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Improvement of Productivity in Acetic Acid Fermentation with *Clostridium thermoaceticum*

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ABSTRACT

Production of acetic acid by a mutant strain of *Clostridium thermoaceticum* was compared in three types of membrane cell-recycle bioreactors. A modified fed-batch bioreactor (where the product is partially removed at the end of fermentation, but the cells are retained), and a two-stage CSTR (with product being removed continuously and the cells being recycled from the second to the first stage) resulted in better performance than a one-stage CSTR or batch fermenter. The difference in performance was greater at higher acetate concentration. With 45 g/L of glucose in the feed, productivity was 0.75–1.12 g/L·h and acetic acid concentrations were 34–38 g/L. This is more than double the batch system. The nutrient supply rate also appeared to have a strong influence on productivity of the microorganism.

Index Entries: *Clostridium thermoaceticum;* calcium magnesium acetate; membrane bioreactor; acetic acid; fermentation.

INTRODUCTION

Acetates have a multitude of industrial uses. Its demand is expected to increase over the next few years because of several new acetate derivatives appearing on the market, among them calcium magnesium acetate (CMA), which is an environment-friendly and noncorrosive alternative to chloride salts for deicing roads in winter (*1*), potassium acetate (PA), which can be used as a heat-exchanger fluid, and sodium acetate, an acidulant in the meat-packing and food industries.

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At present, CMA and PA are produced from petroleum-derived acetic acid. Because of their high cost, their use has been limited. A fermentation route based on renewable resources could be cheaper, since the acetate is not required in high purity. Of the two fermentation routes, the anaerobic process is receiving increasing attention owing to the higher theoretical yields compared to the aerobic (e.g., vinegar) process. The most common organism is *Clostridium thermoaceticum,* which has several attractive features, such as its ability to utilize several sugars and its high fermentation temperatures. However, its main limitations are the relatively low acetate concentrations (~20 g/L) and low productivity (<0.1 g/L·h) produced by the wild strain (*2,3*). Acid-tolerant strains have been developed (*4,5*), as well as strains with tolerance to higher acetate concentration, by gradual adaptation (*6,7*). Mutant strains of *C. thermoaceticum* with tolerance to high concentration of CMA (~100 g/L) have been reported by Ljungdahl et al. (*8*) and our laboratory (*9,10*). Our strain is now available as ATCC 47907 and DSM 6867. Such strains should favorably impact the eonomics of producing acetate by fermentation.

To overcome the limitation of low productivity, the most common approach is to use continuous bioreactors with high cell densities. Immobilized-cell bioreactors (*11,12*) and cell-recycle bioreactors (*13–15*) have been investigated. In most cases, these continuous bioreactors resulted in acetate productivities of 2–8 g/L/h, but at low acetate concentrations of 6–15 g/L. To exploit the full potential of the mutant strain, however, high productivities and high product concentration must be achieved together.

We have been studying the use of membrane-based cell-recycle bioreactors for improving the productivity of fermentations because of the following benefits: high concentration of free cells, excellent mixing (no diffusion limitation and precise pH control), and ease of scale-up. This article reports on studies with modified membrane bioreactors in an attempt to increase both acetate concentration and productivity simultaneously. The following membrane bioreactors were evaluated: a continuous, stirred-tank reactor (CSTR), two-stage CSTR, and fed-batch reactor. The purpose of the membrane was to retain the cells within the bioreactor and to increase cell concentration as needed. These bioreactors were compared to a conventional batch and fed-batch fermenter for their capability to achieve high acetate concentration and high productivity simultaneously.

MATERIALS AND METHODS

Microorganism and Media

A mutant strain of *C. thermoaceticum* (ATCC 49707) was used in this study. The medium used for culture maintenance and fermentation experiments has been described earlier (*7,9,16*). Its composition is shown in

Table 1
Composition of Media (Concentration Expressed in g/L)

Component	For culture maintenance	Fermentation
Glucose	20.0	45.0
Yeast extract	5.0	10.0
$NaHCO_3$	7.5	0.0
KH_2PO_4	7.0	1.4
K_2HPO_4	5.5	1.1
$(NH_4)_2SO_4$	1.0	2.0
$MgSO_47H_2O$	0.25	0.5
$Fe(NH_4)_2(SO_4)_26H_2O$	0.04	0.08
$CoSO_4 \cdot 7.5H_2O$	0.03	0.06
$Na_2WO_42H_2O$	0.0033	0.0066
$Na_2MoO_42H_2O$	0.0024	0.0048
$NiCl_26H_2O$	0.00024	0.00048
$ZnSO_47H_2O$	0.00029	0.00058
Na_2SeO_3	0.000017	0.000034
Cystein	0.25	0.25

Table 1. The culture was maintained in an active state by alternative transfers to nutrient medium (without any acetate) and the medium containing 6% sodium acetate.

Membrane Bioreactor

Fermentation studies were done in a 2-L reaction vessel with 1-L broth volumes. Temperature was controlled at 60°C and pH was maintained at 6–6.2 using 10*N* NaOH. Sterile CO_2 was continuously added in the head space of the fermenter and vented through a 0.45 μm sterile filter. A hollow-fiber ultrafiltration membrane (UFP500-E4, A/G Technology, Needham, MA) was used for cell recycle. This membrane had a 0.033-m^2 area and a mol-wt cutoff of 500,000. The membrane was cleaned using the following procedure:

1. Circulation of 0.2*N* NaOH at 60°C for 30 min;
2. Rinsing with sterile deionized water, followed by;
3. Recirculation of sterile deionized water (with CO_2 sparging) at 75°C for 30 min.

The culture was initially grown in 4-L flasks containing 3 L of broth for 24 h. This culture was concentrated using the membrane to 800 mL and transferred to the fermenter. Concentrated nutrient solution (5X) was added to the culture to bring the total volume of the broth to 1 L. Initially, the fermentation was conducted in a batch mode until the acetate concentration reached 35–40 g/L. Fresh medium (containing 45 g/L glucose and

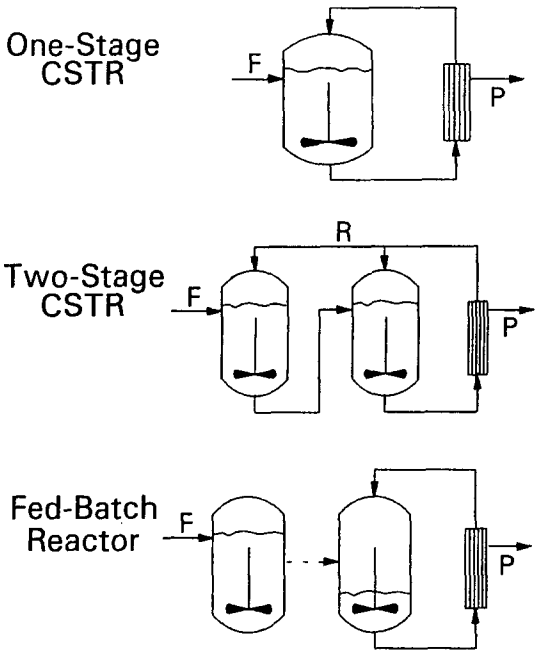

Fig. 1. Schematic of membrane bioreactors.

the nutrients) was then pumped into the system as described below. The concentration of nutrients was the same for continous and fed-batch fermentations (Table 1).

Figure 1 shows schematics of the three reactor systems used in this study. In the one-stage CSTR system, feed solution (F) was pumped in and permeate (P) was pumped out at the same rate to keep the fermentation volume constant. This volume included the reaction vessel contents and the holdup in the membrane module, the pump, and the connecting tubing. Flow rates were adjusted to give dilution rates of 0.020 and 0.033/h (residence times of 50 and 30 h, respectively). Dilution rate is defined as feed-flow rate divided by total volume. The system was operated at constant conditions for at least five reactor volumes before samples were taken. Usually it took only two volume changes to reach a steady state with respect to fermentation parameters.

In the two-stage CSTR system, the membrane module was connected to the second stage. The feed (containing glucose and nutrients) was introduced into the first stage. Broth from the first stage was pumped continously into the second stage at the same rate as the feed into the first stage, to keep the volume in each stage constant at 1 L. The fermentation broth from the second stage was recycled (R) to the first stage at 25% of the feed flow rate (F) to first stage. The dilution rate was based on the total volume of the two stages.

In the fed-batch system, when acetate concentration reached 35–40 g/L, 80% of the broth volume was filtered through the membrane and

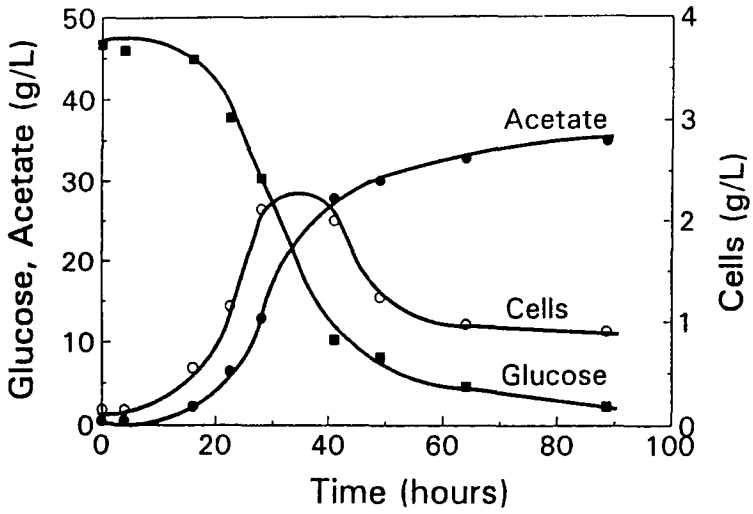

Fig. 2. Batch fermentation of glucose by *C. thermoaceticum.*

cells were recycled. Fresh nutrient solution was added to the fermenter to the original volume, and fermentation was allowed to proceed in batch mode. This procedure was repeated several times. The membrane was cleaned between each cycle as described earlier. Residence time for fed-batch fermentation was determined by dividing the time taken for one cycle by 0.8 because only 80% of reactor volume was removed, e.g., if the cycle was completed in 24 h, residence time was taken as 30 h.

Analytical Methods

Glucose and acetate were analyzed by HPLC using a Bio-Rad (Richmond, CA) Aminex HPX-87H column and RI detector. All results of productivity, concentration, and yield are reported in terms of acetic acid, with a mol wt of 60. Productivity is reported as volumetric productivity (g/L·h) unless otherwise noted. Cell concentration was monitored by measuring optical density at 600 nm. To determine dry weight of the cells, broth samples were centrifuged at 4000 rpm. The supernatant was discarded, and the cell pellet was resuspended in deionized water and centrifuged again. After a total of three such water washes, the cells were dried at 80°C.

RESULTS AND DISCUSSION

Figure 2 shows a typical batch fermentation with this strain of *C. thermoaceticum.* There was usually a lag period before both growth and acetate production commenced. This was followed by a significant drop in cell numbers, as indicated by OD measurements, and a simultaneous decrease in the rate of acetate production.

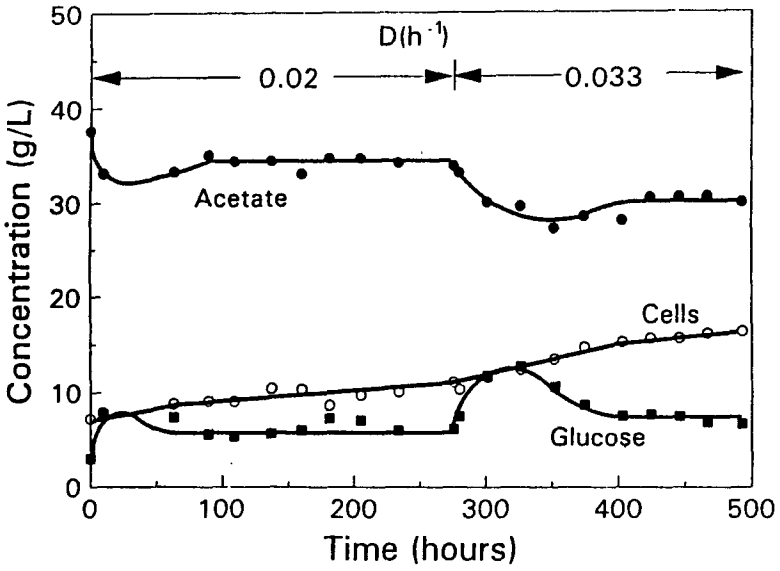

Fig. 3. Continuous fermentation in a one-stage CSTR with cell recycle.

Figure 3 shows the concentration profiles in a one-stage CSTR. With a cell concentration of 9.5 g/L and a dilution rate of 0.020/h, acetate concentration was 34 g/L and productivity was 0.68 g/L·h. When the dilution rate was increased to 0.033/h, acetate concentration decreased to 30 g/L. However, productivity increased to 1.00 g/L·h. The cell concentration increased to 15.5 g/L when dilution rate was raised to 0.033/h. These data confirmed previous reports that high productivity and high product concentration are mutually exclusive in high-rate fermenters, such as the membrane bioreactor (*13*). The yield of acetate was 0.85–0.90 g/g glucose consumed.

The results for the two-stage CSTR are shown in Fig. 4. At a dilution rate of 0.020/h, a final acetate concentration of 37.5 g/L was achieved. This was 10% higher than that obtained in the one-stage CSTR. Productivity was also higher by 10%. Thus, by adding one stage in series, both acetate concentration and productivity could be increased simultaneously. Cell concentrations in first and second stages were 4.5 and 12.5 g/L, respectively. At a dilution rate of 0.033/h, the two-stage CSTR resulted in both acetate concentration and productivity that were 11% higher than the one-stage CSTR.

With the fed-batch bioreactor (Fig. 5), fermentation was usually complete in about 34 h. Since 80% of the volume is removed in each cycle, the effective residence time was 43 h. The acetate concentration was 38.0 g/L with a productivity of 0.93 g/L·h. The acetate concentration was 11% higher, and productivity was 36% higher than the one-stage CSTR and 24% higher than the two-stage CSTR.

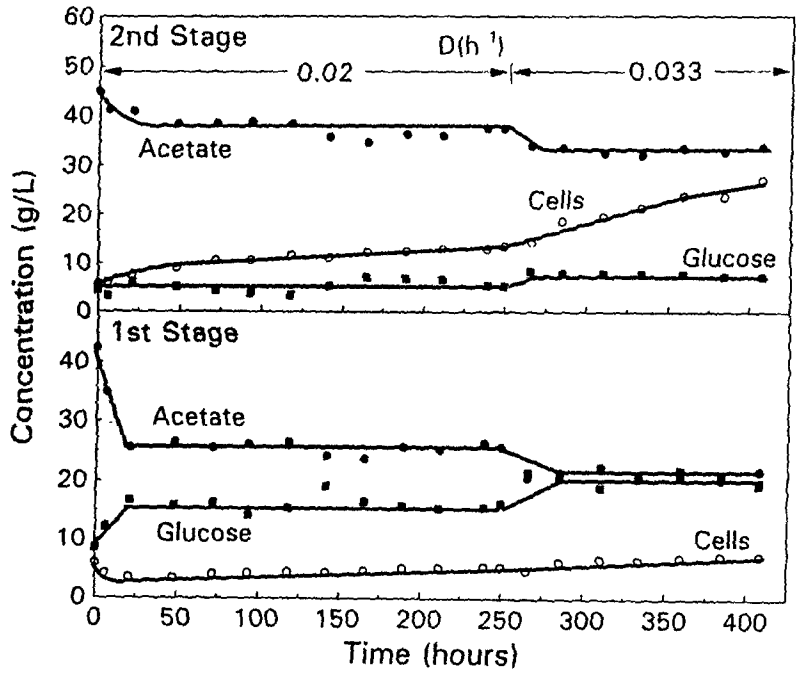

Fig. 4. Continuous fermentation in a two-stage CSTR with cell recycle.

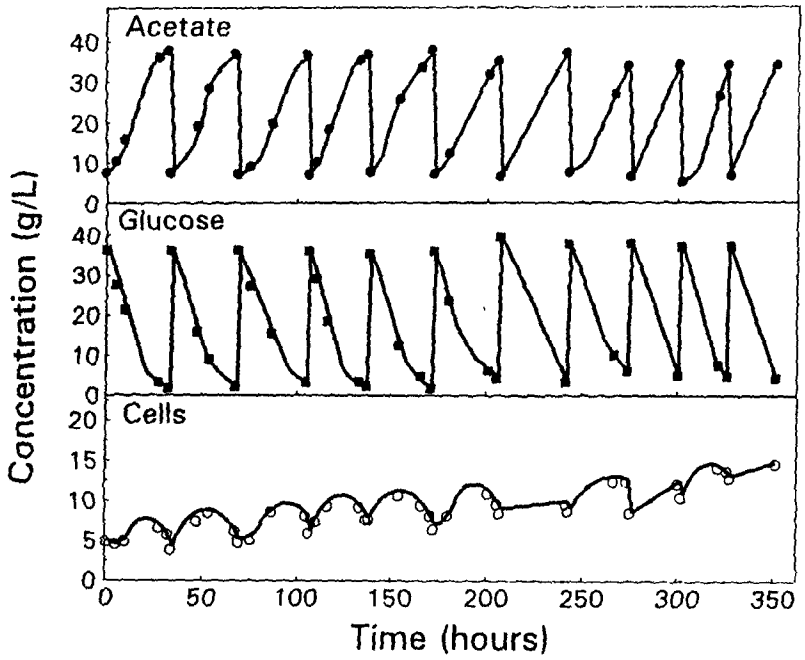

Fig. 5. Fermentation in a fed-batch bioreactor with cell recycle.

The concentration profiles of acetic acid production indicated a lag period in the early phase of the cycle. Also, in each cycle of fed-batch opeation, the cells passed through a growth phase, followed by a significant decrease in OD, probably because of cell lysis caused by nutrient depletion. These characteristics were similar to those observed in conventional batch fermentations (Fig. 1). As a result, in the initial phase of some cycles, acetate productivities were much lower than expected, averaging about 0.88 g/L·h over seven cycles. In the last three cycles, broth was filtered every 24 h (equivalent to 30 h of residence time), and acetate concentration for these three cycles was 34 g/L. The two-stage CSTR with the same residence time (D = 0.033/h) also resulted in acetate concentrations of 33.5 g/L. Thus, at lower acetate concentration, there was no advantage to fed-batch operation compared to the two-stage CSTR. This may be because of the lag phase in the fed-batch reactor, which was not present in the continuous membrane reactors.

The fed-batch reactor would have one drawback when scaled up. For large fermenters, the time to filter 80% of the broth and to refill the fermenter with fresh medium can be significant. This will increase the overall cycle time and lower the effective productivity. Continuous bioreactors would not have this problem.

Influence of Acetic Acid on Productivity

Acetic acid is inhibitory to *C. thermoaceticum* (*17,18*). Therefore, productivity of the microorganism decreases with increase in acetate concentration. When the membrane is operated as a CSTR, acetate concentration in the fermenter is at the highest level, which means that the productivity of the cells is at their lowest level. Therefore, it is not possible to achieve high product concentration and high productivity simultaneously in a CSTR. A plug-flow reactor with high cell density can achieve higher productivity for the same final concentration of acetate. However, such a reactor is impractical for this fermentation because of the need to control pH at a precise value. On the other hand, fed-batch reactors perform similar to plug-flow reactors. At the beginning of the fermentation, acetate concentration and inhibition of the bacteria are lowest. As fermentation proceeds, acetate concentration and inhibition increases. Maximum inhibition occurs only in the final phase of the fermentation. Thus, as shown in this study, the overall productivity in the fed-batch reactor was higher than the CSTR. Similarly, productivity in the first stage of the two-stage CSTR is higher because of lower acetate concentration (Table 2).

Influence of Nutrient Supply Rate on Productivity

Table 2 clearly indicates that product inhibition alone cannot explain the differences in cell-specific productivities at different acetate concentrations. For example, in the one-stage CSTR at a dilution rate of 0.020/h,

Table 2
Acetate Concentration and Productivity Data for Different Bioreactors

Bioreactor	Residence time, h	Cell concentration, g/L	Acetate concentration, g/L	Volumetric productivity, g/L·h	Specific productivity, g/g cell·h
Batch	70	1.1	35.0	0.39	0.370
CSTR	50	9.5	34.0	0.68	0.071
	30	14.5	30.0	1.00	0.064
Two-stage CSTR					
1st stage	20	4.5	25.5	0.90	0.200
	12	6.5	21.0	1.20	0.185
2nd stage	20	12.5	37.5	0.60	0.048
	12	22.0	33.5	1.04	0.047
Overall	50	8.8	37.5	0.75	0.085
	30	14.2	33.5	1.12	0.079
Fed-batch reactor	43	8.0	38.0	0.88	0.110
	30	12.0	33.5	1.12	0.094

acetate concentration was 34.0 g/L, and specific productivity was 0.071 g/g cells·h. The second stage of the two-stage CSTR had a similar acetate concentration of 33.5 g/L, but the dilution rate was 0.033/h and its specific productivity was 0.048 g/g cells·h. If product inhibition was the sole factor in determining the productivity, then specific productivities in both bioreactors would have been similar. The difference in specific productivities at the same acetate concentration suggests that nutrient supply rate also influences specific productivity. Parekh and Cheryan (*19*) have shown that excess nutrients (yeast extract and salts) are needed to maintain productivity of *C. thermoaceticum*. In a one-stage CSTR, all the cells obtain fresh nutrients. In a two-stage CSTR, fresh feed goes only to the first stage, where a portion of the nutrients is used up. When the broth enters the second stage, the lower nutrient concentration would decrease cell viability and productivity. On the other hand, the same nutrient-rich medium used in batch fermentation (without cell recycle) resulted in acetate concentration of 35 g/L in 90 h. The maximum cell concentration and specific productivity were 2 g/L and 0.2–0.5 g/g cells·h, respectively. In the fed-batch reactor with cell recycle, specific productivity was 0.11 g/g cells·h, which is in between the batch and continuous membrane bioreactors. Because more cells have to share the same amount of nutrients, individual cells obtain less nutrients in a membrane bioreactor.

In batch fermentation without cell recycle, acetic acid production is proportional to amount of yeast extract and trace salts supplied in the medium. For all three modes of operation, when dilution rate was increased, volumetric productivity increased, but specific productivity decreased. These results imply that membrane bioreactors need a higher nutrient supply rate, in proportion to cell concentration, to realize the full

potential of the microorganism. Research is under way in our laboratory to determine which component of the nutrient medium has the maximum impact on the productivity of *C. thermoaceticum*.

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