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Improvements in Lipase Production and Recovery from *Acinetobacter radioresistens* in Presence of Polypropylene Powders Filled with Carbon Sources

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Abstract

Polypropylene powders as the adsorbent for organic solution containing *n*-hexadecane and olive oil were employed as the carbon source for producing an alkaline lipase from *Acinetobacter radioresistens*. The best volumetric ratio of *n*-hexadecane to olive oil around 5 for lipase production was determined from shake-flask and fermentation cultivations. The existence of a maximum time course lipase activity of the aqueous phase was attributed to the compensation effects of olive oil on cell growth and lipase production, repression of lipase synthesis by oleic acid, and lipase adsorption on the supports. A linear relationship between the average cell growth rate in the exponential phase and the ratio of surface areas of the supports was found. The benefits of using the present fermentation process include less foaming and emulsion of the broth, less organic phase used, higher lipase production, and easy recovery of the lipase in the centrifugation step.

Index Entries: Alkaline lipase fermentation; polypropylene powders; *Acinetobacter radioresistens*.

Introduction

Nature has designed lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) to hydrolyze oils and fats into fatty acids and glycerol at the interface between the insoluble substrate and water. Owing to the flexible

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backbone and stability in organic solvents, they have been employed in the enantio- and regioselective hydrolysis or synthesis of a wide range of natural and unnatural esters and amides *(1–3)*. Commercially available lipases are currently produced in large quantities from animals and microorganisms and are primarily employed in food and detergent industries *(4)*. Continuous interest in isolating new lipase-secreting microorganisms and studying their enzyme production, purification, and characterization is increasing, because this could provide new lipases with better quality and a wide range of applications.

The optimal activity for hydrolysis of most of the extracellular lipases from microbial sources is near neutral pH. However, to meet the requirement of more severe cleaning conditions, the lipase included in the detergent formulation should maintain its conformation and activity in the alkaline range. Relatively rare species of microorganisms reported in the literature can secrete alkaline lipases *(5–11)*. Recently, a new strain of *Acinetobacter radioresistens* capable of secreting an alkaline lipase was isolated from the sludge of wastewater *(12)*. The lipase has a molecular weight of 38,000 and an isoelectric point of 4.5 with the optimal pH of 9.5 for hydrolysis of olive oil *(13)*. Investigations of the cultivation condition from fermentation indicated that the optimal temperature and pH were 30°C and 7.0, respectively, for cell growth and lipase production. The microorganism prefers to use *n*-hexadecane, oleic acid, or olive oil as the carbon source and NH_4Cl as the nitrogen source. However, a prolonged cultivation time was required when *n*-hexadecane was the carbon source. Supplement with olive oil will decrease the lag phase on digesting *n*-hexadecane. However, oleic acid releasing from the hydrolysis of olive oil might repress the lipase synthesis, although it also acts as a surfactant to emulsify and hence enhances the digestion of *n*-hexadecane *(14)*.

Therefore, formulation of the medium composition of the organic solution needs to be optimized although the variation in composition usually results in the change of physical properties and hence the dispersion or interfacial area of the organic phase. Different strategies such as the addition of nonwoven fabric, the addition of surfactants, and induction of cell-surface hydrophobicity were proposed to overcome serious foaming, enhancement of the dispersion of organic phase for cell attachment, and long lag phase, respectively *(15,16)*. Since it is difficult to predict the drop size distribution of the organic phase, further study on finding the exact effect of medium composition on cell growth and lipase production is needed.

Hydrophobic polypropylene powders with high porosity and internal surface area have been employed as the adsorbent for lipase immobilization. Good to high selectivity was obtained when compared with the adsorption of contaminant proteins *(17–19)*. By adding the support filling with an organic solution as the carbon source, one can not only control the aqueous-organic interfacial area for cell attachment but also reduce the foaming and hence the loses of lipase from the fermentor. Therefore, we

studied the effects of polypropylene powders containing different volumetric ratios of *n*-hexadecane to olive oil on cell growth and lipase production. We also studied the concentration of the lipase from the broth to show the enhanced enzyme recovery in comparison with that without adding the supports.

Materials and Methods

Microorganism and Culture Conditions

A. radioresistens CMC-2 was kindly provided by Professor Ming-Cheung Chang from the Department of Biochemistry of National Cheng Kung University. The culture conditions were similar to those previously reported *(13)*. Briefly, the strain was maintained at –20°C in glycerol. Prior to preparation of preculture, the cells were transferred to a Luria-Bertani (LB) agar slant and inoculated for 10 h at 30°C. The LB slant contained 10 g/L of tryptone, 10 g/L of NaCl, 15 g/L of agar, and 5 g/L of yeast extract (Difco, Detroit, MI). Preculture was prepared by inoculating one loopful of cells from the agar slant into a 500-mL Erlenmeyer flask containing 100 mL of LB broth and incubating for 10 h at 30°C in a rotary shaker at 180 rpm. The basal medium for lipase production contained 10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, and 1 g/L of NH_4Cl. Shake-flask cultivation was carried out at 30°C and 180 rpm with the initial pH adjusted to neutral by adding 1 *N* NaOH. During the cultivation, the basal medium was supplemented with the supports filled with the organic phase consisting of various volumetric ratios of *n*-hexadecane to olive oil, as indicated. A blank cultivation was also carried out in which the basal medium was supplemented with 2 mL of *n*-hexadecane and 0.1 mL of olive oil. Tank fermentation was performed in a 3.0-L fermentor (Bioflo 2000; New Brunswick, Edison, NJ) with a working volume of 1.2 L. The inoculum volume was 50 mL. Temperature, agitation speed, and aeration rate were controlled at 30°C, 500 rpm, and 1 vvm, respectively. The pH of the broth was controlled at 7.0 by using 1 *N* NaOH and 1 *N* HCl solutions. All other chemicals of analytical quality were used without further purification in the following experiments.

Preparation of Polypropylene Powders Filled with Carbon Source

Organic solutions of 20 mL containing different volume ratios of *n*-hexadecane to olive oil were prepared and weighted. Polypropylene powders (Accurel MP 1000), kindly donated from Akzo Nobel (Obernburg, Germany), were screened and the particles with size distribution between 400 and 600 μm were selected as the adsorbent. To 20 mL of the organic solution was added 1 g of the support. After stirring for 1.5 h, the particles were separated by suction for 5 min, then were put on Whatman No. 1 filter paper for 5 min to remove the solution adhering on the external particle surface, and finally weighted to calculate the volume of adsorbed solution.

The residual organic solution between the particles under shear were further removed by putting the supports in the fermentor containing 1.2 L of deionized water. After stirring at 500 rpm for 2 h at 30°C, the particles were filtered, dried in an oven at 50°C for 3 h, and then weighted to calculate the organic solution remaining in the adsorbent.

Analysis

Biomass

Cell concentration in the aqueous phase was monitored by turbidimentry at 600 nm (UV-160A; Shimadzu, Tokyo, Japan) and correlated to the dry cell weight. Interference by the residual organic phase in the turbidity measurement was eliminated by centrifuging a 1-mL sample without the adsorbent twice in a DuPont Sorvall RC-5B centrifuge for 5 min at 192*g* and resuspending the cells in deionized water.

Lipase Activity and Fatty Acid Concentration

Lipase activity was measured by the pH-stat method. The substrate solution was prepared by stirring 20 mL of olive oil and 10 g of gum arabic (Sigma, St. Louis, MO) in 200 mL of deionized water. On carrying out the lipase activity assay, 1 mL of enzyme solution (or a given amount of adsorbents containing lipases) was added to 15 mL of the substrate solution and the pH adjusted at 10.0 by using 3 *N* NaOH. Titration was performed in a Mettler DL-25 titrator using appropriate NaOH solutions. One unit of lipase activity was defined as 1 μmol of fatty acid released/min at 37°C and pH 10.0.

Fatty acid releasing from the hydrolysis of olive oil was determined using the spectrophotometric method derived from Lowry and Tinsley *(20)*. Briefly, a screw-cap culture tube containing 4.6 mL of isooctane and 1 mL of 5% cupric acetate–pyridine solution at pH 6.0 was added to 0.4 mL of isooctane containing different amounts of oleic acid. After vortexing for 90 s and settling for 60 s, the absorption intensity of the organic phase was measured at 715 nm to prepare the calibration curve. The fatty acid contained in a 10-mL sample was extracted into 10 mL of isooctane by vortexing for 2 min. The mixture was centrifuged for 5 min at 192*g* to separate the organic phase. To 1 mL of the resultant solution was added 4 mL of isooctane and 1 mL of the cupric acetate–pyridine solution, and the fatty acid concentration was determined as just described.

Protease Activity

Protease activity was measured by proteolysis of azocasein (Sigma) according to Leighton et al. *(21)*. Briefly, an equal volume of sample from the fermentation broth was added to the solution containing 5 g/L of azocasein. The resultant solution was incubated for 15 min at 37°C, and the reaction was terminated by adding 5% (w/v) of trichloroacetic acid. One unit of protease activity was defined as the increase in 0.01 absorbancy unit/min at 440 nm.

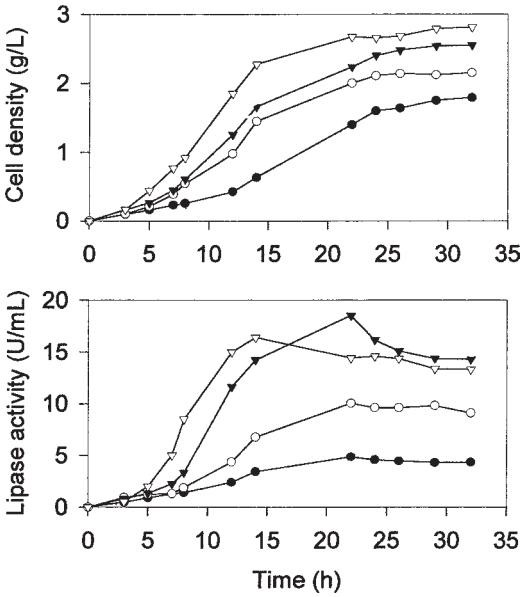

Fig. 1. Time course cell density and lipase activity in shake-flask cultivation at volumetric ratio of 20 without supports and adding 2.1 mL of organic phase (●) and by adding wet supports of 2.22 g (○), 3 g (▼), and 4 g (▽).

Results and Discussion

Effects of Composition and Mass of Organic Phase in Shake-Flask Cultivation

Depending on the composition of the organic phase, 1 g of dry powders adsorbed 2.78–3.48 g of the organic solution owing to the high porosity of the supports. From the previous shake-flask cultivation *(13)*, the best volumetric ratio of 20 was found to give a maximum lipase production. Therefore, by fixing at this ratio, the time course cell growth and lipase activity of the aqueous phase with or without adding the supports were determined (Fig. 1). Higher cell growth rates and lipase production were found with the supports. For example, the cell density and lipase activity at 32 h increased from 1.79 to 2.15 g/L and 4.35 to 9.13 U/mL, respectively, when 2.22 g of wet supports containing 2.2 mL of the organic solution were employed. This implied that by employing almost the same organic solution, the total interfacial area for cell attachment was higher when dispersing the organic phase via the supports. Inspection of the data also indicates that the cultivation is growth associated for product formation. Since olive oil and *n*-hexadecane are good carbon sources for cell growth *(14)*, adding more supports indeed enhances cell growth and lipase production in the exponential phase. However, the time course lipase activity started to decrease at 22 h when 3 g of wet supports was added. A similar behavior was observed at 14 h when 4 g of the supports was

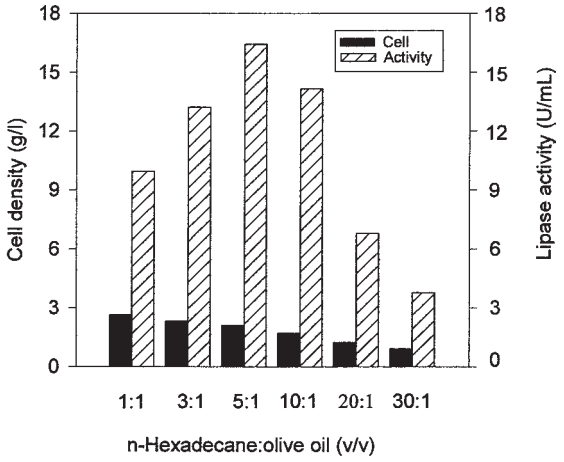

Fig. 2. Effects of composition of organic solution on cell density and lipase activity in shake-flask cultivation at 24 h by adding 1 g of wet supports.

employed. These behaviors might be attributed to the protease formation to degrade the lipase, repression of lipase synthesis by oleic acid releasing from the olive oil, and lipase adsorption on the supports, as elucidated later.

Figure 2 illustrates the effects of the volumetric ratio on cell density and lipase activity at 24 h when 1 g of wet supports was added. Since a prolonged lag time of more than 50 h was needed when using *n*-hexadecane as the carbon source *(14)*, increasing the ratio from 1 to 30 resulted in a decrease in cell density from 2.63 to 0.90 g/L. However, a maximum lipase activity of 16.43 U/mL was obtained at a ratio of 5, but not 20, without adding the supports. Since the interfacial area for the cell attachment in Fig. 2 was regarded as constant, these results implied that the content of olive oil must play an important role in cell growth and lipase production. Therefore, in the following cultivation, the effects of volumetric ratio varied from 5 to 10 on lipase production were investigated.

Effects of Composition and Mass of Organic Phase in Fermentation Cultivation

Figure 3 illustrates the effects of volumetric ratio on time course cell density and lipase activity when 6 g of wet supports were employed. Behaviors very similar to those in the shake-flask cultivation were seen when the ratio increased. However, a maximum lipase activity of 20.13 U/mL of broth at 16 h at a ratio of 5 (or 21.86 U/mL at 24 h for a ratio of 7) was obtained. Therefore, the ratio was set at 7 in studying the effects of powders on lipase production. Much less foaming was observed, which implies that antifoam does not need to be added during the fermentation and thus the loss of lipase owing to foaming is avoided. Of course, the emulsion of the broth was also greatly reduced, which may be attributed to

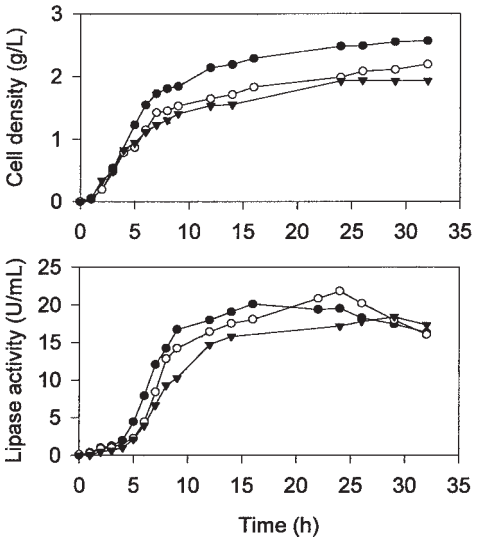

Fig. 3. Time course cell density and lipase activity in fermentation cultivation by adding 6 g of wet supports at volumetric ratios of 5 (●), 7 (○), and 10 (▼).

the hydrophobicity of the support in adsorbing oleic acid and biosurfactants secreted by the microorganism.

Figure 4 illustrates the time course cell growth and cell density by using wet supports as the parameter. The average cell growth rate between 3 and 8 h in the exponential phase was further calculated and is represented in Fig. 5. Except for the system of adding 6 g of wet supports, a linear relationship between the average cell growth rate and the ratio of surface areas was demonstrated. Under the shear, the organic phase originally adhered on the support surface was deprived into the broth to form oil drops with diameters varying between 5 and 20 μm by microscopy. Loss of the organic phase was estimated as 18.3% (w/w) with a standard deviation of 3.3% when 3–9 g of wet supports was employed. Therefore, the result of linearity in Fig. 5 implies that the total interfacial area might act as an index of substrate availability for cell growth.

As in Fig. 1 for the shake-flask cultivation, Fig. 4 illustrates the increase in lipase production with the supports in the exponential phase. However, a maximum lipase activity of 44.3 U/mL occurred at 16 h when 9 g of wet supports was employed. Protease activity was detectable at 16 h (Fig. 6) when 6 g of the supports, or at 3 h when 30 g of supports, was added. This is in correspondence with the decrease in time for the maximum lipase activity when increasing the supports. Oleic acid dissolving in the oil drops suspending in the broth was detectable at 4 h except for the system containing 6 g of wet supports. Therefore, the higher the oleic acid concentration was at any specific time, the greater the lipase synthesis was inhibited *(14,22)*. The lipase activities of the aqueous solution and the supports were further compared at the harvest time of 32 h. As represented in Table 1, the

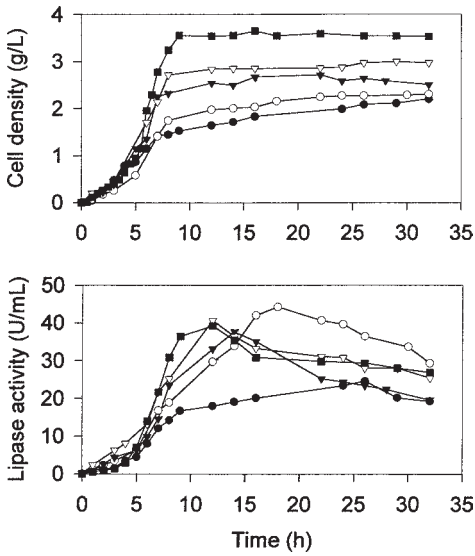

Fig. 4. Time course cell density and lipase activity in fermentation cultivation at volumetric ratio of 7 by adding wet supports of 6 (●), 9 (○), 15 (▼), 18 (▽), and 30 g (■).

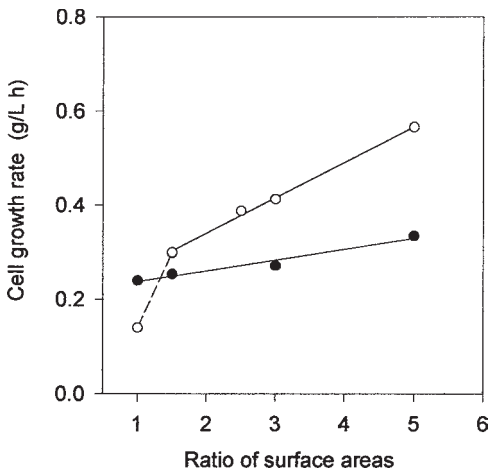

Fig. 5. Variations in average cell growth rate with ratio of surface areas of wet supports for supports without (○) and with (●) pretreatment under shear.

more the wet powders were employed, the higher was the percentage of lipase activity on the supports. Lipase activities of 141–207 U/g of the wet supports and 19.26–29.37 U/mL of the broth were obtained. The cultivation using 9 g of wet supports was repeated and stopped at 16 h. The total lipase activity for the recovered supports was measured as 643 U, which was 66% of that at 32 h in Table 1. Therefore, by comparing the loss of total lipase activity of the aqueous phase (i.e., 15,252 U) to the gain on the supports (i.e., 330 U) between 16 and 32 h, the loss of total lipase activity must

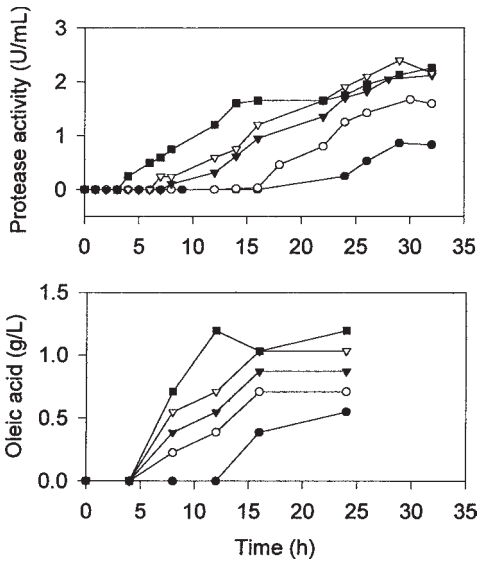

Fig. 6. Time course protease activity and oleic acid concentration of broth at volumetric ratio of 7 by adding wet supports of 6 (●), 9 (○), 15 (▼), 18 (▽), and 30 g (■).

Table 1
Lipase Activities of Aqueous Phase and Supports at 32 h

Wet supports added (g)	6	9	15	18	30
Wet supports recovered (g)	1.91	5.51	9.33	14.39	23.64
Total activity of supports (U)	267	973	1929	2502	3894
Total activity of broth (U)	23,112	35,244	23,568	30,504	32,244
Total activity (U)	23,379	36,217	25,497	33,006	36,138
Lipase activity on supports (%)	1.14	2.69	7.57	7.58	10.8

be owing to the lipase degradation by proteases. Moreover, the increase in lipase activity on the supports implied that the adsorbed lipases might resist the degradation by proteases.

Effects of Composition and Mass of Organic Phase in Fermentation Cultivation by Adding Pretreated Supports

To eliminate the formation of oil drops under the shear, the wet supports were pretreated in the fermentor containing deionized water for 2 h. Behaviors for cell growth and lipase activity similar to those shown in Fig. 3 were obtained when 6 g of the pretreated supports at volumetric ratios of 5 and 7 were employed (data not shown). However, a longer lag phase was observed for the system at a ratio of 7. Figure 7 illustrates the time course cell growth and lipase activity with the supports as the parameter at a volumetric ratio of 5. A maximum lipase activity of 31.1 U/mL at 11 h was found when adding 9 g of wet supports. Moreover, the average cell

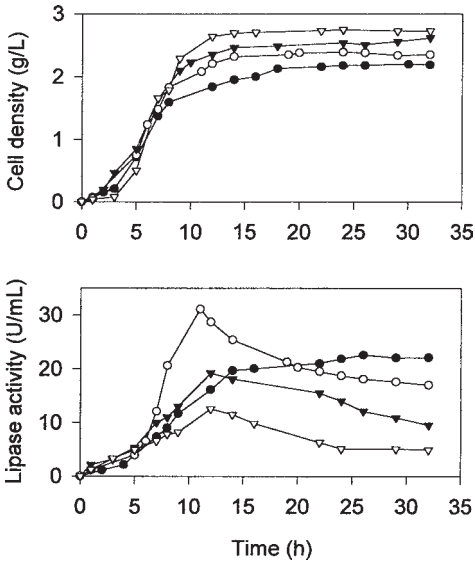

Fig. 7. Time course cell density and lipase activity in fermentation cultivation at volumetric ratio of 7 by adding pretreated wet supports of 6 (●), 9 (○), 15 (▼), and 30 g (▽).

Table 2
Lipase Activities of Aqueous Phase and Pretreated Supports at 32 h

Wet supports added (g)	6	9	18	30
Wet supports recovered (g)	1.78	6.12	16.48	24.89
Total activity of supports (U)	323	1005	2532	4793
Total activity of broth (U)	26,532	20,940	11,400	5868
Total activity (U)	26,855	21,945	13,932	10,660
Lipase activity on supports (%)	1.20	4.58	18.2	45.0

growth rates between 3 and 8 h were calculated and are represented in Fig. 5, in which a good linear relationship to the ratio of surface areas is illustrated. This indicates that the interfacial area is a critical parameter for cell attachment to uptake the carbon source. Protease formation was detectable at 12 h when adding 18 or 30 g of supports, but at 22 and 24 h for 9 and 6 g of supports, respectively. This result explained why a lower maximum lipase activity and lipase activity at 32 h in Fig. 7 was observed in the system containing 18 or 30 g of supports. Of course, more lipases adsorbed on the supports in the exponential phase might also contribute to the lower maximum activities.

Table 2 presents the lipase activities of the aqueous phase (i.e., 153–192 U/g of wet supports) and the supports (i.e., 4.89–22.11 U/mL) at 32 h. A higher percentage of lipase activity on the supports was found when 30 g of pretreated supports were employed. Therefore, the best strategies to obtain high lipase production include providing as much more

interfacial area as possible (i.e., using more finer powders), controlling the release of oleic acid, avoiding the formation of protease (i.e., performing fed-batch fermentation), or adding protease inhibitors.

Conclusion

Polypropylene powders were employed as the adsorbent for the organic solution containing *n*-hexadecane and olive oil in producing *A. radioresistens* lipase. From the time course cell growth and lipase activity in the shake-flask cultivation, the best volumetric ratio of 5 was determined. When more wet supports were added, a higher cell density with a maximum time course lipase activity was found. Similar results were obtained when cultivations containing the wet supports with or without pretreatment in a fermentor were carried out. The existence of a maximum time course lipase activity was attributed to the compensation effects of olive oil on cell growth and lipase production, repression for lipase synthesis by oleic acid, and lipase adsorption on the supports. A linear relationship between the average cell growth rate in the exponential phase and the ratio of surface areas of the supports was found. Less foaming and emulsion of the broth, higher total lipase production with less organic solution employed, and enhanced lipase recovery in the centrifugation step were observed when compared with cultivations without the use of supports.

Acknowledgment

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References

1. Wong, C. H. and Whitesides, G. M. (1994), *Enzymes in Synthetic Organic Chemistry*, Elsevier, Amsterdam.
2. Koskinen, A. M. P. and Klibanov, A. M. (1996), *Enzymatic Reactions in Organic Media*, Chapman & Hall, New York.
3. Faber, K. (1997), *Biotransformations in Organic Chemistry*, Springer-Verlag, Berlin.
4. Schmidt, R. D. and Verger, R. (1998), *Angew. Chem. Int. Ed*. **37**, 1608–1633.
5. Watanabe, N. Y., Minoda, O. Y., and Yamada, K. (1997), *Agric. Biol. Chem.* **41**, 1353–1358.
6. Nishio, T., Chikano, T., and Kamimura, M. (1987), *Agric. Biol. Chem.* **51**, 181–186.
7. Lesusse, E., Schanck, K., and Colson, C. (1993), *Eur. J. Biochem*. **216**, 155–160.
8. Kojima, Y., Yokoe, M., and Mase, T. (1994), *Biosci. Biotechnol. Biochem.* **58**, 1564–1568.
9. Wang, Y. X., Strivastava, K. C., Shen, G. Y., and Wang, H. Y. (1995), *J. Ferment. Bioeng*. **79,** 433–438.
10. Lin, S. F. (1996), *J. Ferment. Bioeng*. **82,** 448–451.
11. Lin, S. F., Chiou, C. M., Yeh, C. M., and Tsai, T. C. (1996), *Appl. Environ. Microbiol*. **62,** 1093–1095.
12. Hong, M. C. and Chang, M. C. (1998), *Biotechnol. Lett*. **20,** 1027–1029.
13. Ng, I. S., Tsai, S. W., and Chen S. J. (1999), *J. Chin. Inst. Chem. Engrs*. **30,** 355–362.
14. Chen, S. J., Cheng, C. Y., and Chen, T. L. (1998), *J. Ferment. Bioeng*. **86,** 308–312.
15. Shen, C. C., Wu, J. Y., Chen, C. Y., and Chen, T. L. (1999), *Biotechnol. Prog*. **15,** 919–922.

16. Chen, S. J., Cheng, C. Y., and Chen, T. L. (1999), *J. Chin. Inst. Chem. Engrs.* **30,** 283–288.
17. Gitlesen, T., Bauer, M., and Adlercreutz, P. (1997), *Biochim. Biophys. Acta* **1345,** 188–196.
18. Pencreach, G., Leullier, M., and Baratti, J. C. (1997), *Biotechnol. Bioeng.* **56,** 181–189.
19. Al-Duri, B. and Yong, Y. P. (2000), *Biochem. Eng. J.* **4,** 207–215.
20. Lowry, R. R. and Tinsley, I. J. (1976), *J. Am. Oil Chem. Soc.* **53,** 470–472.
21. Leighton, T. J., Doi, R. H., Warren, R. A. J., and Kelln, R. A. (1973), *J. Mol. Biol.* **76,** 103–122.
22. Kok, R. G., Nudel, C. B., and Gonzalez, R. H. (1996), *J. Bacteriol.* **178,** 6025–6035.