Journal of Microbiology and Biotechnology
Vol. 7. No. 1, 62-67, 1997

Characterization of Ethanol Fermentation Using Alginate Immobilized Thermotolerant Yeast Cells

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To enhance the hyperproductive and low energy-consuming ethanol fermentation rate, the thermotolerant yeast *S. cerevisiae* RA-74-2 cells were immobilized. An efficient immobilization condition was proved to be 1.5% (w/v) alginate solution, neutral pH and 20 h activation of beads. The fermentation characteristics and stability at various temperatures were examined as compared with free *S. cerevisiae* RA-74-2 cells. The immobilized cells had excellent fermentation rate at the range of pH 3~7 at 30~42°C in 15~20% glucose media. When the seed volume was adjusted to 0.12 (v/v) (6 ml bead/50 ml medium), 11% (w/v) ethanol was produced during the first 34 h and 12.15% (w/v) ethanol [95% (w/v) of theoretical yield] during the first 60 h in 25% glucose medium. In repetitive fermentation using a 2 litre fermentor, 5.79-7.27% (w/v) ethanol [76-95% (w/v) of theoretical yield] was produced during the 40~55 h in 15% glucose media. These data suggested the fact that alginate beads of thermotolerant *S. cerevisiae* RA-74-2 cells would contribute to economic and hyperproductive ethanol fermentation at high temperature.

Ethanol has recently attracted much attention as alternative fuel (8, 13). However, the road to commercialization of fuel ethanol requires a hyperproductive process and an efficient ethanol-producing yeast. Conventional batch production of ethanol is not economical due to its low productivity (10). High cell density rapid fermentation (HCDRF) could be used for efficient and hyperproductive ethanol fermentation because more viable cells in the reactor contribute to increased substrate turnover rate. HCDRF methods can be classified into many types, such as cell recycled fermentation (11), immobilized cell fermentation (16), flocculant yeast fermentation (19), hollow fiber bioreactor (12), etc. Among them, recent advances in immobilized cell technology have suggested new methods for producing biochemicals and biofuels such as ethanol. The industrial application of immobilized cells for ethanol fermentation has not yet been demonstrated, but immobilized cell fermentation processes have advantages such as high productivity, simplified operation, lower labour and seed requirements (6). Use of thermotolerant ethanol-producing yeasts could prevent contamination during the fermentation period, and ensure that produced ethanol was easily recovered and minimize the amount of cooling and energy required (9, 17, 18). Therefore, ethanol fermentation by immobilized thermotolerant yeast would bring about economical and efficient production of fuel ethanol. Despite these assets, there are surprisingly few publications on high-temperature immobilized cell fermentation.

In this study, *S. cerevisiae* RA-74-2 cells were used for high-temperature immobilized cell fermentation of HCDRF. The carrier used was sodium alginate, which forms gels with divalent ions like calcium. The gel forming properties are also correlated with the proportion and length of the blocks of contiguous L-guluronic acid residue in the polymeric chains. Galazzo *et al.* (4) reported that alginate-grown cells induce cell alterations which accelerate glucose conversion to ethanol. The objective of the present investigation was to study the effect of temperature and pH on the productivity and stability of *S. cerevisiae* RA-74-2 cells immobilized for economical ethanol fermentation.

MATERIALS AND METHODS

Microorganism and Media

The thermotolerant *S. cerevisiae* RA-74-2 cells as previously reported (17, 18) were used for high-temperature immobilized cell fermentation. The YPD medium for cell cultivation was composed of glucose 20 g/l, yeast ex-

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Key words: thermotolerant yeast, *S. cerevisiae* cells, immobilized fermentation

tract 5 g/l, and polypeptone 5 g/l. The composition of medium for ethanol fermentation was glucose 150~200 g/l and yeast extract 5 g/l. $CaCl_2$ 1.4 g/l was added at 1.4 g/l of fermentation medium as required in repetitive fermentation. A potato dextrose agar (PDA) medium was used for preserving the strain at 4°C.

Immobilization of Cells in Alginates

The yeast was cultivated at 37°C for 18~22 h in YPD liquid medium and collected by centrifugation (4,000×g for 5 min). The collected cells were washed twice with distilled water. Sodium alginate (Junsei Chemical Co. Japan) was used as a carrier in this experiment. 5 ml of cell suspension (1 g dry cell weight/5 ml distilled water) was mixed with 95 ml of 1.5% (w/v) alginate. The mixture was dropped through a syringe into 50 mM $CaCl_2$ solution, forming beads 2.8-3.1 mm in diameter. The beads were allowed to cure at 4°C for 1 h, and were then rinsed with distilled water and equilibrated overnight in 50 mM $CaCl_2$ solution at 4°C until used. The microcapsules were prepared according to the methods of Cheong *et al* (3).

Activation of Beads

The beads prepared for fermentation were activated at 37°C for 20 h in YPD medium. Agitation speed during the activation was adjusted to 100 rpm.

Ethanol Fermentation

Fermentation was carried out in 250 ml flasks and 2 litre jar fermentors at various temperatures. Other conditions of fermentation were the same as previously reported (18).

Analytical Methods

Glucose concentration was measured using the Somogyi-Nelson method. Ethanol content was determined by gas chromatography (HP-1 Hewlett Packard: crosslinked methyl silicone gum; FID; 25 m×0.2 mm×0.11 μm; oven temp, 50°C; Injector 150°C; Detector, 180°C; H_2 gas, 35 ml/min; Aux gas N_2, 20 ml/min) and alcohol hydrometer. Free cell concentrations of the fermentation broths were measured by Optical Density (OD) and converted to Dry Cell Weight (D.C.W). For biomass assay in beads, 10 beads were withdrawn and washed twice with saline solution. Subsequently, the beads were submerged in 10 ml of 0.2 M Sörensen's citrate-sodium citrate buffer (pH 5.0) and agitated in an ice bath until the alginate beads were dissolved (7). This liquefied alginate cell suspension was then examined for total cell counts using haemacytometer.

RESULTS

Immobilization of Thermotolerant Yeast Cells

Optimum concentration of gel matrix and activation. The carrier concentration used was 1~3% (w/v) alginate. Microencapsulation, more load to cell and less dead zone formation as compared to bead formation, was the preferred immobilization method for *S. cerevisiae* ATCC 24858, but was not suitable for industrial ethanol-producing strains and this strain, *S. cerevisiae* RA-74-2. Because high temperature conditions and rapid carbon dioxide production rates in initial period broke the microcapsule, bead formation was rather suitable for *S. cerevisiae* RA-74-2 cells. Beads of 1% (w/v) alginate did not have enough rigidity and 3% (w/v) alginate beads did not allow sufficiently making for high viscosity. In addition the increased alginate concentration will reduce the average pore size (20). As shown in Table 1, 1.5% (w/v) alginate was found to be the optimum carrier concentration for efficient fermentation, rigidity and easy production. The initial cell count for beads was 9.61×10^7 cells/bead. After 20 h in YPD, cell counts were increased to 1.57×10^8 cells/bead and maintained to prolong the culture. This result suggests that 20 h were sufficient to activate beads of *S. cerevisiae* RA-74-2 cells in YPD medium (Table 2).

Effect of pH on immobilization. To investigate the effect of pH on bead preparation, thermotolerant yeast cell suspensions were mixed with various pH of 1.5% (w/v) alginate solution. The problem did not occur at pH 6~8 in both bead formation and high-temperature ethanol fermentation. Beads were not formed above pH 9 and below pH 5.0 as previous reported (7). Therefore, we used 1.5% alginate solution without pH adjustment (data not shown).

Table 1. Effect of carrier concentration on immobilized cell fermentation at 40°C.

Alginate concentration (%)	Ferm. time (h)	Ethanol. conc. %(w/v)	Free cell (g/l)	Ep (g/l.h)	Yp/s
1.0	12	6.656	0.30	5.55	0.408
1.5	12	6.926	0.13	5.77	0.422
2.0	12	6.783	0.14	5.65	0.416
2.5	12	6.815	0.13	5.68	0.408
3.0	12	6.656	0.12	5.55	0.414

Abbreviation: Ferm. time, Fermentation time; Ep, Ethanol productivity; Ethanol. conc, Ethanol concentration; Yp/s, Ethanol yield (g-Ethanol/g-consumed glucose). Fermentation was conducted in a 250 ml flask with 50 ml broth containing glucose 175 g/l, yeast extract 5 g/l. Agitation speed was adjusted to 100 rpm.

Table 2. Effect of activation time on cell growth to immobilized *Saccharomyces cerevisiae* RA-74-2 cells in 1.5% (w/v) alginate beads at 40°C.

Time (h)	0	10	20	30
Cell numbers (/bead)	9.6×10^7	1.12×10^8	1.57×10^8	1.55×10^8

Cell numbers were measured by haemacytometer after dissolving the beads.

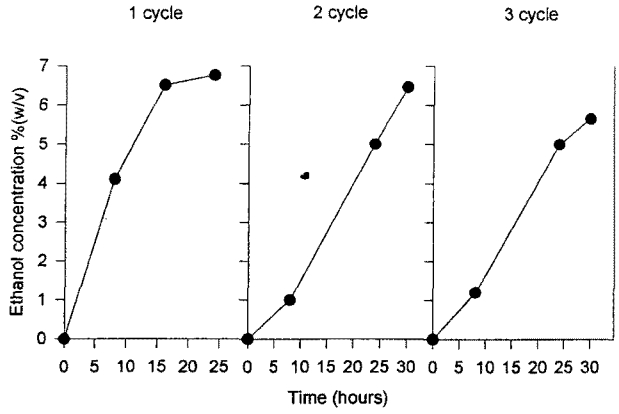

Fig. 1. Stability of the immobilized RA-74-2 cells.
Repetitive fermentation was carried out in a 250 ml flask with 50 ml fermentation broth at 40°C. The media composition was glucose 175 g/l, yeast extract 5 g/l and $CaCl_2$ 1/4 g/l.

Stability of beads. The beads were examined for stability and rigidity by repetitive fermentation at 40°C. The results are shown in Fig. 1. The beads showed stable fermentation and rigidity at 40°C. However, as the high-temperature fermentation was repeated, ethanol productivity decreased. These results suggest that the yeasts in beads were damaged by high concentration of ethanol at 40°C and intrinsic viscosity of alginate gel matrix decreased below the pH 4 because pH changes of culture media were concomitant with fermentation proceeding (20). Since the time of cell activation in second fermentation was longer than that of initial fermentation, the concentration of produced ethanol and final pH should be considered efficient for ethanol fermentation at high temperature (Fig. 1).

Fermentation Conditions of Immobilized Thermotolerant Yeast Cells

Optimum seed volume in fermentation. To investigate the effect of seed volume, 0.04, 0.08, 0.12 and 0.16 (ml bead/ml medium) samples were tested at 40°C for 42 h in 250 ml flasks with 50 ml fermentation broth. The results are shown in Fig. 2. As seed volume was increased, initial fermentation rate was also increased. Because the final fermentation ratio using 0.16 (v/v) was similar to that of 0.12 (v/v), 0.12 (ml bead/ml medium) was chosen for optimum seed volume.

Effect of initial pH of media on high-temperature fermentation. The effect of pH on ethanol production with free and immobilized cells was examined. Fermentation was conducted for 40 h at 40°C in 17.5% glucose medium. Seed volume was adjusted to 0.12 (v/v). The fermentation ratio at pH 4-7 was similar to that of free and immobilized cells. At pH 3, immobilized cells showed 83% fermentation ratio, but free cells showed only 66% (Fig. 3). These results suggest that alginate gel matrix have negative charge and has a physicochemical barrier effect against hydrogen ions as previous reported

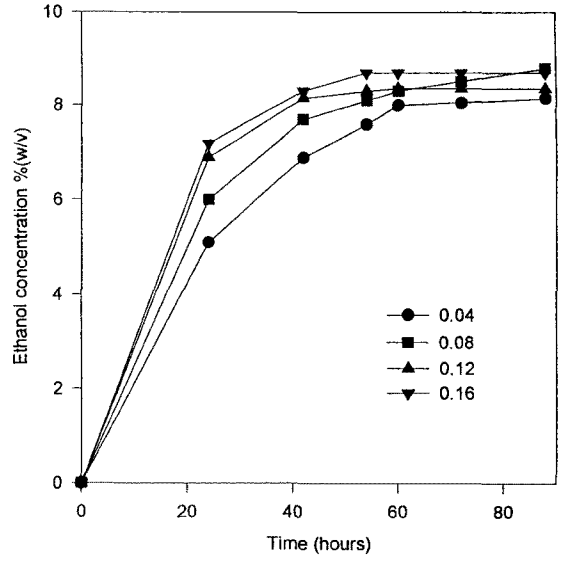

Fig. 2. Effect of seed volume on high temperature-immobilized cell fermentation.
Fermentation was conducted in a 250 ml flask with 50 ml fermentation broth at 40°C. The media composition was glucose 200 g/l, yeast extract 5 g/l. The seed volume was expressed as the bead volume/medium volume ratio. 0.04 (v/v), 2 ml bead/50 ml medium; 0.08 (v/v), 4 ml bead/50 ml medium; 0.12 (v/v), 6 ml bead/50 ml medium; 0.16 (v/v), 8 ml bead/50 ml medium.

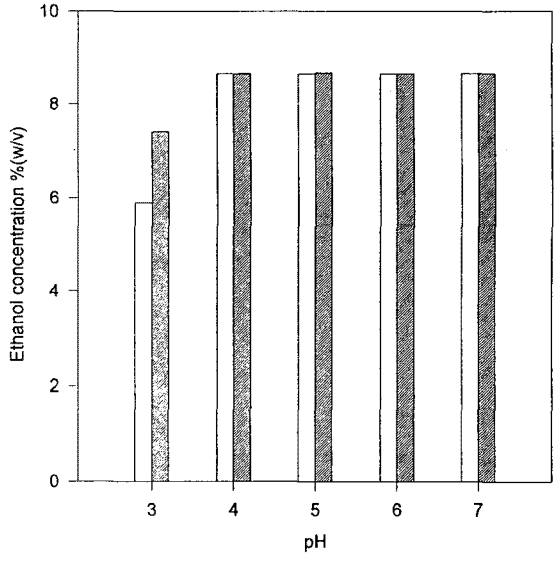

Fig. 3. Effect of initial pH on high temperature-immobilized cell fermentation.
Fermentation was conducted in a 250 ml flask with 50 ml fermentation broth for 40 h at 40°C. The media composition was glucose 175 g/l and yeast extract 5 g/l. The pH adjustment was carried out by 2N-HCl or NaOH after sterilization. □, Free cell; ▨, Immobilized cell.

(15, 2).

Effect of temperature on high-temperature fermentation. The effect of temperature on ethanol production with free and immobilized cells was examined. Fermentation was conducted at various temperatures in 20% glucose medium and seed volume was adjusted to 0.12 (v/v). The result is shown in Fig. 4. Ethanol productivity in 40 h or longer fermentation was the best at 40°C for free and immobilized cells (1.89 g/l.h ethanol in free cell and 2.06 g/l.h ethanol for immobilized cells, respectively). These results suggest that fermentation on high temperature has a merit by rapid metabolic rate, especially for this yeast strain. In case of prolonged culture of immobilized cells, ethanol production was similar to that expected for free cells at 30~40°C. However, immobilized cells were able to produce 7.15% (w/v) ethanol at 42°C, but free cells failed to ferment efficiently at 41°C. These results suggest that alginate gel matrix has a physical barrier effect against heat transfer as previous reported (15, 2). In Fig. 4B, ethanol concentration produced during over 120 h fermentation was similar to that of 40 h fermentation. Although high temperature and rapid carbon dioxide production accelerated simple diffusion of small molecules such as glucose and ethanol, toxicity of ethanol at high temperature was considered to be more important factor for alleviated ethanol production.

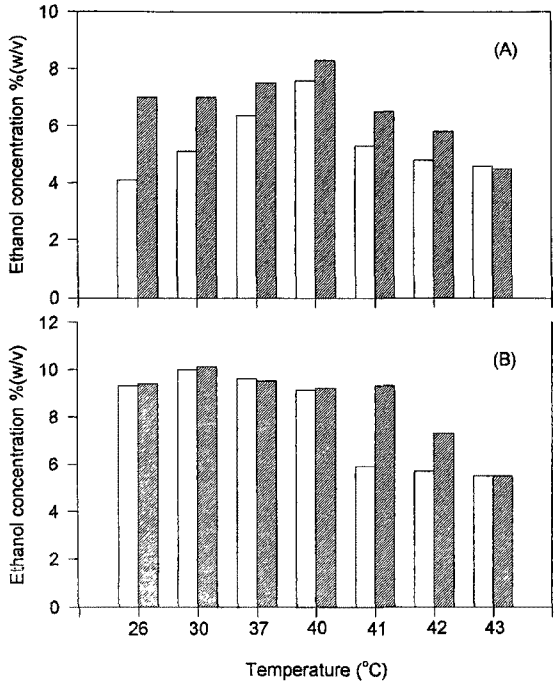

Fig. 4. Effect of temperature on high temperature-immobilized cell fermentation.

Fermentation was conducted in a 250 ml flask with 50 ml fermentation broth for 40 h (A) or 120 h (B) at 40°C. The media composition was glucose 200 g/l and yeast extract 5 g/l. □, Free cell; ▨, Immobilized cell.

Effect of glucose concentration on high-temperature fermentation. The effect of glucose concentration on ethanol production with immobilized cells was examined. Fermentation was conducted at 40°C and seed volume was adjusted to 0.12 (v/v). In this case, ethanol productivity was increased at 40°C. As shown in Table 3, the lower the glucose concentration, the faster the fermentation rate and the higher the ethanol productivity. For prolonged fermentation, the final fermentation ratio reached to 85~90% and remaining sugar and ethanol productivity decreased. Generally, 10% glucose medium was used to ferment ethanol with immobilized cells and the ethanol productivity was about 1.5~2.5 g/l.h as previous reported (5, 14). However, low concentration of ethanol produced in fermentation broth requires more cost of recovery. A 15~20% concentration of glucose was selected for efficient production and economic recovery of ethanol in high temperature vacuum fermentation.

Repetitive fermentation at various temperatures by immobilized cells. To examine the stability of beads at various temperatures and the feasibility of continuous fermentation, repetitive fermentation was carried out at 2 litre (l) fermentor scale with 1.5 liter of 15% glucose media. The initial cell number was 1.57×10^8/bead and seed volume was adjusted to 0.04 (v/v). Agitation speed was adjusted to 50 rpm for maintenance of accurate temperature, substrate transfer and ethanol diffusion. The first fermentation was carried out at 40°C and the second at 43°C. The third at 37°C, and the fourth at 30°C. The beads were withdrawn and used to check biomass and ethanol concentrations. Free cell mass was measured at various intervals. As shown in Fig. 5, ethanol fermentation was efficient at 30~43°C. Cell numbers increased up to 2.45×10^8/bead and 6.75% (w/v) ethanol [88% (w/v) of theoretical yield] was produced over the 48 h at 40°C in the first fermentation. During the second fermentation, 5.8% (w/v) ethanol (76% of theoretical yield) was produced at 43°C over 60 h and cell growth was stopped at 2.43×10^8/bead. This indicates that cells

Table 3. Effect of glucose concentration on immobilized cell fermentation at 40°C.

Glucose conc. (%)	Fermenation time (h)	Ethanol conc. % (w/v)	Fermenation ratio (%)	Ep (g/l.h)
10.0	5	3.93	78	7.87
15.0	8	4.85	64	6.06
17.5	16	7.43	84	4.64
20.0	25	8.62	85	3.44
25.0	34	11.00	87	3.23

Composition of basal media was yeast extract (5 g/l). Abbreviation: conc, concentration; Ep, ethanol productivity.

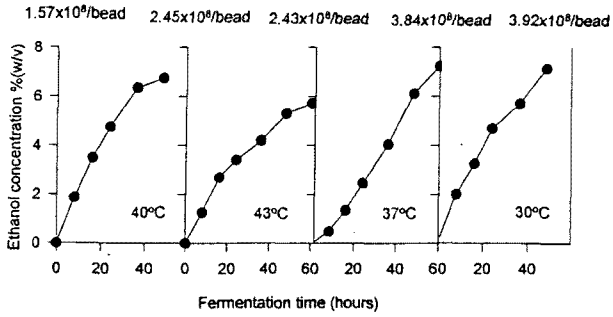

Fig. 5. Repetitive fermentation in 15% glucose media by immobilized thermotolerant yeast cells at various temperatures.
Cell number in beads was counted by haemacytometer after liquefaction of beads with 0.2 M Sörensen's citrate-sodium citrate buffer (pH 5.0).

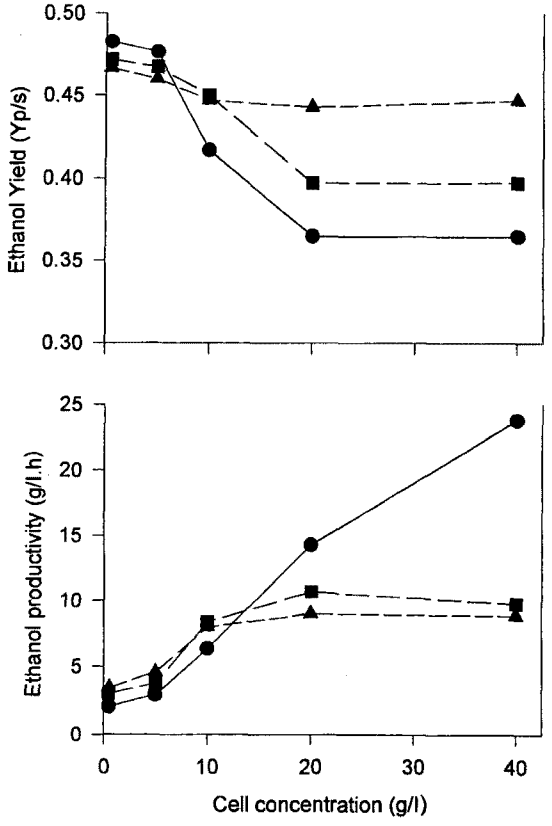

Fig. 6. Relativity of cell concentration versus ethanol yield (Yp/s) and ethanol productivity.
Fermentation was conducted by free cells at 40°C and the seed volume used was 0.5~40 g/l. —●—, glucose 100 g/l; —■—, glucose 150 g/l; —▲—, glucose 200 g/l.

were not grown at 43°C in 15% glucose medium. In the third fermentation at 37°C, cell numbers were increased up to 3.84×10^8/bead and 7.27% ethanol was produced (95% of theoretical yield). These results suggest that cell damages occurred during the second fermentation were ameliorated within 10 h at 37°C. In the fourth fermentation, 6.91% (w/v) ethanol (91% of theoretical yield) was produced during the 50 h and cell numbers were 3.92×10^8/bead. The free cell concentration was maintained to 0.4-0.6 g/l during repetitive fermentation. For fermentation at 37°C, free cell concentration in the fermentation broth was 0.6 g/l. These results suggest that the alginate bead was unstable at 43°C. However, immobilized thermotolerant yeast cells were able to ferment ethanol efficiently at 30~43°C (Fig. 5).

DISCUSSION

Higher productivity of ethanol fermentation could be achieved by use of high cell density rapid fermentation, and a thermotolerant yeast could minimize the amount of cooling water and energy required (1). To develop hyperproductive and low energy-consuming fermentation system, thermotolerant yeast *S. cerevisiae* RA-74-2 cells were immobilized. The carrier used was 1.5% (w/v) alginate solution without pH adjustment. The alginate bead has stability at 40°C in 3 cycle repetitive fermentation. The activation of the cells in the beads was sufficient within 20 h in YPD and 0.12 (6 ml bead/50 ml medium) was efficient for high temperature fermentation. This result coincided with Table 3. The higher concentration of yeast consumed more substrate for cell maintenace energy and a suitable cell concentration is essential for efficient fermentation (Fig. 2, Table 3). Alginate beads of *S. cerevisiae* RA-74-2 cells made a more efficient fermentation at a broad range of pH and temperature than free cell of *S. cerevisiae* RA-74-2 (Fig. 3, 4). These results suggest that alginate matrix has a physicochemical barrier effect against heat and hydrogen ions transfer into the cell (15, 2). In repetitive fermentation, ethanol production was stable at 30~43°C (88~95% of theoretical yield at 30~40°C) and the maximum productivity was shown at 37°C in a lab scale fermentor (Fig. 5). High cell density fermentation with free cells showed higher productivity than with immobilized cell's (Fig. 6). Economic fermentation with free cells was carried out in 15~20% glucose medium at 10 g/l cell concentration at 40°C. Ethanol productivity in this condition was 9~11 g/l.h, but in the case of immobilized cells (10 g/l cell concentration) 5.5~5.8 g/l in 17.5% glucose medium. This result matched the productivity of free cell fermentation at cell concentration of 5 g/l. These data indicated that only a half, 5 g cells/l, of immobilized cells (10 g/l cell concentration) actively participated in fermentation. These suggested that the interior parts of the bead were inhibited against glucose uptake and diffusion of ethanol and that productivity would be increased if a more porous and stable carrier were used.

Acknowledgement

This work was supported by a research grant from the Research and Development Center for Energy and Resources.

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(Received June 20, 1996)