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Growth Characteristics of Immobilized Yeast Cells in Continuous Ethanol Fermentation with Forced Substrate Supply

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The cell density distribution of *Saccharomyces cerevisiae* IFO 2347 in an immobilized cell layer was analyzed during ethanol fermentation with forced substrate supply. The cells were immobilized in a mixed gel composed of κ-carrageenan, locust bean gum and celite (2 : 0.5 : 40 wt/vol %) with an initial cell concentration of 4×10^8 cells/ml gel.

When a medium containing 200 g/*l* of glucose was forced through the immobilized cell layer (thickness = 14 mm) with a retention time of 1.5 h, the yeast cells grew homogeneously, reaching 2×10^9 cells/ml gel in the first 24 h. After 24 h, the cells continued to grow, and a cell density gradient was formed in the vertical direction of the gel layer. Finally, a steady state was attained at 250 h of operation, with 1.7×10^{10} cells/ml at the surface and 4×10^9 cells/ml at the bottom of the gel layer. At the steady state, glucose supplied was almost completely consumed, and 96 g/*l* of ethanol was produced.

The cellular distribution in the gel layer was analyzed by model equations taking in account the inhibitory and toxic effects of ethanol on the growth of yeast cells.

In the previous work,[1] we proposed a novel system in which yeast cells were immobilized in a gel layer with forced substrate supply. With this system, it was possible to eliminate the diffusional dependency of the substrate supply that exists in a normal immobilized cell reactor, and as a result, although a vertical gradient of cellular distribution was formed, a high cell density was maintained in the gel layer with an average value of *ca.* 8×10^9 cells/ml gel. Ethanol productivity was raised 4-fold compared with the normal immobilized cell system.

The aim of this work is to analyze the vertical gradient of cellular distribution in the immobilized cell layer with forced substrate supply.

Materials and Methods

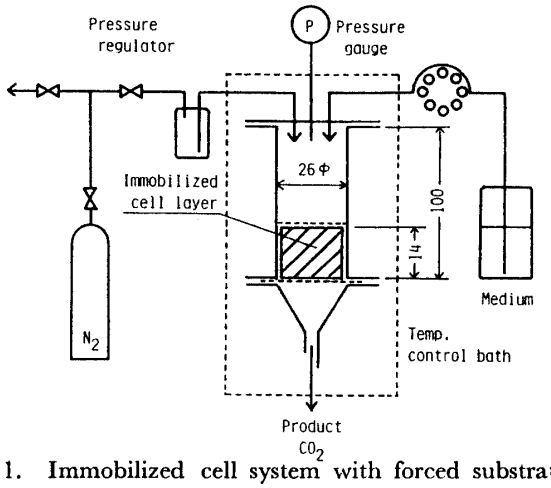
Microorganism *Saccharomyces cerevisiae* IFO 2347 was used.

Medium composition *S. cerevisiae* was precultured in a complex medium composed of 10 g of glucose, 5 g of peptone, 3 g of yeast extract, and 3 g of malt extract in 1 *l* of tap water. For continuous ethanol fermentation, the following medium was used[2] (g/*l* of tap water): glucose, 200; yeast extract, 3.0; NH_4Cl, 2.5; K_2HPO_4, 5.5; $MgSO_4\cdot7H_2O$, 0.25; NaCl, 1.0; $CaCl_2$, 0.01; and citric acid, 3.0.

Immobilization of cells Yeast cells precultured by shaking culture at 28°C for 18 h were immobilized in a mixture of κ-carrageenan, locust bean gum and celite (2 : 0.5 : 40 wt/vol %) as described in the previous paper.[1]

Bioreactor The immobilized cell preparation (26 mmφ, 14 mm thickness) was sandwiched between two stainless steel nets (26 mmφ, 1 mm meshes) in the cylindrical glass reactor (26 mmφ, 100 mm length; Fig. 1). The reactor was set in a chamber with controlled temperature (28°C), N_2 gas charged into the reactor to give a pressure of 0.2 kg/cm², and the feeding of medium was started. Fresh medium was supplied continuously to the top of the reactor *via* a peristaltic pump, and the culture liquid and CO_2 evolved were discharged from the bottom of the reactor due to the N_2 pressure. Subsequently, the pressure in the reactor was manually controlled to keep a steady flow of liquid through the immobilized cell layer.

Continuous fermentation A series of continuous fermentations[1] was carried out at a constant retention time of 1.5 h, with a glucose concentration in



Fi. 1. Immobilized cell system with forced substrate supply.

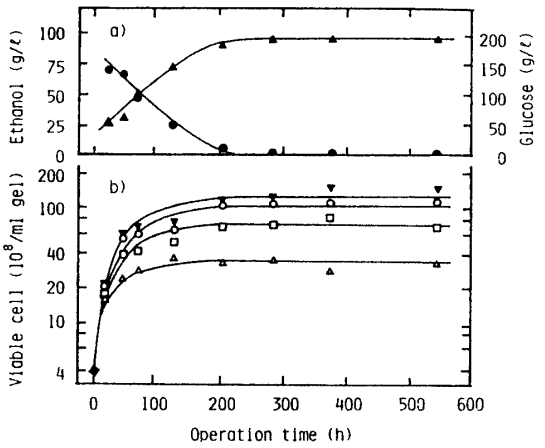


Fig. 2. Concentrations of glucose and ethanol discharged in the effluent (a) and cell density distribution in the gel layer (b) against operation time (data from Table 1).
●, Residual glucose; ▲, Ethanol produced.
◆, Initial cell concentration. Viable cell concentration in the gel layer at the following depth: ▼, 1 mm; ○, 2 mm; □, 6 mm; △, 13 mm.

the feed medium of 200 g/*l* and a gel thickness of 14 mm.

Analyses The distribution of the immobilized cells was measured as follows: after a continuous fermentation, the immobilized cell layer was cut into horizontal slices of 0.5–4 mm in thickness, and each slice was crushed in physiological saline and melted at 40°C. The total cell number in each suspension was counted under the microscope, and the cell viability was measured by the Fink-Kühles method.[3] Concentrations of glucose and ethanol were measured by methods described elsewhere.[1]

Results and Discussion

Yeast cell growth in the gel layer To examine the cell density gradient in the immobilized gel layer, eight runs of continuous ethanol fermentation were carried out at a retention time of 1.5 h (see Table 1). Plots of cell density distribution in the gel layer, estimated graphically from the data in Table 1, against the operation period are depicted in Fig. 2.

The residual glucose concentration in the effluent gradually decreased to almost zero after 200 h of operation, whereas ethanol production rose over the same period, attaining a stable ethanol yield from glucose, $Y_{p/s}$, of 0.48 g ethanol/g glucose (Table 1 and Fig. 2a).

Yeast cells in the gel layer grew almost homogeneously in the layer over the first 24 h of operation (Run 1), reaching 2×10^9 cells/ml gel from the initial concentration of 4×10^8 cells/ml (Fig. 2b). After 24 h, cell growth continued but a cell density gradient was formed in the vertical direction. Finally, a steady state was established at about 250 h, with 1.7×10^{10} cells/ml in the surface zone and 4×10^9 cells/ml in the bottom zone. The average cell density of the gel layer was about 8×10^9 cells/ml gel, which was 2-4-fold higher than that in a normal immobilized cell system,[4] and the cell concentration in the effluent was about 1×10^5 cells/ml at the steady state (Runs 5–8 in Table 1) which is appreciably lower compared to that in the normal system.[4]

It was interesting that the cellular distribution in the gel was maintained without significant leakage of cells from the bottom of the gel layer in spite of the continuous supply of substrate into the gel. Therefore, kinetic analysis was conducted to examine why this cell density gradient was maintained.

Estimation of ethanol and glucose in the gel layer Before analysis of cell density gradient, the profile of ethanol and glucose concentrations in the gel layer was estimated on the basis of the cell density observed in Table 1.

Ethanol production rate is expressed by Eq. 1.

$$\frac{dC_p}{dt}=Q_pC_x \qquad (1)$$

Table 1. Cell density distribution in the gel layer and concentrations of residual glucose and ethanol produced in the effluent as a function of operation period in continuous ethanol production with forced substrate supply.

Run	Operation period (h)	Gel depth (mm)	Cell density (10^8 cells/ml gel) Total	Viable	Residual glucose (g/l)	Ethanol produced (g/l)
1	24	0- 1.38	21.6	20.9	139.3	28.9
		1.38- 3.12	21.6	21.0		
		3.12- 7.11	20.4	18.6		
		7.11-13.80	18.7	16.9		
2	48	0- 1.12	67.5	65.0	134.3	32.3
		1.12- 2.68	54.2	52.2		
		2.68- 7.33	49.2	47.0		
		7.33-13.65	29.3	28.3		
3	72	0- 1.12	78.3	74.9	96.8	51.3
		1.12- 2.94	63.3	58.3		
		2.94- 7.81	50.4	45.4		
		7.81-13.54	39.0	32.3		
4	138	0- 1.53	78.0	75.0	53.1	73.1
		1.53- 3.42	63.7	60.8		
		3.42- 8.26	51.9	47.4		
		8.26-13.28	48.1	41.2		
5	212	0- 1.20	180	170	14.6	88.9
		1.20- 3.68	141	122		
		3.68- 5.17	86.3	71.9		
		5.17- 8.87	43.5	35.1		
		8.87-13.96	34.9	27.5		
6	280	0- 0.50	178	173	1.0	96.0
		0.50- 3.13	146	132		
		3.13- 6.71	91.3	78.9		
		6.71-10.81	80.4	60.3		
		10.81-14.84	63.3	40.0		
7	382	0- 1.10	188	178	1.7	95.6
		1.10- 4.84	166	155		
		4.84- 7.07	122	99.8		
		7.07- 9.64	66.7	53.6		
		9.64-13.36	36.0	31.6		
8	552	0- 0.60	170	164	1.0	97.6
		0.60- 1.45	141	130		
		1.45- 3.01	119	104		
		3.01- 7.66	95.2	76.2		
		7.66-13.91	55.0	40.6		

In each run, the initial cell concentration in the gel was $3\text{–}4\times10^8$ cells/ml gel, and 200 g/l of glucose medium was supplied with a retention time of 1.5 h.

where C_p : ethanol concentration, g/l
Q_p : specific rate of ethanol production, h^{-1}
C_x : viable cell concentration in the gel layer, g/l
t : time, h

If it is assumed that the liquid passes through the gel layer with plug flow at a constant velocity, $\bar{U}$, the retention time of the liquid in the i-th small slice of the gel, Δt_i, which is equivalent to the time liquid takes to pass through the i-th gel slice, is defined as

$$\Delta t_i=\frac{\Delta\delta_i}{\bar{U}}=t_i-t_{i-1} \quad (2)$$

where $\Delta\delta_i$: thickness of i-th small slice of the gel layer, mm
$\bar{U}$: linear velocity of the liquid in the gel layer, mm/h

Therefore, by assuming that the cell density in the i-th slice, C_{xi}, is constant, ethanol concentration at the end of the i-th slice in the gel layer, C_{pi}, is given by the following equation, which is based on Eqs. 1 and 2.

$$C_{pi}=\int_0^{t_1} Q_p C_{x1}\mathrm{d}t + \int_{t_1}^{t_2} Q_p C_{x2}\mathrm{d}t+\cdots+\int_{t_{i-1}}^{t_i} Q_p C_{xi}\mathrm{d}t \quad (3)$$

For the specific rate of ethanol production, Q_p, the following experimental equations have been obtained in the ethanol fermentation by this organism.[5]

$$Q_p=0.6 \qquad (0\leqslant C_p<30) \quad (4)$$

and

$$Q_p=0.84-0.008C_p \quad (30\leqslant C_p\leqslant 100) \quad (5)$$

Hence, by substituting Eqs. 4 and 5 into Eq. 3, the values of C_{pi} can be calculated.

On the other hand, glucose concentration at the end of the i-th slice in the gel layer, C_{si}, is given by Eq. 6 because the ethanol yield from glucose, $Y_{p/s}$, was constant at 0.48 g ethanol/g glucose (see Table 1).

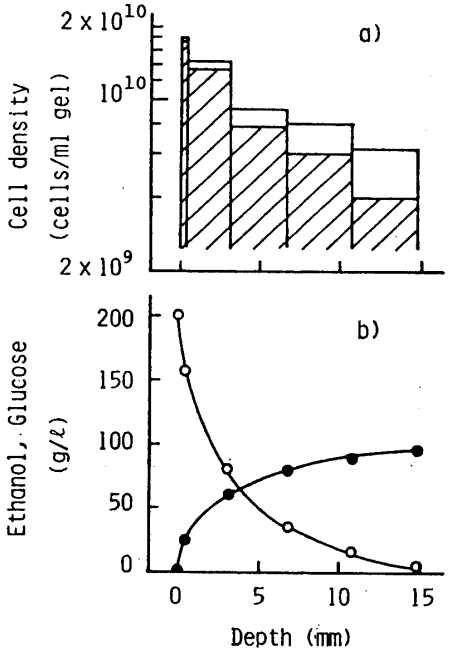


Fig. 3. Typical distribution of total and viable cells (a) and profile of ethanol and glucose (b) in the gel layer at steady state of the continuous ethanol fermentation with forced substrate supply. The histogram shows the total cell number of unit gel volume in a gel slice and the shaded portion indicates the viable cell number (data from Run 6 in Table 1).
●, Ethanol concentration at the end of each gel slice, calculated from Eq. 3; ○, Glucose concentration corresponding to ethanol produced, calculated from Eq. 6.

$$C_{si}=C_{s0}-\frac{C_{pi}}{Y_{p/s}} \quad (6)$$

where C_{s0}: glucose concentration in the feed medium, g/l

As an example of the estimation of C_{pi} and C_{si}, Run 6 in Table 1 was adopted (see Fig. 3). By using the viable cell concentration in each slice (10^9 cells=25 g dry cell), the ethanol concentration at the end of each slice was calculated from Eq. 3. With these values, and the assumption that C_{s0}=200 g/l and $Y_{p/s}$=0.48, glucose concentration was estimated from Eq. 6. The calculated values of ethanol and glucose at the end of the bottom slice (15 mm depth in Fig. 3b) were 95.6 g/l and 0.8 g/l, almost the same as the experimental values observed (Table 1). This suggested that this method is valid for estimating the profile of ethanol and glucose concentrations in the gel layer.

Estimation of cell distribution in the gel layer The results in Fig. 3b suggested that there might be enough glucose running through the gel layer to support cell growth. We therefore examined why the cell density gradient was formed in the gel layer despite the sufficient glucose supply.

If the cellular growth rate is a function of the concentrations of glucose, ethanol and cell density in the gel layer, it can be expressed as follows:

$$\frac{\mathrm{d}C_x}{\mathrm{d}t}=\mu_r C_x \quad (7)$$

$$\mu_r=\mu_{r0} f(C_s)g(C_x)h(C_p) \quad (8)$$

where μ_r : real specific growth rate, h^{-1}
μ_{r0}: maximum value of μ_r, h^{-1}

First, Monod's equation was assumed for $f(C_s)$ in Eq. 8. The glucose concentration in this process (Fig. 3b) is sufficiently high compared with the value of the saturation constant, K_s, of the Monod equation, which has been estimated at, for example, 0.22 g/l.[6]

Second, for the effect of C_x on the growth in the ethanol fermentation, a critical biological space was considered since it was observed that the growth rate decreased as cell concentration approached the maximum value, C_{xm}, in the continuous fermentation

with cell recycling.[7] In fact, this was also observed in our previous work[5] on repeated batch culture of *S. cerevisiae* for ethanol production. Therefore, $g(C_x)$ in Eq. 8 can be expressed by Eq. 9.

$$g(C_x)=\left(1-\frac{C_x}{C_{xm}}\right) \tag{9}$$

Finally, for the inhibitory effect of C_p on the growth, the following empirical equation[6,8] was adopted.

$$h(C_p)=e^{-k_1 C_p} \tag{10}$$

where k_1: inhibition coefficient for growth, l/g

By substituting Eqs. 9 and 10 into Eq. 8, with the condition that $C_s \gg K_s$, the real specific growth rate can be expressed as

$$\mu_r=\mu_{r0}\left(1-\frac{C_x}{C_{xm}}\right)e^{-k_1 C_p} \tag{11}$$

On the other hand, for the death rate of yeast cells due to ethanol, the following equation has been proposed.[9]

$$-\frac{dC_x}{dt}=k_d C_x \tag{12}$$

$$k_d=k_{d0}e^{k_2 C_p} \tag{13}$$

where k_d : specific death rate, h^{-1}
k_{d0} : k_d at $C_p=0$, h^{-1}
k_2 : empirical coefficient for death, l/g

As the cell density in each gel layer remained unchanged at the steady state (Fig. 2b) and the leakage of viable cells from the gel layer was negligible, as indicated by the low concentration of viable cells in the effluent, it was considered that the rates of growth (Eq. 11) and death of the cells (Eq. 13) had to be balanced, *i.e.*, $\mu_r=k_d$. Therefore, from Eqs. 11 and 13, one obtains that

$$\mu_{r0}\left(1-\frac{C_x}{C_{xm}}\right)e^{-k_1 C_p}=k_{d0}e^{k_2 C_p} \tag{14}$$

Equation 14 can be rearranged in logarithmic form, as follows:

$$\ln\left(1-\frac{C_x}{C_{xm}}\right)=\ln\left(\frac{k_{d0}}{\mu_{r0}}\right)+(k_1+k_2)C_p \tag{15}$$

This predicts a linear relationship between $\ln(1-C_x/C_{xm})$ and C_p, through which cellular growth in the gel layer might be analyzed.

Data analysis To examine Eq. 15, the data of four runs at steady state (Runs 5–8 in Table 1) were used. For the calculation of $\ln(1-C_x/C_{xm})$ in Eq. 15, 1.8×10^{10} cells/ml gel (=350 mg dry cell/ml gel) was adopted as C_{xm}, since the viable cell concentration at the surface zone of the gel were about 1.75×10^{10} cells/ml gel (see Table 1); and the average value of viable cell density in each gel slice was used as C_x in each slice of the gel layer (see Fig. 3a). On the other hand, the values of C_p corresponding to these values of C_x were obtained at the middle point of each slice (see Fig. 3b). The results analyzed by Eq. 15 are shown in Fig. 4.

The figure shows a linear relationship between $\ln(1-C_x/C_{xm})$ and C_p, indicating that lower C_x would be caused by higher C_p. From this linear correlation, (k_1+k_2) and $\ln(k_{d0}/\mu_{r0})$ in Eq. 15 were estimated as 0.028 and −2.7, respectively. This value of (k_1+k_2) was comparable to those obtained for ethanol production by yeast cells.[8,9] However, further kinetic experiments will be required to characterize the individual constant.

It can be concluded that the yeast cells grew in the gel layer with forced substrate supply to reach 1.7×10^{10} cells/ml gel at the surface zone and 4×10^{9} cells/ml at the bottom zone, but that the cells could not reach the maximum level in the middle part of the gel, in spite of the presence of enough glucose, due to the toxic effect of ethanol on the cell

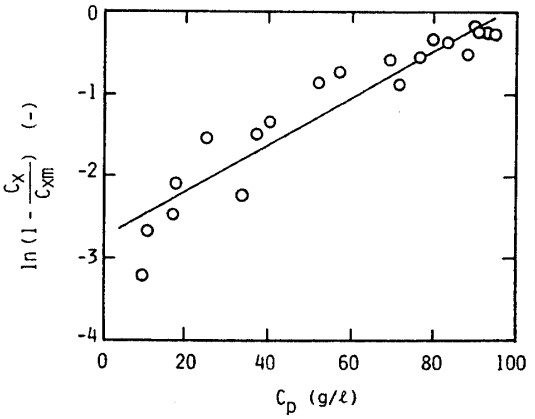


Fig. 4. Relationship between $\ln(1-C_x/C_{xm})$ and C_p based on Eq. 15 (data from Runs 5–8 in Table 1).

growth. The cell density gradient in the gel layer could be analyzed by mathematical model based on the inhibitory and perish effects of ethanol on the growth of yeast cells. In other words, the presence of the cell density gradient in the gel layer appears to be directly caused by the concentration of ethanol produced in the gel. In addition, it is indicated by this analysis that the cell density in a normal immobilized cell system, even in the surface zone of the gel beads, might be limited to around $3–5\times10^9$ cells/ml gel by the *ca.* 90 g/*l* of ethanol produced,[4] since the ethanol produced in the gel beads would stagnate around the gel beads.

Nomenclature

C_p : ethanol concentration, g/*l*
C_s : residual glucose concentration, g/*l*
C_{s0} : glucose concentration in the feed medium, g/*l*
C_x : viable cell concentration in the gel layer, cells/ml gel or mg/ml gel
C_{xm} : maximum value of C_x, cells/ml gel or mg/ml gel
k_1 : inhibition coefficient for growth, *l*/g
k_2 : empirical coefficient for death, *l*/g
k_d : specific death rate, h^{-1}
k_{d0} : k_d at $C_p=0$, h^{-1}
K_s : saturation constant, g/*l*
μ_r : real specific growth rate, h^{-1}
μ_{r0} : maximum value of μ_r, h^{-1}

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