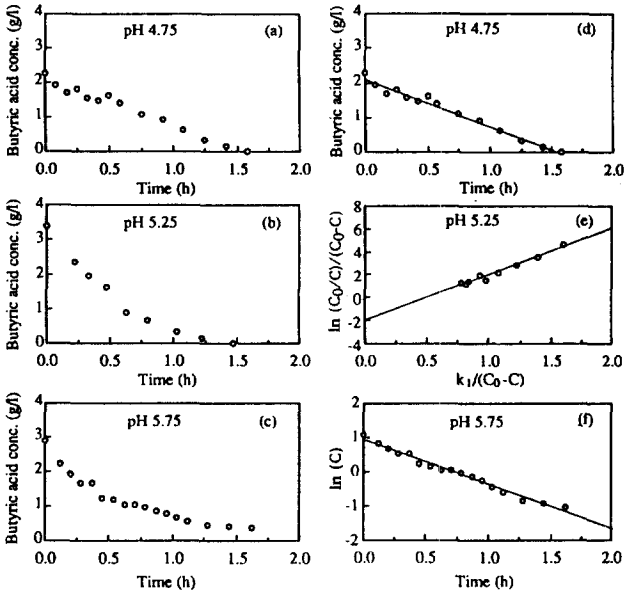

Fig. 2. Butyric acid concentration profiles during butyric acid uptake experiments and their kinetic analysis: (a) pH 4.75, (b) pH 5.25, and (c) pH 5.75. Fig. (d), (e), and (f) show the corresponding data fitted using Eq. (1), (4), and (2), respectively.

Table 1. Kinetic constants for acid uptake experiments

pH	Butyrate				Acetate			
	Order	K_b	Cell mass	k_b	Order	K_a	Cell mass	k_a
4.75	0	0.74	3.75	0.20	1	0.70	5.41	0.13
5.25	0/1	2.0/4.0	3.21	0.6/1.3	1	0.10	4.50	0.02
5.75	1	1.28	2.87	0.45	1	0.25	4.27	0.06

Note: K_b is k_0 in Eq. (1), k_1 in Eq. (2), and k_2 or k_2/k_3 in Eq. (4). K_a is k_1 in Eq. (2). k_a and k_b are normalized values by dividing K_a and K_b by respective cell mass (g/l).

where k_1 and k_2 are kinetic constants. This kinetics is reduced to zero order kinetics with a rate constant k_1/k_2 when butyric acid concentration is high ($k_2C \gg 1$). This kinetics approaches a first order kinetics with a rate constant k_1 when butyric acid concentration is low ($k_2C \ll 1$). Upon integration of Eq. (4) we obtain the following expression

$$\frac{\ln(C_o/C)}{C_o - C} = -k_2 + \frac{k_1 t}{C_o - C} \quad (5)$$

where C_0 is the initial acid concentration. The undissociated butyric acid concentration profile at pH 5.25 were fitted well to this expression (Fig. 2e). The rate constants (k_1 and k_2) were obtained from the slope and y-intercept of the profile. The values are listed in Table 1. The change in kinetic order at different pH (4.75 and 5.75) and the shift in kinetic order at the same pH (5.25) indicate that butyric acid uptake mechanism is dependent upon pH and acid concentration.

2. Acetic Acid Uptake

Unlike butyric acid, uptake of acetic acid followed first order kinetics regardless of pH (Fig. 3). Lower pH increased acetic acid assimilation rate. The rate of acetic acid uptake was slower than butyric acid uptake rate. At pH 4.75 acetic acid concentration was 0.6 g/L after two hours of acid uptake. At this pH acetic acid

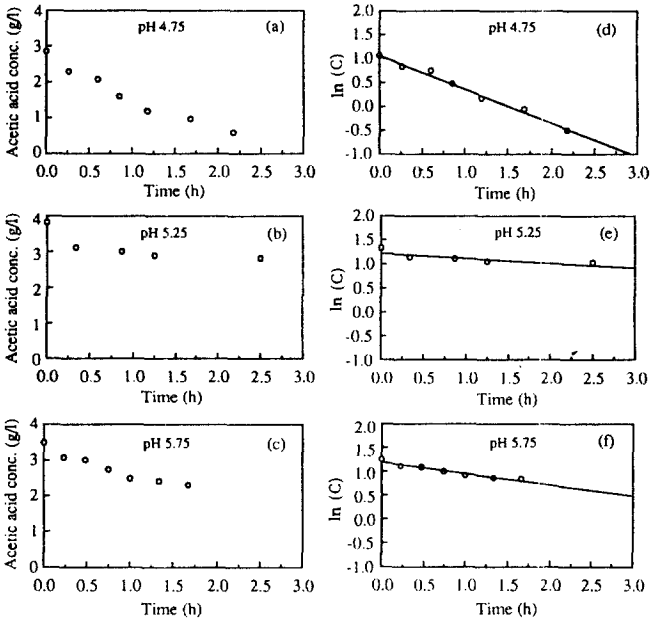

Fig. 3. Acetic acid concentration profiles during acetic acid uptake experiments and their kinetic analysis: (a) pH 4.75, (b) pH 5.25, and (c) pH 5.75. Fig. (d), (e), and (f) show the corresponding data fitted using Eq. (2).

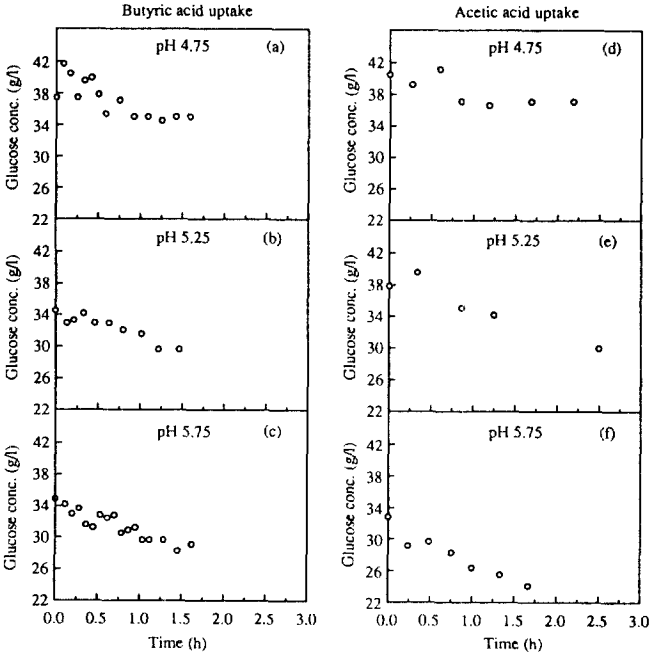

Fig. 4. Glucose concentration profiles during acid uptake experiments. For butyric acid uptake, (a) pH 4.75, (b) pH 5.25, and (c) 5.75. For acetic acid uptake, (d) pH 4.75, (e) pH 5.25, (f) pH 5.75.

concentration at the end of fermentation decreased to 0.26 g/L. At pH 5.25 and 5.75 acetic acid concentration was 2.8 g/L and 2.3 g/L after 2.5 and 1.7 hrs, respectively. At the end of fermentation acetic acid concentration decreased to 1.28 and 0.90 g/L for pH 5.25 and 5.75, respectively. The rate constants were determined using Eq. (2) and are listed in Table 1.

The overall kinetic expressions for acid uptake [Eqs. (1), (2) and (4)] are combined results of acid transport across the cell membrane and intracellular enzymatic reaction. Literature data indicate that acid transport across cell membrane seems to be driven by simple diffusion. Weak acids cross the cytoplasmic membrane predominantly in their uncharged form [Kell et al., 1981; Gottschalk, 1986]. The enzymatic reaction can be expressed by Monod kinetics. Monod kinetics predicts a linear relationship between reaction rate and intracellular acid concentration when the acid concentration is low. When the acid concentration reaches a saturation level, reaction rate becomes independent of acid concentration.

At pH 4.75 greater than 50% of the butyric acid was in undissociated form and sufficient amount of acid was available for diffusion into the cells. Intracellular acid concentration probably reached and remained at a saturation level and the overall acid uptake kinetics was independent of the concentration (zero order kinetics). At pH 5.75 10% of the butyric acid was in the undissociated form and only a small amount of acid was able to diffuse into the cells. Intracellular acid concentration was probably below the saturation level and the overall acid uptake kinetics was concentration dependent (first order kinetics).

At pH 5.25 butyric acid uptake kinetics shifted from zero order to first order as the concentration of butyric acid decreased. UBA concentration at the shift point was 0.5 g/L. This suggested that the saturation level of UBA was 0.5 g/L at pH 5.25. The same UBA saturation level can explain why we observed only first order kinetics at pH 5.75. At pH 5.75, UBA at the beginning of acid uptake experiment was 0.3 g/L and UBA concentration was always less than the saturation level (0.5 g/L). However, the same UBA saturation level (0.5 g/L) can not explain why we observed only zero order kinetics at pH 4.75. At pH 4.75, UBA level at the beginning of acid uptake experiment was 1.17 g/L and zero order kinetics is expected. However, we did not observe kinetic order shift to first order when UBA decreased to 0.5 g/L. This implies that UBA concentration for kinetic switch was much lower than 0.5 g/L at pH 4.75. This differences in UBA concentrations for kinetic order shift at different pH might be due to the effect of pH on saturation level of enzyme activity in Monod kinetics.

Acetic acid uptake followed first order kinetics regardless of pH. This implied that intracellular level of undissociated acetic acid (UAA) did not reach the saturation level at all pH. Even though the amount of extracellular UAA was comparable to that of UBA because both pK's were similar, diffusion of UAA across the cell membrane seemed to be less efficient compared with UBA diffusion.

During acid uptake experiments glucose was consumed simultaneously (Fig. 4). For butyric acid uptake experiments, the amount of glucose consumed during the first one and a half hours was approximately 6.0, 5.0 and 7.0 g at pH 4.75, 5.25 and 5.75, respectively. For acetic acid uptake experiments, 4.0, 7.0 and 8.0 g of glucose was consumed during the first one and a half hours at pH 4.75, 5.25 and 5.75, respectively. The consumption ratio (wt) of glucose to acid was 2 : 1 to 3 : 1 for butyric acid at pH 4.75, 5.25 and 5.75 and acetic acid at pH 4.75. The ratio increased to 7:1 to 9:1 for acetic acid at higher pH (5.25 and 5.75). This indicates that at higher pH acetic acid uptake becomes significantly less efficient as compared to glucose uptake.

During acid uptake experiments glucose concentration was maintained at above a level (15 g/L) required for complete recy-

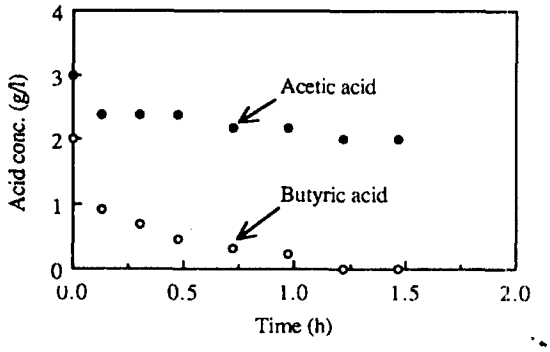

Fig. 5. Butyric acid (○) and acetic acid (●) profiles during simultaneous acid uptake experiment.

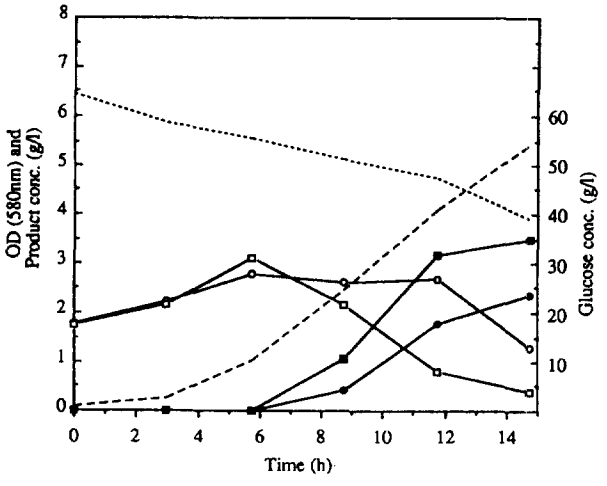

Fig. 6. Profiles of cell mass —, glucose ···, acetone —●—, butanol —■—, acetate —○—, and butyrate —□—, concentrations during initial acid addition at pH 5.25.

cling of butyric acid by strain B18. In a previous study [Park et al., 1993] we observed that only part of the butyric acid was recycled when the residual glucose was not sufficient. High glucose flux into glycolysis for ATP generation seems to be necessary to supply energy required for an efficient conversion of butyrate to butanol via both reversal of butyrate producing enzymes [Hüsemann and Papoutsakis, 1989] and CoA transferase [Hartmanis et al., 1984].

C. acetobutylicum B18 assimilated added acetic and butyric acids at concentrations (below 3.5 g/L) less than reported for *C. acetobutylicum* ATCC 824 (4.5 g/L) at high rate of metabolism using fed-batch fermentation [Fond et al., 1985].

3. Simultaneous Uptake of Butyric and Acetic Acids

After cells produced both organic acids themselves at pH 5.25, both butyric and acetic acids were added. Added acids were consumed at the same time (Fig. 5). Butyric acid was consumed at a faster rate. Simultaneous uptake of both acids implies that the delay in acetic acid recycling as compared to butyric acid at the onset of solvent production under normal fermentation without acid addition [Park et al., 1993; Geng and Park, 1993] was due to a delayed expression of CoA transferase as compared to butyrate producing enzymes.

4. Initial Addition of Organic Acids

When butyric and acetic acids were added at the beginning of fermentation, butanol production started earlier (6 hrs after fermentation start up) (Fig. 6). Without initial acid addition, butanol production commenced 12-15 hrs after fermentation start up [Park et al., 1993; Geng and Park, 1993]. The amount of added organic acids (1.75 g/L each) was slightly higher than the level at which butanol production commenced during pH controlled (at 5.0 and 5.5) batch experiments without initial organic acid addition. However, solvent production did not initiate immediately. Butyric acid was further produced and its concentration increased to at least 3.1 g/L (UBA 0.85 g/L) before butanol production commenced. This UBA concentration (0.85 g/L) was 2.5 times higher than concentrations in experiments without acid addition. Pervaporation did not influence early butanol production because the pervaporation system was turned on after the onset of solvent production. It was also reported that solvent synthesis was rapidly induced by *C. acetobutylicum* ATCC 824 when 0.6 g/L of acetate plus 0.88 g/L of butyrate were added at the beginning of fermentation at controlled pH 5.0 [Gottschal and Morris, 1981].

CONCLUSION

The kinetics of organic acid (acetic and butyric acids) uptake by *Clostridium acetobutylicum* B18 was dependent upon pH, acid concentration and the kind of acid. Both cell membrane transport and enzymatic reaction played key roles in overall acid uptake kinetics. For butyric acid, under the conditions where intracellular acid concentrations reached the saturation level for Monod kinetics (pH 4.75), the overall kinetics followed zero order expression. At pH 5.75 the kinetics followed first order expression. At pH 5.25 conditions switched from one to the other and acid uptake kinetics switched from zero to first order. For acetic acid, first order kinetics was observed regardless of pH probably because intracellular acid concentration never reached the saturation level. Cells consumed both organic acids simultaneously but butyric acid uptake was faster. Glucose was also consumed at the same time as acid uptake. Butanol production started earlier when organic acids were added at the beginning of fermentation.

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