Trans. Nat. Acad. Sci. Technol. 14: 207-218. 1992

CONTINUOUS FERMENTATION OF ACETIC ACID PRODUCTION FROM ETHANOL USING CELLS IMMOBILIZED IN CALCIUM-ALGINATE GEL BEADS

Chay B. Pham and Amaya B. Mata

National Institute of Biotechnology and Applied Microbiology (BIOTECH), University of the Philippines at Los Banos, College, Laguna, 4031, Philippines

ABSTRACT

A continuous process for acetic acid production in a tower bioreactor was developed using ethanol and *Acetobacter* sp. cells immobilized in calcium-alginate gel beads. The optimum initial ethanol concentration was determined. The maximum acetic acid concentration, productivity and fermentation efficiency were achieved at an initial ethanol concentration of 40 g/l and aeration rate of 1.348 vvm.

Increasing the ethanol concentration up to 70 g/l and decreasing the aeration rate up to 0.434 vvm reduced the production of acetic acid

The process was improved with an increase in initial ethanol and acetic acid concentrations using oxygen-enriched air at low aeration rate. The final acetic acid concentration of 3.95% (w/v) was obtained in a continuous fermentation process using 0.1 vvm oxygen-enriched air and 4.2% (w/v) ethanol concentration.

Introduction

The organic chemical acetic acid has versatile utilization as a raw material in pharmaceuticals and food industries. Thus, the bioconversion of ethanol into acetic acid is important to study. Microbial production of acetic acid by a batch fermentation process results in low conversion yield, low productivity and high cost of final products. Two major problems associated with a batch fermentation are substrate inhibition and frequent use of low aeration rate in the batch process. It was constrictive due to the requirement of oxygen for the reaction of the oxidation in microoganism.

Since the development of the submerged culture process in the vinegar industry during the 50's, there has been no major breakthrough in acetic acid fermentation technology (4). A number of processes were developed: a) the Yeomans cavitator used in the US and Japan; b) the Bourgeois process used in Spain and Italy; and c) the Fardon process used mainly in Africa for making malt vinegar. These processes were not used extensively on a commercial scale because of the low oxy-

gen transfer rate. A very low air flow input (0.1 vvm) is usually used to limit evaporation losses (8). To improve the productivity of acetic acid fermentation, several researchers(2,9) have used cell immobilization techniques, but with limited success. Production of vinegar by live cells of *Acetobacter aceti* immobilized on fibrous and other carriers has been studied. Both adsorption and covalent binding techniques were employed for the cell immobilization. In the case of *Acetobacter aceti* immobilized on an adsorbed Ti/Zn hydroxide bed, the oxygen consumption takes place at a rate of 30% of that used by free cells (6).

Recently, a modification of the tower fermentation has been applied to the acetification of alcoholic mash and a volumentric efficiency of up to 1.0 fermentor volume output per day has been achieved (2). The oxidation of ethanol by *Acetobacter* strain is a two-step process: the first is an oxidation of ethanol to acetaldehyde and the second completes the oxidation of acetaldehyde to acetic acid (7) (Equations 1 and 2).

$$C_2H_5OH \xrightarrow[\text{alcohol dehydrogenase}]{[0]} CH_3CHO + H_2O \quad CH_3\ CH\ (OH)_2 \qquad \text{Eq. 1}$$

$$CHP_3CH\ (OH)_2 \xrightarrow[\text{acetaldehyde dehydrogenase}]{[0]} CH_3CO_2H + H_2O \qquad \text{Eq. 2}$$

The effects of ethanol and oxygen concentrations are therefore important in the bioconversion of ethanol into acetic acid(5,8). The objectives of this study were to develop the process of a continuous production of acetic acid from ethanol using *Acetobacter* sp. cells immobilized in calcium-alginate gel beads, and to optimize the ethanol concentration and aeration rate.

Materials and Methods

Materials

Ethanol (95% v/v) was purchased from Janija Trading, Mandaluyong, Metro Manila; all the other chemicals, such as glucose and agar (analytical reagent grade) were obtained from Sigma Chemical Co.

Microorganisms

Five strains of *Acetobacter,* namely: *Acetobacter aceti* GA16, *Acetobacter aceti* 6B1, *Acetobacter sp.* BSP2, *Acetobacter sp.* CALF and *Acetobacter sp.* V film were used in the experiment. Pure cultures of these microoganisms were provided by the BIOTECH Culture Collection.

The strains were subcultured on the agar slants and maintained at refrigerated temperature (4°C).

Inoculum

The composition of inoculum or fermentation medium were as follows (g/l):

K_2HPO_4, 0.1; KH_2PO_4, 0.9; $(NH_4)_2SO_4$, 1.4; $MgSO_4.7H_2O$, 0.25; NaCl, 0.01; $FeSO_4.7H_2O$, 0.01; $MnSO_4.H_2O$, 0.01; yeast extract, 1.0; and ethanol. 40.

A 150 ml inoculum medium was prepared by dissolving the required amounts of all the components, except ethanol, in 142 ml of distilled water. The pH of the medium was adjusted to 6.2 using 10% NaOH solution. The medium was sterilized at 15 psi for 15 min., then cooled to room temperature, and after which 8 ml of 95% (v/v) ethanol was added aseptically. The medium (25 ml) was dispensed into five sterile 250 ml erlenmeyer flasks covered with cotton plugs. A loopful of culture of each of the five *Acetobacter* strains was inoculated into these flasks. The flasks were shaken in a reciprocal shaker at room temperature for 24 hours.

Batch Fermentation of Acetic Acid Production

The batch process was carried out either in an erlenmeyer flask or tower fermentor. In the selection of *Acetobacter* strains, the inoculum (15 ml) was inoculated into a sterile 500 ml erlenmeyer flask containing 150 ml fermentation medium and shaken in the reciprocal shaker at room temperature for three days.

For comparison with the continuous fermentation process, the batch fermentation of acetic acid by free cells of *Acetobacter sp.* strain was conducted using the tower fermentor. The fermentation conditions used in batch process are the same as in the continuous process, except that the fermentation time was five days.

Continuous Fermentation of Acetic Acid

Immobilized process

The cells from the 24 h inoculum culture of *Acetobacter sp.* were harvested by centrifugation at 3000 rpm and 5°C for 20 minutes. They were well mixed with 2% (w/v) of sterilized soldium alginate.

The solution was added dropwise to a gently stirred 0.2M $CaCl_2$ solution using a microtube peristaltic pump. The spherical gel beads were collected by decanting the $CaCl_2$ solution.

Experimental set-up

Figure 1 illustrates the schematic diagram for the continuous production of acetic acid. The cell-gel beads were transferred aseptically to the fermentor. The fermentation medium was continuously fed to the bottom of the fermentor by a microtube peristaltic pump while filtered air was supplied through a sparger. The product was overflowed and collected by a product vessel. The gases left the fermentor

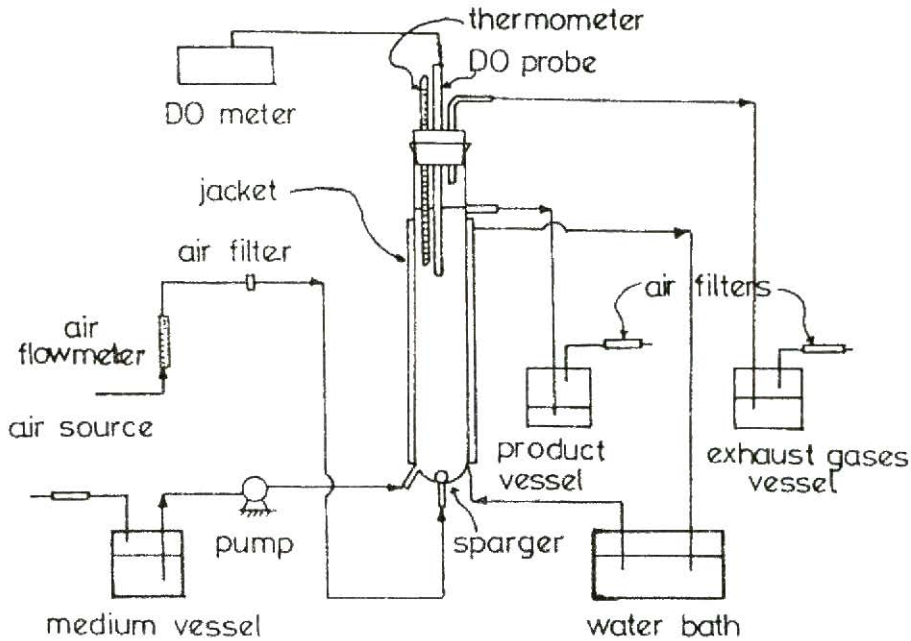

Figure 1. Schematic diagram of the experimental set-up.

through the outlet at the top of the column and were passed into an absorbing vessel containing distilled water. The temperature inside the fermentor was maintained by a jacket through which water from a water bath circulated, while the dissolved oxygen (DO) concentration in the fermentation broth was measured by a digital DO meter.

Studies on initial ethanol concentration

Three values of ethanol concentration were used: 40, 55 and 71 g/l. The aeration rate used in three experiments was 1.348 vvm and the dilution rate was 0.03 h^{-1} at 30°C.

Studies on aeration rate

The effects of aeration on the continuous fermentation of acetic acid were studied. The aeration rates used were 1.348 vvm, 0.842 vvm, and 0.434 vvm. The initial ethanol concentration was 40 g/l and the dilution rate was 0.03 h^{-1} at 30°C.

Studies on oxgyen-enriched air

The DO concentration of fermentation broth was kept at about 8.2 ppm by the DO controller which monitored intermittently the addition of industrial grade oxygen into the fermentor. The continuous fermentation was carried out at 40 g/l ethanol concentration, at 30°C and aeration rate of 0.1 vvm.

Analytical Methods

Ethanol determination

Ethanol concentration was determined by gas chromatography using a Shimadzu Model GC-7A gas chromatograph. The glass column was packed with Porapak Q (80-120 mesh) and the injection and oven temperatures were 200°C and 180°C, respectively. Isopropanol solution was used as the internal standard.

Acetic Acid determination

The concentration of acetic acid in the products was determined by titration method using 0.05 M NaOH solution as a standard solution with 10% phenolphthalein indicator (1).

pH determination

The pH of the products was measured with a digital pH meter.

Results and Discussion

Comparison of the Ability of Acetobacter Strains for Acetic Acid Production

Figure 2 shows the relative performance of the five *Acetobacter* strains in terms of acetic acid production by batch fermentation in shake flasks. Results show that *Acetobacter sp.* V film obtained the highest final acetic acid concentration (8.34 g/l), followed by *Acetobacter sp.* CALF (5.85 g/l) and *Acetobacter sp* BSP2 (3.10 g/l). *Acetobacter aceti* 6A16 and *Acetobacter aceti* 6B1 yielded the lowest final acetic acid concentration of 0.64 g/l.

It can be seen that *Acetobacter sp.* V film achieved the highest conversion ratio (20.85%) followed by *Acetobacter sp.* CALF (14.62%) and *Acetobacter sp.* BSP2 (7.75%). Therefore, *Acetobacter sp.* V film was the most suitable microorganism to use in this experiment.

Effect of Initial Ethanol Concentration on the Continuous Acetic Acid Fermentation

The acetic acid, ethanol and D. O. concentrations and pH in the fermentation broth were obtained in a continuous fermentation process at an initial ethanol concentration of 40 g/l and aeration rate of 1.348 vvm at 30°C (Figure 3). The maximum acetic acid concentration obtained was 18.5 g/l after eight days of fermentation. The ethanol concentration decreased from 40 g/l to 5.5 g/l, while pH of fermentation broth also decreased from 6.2 to 3.

It is important to note that the D.O. concentration was almost depleted, indicating that the aeration rate was not adequate for the oxidation reaction to convert the ethanol into acetic acid by the *Acetobacter sp.* strain.

The effect of ethanol concentration on the acetic acid production was optimized in the continuous fermentation process. The initial ethanol concentra-

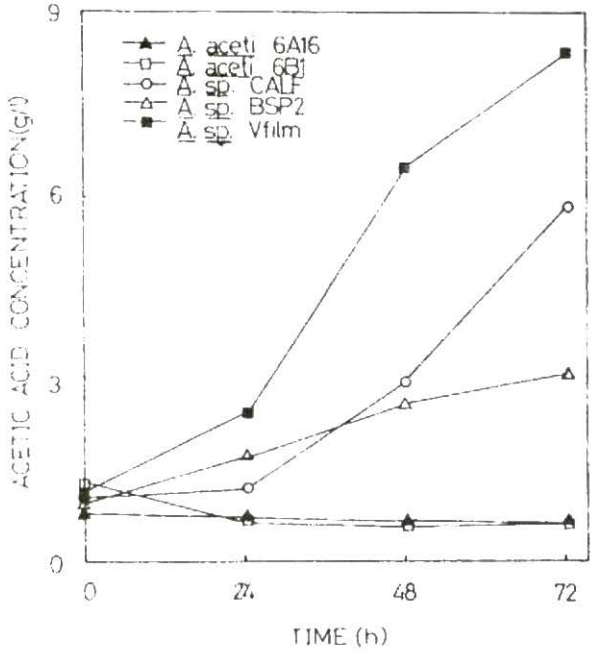

Figure 2. Batch fermentation of acetic acid by different strains of Acetobacter.

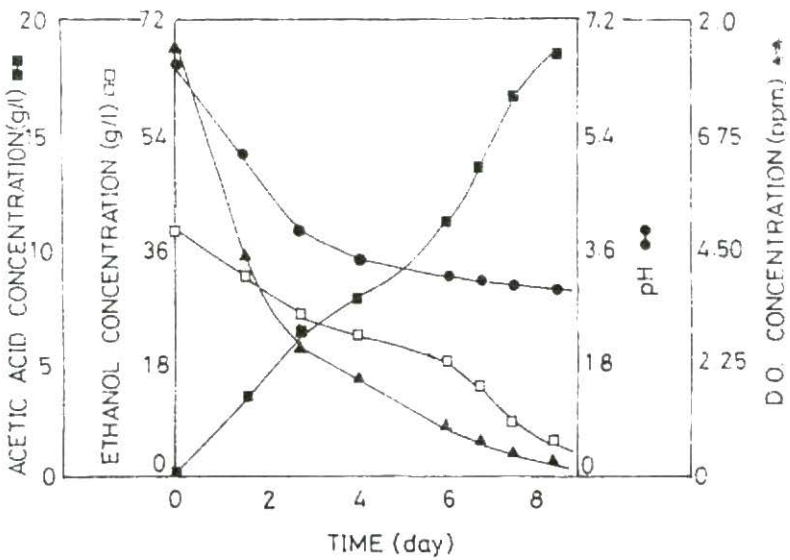

Figure 3. Continuous fermentation of acetic acid at an initial ethanol concentration of 40g/l and aeration rate of 1.348 VVm.

tion of 40 g/l gave the highest acetic acid concentration as compared to those of 55 g/l and 71 g/l initial ethanol concentrations (Figure 4), possibly owing to the occurrence of substrate inhibition of acetic acid production at high initial ethanol concentrations. These findings are similar to the previous reports (8) that ethanol up to about 40 g/l concentration showed no significant inhibitory effect on acetic acid production. Therefore, 40 g/l initial ethanol concentration is the highest ethanol concentration that can be tolerated by *Acetobacter sp.* V film.

Effect of Aeration Rate on Continuous Acetic Acid Production

Figure 5 shows the acetic acid produced in the continuous fermentation using 40 g/l initial ethanol concentration at different aeration rates. The highest acetic acid concentration of 18.5 g/l was achieved using an aeration rate of 1.348 vvm, while the lowest concentration of 5.41 g/l was obtained at an aeration rate of 0.434 vvm. It can be concluded that the decrease of aeration rate also decreased the production of acetic acid in the fermentor.

For mass balance at steady state of the continuous fermentation, ethanol evaporation was calculated for each experiment (Table 1)

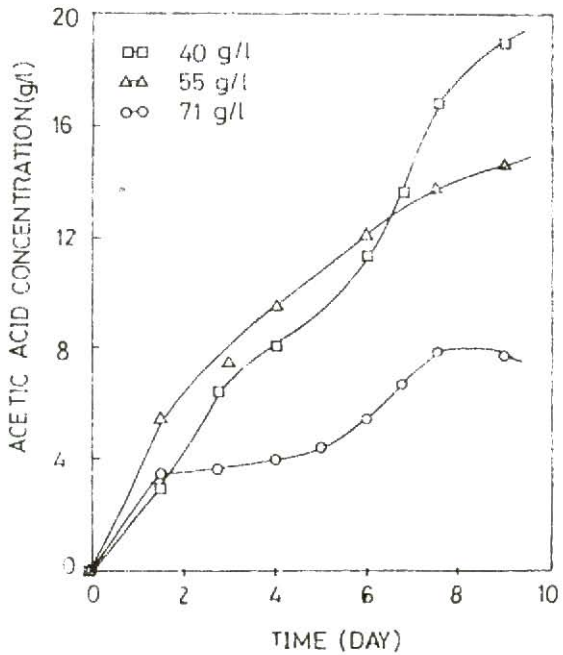

Figure 4. Continuous fermentation of acetic acid at different ethanol concentration at aeration ratio of 1.348 ppm

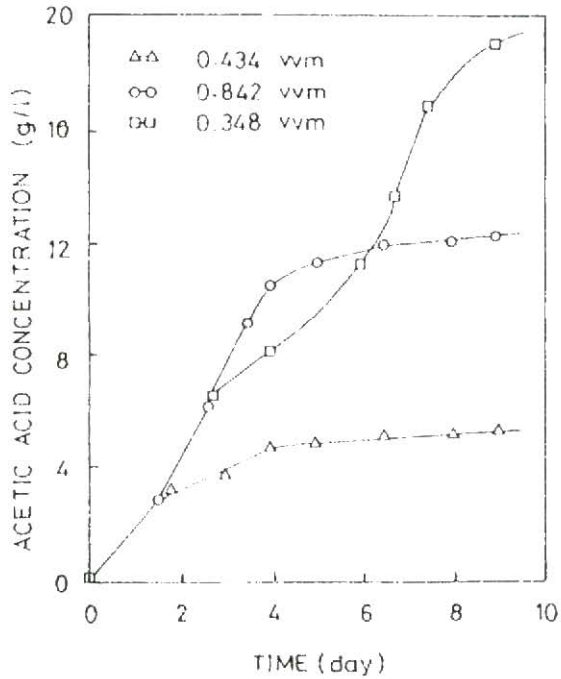

Figure 5. Continuous fermentation of acetic acid at different aeration rates at 40 g/l ethanol concentration

Table 1. Rate of ethanol evaporation and percent loss of ethanol at different aeration rates[1]

Aeration Rate (vvm)	*Rate of Ethanol Evaporation (g/h)*	*% Loss of Ethanol*
1.348	0.14	50
0.842	0.09	32
0.434	0.08	28

[1]Fermentation conditions: initial ethanol concentration, 40 g/l; dilution rate, 0.03 h^{-1}; temperature, 30°C.

The rate of ethanol evapoaration and the percent loss of ethanol were highest at an aeration rate of 1.348 vvm. Despite the significant losses of ethanol at 1.348 vvm, the highest acetic acid concentration was achieved at this aeration rate. It could be due to the greater availability of dissolved oxygen in the fermentation broth at higher aeration rates. As seen in Figure 2, the concentrations of dissolved oxygen in the culture aerated at 1.348 vvm were relatively higher than those in the culture aerated

at 0.842 vvm and 0.434 vvm. This implies that the available oxygen in the fermentation broth is related to the conversion of ethanol into acetic acid. Hence, a higher aeration rate is more favorable for acetic acid formation.

The calculation of productivity and fermentation efficiency achieved in all the continuous experiments are tabulated in Table 2.

The definition of the productivity and fermentation efficiency is as follows:

Let S_i = initial ethanol concentration, g/l
S_t = ethanol concentration at time, t
P_i = initial acetic acid concentration, g/l
P_t = acetic acid concentration at time, t

$$Y_{p/s} = \frac{P_t - P_i}{S_i - S_t} = \frac{P}{S} \qquad \text{Eq. 3}$$

where $Y_{p/s}$ = stoichiometric product yield

$$\text{Fermentation efficiency (\%)} = \frac{\text{Actual } Y_{p/s}}{\text{Theoretical } Y_{p/s}} \times 100 \qquad \text{Eq. 4}$$

Mass balance of acetic acid in the fermentor:

$$r_p \text{ total} = r_p + D(P_i - P_t) \qquad \text{Eq. 5}$$

Where r_p = rate of acetic acid formation or productivity, $g/l^{-1}h^{-1}$

At steady rate, r_p total = 0

Equation 5 becomes:

$$r_p + D(P_i - P_t) = 0 \qquad \text{Eq. 6}$$

or

$$r_p = D(P_t - P_i) \qquad \text{Eq. 7}$$

Using the values calculated from Equations 4 and 7 as criteria for evaluation, the continuous fermentation of acetic acid yielded a final acetic acid concentration of 18.47 g/l, productivity of 0.55 $g/l^{-1}h^{-1}$ and fermentation efficiency of 41.54%. These values are comparatively lower than those reported in previous researches. Zhou and Li(13) studied the continuous fermentation of acetic acid at 30°C using immobilized

Table 2. Productivity and fermentation efficiency of acetic acid production in continuous process.[1]

S_1 (g/l)	Aeration rate (vvm)	S_t (g/l)	P_i (g/l)	P_t (g/l)	$(g.l^{-1}h^{-1})$	Fermentation Efficiency (%)
40	1.348	6.08	0.00	18.47	0.55	41.54
55	1.348	31.99	0.75	9.44	0.28	20.23
71	1.348	43.22	0.47	7.33	0.22	19.23
40	0.842	19.90	0.00	7.54	0.29	36.15
40	0.434	25.00	0.87	5.41	0.16	23.08

[1]Culture conditions: dilution rate 0.03 h^{-1}; temperature, 30°C.

Acetobacter rancens As 41 in sodium alginate. They obtained a maximum acetic acid concentration of 42.6 g/l and a fermentation efficiency of 80%.

The difference of the results might be attributed to several factors, such as microoganism strains and DO level concentrations used in the fermentation broth

Acetic Acid Fermentation Using Oxygen-Enriched Air

The improvement of a process for the continuous fermentation of acetic acid production by immobilized cells-calcium alginate gel beads was performed using oxygen-enriched air (Fig. 6). The acetic acid concentration reached 39.5 g/l and ethanol concentration was almost consumed. The DO concentration of 8.2 ppm was kept constant during the continuous fermentation process. The pH of fermentation broth decreased from 6.2 to 3.2 after six days of fermentation and then remained constant up to the end of the fermentation process. This indicates that the oxygen concentration plays a very important role in the bioconversion of ethanol into acetic acid using immobilized cells in calcium gel beads.

The fermentation conditions used in the continuous fermentation process were also applied in the batch fermentation using free cells. Figure 7 shows results of the batch fermentation of acetic acid using ethanol and free cells of *Acetobacter sp.* V film in oxygen-enriched air at 0.1 vvm. The maximal acetic acid concentration of 39.2 g/l was obtained after five days of fermentation.

The batch process using free cells and the continuous process immobilized cells in calcium-alginate gel beads for acetic acid production were compared. The productivity was 1.185 $g/l^{-1}h^{-1}$ for the continuous fermentation process and the batch process, respectively. The fermentation efficiency was also higher in the continuous process (76.2%) than in the batch process (75.3%).

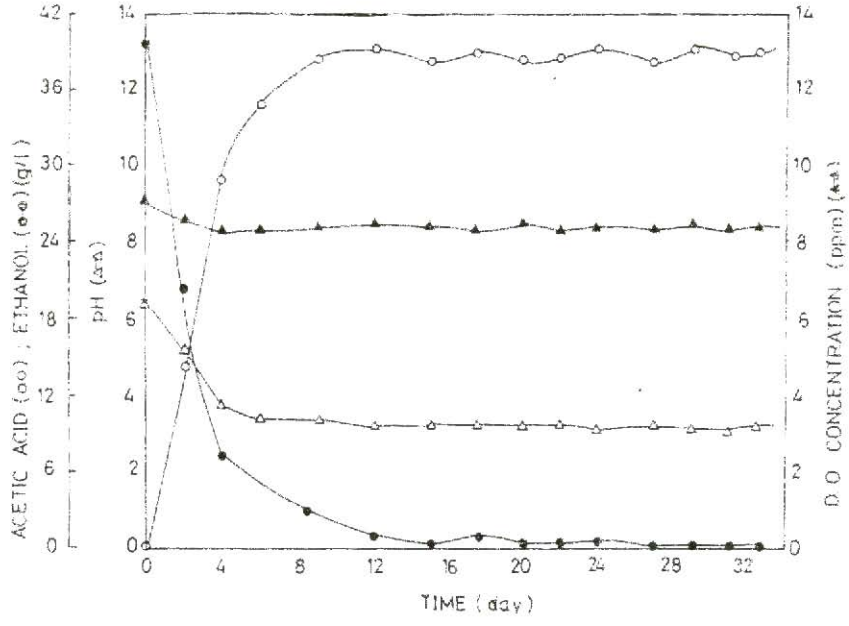

Figure 6. Continuous fermentation of acetic acid using ethanol and Acetobactor sp cells immobilized in calcium-alginate beads in oxygen-enriched air at 0.1 vvm

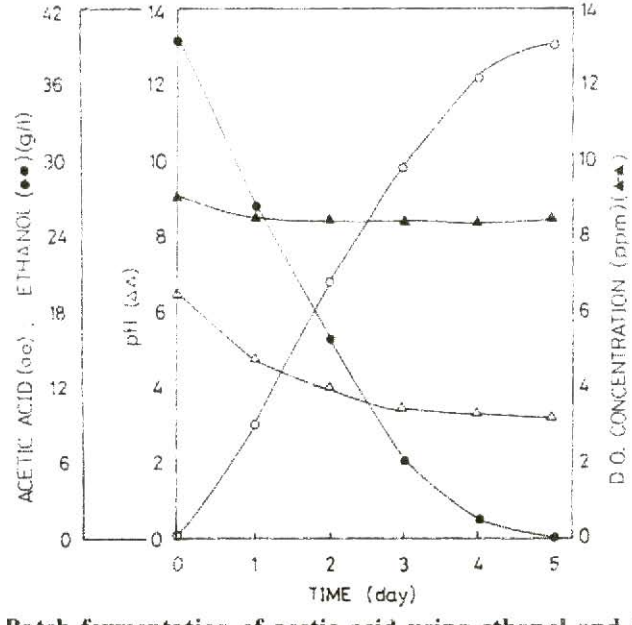

Figure 7. Batch fermentation of acetic acid using ethanol and free cells of Acetobacter sp. in oxygen-enriched air at 0.1 vvm.

Conclusion

The oxidative reaction of *Acetobacter sp.* to convert ethanol into acetic acid required an optimum of 40-45 g/l ethanol concentration and oxygen-enriched air at 0.1 vvm. The process development could be scaled to pilot scale production. The product was clear. It needs no application of the filtration or centrifugation process because the microbial cells are immobilized in calcium-alginate gel beads in the continuous fermentation process.

References

1. AOAC. 1980. Official Methods of Analysis, 13th Ed. Association of Official Analytical Chemists, Washington, D.C.
2. Brown, W. V. 1963. Acetic acid production from ethanol mash. *Chemical Engineering Progress* 59: 10-18.
3. Chattapodhyay, P. K. 1980. Master Technology Thesis. BERC, III Delhi, India.
4. Ghommidh, C., J. M. Navarro and G. Durand. 1981. Acetic acid production by immobilized actinobacter cells. *Biotechnology Letters.* 3:93-98.
5. Ghommidh, C., J. M. Navarro and G. Durand. 1982. A study of acetic acid production by immobilized *Acetobacter* cells-oxygen transfer. *Biotechnology Bioengineering,* 24:605-617.
6. Kennedy, J. F., S. A. Barker and J. D. Humphrey. 1976. Microbial cells living immobilized on metal hydroxides. *Nature* 261:242-244.
7. Levonenmunoz, E. and M. D. Cabezudo. 1981. Influence of oxygen-transfer rate on vinegar production by *Acetobacter aceti* in submerged fermentation. *Biotechnology Letters* 3:27-32.
8. Mori, A. and G. Terui. 1978. Kinetic studies on submerged acetic acid fermentation process. *Journal of Fermentation Technology* 50: 776-781.
9. Nakayama, T. 1959. Studies on acetic acid bacteria, I. Biochemical studies on ethanol oxidation. *Biochemistry Journal* 46: 1217-1225.
10. Nickol, G. B. 1979. In Microbial Technology. ed. H. J. Peppler and D. Perlman. Academic, New York, Vol. 2, p. 155-174.
11. Prescott, S. C. and C. G. Dunn. 1959. Industrial Microbiology, McGraw-Hill, Kogakusha, Tokyo, pp. 428-492.
12. Tyagi, R. D. and T. K. Ghose. 1982. Studies on immobilized *Saccharomyces cerevisiae.* I. Analysis of continuous rapid ethanol fermentation in an immobilized cell reactor. Biotechnology Bioengineering. 24: 781-795.
13. Thous and Li. 1989. Immobilized *Acetobacter* for production of acetic acid. *Zhonqquo Nianqzao.* 2: 22-24.