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Shear Effects on Production of Lignin Peroxidase by *Phanerochaete chrysosporium*

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Since biosynthesis of lignin peroxidase from *Phanerochaete chrysosporium* was known to be sensitive to shear, it is interesting to understand the effects of the shear sensitivity for the overproduction of lignin peroxidase. In stirred-tank fermentor, the shear-sensitivity in lignin peroxidase biosynthesis was quantified by using Kolmogorov length scale. It was found that agitation at 80 μm Kolmogorov length scale is advantageous for the production of lignin peroxidase from *P. chrysosporium*. To overcome the shear sensitivity in lignin peroxidase biosynthesis caused by the agitation, *P. chrysosporium* was immobilized on various solid carriers. The nylon-immobilized *P. chrysosporium* was chosen in the present study as a way to overcome the shear sensitivity at the ranges of above 50 μm Kolmogorov length scale. The adhesion force between immobilized cell and carrier can be predicted by thermodynamic approach and used as a criteria to select an adequate carrier materials for immobilization.

Key words: Phanerochaete chrysosporium, shear effect, lignin peroxidase, Kolmogorov length scale, cell immobilization

INTRODUCTION

The biodegradation of lignin has been extensively studied with white rot fungi, and in particular with a filamentous fungus, *Phanerochaete chrysosporium*.

Different cultivation systems have been used to date for the cultivation of *P. chrysosporium* and the production of ligninolytic enzymes. Attempts to produce lignin peroxidase in conventional stirred tank reactors or to scale up in shallow stationary cultures in large flasks or in trays have been unsuccessful [1]. Comprehensive data on kinetics of *P. chrysosporium* and on factors affecting the growth and the production of lignin peroxidase production using this fungus are not readily available.

In the present study, physiological characteristics of *P. chrysosporium* in a stirred tank reactor are examined. The Kolmogorov length scale was used to define the shear effects on the biosynthesis of lignin peroxidase from *P. chrysosporium* and it was suggested that immobilized cell system is a mean to overcome the shear effects.

MATERIALS AND METHODS

Microorganism and medium

The microorganism used in the lignin peroxidase production studies was *Phanerochaete chrysosporium* ME-446 (ATCC 34541). Cultures were stored at 4°C. The cultures for lignin peroxidase production in jar fermenter were grown in liquid medium of Tien and Kirk [2] except that 0.5% (w/v) glucose, 1.2 mM ammonium tatrate, 0.1 M sodium succinate buffer (pH 4.75) and 0.04% (w/v) oleic acid (from Junsei, Japan) emulsified with 0.05% (w/v) Tween 80 (from Sigma, USA) were added. To protect the secreted lignin peroxidase in the fermenter, 0.05% (w/v) Tween 80 was added in the broth at the beginning of carbon-limited phase. The flask cultures were made in 500 ml Erlenmeyer flasks containing 200 ml of the medium. After inoculation with approximately $(4\pm2)\times10^6$ spores/ml, cultures were flushed with 100% O_2 for 5 min. Agitation speed of incubation shaker was 150 rpm at 37°C. The pelleted inoculum for fermentation was prepared by growing the fungus from spore suspension ($(4\pm2)\times10^6$ spores/ml) in shaking 500 ml Erlenmeyer flask containing 200 ml of the above medium at 150 rpm, 37°C. At the beginning of the carbon-limited phase, veratryl alcohol (2 mM final concentration, from Aldrich, USA) and Remazol brilliant blue R dye (3 mg/l, from Sigma) were added aseptically to the cultures. These compounds were known to stimulate the lignin peroxidase synthesis [3]. The decolorization of dye could be an indication of the presence of ligninolytic activity. The initial pH was 4.75 and the pH was allowed to change freely during the culture. To select nitrogen source, ammonium chloride, ammonium nitrate and ammonium tartrate (from Sigma) were used. To optimize initial pH, the following buffers were used: 0.2 M sodium citrate buffer (pH 3 and 5); 0.2 M sodium succinate buffer (pH 4 and 4.75); 0.2 M sodium phosphate buffer (pH 6 and 7); 0.2 M sodium borate buffer (pH 8).

Bioreactor

The cultivation of *Phanerochaete chrysosporium* for the production of lignin peroxidase was made in a 5 l stirred tank reactor without baffles, which had a single six-flat-blade turbine impeller and the following scales: tank diameter, 0.17 m; impeller diameter, 0.08 m; im-

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peller width, 0.0167 m; reactor content, 2 L; aeration rate, 1.1 vvm.

Assays

Cell concentration

Samples of 20 ml were taken aseptically from the reactor and centrifuged for 5 min. The cell was washed with 0.85% (w/v) NaCl solution and centrifuged again. Then the cell was washed with distilled water and dried at 90°C for 24 hr. The supernatant was used for glucose, nitrogen and enzyme assays. Centrifugal separation did not change the lignin peroxidase activity.

In the case of the immobilized cell, cell concentration was determined by the following method: after dried carrier-cell weight was measured, the immobilized cell was removed by 10% (v/v) sodium hypochloride solution; then, dried carrier weight was determined; cell weight was measured from the difference between carrier-cell weight and carrier weight.

Glucose concentration

Glucose in culture medium was determined by DNS (dinitrosalicylic acid) method. 1 ml of the supernatant of the centrifuged medium was reacted with 1 ml of DNS color reagent at 100°C for 5 min. 10 ml of distilled water was added after the reaction and optical density was measured at 546 nm.

Lignin peroxidase activity

Broth was centrifuged at 13,000 rpm for 5 minutes and the supernatant was used for the assay of lignin peroxidase activity. All steps were performed at 4°C. Lignin peroxidase activity was determined at room temperature with a spectrophotometer by detecting the H_2O_2-dependent oxidation rate of veratryl alcohol to veratraldehyde (extinction coefficient 9,300 $M^{-1}cm^{-1}$) [4]. The reaction mixture contained 0.2 M sodium tatrate buffer (pH 2.9), 2 mM veratryl alcohol and 0.27 mM H_2O_2 in a final volume of 3 ml. One unit of lignin peroxidase activity (U) represents the amount of enzyme catalyzing the oxidation of one mole of veratryl alcohol in one minute.

Spore concentration

The spore concentration was determined by Haemocytometer using a microscope and by the optical density at 650 nm. The spore suspension was prepared by suspending spores in sterilized 0.02% (w/v) Tween 80 solution. After the samples of several different spore concentrations were made, the spore concentration was measured by the Haemocytometer. Then, the samples' optical densities were measured at 650 nm and compared with the standard curve.

Immobilization

Fibrous nylon cubes (~1 cm) were used as the carrier. In the case of flask cultures, 2.0 g of washed and dried fibrous nylon cubes were added in 190 ml of the growth medium without vitamins during autoclave. After the sterilization, 5 ml of spore suspension ($(4\pm2)\times10^6$ spores/ml) and 5 ml of vitamin solution were added. Incubation was carried out at 37°C. For shaking, a rotary shaker operating at 80 rpm was used. In case of jar fermenter, 12.3 g of washed and dried cubes were added in 1480 ml of the growth medium without vitamins in the 5 L fermenter. After the sterilization, 6.0 g of pre-immobilized cell (prepared by cultivation under 500 ml medium in 1 L flask at 37°C, 80 rpm) and 20 ml of vitamin solution were added. The fermenter was aerated at 1.1 vvm. Unless otherwise stated, the agitation rate was maintained at 150 rpm and the pH was not controlled during the immobilization and growth phase.

Adhesion force

Characterization of surfaces

Surface free energy of various solid carriers (nylon cube, NY; polyurethane, PU; celite, CL) was determined from contact angle measurements using the sessile drop technique at room temperature with distilled water and α-bromonaphtalene (from Janssen, UK). For polyurethane, contact angles were measured on nonporous heat-pressed solid film of the corresponding polymer. To measure the contact angle of *P. chrysosporium* cells, conidiospores and mycelium were harvested by centrifugation (5 min at 9,000 g), washed twice and suspended in distilled water. Coniodiospore surfaces were prepared by spreading a thick layer of conidiospores on glass slides. According to theory, given the known two components (i.e. γ_L^d, γ_L^p) of the surface energy of a liquid (γ_L), the contact angle of the liquid on a single solid surface has the following relationship (Equation(1) and (2)) with the two components (i.e., γ_s^d, γ_s^p) of the surface free energy of the solid surface (γ_s). Here measuring the contact angle of two liquids on a single solid surface gives an accurate estimation of the two components of the surface free energy of the solid surface.

$$\cos\theta = -1 + \frac{2\sqrt{(\gamma_s^d \gamma_L^d)}}{\gamma_L} + \frac{2\sqrt{(\gamma_s^p \gamma_L^p)}}{\gamma_L} \quad (1)$$

$$\gamma_s = \gamma_s^d + \gamma_s^p \quad (2)$$

Characterization of liquids

Two components of the surface free energy components of a liquid, γ_L^d and γ_L^p, were calculated using Equation (1). Contact angles were measured on parafilm (from Whatman), assuming it completely apolar ($\gamma_s^p=0$) and has a surface free energy of $\gamma_s=\gamma_s^d=25.9$ mJ/m^2 at 20°C.

Adhesion experiments

All adhesion experiments were made in 500 ml Erlenmeyer flasks containing 200 ml of the medium. Inoculation of the spores of *P. chrysosporium* was made at a concentration of $(4\pm2)\times10^6$ spores/ml. For shaking, a rotary shaker operating at 80 rpm was used at 37°C. Immobilized and nonimmobilized mycelium were measured in terms of dry weight of mycelium after centrifugation and drying overnight at 90°C.

RESULTS AND DISCUSSION

The shear effects on enzyme production in stirred-tank fermenter

For fermentation processes using fungi, a sufficiently high power input is required to ensure the necessary oxygen transfer and mixing requirements. However, excessive shear force causes the damage to the mycelial cells, thus setting an upper limit to the power dissipation is important to perform a successful fermentation. Therefore it is interesting to quantify

the effects of shearing forces on mycelium to optimize the process.

Fig. 1(a) shows the dry cell weight at various impeller speeds. As shown in Fig. 1(a), the cell growth was not damaged below 300 r.p.m. but the growth of the cell was seriously damaged at 600 rpm. Shear forces were given by the integrated shear factor (s^{-1}), averaged shear rate (s^{-1}) and impeller tip speed (m/s) as in Fig. 1(b). As shown in Fig. 1(b), it was found that specific growth rate was dependent on shear force. The specific growth rate slightly decreased with agitation speed until 300 rpm and after then seriously decreased.

There are many ways to describe shear effects on fermentation but they vary according to geometrical dimensions. Since Kolmogorov length scale is little influenced by reactor's geometrical dimensions, it is useful to describe shear effects using this length scale even when the operation condition of bioreactor varies. However, Kolmogorov length scale is significantly affected by aeration, since the hydrodynamic phenomena around the stirrer blades changes dramatically during aeration. Therefore two models, Hughmark's equation [6] and Michel's equation [7], were used to consider the effect of air. Because Hughmark [6] correlated a large number of data for turbine stirrers including those of Michel and Miller [7], Hughmark's equation was widely accepted. So, Kolmogorov length scale was calculated based on the Hughmark's equation. As shown in Fig. 2., specific growth rate was nearly constant down to 50 μm Kolmogorov length scale and after then abruptly decreased. It was found that the growth of *Phanerochaete chrysosporium* was seriously damaged below 50 μm kolmogorov length scale. This data suggests that the cultivation of *P. chrysosporium* is carried out above 50 μm Kolmogorov length scale.

Fig. 3(a) shows the production of lignin peroxidase at various agitation speeds. Enzyme activity per maximum dry cell weight decreased against agitation speed, indicating that production mechanisms of the lignin peroxidase are somewhat damaged. Although there exists a possibility that the secreted lignin peroxidase was inactivated by shear force, the inactivation of lignin peroxidase in culture medium by shear force was negligible from separate experiments (data not shown). Shimada *et al.* [8] postulated that pellet formation due to the changes in agitation speed might have serious physiological effects on the metabolism of *Phanerochaete chrysosporium* and hence affect its secondary metabolism. But the deleterious effect of agitation on the metabolism of the cell has not been explained satisfactorily. Fig. 3.(b) clearly shows that the production of lignin peroxidase was damaged more severely with the decrease in the Kolmogorov length scale compared with specific growth rate of *Phanerochaete chrysosporium*. This data suggests that the cultivation for lignin peroxidase production should be carried out above about 80 μm Kolmogorov length scale.

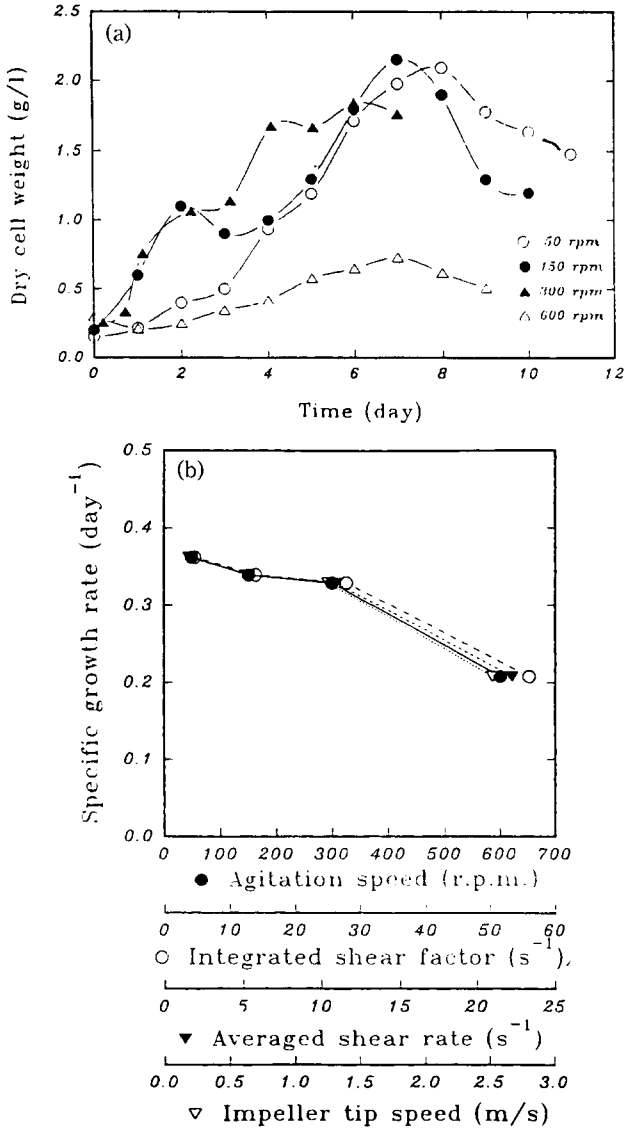

Fig. 1. The shear effects on *Phanerochaete chrysosporium* growth in 5 l fermenter.
(a) Time course of *Phanerochaete chrysosporium* growth in 5 l fermenter at various agitation speeds. (b) Specific growth rate versus calculated shear terms.

Immobilization of *Phanerochaete chrysosporium* using carriers

In order to overcome the sensitive lignin peroxidase synthesis to agitation, *P. chrysosporium* was immobilized on various solid carriers. Fig. 4. illustrates the time courses of lignin peroxidase production in flask cultures using the various immobilized carriers

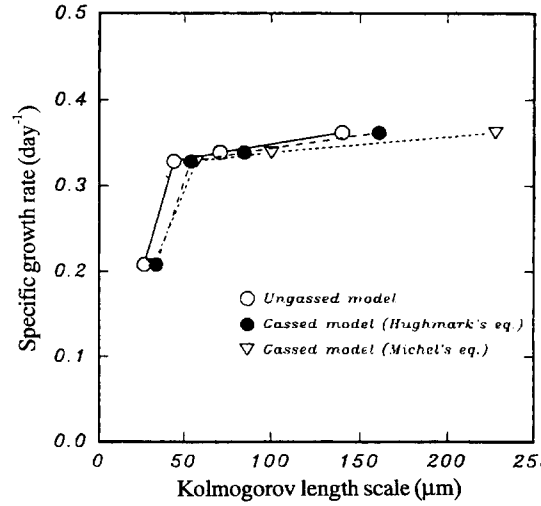

Fig. 2. Specific growth rate versus Kolmogorov length scale.

compared to using that of free pellets. The rate of glucose consumption was faster in the immobilized system than in the free pellets system except immobilized on celite system. Nylon was superior to the other carriers in the lignin peroxidase production, and the lignin peroxidase activity produced by nylon-immobilized cells reached the maximum value much earlier than that of the freely suspended pellets. The initial enzyme production rate with the polyurethane foam-immobilized fungus was almost as high as with the nylon-attached *Phanerochaete chrysosporium*, but the maximum activity was markedly lower than that obtained both with the nylon-attached fungus and with the free pellets.

The surface thermodynamic model was introduced to explain the cell attachment on the carriers. The hydrophobic-hydrophilic nature and the surface free energy of the solid carrier surfaces were characterized by contact angles of distilled water (D.W.) and α-bromonaphtalene (see Table 1). Carriers can be classified into two ctegories: hydrophobic polyurethane (PU) and nylon (NY), and hydrophilic celite. The polar component increased with hydrophilicity of the material. As shown in Fig. 5, the adhesion of *P. chrysosporium* decreased with increase in surface free energy of solid carriers. However, in all the cases except celite adhesion was favorable for spores. The adhesion value becomes positive when γ_s=67.08 mJ/m^2, implying that the adhesion could be unfavorable when celite was used as a carrier. The results are summarized in Fig. 6. The fractions of attached cell mass and final dry cell mass using nylon were higher than using polyurethane. When

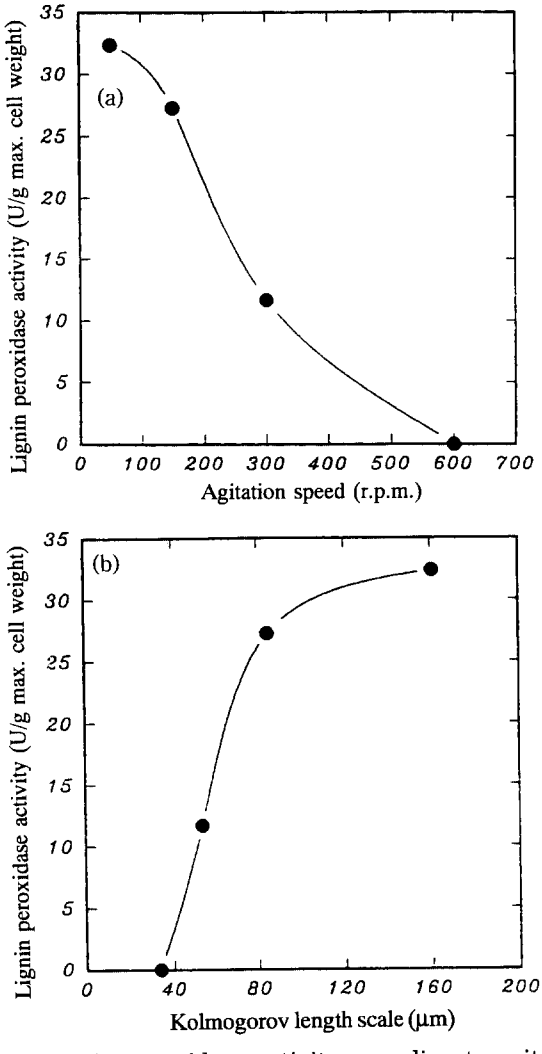

Fig. 3. Lignin peroxidase activity according to agitation speed and Kolmogorov length scale.
(a) Effect of agitation speed on lignin peroxidase production.
(b) Kolmogorov length scale versus lignin peroxidase production.

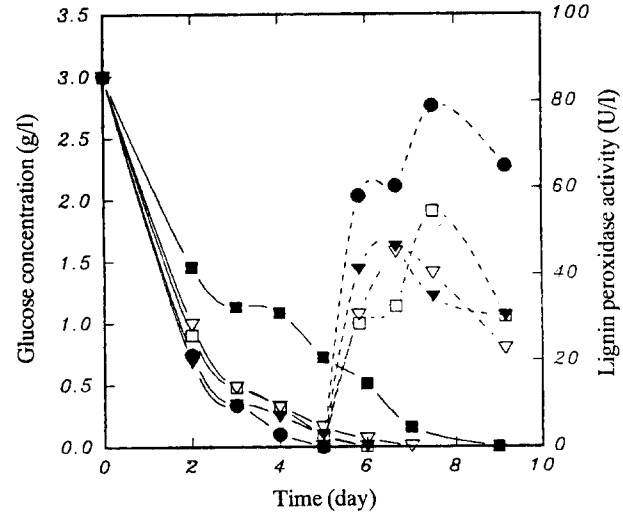

Fig. 4. Time courses of glucose concentration and lignin peroxidase production using various immobilizing carriers in flask cultures.
——: glucose consumption, ----: lignin peroxidase production, ●: nylon, □: control, ▽: polyurethane foam 1, ▼: polyurethane foam 2, ■: celite.

Table 1. Surface free energies of carriers, P. chrysosporium, and liquids

Solid carriers	Contact angle (degrees)		Surface free energy (mJ/m^2)			Remark
	Distilled water	α-bromonaphtalene	γ_s^d	γ_s^p	γ_s	
PP	85	45	32.0	3.5	35.5	ref.[9]
PU	71	15	41.3	6.9	48.2	this study
NY	59	14	42.7	12.2	54.9	this study
SS	50	0	43.9	16.5	60.4	ref.[9]
CL	30	0	43.9	27.0	70.9	this study
			γ_c^d	γ_c^p	γ_c	ref.[9]
P. chrysosporium						
Spores	105	53.5	27.9	0.12	28.0	this study
Mycelia			43.9	0.1	44.0	ref.[9]
			γ_L^d	γ_L^p	γ_L	
Liquids						
Distilled water			21.4	51.3	72.7	ref.[9]
α-bromonaphtalene			43.9	0	43.9	ref.[9]
Broth (4 days)			18.0	24.1	42.1	this study

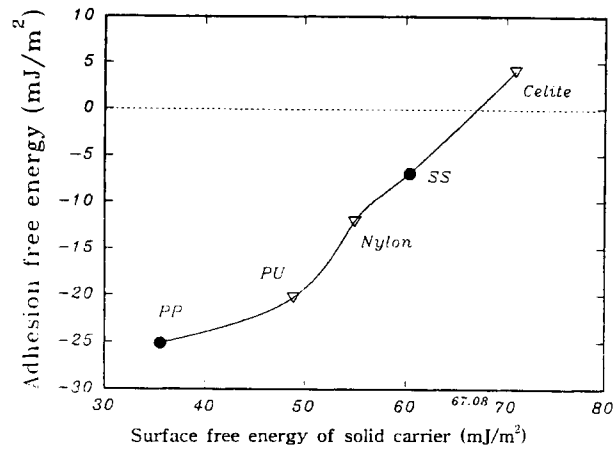

Fig. 5. Theoretical predictions of the free energy of adhesion for *Phanerochaete chrysosporium* as spores as a function of the surface free energy of solid carrier.
●: from literature [9], ▽: experimental data

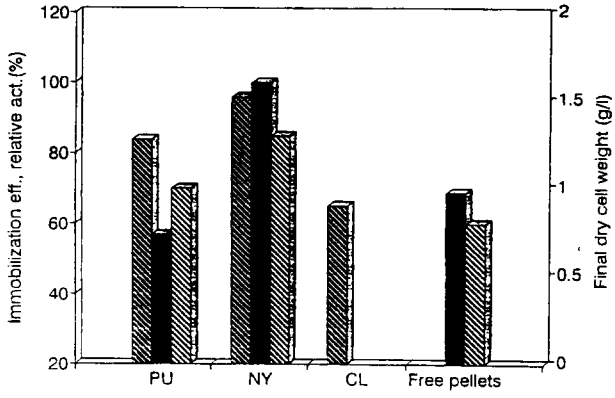

Fig. 6. Immobilization efficiency, relative lignin peroxidase activity and final dry cell weight for *Phanerochaete chrysosporium* immobilized on polyurethane (PU), nylon (NY) and celite (CL) as compared to those for free pellets.
▩: immobilization efficiency, ■: relative lignin peroxidase activity compared with that using nylon-immobilization, ▨: final dry cell weight.

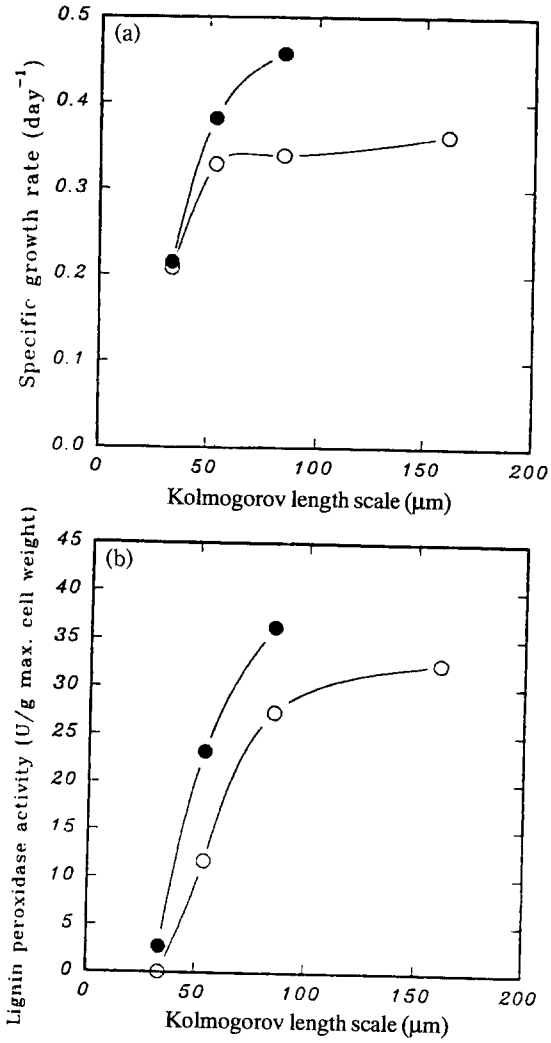

Fig. 7. Comparison of specific growth rate and lignin peroxidase activity of immobilized cell and free cell in 5 l fermentor.
(a) specific growth rate versus Kolmogorov length scale, ○: Free cell, ●: Immobilized cell. (b) lignin peroxidase activity versus Kolmogorov length scale, ○: Free cell, ●: Immobilized cell.

immobilized on polyurethane, fungal growth was restricted to the pores of the foam matrix. On the contrary, plate type, nonporous nylon carrier appears to give higher surface area per unit volume ratio than that of polyurethane and hence better results.

As shown in Fig. 7(a), the immobilized *P. chrysosporium* showed higher specific growth rate than the free cell. The specific growth rate in the immobilized system rapidly increased as the increase in Kolmogorov length scale, whereas in the case of free cell the specific growth rate was nearly constant above 50 μm Kolmogorov length scale. Because of the higher surface area per volume ratio of nylon carrier, nylon-immobilized cells appears to consume more nutrient such as glucose as well as oxygen compared to free cells. In fact oxygen transfer rate in immobilized cell system was enhanced in comparison with free cell system (data not shown). It was found that the immobilized cell system could protect cell growth inhibition by agitation, particularly at high Kolmogorov length scale. As shown in Fig. 7(b), the activity of lignin peroxidase produced from the immobilized *P. chrysosporium* increased more rapidly with the increase of Kolmogorov length scale than free pellet. The results suggest that the immobilized cell system can enhance lignin peroxidase production, particularly at high level of Kolmogorov length scale.

CONCLUSIONS

In stirred-tank fermenter, it is advisable to perform the cultivation for production of lignin peroxidase using *Phanerochaete chrysosporium* beyond 80 μm Kolmogorov length scale. To overcome the sensitivity of lignin peroxidase biosynthesis to agitation, *P. chrysosporium* was immobilized on various solid carriers. The adhesion force of immobilized cell-carrier could be predicted by surface thermodynamic equation. And the equation could be used to select an adequate carrier material. The immobilized cell system was a good means to overcome the shear against cell growth and lignin peroxidase biosynthesis, caused by agitation at the range of above 50 μm Kolmogorov length scale. However, at very high rpm the immobilized cell system was no more advantageous to overcome the sensitivity to shear.

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