

# The Effect of pH on Nitrogen Supply, Cell Lysis, and Solvent Production in Fermentations of *Clostridium acetobutylicum*

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In batch fermentations of *C. acetobutylicum*, with 5 g/L yeast extract and 50mM glucose, the ratio of ammonium to glucose affected solvent production when the pH was left to vary uncontrolled from 4.5 to 3.65. High solvent production was observed for a low ratio. When the pH was controlled at 4.5, only acids were produced for all ratio values. At a low ammonium-to-glucose ratio, solvents were produced when the pH was controlled at 3.7. Acids only were produced for a low ratio value at pH 4.0 or for a high ratio value at pH 3.7. In continuous cultures, mostly acids were produced under glucose limitation, but solvents were produced under nitrogen limitation. It was concluded that the nitrogen availability controls solvent production and that the pH affects the availability of organic nitrogen. Biomass autolysis at the stationary phase of batch cultures was reversibly inhibited at pH values less than 3.8. In batch fermentations, the overall molar growth yields on ATP ( $Y_{ATP}$ ) varied from 5.5 to 9.0 and the transient yields from 5.5 to 15.5. In continuous cultures, the  $Y_{ATP}$  values varied from 5.5 to 14.7 under glucose limitation, and from 6.1 to 9.3 under nitrogen limitation. The  $Y_{ATP}$  depended on the ammonium to glucose ratio and the culture pH, but did not show the usual dependence on the specific growth rate in batch cultures. The experiments seem to confirm the hypothesis that solvent production is controlled by the demand and availability of ATP.

## INTRODUCTION

The renewed interest in the butanol/acetone fermentation in recent years<sup>1</sup> has yielded a wealth of new information on this important and fundamentally interesting fermentation.<sup>2-15</sup> Despite this renewed intensive and well focused research activity, the true kinetics and biochemical mechanisms which regulate the production of acids and solvents still elude us. Various reactor operations (batch, continuous, nitrogen,

phosphate, or carbon-source limited), the culture pH and key intermediates (acetate and butyrate) have been examined, among others, for their effect on solvent production.<sup>2-10,13</sup> Curiously, the results of these investigations have been often diametrically conflicting. Then, we must accept all these often conflicting results as revelations or facets (under the specific conditions of each experiment) of the biochemical cybernetic scheme according to which the cells regulate their metabolism in response to the prevailing culture conditions. What is that cybernetic scheme is still not known. Ultimately, exact answers will be given by a detailed study of the molecular biology of butyric-acid clostridia, but this approach is very tedious and requires a substantial long-term commitment. On the other hand, macroscopic experiments are more easily employed to test various hypotheses or cybernetic schemes. Such experimentally tested cybernetic schemes are useful for devising better reactor configurations or policies to improve the yields and selectivities of the fermentation. They are also useful in the study of the molecular biology of butyric-acid bacteria.

Andersch and co-workers<sup>2</sup> have found that in continuous, nitrogen-limited cultures of *C. acetobutylicum* on a totally synthetic medium, solvents were produced only if the medium pH was below 5.2. Gottschal and Morris<sup>3</sup> observed no solvent production in a nitrogen-limited continuous culture of *C. acetobutylicum* on a totally synthetic medium at pH 5.7. Their glucose-limited continuous cultures did not produce any solvents. However, solvents were produced in turbidostatic cultures at high cell densities.<sup>4</sup> Jöbses and Roels<sup>5</sup> found that continuous nitrogen-limited cultures of *C. beijerinckii* at pH 4.5 on a complex medium produce solvents, while glucose-limited continuous cultures do not. A low pH was found necessary for solvent production, while the addition of butyrate and acetate in the feed medium did not stimulate solvent production in glucose-limited continuous cultures.<sup>8</sup> Monot and

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Engasser<sup>9</sup> found that nitrogen-limited continuous and batch cultures of *C. acetobutylicum* on a totally synthetic medium result in production of solvents. Monot and co-workers<sup>10</sup> found that at high pH and low glucose concentrations only acids are produced in batch and continuous cultures, while at low pH and high glucose concentrations solvents can be produced. It was concluded that solvents are produced if the concentration of undissociated butyric acid in the medium reaches a critical level. On the contrary, George and Chen<sup>13</sup> found that acidic conditions are not obligatory for solvent production in fermentations of *C. beijerinckii*. Cultures maintained at pH 6.8 produced nearly as much butanol as those incubated without pH control.<sup>13</sup> It was also found that a combined addition of acetate and butyrate accelerated solvent production but neither acid alone had an effect on solvent production.<sup>13</sup> Bahl et al.<sup>5</sup> found that continuous glucose-limited cultures of *C. acetobutylicum* produce only acids at culture pH values higher than 4.7 and small amounts of solvents at pH lower than 4.7. Good solvent production was achieved, however, in continuous cultures with high feed glucose and yeast-extract concentrations.<sup>14</sup> Phosphate-limited continuous cultures<sup>15</sup> resulted in good solvent production. Monot et al.<sup>6</sup> found that excess amounts of ammonium acetate in batch cultures of *C. acetobutylicum* on a totally synthetic medium resulted in the production of acids only. It was also concluded<sup>6</sup> that low initial glucose concentrations resulted in acid formation only, and that good solvent production requires high initial glucose concentrations. Finally, Martin et al.<sup>7</sup> found that addition of acetate and butyrate separately alters the distribution of solvent products in batch cultures, but not the combined solvent yield.

From the preceding brief and limited literature overview, it appears that the effects on solvent production of the culture pH, acetate, butyrate, and glucose or nonglucose limiting growth conditions, among others, are still not clearly understood and often contradicting. If we accept that all these effects are different facets of the same biochemical cybernetic scheme under the different culture conditions, we should conclude that many if not all of these factors affect solvent production in an indirect and synergetic fashion.

A careful consideration of the various events which take place in batch and continuous cultures<sup>2-10,12-15</sup> and our calculations based on the fermentation equation we have recently proposed,<sup>1</sup> led us to propose that solvent production is predominantly regulated by the availability and demand of biosynthetic (ATP) and reduction [ $\text{NAD(P)H}_2$ ,  $\text{FdH}_2$ ] energy.<sup>16</sup> Glycolysis provides less ATP and more  $\text{NAD(P)H}_2$  than the cells need for growth, while acid production is the main source of ATP, but uses very little of the available reduction energy.<sup>1,16</sup> This excess reduction energy is released in the form of hydrogen. Solvent formation requires significant amounts of reduction energy and produces very little ATP.<sup>1,16</sup> Thus, as observed, glucose-

limited (and thus probably ATP limited, see below) cultures result in the formation of acids only. If the culture is non-ATP limited (as in nitrogen-limited cultures), a mixture of solvents and acids is produced in response to a required lower rate of ATP production per unit of sugar utilized. Similarly, when growth is inhibited by high acid concentrations<sup>12</sup> or low pH, for example, the demand for a lower rate of ATP production per unit substrate utilized is apparently met by the production of a mixture of solvents and acids. Reuptake of acids may be viewed as a means of lessening their inhibitory effect on growth.<sup>12,16</sup> Phosphate limitation of continuous growth may or may not imply ATP limitation, in view of the fact that phosphate is a constituent of nucleic acids, phospholipids, and coenzymes, and only a small percentage of phosphate is employed in the phosphorylation of adenine nucleotides. As such, phosphate limitation slows dramatically biosynthesis and thus the rate of ATP demand in the presence of excess carbon source (glucose). Thus, solvent production under phosphate-limited conditions<sup>15</sup> may be viewed as the result of a lower required rate of ATP production per unit of sugar utilized.

Within the overall objective of testing the above hypotheses, this article examines the combined effect of the culture pH and the ratio of nitrogen to carbon sources on solvent production in fermentations of *C. acetobutylicum*. It also examines the effect of pH on the growth rate and autolysis of biomass.

Before we proceed any further we would like to define clearly a number of terms pertaining to growth limiting or determining nutrients or factors. For fixed temperature and pH conditions, a batch culture is termed *unrestricted* when every nutrient is in excess but not inhibitory supply (or concentration).<sup>17</sup> Unrestricted batch cultures are characterized by exponential growth, which is terminated and the culture enters the stationary phase if a nutrient becomes exhausted (thus the nutrient becomes growth limiting), or a produced metabolite or unfavorable growth factor (such as pH or temperature) inhibit growth. These metabolites or factors will be termed growth limiting.<sup>17</sup> Presently, we will be concerned with carbon substrate (glucose) and total nitrogen and pH limitations of growth. As is subsequently described, all other nutrients will be provided in excess, but not inhibitory concentrations, while butyrate, acetate, and butanol inhibition of growth will be avoided by employing low glucose concentrations (see below). Glucose or nitrogen limitations in continuous growth are easily judged by very low or relatively high residual glucose concentrations, respectively. What nutrient or factor limits eventually an unrestricted batch culture is a more difficult question to answer. With all other nutrients and factors kept practically constant, if a lower concentration of a nutrient results in significant changes on the growth or product-formation characteristics, this nutrient is eventually limiting the unrestricted batch culture. Since

the only source of ATP for growth is substrate-level phosphorylation, and since ca. 15 mol of ATP are needed per 1 mol glucose incorporated in biomass,<sup>1</sup> if a culture is glucose limited, it is probably ATP limited, as well. This seems to be supported by the fact that the highest values for the  $Y_{ATP}$  are obtained under glucose-limited growth conditions.<sup>5</sup> These continuous, steady-state  $Y_{ATP}$  values, under glucose limited conditions in a defined medium, vary from 14.9 to 21.2 and are considerably higher than the expected average value of 10.5. If the cultures were not ATP limited, more glucose would be converted into biomass and/or products (solvents) which yield less ATP,<sup>1</sup> and also lower  $Y_{ATP}$  values would be obtained. The question of whether glucose limitation is accompanied by ATP limitation will be considered again in the Discussion and Conclusions section.

## MATERIALS AND METHODS

### Organism and Growth Conditions

The organism used was *Clostridium acetobutylicum* strain ATCC 824. The cultures were stored as spores at 2–14°C under a nitrogen atmosphere in a 5% (w/v) corn mash/0.5% (w/v) glucose medium.<sup>18</sup> Spore suspensions [10% (v/v)] were transferred to screw-cap tubes containing heat-sterilized soluble medium prepared by dissolving in 1 L distilled water:  $KH_2PO_4$ , 0.75 g;  $K_2HPO_4$ , 0.75 g;  $MgSO_4 \cdot 7H_2O$ , 0.4 g;  $MnSO_4 \cdot H_2O$ , 0.01 g;  $FeSO_4 \cdot H_2O$ , 0.01 g; asparagine, 2.0 g; yeast extract (Sigma Chemical Co.), 5.0 g;  $(NH_4)_2SO_4$ , 2.0 g; cysteine, 0.5 g; and glucose, 10 g. The culture pH was adjusted to 6.2–6.6 with 1N NaOH before the addition of spores. The cultures were sparged with sterile purified ( $O_2$  free) nitrogen, were tightly capped, pasteurized at 70–80°C for 10 min, and then incubated at 37°C. Vegetative cultures prepared in this way were used as inocula for subsequent fermentations. The media used in all batch and continuous cultures were as above except for the amounts of  $(NH_4)_2SO_4$  and glucose which were varied as is described below.

Uncontrolled-pH batch cultures were established in 1-L Erlenmeyer flasks with a working volume of 500 mL, initially under a nitrogen atmosphere. The cultures were incubated at 37°C. The vigorous gas production of vegetative growth kept the cultures well mixed. The gas pressure in the flasks was slightly higher than the atmospheric and the gas formed was vented. Controlled-pH batch fermentations were established either in a 1-L Bioflo C-30 fermentor (New Brunswick Scientific) with a working volume of 500 mL, or in a 2-L BIOSTAT M fermentor (Braun Instruments) with a working volume of 1.4 L. The pH was controlled by the addition of 2N NaOH or 0.5N HCl. The cultures were maintained at 37°C and were stirred at 200 rpm. Anaerobiosis was ensured by a continuous flow of purified, sterile nitrogen. Continuous fermentations were established in the Bioflo C-30 fermentor using a peristaltic pump

for media feeding. An overflow side arm maintained the reactor broth volume constant at ca. 350 mL. Except for Viton (Cole Parmer) tubing employed in the peristaltic pumps, latex tubing was used in all continuous and batch experiments.

### Analytical Procedures

Cell density was determined by measuring the OD (optical density) of the culture broth at 600 nm in a Gilford 250 spectrophotometer. The OD readings were converted to biomass concentrations using a mass extinction coefficient of  $5.03 \pm 0.2$  L/g biomass cm at 600 nm. The latter was established by dry-weight determinations of broth samples as is detailed in ref. 18. Glucose was determined by a peroxidase glucose-oxidase enzymatic assay (Sigma Chemical Co.).

Concentrations of ethanol, acetone, acetate, butanol, acetoin, and butyrate were determined using a Vista 6000 gas chromatograph (GC) and an 8000 autosampler (Varian Instrument Group). Fermentation samples were centrifuged at 4°C for 10 min at  $1.5 \times 10^4$  rpm (SS-34 rotor) using a Sorvall RC-5B centrifuge. The clear supernatants were frozen until further analysis. Chromatographic samples were prepared by mixing 0.1 mL of 3N HCl with 1.0 mL supernatants in 1.4-mL screw-cap septum vials which were then loaded into the autosampler. A 2-mm-i.d., 2-m-long glass column packed with 80-100 mesh Porapak Q and nitrogen carrier gas (30 mL/min) were employed for the separation. The column temperature was held at 115°C for 10 min after injection and was then increased at a rate of 2°C/min to 170°C where it was held until the last component (butyrate) eluted. The injector was kept at 220°C and the flame ionization detector at 270°C. The composition of the gas phase ( $CO_2$ ,  $H_2$ , and  $N_2$ ) was either calculated according to the fermentation equation as is described below, or was determined on-line by measuring the  $N_2$  flow rate and gas composition by gas chromatography. A 3.1-mm, 2-m-long stainless-steel column packed with washed molecular sieve 5A (Alltech) and a thermal conductivity detector were employed on the Varian model 6000 gas chromatograph (GC). On-line gas and liquid analyses were performed simultaneously employing the same temperature programming as described above. The injection, rotary type (Valco six-port), valve was located in the oven. Further details can be found elsewhere.<sup>19,20</sup>

### Calculations

A number of fermentation parameters can be calculated from measured ones using the fermentation equation which has been derived and tested in ref. 1. The fermentation equation is a stoichiometric equation which describes the interrelations among the various products, intracellular intermediates, biomass, and consumed substrates of the fermentation. The equation obeys the constraints imposed on growth and product

formation by thermodynamics and the biochemical topology.<sup>1</sup> The equation has been found valid for completed, steady-state and transient fermentations.<sup>1,19,20</sup> It can be, thus, used to calculate both observable parameters (such as product concentrations or amounts) but also important intracellular parameters, such as the molar growth yield on ATP ( $Y_{ATP}$ ) and the amount of  $NADH_2$  formed through the oxidation of  $FdH_2$  (NfF) via the  $NADH$ : ferredoxin oxidoreductase.<sup>1</sup> Negative NfF values indicate a net oxidation of  $NADH_2$  to produce  $FdH_2$  which is oxidized by the hydrogenase to produce  $H_2$ .<sup>1</sup> We briefly summarize the procedure for the  $Y_{ATP}$  calculation.<sup>1</sup> Two moles of ATP are produced via the Embden–Meyerhof–Parnas pathway for the conversion of glucose to pyruvate. One mole ATP is produced for the production of either acetate or butyrate from pyruvate. Finally, since one mole of either acetate or butyrate is necessary for the production of acetone, one mole ATP is produced per mole acetone formed. The  $Y_{ATP}$  is calculated by dividing the gram of biomass produced by the total number of moles ATP produced. The latter is equal to two times the moles of glucose converted into products plus the number of moles of acetate, butyrate, and acetone produced. This method was also apparently used by Bahl et al.<sup>5</sup> The weight fraction of carbon in biomass,  $\sigma_b$ , was taken to be 0.462 according to a well accepted biological regularity.<sup>1</sup> Measured values of  $\sigma_b$  for *C. beijerinckii* were found to be 0.48, 0.45, and 0.46 for growth on minimal, complex, and nitrogen-limited media, respectively.<sup>8</sup> The degree of reductance of biomass,  $\gamma_b$ , was taken to be 4.291 according to a well accepted biological regularity.<sup>1</sup> Measured values of  $\gamma_b$  for *C. beijerinckii* were found to be 4.68, 3.69, and 4.01 for growth on minimal, complex, and nitrogen-limited media, respectively.<sup>8</sup> As is discussed in detail in ref. 1, 10–20% variations in the value of  $\gamma_b$  have an insignificant effect on the calculations based on the fermentation equation because of the small percentage of carbon converted into biomass. In any case, the calculation of the  $Y_{ATP}$  is independent of either  $\gamma_b$  or  $\sigma_b$ . The gamma ratio is the ratio of the degree of reductance of biomass and other fermentation products divided by the degree of reductance of carbon substrate(s), assuming that the degree of reductance of the nitrogen source is the same as that of  $NH_3$ , namely zero.<sup>1</sup> For totally synthetic media, a  $\gamma$ -ratio value close to the ideal value of 1.0 is an indication of good data consistency. For fermentations on complex media, if only a small percentage of the biomass and product carbon is derived from the complex carbon source, a  $\gamma$ -ratio value close to 1.0 is again ideally expected.

## RESULTS

The experiments presented below were sequentially designed to examine the effect of inorganic nitrogen ( $NH_4^+$ ) and organic nitrogen (yeast extract and aspar-

agine) on solvent production under various fermentation conditions. In all batch fermentations and the glucose-limited continuous fermentation, we kept the initial glucose concentration low (50mM–62mM) to minimize the inhibitory effect of high acid concentrations on the cell metabolism.<sup>12</sup> As was mentioned in the Materials and Methods section, the concentrations of yeast extract and asparagine were kept constant. The amount of  $NH_4^+$  in the medium was varied by varying the concentration of  $(NH_4)_2SO_4$ . An adequate supply of  $SO_4^{2-}$  was provided by its  $Mg^{2+}$ ,  $Mn^{2+}$ , and  $Fe^{2+}$  salts to assure that sulfate is not growth limiting under any of the employed growth conditions. Yeast extract was also an additional source of sulfur for growth.

## Uncontrolled-pH Batch Fermentations

Three uncontrolled-pH batch fermentations were carried out corresponding to molar ratios of ammonium to glucose of 0.16, 0.60, and 1.52. In all three fermentations, the initial pH was 4.5 which dropped to 3.65 by the onset of solvent production and remained at the 3.65–3.7 level throughout the fermentation. There was little variation in the pH profile of the three fermentations. The time courses of two of the three fermentations are shown in Figures 1 and 2. The fermentation balances for all three fermentations are displayed in Table I. Since the  $H_2$  and  $CO_2$  amounts were not measured directly, the fermentation equation can be used to calculate the two gases and an additional product for checking the consistency of the data.<sup>1</sup> It can be seen from Table I that the calculated values are in good agreement with their experimental counterparts.

It can be observed from Table I that the fermentations with the higher two ammonium concentrations resulted in higher glucose utilization and lower  $Y_{ATP}$  values. The  $Y_{ATP}$  values shown in all tables are integrated (or overall) values calculated either at the point of the maximal biomass concentration (for the cases where biomass lysis is observed) or at the point where no more changes occur in the concentrations of substrate and products. Differences in lag times could not be related to the ammonium concentrations and were attributed to the state of the inocula. Production of acids began at about the same time for all three fermentations. Acetoin, acetone, butanol, and ethanol production were also induced at about the same time. Solvent formation and glucose uptake are seen to continue after growth has ceased.

With the increase of the ammonium concentration, the cultures were found to produce more ATP per mole of glucose. This was accomplished by increased acid formation. Acetate yields were not found to vary greatly across the range of ammonium to glucose ratios studied. However, as this ratio increased, the butyrate yields were found to increase appreciably. The higher butyrate yields resulted in the rise in ATP production.

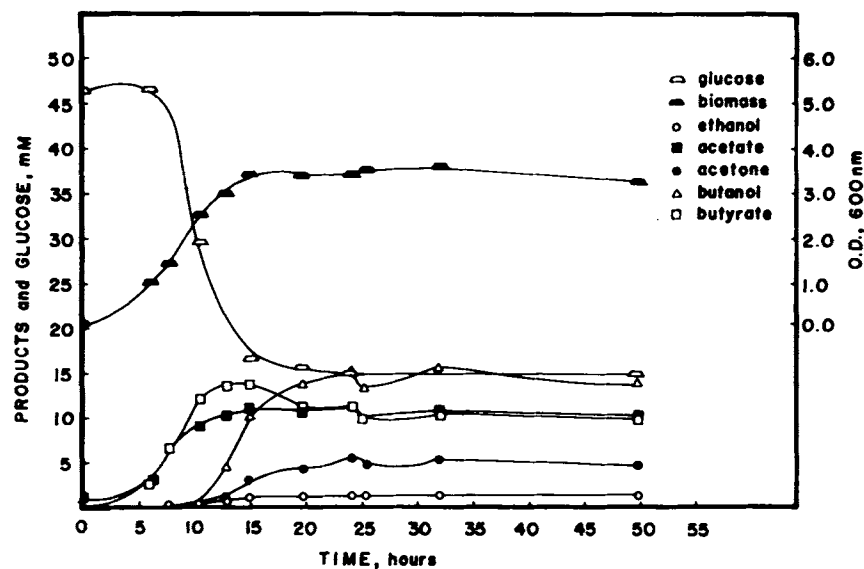


Figure 1. Uncontrolled-pH batch fermentation of *C. acetobutylicum*. Initial pH was 4.5; final pH was 3.70; initial glucose concentration was 51.3mM; and initial molar  $(\text{NH}_4^+)/(\text{glucose})$  was 0.16.

Along with the increasing butyrate yields, decreases in the butanol and acetone yields were observed. The effect of the ammonium to glucose ratio on the time course and final values of the butyrate and butanol yields is demonstrated better in Figures 3 and 4, respectively. The final butyrate and butanol yields show a linear dependence on the ammonium to glucose ratio under the present conditions and in the range studied. In Figure 3, time zero was arbitrarily taken as the time at which butyrate production begins. It was assumed that the initiation of butyrate formation is not affected by the ammonium levels. This is reasonable since bio-

mass and acid formation are initiated together and the lag phase is not affected by the ammonium to glucose ratio in this system. The initial rate of butyrate formation appears similar for the three ammonium to glucose ratios, while the butyrate concentration increases with this ratio. At an ammonium to glucose ratio of 0.16, butyrate is observed to be taken up by the cells and used for solvent production. This butyrate uptake is less pronounced at the higher ammonium to glucose ratios.

Butanol formation is initiated at approximately the same time in the three runs. As the ammonium-to-

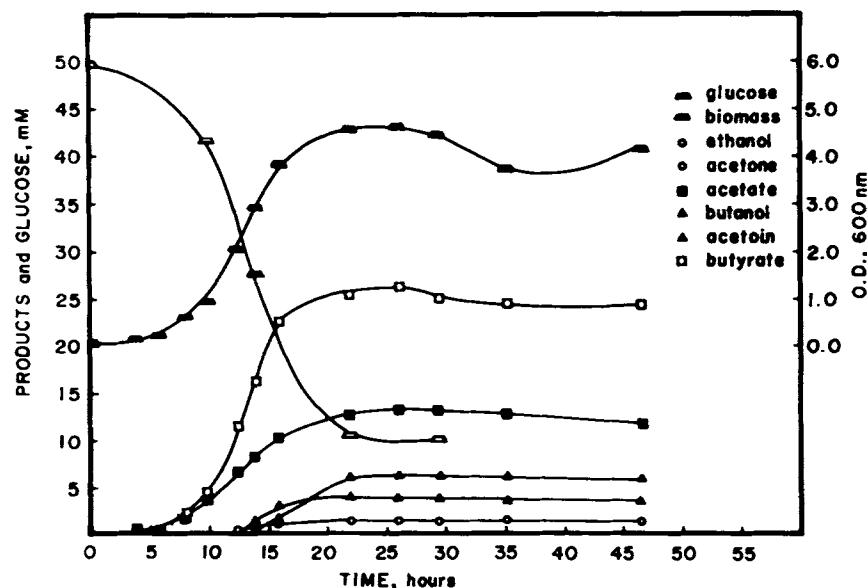


Figure 2. Uncontrolled-pH batch fermentation of *C. acetobutylicum*. Initial pH was 4.5; final pH was 3.70; initial glucose concentration was 54mM; and initial molar  $(\text{NH}_4^+)/(\text{glucose})$  was 1.52.

**Table I.** Fermentation balances for batch fermentations with different initial molar ammonium to glucose ratios (uncontrolled pH).

$(\text{NH}_4^+)/(\text{glucose})$	0.16	0.60	1.52
Biomass (g/L)	0.70	0.77	0.90
Initial glucose (mM)	51.3	51.5	54.0
Glucose utilized (mM)	36.5	43.8	43.6
Residual glucose (mM)	14.8	7.5	10.4
$\mu$ ( $\text{h}^{-1}$ )	0.2	0.46	0.48
$Y_{\text{ATP}}^a$	7.47	6.24	7.05
Gamma ratio <sup>a</sup>	1.001	0.995	1.014

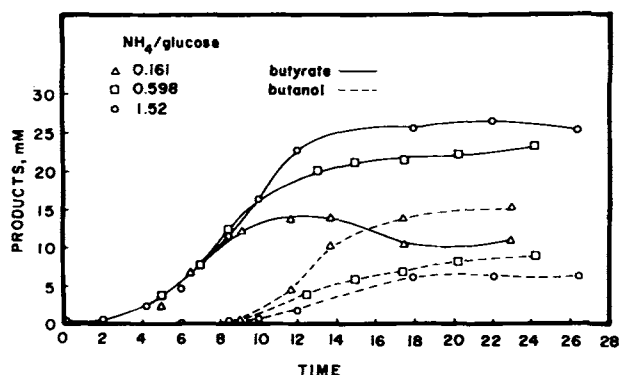
Substance	Amount (mol/100 mol glucose fermented)		
Butyrate	27.0	52.1	58.3(56.6 <sup>a</sup> )
Acetate	28.0(27.9 <sup>a</sup> )	30.4(31.2 <sup>a</sup> )	30.1
$\text{CO}_2^a$	205	203	203
$\text{H}_2^a$	163	196	205
Ethanol	3.3	2.7	3.2
Butanol	37.9	19.5	14.5
Acetone	13.3	6.8	3.4
Acetoin	2.0	nd	8.8
Carbon recovered in products (as glucose)	95.8	96.1	101.4
NfF <sup>a</sup>	24.7	-0.2	-23.0
$\text{H}_2/\text{CO}_2^a$	0.80	0.96	1.01

Note: nd means not determined.

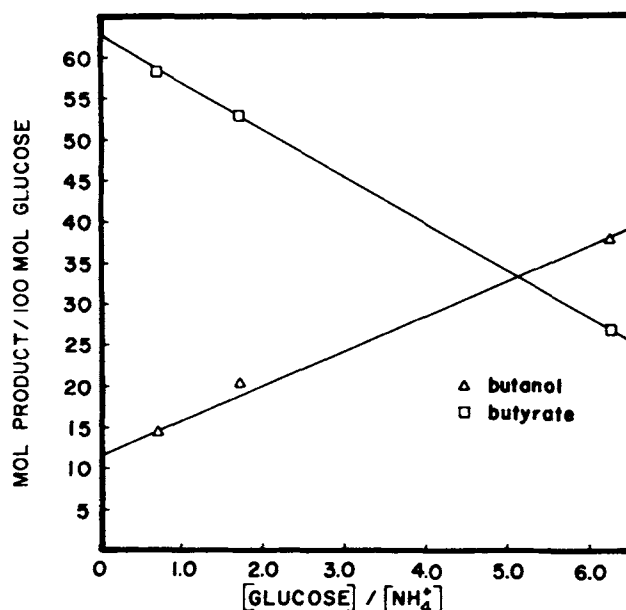
<sup>a</sup> Calculated quantities are according to the fermentation equation (see the Materials and Methods section).

glucose ratio decreases, the rate of butanol formation appears to increase. Also, higher butanol concentrations are obtained at the lower ammonium levels. Acetone formation displays the same behavior as butanol in response to changes in the ammonium to glucose ratio. Ethanol formation does not appear to be affected by changes in this ratio.

Along with increased solvent formation, lower ammonium concentrations result in lower values of the



**Figure 3.** Effect of ammonium to glucose ratio on butanol and butyrate formation profiles in uncontrolled-pH batch fermentations of *C. acetobutylicum*. Initial pH was 4.5; final pH was 3.7; and initial glucose concentration was ca. 51mM.



**Figure 4.** Effect of glucose to ammonium ratio on total butanol and butyrate formation in uncontrolled-pH batch fermentations of *C. acetobutylicum*. Initial pH was 4.5; final pH was 3.7; and glucose concentration was ca. 51mM.

$\text{H}_2$  to  $\text{CO}_2$  ratio and higher values for NfF (see the Materials and Methods section). In fact, the NfF changes from a large negative value to a large positive value as the ammonium to glucose ratio decreases. Negative NfF values indicate a net oxidation of  $\text{NADH}_2$  to form  $\text{FdH}_2$ , which is used to produce  $\text{H}_2$ . A positive NfF value indicates a net oxidation of  $\text{FdH}_2$  to produce  $\text{NADH}_2$  which is used to produce butanol and ethanol. Higher NfF values are seen to be accompanied by lower amounts of  $\text{H}_2$  production. It appears then that the ammonium-to-glucose ratio changes the mechanism by which excess (from what is necessary for biomass synthesis) reduction energy is released by the cells. At high ammonium concentrations, this excess reduction energy is released in the form of  $\text{H}_2$  and is accompanied by higher acid production and negative NfF values. At low ammonium concentrations, the excess reduction energy is used to produce solvents at the expense of acid production and is accompanied by a positive NfF value.

The  $\gamma$ -ratio values close to one indicate good data consistency for all three fermentations. No significant biomass lysis at the stationary phase was observed for any of the three fermentations.

### Controlled-pH Batch Fermentations

The effect of ammonium concentration on batch fermentations at constant pH was also investigated. In these experiments, a constant nitrogen sparge was maintained to ensure anaerobic conditions. It was assumed that the reduction of molecular nitrogen did not contribute significantly to the nitrogen requirements

of the cells (*C. acetobutylicum* is known to possess nitrogen-fixation capabilities.<sup>21</sup>) Control fermentations run under a helium atmosphere indicated that this assumption was valid.

Fermentations were run at three ammonium concentrations: 7.58mM, 30.27mM, and 75.68mM. The pH of the cultures was held constant at 4.5. The biomass concentration, product yields, the amount of glucose utilized, and other fermentation parameters are presented in Table II. At every ammonium level, the total utilization of glucose was realized. The biomass concentration and  $Y_{ATP}$  decrease as the ammonium-to-glucose ratio increases.

Butyrate and acetate were produced almost exclusively at all ammonium-to-glucose ratios tested. Butyrate yields do not follow any discernible trend; however, the acetate yields are observed to rise slightly with the ammonium-to-glucose ratio. The acetone, butanol, and ethanol yields are consistently low at all ammonium levels. Acetoin yields do not vary greatly with the ammonium concentration.

The negative NfF values in Table II indicate that in all cases ferredoxin is reduced through the oxidation of NADH<sub>2</sub>. The quantity of ferredoxin reduced by NADH<sub>2</sub> increases as the ammonium levels rise.

The time courses of product formation and glucose utilization for an ammonium to glucose ratio of 0.49 are presented in Figure 5. This behavior is typical of the other fermentations run at a controlled pH of 4.5.

Glucose concentration drops quickly as biomass and acids are formed. The short period of increasing acid and biomass concentrations after glucose depletion indicates that perhaps the cells are using yeast extract as a carbon source. At the end of growth, rapid lysis of cells was observed for all three fermentations. Glucose was completely used by all three fermentations at pH 4.5.

As we have mentioned earlier, the growth medium composition was such that no noncarbon nutrient, except nitrogen perhaps, could be growth limiting. Thus, since variation of the NH<sub>4</sub><sup>+</sup> concentration did not have any effect on the pH 4.5 fermentations, and since glucose was completely utilized, one may conclude that the pH 4.5 fermentations were not limited by the nitrogen or any other noncarbon nutrient supply. Therefore, when glucose was almost exhausted, the unrestricted batch cultures became glucose limited.

It is seen, therefore, that the ammonium to glucose ratio has a decisive effect on the product distribution of the uncontrolled-pH fermentations (where the pH is at the level of pH 3.65–3.7 for more than 50% of the fermentation time), while it has a small or no effect on the product distribution and other macroscopic fermentation characteristics when the pH is controlled at 4.5. It appears then that the lower pH is responsible for limiting the availability of the medium nitrogen (inorganic and organic) to the cells. The complete reasoning for this conclusion is as follows. The growth

**Table II.** Fermentation balances for batch fermentations with different initial molar ammonium to glucose ratios. The pH was controlled at 4.5.

(NH <sub>4</sub> <sup>+</sup> /(glucose))	0.13	0.49	1.44
Biomass (g/L)	1.52	1.23	1.16
Initial glucose (mM)	58.3	61.4	52.7
Residual glucose (mM)	0.0	0.0	0.0
$\mu$ (h <sup>-1</sup> )	0.69	0.62	0.57
$Y_{ATP}$ <sup>a</sup>	8.40	6.96	6.73
Gamma ratio <sup>a</sup>	1.002	0.998	1.037

Substance	Amount (mol/100 mol glucose fermented)		
	0.13	0.49	1.44
Butyrate	67.5(68.3 <sup>a</sup> )	64.6	73.7(69.2 <sup>a</sup> )
Acetate	39.4	43.9(44.0 <sup>a</sup> )	44.0
CO <sub>2</sub> <sup>a</sup>	187	179	199
H <sub>2</sub> <sup>a</sup>	217	213	239
Ethanol	1.1	nd	1.5
Butanol	1.2	0.4	2.1
Acetone	0	0	0.2
Acetoin	3.7	2.6	5.7
Carbon recovered in products (as glucose)	92.6	89.7	104.4
NfF <sup>a</sup>	-37.4	-39.2	-51.6
H <sub>2</sub> /CO <sub>2</sub> <sup>a</sup>	1.16	1.19	1.19

Note: nd means not determined.

<sup>a</sup> Calculated quantities are according to the fermentation equation (see the Materials and Methods section).

medium formulation assured that no noncarbon nutrient, except nitrogen perhaps, could be limiting the unrestricted growth at some stage of the fermentation. Glucose concentration was the same for all three fermentations of Table I. The pH profiles were virtually identical for all three fermentations of Table I. Since the cells can utilize effectively both or either of NH<sub>4</sub><sup>+</sup> and organic nitrogen as nitrogen sources, from Table I we conclude that reduced availability of nitrogen enhances solvent production. When the pH was kept constant at 4.5, variation of the NH<sub>4</sub><sup>+</sup> concentration did not affect solvent production or any other macroscopic characteristic of the fermentation (Table II). Since the only factor that is different in the fermentations of Table I from those of Table II is the pH variation (from 4.5 to 3.65), one may conclude that the pH in combination with the lower nitrogen-source concentration enhances solvent production. At pH 4.5, the reduced availability of the nitrogen source, *in the range studied*, did not induce solvent production. If the lower pH values affected any factors, other than the nitrogen source, that have an effect on solvent production, or if the lower pH values alone induced solvent production, the same amounts of solvents would have been produced independent of the nitrogen-source variation. Table I shows that this did not happen. Therefore, the lower pH values affect the availability of nitrogen to

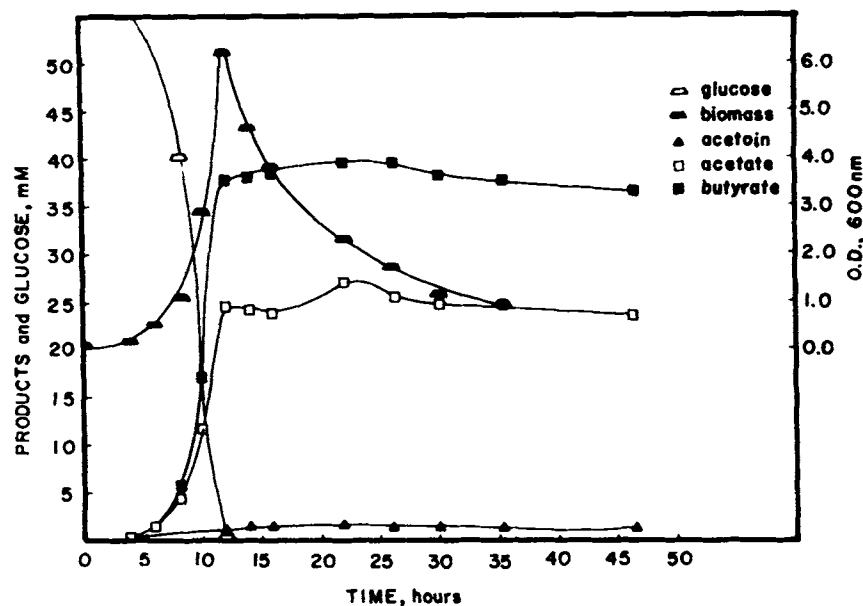


Figure 5. Batch fermentation of *C. acetobutylicum* with culture pH controlled at 4.5. Initial glucose concentration was 61.4mM and initial molar  $(\text{NH}_4^+)/(\text{glucose})$  was 0.49.

the cells. This conclusion will be verified by the pH 3.7 fermentations performed with low and high  $\text{NH}_4^+$  concentrations as is subsequently described.

To summarize, from the experiments of Tables I and II, it appears that limited nitrogen availability results in enhanced solvent production. Also, when an unrestricted batch fermentation is not limited by the availability of any noncarbon nutrient, and is thus eventually limited by the glucose concentration, acids are exclusively produced (Table II).

In order to investigate further the effect of pH on nitrogen availability and solvent production, we performed three more controlled-pH fermentations. Two fermentations with an ammonium to glucose ratio of 0.136 were performed at pH values of 4.0 and 3.7. Their time profiles are shown in Figures 6 and 7, respectively, and their fermentation balances are summarized in Table III, where the balance for the fermentation with an ammonium to glucose ratio of 0.13 and pH 4.5 is repeated for comparison. The biomass profiles of the three fermentations are also shown in Figure 8 for comparison. The third fermentation with an ammonium to glucose ratio of 1.5 and pH 3.7 was an almost exclusively acid fermentation and thus identical to the corresponding fermentation at pH 4.5 shown in Table II, except that no biomass lysis occurred at the stationary phase. The first fermentation was started and controlled at pH 4.0. The other two fermentations were started at pH 4.5 and their pH was lowered gradually but quickly to pH 3.7 at  $\text{OD}_{600}$  values between 0.5 and 0.8.

It can be seen from Figure 8 and Table III that the lower pH results in a lower specific growth rate, a lower biomass yield,  $Y_s$  (g biomass/g glucose utilized), and a lower overall  $Y_{\text{ATP}}$ . It appears that  $Y_s$  is decreasing

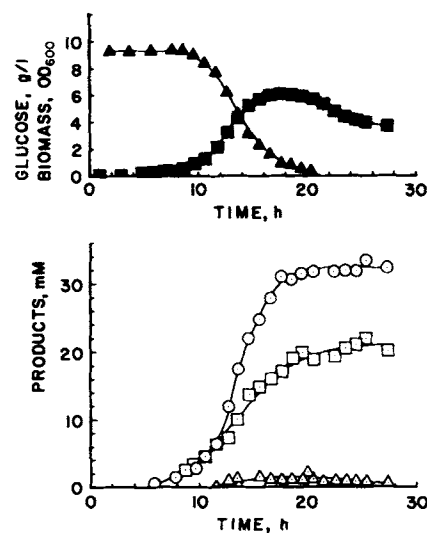
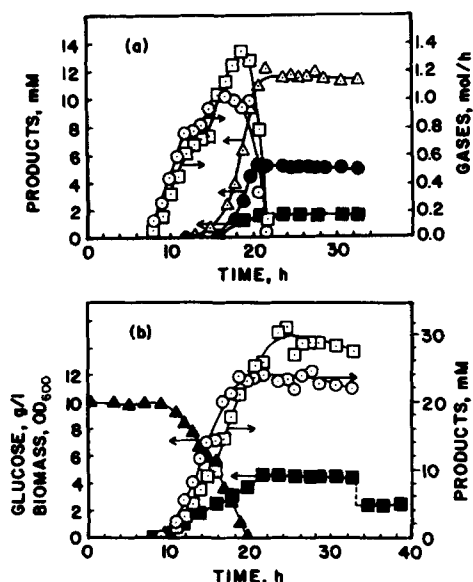


Figure 6. Batch fermentation of *C. acetobutylicum* with culture pH controlled at 4.0. Initial glucose concentration was 51.6mM and initial molar  $(\text{NH}_4^+)/(\text{glucose})$  was 0.136. The symbols show (▲) glucose, (■) biomass, (○) butyrate, (□) acetate, and (△) acetoin.

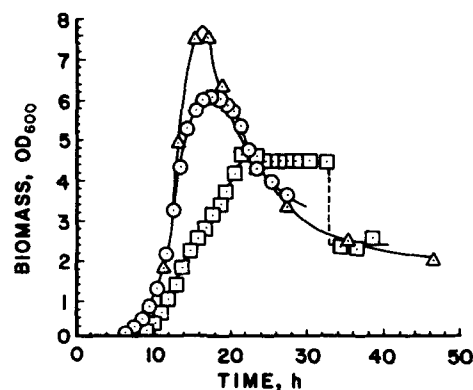
with decreasing pH, while there is no clear trend in the  $Y_{\text{ATP}}$  with decreasing pH (Table III). Figures 5, 6, and 7 are also useful for observing the changes in the acetate and butyrate profiles as the pH varies from 4.5 to 3.7. The higher the pH, the more butyrate is produced and the larger the difference between butyrate and acetate concentrations. When significant amounts of solvents are produced, acetate concentration becomes at least equal or higher than the butyrate concentration (Figs. 1 and 7, and unpublished results). Another interesting observation that can be made from Figure 7 is that solvent production begins when  $\text{H}_2$





**Figure 7.** Batch fermentation of *C. acetobutylicum* with culture pH controlled at 3.7. Initial glucose concentration was 54.4mM and initial molar  $(\text{NH}_4^+)/(\text{glucose})$  was 0.136. The symbols show (a): (Δ) butanol, (●) acetone, (■) ethanol, (□) CO<sub>2</sub>, and (○) H<sub>2</sub>, and (b): (▲) glucose, (■) biomass, (□) acetate, and (○) butyrate.

production slows down. This, we found, is typical of solvent-producing fermentations. Also, in solvent producing fermentations, we have observed that H<sub>2</sub> production peaks a few hours earlier than CO<sub>2</sub> production as is shown in Figure 7. In acid fermentations, H<sub>2</sub> and CO<sub>2</sub> productions peak together (unpublished results). From Figure 8 it can be observed that while biomass lysis occurred immediately after the cultures at pH 4.5 and 4.0 reached the stationary phase, the biomass lysis was abolished at pH 3.7. For the latter fermentation, we kept the cells in the stationary phase at pH 3.7 for 11 h without observing any significant lysis. Then, we raised the culture pH to 5.0 and this resulted in an immediate biomass lysis. The new OD<sub>600</sub> reading was 55% of the original reading and remained unchanged



**Figure 8.** Biomass profiles of batch fermentations of *C. acetobutylicum* with culture pH controlled at (Δ) 4.5, (○) 4.0, and (□) 3.7. The initial glucose concentration was ca. 55mM and the initial molar  $(\text{NH}_4^+)/(\text{glucose})$  was ca. 0.136.

**Table III.** Fermentation balances for batch fermentations with (approximately) the same initial ammonium to glucose ratio but different controlled-pH values.

pH	4.5	4.0	3.7
$(\text{NH}_4^+)/(\text{glucose})$	0.13	0.136	0.136
Biomass (g/L)	1.52	1.21	0.95
Initial glucose (mM)	58.3	51.6	54.4
Residual glucose (mM)	0.0	0.0	0.0
$\mu$ (h <sup>-1</sup> )	0.69	0.62	0.37
$Y_s$	0.145	0.130	0.097
$Y_{\text{ATP}}^a$	8.40	9.00	5.5
Gamma ratio <sup>a</sup>	1.002	1.013	1.002

Substance	Amount (mol/100 mol glucose fermented)		
Butyrate	67.5	61.3	36.4
Acetate	39.4	33.8	43.7
CO <sub>2</sub>	187 <sup>a</sup>	208	180
H <sub>2</sub>	217 <sup>a</sup>	194	183
Ethanol	1.1	1.6	2.8
Butanol	1.2	2.2	18.8
Acetone	0.0	1.3	9.0
Acetoin	3.7	2.2	1.8
Carbon recovered in products (as glucose)	92.6	93.0	90.0
Nff <sup>a</sup>	-37.4	-26.0 <sup>b</sup>	-15.4 <sup>c</sup>
H <sub>2</sub> /CO <sub>2</sub>	1.16 <sup>a</sup>	0.93	1.02

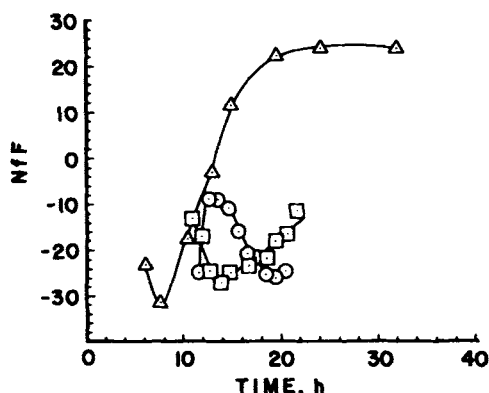
<sup>a</sup> Calculated quantities are according to the fermentation equation (see the Materials and Methods section).

<sup>b</sup> This was calculated at  $t = 20$  h.

<sup>c</sup> This was calculated at  $t = 21$  h.

for at least six hours after we raised the pH. We concluded, thus, that the biomass autolytic activity was strictly pH dependent. We have estimated that biomass lysis is abolished at pH values lower than 3.8. We also found that growth-rate inhibition was pH dependent but virtually independent of the ammonium to glucose ratio. Below a pH value of 3.6, we found that growth was totally inhibited.

It can be seen from Table III that at the low ammonium-to-glucose ratios, the lower the pH, the lower are the butyrate yield and the higher the butanol and acetone yields. The acetate yield remains virtually unchanged while the acetoin yield decreases with decreasing pH. In any case, the fermentations at pH values of 4.5 and 4.0 are almost exclusively acid producing, while solvents are produced in the pH 3.7 fermentation. It should be noted, however, that the solvent yields were lower and the acid yields higher compared to the uncontrolled-pH fermentation (Table I). Table III also shows that the lower the pH, the larger the Nff value, which in all cases is negative. The H<sub>2</sub>-to-CO<sub>2</sub> ratio apparently does not vary in any systematic way with the pH. The effect of pH on solvent versus acid production in these low ammonium concentration fermentations can be further illuminated by observing the behavior of the calculated Nff values as a function of time, as is shown in Figure 9. Note



**Figure 9.** Time profiles of NfF (mol NAD reduced by FdH<sub>2</sub> per 100 mol glucose fermented) for three batch fermentations of *C. acetobutylicum* with (Δ) uncontrolled culture pH, or culture pH controlled at (○) 4.0 and (□) 3.7. Initial glucose concentration was ca. 52mM and initial molar (NH<sub>4</sub><sup>+</sup>)/(glucose) was ca. 0.14.

that these NfF values are cumulative, that is, they represent the total amount of NADH<sub>2</sub> reduced by ferredoxin up to a given fermentation time. The instantaneous NfF values can be measured or judged from the slopes of the curves in Figure 9. Note also that the NfF values are normalized per 100 moles of glucose fermented. For the pH 4.0 fermentation (where only acids were produced), the NfF shows an early increase and then it continuously decreases, remaining negative at all times (in the early part of the fermentation its instantaneous values are positive). At pH 3.7, NfF decreases in the early part of the fermentation (where only acids are produced) and increases thereafter corresponding to the solvent-producing phase. In this latter phase, the NfF instantaneous values are positive, but the cumulative NfF values remain negative. For the uncontrolled-pH fermentation, NfF decreases in the early acid-producing phase of the fermentation and increases thereafter to attain a large positive value which corresponds to significant solvent production.

In summary, it appears that when the ammonium-to-glucose ratio is low (0.136), the lower pH results in solvent production, while when that ratio is high (1.5) and the pH low, only acids are produced and the product distribution is very little affected. One would conclude, then, that the reduced nitrogen availability which results in solvent production is primarily effected through the reduced availability of organic nitrogen (yeast extract and asparagine).

The effect of the low pH on the  $Y_{ATP}$  and the availability of organic nitrogen to the cells may be further illuminated by comparing the overall and transient  $Y_{ATP}$  values of the fermentations presented in Tables I, II, and III. To this effect, we have plotted in Figure 10 the cumulative  $Y_{ATP}$  as a function of fermentation time for the three low ammonium concentration fermentations corresponding to uncontrolled pH and controlled-pH values 4.0 and 3.7.

From Table I, it can be seen that the  $Y_{ATP}$  has the

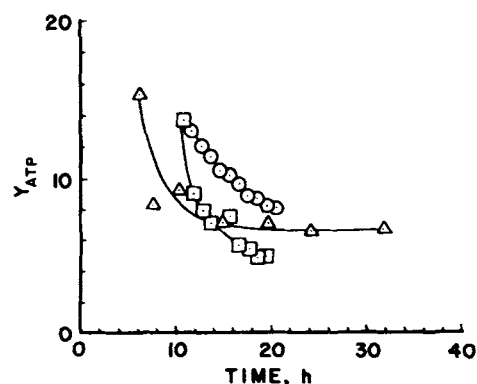
highest value at the lowest ammonium concentration, which corresponds to the lowest specific growth rate. From Table II, for the controlled pH 4.5 fermentations, the  $Y_{ATP}$  appears inversely proportional to the ammonium to glucose ratio, decreasing with decreasing  $\mu$ . For the case of a low ammonium-to-glucose ratio, the lowest  $Y_{ATP}$  was observed for the fermentation with the lowest pH (Table III). From Figure 10, we observe that the  $Y_{ATP}$  varies dramatically during all three batch fermentations. This  $Y_{ATP}$  variation we found to be true for all fermentations we have carried out in our laboratory. Curiously, the most significant drop in the  $Y_{ATP}$  values occurs during the exponential growth phase where the specific growth rate is constant.

The fact that the highest  $Y_{ATP}$  values were observed for the two fermentations with the lowest ammonium concentrations may be explained by the early depletion of the medium NH<sub>4</sub><sup>+</sup> and thus the larger percentage of organic nitrogen used by the cells. Lower utilization of NH<sub>4</sub><sup>+</sup> and higher utilization of organic nitrogen result in higher  $Y_{ATP}$  values.<sup>23</sup> The low  $Y_{ATP}$  observed for the low ammonium-to-glucose ratio and low pH we can only attribute to the effect of pH on the membrane function and cell metabolism.

## Continuous Cultures

The effect of the ammonium-to-glucose ratio on continuous cultures with controlled pH at 4.5 was also examined. Two fermentations were conducted with ammonium concentrations of 30.3mM. The glucose concentrations in the feed for the two runs were 50mM and 100mM, respectively, giving ammonium-to-glucose ratios of 0.61 and 0.30, respectively.

At the ammonium-to-glucose ratio of 0.61, the continuous fermentation was run at a dilution rate of 0.18 h<sup>-1</sup>. The time course of this fermentation is shown in Figure 11. Total glucose utilization was observed so that growth was glucose limited, irrespective of the steady-state or non-steady-state conditions. Under the



**Figure 10.** Time profiles of the  $Y_{ATP}$  for three batch fermentations of *C. acetobutylicum* with (Δ) uncontrolled culture pH or culture pH controlled at (○) 4.0 and (□) 3.7. Initial glucose concentration was ca. 52mM and initial molar (NH<sub>4</sub><sup>+</sup>)/(glucose) was ca. 0.14.

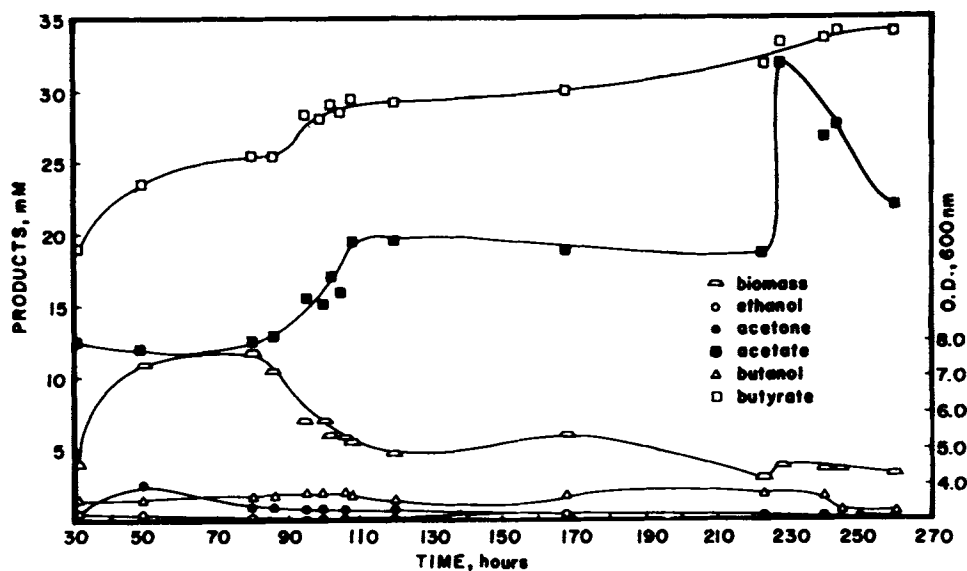


Figure 11. Continuous, glucose-limited fermentation of *C. acetobutylicum* at  $D = 0.18 \pm 0.005$   $\text{h}^{-1}$  and pH controlled at 4.5. Feed glucose concentration was 50mM. Time was zero at inoculation.

technical restrictions of the equipment employed, the fermentation period between hours 110 and 170 is as close to steady state as we could achieve.<sup>18</sup> The transient at the end of the fermentation was induced externally by a small change in the glucose feed concentration.

This glucose-limited continuous culture produced acids almost exclusively. High concentrations of acetate and butyrate were observed. Small quantities of butanol, ethanol, and acetone were also formed, irrespective of steady-state or non-steady-state conditions. The carbon recovery at the steady-state was 84% and the  $Y_{\text{ATP}}$  was 7.6. Over the whole time course, the glucose carbon recovery into products increased monotonically with time from 54 to 98.3% and the  $Y_{\text{ATP}}$  decreased, almost monotonically, from 14.7 to 5.5.

When the cells were grown continuously with a dilution rate of  $0.16 \text{ h}^{-1}$  and an ammonium-to-glucose ratio of 0.30, glucose accumulated in the reactor (8mM–34mM) and thus it was not the growth-limiting nutrient. In this continuous nitrogen-limited fermentation (Fig. 12), acid yields were significantly lower than those observed in the glucose-limited culture of Figure 11. Both acetate and butyrate yields were observed to be significantly lower in the non-glucose-limited continuous culture. An increase in solvent yields, in particular the butanol and acetone yields, was also observed. The ability to produce solvents did not appear to depend on achieving steady-state conditions, which strictly speaking were never achieved. This nitrogen-limited culture was considerably more sensitive to small ex-

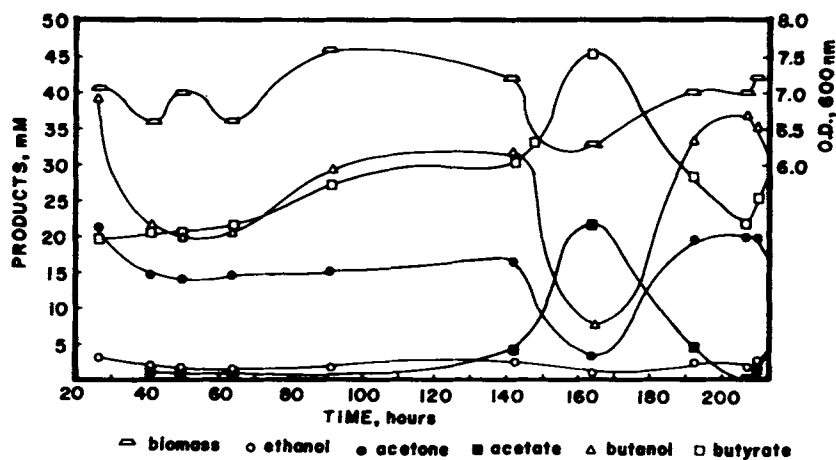


Figure 12. Continuous, nitrogen-limited fermentation of *C. acetobutylicum* at  $D = 0.16 \pm 0.013$   $\text{h}^{-1}$  and pH controlled at 4.5. Feed glucose concentration was 100mM. Time was zero at inoculation.

ternal perturbations than the glucose-limited culture. The perturbation at 150 h was induced by a small change in the feed medium, which led to sustained oscillations in the fermentation parameters. The  $Y_{ATP}$  values varied from 6.1 to 9.3 and the glucose carbon recovery into products from 57 to 86% but not monotonically with time.

## DISCUSSION AND CONCLUSIONS

From both the batch and continuous fermentations presented above, it appears that solvent production takes place only under conditions of reduced nitrogen availability or of nitrogen limitation, at least in the range of fermentation conditions that was investigated. We have also observed that the culture pH below 4.0 inhibits the biomass growth rate (Table III and Fig. 8). Below 3.8, the culture pH also reduces or totally inhibits the availability of organic nitrogen from the yeast extract to the cells. Thus, we found that the effect of pH on solvent production is indirect, through inhibition of the growth rate and the nitrogen availability. It was found that at pH 3.7 solvents may or may not be produced depending upon the amount of the ammonium concentration in the medium. Also, in uncontrolled-pH batch cultures (which display similar pH profiles), the amounts of solvents produced increase with decreasing ammonium-to-glucose ratios in the medium.

The inhibition of the nitrogen availability at low pH may be the result of the inhibition either of the proteolytic and other enzymes,<sup>22</sup> which are responsible for breaking down the biopolymers of the complex carbon and nitrogen source (yeast extract) into monomeric units, or of the transport mechanisms of these monomers and asparagine. If we observe that the low pH inhibits also the proteolytic protein(s) responsible for the biomass autolysis, inhibition of the nucleases and the proteolytic enzymes appears as a likely mechanism of the reduced organic-nitrogen availability.

We have also observed that reduced nitrogen availability is not necessarily displayed by high residual glucose concentrations (compare the low-ammonium-concentration fermentations of Tables I and III). The concentration of glucose at the stage of the fermentation where nitrogen limitation occurs may or may not allow complete glucose conversion into products (primarily) and biomass (cf. Figs. 1 and 7). Complete glucose utilization (in batch cultures), whether solvents are produced or not, apparently depends on the past history of the fermentation, that is, on the state of the cell metabolic machinery and on any possible inhibitory effects.

We have noted that nitrogen limitation does not depend only on the total concentration of nitrogen sources in the medium and the pH, but also on the growth rate and the mode of fermentation, i.e., batch versus continuous (compare the continuous fermentations of Figure 12 with the fermentations of Table II).

The  $Y_{ATP}$  yield for growth on glucose is known to be affected by the growth supplements (nucleic-acid bases and amino acids), the energy requiring transport of  $NH_4^+$  and the specific growth rate.<sup>23</sup> The latter dependence derives from the energy requirements for cell maintenance and has the following form:

$$\frac{1}{Y_{ATP}} = \frac{1}{Y_{ATP}^{max}} + \frac{m_e}{\mu} \quad (1)$$

Equation (1) was first used by de Vries et al.<sup>24</sup> as an improvement to the corresponding equation for  $Y_s$  (Pirt's equation). Although never "thoroughly" derived, eq. (1) can be obtained from an equation of an ATP balance stating that all ATP produced is used for biomass growth (with a constant  $Y_{ATP}^{max}$ ) and biomass maintenance (with a constant coefficient,  $m_e$ ). Equation (1) would therefore be valid under the conditions that the above ATP balance is satisfied, and  $Y_{ATP}^{max}$  and  $m_e$  are constant. Those conditions have been extensively discussed by Stouthamer and co-workers<sup>23-26</sup> for continuous and batch cultures. The constancy of  $Y_{ATP}^{max}$  is essentially affected only by the presence of nucleic-acid bases in the medium.<sup>23</sup> Since much of maintenance energy is assumed to be necessary for the maintenance of the right ionic composition of the cells and the maintenance of the right intracellular pH and thus for the transport of ions,<sup>23,25,27</sup>  $m_e$  would be affected by the culture pH,  $NH_4^+$  concentration, the transport of carboxylic acids, and temperature. The validity of the ATP balance requires that growth is ATP limited, such that ATP is neither accumulated nor hydrolyzed unnecessarily. When the latter happens, the growth has been termed "uncoupled" and the ATP is wasted into "energy-spilling" or "slip" reactions.<sup>26</sup> It is under continuous, steady-state, carbon and energy-limited conditions that all the necessary conditions seem to be satisfied and eq. (1) appears to be valid.<sup>26</sup> However, even continuous steady-state data do not always obey eq. (1).<sup>26</sup> The validity of eq. (1) under unrestricted batch growth conditions, where  $\mu$  is constant, has not been examined before. The data of Figure 10 for the constant pH fermentations indicate that eq. (1) is not valid. Since  $Y_{ATP}$  varies while  $\mu$  is constant, and the growth proceeds under unrestricted conditions, growth is apparently uncoupled. It appears then justified to ignore the maintenance energy requirements in the derivation and use of the fermentation equation<sup>1</sup> in batch cultures. It has been suggested earlier<sup>17</sup> that under unrestricted growth conditions the maintenance energy may be considered negligible. As we have discussed already, the  $Y_{ATP}$ , in the present experiments, appears to be affected by the  $NH_4^+$  concentration and the pH. In view of the fact that different amounts of ATP are produced from different products, and since acids act as energy uncouplers,<sup>28</sup> many culture parameters may affect  $Y_{ATP}$ . A variable degree of "uncoupled" growth resulting from the variation of a number of culture parameters

is the most probable reason for the highly variable  $Y_{\text{ATP}}$  values.

The data of Table II and Figure 11 indicate that a minimum total or undissociated butyric acid concentration is not sufficient for solvent production. It has been suggested recently,<sup>10</sup> that a minimum of 18mM undissociated butyric acid was necessary for solvent production. Figure 1 shows that solvent production is initiated in the presence of 12mM undissociated (12.5mM total, pH 3.7) butyric acid. In another pH 4.5 batch fermentation (under culture conditions different from those of Table II), solvent production was initiated at a 5.5mM undissociated (8.2mM total) butyric-acid concentration (unpublished results). Thus, it appears doubtful that there exists a minimum butyric-acid concentration which is necessary for solvent production.

The autolytic behavior of stationary cultures of *C. acetobutylicum* has been found<sup>11,29,30</sup> to be due to a glycoprotein referred to as an autolysin. The autolysin was found to have a maximal stability at the pH range between 4.0 and 5.0, to be partially inactivated by proteases,<sup>29,30</sup> and to be produced at the end of the exponential-growth phase.<sup>29</sup> The autolytic activity has been also found to be associated with high butanol concentrations.<sup>11</sup> In the present work, we found that the autolytic activity is not necessarily linked to solvent production (Figs. 5, 6, and 8) and that this activity is totally inhibited at culture pH values below 3.8 (Figs. 1, 2, 7, and 8). This autolytic activity inhibition was reversible when the culture pH was raised (Figs. 7 and 8), indicating that the lower pH values inhibit the action rather than the production of the autolysin.

For batch fermentations, the values of the overall  $Y_{\text{ATP}}$  were found to vary from 5.5 to 9.0, while the transient yields varied from 5.5 to 15.5. In the continuous glucose-limited culture, the  $Y_{\text{ATP}}$  varied between 5.5 and 14.7. In the continuous nitrogen-limited culture, the  $Y_{\text{ATP}}$  was found to vary between 6.1 and 9.3.

The experiment of Figure 12 has shown that for the chosen temperature and pH values, nitrogen and thus non-ATP limitation leads to solvent production. Those unrestricted batch cultures that were eventually limited by the nitrogen availability (Tables I and III), produced solvents in amounts increasing with decreasing nitrogen availability. Since limitation by the nitrogen availability in the presence of nonlimiting glucose amounts implies non-ATP limitation, we may conclude that under the present culture conditions, non-ATP-limited fermentations lead to solvent production. Under conditions of glucose limitation (Fig. 11) or in unrestricted batch cultures that were eventually limited by the glucose concentration (Table II), acids are exclusively or predominantly produced.

The question of whether glucose limitation implies carbon or ATP limitation perhaps cannot be, strictly speaking, answered definitely, in view of the fact that the cell needs for ATP vary considerably with the

culture conditions. Throughout the literature, sugar-substrate limitation is typically taken to imply both carbon and ATP limitation in anaerobic saccharolytic fermentations. The highest  $Y_{\text{ATP}}$  values [increasing according to eq. (1) from 14.9 to 21.2 as  $D$  increased from 0.14 to 0.72 h<sup>-1</sup>] were obtained under glucose-limited, continuous, steady-state conditions.<sup>5</sup> Since ATP limitation is a necessary condition for the validity of eq. (1), it appears that under continuous steady-state conditions, glucose limitation implies ATP limitation. In the herein reported experiments and overall, the  $Y_{\text{ATP}}$  values were higher under glucose-limited (Fig. 11) conditions than under nitrogen-limited conditions (Fig. 12). Also, from Figures 6, 7, and 10, it can be seen that the highest  $Y_{\text{ATP}}$  values were obtained under exponential-growth conditions when the rates of acid production were highest. Generally, the  $Y_{\text{ATP}}$  drops faster and lower with increasing solvent production (Fig. 10).

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## NOMENCLATURE

$D$	dilution rate (h <sup>-1</sup> )
$\text{FdH}_2$	reduced ferredoxin
$m_e$	maintenance coefficient (mol ATP/g dry biomass/h)
$\text{Nff}$	mol NAD reduced by $\text{FdH}_2$ (via the NADH-ferredoxin oxidoreductase) per 100 mol glucose fermented
$Y_{\text{ATP}}$	molar growth yield on ATP (g dry biomass/mol ATP utilized in biomass synthesis)
$Y_{\text{ATP}}^{\text{max}}$	maximum $Y_{\text{ATP}}$ , in equation (1)
$Y_s$	biomass yield (g biomass/g glucose fermented)
$\gamma_b$	reductance degree of biomass
$\mu$	specific growth rate (h <sup>-1</sup> )
$\sigma_b$	weight fraction of carbon in biomass

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