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Continuous Ethanol Production Using Immobilized Baker's Yeast

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고정화 효모를 이용한 연속적 에탄올 생산

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Abstract — Ethanol production by calcium alginate-immobilized baker's yeast was studied in the continuous shaked-flask reactor (CSFR) using glucose medium as a feed. Immobilized cells were stable at 30~37℃ and pH 4~8. Fermentation characteristics of immobilized baker's yeast were examined changing the initial glucose concentration employed were 50, 100 and 150 g/*l*, respectively. It was investigated that the influent glucose concentration and the dilution rate have an influence on the ethanol fermentation characteristics at steady state in continuous culture of immobilized baker's yeast. The optimum conditions for high ethanol productivity and low residual glucose output in ethanol prodution were shown to be 0.2 h^{-1} for the dilution rate and 150 g/*l* for the influent glucose concentration. The maximum ethanol productivity, ethanol yield, specific growth rate and glucose conversion rate were around 7.12 g/*l*·h, 0.23, 0.366 g/*l*·h and 78.43, respectively.

There is a need at the present time to consider viable alternatives to fossil resources for the production of fuels and chemicals. One of the possible alternatives is the utilization of microbial metabolism. Metabolic pathways of microbial cells may be changed by manipulating growth conditions so as to enhance (or reduce) the production of metabolites that may be useful as fuels or chemical resources (1).

Ethanol production from renewable resources by microbial technology seems promising and has recently commanded a great deal of attention (2). Recent advances in immobilized cell technology have suggested new methods for producing biochemical and biofuels such as ethanol (3).

During recent years considerable attention has been given to the continuous production of ethanol with immobilized cells (14). Several Immobilization techniques that maintain high cell density in the fermentor have been proposed for continuous ethanol fermentation. In these proposal, high productivity was attained with increasing high cell density.

Na-alginate is popular as a support for immobilization. Alginate is a collective term for a family of copolymers cotaining 1,4-linked β-D-mannuronic and α-L-gluconic acid residues in varying propertions and sequential arrangement. It forms gels with divalent ions like calcium and the gel forming properties are strongly correlated with the proportion and lenths of the blocks of contiguous L-guluronic acid residues in the polymeric chains.

Experiments for continuous alcohol fermentation using yeast cells bound in Ca-alginate beads have been conducted to measure alcohol productivity and cell densities and to design a bioreactor. The problem associated with Calcium alginate is that it is unstable in the presence of phosphate buffer and certain cations such as Mg^{2+} or K^{+}. Unfortunately,

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phosphate saot is one of the major nutrients of living microbial cells. So $CaCl_2$ was included in fermentation media as gel stabilizer (4).

In this work, baker's yeast were used. Application of yeast cells immobilized in alginate beads for continuous production of ethanol has been intensively studied. when yeast cells immobilized in alginate beads are used in a packed bed (6) for ethanol fermentations, however, problems with gas hold-up and weaking of the gel beads due to CO_2-development often occur (7). To overcome the gas hold-up problem, different designs of stirred-or multistage-reactor have been proposed (7~10). These solutions, however, will cause the increase of capital cost and energy consumption for a fermentation plant.

Bioreactor using free cells have inherent disadvantages as follows : low ethanol productivities, large bioreactor volumes, low cell concentrations, and appreciable agitation energy requirements. The main advantage of immobilized cell bioreactor are : 1) the immobilized cells can be used for long periods of time without replacement, 2) the immobilized cell bioreactor can be operated at high dilution rates without washing out ; also, high ethanol productivities are achieved 3) lower bioreactor capital cost (11).

This work presents data on the kinetics of growth and ethanol production by immobilized yeast cells in Ca-alginate in continuous fermentation using shaked-flask reactor (CSFR).

Materials and Methods

Microorganism

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

Fermentation medium

The fermentation medium was similar to composition of Rose and Harrison(12).

The composition of fermentation medium 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% $CaCl_2$, and 100 ml 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 ml 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 hr, rinsed with water and equilibrated overnight in 0.05M $CaCl_2$ solution at 4℃ until used (13).

Contionuous Fermentation

Continuous fermentation was carried out in continuous flow shaked-flask reactor(CSFR). A schematic layout of the CSFR was shown in Fig. 1. Immobilized yeast were transferred into a CSFR with 200 m*l* working volume under shaking(100rpm). Enough fresh feed medium was pumped continuously to the reactor to bring the total culture volume to 200 m*l* (180 m*l* of broth plus 20 m*l* of beads).

This volume was maintained throughout the fermentation using peristaltic pump continuously delivered fresh medium at a controlled rate while drawing off spent medium at an equal rete. The flow

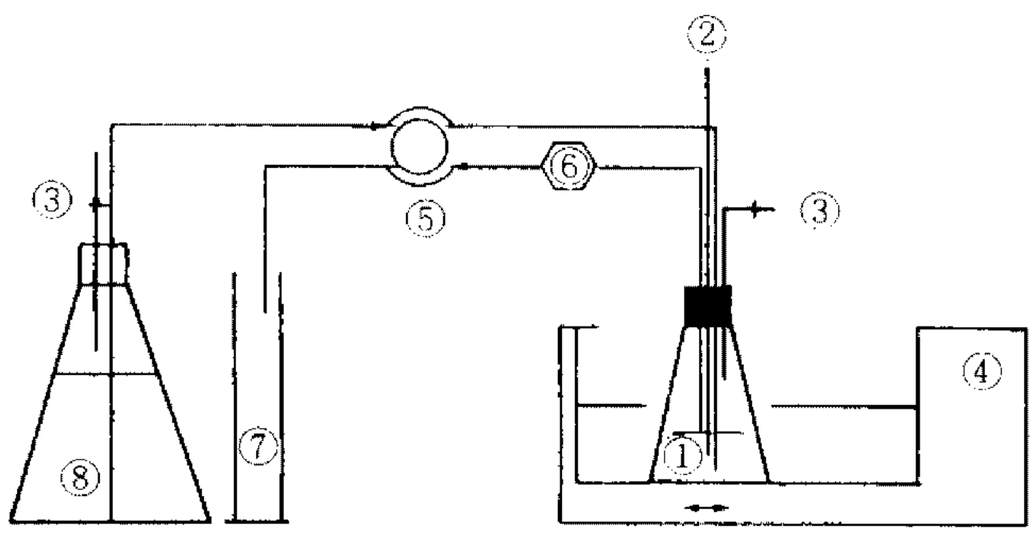

Fig. 1. Diagram of continuous shaked-flask reactor (CSFR)

① Flask reactor
② Thermometer
③ Membrane filter
④ Shaking water bath
⑤ Peristaltic pump
⑥ pH monitor
⑦ Effluent collector
⑧ Fermentation medium

Table 1. Operating conditions of shaked flask reactor

Reactor	Total volume (m*l*)	1,000
	Working volume (m*l*)	200
	Liquid volume (m*l*)	180
	Bead volume (m*l*)	20
Flow rate (m*l*/h)		20~140
Inlet glucose concentration (g/*l*)		50~200
Inlet pH		5.0~6.0
Reaction temperature (℃)		25~40

Table 2. Operating conditions of gas chromatography

Model	Hwelette packard
Detector	Flame Ion Detector
olumn material	Porapack Q(80~100 mesh)
Control temp.	Oven : 200℃
	Detector : 220℃
	Injector : 210℃
Carrier gas	Helium

from the CSFR was found to be very stable. Temperature was controlled by shaking incubator. All reactor accessorys were used after autoclaving at 121℃ for 15 min. $CaCl_2$ (0.147%) was added in substrate as a gel stabilizer (14).

In most cases the activation was performed with the fermentor medium. In the glucose fermentation, up to 4~5 cycle were required to attain steady state.

Analytical methods

Glucose concentration was measured by DNS (dinitrosalicylic acid) method (15).

Ethanol contents in the product were determined by gas chromatography (GC) were shown in Table 2.

Free cell number was counted by Haemacytometer. For biomass assay in beads (10 each) were withdrawn and submerged in 20ml of 0.2 M Sörensen's citrate−sodium citrate buffer (pH 5.7) and gently agitated in ice bath until the alginate beads are disolved. This liquified alginate cell suspension was taken for total cell counts using Haemacytometer (16).

The pH was continuously measured by pH/Ion monitor (LKB 2195) connected to bioreactor.

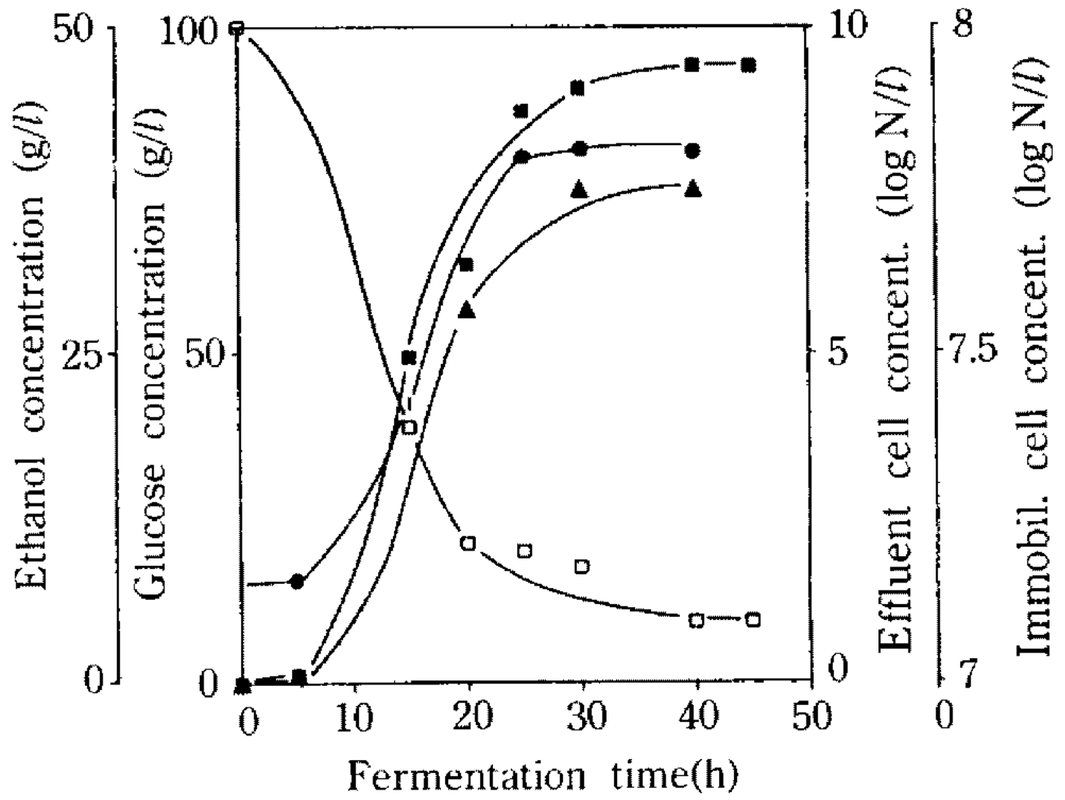

Fig. 2. Ethanol fermentation characteristics as function of time using immobilized yeast.
−■− : ethanol, −□− : glucose, −▲− : effluent, and −●− : immobilized cell

Results and Discussions

Continuous fermentation

The activation of gel beads : The fermentation conditions (10 m*l* of beads with shaking) were used in these experiments. In batch fermentation, the change of ethanol concentration, glucose concentration and cell concentration with increasing time were shown in Fig. 2.

After 20~25 hours, fermentation was almost terminated, 43 g/*l* of alcohol concentration were produced, 90% of glucose were used, and cell growth was almost paralleled. These were similar with results of Borghi *et al.* (12). and Lee *et al.* (5). The activation time described by Kim *et al.* (18) was faster than that of this result.

Kinetic parameters : The beads activated for 24 hr were used in these experiments.

The mean residence time of the liquid phase(h^{-1})

$$\tau = (\pi d^2/4L) \times \varepsilon/F \quad (\varepsilon = V_L/V_T)$$
$$= V_T \times \varepsilon/F$$
$$= V_L/F$$

V_L : Liquid volume (m*l*)
F : Flow rate (m*l*/h)

Dilution rate (h^{-1})

$$D = F/V_L$$

Ethanol productivity

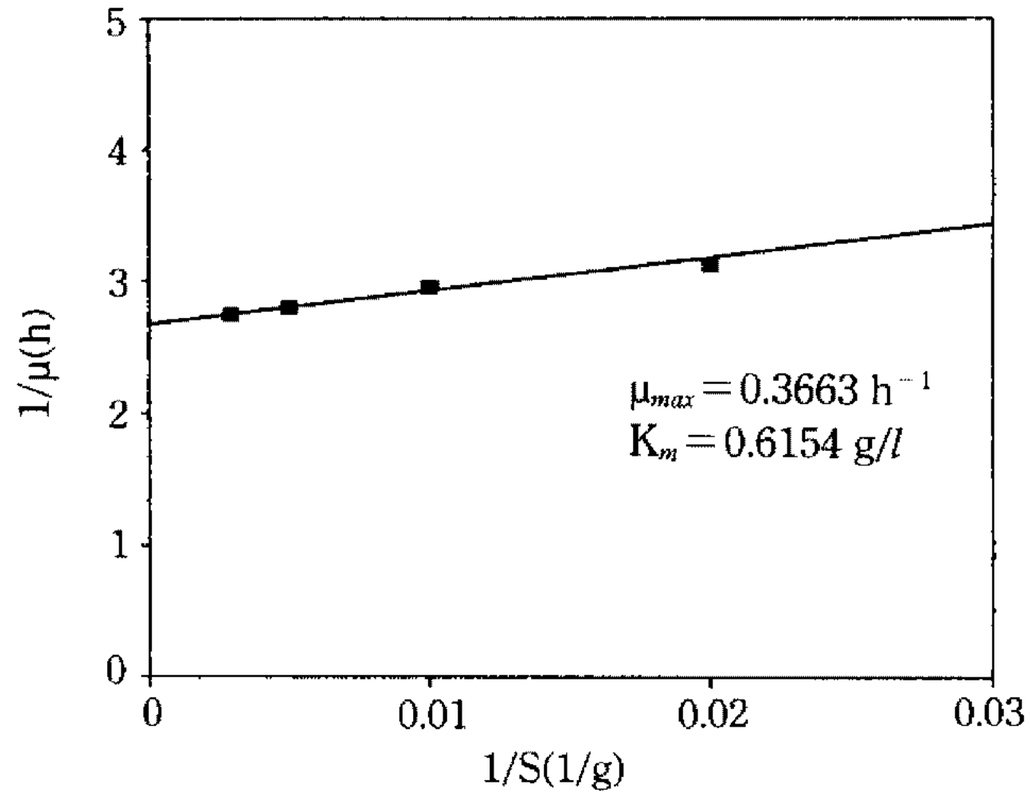

Fig. 3. Determination of μ_{max} and k_s value of immobilized yeast (Lineweavery−Burk plot of batch fermentation data using immobilized yeast).

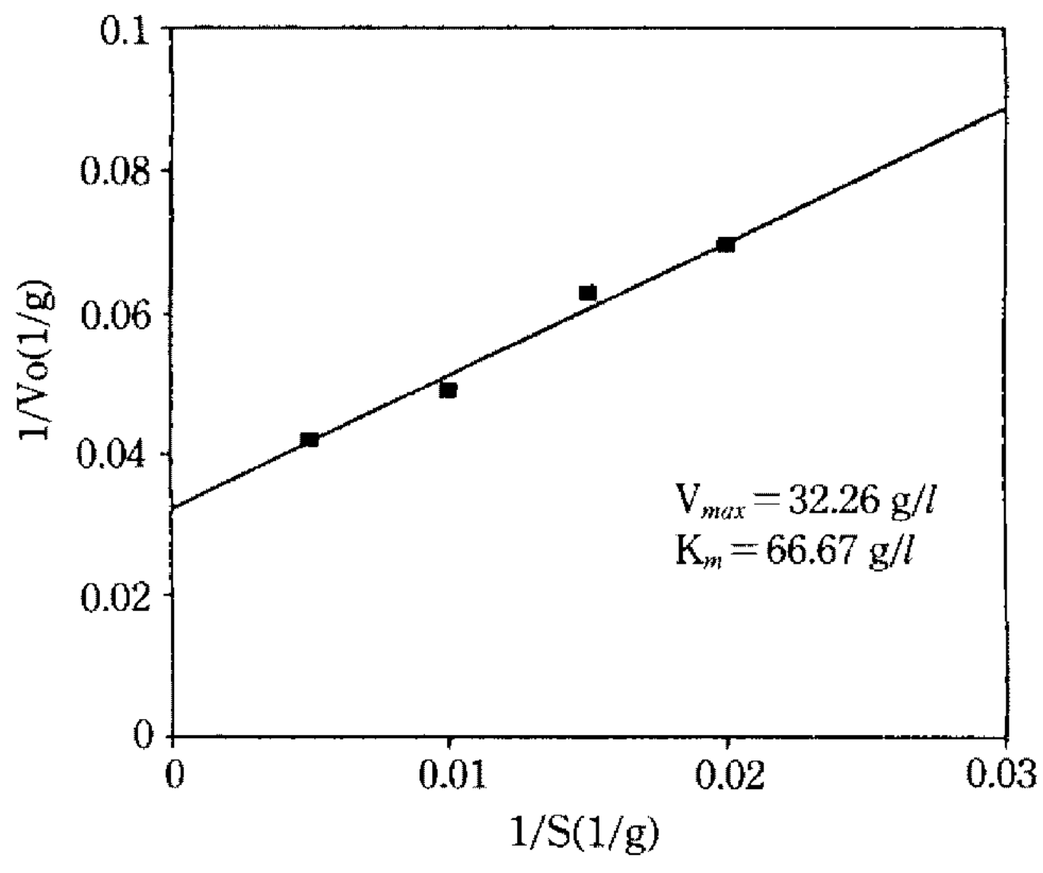

Fig. 4. Determination of k_m and V_{max} value of immobilized yeast (Lineweaver-Burk plot of continuous fermentation data using immobilized yeast).

$$P_E = P \times D$$

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

$\mu_n = \mu_{max}\ (S/(k_s + S))$

- μ_{max} : Maximum specific growth rate
- S : Substrate concentration (g/*l*)
- k_s : Michalis constant

Maximum specific growth rate for immobilized cells (μ_{max}) was computed by below plotting (Fig. 3).

Effective diffusion coefficient

$$D_e/D_o = k_1(1 - K_2 \times a1/C_c)^2$$

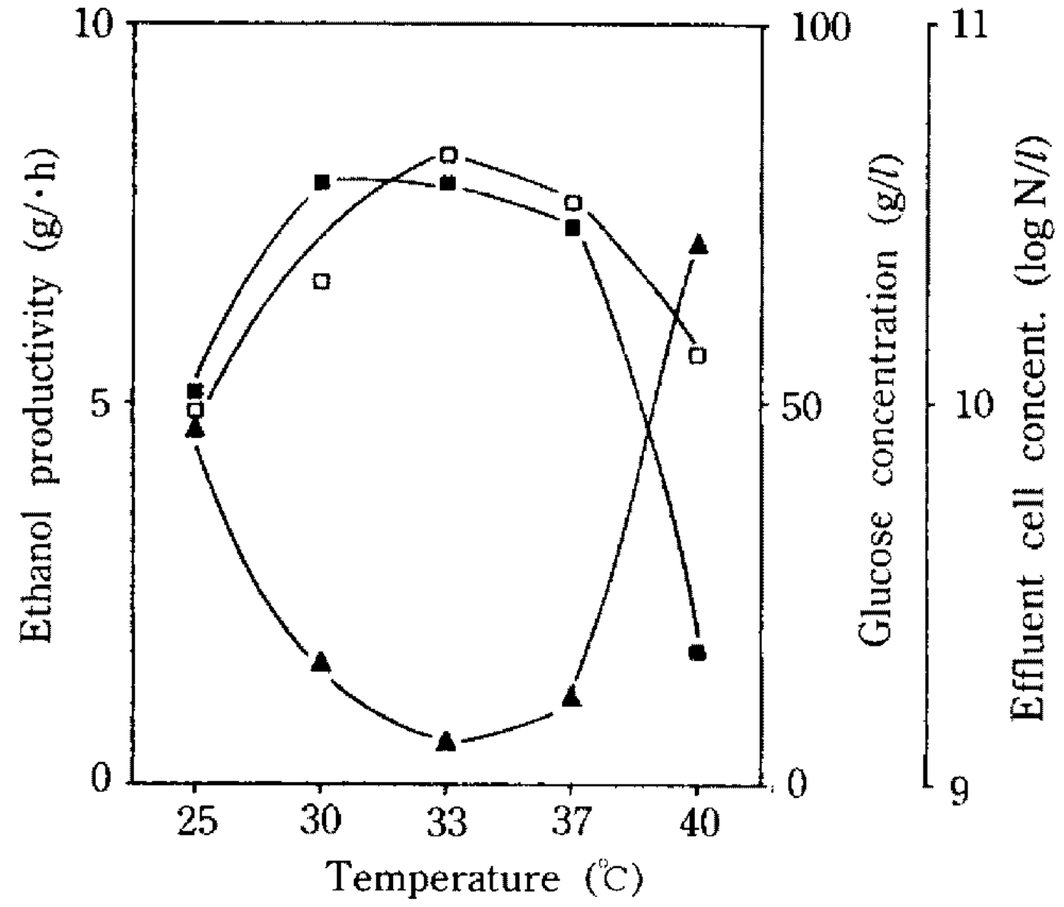

Fig. 5. Effect of temperature in DR 0.2 h^{-1} of the continuous fermentation.
−■− : ethanol, −□− : glucose and −▲− : cell

- D_e : Substrate effective diffusion coefficient in bead gel
- D_o : Diffusion coefficient of glucose at 30 ℃ ($5.01 \times 10^{-4}\ cm^2/min.$)
- K_1 : Constant
- k_2 : Constant
- C_c : Cell concentration in bead gel

Substrate uptake rate of immobilized cell (h^{-1})
$-ts = (V_m \times S)/(K_m + S)$: Michaelis−Menten equation (Fig. 4).

Effect of temperature

The effect of temperature on the ethanol productivity, glucose and cell concentration with immobilized cell systems was examined. In continuous fermentation, the effect of temperature are shown in Fig. 5. For temperature range between 25℃ and 40℃, maximum ethanol productivity was shown in 30 and 33℃, maximum cell concentration and glucose uptake rate was shown at 33℃.

Considering the energy cost, 30℃ was selected as an optimum temperature. These were similar with the results described by Lee *et al.* (4). But the result described by Bajpai *et al.* (19) using *Zymomonas mobilis* showed optimum temperature for ethanol production at 37℃.

Effect of initial pH of glucose solution

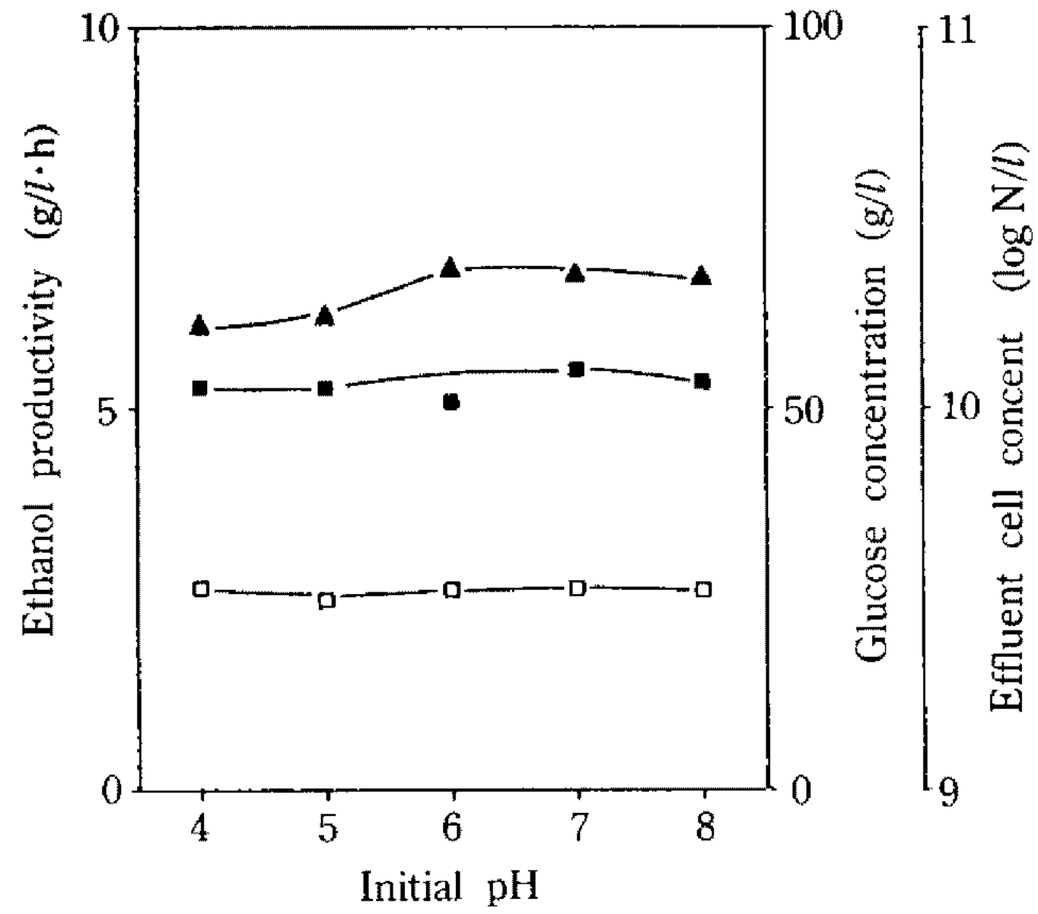

Fig. 6. Effect of initial pH of glucose concentration in DR 0.2 h^{-1} of the continuous fermentation.
—■— : ethanol, —□— : glucose and —▲— : cell

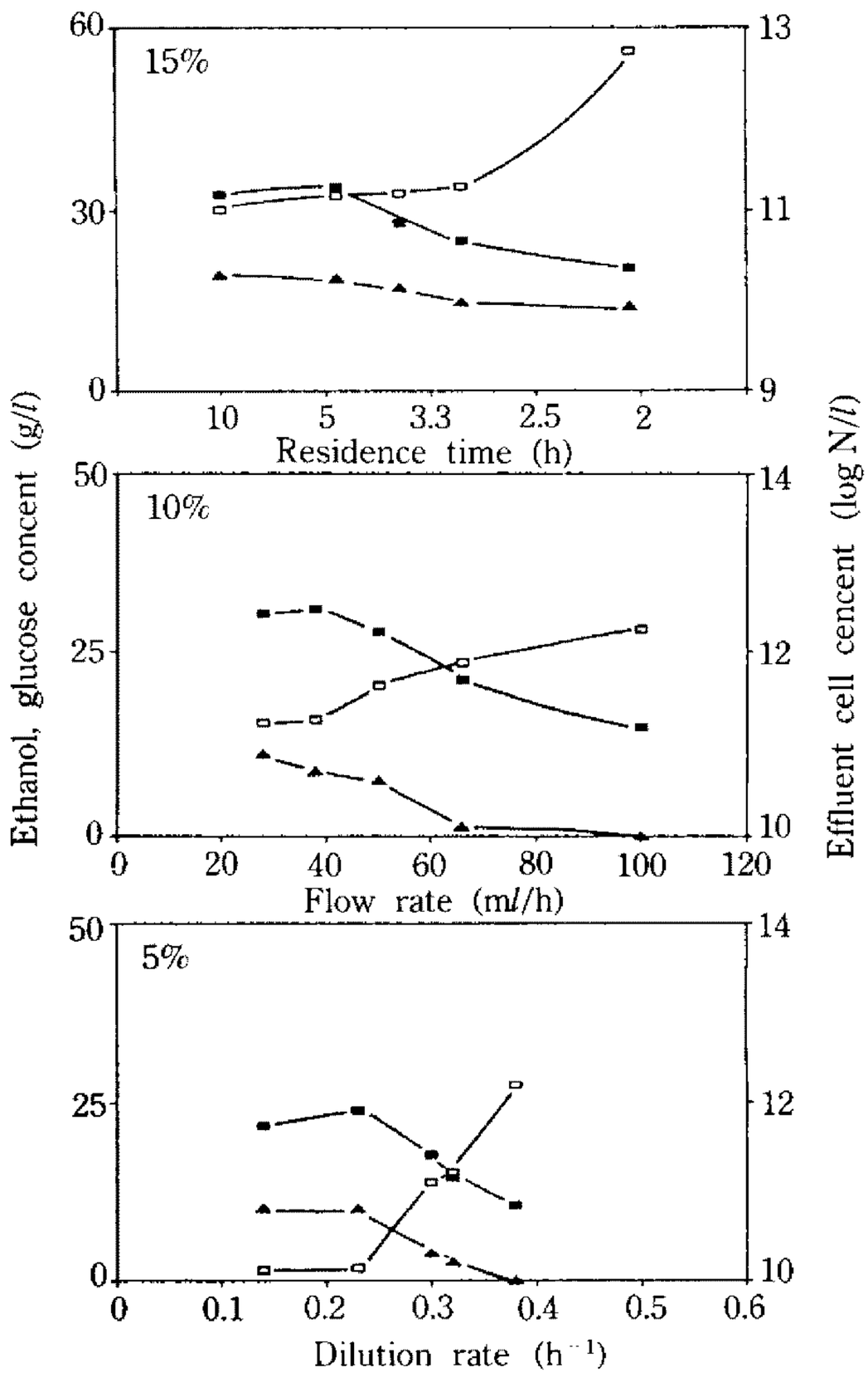

Fig. 7. Effect of dilution rate and glucose concentration in the continuous fermentation.
—■— : ethanol, —□— : glucose and —▲— : cell

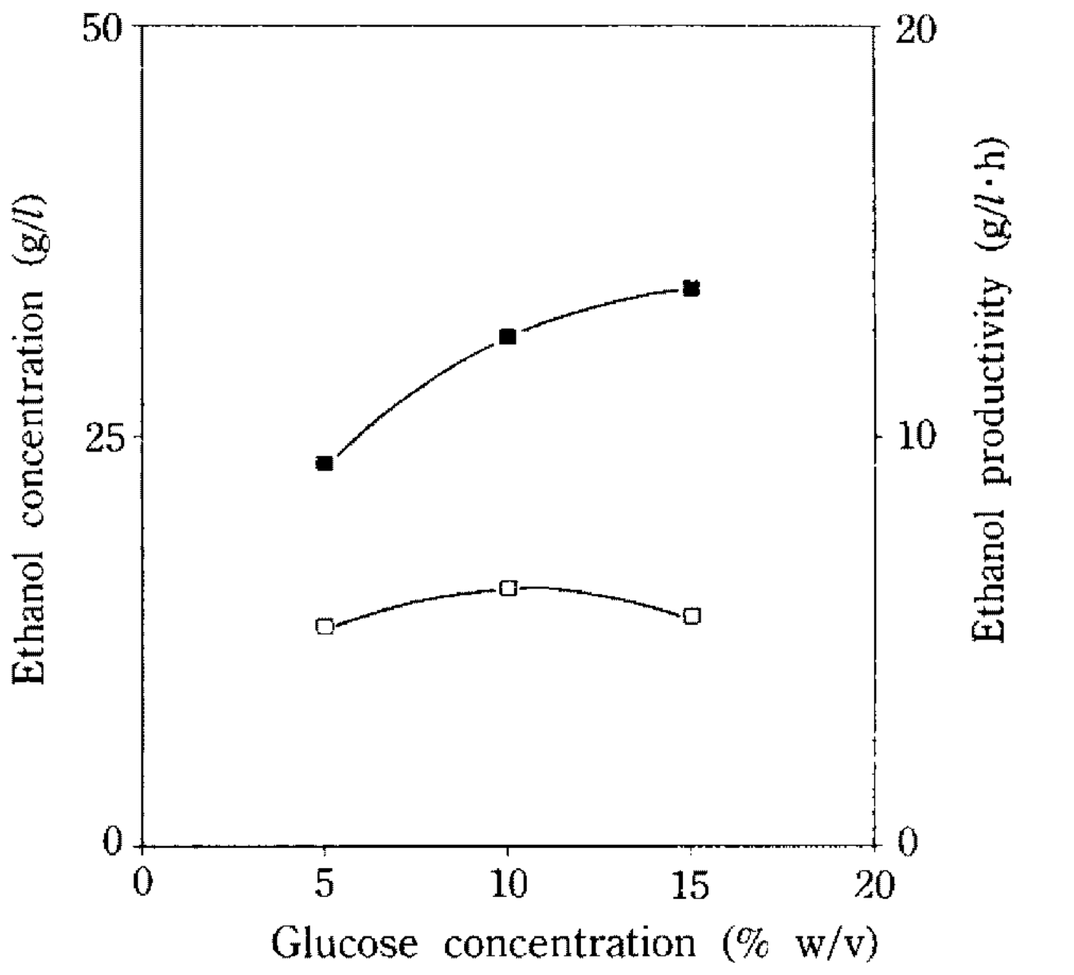

Fig. 8. The relationship of ethanol concentration and ethanol productivity according to glucose concentration (D. R. 0.2 h^{-1}) in the continuous fermentation.
—■— : concentration and —□— : yield

The effect of initial pH of glucose solution on the ethanol productivity, glucose and cell concentration with immobilized cell systems was examimed. In continuous fermentation, the effect of initial pH of glucose solution are shown in Fig. 6. Ethanol production was stable at the pH range between pH 4.0 and pH 8.0. But in the result of Bajpai *et al.* (19). Using *Zymomonas. mobilis*, pH 4, 7 and 8.0 were significantly lower than pH 5.0 on ethanol productivity.

Effect of dilution rate and glucose concentration

The effect of dilution rate and glucose concentration on the ethanol productivity, glucose concentration, cell concentration, ethanol yeild coefficient, specific glucose uptake rate and ethanol productivity in the immobilized cell systems was examined. In continuous fermentation, the results to find effect of dilution rate and glucose concentration are shown in Fig. 7~9 and Table 2.

In Fig. 7, Optimum dilution rate (DR) was shown in D.R. about 0.2 h^{-1}. These were similar with described by Del Rosario *et al.* (20) and Lee *et al.* (5). The result described by Tyagi *et al.* (7) using molasses as a substrate showed DR. 0.35 h^{-1} of optimum dilution rate on ethanol production. But optimum

dilution rate of DR. 4.0, 5.0 and 6.0 h^{-1} were shown in the result of Dostalek *et al.* (21), Prince *et al.* (22) and Cysewski *et al.* (23), respectively. In Fig. 8 and Fig. 9, optimum glucose concentration was shown in 15% of glucose solution which gave the Maximum specific ethanol productivity.

Table 3 showed kinetic parameters on dilution rate and glucose concentration.

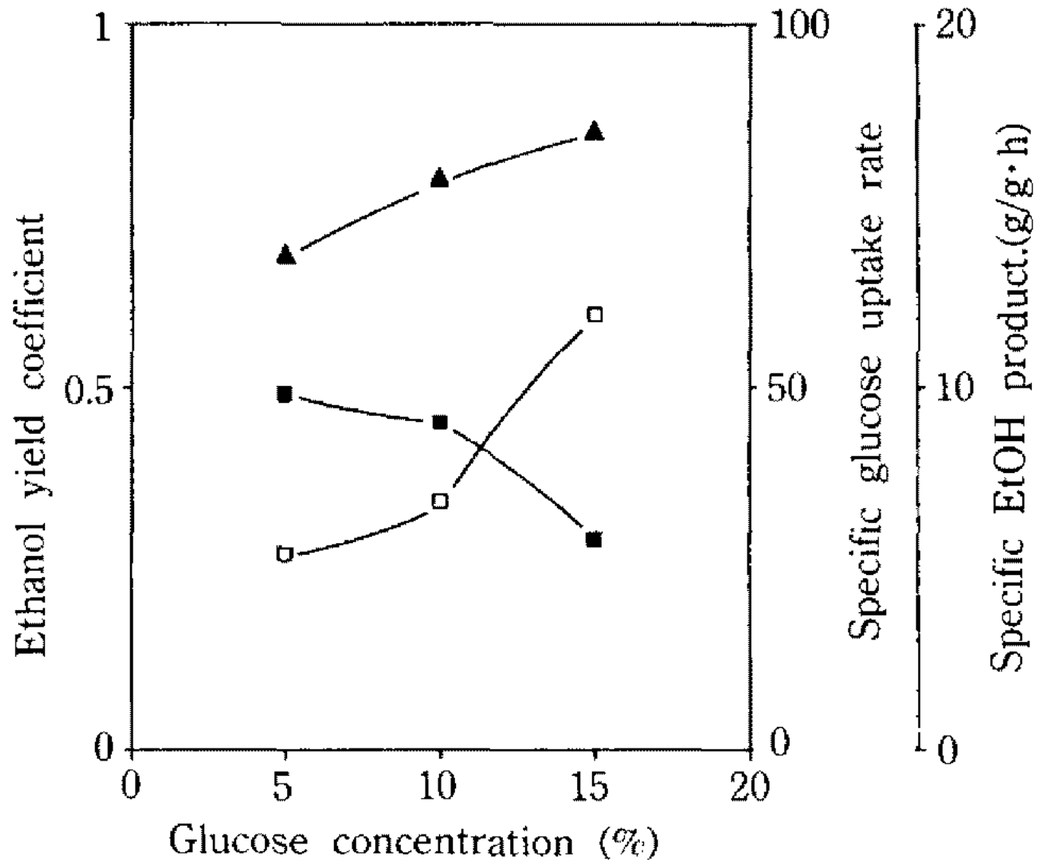

Fig. 9. Effect of glucose concentration (DR. 0.2) in the continuous fermentation.
—■— : ethanol yield, —□— : productivity and —■— : glucose

Compare of Ethanol fermentation at optimum condition

The fermentation was carried out in batch and continuous systems under the optimum conditions. Table 4 showed kinetic parameters of batch and

Table 4. Kinetics of batch and continuous ethanol fermentation at optimum condition

Parameters	Batch	CSFR
q_s	2.38	1.93
q_p	1.08	0.56
%C	99.20	78.43
P	67.50	33.90
P_E	1.99	7.12
η(%)	88.24	68.86
Y	0.45	0.23
G	-	1.62
$Y_{p/s}$	0.46	0.28
X_f	10.06	10.28
μ_m	0.09	0.365
-rs	34.09	22.334
X_b	6.95	7.0

Batch : 15% glucose solution, pH 5.0, temperature 30℃, fermentation time 34 hr. (17)
Continuous : 15% glucose solution, pH 5.0, temperature 30℃, dilution rate 0.21

Table 3. Kinetic parameters on effect of dilution rate and glucose concentration

Parameters G	DR	q_s g/g·h	q_p g/g·h	%C %	P g/*l*	P_E g/g·*l*	η %	Y	G g/*l*·h	$Y_{p/s}$	X_f log N/*l*
5%	0.14	0.12	0.05	97.88	21.7	3.04	84.93	0.43	0.11	0.44	10.82
	0.23	0.23	0.12	96.34	23.84	5.48	93.49	0.48	0.09	0.50	10.81
	0.3	0.84	0.41	72.32	17.50	5.25	68.63	0.35	1.15	0.48	10.35
	0.32	1.24	0.52	69.24	14.54	4.65	57.02	0.29	1.53	0.42	10.24
	0.38	1.66	0.76	45.08	10.51	3.99	41.22	0.21	3.43	0.46	10.03
10%	0.14	0.16	0.06	84.59	30.22	4.23	59.14	0.30	0.38	0.34	10.90
	0.19	0.42	0.16	84.18	30.98	5.88	60.62	0.31	0.79	0.37	10.79
	0.25	0.87	0.31	79.58	27.82	6.96	54.44	0.29	1.70	0.35	10.68
	0.33	3.82	1.06	76.44	21.16	6.98	41.41	0.21	2.35	0.27	10.13
	0.5	5.62	1.15	71.94	14.72	7.36	28.81	0.14	3.51	0.20	10.00
15%	0.1	0.94	0.26	79.92	32.68	3.27	42.64	0.21	0.75	0.27	10.29
	0.21	1.94	0.56	78.43	33.90	7.12	44.23	0.23	1.60	0.29	10.29
	0.27	3.88	0.92	78.15	27.86	7.52	36.34	0.19	2.52	0.24	10.16
	0.33	7.48	1.61	77.41	25.06	8.27	32.69	0.16	3.39	0.22	9.99
	0.49	8.43	1.84	62.61	20.52	10.05	26.77	0.14	7.00	0.21	9.94

*G : glucose concentration
*DR : dilution rate

continuous fermentation at optimum condition.

Considering the conversion rate and ethanol concentration, batch fermentation was superior to continuous fermentation. But in the view of ethanol productivity, 7.12 (g/*l*·h) of continuous fermentation was higher than 1.99 (g/*l*·h) batch fermentation.

Therefore, continuous fermentation was superior to batch fermentation because ethanol productivity is the most important parameter.

요 약

효모를 Na-alginate에 고정화한 후 연속반응기를 이용한 glucose 발효로 에탄올을 생산하였다.

그 결과 고정화 효모의 활성화 시간은 20~25시간이었다. 연속발효에서 고정화효모의 온도안정성은 30~37℃였으며 pH 안정성은 pH 4.0~pH 8.0, 최적 희석속도는 0.2 h^{-1}이었고 에탄올생산 최적 당농도는 15%였다. 최적조건에서 에탄올수율은 0.23, 생산된 에탄올 농도는 33.90 g/*l* 그리고 에탄올생산성은 7.12 g/*l*·h로 각각 나타났다.

Nomenclature

S : Substrate concentration (g/*l*)
T : Time (h)
D : Dilution rate (h^{-1})
G : Glucose output rate (g/*l*·h)
P : Ethanol concentration (g/*l*)
P_E : Ethanol productivity (g/*l*·h)
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 (logN/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 cofficient
μ : Specific growth rate
K_s : Michaelis constant
μ_{max} : Maximum specific growth rate
$-r_s$: substrate uptake rate of immobilized cell (h^{-1})
V_{max} : maximum specific reaction rate (g/*l*·h)
K_m : Michaelis constant (g/*l*)

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