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Studies on the Immobilization of *Saccharomyces cerevisiae* for Ethanol Production

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효모의 Alginate 고정화에 관한 연구

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Abstract — Ethanol production by calcium alginate-immobilized baker's yeast (*Saccharomyces cerevisiae*) was studied in the batch fermentation using glucose medium as a feed. Immobilied cells were stable between 30℃ and 40℃ whereas free cells were stable between 30℃ and 37℃. The beads were showed constant ethanol productivity during 720 hours (30 days) over. Fermentation characteristics of immobilized baker's yeast were examined changing the initial glucose concentration of broth in fermentation. Initial glucose concentrations employed were 50, 100, 150 and 200 g/*l*, respectively. In 15% gucose medium, maximum specific growth rate, maximum ethanol yield and ethanol concentration were observed as 0.092 h^{-1}, 0.45, 67.5 g/*l*, respectively.

The current scarcity of petroleum in the world has stimulated the search for alternate sources of energy. Ethanol produced from renewable resources by microbial fermentation seems promising and has recently commanded a great deal of attention (1). The interest in industrial alcohol fermentation has been increased during the last 20th years because of the oil shortage, which pushed many non-oil producing countries to carry out research into other possible alternatives of energy sources (2). Among these, alcohol fermentation is most important because it uses renewable resources.

Alcohol production used mainly batch or very few continuous fermentor with free cells (3). During recent years considerable attention has been given to the production of ethanol with immobilized cells. Several immobilization techniques that maintain high cell density in the fermentor have been proposed for ethanol production. These are classified into three types (4) : 1) physical immobilization by inert carrier, 2) entrapping methods by various hydrogels and 3) a method which uses floculent microorganisms.

Among those types, entrapping was the most popular method. Many kinds of carriers, κ-carrageenan, Na-alginate, gelatin, chitosan, polyacrylamide, agar and photo-crosslinkable resin oligomer, have been investigated (5).

Supporting materials frequently used are different kinds of gels such as κ-carrageenan and Ca-alginate with the cells immobilized in small beads. The advantages quoted are numerous : high productivity, low capital costs due to reduced equipment size and low separation costs as cell separation is not necessary after the fermentation step. However, there are as yet no large scale applications. The problem associated with κ-carrageenan is its high cost of separation from λ-carrageenan and high temperature is required immobilization (6). In the case of polyacrylamide,

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there are many problems associated with high heat occurrence by free radicals during polymerization, toxic character, high defacement and irregular shape (6). In the case of agar, its gel property is weaken by cell growth and high temperature is required for immobilization. Entrapment in beads of Ca-alginate gel is one of the most widely used techniques for immobilizing living microbial and animal cell (7), because the problem associated with κ-carrageenan, agar and polyacrylamide are not. Such gel beads can easily be made by allowing droplets of sodium-alginate solution fall into a calcium chloride bath (8).

This work presents data on the condition of alginate immobilization and the kinetics of growth and ethanol production by immobilized baker's yeast in batch fermentation.

Materials and Methods

Microorganism

Commerical baker's yeast (*Saccharomyces cerevisiae*) purchased in local market was used in this experiment.

Fermentation medium

The composition of fermentation medium (2) is as follow : 10% glucose, 0.1% KH_2PO_4, 0.1% NaCl, 0.07% $MgSO_4 \cdot 7H_2O$, 0.4% $(NH_4)_2SO_4$, 0.2% yeast extract, 0.147% $CaCl_2$ (repeat fermentation only), and 100 m*l* tap water. The fermentation medium was dispended in flask and was autoclaved at 121℃ for 15 min. The pH was adjusted to pH 5.0~6.0 before autoclaving.

Entrapment of cells in alginate

Sodium-alginate (Junsei Chemical Co., Japan) was used in this experiment.

Ten m*l* of 4% (w/v) Na-alginate was mixed with 10 m*l* of cell suspension (1g cell, wet weight/10 m*l* of physiological saline). The mixture was passed through a syringe and dropped into 0.2 M $CaCl_2$ solution, forming beads 2.8 to 3.0 mm in diameter.

The beads were allowed to "cure" at 20 to 22℃ for 1 hour, rinsed with water and equilibrated overnight in 0.05 M $CaCl_2$ solution at 4℃ until used (9).

Batch Fermentation

Batch fermentations were carried out in 300 m*l* Erlenmeyer flask contained 100 m*l* of 10% glucose medium adjusted to pH 5.0~6.0. The fermentor containing the medium was sterilized in an autoclave at 121℃, 115 lbs for 15 min. The media were inoculated with free or immobilzied cells and incubated at 30℃ under shaking (100 rpm).

The fermentor was operated by controlling glucose conclentration, temperature and pH.

Analytical methods

Glucose concentration was measured by DNS method (10). Ethanol analysis was carried out by gas chromatography (GC) in Hwlette Packard equipped with a flame ionization detector. One microliter of the diluted sample, with isopropanol as internal standard, was injected on Porapack Q (80~100 mesh) column isothermically operated at 200℃. Helium was used as carrier gas. The injector and detector temperature were 220 and 210℃.

Free cell number was counted by Haemacytometer. For biomass assay in beads, beads (10 each) were withdrawn and submerged in 20 m*l* of 0.2 M Sorensen's citrate-sodium citrate buffer (pH 5.7) and gently agitated in ice bath until the alginate beads is dissolved. This liquified alginate cell suspension was taken for total cell counts using Haemacytometer (11).

Results and Discussions

Conditions of Immobilization

Optimum concentration of Na-alginate for cell immobilization : A living yeast suspension concentrated to 100g wet weight/*l* was mxied with various concentration of Na-alginate (total 1.0, 1.5 and 2.0%).

In batch fermentation, the experimental data to find optimum concentration of Na-alginate are shown in Fig. 1. Concentrations less than 1% did not gel proprely and above 2% the alginate-yeast slurry was too viscous to form beads when extruded. The resulting concentrations were 1.0%, 1.5% and 2.0% (w/v) Na-alginate solution. Even Though similar ethanol productivity was observed with 1.0%, 1.5% and 2.0% Na-alginate respectively, 2% Na-alginate was selected as because of good shape and high rigidity.

Optimum pH of Na-alginate on cell immobiliza-

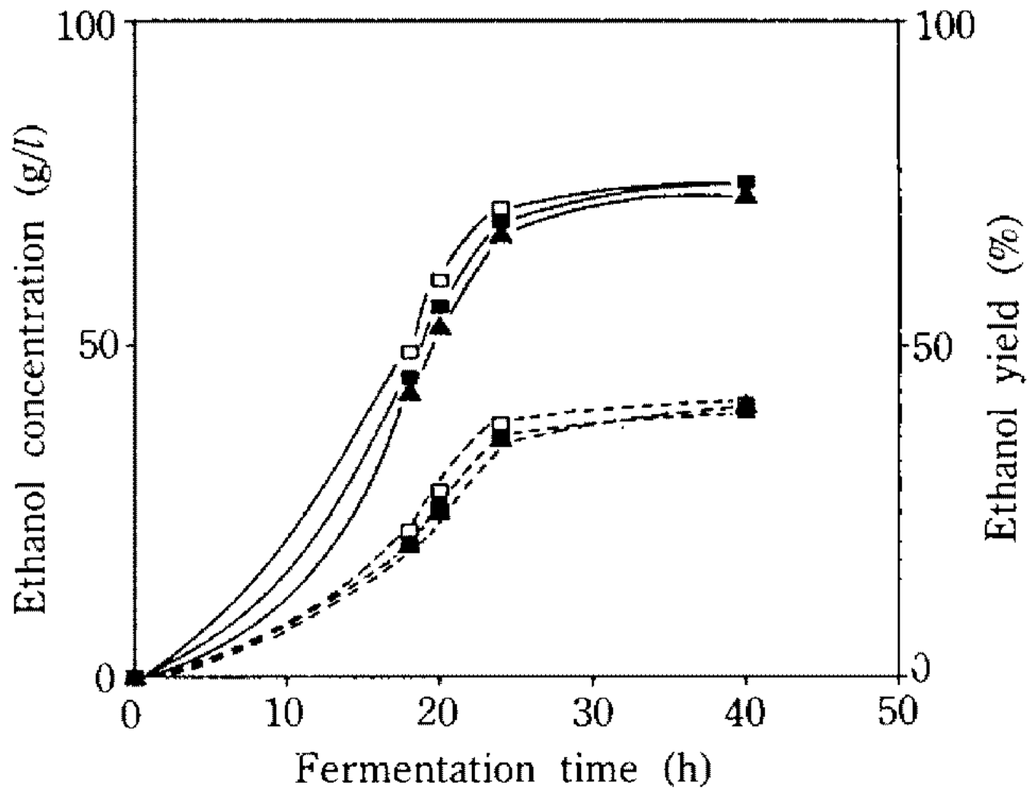

Fig. 1. Effects of Na-alginate concentration on ethanol production.
−■− : 1.0%, −□− : 1.5% and −▲− : 2.0% Na-alginate

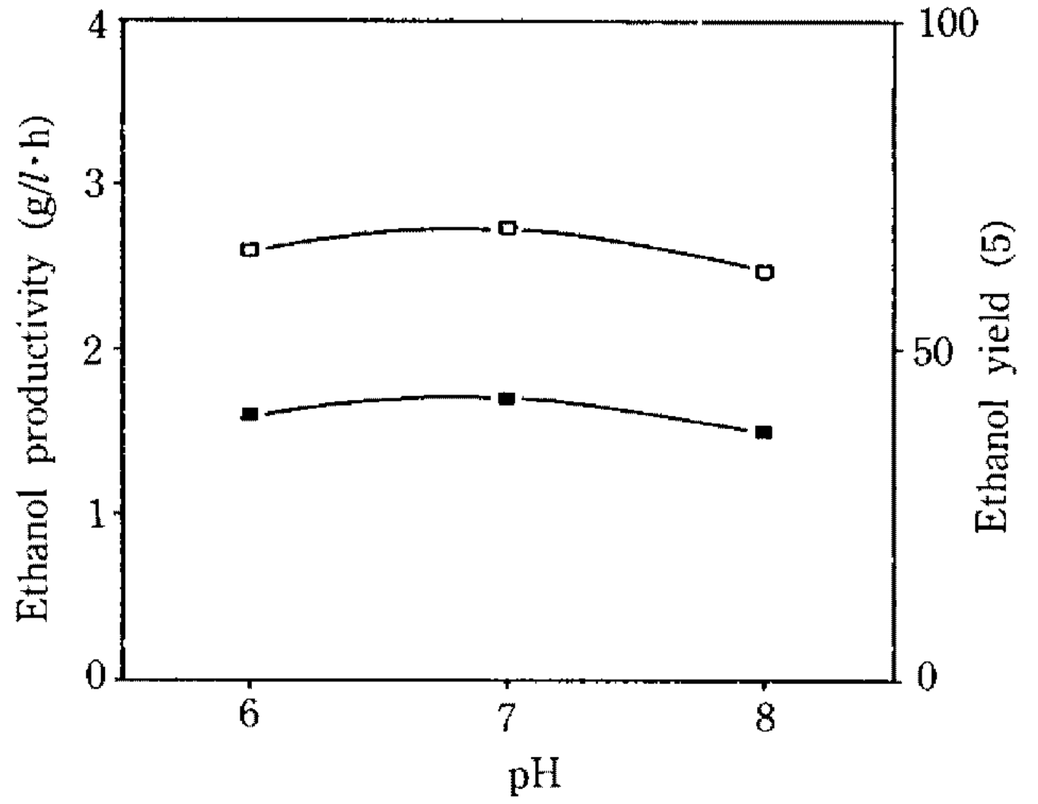

Fig. 2. Effect of immobilization pH on ethanol production.
−■− : productivity and −□− : yield.

tion : A living yeast cell suspension concentrated to 100g wet weight/*l* was mixed with various pH (pH 6, 7 and 8) of 2% Na-alginate. These were filled in batch fermentor. In batch fermentation, the optimum pH of Na-alginate are shown in Fig. 2. Beads were not formed above pH 9 and below pH 5.0. pH 7.0 was selected as an optimum pH of Na-alginate solution because it gives higher ethanol productivity after 20 hour fermentation than others.

Optimum cell concentration on immobilization : Different concentrations of yeast (1g, 2g, 3g and 4g wet weight/10 m*l* (4.3×10^9 cells/*l*) were added to the same volume of 2% Na-alginate solution (pH 7.0). These were filled in batch fermentor.

Fig. 3 shows the optimum concentration of cells for ethanol production in batch fermentation. This beads containing 0.1 g/m*l* of cells were selected as an optimum cell concentration because it gives higher ethanol productivity after 20 hours fermentation than others and it has an economical benefits.

Conditions of fermentation

The beads made under the optimum conditions of immobilization (pH 7.0, 2% Na-alginate containing living yeast cells suspension concentrated to 100g wet weight/*l*) were used in these experiments.

Optimum bead volume in fermentation : Different volumes (5, 10, 20, 30 and 40 m*l*/100 m*l* of medium) of beads were filled in batch fermentor. Fig. 4 shows

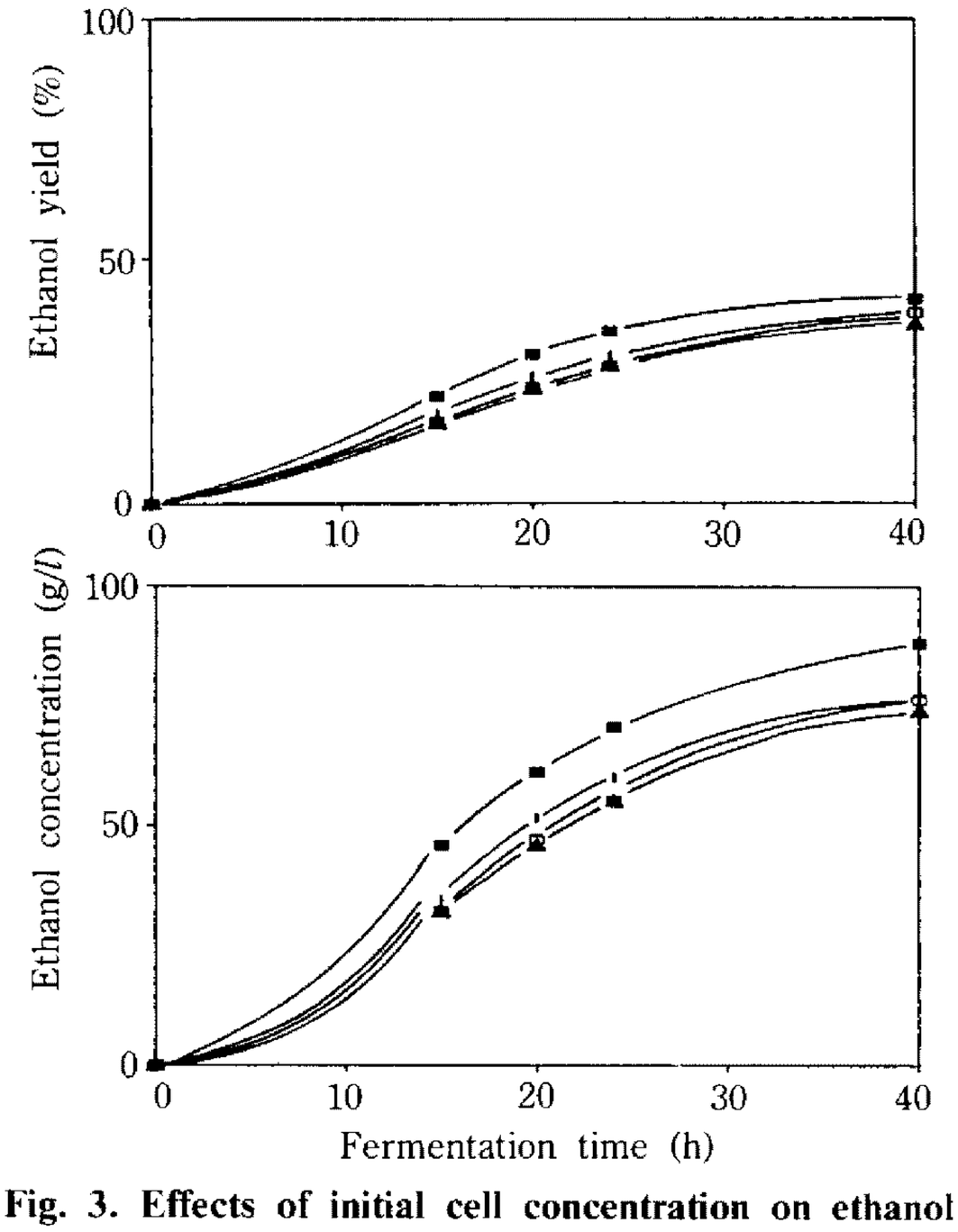

Fig. 3. Effects of initial cell concentration on ethanol production.
−■− : 1g (4.3×10^9 cell/*l*) −□− : 2g (8.6×10^9 cell/*l*)
−▲− : 3g (1.3×10^{10} cell/*l*) −|− : 4g (1.7×10^{10} cell/*l*)

the optimum bead concentration for ethanol production in batch fermentation. In the case of 5 m*l* of baeds volume, ethanol productivity was 1.53 g/*l*·h remarkably decreased after 20 hr of fermentation. In the case of 10, 20, 30 and 40 m*l* bead volume, similar ethanol prodictivity was observed. Considering the

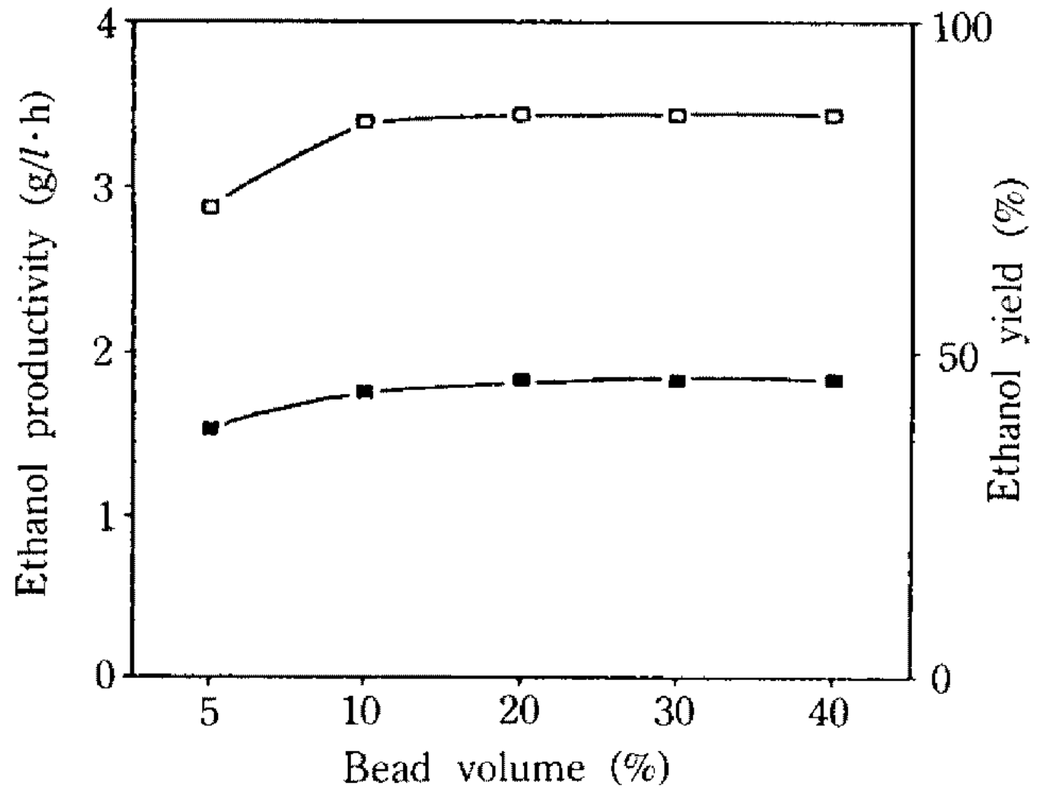

Fig. 4. Effect of bead volume on ethanol production.
—■— : productivity and —□— : yield.

economical benefits, 10 m*l* of beads volume was selected as an optimum.

Effect of shaking in batch fermentation : One gram of free cell and 10 m*l* of beads were filled in batch fermentor. Fig. 5 shows the shaking effects on ethanol production during the time course in batch fermentation. Free cells have higher ethanol productivity than immobilized cell and ethanol productivity was significantly increased by shaking.

The bead stability for recycling in batch fermentation : Ten m*l* of beads entrapped in 2% Na-alginate (pH 7) containing cell suspension concentrated to 100g wet weight/*l* were filled in batch fermentor. In batch fermentation, Fig. 6 shows the driving stability. In repeated batch fermentation, one cycle is equal to two days (48 hr). The beads were stable and showed constant ethanol productivity during 15 cycles (720 hr) (12). According to Ghose *et al.* (13) stability of immobilized cells was maintained for 1800 hours.

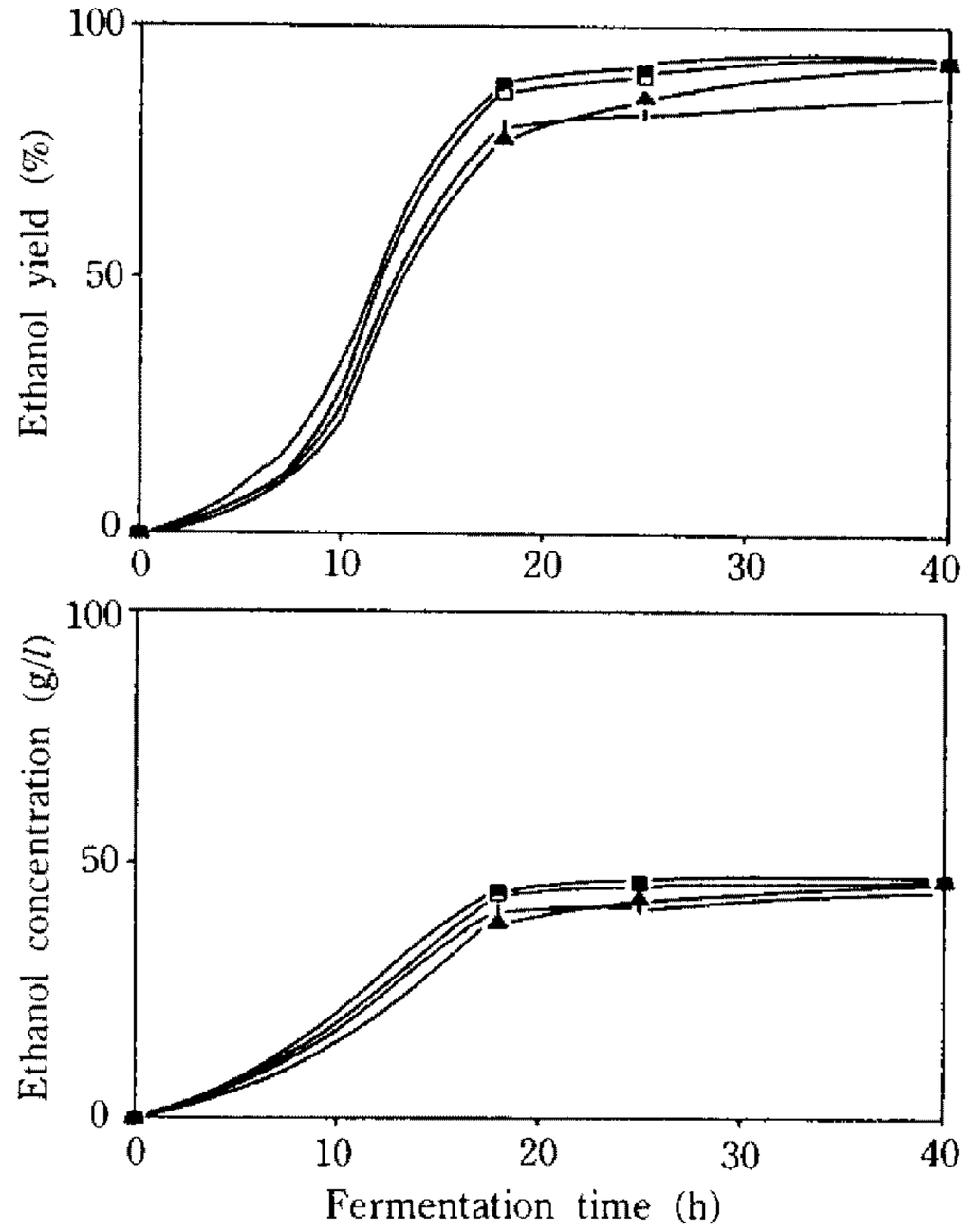

Fig. 5. Effect of shaking on ethanol production.
—■— : Free cell, 4.3×10^9 cell (shaking)
—□— : Free cell (stational)
—▲— : Immobilized cell, 10 m*l* bead gel (shaking)
— | — : Immobilized cell (stational)

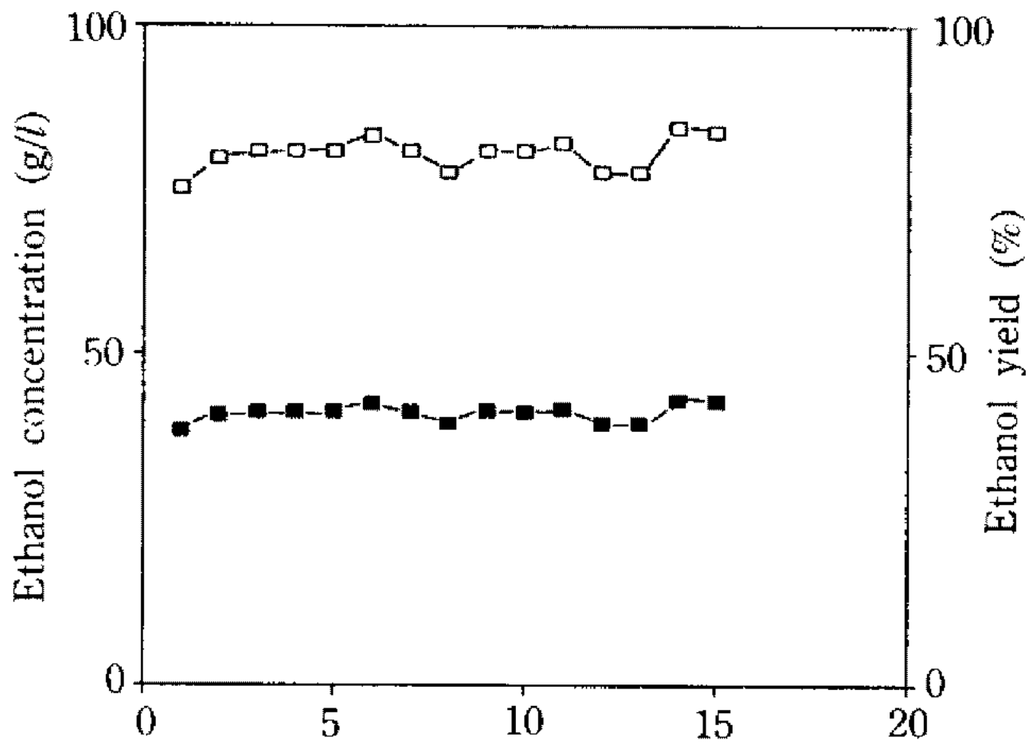

Fig. 6. The bead stability for recycling in batch fermentation.
—■— : ethanol and —□— : yield.

Batch fermentation

Kinetic parameters

Glucose + Nutrient ⟶ Ethanol + Cell + by-product
(S) Cell as catalyst (P) (X)

1.0g Glucose ⟶ 0.51g Ethanol + 0.49g CO_2

Ethanol productivity

$$P_E = P/\Delta t$$

| Δt : Fermentation time (h)
| P : Ethanol concentration (g/*l*)

Theoritical ethanol yield (%)

$$\eta = P/E \times 100\ (\%)$$

| E : Theoretical total ethanol weight produced from initial substrate

Ethanol yield

$$Y = P/G$$

| G : Glucose concentration (g/*l*)

Specific glucose uptake rate (g·glu/g cell·h)

$$q_s = \frac{1}{X} \cdot \frac{ds}{dt}$$

| X : Cell weight (g)
| dS : Glucose uptake (g/*l*)
| dt : Fermentation time (h)

Specific ethanol productivity (g·EtOH/g cell·h)

$$q_s = \frac{1}{X} \cdot \frac{dP}{dt}$$

| dP : Ethanol concentration (g/*l*)

Conversion rate of glucose (%)

$$\%C = (S_o - S)/S_o \times 100 \ (\%)$$

| S_o : Initial substrate concentration (g/*l*)
| S : Final substrate concentration (g/*l*)

Ethanol yield coefficient

$$Y_{p/s} = \Delta P/\Delta S = (P - P_o)/(S - S_o)$$
$$= (\text{g of EtOH produced})/(\text{g of glucose consumed})$$
$$= q_p/q_s$$

Specific growth rate (cell number, h^{-1})

$$\mu_n = \ln(X_n/X_{no}) \times (1/t)$$

| X_{no} : Initial cell number
| X_n : Cell number after time, t

Substrate uptake rate of immobilized cell (h^{-1})

$-rs = (V_{max} \times S)/(k_m + S)$: Michaelis-Menten equation (Fig. 7)

| V_{max} : Maximum specific reaction rate (g/*l*·h)
| K_m : Michaelis constant (g/*l*)
| S : Substrate concentration (g/*l*)

Effect of temperature in batch fermentation : The effect of temperature on the ethanol productivity and yield with free and immobilized cell systems was examined. In batch fermentation, the results to find effect of temperature are shown in Fig. 8. For temperature range between 25℃ and 40℃, free cell showed a broad optimum temperature range with the best ethanol procuctivity and yield between 30℃ and 37℃ but immobilized cell showed between 30℃ and 40℃. At 25℃, both free and immobilized cell showed similar ethanol productivity. At 40℃, free cell showed rapid decrease of ethanol productivity, but immobilized cell showed similar ethanol productivity with 30~37℃.

These results showed that immobilized cell was more stable than free cell on temperature because alginate is stable even at thermophiloic fermenta-

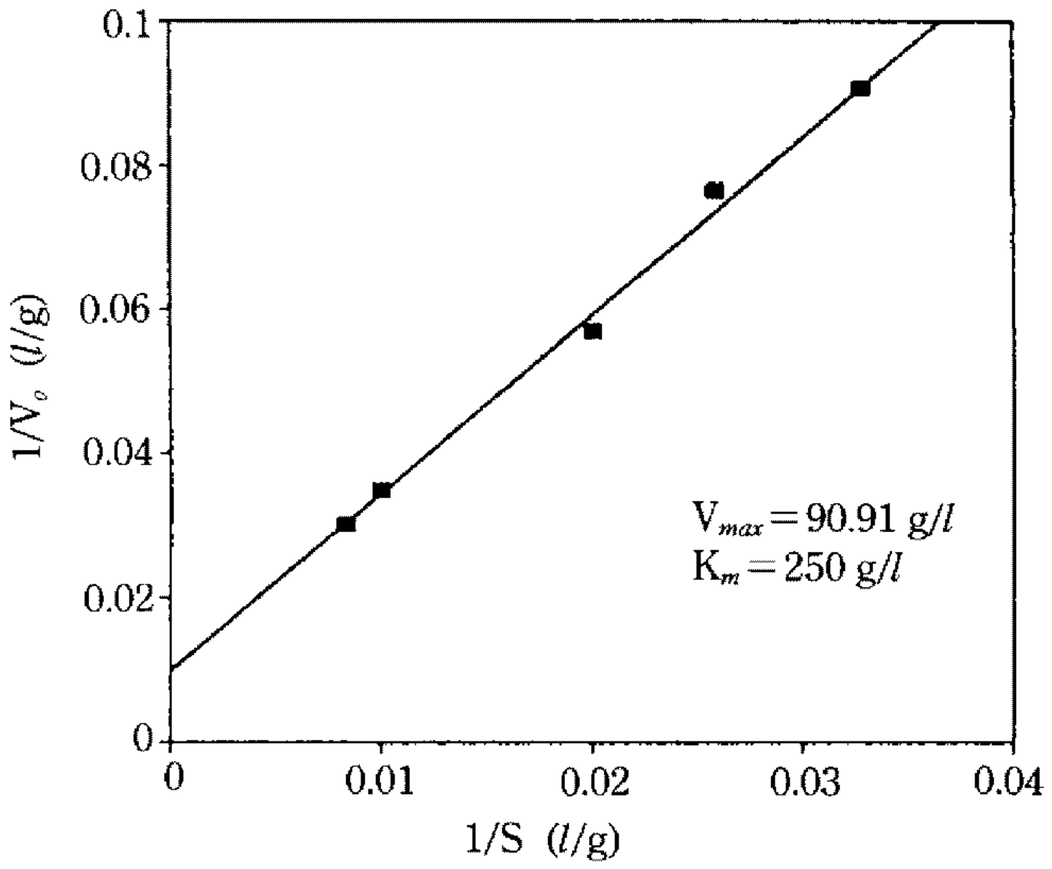

Fig. 7. Determination of K_m and V_{max} value of immobilized yeast (Lineweaver-Burk plot of batch fermentation data using immobilized yeast)

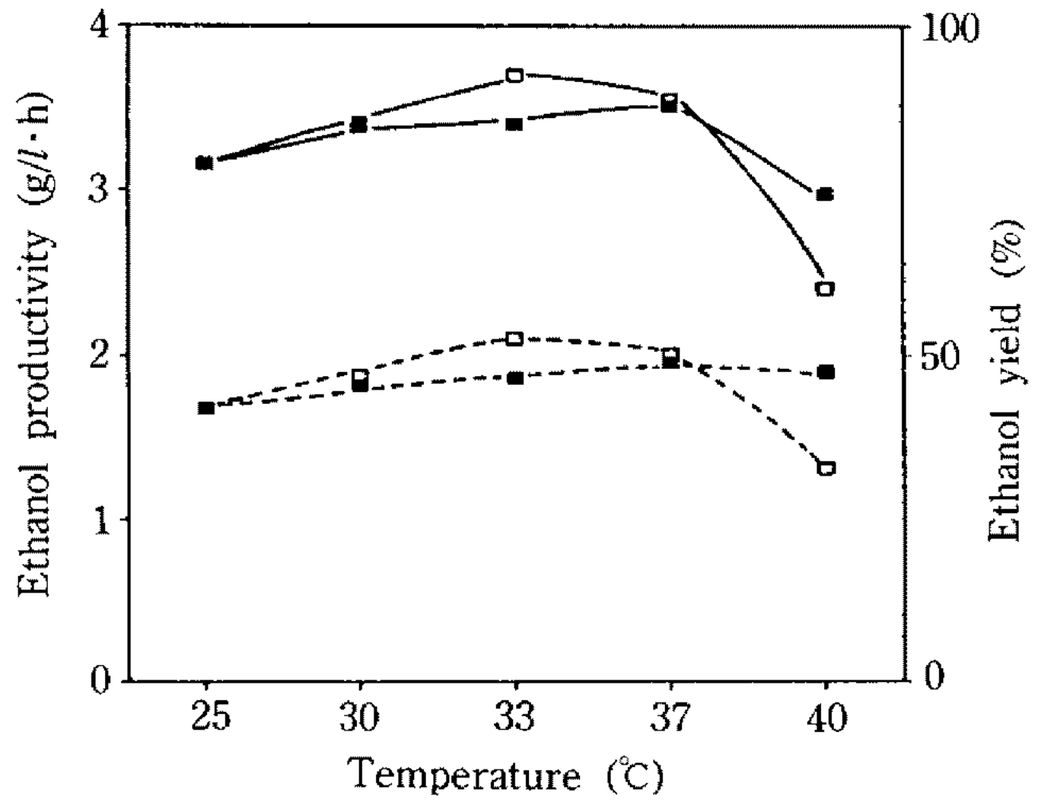

Fig. 8. Effect of temperature in the batch fermentation.
—■— : Immobilized cell —□— : Free cell
--- : productivity — : yield

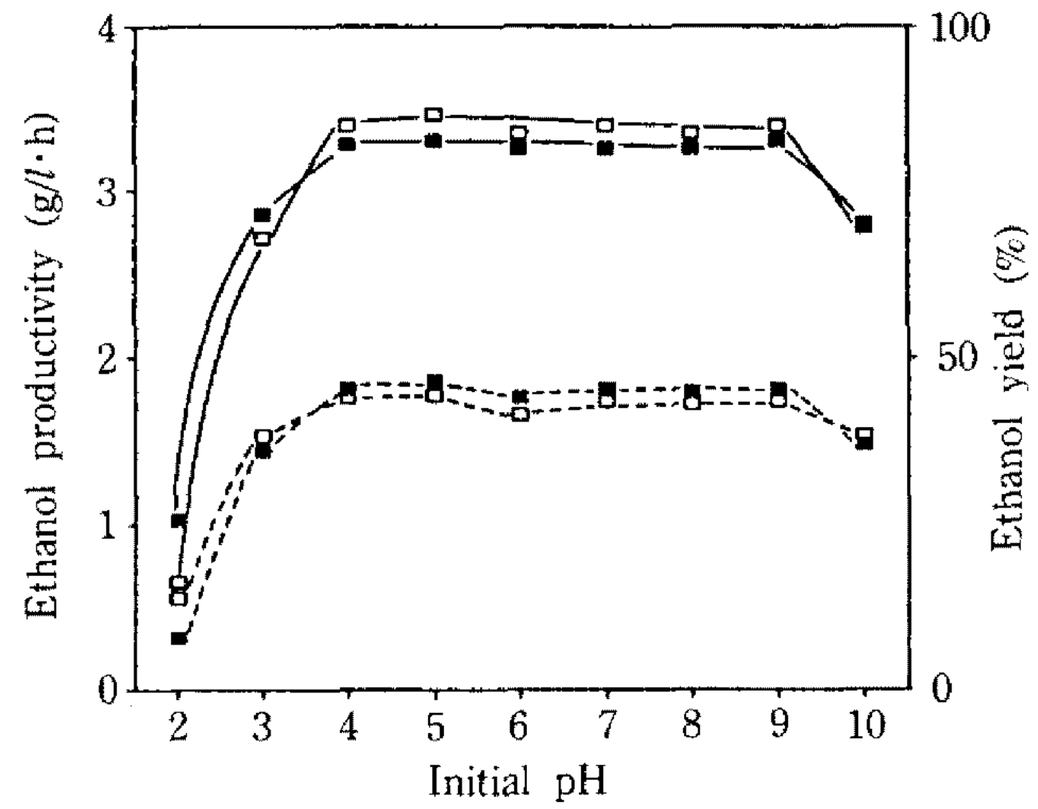

Fig. 9. Effect of Initial pH of glucose solution in batch fermentation.
−■− : Immobilized cell −□− : Free cell
--- : productivity — : yield

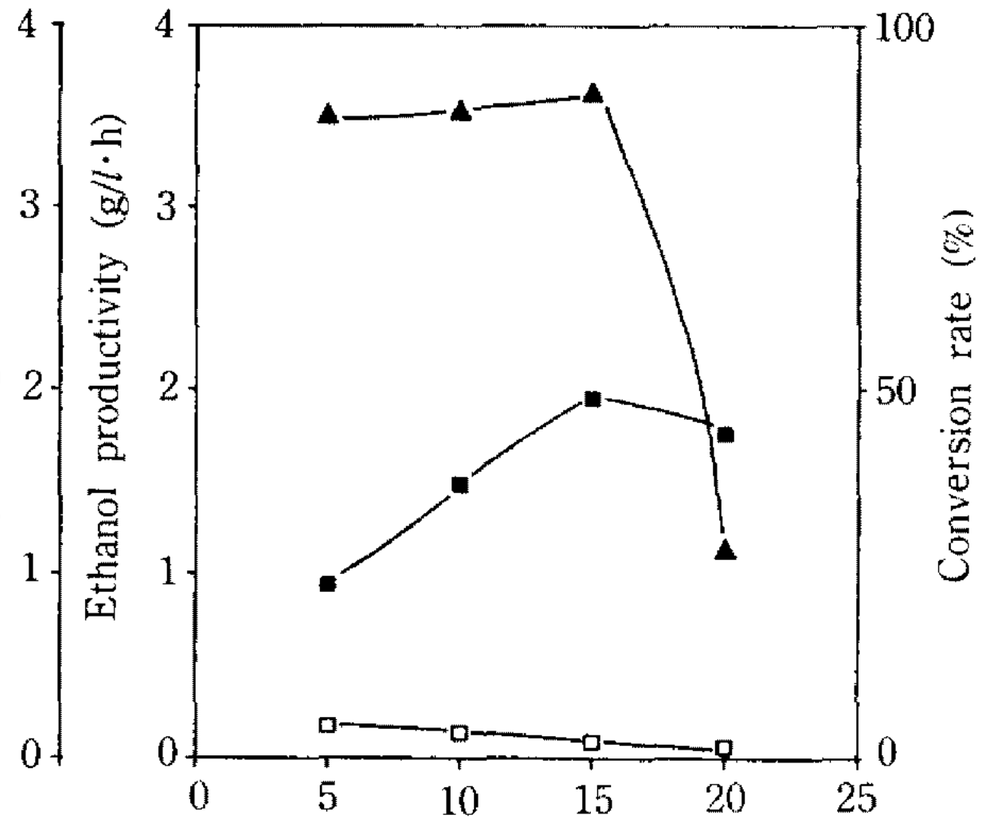

Fig. 10. The relationship of conversion rate, ethanol productivity, and specific growth rate according to initial glucose concentration in the batch fermentation.
−■− : ethanol, −▲− : glucose and −□− : cell

tion temperature (14). These were similar with results decribed by Park *et al.* (15).

Effect of Initial pH of glucose solution in batch fermentation : The effect of pH on the ethanol productivity and yield with free and immobilized cell systems was examined. In batch fermentation, the results to find effect of initial pH of glucose solution are shown in Fig. 9. For pH range between 2.0 and 10.0, both free and immobilizeds cell showed a broad pH for the best ethanol procuctivity and yield between pH 4.0 and pH 9.0. Between pH 4.0 and pH 9.0, both immobilized and free cells showed almost similar ethanol productivity. But in case of pH 2.0, 3.0, and 10.0, immobilized cells showed higher ethanol productivity than that of free cells.

These results showed that immobilized cell was more stable than feee cell on effect of pH as decribed by Buzas *et al.* (16) and Williams *et al.* (14).

Effect of initial glucose concentration in batch fermentation : The effect of gluocse concentration on the ethanol and glucose concentration, conversion rate, ethanol productivity, and specific growth rate with immobilized cell systems according to time were examined. In fermentation, the results to find effect of glucose concentration are shown in Fig. 10, 11 and Table 1. In Fig. 10, the lower initial glucose concentration, the faster glucose uptake time. In Fig. 11, conversion rate was similar in 5%, 10% and 15% initial glucose concentration

Table 1. Kinetic parameters on effect of initial glucose concentration in the batch fermentation using immobilized baker's yeast

Parameters	Concentration of gluocse			
	5%	10%	15%	20%
q_s (g/g·h)	0.86	0.65	2.38	0.98
q_p (g/g·h)	0.20	0.30	1.08	0.46
%C	98.88	98.90	99.20	92.90
P (g/*l*)	22.10	44.60	1.99	1.77
P_E (g/*l*·h)	0.92	1.49	1.99	1.77
η (%)	86.67	87.45	88.24	86.57
$Y_{p/s}$	0.45	0.45	0.46	0.48
X_f (longN/*l*)	10.47	10.50	10.06	10.06
X_b (LogN/bead)	6.81	6.90	6.95	6.85
μ_m (h^{-1})	0.170	0.138	0.092	0.063
Y	0.44	0.45	0.45	0.44
T (h)	24	30	34	50

*T : Time required to reach maximum ethanol concentration (hr)

but 20% was decreased. Specific growth rate was decreased according to increase of initial glucose concentration (17). Maximum ethanol productivity was shown in 15% initial glucose solution. Strong inhibition is indicated at high glucose concentration (200 g/*l*) in the medium, as shown by Agrawal *et al.* (18).

In these results, optimum initial glucose concen-

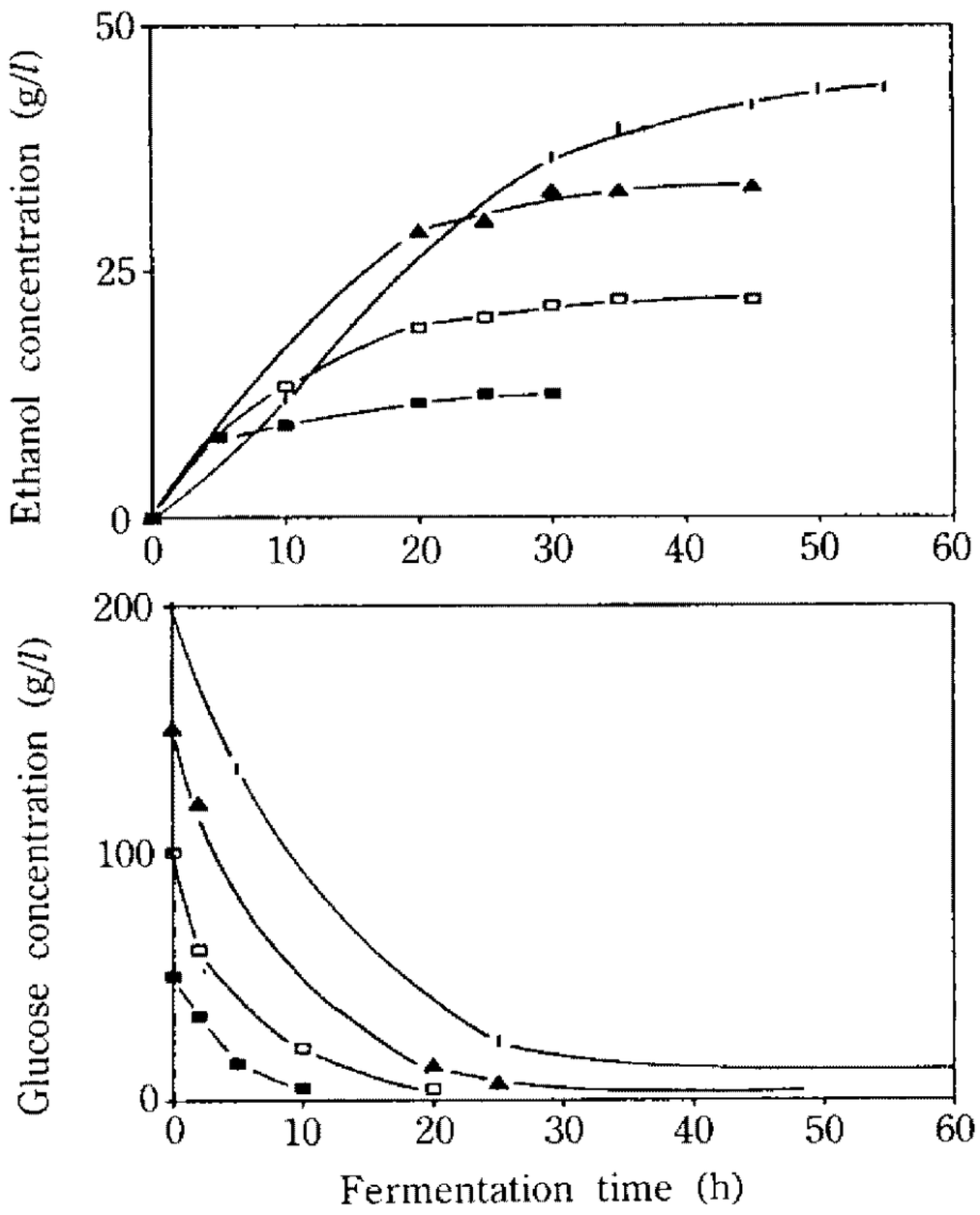

Fig. 11. Effect of initial glucose concentration in the batch fermentation.
−■− : 5%, −□− : 10%, −▲− : 15% and | : 20% glucose solution

tartion was turned out to be 15% of glucose. Time required to reach maximum ethanol concentration was facter than that of Borghi *et al.* (2). This ethanol concentration (67.5 g/*l*) were higher than the result (55.0 g/*l*) described by Agrawal *et al.* (18) in the 15% glucose. Optimum glucose concentration of 11% was shown in Torres *et al.* (21). In the result of Sedha *et al.* (19), a sugar concentration of 18% used in molasses wash was found to be optimum for maximum fermentation efficiency.

요 약

효모를 Ca-alginate에 고정화하여 회분발효에서 glucose로부터 에탄올을 생산하여 다음의 결과를 얻었다.

100g wet weight/*l*(4.3×10^9 cell/*l*)의 효모를 pH 7.0, 2% 농도의 Ca-alginate에 고정화하였다. 10% beads volume이 에탄올 생산에 최적이었고 30일(720시간) 동안 bead의 수명이 지속되었다. 회분식 발효에서 온도안정성은 고정화 효모의 경우 30~40℃였으며 free cell의 경우 30~37℃였다. pH 안정성은 pH 4.0~9.0였으며, 에탄올생산 최적 당농도는 15%였다. 최적조건에서 에탄올수율은 0.45, 생산된 에탄올 농도는 67.5 g/*l* 그리고 에탄올 생산성은 1.99 g/*l*·h로 각각 나타났다.

Nomenclature

S : Substrate concentration (g/*l*)
T : Time (h)
P : Ethanol concentration (g/*l*)
P_E : Ethanol productivity
η : Theoretical ethanol yield (%)
Y : Ethanol yield
X : Cell weight (g/*l*)
X_f : Free cell number (log N/*l*)
X_b : Cell number in bead (log N/bead)
q_s : Specific glucose uptake rate (g/g·h)
q_p : Specific ethanol productivity (g/g·h)
%C : Conversion rate of glucose (%)
$Y_{p/s}$: Ethanol yield coefficient
μ_n : Specific growth rate (h^{-1})
K_s : Michaelis constant
μ_{max} : Maximum specific growth rate
$-r_s$: Substrate uptake rate of immobilized cell (h^{-1})
V_{max} : Maximum specific reation rate (g/*l*·h)
K_m : Michaelis constant (g/*l*)

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