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Production of butyric acid by batch fermentation of cheese whey with *Clostridium beijerinckii*

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SUMMARY

The effect of pH on the fermentation of butyric acid by *Clostridium beijerinckii* using cheese whey as a substrate was studied. Maximum concentrations of the acid were produced when the pH was controlled at 5.5. Raising or lowering of pH was found to reduce the total acid formation. This particular strain of *C. beijerinckii* produced insignificant amounts of butanol in all the pure culture cases investigated. A comparative study of the fermentation in a synthetic glucose medium and in cheese whey showed the whey to produce more butyric acid.

INTRODUCTION

Cheese whey constitutes a major waste disposal problem for the dairy industry throughout the world [20], except for places where the industry is decentralized and local utilization is possible. Part of the whey is presently utilized as a human food source and also for feeding animals. Considerable amounts are processed into lactose and protein in the U.S.A. Among emerging technologies, alcohol and single-cell-protein production seem to be reasonable alternatives to traditional disposal procedures [4,26]. The acetone/butanol fermentation has been shown to be economically attractive [15]. Considering the toxicity of the products of this fermentation [3], cheese whey with its 5–6% lactose concentration represents the maximum carbohydrate levels that can be utilized in this system.

Butyric acid production by *Clostridium* species has a significant bearing on acetone/butanol fermentation, as the onset of butanol production has been associated with the concentration of acids present in the system [2,10]. An understanding of physiological factors that govern the acid formation is, therefore, important for future industrial applications. The production of organic acids such as butyric, propionic [5] and acetic acids represents another possibility for whey utilization. Though there have been few organized attempts to maxi-

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mize fermentative production of butyric acid [24], the economics of butyric acid is attractive for commercial production with genetically engineered bacteria [8]. It may also be possible to esterify the organic acids with alcohols to produce carboxylic acid esters that have better properties as fuels than alcohols alone [7].

This study involves the production of butyric acid from cheese whey using *C. beijerinckii* (formerly known as *C. butylicum*). Specifically, the objectives were to investigate the influence of pH on the metabolism of an industrial strain and identify conditions for the maximum production of butyric acid utilizing cheese whey.

Published literature dealing with butyric acid fermentation is relatively scarce [24]. A number of strains of *C. beijerinckii* have been investigated in recent years for the production of butanol and isopropanol [12,14]. The reported levels of acid production in these studies were always very low. Fermentation of cheese whey by *C. acetobutylicum* has also been studied, but for solvent production only [17,18]. It has been shown that considerable amounts of butyric acid could be produced by *C. acetobutylicum* under suitable pH conditions using glucose as substrate [21].

MATERIALS AND METHODS

Microorganisms. Spores of a strain of *C. beijerinckii* were supplied by the Bio-diesel Corporation of Iowa, U.S.A. These were stored at 4°C in reinforced clostridial medium (Oxoid) with 1% calcium carbonate added to it. The spores were germinated by heat-shocking for 1 min at 100°C.

Cheese whey. Acid cheese whey was obtained from Mid America Dairy, Kirksville, MO, U.S.A. It contained between 50 and 65 g/l lactose and had a pH between 4.11 and 4.25. Raw whey was stored frozen in 1 gallon plastic containers.

Media. Unsupplemented heat-sterilized cheese whey was used as fermentation medium. Leung's [16] synthetic medium for *C. acetobutylicum* was also used in some experiments.

Batch fermentation. A 14 liter New Brunswick fermenter (working volume 6–6.5 liters) was used in this study. The broth was agitated at 100 rpm to maintain uniformity and temperature was held constant at 37°C. pH was automatically controlled by addition of 5 N NaOH or 1 N H_2SO_4 when desired. A 5% (v/v) actively growing culture was used as inoculum. The broth was kept anaerobic by flowing sterile nitrogen through it before and after inoculation. The flow of nitrogen was stopped once the microorganisms were observed to be growing.

Product analysis. Concentrations of acids (butyric and acetic) and solvents (acetone, butanol, ethanol) in sampled broth were measured by a Varian 1520 gas chromatograph using a flame ionization detector. The gas chromatograph was equipped with a 183 cm column of teflon-coated steel (internal diameter 2 mm), packed with 80/100 mesh Chromosorb WAW AT 1000 packing material (Alltech). The oven temperature was programmed from 90°C to 160°C at a rate of 10°C/min. Detector temperature was maintained at 230°C.

Lactose concentration in the samples was measured by the Nelson-Somagi method [23].

RESULTS AND DISCUSSION

Batch fermentations were conducted to investigate the cellular control of butyric acid fermentation by this strain of *C. beijerinckii.* The specific objective was to determine the physiological conditions under which the microorganism produces maximum concentrations of butyric acid from cheese whey.

Uncontrolled pH experiments with unsupplemented cheese whey (initial pH 6.2) in Hungate tubes resulted in butyric acid, acetic acid and butanol concentrations of 6.5, 2.3 and 0.8 g/l, respectively, after 7 days. Uncontrolled experiments in a fermenter noted a pH drop from 6.5 to 4.5 in 200 h with similar product concentrations (butyric acid 6.5 g/l and acetic acid 2.0 g/l). No butanol was observed in the fermenter.

The concentration of hydrogen ions in culture broth is an important variable that influences the relative rates of growth and product formation in

microbial systems. Growth of bacteria has been reported to be optimal under neutral to slightly acidic conditions. Growth of cells is strongly impaired at low pH. On the other hand, induction of solventogenesis in solvent-producing microorganisms has been correlated with the concentration of undissociated butyric acid in the system, which increases under acidic conditions [22]. Hence, profiling of pH is an important tool in controlling the patterns of product formation. Therefore, a number of experiments were conducted with different pH profiles in the fermenter. Figs. 1 to 4 show results of these experiments. In Fig. 1, the pH of the fermentation medium was controlled at 5.5 throughout the experiment. The experiment in Fig. 2 was initiated at a pH of 6.3 which was allowed to fall naturally to 5.5 and was then controlled at 5.5 ± 0.15 for the rest of the experiment. Fig. 3 shows the case where the initial pH was maintained at 6.0 ± 0.1 for 44 h, then forced to fall to 5.5, and once again to 5.0 after 6 h. From this time on, the pH was left uncontrolled; it eventually dropped to 4.5 in 150 h. Fig. 4 represents the case where the initial pH was 6.3 and was allowed to fall to 5.0, where it was con-

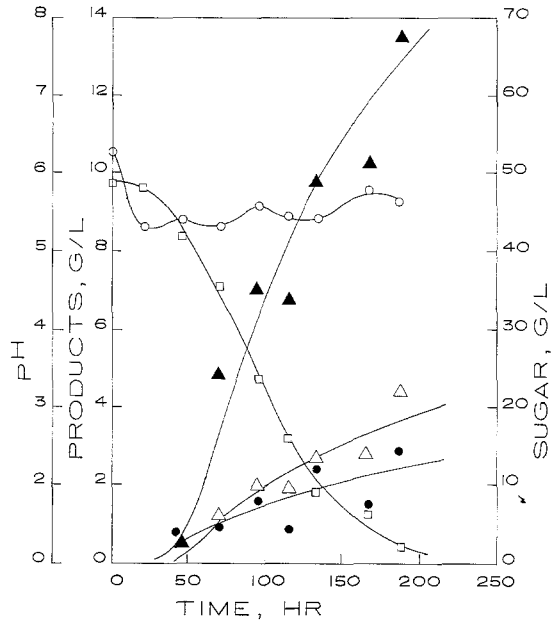

Fig. 2. The time course of substrate utilization and product formation by *C. beijerinckii*. Initial pH 6.3 and controlled at 5.5 ± 0.15 after 30 h. The symbols are the same as in Fig. 1.

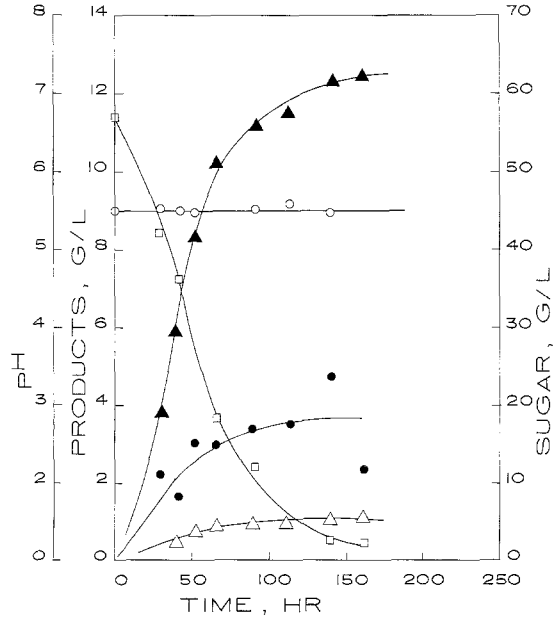

Fig. 1. The time course of substrate utilization and product formation by *C. beijerinckii* at a controlled pH of 5.5. Butyric acid (▲); acetic acid (●); butanol (△); lactose (□); pH (○).

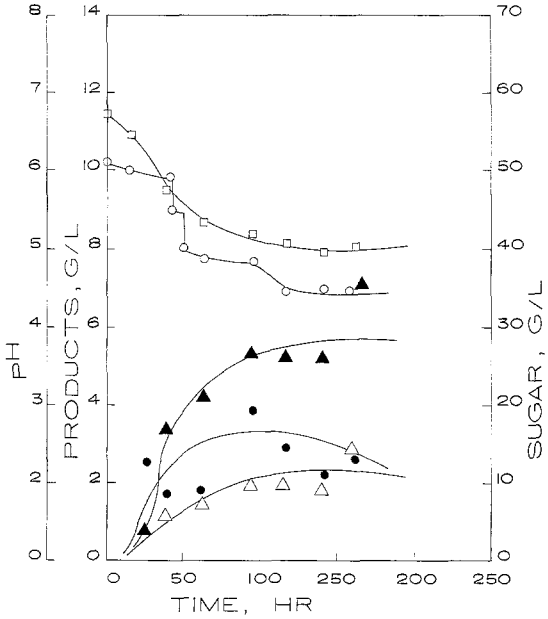

Fig. 3. The time course of substrate utilization and product formation by *C. beijerinckii*. pH dropped in stages from 6 to 5.5 and then to 5.0. The symbols are the same as in Fig. 1.

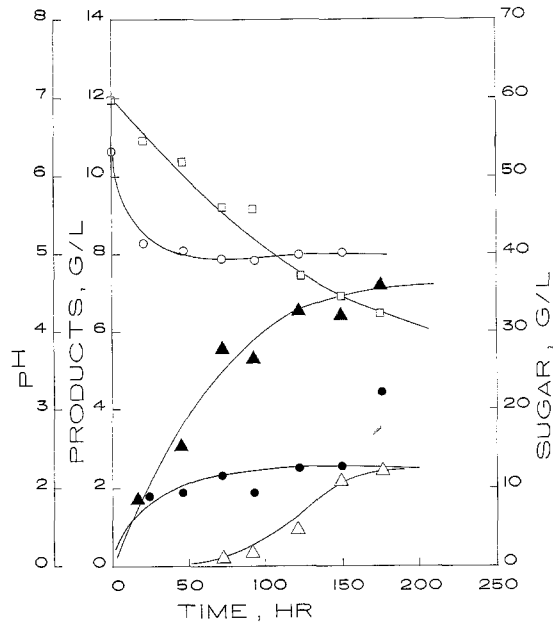

Fig. 4. The time course of substrate utilization and product formation by *C. beijerinckii*. Initial pH 6.3 and controlled above 5.0. The symbols are the same as in Fig. 1.

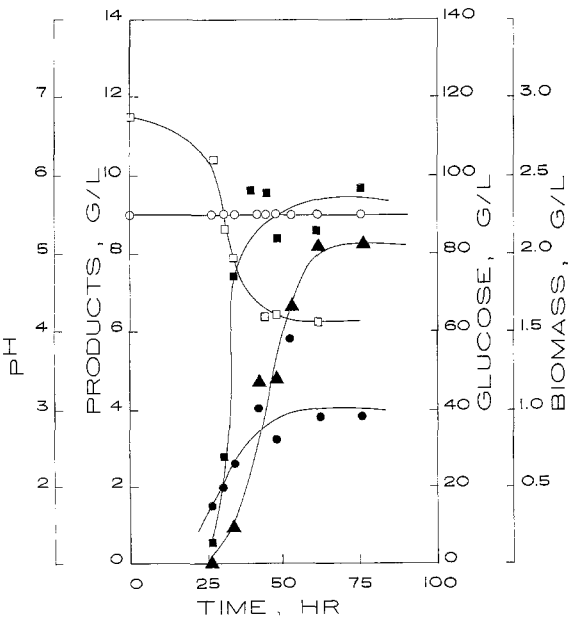

Fig. 5. Profiles of substrate, biomass and products in a controlled-pH butyric acid fermentation utilizing *C. beijerinckii* on a glucose synthetic medium. Biomass (■); butyric acid (▲); acetic acid (●); glucose (□); pH (○).

trolled for the rest of the experiment. For the purpose of comparison, an experiment was also performed with a glucose synthetic medium. Here, the pH was held constant at 5.5 and the initial sugar concentration was 115 g/l. Results of this experiment are shown in Fig. 5.

Cheese whey fermentations were accompanied by the formation of precipitates; as a result, biomass concentrations could not be monitored. In all of the experiments, butyric acid was the major product formed, characterizing a 'butyric fermentation' [19]. Although this microorganism is capable of producing butanol [30], no significant amounts were formed under these conditions. In all cases, the concentrations of acetic acid and butanol remained between 1 and 4 g/l and no correlation was observed with the pH of the system. Acetone did not show up in any of these experiments. Such behavior has been reported in the past both with *C. beijerinckii* [9,31] and with *C. acetobutylicum* [31]. Since the objective of this work was to promote butyric acid production, the reasons for the low levels of solvents produced were not explored. Nonetheless, several recent publications throw light upon this aspect in the form of activities of relevant enzyme systems and the redox potential of the system. It has been documented [1,6,11] that enzymes involved in the synthesis of acids, namely phosphotransacetylase, acetate kinase, phosphotransbutyrylase and butyrate kinase, have their highest activity in the acid production phase. On the other hand, the terminal enzymes catalyzing solvent production (butyraldehyde dehydrogenase and butanol dehydrogenase) have 70–90-fold higher activity in solvent-producing cells than in the acid-producing stage [27,28]. The levels of reduced nucleotides such as NADH and NADPH have also been shown to play an important role in controlling solvent production [13] and have been used to control the metabolic flow [25,29]. Since the current strain of *C. beijerinckii* has been shown [30] to be capable of producing butanol in the presence of *Bacillus cereus*, which is not a solvent producer, it is suspected that acids were primarily produced here not because of a lack of required enzymes to produce solvents but because of the modulating influence of

the redox potential in this system.

The concentration of butyric acid was greater than 12 g/l in Figs. 1 and 2. However, the production rate was faster at a constant pH of 5.5, implying a negative influence of higher initial pH. This was verified by additional experiments (data not shown) where the pH was controlled above 6.0 and poor product formation was observed. Lower pH values were also found to be unfavorable for butyric acid production (Figs. 3 and 4). For this strain, a pH of 5.5 appears to be optimal for growth as well as for butyric acid production.

A comparison of product formation profiles on whey (Fig. 1) with those on glucose synthetic medium (Fig. 5) shows that even though the growth rate on glucose is considerably higher than that on whey, the concentration of total acids is higher in whey. The reason for this discrepancy remains unclear, although it may be related to differences in toxicity of the organic acids to the transport systems of the two sugars [32], The total amount of carbohydrates utilized is approximately the same in both cases (roughly 6%), suggesting that the adverse effects of the acids on metabolism is the primary limitation of this fermentation system. This view is supported by a number of observations reported in the literature [3].

In conclusion, *C. beijerinckii* produced the highest level of butyric acid when the pH was maintained at a constant value of 5.5. An initial pH of greater than 6.0 caused a sluggishness in product formation and production was further impaired when the pH fell below 5.0. This particular strain produced very little butanol when present in pure culture, which is contrary to the findings with others of the same type [9]. In mixed culture with a strain of *B. cereus,* it produced substantial amounts of butanol as well as butyric acid [30].

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