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Production of Acetic Acid by Immobilized Whole Cells of *Clostridium thermoaceticum*

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Abstract

Immobilized cells of *Clostridium thermoaceticum* for acetic acid production has been investigated. Using κ-carrageenan gel as the immobilization-matrix, high cell concentration within the gel could be achieved and thus lead to high volumetric acetic acid productivity. Batch experiments using 3% gel showed that cell concentration up to 65 g (dry cell weight)/L gel could be achieved. These dry weight cell concentrations in the gel through immobilization are typically 10–15 times greater than what can be obtained in free-cell fermentations. The specific growth rate and acetic acid formation rate were similar to those observed for the free cells. Continuous culture experiments using a feed medium containing 20 g/L of glucose were performed where the reactor contained 50% by volume of the carrageenan gel and the pH was controlled at 6.9. Different steady states were acheived at dilution rates ranging from 0.061 to 0.399 h^{-1}. Cells grew mainly near the surface of the gel and reached maximum concentration within the matrix of approximately 35 g/L. Dilution rates much greater than the maximum specific growth rate were obtained, which resulted in volumetric productivity up to 4.9 g/L-h. This value was significantly greater than that for the conventional continuous culture with free cells. Using a 40 g/L feed glucose concentration, steady states could be achieved between dilution rates of 0.12–0.4 h^{-1}. The maximum productivity further increased to 6.9 g/L-h at a dilution rate of 0.37 h^{-1} and at an acetic acid concentration of 19 g/L. The cell concentration was 60 g (dry weight)/L gel at steady state.

Index Entries: Acetic acid, production by immobilized whole cells; *Clostridium thermoaceticum*, acetic acid production by; carrageenan gel, as carrier for *Clostridium thermoaceticum;* immobilized cells, of *Clostridium thermoaceticum*.

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Introduction

The gradual depletion of petroleum fossil fuel has led to progressive increases in the prices of petrochemical feedstocks. This has resulted in a renewal of interest in alternative routes to the production of chemicals. Specifically, the conversion of renewable resources such as carbohydrates and cellulosic biomass to chemicals through fermentation offers alternatives to petroleum feedstocks.

Studies are in progress in our laboratory on a novel route for acetic acid production using a thermophilic anaerobe, *Clostridium thermoaceticum*. The main advantage of this organism is its unique ability to use carbon dioxide as an electron acceptor and to regenerate an additional mole of acetic acid (*1–3*). The maximum theoretical yield is therefore 3 mol acetic acid/mol glucose. Our earlier pH-controlled experiments have confirmed an experimental yield of 2.5 mol acetic acid/mol glucose at an average volumetric productivity of 0.5 g/L-h and a final maximum acetic acid concentration of 56 g/L.

The main objective of this paper is to examine the feasibility of employing immobilized cells to increase the overall volumetric productivity of the fermentation. Until recently, the application of immobilized cells has been limited to resting cell systems for single enzyme reactions and noncofactor linked reactions in degradative pathways. Wada et al. (*4–6*) were among the first to introduce immobilized growing cells and showed that high yeast cell concentration could be obtained in carrageenan gel and lead to higher volumetric productivities. The production of acetic acid by *Clostridium thermoaceticum*, similar to other anaerobic fermentations, is linked to the cell concentration and the growth rate. The accumulation of acetic acid, however, will inhibit cell growth as well as the rate of product formation. The application of immobilized cells to this fermentation could increase the cell concentration in the reactor, and therefore increase the overall productivity.

Materials and Methods

Microorganism and Culture Media

The description of the microorganism, *Clostridium thermoaceticum* (DSM.521-S3), the compositions, and the procedures for the preparation of the media have already been reported (*1*). Only the medium composition will be repeated for completeness and is shown in Table 1.

Immobilization Procedure

κ-Carrageenan (NJAL 724) was a gift from Marine Colloids (Rockland, Maine). For the batch studies, the immobilization procedure was similar to that described by Wada et al. (*4*) and Tosa et al. (*5*). Carrageenan gels (3%) were used with 2% KCl as the gelation agent; the resulting gel was cut into cubes.

For the continuous culture experiments, a modified gel preparation procedure especially useful for thermophilic anaerobe(s) was used. κ-Carrageenan gel was

TABLE 1
Medium Composition for the Growth and the Production of Acetic Acid by *Clostridium Thermoaceticum*

Component	Concentration, g/L
Glucose	Variable
$NaHCO_3$	16.8
K_2HPO_4	7.0
KH_2PO_4	5.5
Yeast extract (Difco)	5.0
Tryptone (Difco)	5.0
$(NH_4)_2SO_4$	1.0
$MgSO_4 \cdot 7H_2O$	9.25
$Co(NO_3) \cdot 6H_2O$	0.029
Sodium thioglycolate	0.5
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.12
$NaMoO_4 \cdot 2H_2O$	0.12
$CaCl_2$	0.016
Resazurin (0.2% solution)	1 mL
Trace salts[a]	1 mL

[a]Trace salts composition in g/L: $MgCl_2 \cdot 4H_2O$ = 5; $NaSeO_3$ = 0.172; H_2BO_3 = 0.01; $ZnCl_2$ = 0.05; $Al_3(SO_4)_2 \cdot 12H_2O$ = 0.1; $NiCl_2 \cdot 6H_2O$ = 0.02; $CuCl_2 \cdot 2H_2O$ = 0.01; EDTA = 5.0.

first dissolved in water, sterilized, and maintained at 60°C under anaerobic conditions with constant sparging of carbon dioxide. The growth medium was prepared separately in the reactor and maintained at room temperature. Cells for immobilization were harvested by centrifugation and placed into phosphate buffer solution. This was then added to the gel solution so that the final gel concentration was 4.5% and the final cell concentration was 0.5 g/L. The gel solution was then added dropwise into the fermentation medium with a pump (Masterflex #7014) through a ⅛″ silicone tubing. The medium was stirred gently using a magnetic bar and under CO_2 atmosphere. The outlet of the tubing was maintained 5 cm above the liquid surface so that the resulting beads were uniformly spherical. The average diameter of the beads was about 5 mm.

The average volume of the beads was determined by placing 30 beads into 5 mL of medium in a graduated cylinder. The volume displaced by the beads could then be readily measured. The average volume of a bead was found to be 0.06 mL.

The cell concentration in the beads was determined as follows. Eight beads were removed from the fermentor for each determination and the beads were dissolved in 5 mL of water at 60°C for 10 min. The optical density at 660 nm was measured using water as a blank. The average value of eight beads was used for each measurement. The values can be doubled checked with pellet protein assays (*7*).

Equipment

Batch experiments were performed either in 500 mL anaerobic flasks or 10 mL Hungate tubes. The flasks or tubes were incubated at 60°C without agitation.

Continuous culture experiments were performed in a 1-L Pyrex fermentor with a 500-mL working volume as shown schematically in Fig. 1. The reactor was immersed in a water bath with the temperature controlled at 58°C using a Haake heater (Model E52, Saddle Brook, NJ). The pH was controlled at 6.95 using 5*N* NaOH through the use of a sterilizable Ingold pH electrode (Ingold Co., Newark, NJ) and an automatic pH controller activating a Masterflex 7014 pump. Feed medium was pumped from the inlet reservoir through a variable speed Masterflex 7013 pump. The volume of the reactor was kept constant through a constant level outlet tube connected to a Masterflex pump for the medium overflow. In order to avoid clogging of the outlet line by the gel, a stainless steel screen was used.

Analytical Procedures

The determination of cell growth and glucose concentration has already been reported (*1*).

Analysis of acetic acid was performed with a Hewlett-Packard 5803A Gas Chromatograph with a dual column flame ionization detector. For 1 mL of appropriately diluted sample, 2 mL of 2*N* hydrochloric acid was added together with 1 mL of propionic acid, the internal standard. A Telfon column (⅛-inch by 8 ft) packed with Chromosorb 101 was used. The following operating conditions were used: column temperature, 175°C (isothermal); injector temperature, 200°C; helium (carrier gas) flow rate, 60 mL/min. The retention times for acetic and propi-

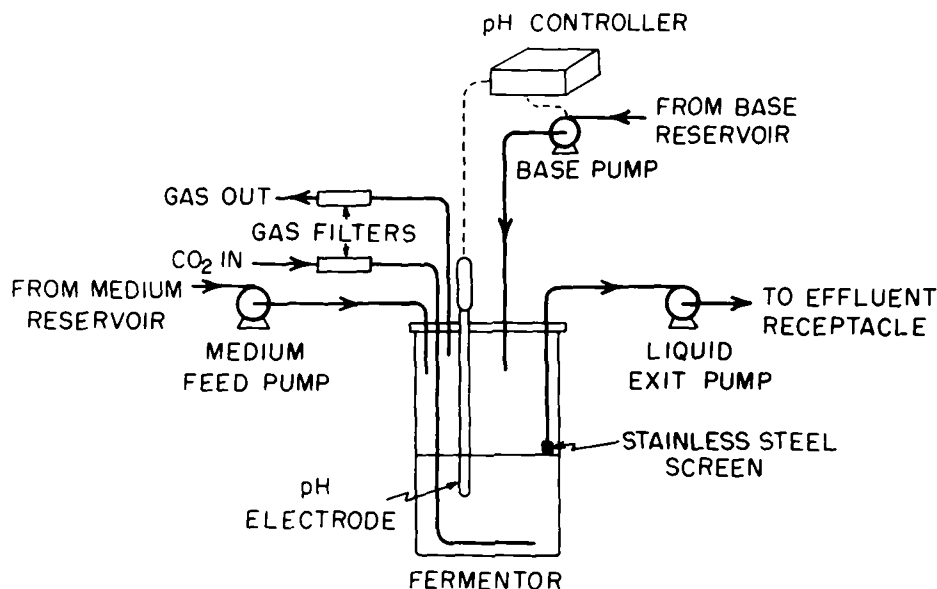

Fig. 1. Schematic diagram of the reactor system for the continuous culture of immobilized cells.

onic acids were 2.12 and 3.43 min, respectively. In order to avoid "tailing and ghosting" of the acetic acid peak, it was necessary to inject three microliters of double distilled formic acid between each sample.

Results and Discussion

Studies with Resting Cells

Initial experiments were performed to determine the necessary information needed for implementing continuous acetic acid production. The ability of resting cells to produce acetic acid was first studied and the results shown in Fig. 2. Cells from the exponential growth phase were harvested from the fermentor, washed, and resuspended into glucose buffer with the pH controlled at 7.0.

The acetic acid production rate was highest initially at 0.5 g/L-h and decreased gradually to zero after about 60 h. The cell mass, measured optically, also de-

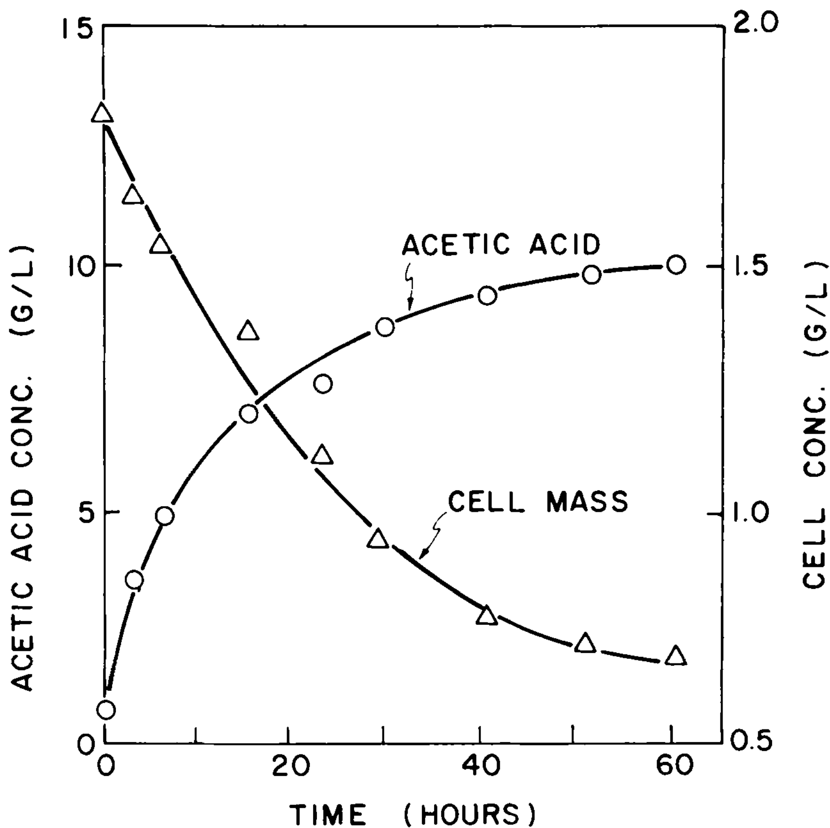

Fig. 2. Production of acetic acid by resting cells of *Clostridium thermoaceticum*. The cells were harvested as described in the text. The composition of the glucose buffer is: glucose, 20 g/L; $NaHCO_3$, 16.8 g/L; KH_2HPO_4, 7 g/L; $MgSO_4 \cdot 7H_2O$, 0.25 g/L. The pH was controlled at 7.0 by addition of sodium hydroxide.

creased throughout the fermentation from an initial value of 1.9 to 0.7 g/L at the end. This decrease of cell mass is thought to be caused by cell lysis. Since the decrease of acetic acid production was greater than the rate of cell disappearance, it is hypothesized that cell inactivation was also responsible for the decreased rate of acetic acid production.

The results from the resting cell experiment suggest that to sustain acetic acid production, growth of the organism might be required. To test this hypothesis, an experiment using chloramphenicol (CAM), a protein synthesis inhibitor, was performed and the results shown in Fig. 3. At a concentration of 200 μg/mL of CAM cell growth was completely inhibited. It can also be seen that inhibition of the acetic acid production also resulted, except for a low basal level of production. However, the basal activity also ceased ater 80 h, although not shown in Fig. 2. The results indicated that cell growth or new protein synthesis is needed for continuous acetic acid production.

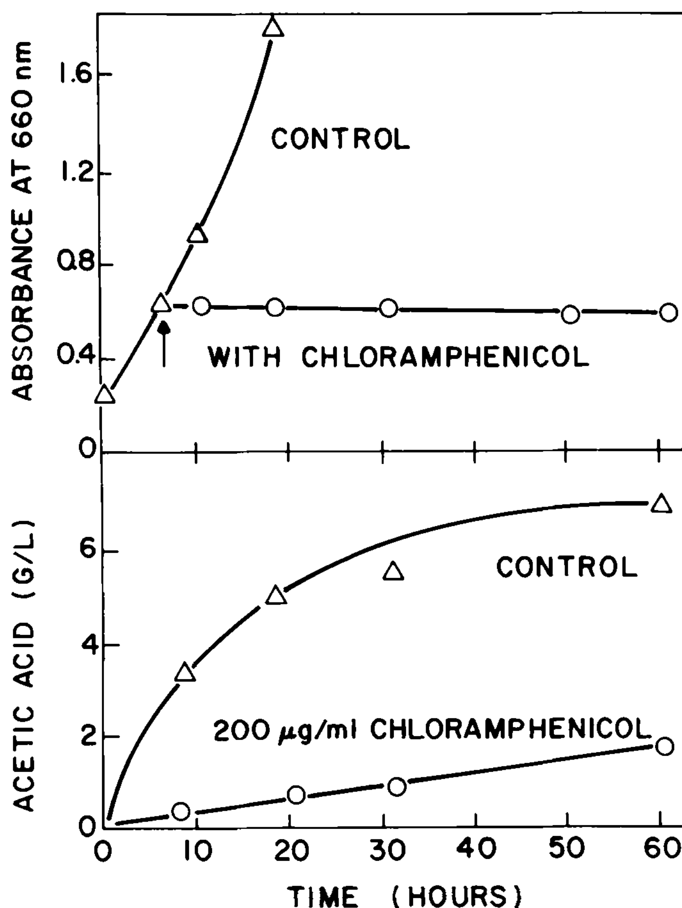

Fig. 3. Effect of chloramphenicol on cell growth and acetic acid production.

Batch Studies with Immobilized-Growing Cells

The batch experiments were performed in 500 mL anaerobic flasks and with cells entrapped in 3% carrageenan gel. A comparison of acetic acid production and cell growth of the immobilized cells in carrageenan gel with that of free cells is shown in Fig. 4. The kinetics of cell growth paralleled those of acetic acid production in both cases. A longer lag period of 5 h was found for the immobilized cells. The maximum acetic acid production rate was 0.8 g/L-h in both cases. The acetic acid concentration after 35 h was 16 g/L for the free cells and 13 g/L for the immobilized cells.

The specific growth rates were also very similar, with a maximum value of $0.14\ h^{-1}$ for the immobilized cells. In this experiment, 30 mL of gel was used in

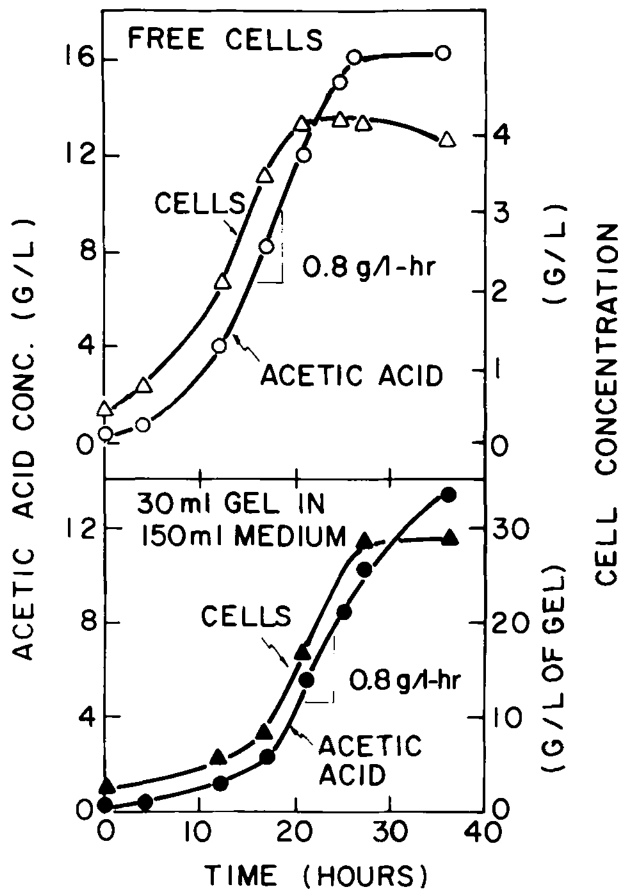

Fig. 4. Comparison of cell growth and acetic acid production by batch cultures of free cells and immobilized cells. The experiment was performed using 500 mL anaerobic flasks with inocula from the same source. For the immobilized cells, cells were entrapped into 30 mL of 3% carrageenan gel as described in the text. The free cells were inoculated directly.

150 mL medium, and all cell growth as found inside the gel reached final concentration of 28 g cells/L gel at the end of the fermentation. Cell growth was distributed uniformly throughout the gel, and no detectable cell growth was observed in the medium.

Continuous Culture Studies with Immobilized Cells

To test the ability of the organism for continuous acetic acid production, a continuous culture experiment was performed. Cells immobilized into the gel (6 mL of gel) were placed into 10 mL of medium with total medium replaced every 6–8 h. The results are shown in Fig. 5. Over a period of 300 h, the acetic acid productivity was constant at 2.2. g/L-h, acetic acid concentration at 14 g/L, and cell concentration at 65 g/L of gel. These results show that through immobilization, significantly higher cell concentrations can be achieved when compared to free-cell fermentations. For example, if one examines the cell concentration using free cells, as shown in Fig. 4, the maximum cell density was 4 g/L. On the other hand, as seen in Fig. 5, when the organism is immobilized the cell concentration within the gel was 65 g/L. This shows a 16-fold increase in the cell concentration that is caused by immobilization.

Additional experiments were initiated using the equipment shown schematically in Fig. 1. These experiments differed from an operational point of view from those experiments shown in Fig. 5 in that we wanted to examine the effect of the dilution rate and inlet glucose concentration on acetic acid productivity. These

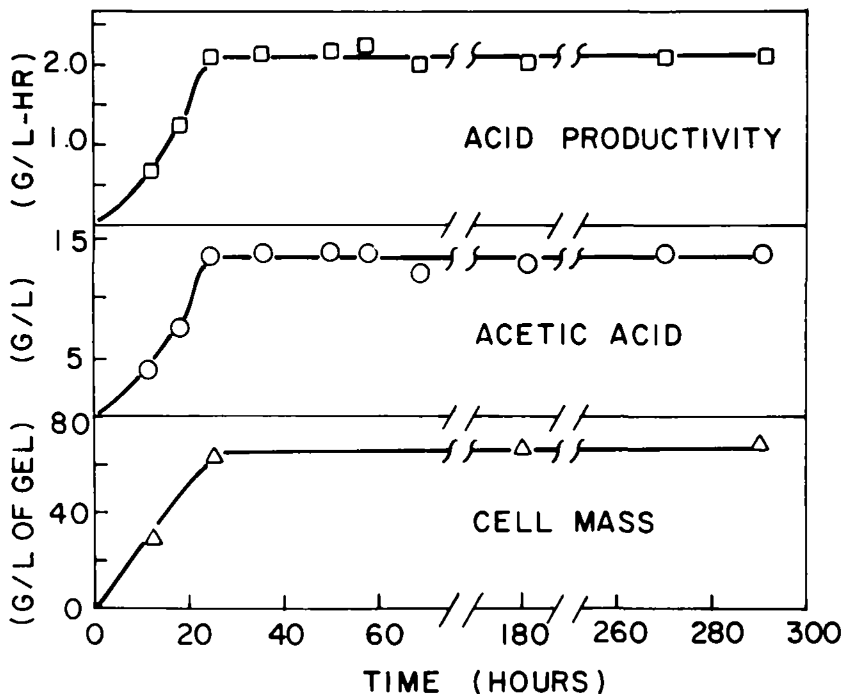

Fig. 5. Batch-fed and withdrawal fermentation of immobilized cells. The experiment was performed in Hungate tubes with 6 mL of gel in 10 mL of medium.

initial experiments were performed using a 3% carrageenan gel where mixing was achieved by a magnetic stirrer during fermentation. After 2 d the gel dissolved and the mean bead diameter decreased from 5 to 2 mm. A slight procedural modification was therefore devised and is presented below.

In order to increase the operational stability of the gel, its concentration was increased to 4.5%, and mixing was achieved during fermentation by CO_2 gas sparged at 250 mL/min. The experiments were performed in a 1-L reactor, as shown in Fig. 1, with 250 mL of medium and 250 mL of the 4.5% carrageenan gel. The experiment was first performed at a low dilution rate of 0.06 h^{-1} and gradually increased to higher values. Steady state was defined when the exit cell concentration (measured optically) or the exit acetic acid concentration varied less than 5% over a period where three times the reactor volume of medium had passed. The dilution rate is defined by the medium flow rate (mL/h) divided by the total reactor volume (gel volume + medium volume, in mL).

The summary of these continuous culture experiments using 20 g/L of inlet glucose is shown in Fig. 6. The acetic acid concentration at steady state was between 15 and 16 g/L at a dilution rate below 0.25 h^{-1} and decreased to 12.2 g/L at a dilution rate of 0.40 h^{-1}. The apparent yield of acetic acid was constant at all dilution rates between 0.80 and 0.84 g acetic acid/g glucose consumed. The acetic acid productivity increased with an increase of the dilution rate from a value of 0.8 g/L-h at 0.062 h^{-1} to 4.9 g/L-h at 0.4 h^{-1}.

The results shown in Fig. 6 were performed over a period of 3 wk. The gel was very stable, and 235 mL of gel was recovered from an initial gel volume of 250 mL at the end of the fermentation. The cell concentration inisde the gel reached only 30–40 g/L of gel and cell growth was observed to be predominantly on the outer layer of the gel. Exit cell concentration was less than 0.9 g/L at all dilution rates.

A second series of immobilized cell experiments were performed using a higher inlet glucose concentration of 40 g/L. In addition to the increased glucose concentration, the yeast extract and tryptone concentrations were both increased to 7.5 g/L. The objectives of these experiments were to determine whether higher steady-state acetic acid concentration and higher volumetric productivity could be obtained using a continuous immobilized cell system. Similar to the earlier experiments, a low dilution rate was first examined with the hope of increasing this value during the course of the experiments.

It was interesting to note that when a low dilution rate was employed, it was not possible to attain a steady state. Some typical results are shown in Fig. 7. The experiments were performed using 250 mL of gel and 250 mL of medium with CO_2 sparging similar to that reported earlier. The cells were first allowed to grow up to a cell concentration of about 60 g/L of gel in batch culture before continuous culture conditions were imposed. It can be seen from Fig. 7 that the experiment was initiated at a dilution rate of 0.1 h^{-1}. After 20 h of operation, the dilution rate was decreased to 0.03 h^{-1}. The acetic acid began to increase from 20 g/L and reaches a maximum value of 30 g/L after about 60 h of experimentation. During this period (20th to 60th h), the free cell concentration in the reactor exit also increased.

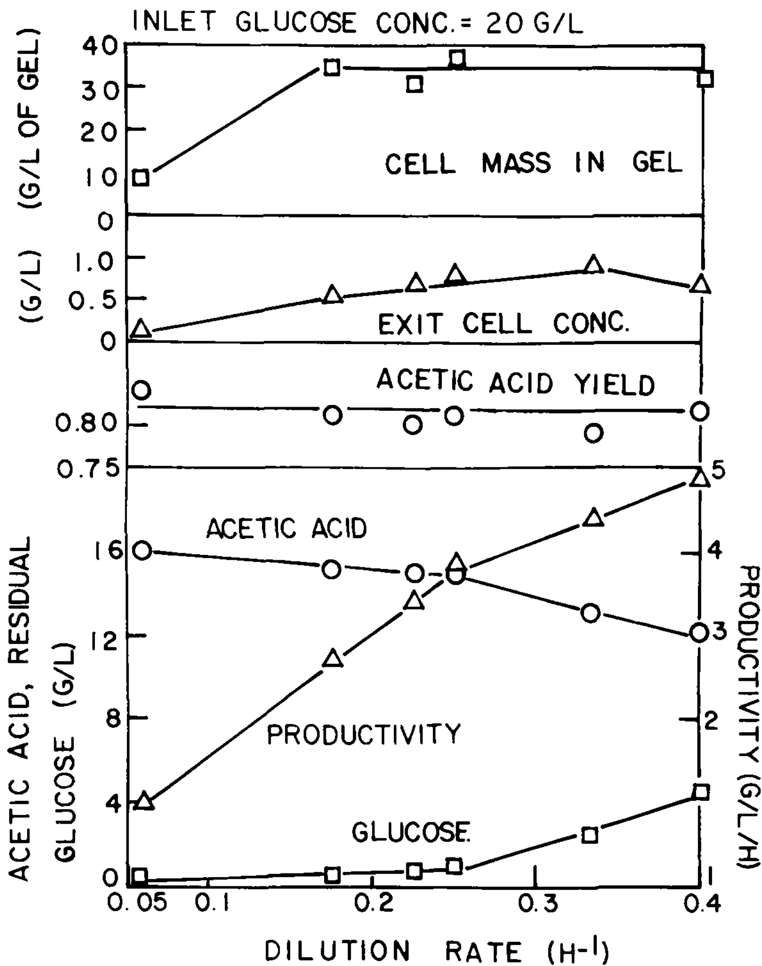

Fig. 6. Continuous culture of immobilized cells (inlet glucose concentration = 20 g/L). The experiment started at the lowest dilution rate, 0.061 h^{-1}, and when steady state was reached, it was stepped up to the next dilution rate.

However, after attaining the maximum acetic acid concentration, one observes a continual decrease in the exit acetic acid concentration. We interpret this behavior to be caused by the inhibition of growth to the organism caused by the high acetic acid concentration. This behavior has been quantitatively assessed in our earlier batch culture fermentations (*1*). Because of the decreasing of the acetic acid concentration with time, the dilution rate was increased at the 110th h to a value of 0.18 h^{-1}.

At higher dilution rates, it was found that steady-states can be attained. It should be mentioned that a slight relaxation of the steady-state criterion was imposed. In the subsequent experiments, steady state was defined as a variation of less than 10% of the exit acetic acid concentration over a period of three volumes (1500 mL) replacement of the medium from the reactor. The results from these continuous immobilized cell experiments are shown in Fig. 8 for dilution rates ranging from 0.11 to 0.40 h^{-1}.

The cell concentration inside the gel reached above 60 g/L, and the exit cell concentration increased to 2 g/L at the higher dilution rates. Glucose concentration in excess of 5 g/L was found in the medium at all dilution rates. The overall product yields were similar to the previous runs, but not shown in Fig. 8. The acetic acid concentration was between 19 and 22 g/L for dilution rates below 0.37 h^{-1} and decreased to 17 g/L at 0.40 h^{-1}. The volumetric productivity also increased with the dilution rate, and the highest volumetric productivity was 6.9 g/L-h at a dilution rate of 0.37 h^{-1}.

Conclusions

The immobilized growing cell reactor has been shown to be an excellent system to achieve internal cell recycle. High cell loading (60 g/L of gel) can be achieved inside the gel. These results show that through immobilization, the cell concentration can be increased significantly as compared with free-cell fermentations. Consequently, dilution rates much greater than the maximum growth rate could be achieved resulting in high overall productivity. The highest volumetric acetic acid productivity achieved was 6.9 g/L-h at a dilution rate of 0.40 h^{-1} at an acetic acid concentration of 19 g/L.

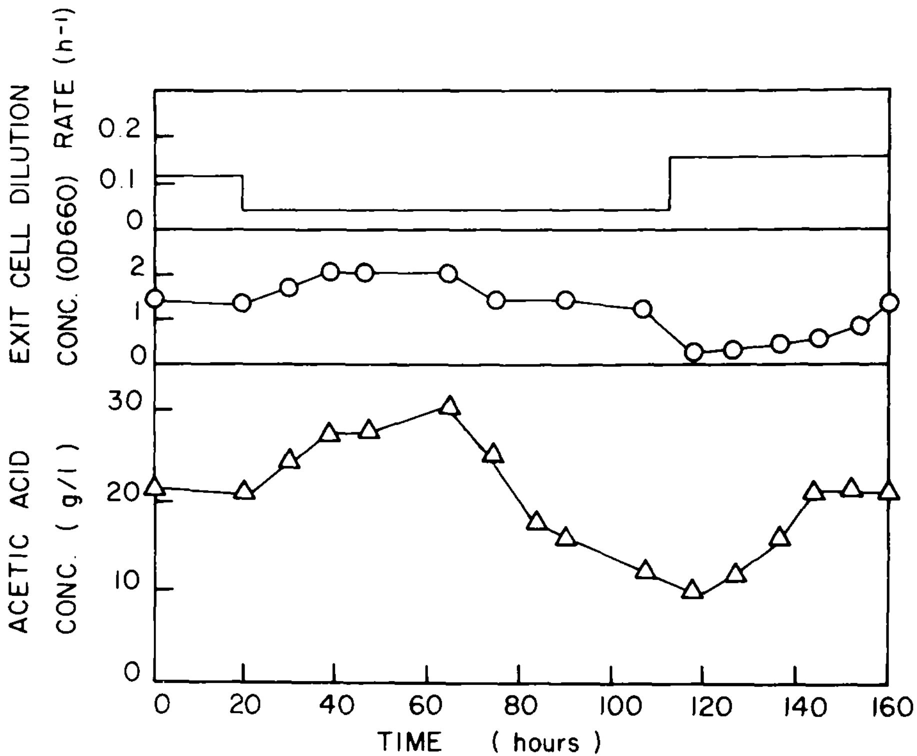

Fig. 7. Behavior of continuous culture of immobilized cells at low dilution rate of 0.03 h^{-1} (inlet glucose concentration = 40 g/L).

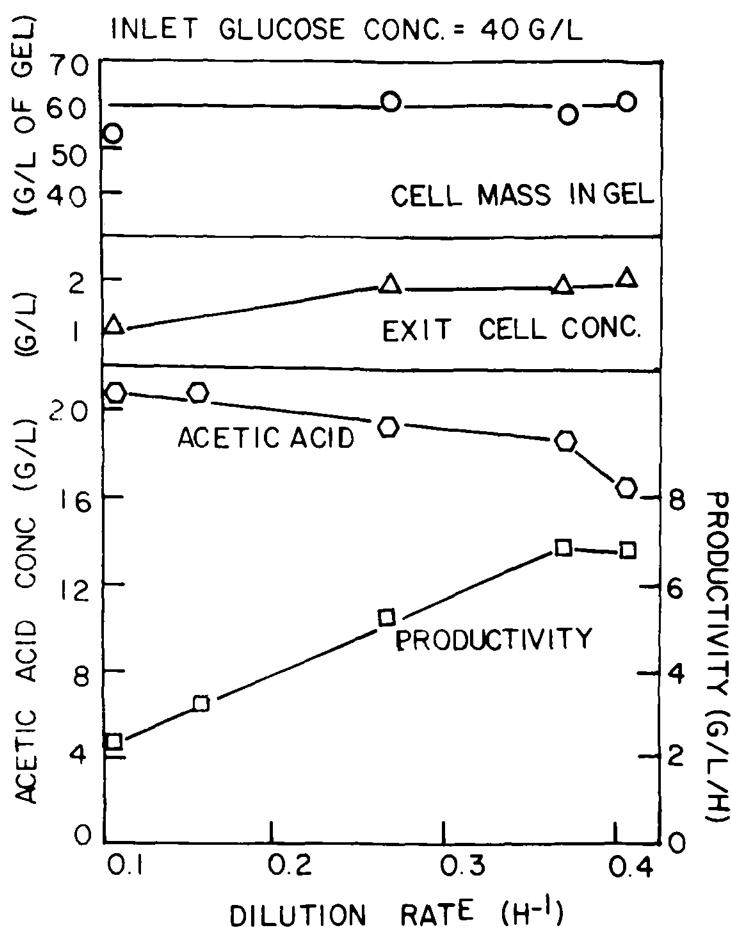

Fig. 8. Continuous culture of immobilized cells (inlet glucose concentration = 40 g/L).

The behavior of cell growth inside the gel for this system is different from that of yeast cell growth previously reported by Wada et al. (*8*). A higher cell loading was obtained, and cells grew inside the gel not only on the outer layer. In this system the high cell concentration inside the gel is the result of physical entrapment of cells rather than better nutrient availability as proposed by the above authors.

Improvement of the reactor system can be obtained from more systematic and fundamental studies on the mechanism of cell release from the gel, and the diffusion of nutrients and products out of the gel.

Acknowledgments

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