Journal of Industrial Microbiology, 3 (1988) 15–19
Elsevier

SIM 00107

Fermentation of cheese whey by a mixed culture of *Clostridium beijerinckii* and *Bacillus cereus*

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Received 6 July 1987
Revised 2 November 1987
Accepted 5 November 1987

Key words: Cheese whey; Clostridium beijerinckii; Bacillus cereus; Fermentation

SUMMARY

Fermentation of cheese whey to produce butanol and butyric acid was carried out using a mixed culture of *Clostridium beijerinkii* and *Bacillus cereus*. Fermentation selectivities were studied by controlling the pH of the system. Controlled pH values higher than 6.5 as well as those below 5.0 were not conducive to butanol production. Maximum product formation was obtained by controlling the pH at 5.5. When compared with the results obtained using the pure culture of *C. beijerinckii*, a higher butanol concentration was obtained in the mixed culture without sacrificing the level of butyric acid formed.

INTRODUCTION

Currently there is a great deal of interest in the utilization of waste cheese whey. Different schemes of whey utilization have been reported in the literature [5,21,25]. Many of these relate to acetone-butanol fermentation by the genus *Clostridium* [18,19,33]. Butanol fermentation by *Clostridium* sp. has been extensively studied to improve overall productivity. Improved performances were obtained under controlled conditions in batch and continuous fermentation [1,3,6,9,10,31,34], immobilizing [9,17,33] and recycling cells [26,32], and by the use of different extraction schemes [11,15].

Mixed cultures have been used successfully to increase the overall productivity and conversion efficiency of several fermentations [8]. Literature on butanol fermentation utilizing mixed culture is scanty. Bergstrom and Foutch [4] used a series of *Clostridium sp.* in mixed cultures for butanol fermentation. Using a coculture of *C. butylicum* and *C. pasteurianum,* consistently higher product concentrations were obtained when compared with the pure cultures. In their system, the butyric acid produced by an acid-producing species was converted to butanol by a second species. However, the total butanol concentration obtained was low compared to the single-culture fermentations reported in the literature.

Fermentation selectivity of clostridia have been well described in the literature [3,10,31]. *Bacillus cereus,* on the other hand, produces a host of enzymes [27] and small quantities of organic acids

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[28]. No study related to butanol or butyric acid fermentation using a mixed culture of these two species has been reported before, although *Bacillus* is often present with clostridia in natural habitats. The present investigation involves fermentation of cheese whey to butanol utilizing a coculture of *C. beijerinckii* and *B. cereus*. The objective was to study the operating conditions, specifically the pH profile, that are conducive to the production of butanol.

MATERIALS AND METHODS

Microorganism. The mixed culture of *C. beijerinckii* and *B. cereus* was supplied by the Bio-Diesel Corporation of Iowa, U.S.A. The coculture was isolated from soil. Spores of the cultures were stored at 4°C in Reinforced Clostridium Medium (RCM, Oxoid Ltd., U.K.) supplemented with 1% $CaCO_3$. The spores were germinated by heat-shocking at 80°C for 10 min.

Medium. Cheese whey was obtained from Mid America Dairy, Kirksville, MO, U.S.A. It contained between 50 and 65 g/l lactose. The cheese whey was steam-sterilized before fermentation.

Batch fermentation. A 14 liter New Brunswick fermenter was used in this study. The working volume was between 6 and 6.5 liters. The agitation speed was 100 rpm and the temperature was maintained constant at 37°C. Hydrogen ion concentration in the fermenter was controlled by the addition of 5 N NaOH. A 5% (v/v) growing mixed culture was used as an inoculum.

Product analysis. Concentrations of the solvent and acids were determined using a Varian 1520 gas chromatograph equipped with a flame ionization detector. A teflon-coated 1.8 m column with an internal diameter of 0.20 cm, packed with Chromosorb WAW 80/100, was used for separation. The temperature of the column was kept constant at 105°C and the detector temperature was maintained at 230°C. Helium-containing formic acid was used as carrier gas.

Sugar analysis. Lactose concentration in the samples was measured by the Nelson-Somogi technique [24] using lactose as standard.

RESULTS AND DISCUSSION

A number of uncontrolled pH experiments were carried out in Hungate tubes containing whey with an initial pH of 6.27. The results of the analysis of the broth after 7 days of fermentation are presented in Table 1.

Table 1

Effect of coculture on solvent production

Culture	Initial pH	Final pH	Butanol (g/l)	Butyric acid (g/l)	Acetic acid (g/l)
Clostridium sp.	6.27	4.42	0.80	6.50	2.30
Bacillus sp.	6.27	5.62	0.13	2.90	5.90
Coculture	6.27	4.45	2.00	4.50	2.50

These results reveal that coculturing *C. beijerinckii* with *B. cereus* essentially increases the butanol formation from 0.80 to 2.00 g/l.

Further investigations with the coculture were carried out under controlled pH conditions in a 14 liter fermenter. Fig. 1A–D represents the pH and product formation profiles of such experiments. A comparison of Fig. 1A and 1B shows that allowing the controlled (lower level only) pH to fall from 5.5 to 5.0 changes the butanol formation from 4.2 to 2.2 g/l without affecting butyric acid formation. An increase in concentration of butanol is also associated with a slow increase in pH of the system (Fig. 1A). Both the butyric acid and butanol production characteristics significantly changed when the initial pH was controlled at 6.45, as shown in Fig. 1C. The pH of the system controls both the growth and product formation. At pH near neutrality, a good growth of cells is indicated by the rapid decrease in the sugar content (Fig. 1C). The sugar depletion is associated with a decrease in metabolic activity of the cells and thus results in a poor acid/solvent production (Fig. 1C). A significant im-

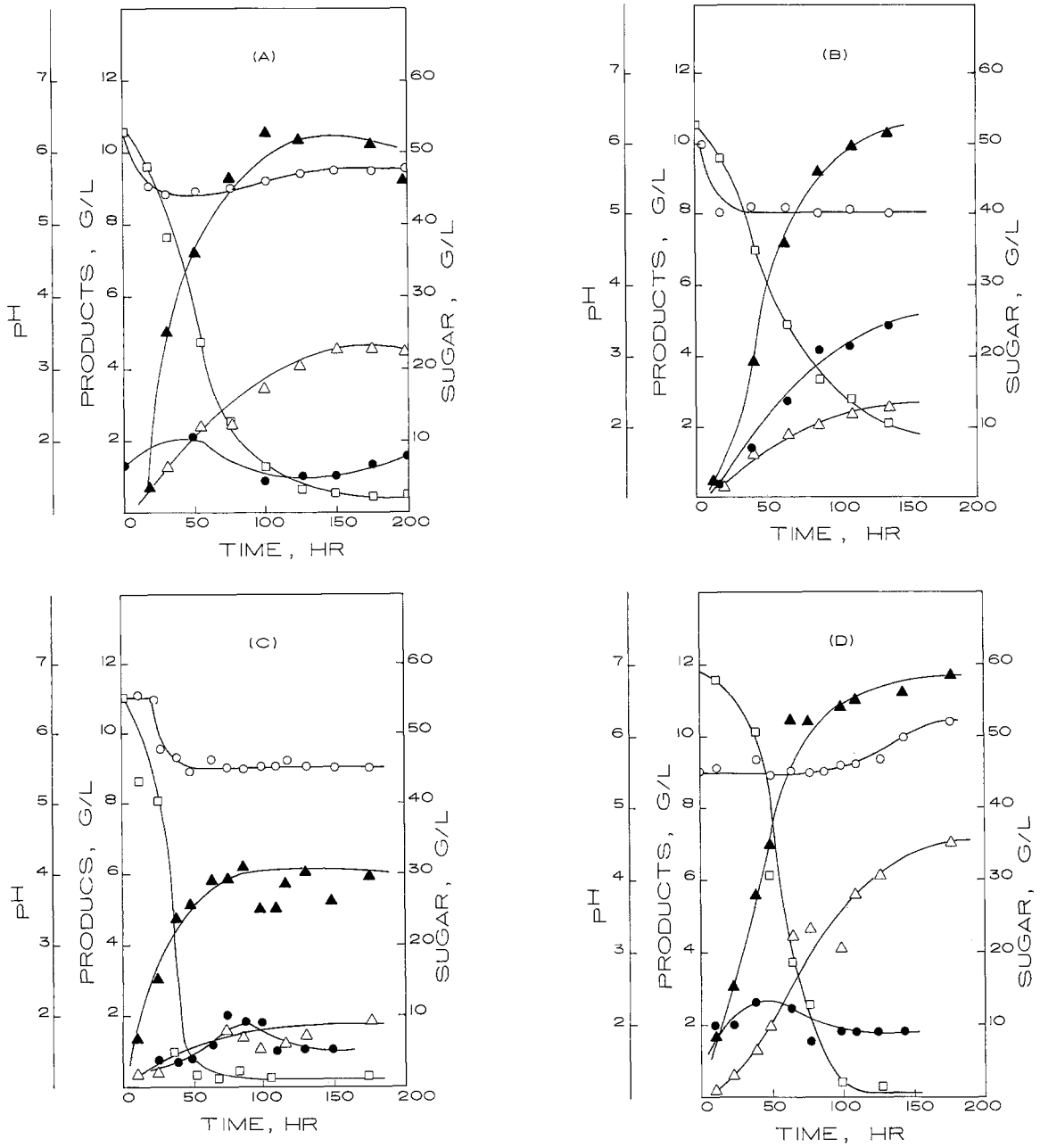

Fig. 1(A–D). The relationship between pH and product formation by a coculture of *C. beijerinckii* and *B. cereus* in a batch fermentation of cheese whey. Butyric acid (▲), acetic acid (●), butanol (△), sugar (□) and pH (○).

provement in butanol formation without affecting the concentration of butyric acid is seen in Fig. 1D, where the initial pH was maintained at 5.5. Here the butanol and butyric acid concentrations were 7.0 and 11.5 g/l, respectively. Comparison of these data with previously reported studies [2] involving the same single strain of *C. beijerinckii* shows a greater than 100% increment in butanol formation without any sacrifice of the butyric acid concentration. The butanol production by *C. beijerinckii* was significantly increased by its association with *B. cereus* without affecting butyric acid production.

In a typical acetone-butanol fermentation, fatty acid production is associated with the growth of the microorganism. The accumulation of acid end products and the associated decrease in pH results in a progressive decrease in the growth rate until cell growth is halted completely, although substrate utilization and cell metabolism continue [12] to produce solvents. Our current knowledge of solventogenesis in clostridia is incomplete [30]. The possible factors that may influence this include: internal undissociated fatty acids and the rate of glucose uptake [7,16,22,23]; the availability and demand for ATP and the reduction energy (NADH) level [20,29,35]; and the intracellular pH [13,19,36]. In our studies, butanol production was found to be concomitant with butyric acid formation. The experimental results indicate that the butanol formation was mediated by the presence of *Bacillus*, but the mode of mediation is unclear to us.

In conclusion, a comparison between uncontrolled and various controlled pH experiments show that the butyl-product formation characteristics can be significantly influenced by controlling the pH of the system. The maximum concentrations of butanol and butyric acid were obtained when the pH of the system was not allowed to fall below 5.5. pH values higher than 6.0 and those below 5.0 are not conducive to the formation of butanol. The relationship between individual microorganisms resulting in an increased butanol formation is unknown, but a clear understanding of it would help to increase the efficiency of butanol fermentation.

ACKNOWLEDGEMENT

This project was supported by a grant from the Bio-Diesel Corporation of Iowa, U.S.A.

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