

Fermentation Performance of Immobilized
Aspergillus niger using Polyelectrolyte Complex

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GENERAL INTRODUCTION

Bioreactor process is able to operate temperate conditions as ordinary temperature and ordinary pressure and an environmental pollution (i.e. safe and clean), although the low reaction efficiency is a problem in general. In order to obtain effectiveness equivalent of a chemical process, either an extensive installation or an immense amount of time is needed. However, it is not practical at all.

There, it is important to operate at high cell density which is very effective to improve the efficiency. As a high cell density fermentation with dry cell concentrations higher than 100 g/l of various bacteria and yeast has been operated. For example, in bacterial cells, Nakano *et al.* reported *Escherichia coli* cell density of 190 g/l (1997) [1], Park *et al.* reported *Bacillus subtilis* cell density of 184 g/l (1992) [2] and Suzuki reported *Lactococcus lactis* cell density of 141 g/l (1996) [3]. Further, in yeast cell, Yano *et al.* reported *Candida brassicae* cell density of 268 g/l (1985) [4]. In contrast, fermentations of mycelial fungi, which are widely used to produce antibiotics, enzymes and many other useful metabolites, are performed at dry cell concentration less than 20 g/l [5-7]. This is associated with the growth of mycelial cells in a highly extended and branched filamentous form. In order to solve the problem, entrapment to the calcium alginate gel beads [8] and adsorption to porosity pumice stones [9] *etc.*, has been performed. These immobilization techniques are effective in reducing the appearance viscosity. However, the problem that the mass transfer into innermost cells is limited and the cells become extinction from

inside. Moreover, support materials are weak to shear stress and cannot be used by high shear stress reactor such as a jar fermenter. For these reasons, it does not necessarily lead to improvement in productivity. Thus, novel technique is required to solve these problems and to improvement of high cell density fermentation of mycelial fungi.

In Chapter 1, an immobilization method by polyelectrolyte complex as a method of solving the decrease of the mass transfer which poses a problem by these immobilizations was established. Moreover, gluconic acid production by *Aspergillus niger* immobilized the method using a jar fermenter was carried out at high cell density (20 ~ 40 g/l) and the obtained data were compared with those free cells. In Chapter 2, I attempted to measure several important parameters in the mycelial fermentation: viscosity of broth, volumetric mass-transfer coefficient ($k_L a$), and diffusion coefficient of glucose (as a substrate) and the obtained data were compared with those free cells.

CHAPTER 1

**“A Mycelium with Polyelectrolyte Complex-Bunched Hyphae:
Preparation and Fermentation Performance”**

1.1 INTRODUCTION

One of the most serious problems in submerged mycelial processes is the very high non-Newtonian viscosity of fermentation broths. This is associated with the growth of mycelial organisms in a highly extended and branched filamentous form. There have been a lot of attempts to solve this problem, which fall into two categories: improvements in the fermentation device [10,11] and in the morphology of mycelial hypha [12-16]. For the latter, a rational approach involves immobilization of mycelial hyphae using porous beads of celite [12] and cellulose [13], gels of κ -carrageenan [14] and calcium alginate [15], and polyurethane foams [16].

A systematic study made in 1980s by Kokufuta et al has demonstrated that polyelectrolytes (PEs) or polyelectrolyte complexes (PECs) are very useful for the immobilization of enzymes and microbial cells [17-29]. In the case of a PEC-immobilized mycelial organism (*Aspergillus terreus*) [27], a marked improvement in sedimentation of mycelia was observed, although there was little difference in itaconic acid production between the immobilized and free cells in a small scale (100 ml) cultivation with an Erlenmeyer flask on a rotary shaker. From this result [27] and also from the results [17-19,21,22,26] of other PEC-immobilized cells, we set up the following working hypothesis for the immobilization of filamentous fungi through mixing of hyphae with the same equivalent of both polyanion and polycation (see Fig. 1): (a) a few parts of polycations would bind on the surface of a hypha, the process of which should

allow to gather the hyphae in part but not all; (b) such a gathering of the hyphae is tightly bunched with PEC resulting from the remaining polycation and the polyanion; (c) as a result, we may obtain filamentous fungi with partially bunched hyphae, the part of which is very stable and does not fall in pieces during the cultivation.

Taking into account this working hypothesis, here we attempted to study the gluconic acid production via a semi-large scale (1 liter) cultivation of PEC-immobilized *Aspergillus niger* cells using a jar fermentor. The present paper reports the excellent sedimentation property and the fermentation performance under a high cell density, together with the morphology of the PEC-immobilized *A. niger* cells.

1.2 MATERIALS AND METHODS

1.2.1 Chemicals

Potassium poly(vinyl alcohol) sulfate (KPVS) and trimethylammonium glycol chitosan iodide (TGCI) were commercially obtained from Wako Pure Chemical Industries (Osaka, Japan). The physical quantities (as nominal data) of both polyelectrolytes are as follows. Equivalent weight (E_W) \sim 162 for KPVS and \sim 375 for TGCI; and the degree of polymerization (DP) \sim 1500 for KPVS and \sim 400 for TGCI. Both polymers were dissolved in distilled water and their concentrations were given in equivalent per volume (eq/ml).

1.2.2 Medium

We used two media; a basal medium for growth of the mycelial organism and a fermentation medium for gluconic acid production. The composition (in g/l) of the former was: glucose, 30; yeast extract, 9; polypeptone, 15; CaCO₃, 4; pH 7. The latter composition (in g/l) was: glucose, 100, MgSO₄·7H₂O, 0.15; KH₂PO₄, 0.2; Na₂HPO₄, 0.4; pH 6. Note that the nitrogen source was not added to the fermentation medium to suppress the cell growth during the gluconic acid production.

1.2.3 Strain and cultivation

Aspergillus niger IFO 31012 was used in this study. The sporulated culture on a malt agar slant in a 500 ml Erlenmeyer flask was mixed with 200 ml of the

basal medium and carefully scraped off by a glass rod. The inoculum was transferred to a 500 ml Erlenmeyer flask and incubated at 30 °C for 2 days on a rotary shaker. The mycelial organism obtained by incubation was harvested by suction filtration and washed well with sterile distilled water.

1.2.4 Immobilization

The harvested microorganism (10 g by dry weight) was suspended into 600 ml of sterile distilled water. The immobilization was performed by addition of aqueous TGCI solution (100 ml; 130 meq/l), soon followed by the KPVS solution of the same volume and the same concentration to the mycelial suspension under stirring (500 rpm). These procedures were completed within 10 min because of a rapid formation rate of PEC. The PEC-immobilized *A. niger* cells were then separated by suction filtration and washed well with sterile distilled water. In the preparation of the immobilized samples for sedimentation measurements, different volumes of the above polyelectrolyte solutions were added to the same cell suspension as used above.

1.2.5 Morphological observations and sedimentation measurements

Morphology was studied by optical and electron microscopes. The specimens were prepared according to the same procedures as those described in our previous papers [19,20,28].

The sedimentation rate was determined as a function of the weight ratio of PEC to dry cells. The measurements were carried out using a graduated test

tube fulfilled with distilled water. A few grams of the immobilized cell were dropped from the top of the test tube, then the drop rate was measured between 5 cm and 15 cm from the top to the bottom. The measurements were repeated ten times separately, and the eight data were averaged after the removal of the most fastest and slowest data.

1.2.6 Gluconic acid production

Different weights of the PEC-immobilized *A. niger* cells were incubated in 1 liter of the fermentation medium at 30 °C under stirring (1000 rpm), using a computer-controlled jar fermentor as shown in Fig. 2. During the incubation, the pH of the fermentation broth was adjusted to ca.6 with 8 N NaOH. Also adjusted by the flow of O₂ was the level (35±3 mg/l) of dissolved oxygen (DO). The concentration of gluconic acid was determined by a HPLC apparatus (Shimazu model HIC-6A) equipped with a UV monitor (Shimazu model SPD-6AV). A column packed with TSK gel SCX(H+) (Tosoh Co., Tokyo, Japan) was employed. The glucose concentration was measured using a Wako Glucose CII-Test kit (Wako Pure Chemical Industries, Osaka, Japan).

1.3 RESULTS AND DISCUSSION

1.3.1 Preparation and morphology of PEC-immobilized filamentous fungi

As was mentioned in the Introduction, the binding of polyions on the surface of a hypha is very important in our immobilization method, because this seems to allow the gathering of the hyphae. Thus, we studied the adsorption of KPVS and TGCI onto the surface of the mycelial cell as a function of pH. As shown in Fig. 3, KPVS preferentially adsorbs on the cell in the acidic pH region; the adsorbed amount decreases with increasing pH and becomes zero at $\text{pH} > 7$. In contrast to the polyanion, an enhanced adsorption of the polycation is observed on the alkaline pH side. These results indicate an electrostatic interaction of the polyions with the acidic or basic groups attached to the surface of mycelial cells; that is, decreasing pH promotes the ionization of the basic groups to enhance the adsorption of the KPVS anions, but increasing pH favors the adsorption of the TGCI cations via the dissociation of the acidic groups. This is because both polyelectrolytes used completely dissociate in the pH 2 to 12 range [31].

These aspects are little different from those of bacterial cells such as *Escherichia coli* [20] and *Nitrosomonas europaea* [19]; that is, at a neutral pH range (6 to 7), the adsorbed amount of polycations is much larger than that of polyanions. From microscopic observations, however, it was found that the addition of polycations to cell suspensions under such pH conditions shows a

marked difference between bacterial and mycelial cells [19,20,28]. The cessation of bacterial cell motion is followed by the aggregation as soon as polycations such as TGCI are added. In the case of mycelial cells [28], the flocculation was observed upon addition of polycations due to the entanglement of branched hyphal filaments. Note that in our experiments [28] using *A. terreus*, polyanion acted as a flocculating agent, but polycation was a dispersing agent, because the addition of the polymer was done at pH 2.2. From these results, it is clear that the adsorbed polyelectrolyte on the cell surface leads to the aggregation or gathering of cells, presumably due to either or both of cell-cell bridging and a decrease in the hydrophilic nature of cell surfaces.

Upon addition of polyanions (KPVS in this case), the polycations which are remaining in the cell suspension without participation in the adsorption form PEC with the added polyions. This allows the entrapment of aggregated or flocculated cells within the resulting PEC (see Fig. 1). In this process, however, there is a clear difference between bacterial and mycelial cells. As can be seen from the optical and electronmicroscopic pictures in Fig. 4, whole bacterial cells aggregated are within a gel-like body of PEC (see (a) to (c) in Fig. 4). Such a whole cell immobilization should not be possible in the case of mycelial cells due to their branched structure. Rather, aggregates of hyphae are adhered by PEC; thus, there are many of PEC-bunched hyphae within a flock of mycelial cells (see (d) to (f) in Fig. 4).

1.3.2 Sedimentation of PEC-immobilized cells

A lowering of sedimentation capability for mycelial cells is a serious problem in their uses for fermentation. Thus, we studied whether or not the sedimentation of *A. niger* cells is improved by our immobilization method using PEC. Fig. 5 shows the sedimentation rate of the PEC-immobilized *A. niger* cells as a function of the weight ratio of dry cells to polymer in the immobilized preparation. It is clear that an increase of the PEC weight in the immobilized cells facilitates their sedimentation. However, the facilitated sedimentation does not show a simple proportion to the PEC weight; in other words, the sedimentation rate trends to level off at the weight ratios larger than 0.5. This seems to be an indication that our immobilized *A. niger* cells contain many of the PEC-bunched hyphae.

1.3.3 Production rate of gluconic acid

Fig. 6 shows a typical example for time courses of glucose consumption and gluconic acid production by free and PEC-immobilized *A. niger* cells. In our experiments, the DO level becomes constant (35 ± 3 mg/l) within a half hour. Under such a condition, the glucose consumption and gluconic acid production shows symmetrical in the case not only of the free cells, but also of the immobilized cells. Thus, we may estimate the glucose oxidizing (GO) activity from a linear slope of glucose consumption or gluconic acid production (see broken lines in Fig. 6).

Table 1 shows the comparison of GO activities for the free and immobilized cells, the values of which were obtained from the fermentation under very high cell density (Note that mycelial fermentations are usually performed at dry cell concentrations <10 g/l [32-34]). We obtain an “apparent” specific GO activity by dividing gluconic acid production rate by dry cell concentration. It is found that there is a decrease in the activity with increasing the cell concentration in both the free and immobilized cell systems. This phenomenon is usual in submerged mycelial processes, and is attributed to the very high viscosity of fermentation broths, from which mass transfer limitations become the serious problem. However, this problem is improved by the immobilization with PEC, because the magnitude of the decrease in the apparent activity is smaller in the immobilized cell system than in the free cell system. Thus, the activity of the immobilized cells becomes 1.44 times that of the free cells even at a cell concentration of 40 g/l .

It has been reported that an apparent specific GO activity is 16.5 mmol/h/g-dry cells (3.2 g/h/g-dray cells) by the fermentation of free *A. niger* cells at a dry cell concentration of 4.9 g/l using a H₂O₂-containing medium under air [35]. This is higher than our data in Table 1; however, the use of high concentrations of the immobilized cells should be advantageous to the mycelial fermentation with respect to the productivity of gluconic acid.

The observed improvement in the mycelial fermentation seems to be due to the fact that our immobilized *A. niger* cells contain many of the PEC-bunched

hyphae. The reasons for this are: (i) there is no change in the bulk pH of the fermentation broths containing the free and immobilized cells because the incubation was carried out under a controlled pH (see the Experimental section); (ii) it is difficult to consider that the metabolic system for oxidizing the glucose in the *A. niger* cell changes due to our immobilization method which is not a whole cell immobilization; and (iii) the PEC by which the hyphae were bunched is electrostatically neutral, so that there are a few possibilities for concentrating the substrate within the PEC phase.

1.4 CONCLUSIONS

In conclusion, it has become apparent that a simple method for mycelial cell immobilization based on our working hypothesis results in a mycelium with PEC-bunched hyphae. Such an immobilized mycelium is useful for the improvement in the problems in submerged mycelial processes which are related to the very high non-Newtonian viscosity of fermentation broths.

TABLES

Table 1

Glucose Oxidizing (GO) Activities of Free and PEC-Immobilized *Aspergillus niger* Cells Obtained by Their Fermentation under High Cell Densities.

cell concentration (g-dry cells/l)	specific GO activity (mmol/h/g-dry cells)		activity ratio (immobilized cells per free cells)
	free cells	immobilized cells	
20	9.18	10.2	1.11
30	5.63	6.96	1.23
40	4.40	6.21	1.44

Figures

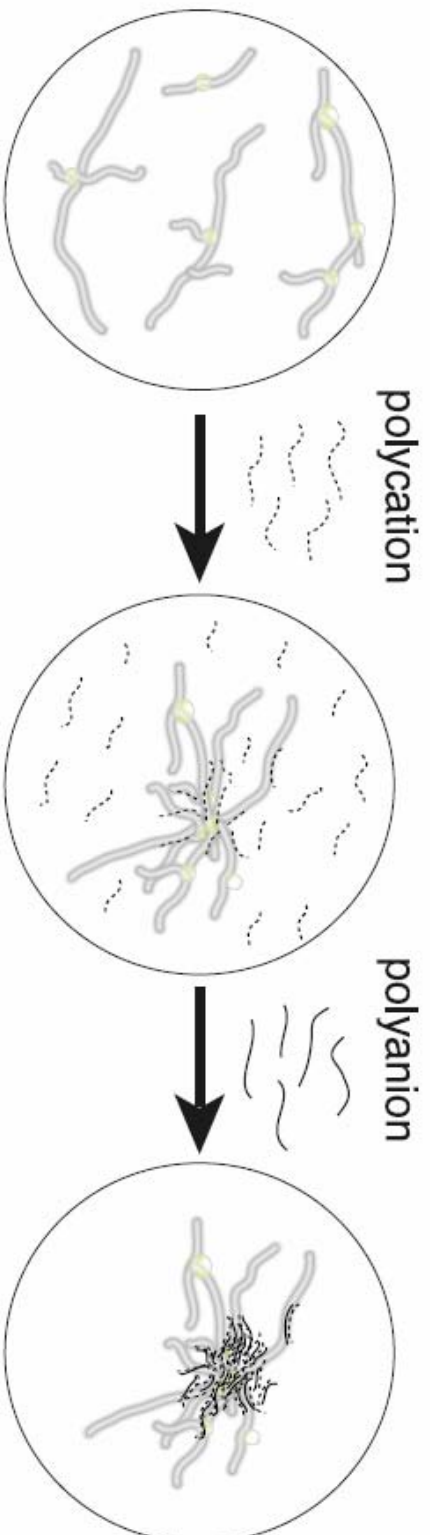


Fig. 1. Schematic illustration for the immobilization of filamentous fungi using polyelectrolyte complex based on our working hypothesis. Broken and solid line denote polycation and poly anion, respectively.

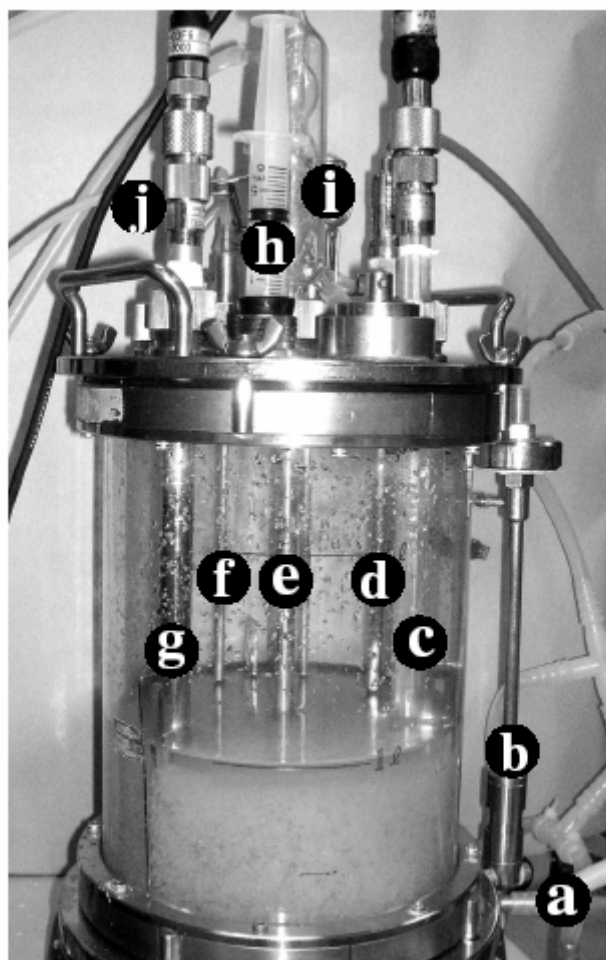


Fig. 2. Jar fermentor used for gluconic acid production using free and PEC-immobilized *Aspergillus niger* cells: a, thermostat water inlet; b, oxygen gas inlet; c, pH sensor; d, thermometer; e, disk turbine; f, sampling port; g, DO sensor; h, antifoamer (10 % silicone emulsion) inlet; i, exhaust; and j, NaOH inlet tube.

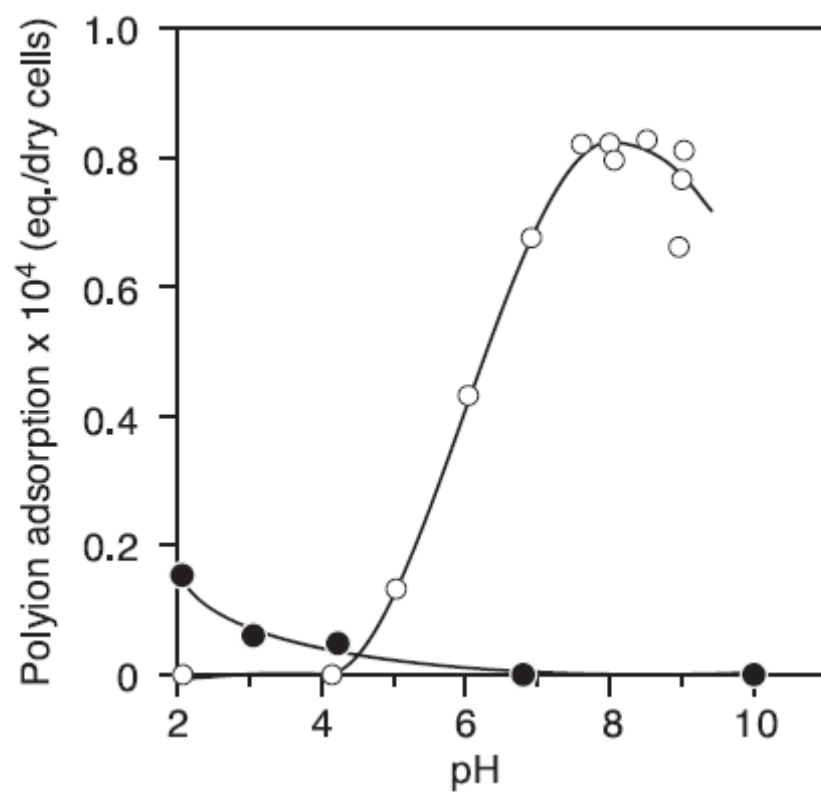


Fig. 3. pH dependence of polyelectrolyte adsorption on the dry cell surface: open circles, polycation (TGCI); and solid circles, polyanion (KPVS).

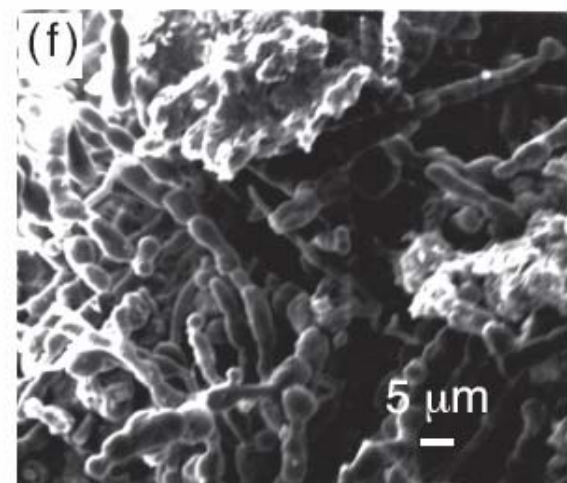
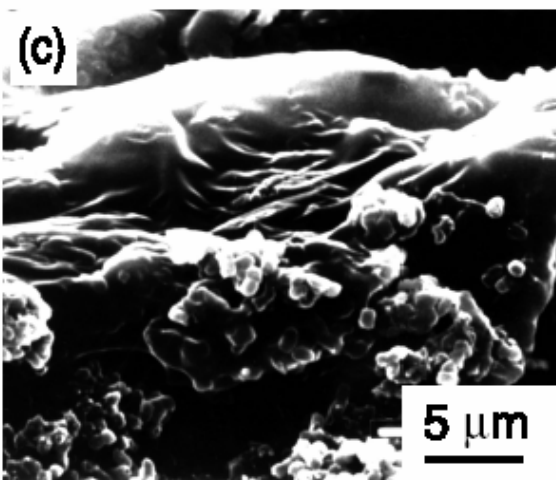
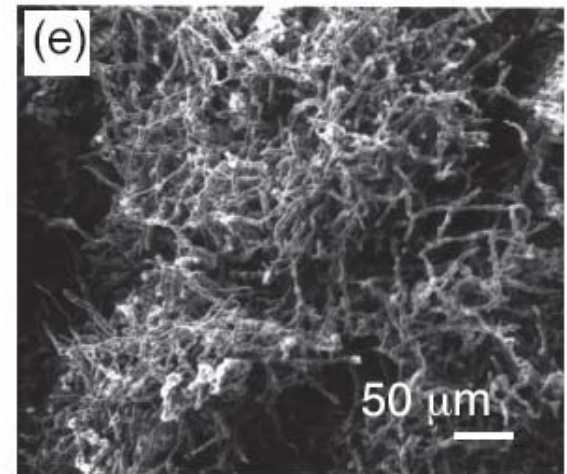
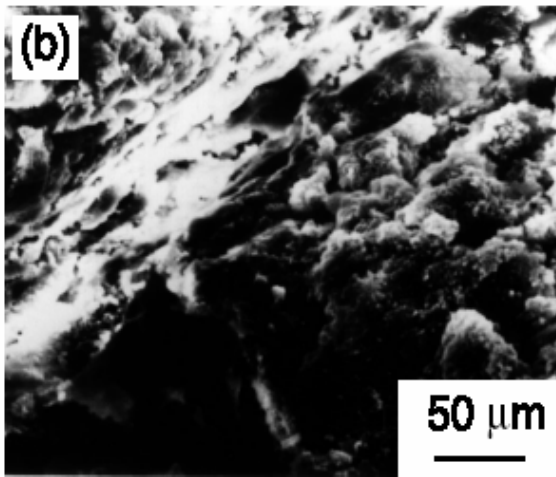
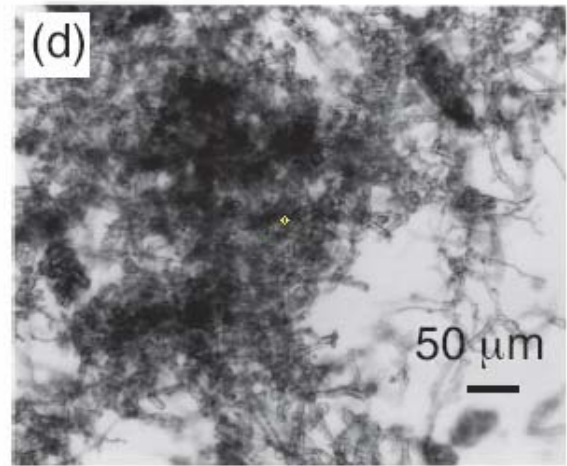
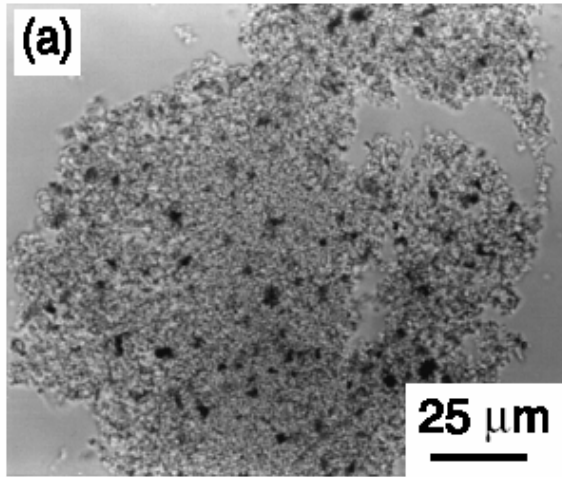


Fig.4. This figure title is next page

Fig. 4. Optical micrographs (a and d) and scanning electron micrographs (b, c, e and f) of PEC-immobilized bacterial (a ~ c) and mycelial cells (d ~ f): Micrographs a ~ c shows PEC-immobilized *Paracoccus denitrificans* (IPO 12442) as an example of bacterial cells (micrographs b and c are cited from [12]). Note that the weight ratio of PEC to dry cells for the bacterial cell immobilization is much larger than that for the mycelial cells. Also note that poly(diallyldimethylammonium chloride) was used as a polycation for the bacterial cell immobilization.

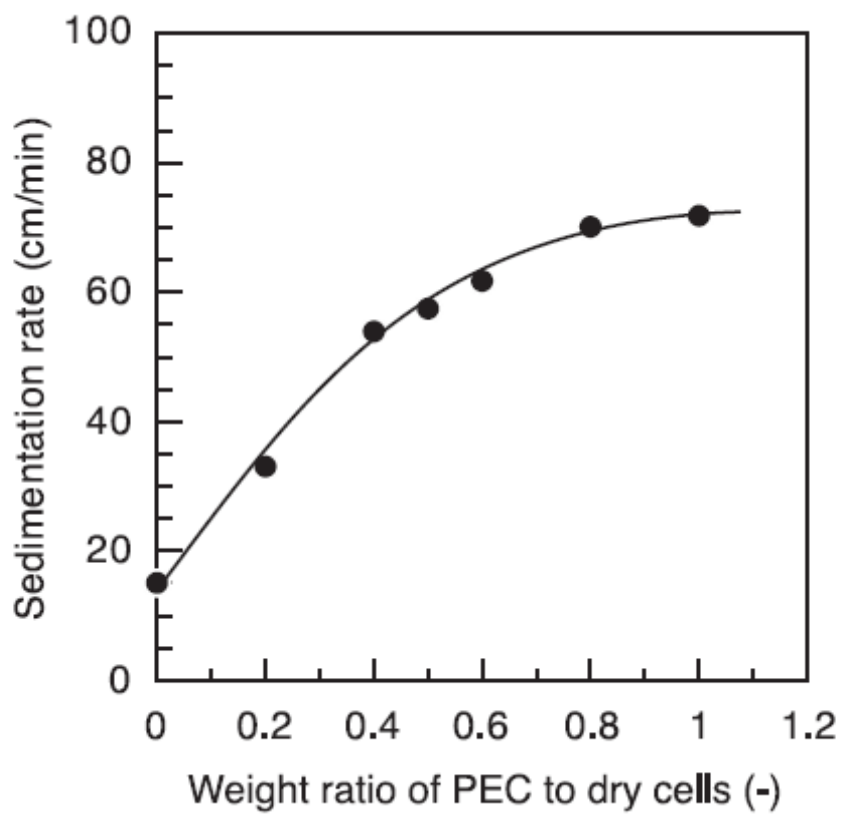


Fig. 5. Results of sedimentation measurements for PEC-immobilized *A. niger* cells. Note that zero weight ratio means the free cells.

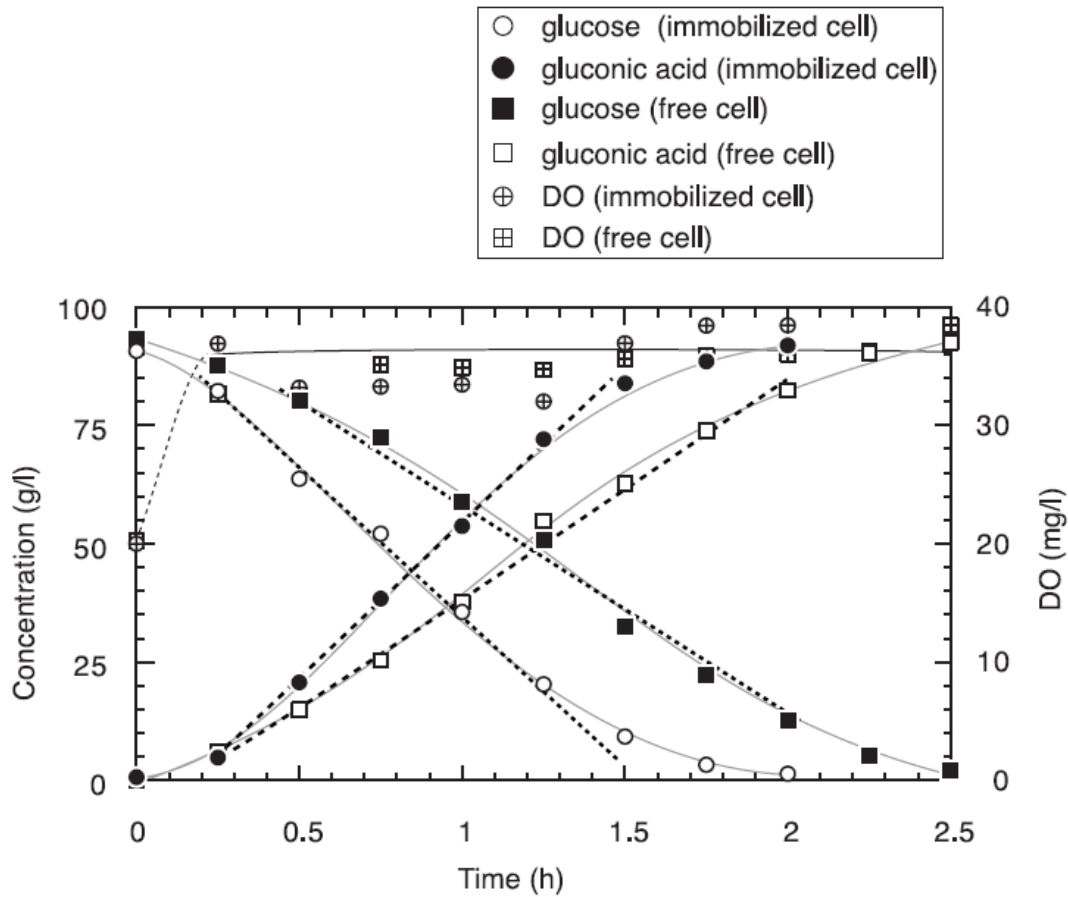


Fig. 6. Time courses of glucose consumption and gluconic acid production by free and PEC-immobilized *A. niger* cells. The cell concentration by dry weight was adjusted to 40 g/l. The other incubation conditions are described in detail in the Experimental section. The rates of glucose consumption and gluconic acid production were determined by the slope of each broken line.

CHAPTER 2

**“A Mycelium with Polyelectrolyte Complex-Bunched Hyphae:
Several important factors affecting on the fermentation Performance
at a very high cell density”**

2.1 INTRODUCTION

In order to make more clearly this enhancement of the activity, we attempted in the present study to measure several important parameters in the mycelial fermentation: viscosity of broth, mass-transfer coefficient ($k_L a$) for oxygen and diffusion coefficient of substrates. The data obtained were compared with those of free cells; then it has become apparent that an elimination of diffusional hindrance plays an important role in an enhancement of glucose oxidizing activity of the PEC-immobilized *A. niger* when the fermentation was performed at a very high cell concentration such as 40 g/l.

2.2 MATERIALS AND METHODS

2.2.1. Preparation of immobilized cells

The harvested microorganism (10 g by dry weight) was suspended into 600 ml of sterile distilled water. The immobilization was performed by addition of aqueous TGCI solution (100 ml; 130 meq/l based on cationic charges), soon followed by the KPVS solution of the same volume and the same concentration, to the mycelial suspension under stirring (500 rpm). These procedures were completed within 10 min because of a rapid formation rate of PEC. The PEC-immobilized *A. niger* cells were then separated by suction filtration and washed well with sterile distilled water.

2.2.2. Measurements of viscosity and k_La

The viscosities of the free and PEC-immobilized mycelial cells were measured using two different methods. One is with a torquemeter equipped with our computer-controlled jar fermentor (see Fig. 2 in Chapter 1), and the other is with a Brookfield viscometer (Tokyo-Keiki Co., Tokyo, Japan). For the latter, the measurements were performed at different cell concentrations and at 30 rpm, using a Tokyo-Keiki rotor #3.

The k_La for oxygen was determined from a continuous monitoring of dissolved oxygen (DO) using a DO sensor equipped with the fermentor. The fermentation

was performed in the same way as reported in chapter 1, other than the use of an air-oxygen mixture in stead of pure oxygen gas. The analysis of the time dependence of DO with the differential method was carried out with the aid of a computer. More detailed explanations will be described in the Results and Discussion section.

2.2.3. Measurements of diffusion coefficient

The diffusion coefficient of glucose (as the substrate) were determined using our own setup as shown in Fig. 1. In order to avoid biochemical changes during the measurement, we used died mycelial cells, the preparation of which was carried out with an autoclave. Our optical and electron microscopic analyses showed that there is no morphological change in the *A. niger* cells before and after this thermal treatment. Thus, the measurements of diffusion coefficient were performed with the died free cells (see *a* of (C) in Fig. 1) and also with the PEC-immobilized cells (*b* of (C) in Fig. 1) which were prepared in the same manner as shown in the section *2.2.1*.

2.3. RESULTS AND DISCUSSION

2.3.1. Viscosity

Initially, we attempted to estimate the viscosity from the measurements of agitation power (P) (i.e., torque $\times 2\pi n$ in $\text{kg}\cdot\text{m}/\text{s}$, where n is impeller rotational speed in s^{-1}) for the free and PEC-immobilized mycelial broths. For this, a calibration graph showing a relation between power number (N_p) and modified Reynolds number (N_{Re}) is needed. We obtained this calibration graph using aqueous glycerol solutions with different viscosities (see Fig. 2). Then, the viscosity (μ in $\text{Pa}\cdot\text{s}$) can be estimated using the following relations:

$$N_p = \frac{\text{external force}}{\text{inertial force}} = \frac{Pg_c \cdot 1}{nD_i \cdot D_i^3} = \frac{Pg_c}{n^3 D_i^5 \rho} \quad (1a)$$

$$N_{Re} = \frac{\text{inertial force}}{\text{viscous force}} = \frac{\rho n^2 D_i}{\frac{\mu n}{D_i}} = \frac{n D_i^2 \rho}{\mu} \quad (1b)$$

Here, g_c denotes the gravitational conversion factor ($\text{kg}\cdot\text{m}/\text{Kg}\cdot\text{s}^2$), D_i is impeller diameter (m) and ρ is density (kg/m^3). Using these relations, we obtained the viscosities: 615 ± 15 $\text{mPa}\cdot\text{s}$ at 600 rpm and 505 ± 15 $\text{mPa}\cdot\text{s}$ at 700 rpm for the free cell broth; and 620 ± 15 $\text{mPa}\cdot\text{s}$ at 600 rpm and 505 ± 15 $\text{mPa}\cdot\text{s}$ at 700 rpm for the PEC-immobilized cell broth. Note that our mycelial fermentation was performed at 1000 rpm; however, this rotary condition caused the air engulfing

in the broth and therefore was difficult to measure the viscosity.

Aqueous glycerol solution does not exhibit a non-Newtonian behavior even when its concentration is very high. Thus, the viscosity obtained from the above technique should be an “apparent” indication. Taking this into account, we further performed the viscosity measurements with a Brookfield viscometer as a function of cell concentration. As can be seen from Fig. 3, there is little difference in the viscosity between the free and immobilized cells. As a result, we were not able to find a significant difference in the viscosity between the free and immobilized mycelial cells within accuracy in our measurements, although we expected an improvement in the rheological properties of the immobilized cell broth.

2.3.2. Oxygen-transfer coefficient (k_La)

The k_La values in s^{-1} for the free and immobilized cells were estimated as follows: (i) The incubation of the free or the PEC-immobilized *A. niger* cells (40 g/l each) was continued at 30 °C and at the dissolved oxygen (DO) level $\sim 10\pm 2$ mg/l until the rate of gluconic acid production becomes a constant; (ii) after that, the aeration was stopped, the procedure of which leads to a decrease of DO due to the respiration (see Fig. 4); and (iii) when the DO level became a minimum, the aeration was restarted (see allow in Fig. 4); and (iv) the k_La was estimated from an increase of DO between 80 s and 120 s using Eq. (2);

$$\frac{dC}{dt} = k_L a (C^* - C) - Q_{O_2} X \quad (2)$$

where C denotes oxygen concentration in kg/m^3 (i.e., $C \sim \text{DO} \times 10^3$), C^* is oxygen concentration at a saturation, t is time, Q_{O_2} is specific oxygen uptake rate (s^{-1}), and X is dry cell concentration in kg/m^3 . The results obtained were: $0.035 \pm 0.004 \text{ s}^{-1}$ for the free cells and $0.033 \pm 0.004 \text{ s}^{-1}$ for the immobilized cells, indicating that there is no difference in the oxygen transfer rate between both the *A. niger* cells in our incubation conditions.

2.3.3. Diffusion coefficient

We employed the method of Daynes [36] and Barrer [37] (i.e., D-B method) for the estimation of diffusion coefficient (D in m^2/s) using our setup shown in Fig. 1 and the following equation:

$$D = \frac{d^2}{6t_L} \quad (3)$$

This method is based on the measurement of a time lag (t_L) which can be obtained from time course of the amount (in weight or mole) of a permeate through a membrane with a thickness of d ($\sim 9 \text{ mm}$ in our measuring device). To examine whether or not this method is appropriate for the present purpose, we initially attempted to estimate the D value of glucose. The result ($D \sim 1.88 \pm 0.3 \times 10^{-7} \text{ m}^2/\text{s}$) which was obtained as $d \sim 9 \times 10^{-3} \text{ m}$ and from $t_L \sim 78 \text{ s}$ as shown in Fig. 5 was 254 times that ($7.65 \pm 0.2 \times 10^{-9} \text{ m}^2/\text{s}$) [38] of glucose in pure

water at 30 °C. This difference should be due to the use of apparent d , because a void between two membranes is fulfilled with water. When trying to calculate d (as a cell constant) from $t_L \sim 78$ s and a known D of the glucose at 30 °C, we obtain $d = 1.89 \times 10^{-3}$ m. The value is about 20 % of the length between two membranes. Nevertheless, we have to pack the free or the immobilized cells in the void between two membranes for the estimation of its diffusion resistance, and therefore have to use $d \sim 9 \times 10^{-3}$ m in Eq. (3). These mean that it is not possible to measure an "absolute" D value using the present technique, but possible to estimate a difference in the D between the free and immobilized cells.

The t_L values obtained from Fig. 5 were: 310 s for the free cells and 220 s for the immobilized cells. By substituting these data in Eq. 3 ($d = 9 \times 10^{-3}$ m), we obtained apparent D values of $4.45 \pm 0.30 \times 10^{-8}$ m²/s for free cells and $6.15 \pm 0.30 \times 10^{-8}$ m²/s for PEC-immobilized cells. These data are larger than the D value of glucose at 30 °C due to the reason as mentioned above; however, we can clearly say that the diffusion limitation by glucose in the submerged mycelial processes is improved by the PEC-based immobilization.

2.4. CONCLUSIONS

We have studied several important factors (viscosity, oxygen transfer coefficient and diffusion coefficient) affecting on the fermentation of the free and PEC-immobilized *A. niger* cells at a very high cell concentration (40 g/l), at which a marked enhancement in the gluconic acid production with the immobilized cells was observed [Chapter 1]. The results of the present study are summarized in Table 1. It has become apparent that there is only a difference in the diffusion coefficient of glucose (as the substrate) between the free and immobilized cells. A similar study [19] that has dealt with PEC-immobilized *A. terreus* showed a decrease in the viscosity caused by the immobilization. However, the viscosity measurements in Ref. 19 was performed in pure water, only using a Brookfield viscometer. Therefore, we believe that the diffusion limitation by substrates as a problem in submerged mycelial processes is improved by immobilization based on polyelectrolyte complexes, although an improvement in the rheological properties could not be demonstrated by our measurements of the viscosity within experimental errors.

TABLES

Table 1. Important factors affecting on the fermentation of the free and PEC-immobilized *Aspergillus niger* cells at a dry cell concentration of 40 g/l, 30 °C and a glucose concentration of 100 g/l.

Factor	Free cells	Immobilized cells
Viscosity (mPa·s)	615±15 ^a	620±15 ^a
	505±15 ^b	505±15 ^b
	890±65 ^c	895±65 ^c
Oxygen-transfer coefficient ($k_L a$) (s ⁻¹)	0.035±0.004	0.033±0.004
Diffusion coefficient (D) (×10 ⁻⁸ m ² /s)	4.45±0.30 ^d	6.15±0.30 ^d

^aDetermined from agitation power at 600 rpm. ^bDetermined from agitation power at 700 rpm. ^cDetermined with a Brookfield viscometer. ^dNote that the data are apparent values obtained by our measuring method (see discussion in the section 2.3.3).

FIGURES

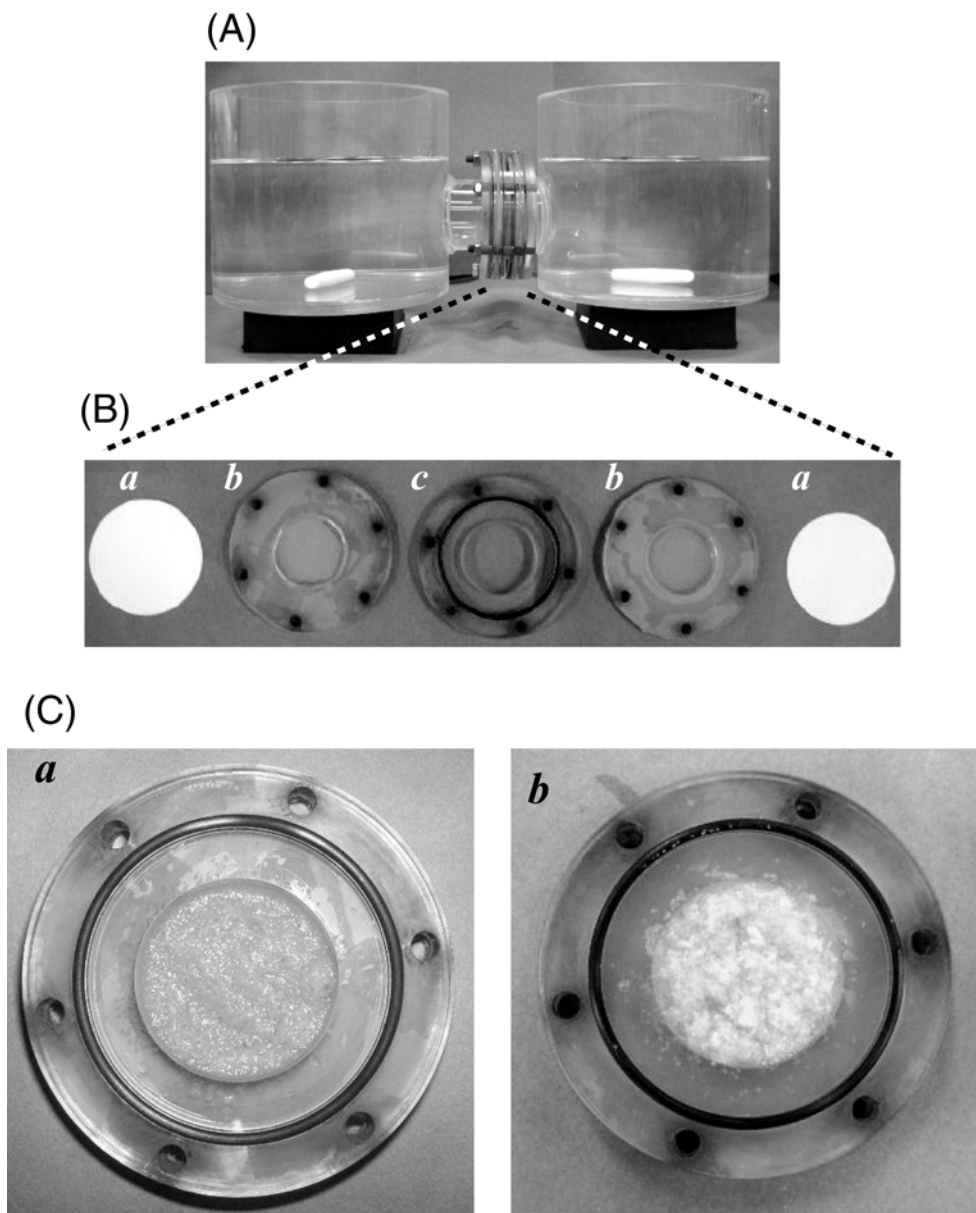


Fig.1. This figure title is next page

Fig. 1. Setup (A) used for measuring the diffusion coefficient of glucose which permeates through the free or the PEC-immobilized *A. niger* cells in a holder (B) composed of a pair of celluloseacetate porous membrane (*a*) with pore size ~ 0.8 μm , a jointer (*b*) with a cavity (30 mm in diameter and 2 mm in thickness) attached with a nylonmesh , and a cell holder (*c*) with thickness ~ 5 mm and cavity diameter ~ 30 mm. All the parts other than the porous membrane were made of PMMA resin. The maximum volume of two vessels on the right and left in (A) is 500 ml. Note that a difference in appearance between *a* and *b* of (C) is due to the morphology of mycelial hyphae complexed with the polyelectrolyte or not (see Fig. 4 of Chapter 1).

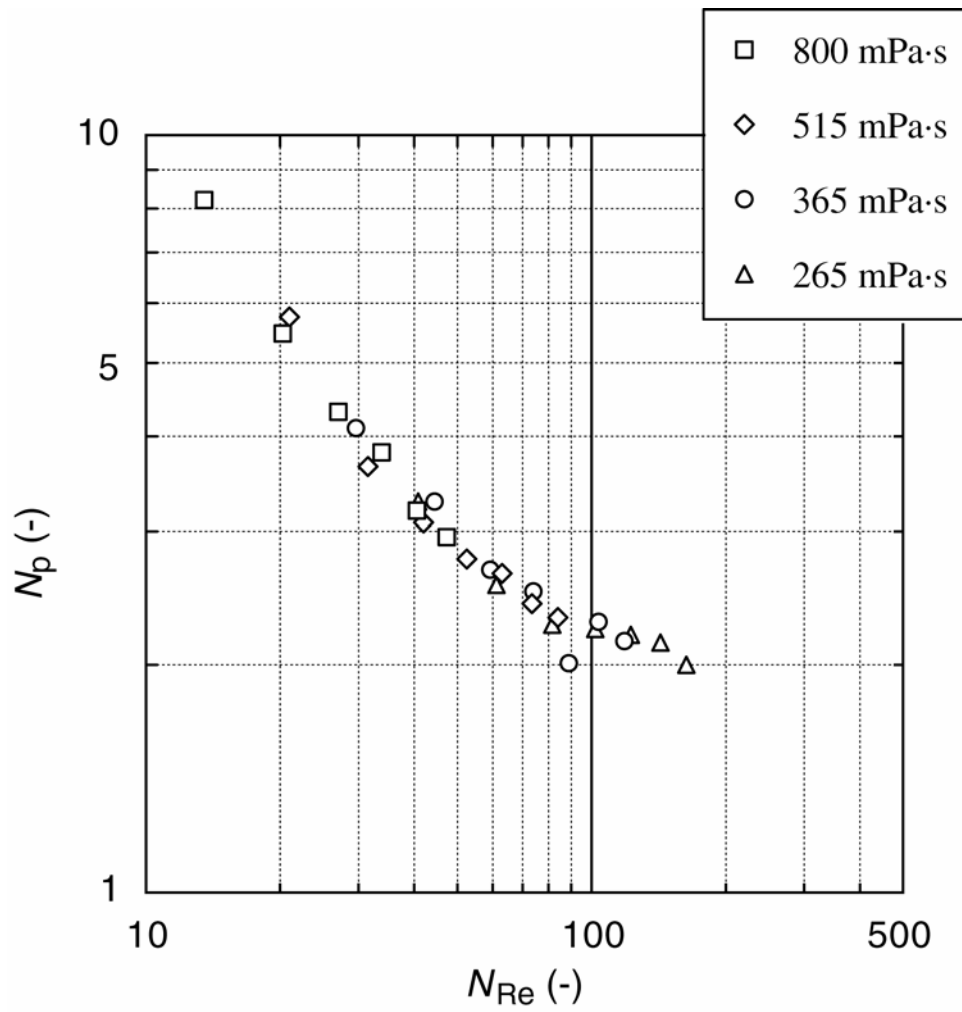


Fig. 2. A relation between power number (N_p) and modified Reynolds number (N_{Re}) obtained with aqueous glycerol solutions at 30 °C. The viscosity of the glycerol solution is shown in the window.

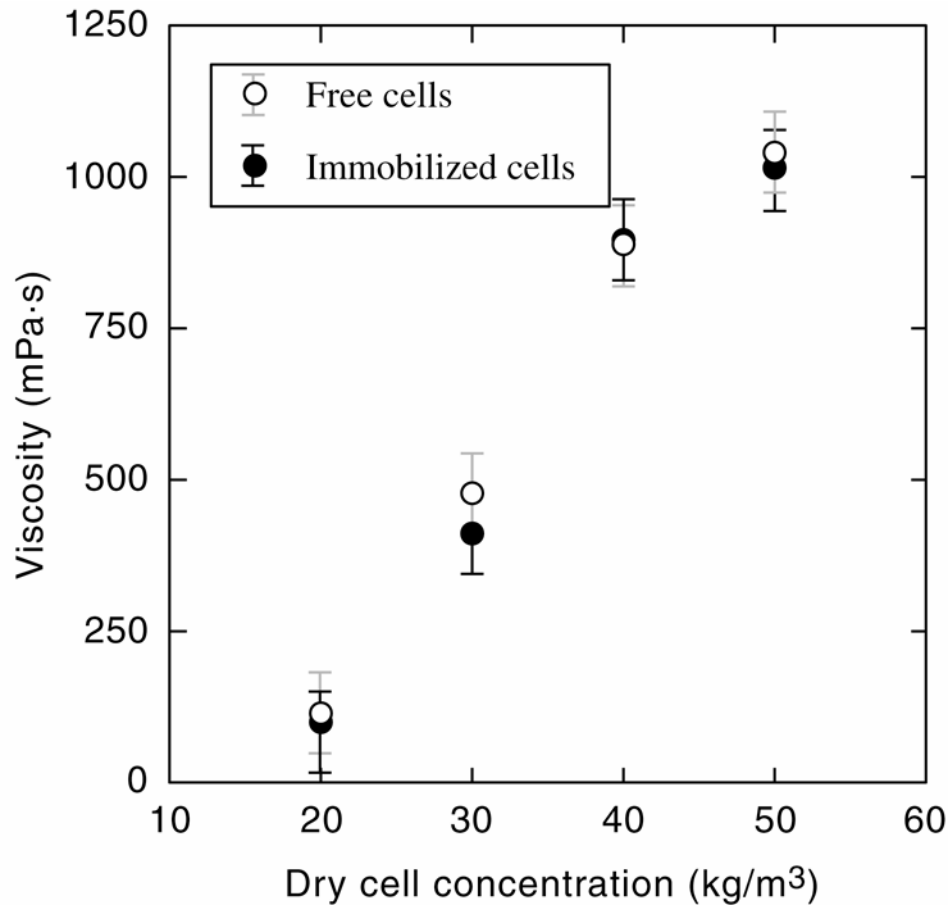


Fig. 3. Dependence of viscosity on dry cell concentration determined by a Brookfield viscometer at 30 °C.

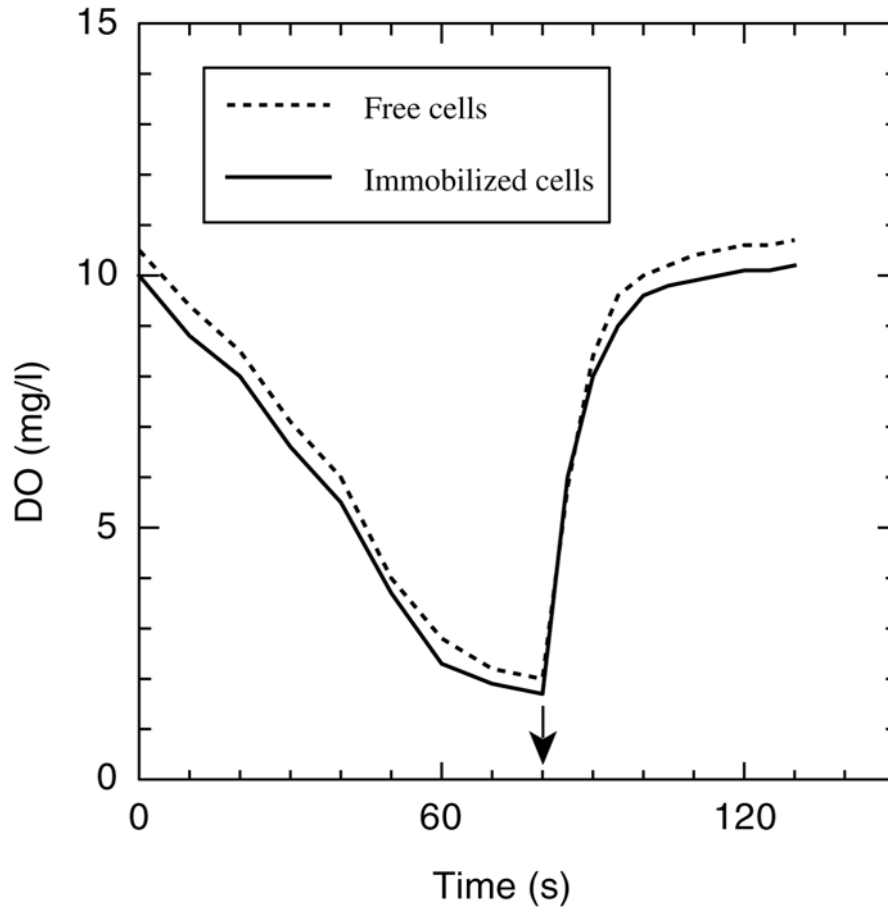


Fig. 4. Time course of dissolved oxygen (DO) for determination of oxygen transfer coefficients ($k_L a$) of the free and immobilized cells. The $k_L a$ values were determined by the differentiation of data between 80 s and 120 s with the aid of a computer. Note that we used an air-oxygen mixed gas, although pure oxygen was used in our previous study [Chapter 1].

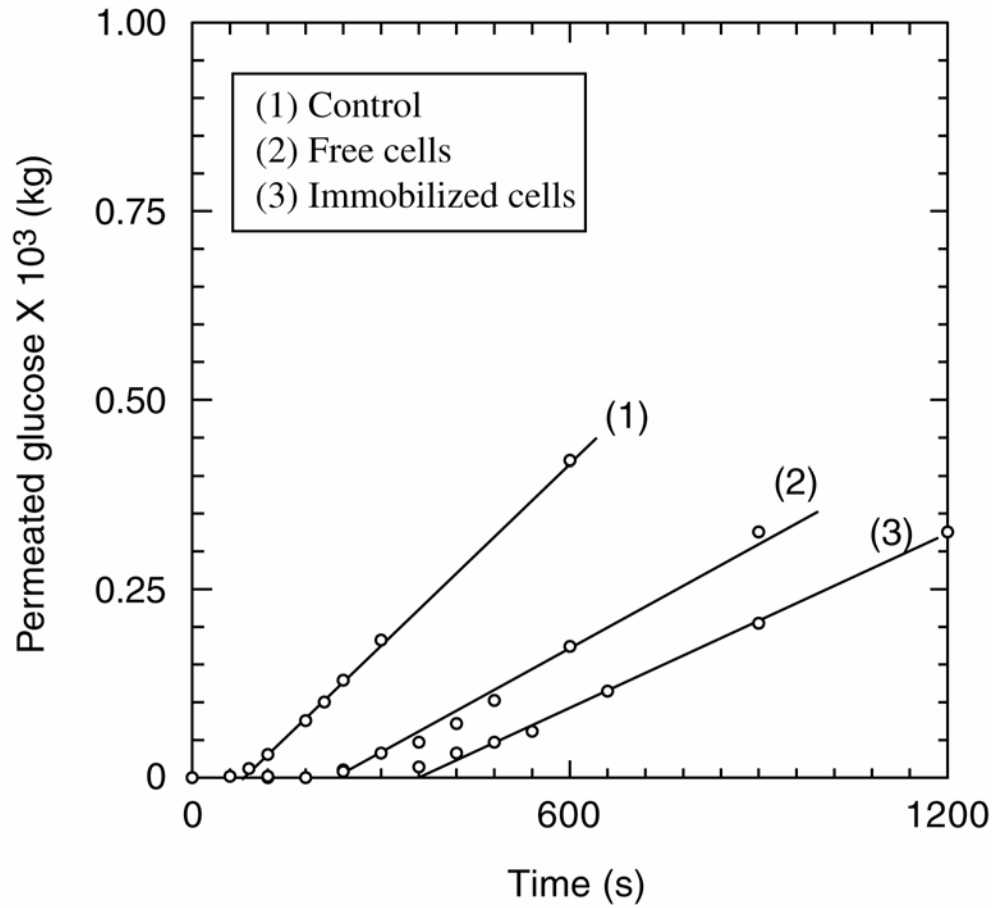


Fig. 5. Estimation of glucose diffusion coefficient at 30 °C using the D-B method. Data fitting was made by least squares method.

CONCLUSIONS

One of the most serious problems in submerged mycelial processes is the very high non-Newtonian viscosity of fermentation broths. This is associated with the growth of mycelial organisms in a highly extended and branched filamentous form. Therefore, in order to solve the problem, we attempted to establish novel immobilization techniques for mycelial organism (*Aspergillus niger*) using polyelectrolyte complex (PEC). We set up the following working hypothesis for the immobilization of filamentous fungi through mixing of hyphae with the same equivalent of both polyanion and polycation: (a) a few parts of polycations would bind on the surface of a hypha, the process of which should allow to gather the hyphae in part but not all; (b) such a gathering of the hyphae is tightly bunched with the PEC resulting from the remaining polycation when a polyanion was further added; (c) as a result, we may obtain filamentous fungi with partially bunched hyphae, the part of which is very stable and does not fall in pieces during the cultivation. Here, Potassium poly(vinyl alcohol) sulfate and trimethylammonium glycol chitosan iodide [6-O-(2-hydroxyethyl-2-(trimethylammonio)-chitosan iodide) were used as the polyanion and the polycation, respectively.

In chapter 1, we studied preparation method of PEC-immobilized cells and carried out the optical and electron microscopic analyses and the sedimentation

rate of the free and immobilized cells. In addition, the gluconic acid production from glucose was studied by a semi-large scale (1 liter) cultivation of the immobilized and free cells using a jar fermentor. As results, the optical and electron microscopic analyses showed that immobilized cell contains many of PEC-bunched hyphae which was not whole entrapped but partially entrapped by gel-like body of PEC. The sedimentation rate increased with the weight ratio of PEC to dry cells and leveled off at the weight ratio larger than 0.5. The gluconic acid production was found that an apparent specific activity of the immobilized cells for glucose oxidation becomes 1.44 times that of the free cells even at a high cell density of 40 g/l.

In chapter 2, we carried out to look at what factors play a crucial role in this enhancement. We measured viscosity of broth, mass-transfer coefficient ($k_L a$) for oxygen and diffusion coefficient of glucose (substrate). It has become apparent that there is only a difference in the diffusion coefficient of glucose between the free and immobilized cells at a very high cell concentration such as 40 g/l dry cell weight. As results, we were not able to find a significant difference in the viscosity and the oxygen transfer coefficient value between the free and PEC-immobilized mycelial cells within accuracy in our measurements, although we expected an improvement in the rheological properties of the

immobilized cell broth. On the other hand, there is a difference in the diffusion coefficient of glucose between the free and PEC-immobilized cells. Therefore, we believe that the diffusion limitation by substrates as a problem in submerged mycelial processes is improved by immobilization based on polyelectrolyte complexes, although an improvement in the rheological properties could not be demonstrated by our measurements of the viscosity within experimental errors.

Finally, the result of this research can apply for not only this experiment system, gluconic acid production by *Aspergillus niger* but fermentative production by other fungus, for example, the high value-added antibiotics production such as neomycin by *Streptomyces marinensis*. Thus this research is significant to fermentation production of a filamentous bacterium.

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