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On-Line Dialysis of Organic Acids from a *Propionibacterium freudenreichii* Fermentation

Evaluation of a New pH Control Strategy

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Abstract

The efficiency of an ion exchange system coupled to a bioreactor to extract on-line inhibitory organic acids produced was evaluated. Batch fermentations without dialysis and fed-batch experiments with and without dialysis of *Propionibacterium freudenreichii* were conducted. It was possible to keep the propionic acid concentration in the reactor at a low level to avoid complete growth inhibition. Improvements in biomass and propionic acid productivities were achieved when the dialysis system was used. The performance of the dialysis system was improved when a new pH control strategy evaluated in this study was used.

Index Entries: Dialysis; propionic acid; *Propionibacterium*; membranes; pH control strategy.

Introduction

Propionic acid is used in poultry feed and bakery products as a preservative and growth enhancer *(1,2)*. It is also used in the manufacture of cellulose-based plastics, perfumes, herbicides, and pharmaceuticals *(2)*. Propionic acid is normally produced by using petrochemical methods. However, it is well known that some bacteria, especially those belonging to

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the *Propionibacterium*, produce this acid in high concentrations *(2)*. The use of this bacteria to produce propionic acid is attractive since the biomass could be used as a source of vitamin B_{12} *(3)*, as a cheese starter *(4)*, in the silage process *(5)*, and as a probiotic *(6)*. Propionibacteria have been indicated as good propionic acid producers from cheap raw materials such as some types of grass *(7)*, whey *(8–10)*, and sugar cane molasses *(11)*. However, owing to their inhibitory effect on cell growth, low acid concentrations are achieved, making the biotechnological production of propionic acid economically unfeasible *(12)*. For this reason, different systems such as cell recycling *(10,11)*, fibrous bed reactors *(13)*, extractive systems with tertiary amines *(14)* or with active charcoal *(15)*, and membrane-based extractive fermentations *(16,17)* have been developed in order to avoid growth inhibition and increase acid productivity. The amount of vitamin B_{12} produced (intracellularly) is inherently related to the biomass produced, which, as has been mentioned, is inhibited by the propionic acid produced. To overcome this constraint, and to separate the acid for the recovery, we have proposed to remove the acid from the bioreactor on-line by neutralization dialysis. This type of dialysis is based on the exchange of acid anions and hydroxyl ions through anion exchange membranes. Neutralization dialysis effectively transfers carboxylic acids, accumulating the corresponding salts outside the fermenter *(18)*. Furthermore, a new pH control strategy was introduced to obtain higher acid concentrations into the dialysate and thus improve productivity.

Materials and Methods

Culture and Media

The organism used was *Propionibacterium freudenreichii* DSM 20271. The methodology and broths were based on the work performed by Quesada-Chanto et al. *(19)*. Deep agar cultures were incubated at 37°C and stored at 8°C. They were transferred to new agar medium every 3 mo. The medium composition per 1 L of deionized water was 2 g KH_2PO_4, 4 g $(NH_4)_2SO_4$, 10 g yeast extract, 11 g glucose, 15 g agar, 1 mL solution A (containing 2.5 g/L $FeSO_4 \cdot 7H_2O$, 2.5 g/L $MnSO_4 \cdot H_2O$, and 5 g/L $MgSO_4 \cdot 7H_2O$), and 1 mL of solution B (containing 10 g/L NaCl, 10 g/L $CoCl_2 \cdot 6H_2O$, and 10 g/L $CaCl_2 \cdot 2H_2O$). The pH was adjusted to 7.0 with 3*M* NaOH solution before autoclaving. The glucose concentration was increased to 60 g/L for fermentation. The composition of the 1500 mL of feeding solution was 9 g KH_2PO_4, 18 g $(NH_4)_2SO_4$, 45 g yeast extract, 4.5 mL solution A, 4.5 mL solution B, and 297 g glucose.

In the preinoculum phase, cells were transferred to 500-mL Erlenmeyer flasks containing 150 mL of the previously described medium, except agar. The flasks were incubated for 5 d at 37°C without shaking. For the inoculum, 50 mL of this suspension were transferred to 1000-mL bottles with 450 mL of the same medium (a total inoculum volume of 500 mL) and incubated at 37°C for 24 h.

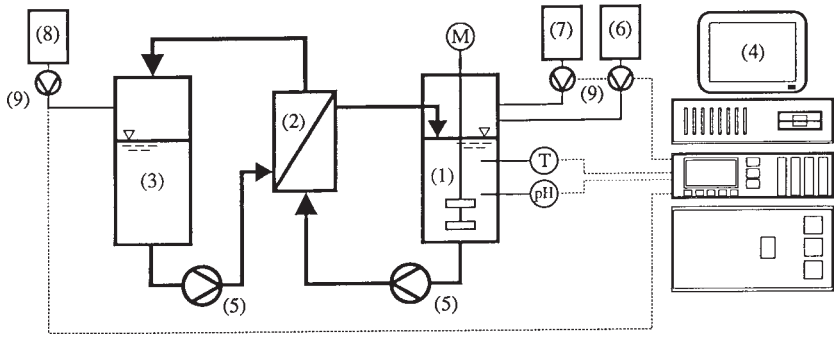

Fig. 1. Flow diagram of the combined fermentation-dialysis process: (1) stirred bioreactor; (2) dialysis module; (3) thermostated dialysate reservoir; (4) bioprocess control system; (5) gear pumps; (6, 8) dosing stations for NaOH; (7) dosing station for H_3PO_4; (9) pH-controlled peristaltic pumps.

Fermentation Process

Fed-batch fermentations were carried out in a 5-L fermentor (MD, B. Braun, Melsungen, Germany). The stirrer was kept at 200 rpm and the reactor temperature at 37°C. A controller system (UBICON, ESD, Hanover, Germany) was used to control the fermentor parameters. The fermentation starting volume was 3 L, including inoculum. The feeding of the reactor was divided into two parts. The first 700 mL were pumped to the reactor after 70 h of fermentation. In 24 h after the first feeding, another 800 mL were fed to the reactor. The dialysis started after 40 h of fermentation. The propionic and acetic acid concentrations in the reactor were 6 and 3 g/L, respectively.

Membrane Module

Two different modules developed for dialysis *(18)* (GKSS, Geesthacht, Germany) were used. The areas available for mass transfer were 0.01 and 0.19 m^2 for the test cell and the "plate and frames" modules, respectively. The area could be increased by the addition of membranes/frames. Although a maximum of about 0.8 m^2 could be reached, such an increase would be limited by the fermentation volume and the pressure of the pump. ADP anion exchange membranes (Solvay, Tavaux, France) were chosen for these experiments. A 5-L solution of 0.05*N* NaOH was used to start the dialysis.

pH Control

Normally, the pH was controlled by pumping 5*N* NaOH into the reactor (Fig. 1; dosing stations 6 and 7 were active). In the second part of this work a new control strategy was carried out. During the first 40 h, the pH was controlled routinely. After the first feeding, dialysis began. Then the pH was controlled by pumping either 10*N* NaOH into the dialysate

Table 1
Concentrations, Yields, and Productivities Achieved by Different Fermentation Processes

Fermentation system	X (g/L)	ΣX (g)	$Y_{X/S}$ (g/g)	P (g/L)	ΣP (g)	P_{inh} (g/L)	Q_X (g/L·h)	Q_P (g/L·h)
Batch[a]	6.5	20	0.12	19	60	14	0.04	0.12
Fed-batch[a]	7.5	36	0.12	20	95	18	0.04	0.10
Fed-batch + dialysis (0.01 m^2)	9.0	40	0.14	25	112	18	0.05	0.13
Fed-batch + dialysis (0.19 m^2)	13.0	62	0.14	11	123	NGI	0.07	0.14
Fed-batch + dialysis (0.19 m^2) + new pH control strategy	15.0	61	0.14	16	120	NGI	0.11	0.22

Note: X, biomass concentration; ΣX, total biomass; $Y_{X/S}$, conversion factor for propionic acid; P, final propionic acid concentration in bioreactor; ΣP, total propionic acid formed; P_{inh}, growth-inhibitory propionic acid concentration; Q_X, biomass productivity; Q_P, propionic acid productivity; NGI, no growth inhibition.

[a]Glucose consumption was not complete.

container (initially containing 0.05*N* NaOH) or 2*N* H_3PO_4 into the reactor (Fig. 1; now dosing stations 7 and 8 were active).

Analytical Methods

Biomass concentration was determined by measuring the optical density (OD_{560}) at 560 nm. Dry biomass was determined by drying the cells after centrifugation (30 min, 10,000*g*) at 80°C for 24 h. Glucose concentration was determined enzymatically (Boehringer Mannheim D-Glucose kit, Boehringer, Mannheim, Germany). Acetic and propionic acids were determined by gas chromatography (column Chromosorb 101, Merck, Darmstadt, Germany). The growth inhibitory propionic acid concentration (P_{inh}) was defined as that concentration of propionic acid at which no further growth of biomass was observed during fermentation.

Results and Discussion

In batch fermentation, 6.5 g/L biomass and 19.2 g/L final propionic acid concentrations were obtained (Table 1). A complete growth inhibition was observed after 72 h when a propionic acid concentration of 14 g/L was achieved (Fig. 2). The propionic acid and acetic acid production continued without cell growth (Fig. 3). Blanc and Goma *(8)* have previously reported this behavior. In the fed-batch fermentation, higher biomass and propionic acid concentrations were produced: 7.5 and 20 g/L, respectively (Table 1). The cell growth was completely inhibited after 140 h when the propionic acid concentration reached 18 g/L (Fig. 2). However, the organic acid production continued after cell growth stopped (Fig. 3). Fed-batch fermenta-

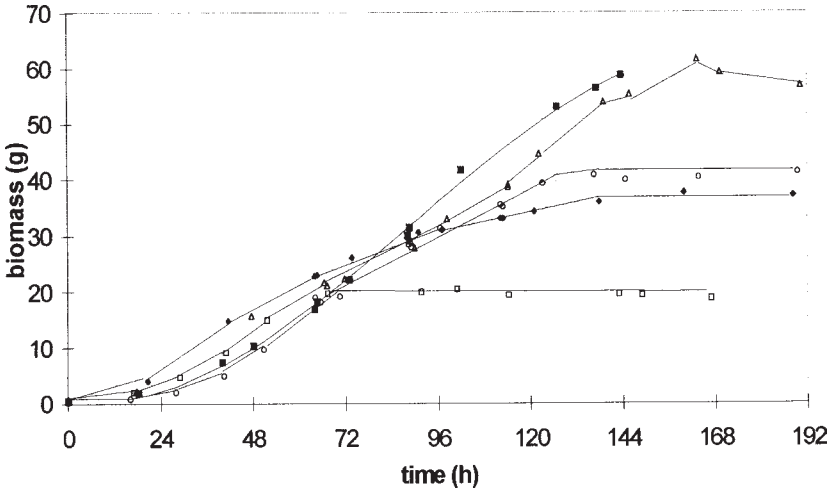

Fig. 2. Total biomass produced in batch (□), fed-batch (◆), and fed-batch with the dialysis module 0.01 m^2 (○), module 0.19 m^2 (△), and module 0.19 m^2 with special pH control (■).

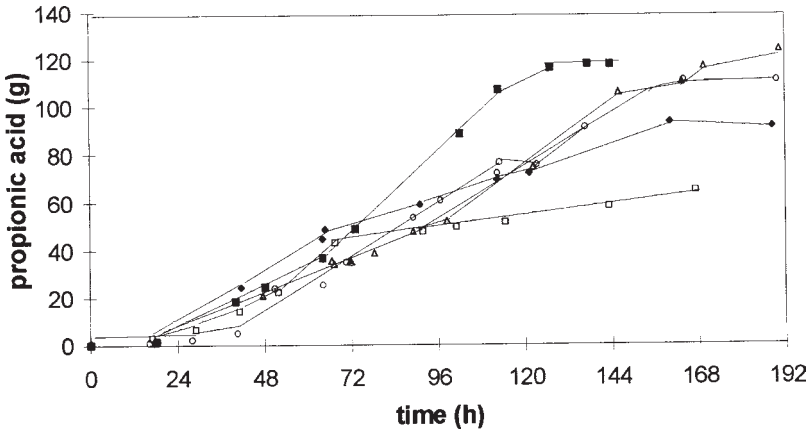

Fig. 3. Total propionic acid mass produced in batch (□), fed-batch (◆), and fed-batch with the dialysis module 0.01 m^2 (○), module 0.19 m^2 (△), and module 0.19 m^2 with special pH control (■).

tions coupled to the dialysis system followed the same feeding strategy. As compared to the normal fed-batch, slightly higher final cell concentrations and productivities with (a) normal fed-batch 7.5 g/L and (b) fed-batch and dialysis (0.01 m^2) 9.0 g/L were achieved with the first membrane module (0.01 m^2) (Table 1). However, the propionic acid reached its inhibitory concentration of 18 g/L and stopped cell growth after 140 h (Fig. 2). To increase the dialysis rate, a membrane module of 0.19 m^2 was used. In this case, no growth inhibitory propionic acid concentration was achieved, and the final cell biomass (13 g/L or 62 g total biomass) and

product productivities were higher (Table 1). Applying a new strategy, the pH was controlled by pumping concentrated NaOH into the dialysate. Then the biomass concentration as well as the acid and biomass productivities increased, showing that such a pH control improved the performance of the process (Table 1). Furthermore, the pH could be maintained at 6.45–6.55 and the fermentation time was reduced by 50 h when compared with the traditional strategies (Figs. 2 and 3). In fed-batch systems, the working volume of the reactor is changed with feeding. For this reason, to compare different processes, the total biomass and propionic acid productions were calculated (concentration × volume). Figures 2 and 3 show these overall productions for the different systems. Fed-batch/dialysis processes showed the best results, especially when the new pH control strategy was used.

In our study, the propionic acid productivity was lower than in cases reported in the literature using *Propionibacterium acidipropionicii* in continuous or fed-batch processes *(11,17)*. However, our process has the advantage that *P. freudenreichii* DSM 20271 is a well-known vitamin B_{12} producer *(20)*; hence, keeping propionic acid below inhibitory concentrations and controlling the pH via dialysate, the cell biomass can be increased, thereby making this process economically attractive for the production of vitamin B_{12}. Also, the fact that organic acids tend to accumulate in the dialysate makes the propionic acid downstream process easier and less expensive. To perform the pH control system, no special devices are necessary, and it can be used easily for similar production systems. This process should be evaluated for higher biomass concentrations in a continuous process to increase the productivities. The vitamin B_{12} content should also be measured under these conditions.

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