Continuous Solvent Production by Immobilized *Clostrictium acetobutylicum*

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INTRODUCTION

As a result of the escalation of oil prices, renewed interest has been generated in the production of fuel and chemicals from renewable resources by fermentative processes (*1–4*). Traditionally, the acetone butanol process has been operated in a batchwise manner with a final concn. of butanol in the range of 13–15 g/L (*5,6*). The conventional batch process suffers from certain disadvantages, such as low productivity, solvent recovery problem, generation of waste cell mass, and so on. To overcome these difficulties, immobilization of cells has been suggested (*7,8*). In the present work, calcium alginate bound ATCC 4259 cells have been used to study some of the basic physiological properties of the immobilized cells.

MATERIALS AND METHODS

Spores were developed by growing the cells in a typical CAB medium containing 4.5% glucose for 60 h. The normal CAB medium has the following composition (expressed in g/L): yeast extract 4; tryptone 1; potassium dihydrogen phosphate 0.7; dipotassium hydrogen phosphate 0.7; DL-aspergine 0.5; $MgSO_4$, $7H_2O$ 0.1; $FeSO_4$, $7H_2O$ 0.015; $MnSO_4$, H_2O 0.1; NaCl 0.1; and resazurin (0.2%), 1 mL. The pH was adjusted to 5.60. The formation of spores was checked microscopically. They were centrifuged out, washed, and then suspended in anaerobic distilled water.

Preparation of the Calcium Alginate Beads

A 4% sodium alginate and a 10% calcium alginate [solution] were sterilized under anaerobic conditions. Ten mL of alginate solution (at 30°C) were mixed with 1 mL of spore suspension. Then, this mixture was extruded through a 23-gage needle into 10% $CaCL_2$ at 5°C. The beads were cured with 1% glutaraldehyde (anaerobic) for at least 2 h at 4°C. The beads were transferred to CAB medium or to column reactor under anaerobic conditions, and heat activated.

Assay of the Products

Glucose was measured by Sigma Diagnostics method by hexokinase. Protein was estimated by Lowry method (*9*). The products of fermentation were measured by Hewlett Packard 5890 gas chromatograph with FID using chromosorb 101 as column packing material. The products could have been assayed by a single injection (190°C) with nitrogen as the carrier gas. Prior to analysis, the samples were acidified to pH 1.0 by phosphoric acid.

Assay of the Hydrogenase Activity

In this experiment, a comparison was made between the total hydrogenase activity of free and immobilized cells. The total hydrogenase activity was determined by tritium exchange method (*10*). The headspace of the anaerobic tubes was filled with H_2/N_2 (2:8). Both the free cell and immobilized cells were grown for 22 h. Then, to the cell and bead suspension, tritium gas was injected. The tubes were shaken at 34°C. At 10-min intervals, samples were withdrawn and radioactivity was measured with a Packard 460 scintilliation counter.

Determination of Cell Viability

This was done by the dye, viz. 2-(*p*-iodo-phenyl)-3-(*p*-nitro-phenyl) -5-phenyl tetrazolium chloride (INT), method (*11–13*). Alginate beads (16 in no.) containing the immobilized cells (16 in no. containing 2.56 mg of dry cells) were contained in an anaerobic vial. Five mL of the INT dye was injected into the vial and incubated in a shaker bath for 150 min at 30°C. Excess INT solution was then removed by syringe. The beads were transferred to an anaerobic vial in a glove box. Fifty mL of acetone was added, and the vial was shaken for 45 min at 30°C to allow complete extraction of the dye. The OD of the extract was measured against an acetone blank. Complete extraction was confirmed when the OD of 2 samples taken at 5-min intervals were the same. The active biomass could be measured from the standard curve. The standard curve was drawn by measuring OD at 490 nm against mg of protein.

Operation of the Immobilized Whole Cell Reactor

It was known earlier that the growth curve of *Cl. acetobutylicum* has 2 distinct phases, viz. acidogenic phase and solventogenic phase (*5,6*). Although the first phase is associated with acid production, in the second phase solvent production starts. In the batch growth of the organisms, the acidogenic phase continues up to 12 h when the solvent production starts wherein the acids are consumed into solvents. It was thought that, by adopting a proper strategy, the immobilized whole cell reactor could be maintained in a solventogenic stage. Furthermore, in the solventogenic stage, cells do not need the original CAB medium. The cells can be maintained in a solventogenic stage by passing only hydrogen (an electron source) and acids (the electron sink).

However, efforts to maintain the immobilized *Cl. acetobutylicum* cells in solventogenic stage proved unsuccessful when natural medium at pH 4.50 was passed for few days. It was observed that cell viability along with solvent production ability dropped. However, stable continuous operation on a glucose medium at pH 5.00 was achieved over a period of several weeks. Thirty-five mL of the beads were packed (anaerobically) into a column having a total volume of 112 mL. When growth was visible in the column, media was starting to be passed at a flow rate of 30 mL/h (residence time = 1.50 h). The temperature of the column was maintained at 34°C. Beads were examined microscopically. The column was operated anaerobically throughout.

RESULTS AND DISCUSSION

Hydrogenase Activities in the Free and Immobilized Cells

The hydrogenase activities of the free and immobilized systems were determined by tritium exchange method. Although there are several methods for determining the hydrogenase activity, this method was chosen because it is based on the interaction of ^3H in the cells. Because of the low mol wt of the tritium, its diffusion rate should not be limiting, and the hydrogenase activity of the immobilized cells can be measured precisely.

The results are shown graphically in Fig. 1. From the figure, the hydrogenase activity of free cells is 1.96×10^3 dpm/min/mg of protein, whereas the corresponding value for the immobilized cells is 1.4×10^3 dpm/min/mg of protein.

Hydrogenase is a key enzyme in the metabolism of *Cl. acetobutylicum*. The lower hydrogenase activity associated with the immobilized cells is

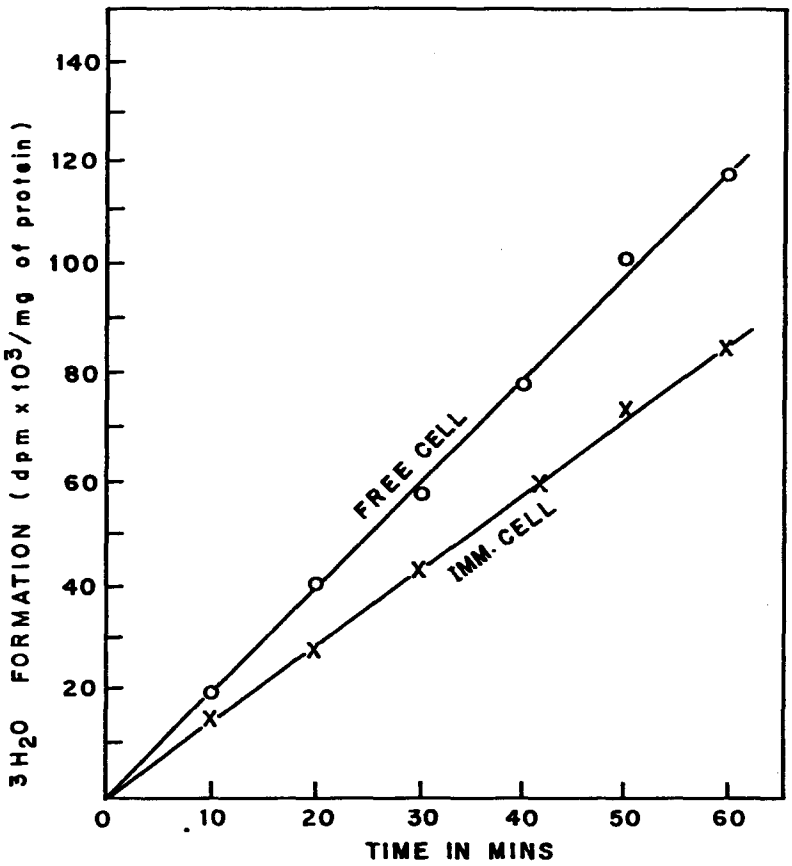

Fig. 1. Comparison of hydrogenase activity of free and immobilized cells of Cl. acetobutylicum by ^3H_2O formation.

possibly the result of different metabolic stages of the cells. The heat-activated immobilized spores take more time to grow than the corresponding free spores. Thus, the free cells may be at a more advanced stage of their growth cycle, which is associated with increased hydrogenase activity.

Operation of Immobilized Whole Cell Reactor

Attempts were made to operate a solventogenic immobilized whole cell reactor by passing CAB medium of pH 4.50. However, cells were found not to be viable after 3–4 d of operation. Even the natural medium (which the organism is expected to see in a batch experiment in solventogenic stage) with a pH 4.5 did not work. Therefore, finally natural CAB medium with a pH of 5.0 was passed in order to run the reactor. The results are presented in Table 1. The reactor was operated under complete anaerobic conditions.

Table 1
Activity of Immobilized *Cl. acetobutylicum* Column with Time

No. of d	Overall condition	pH of the medium	Viability of cells	Butyrate, mm	Acetate, mm	Ethanol, mm	Acetone, mm	Butanol, mm
I	II	III	IV	V	VI	VII	VIII	IX
1	Phase I* med was starting to be passed	5.00	Good	80.11	50.05	16.72	12.89	44.7
2	DO	5.00	Good	71.27	47.81	14.78	16.81	46.13
3	DO	4.80	Good	74.31	40.28	18.73	4.8	54.80
4	DO	4.80	Good	55.33	37.85	10.73	17.26	58.18
5	Phase II medium was introduced with the same flow rate	4.60	Good	65.81	42.89	14.22	18.21	67.17
6	DO	4.60	Good	81.23	56.81	10.27	16.71	34.81
7	DO	4.50	Not good	94	101.9	9.6	3.4	7.23
8	Normal CAB medium with 3% glucose was passed	5.60	Good (improving)	74.31	65.12	8.72	12.81	16.76
9	DO	5.30	Good	76.37	48.12	9.18	16.72	28.19
10	DO	5.30	Good	67.85	45.08	17.89	8.91	35.22
11	DO	5.00	Good	62.81	43.52	19.25	9.24	43.27
12	DO	5.00	Good	57.37	40.69	18.77	10.88	49.12
13	DO	DO	DO	34.21	32.72	19.62	10.28	59.30
14	DO	DO	DO	30.07	26.25	21.78	10.59	62.72
15	DO	DO	DO	26.7	25.8	24.81	9.34	67.89
16	DO	DO	DO	26.26	22.67	26.48	12.82	66.67
17	Normal CAB medium with 3% glucose was passed	5.00	Good	25.6	24.5	25.3	18.74	67.30
18	DO	DO	DO	23.8	27.12	26.42	13.48	65.94
19	DO	DO	DO	24.58	25.18	25.61	12.82	66.89
20	DO	DO	DO	26.14	25.7	24.8	12.5	67.4
21	DO	DO	DO	23.12	26.20	25.32	11.08	67.8
22	DO	DO	DO	23.74	24.18	24.05	11.62	66.25

*The composition of the medium in different phases are as follows:

Medium No.	Glucose, %	Butyrate, m*M*	Acetate, m*M*	Acetone, m*M*	Ethanol, m*M*	Butanol, m*M*
Phase I	3.5%	40	30	7	2.3	10
Phase II	2.25%	37.5	45	12	11	30

On the 21st d, the reactor effluent port became partially plugged, diminishing the liquid flow rate. The problem grew steadily worse, and the reactor was shut down on the 26th d.

On the 26th d, cell viability in the beads was measured by the INT method as described earlier. The INT color production per unit volume of beads was 10% of that observed during active periods of fermentation.

The beads were then cut into thin slices and examined microscopically. The following things were observed:

1. The concentration of the cells was greater near the surface than in the core.
2. The majority of the cells appeared to be in a dormant state, and most of the rest formed spores.
3. The cells were clumped into clusters with voids between the clusters.

Analysis of Table 1 indicates that cell viability was high through 6 d. However, on the 7th d (i.e., after passing the solventogenic medium for 3 d), cell viability decreased. Concomittantly, solvent production was poor. On d 8, normal CAB medium was initiated in order to improve the cell viability and rejuvenate the reactor. Gradually, the pH of the medium was decreased and ultimately, the pH was kept constant at 5.0. Acid production was initially high, but gradually came down. Simultaneously, solvent production went up. The result further indicated that the steady state could be maintained in the reactor from the 14th to the 22nd d. Eventually, the reactor had to be shut down because of operational problems. Thus, long-term continuous operation appears possible.

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