SHORT COMMUNICATION

# Transesterification of Phospholipids by Acetone-Dried Cells of a *Rhizopus* Species Immobilized on Biomass Support Particles

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**ABSTRACT:** Acetone-dried cells of a *Rhizopus* species, immobilized on biomass support particles were applied to the transesterification between phosphatidylcholine and heptadecanoic acid. All eight species of *Rhizopus* tested exhibited good transesterification performance, with the immobilized cells of *R. niveus*, *R. delemar*, and *R. javanicus* showing particularly high reaction rates which were equal to or exceeded that of a commercially available immobilized lipase preparation, Lipozyme IM60.
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**KEY WORDS:** Cell immobilization, lipase, phospholipids, *Rhizopus*, transesterification.

Recently, there has been an increasing interest in the use of phospholipids as natural emulsifiers in the food, cosmetics, pharmaceutical, and other industries. Phospholipids from natural sources contain several fatty acids, with proportions depending on the source. For some practical applications, it is desirable to obtain phospholipids with specific fatty acids. Phospholipids with specified fatty acid compositions can be obtained by solvent fractionation and by chemical or enzymatic conversion. Enzymatic methods appear to have strong advantages in the modification of phospholipids because of mild reaction conditions and high stereochemical or positional specificities. There have been many reports on the acyl exchange of phospholipids with native or immobilized lipase (1–6). However, it has been frequently pointed out that the significantly high cost of lipases or immobilized lipase preparations constitutes a bottleneck to their use in industrial applications (7,8), especially when the product of the lipase-catalyzed reaction is not of high value.

In this work, unique acetone-dried cells of *Rhizopus* species, immobilized on biomass support particles (BSP) (9), were applied to the transesterification reaction between phosphatidylcholine (PC) and heptadecanoic acid. The objective of the study is to evaluate the viability of the BSP-immobilized cells for transesterification of phospholipids in comparison with a commercially available immobilized lipase preparation, Lipozyme (Novo Nordisk, Bagsvaerd, Denmark), which has frequently been used in transesterification reactions (5,6).

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## MATERIALS AND METHODS

*Microorganisms and media.* The experiments were performed with eight *Rhizopus* species, i.e., *R. niveus* IFO 4759, *R. oryzae* IFO 4744, *R. delemar* IFO 4697, *R. japonicus* IFO 4758, *R. javanicus* IFO 5441, *R. chinensis* IFO 4768, *R. oligosporus* IFO 8631, and *R. stolonifer* IFO 4781. All cultures were stocked at 4°C after cultivation on potato sucrose agar. The basal medium contained per 1 L of tap water: meat extract (Wako Pure Chemicals, Osaka, Japan), 5 g; $NaNO_3$, 1.0 g; $KH_2PO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; and oleic acid, 20 g.

*BSP.* Cubic, polyurethane foam BSP 6-mm square (HR-40; Bridgestone, Tokyo, Japan), with a porosity of around 0.97 and a pore size of 40 pores per linear inch, were used in all experiments.

*Chemicals.* Immobilized lipase from *Mucor miehei* (Lipozyme IM20 and IM60) was a gift from Novo Nordisk. Egg yolk PC was from Asahi Chemical Industry (Osaka, Japan). All other chemicals were of analytical grade and were obtained from commercial sources.

*Preparation of acetone-dried BSP-immobilized cells.* Sakaguchi flasks, containing 100 mL of the medium and 150 BSP cubes, were inoculated by aseptically transferring a loopful of spores from an agar slant. These flasks were then incubated at 30°C for about 90 h on a reciprocal shaker. The initial pH was adjusted to 5.6. Immobilized cells, recovered from culture broth by filtration, were washed twice each with tap water and with acetone, followed by drying under vacuum for 48 h, for use as a lipase catalyst (9).

*Determination of lipase activities and cell mass concentration.* The hydrolytic activity of lipase was determined by following the method described by Fukumoto *et al.* (10). The transesterification lipase activity, previously defined by the interesterification reaction between olive oil and methyl stearate (9), was used to compare the reactivity of BSP-immobilized cells for phospholipids with Lipozyme. One unit (U) of the transesterification and hydrolysis of lipase activities was defined as the enzyme amount that liberated 1 μmol of methyl oleate or fatty acid from olive oil per minute at 40°C. The concentration of cell mass immobilized in BSP was determined by measuring the difference in weight between BSP with dried cells and BSP cleaned with NaClO solution (11).

*Transesterification reaction.* To obtain a defined initial water content of the enzyme (BSP-immobilized cells or Lipozyme) and the substrate solution, both were preequilibrated over saturated LiCl solution with a water activity ($a_w$) of 0.11 in a closed vessel for 24 h at 25°C (6). Enzyme equivalent to about 2.5 U of transesterification lipase activity and 5.0 mL of a solution of PC (13 mM) and heptadecanoic acid (111 mM) in hexane were mixed in screw-cap vessels (10 mL), followed by incubation at 40°C. Heptadecanoic acid was selected because it is absent in PC, so its incorporation could be easily detected. Periodically, 400-μL aliquots of the reaction mixture were withdrawn from the vessel and dissolved in 200 μL $CHCl_3$-MeOH (2:1, vol/vol). This sample was then analyzed by the method described next.

*Analysis.* The amount of PC remaining unhydrolyzed was determined in a manner similar to that described by Yagi *et al.* (1) with an Iatroscan TH-10 TLC analyzer, equipped with an SIC chromatorecorder II (Iatron Laboratories, Tokyo, Japan). The fatty acid composition of PC was determined by gas chromatography (GC) as follows: A 400-μL sample was first separated into PC, lysophosphatidylcholine, and fatty acids by thin-layer chromatography (TLC). On a silica gel plate (type 60; Merck, Darmstadt, Germany), it was developed in a mixture of $CHCl_3$/MeOH/$H_2O$ (20:10:1, vol/vol/vol). After visualization with iodine vapor, the PC band was scraped from the TLC plate and put into a test tube. Fatty acid methyl esters were formed by adding 2.0 mL of 2.8% sodium methoxide in methanol to the scrapings, followed by incubation for 5 min at 50°C. Water (0.5 mL) and 0.5 mL of 4 mM methyl decanoate solution in hexane as an internal standard solution were then added to the test tube. After vortexing, the upper layer was taken for GC analysis. Methyl esters of fatty acids were analyzed by an HP5890 Series II gas chromatograph, fitted with a flame-ionization detector (Hewlett-Packard, Avondale, PA). The column used was a DB23 (30-m length, 0.25-mm i.d., 0.25-μm film thickness, J&W Scientific, Folsom, CA), and the temperature was set at 190°C.

## RESULTS AND DISCUSSION

*Lipase production by a* Rhizopus *species immobilized on BSP.* All cells of eight *Rhizopus* species were naturally immobilized in BSP without the formation of freely suspended cells in shake flask culture. Figure 1 shows the cross-sections of BSP with immobilized *R. niveus* and empty BSP. The cells formed a dense film near the BSP surface. The concentrations of cells immobilized in the BSP and the lipase production behavior at 90-h cultivation are summarized in Table 1. Dried cells of all *Rhizopus* species tested showed comparatively high lipase activity. Six species (not *R. chinensis* and *R. stolonifer*) showed greater transesterification activity than Lipozyme IM20, and *R. niveus, R. oryzae, R. delemar,* and *Rh. javanicus* exhibited remarkably high transesterification activity (about 1.6 times or more than Lipozyme IM60). BSP-immobilized cells of these eight *Rhizopus* species and the two Lipozymes (IM20 and IM60) were used in the following experiments for transesterification of phospholipids.

*Transesterification of phospholipids by BSP-immobilized cells and Lipozymes.* Time courses of the transesterification reaction, catalyzed by the eight BSP-immobilized cells and two Lipozymes, are compared in Figure 2. All eight species of *Rhizopus* were capable of incorporating heptadecanoic acid

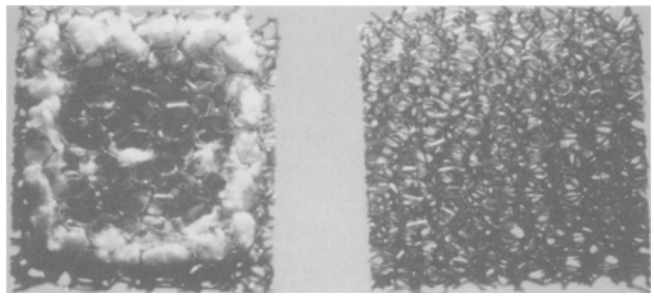

**FIG. 1.** Micrograph of cross-sections of biomass support particles (BSP) with immobilized *Rhizopus niveus* (left) and empty BSP (right).

**TABLE 1**
**Lipase Production by Eight Species of *Rhizopus* Immobilized on BSP[a]**

| Microorganism | Cell concentration (mg/BSP) | Lipase activity in cells (U × $10^2$/mg dried cells) | | Lipase activity in culture supernatant (U × $10^2$/mL) |
|---|---|---|---|---|
| | | Transesterification | Hydrolysis | Hydrolysis |
| *R. niveus* | 1.41 | 39.11 | 110.07 | 137.50 |
| *R. oryzae* | 0.74 | 27.48 | 134.17 | 48.96 |
| *R. delemar* | 1.16 | 29.64 | 110.97 | 35.42 |
| *R. japonicus* | 0.72 | 14.16 | 62.27 | 27.08 |
| *R. javanicus* | 1.02 | 27.79 | 116.01 | 79.17 |
| *R. chinensis* | 1.39 | 4.95 | 19.81 | 26.04 |
| *R. oligosporus* | 1.09 | 10.16 | 76.31 | 41.67 |
| *R. stolonifer* | 1.12 | 7.02 | 50.89 | 35.42 |
| Lipozyme IM20 | | 8.08 | 2.73 | |
| Lipozyme IM60 | | 16.1 | 10.04 | |

[a]Basel medium: meat extract 5 g/L; $NaNO_3$ 1 g/L; $KH_2PO_4$ 1 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; oleic acid 25 mL/L; biomass support particle (BSP) 150 particles/100 mL medium. Culture conditions: temperature, 30°C; initial pH, 5.6; cultivation time, 90 h; reciprocal shaking, 200 oscillations/min; and 70 mm amplitude.

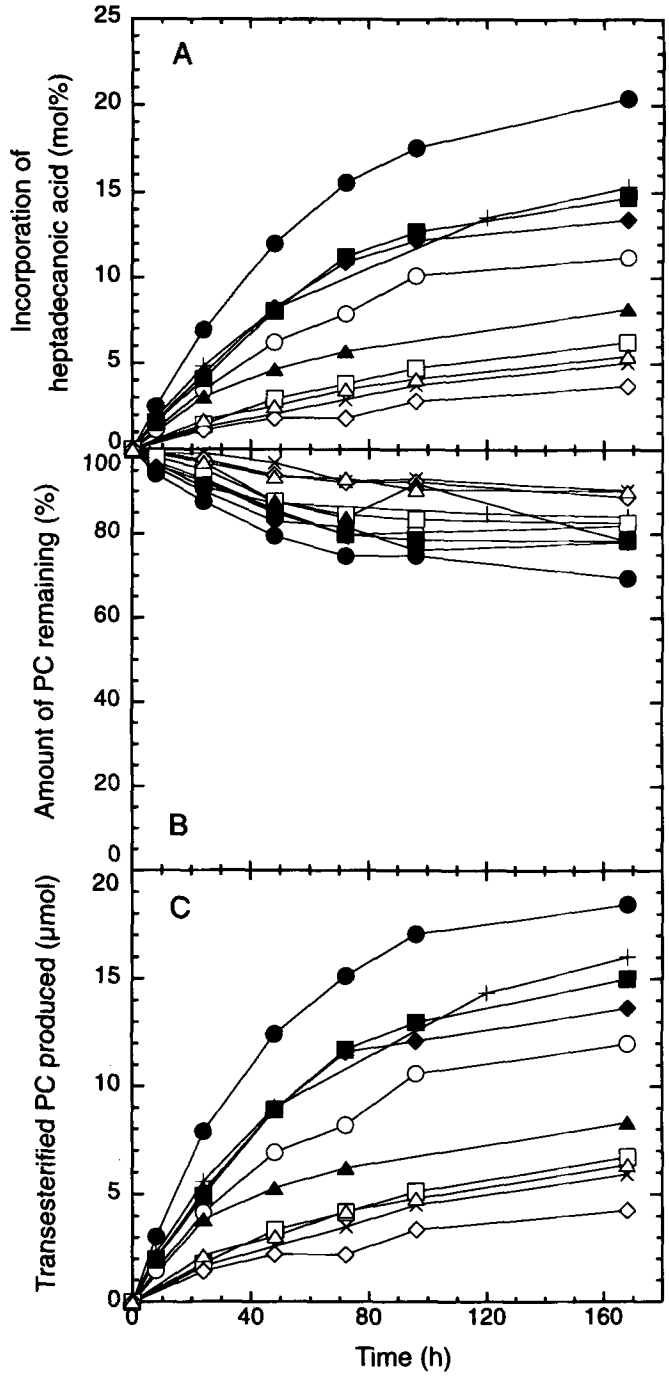


**FIG. 2.** Time courses of transesterification between phosphatidylcholine (PC) and heptadecanoic acid by a *Rhizopus* species immobilized on biomass support particles or Lipozymes. Cells or Lipozymes (Novo Nordisk, Bagsvaerd, Denmark): *R. niveus* (●), *R. oryzae* (○), *R. delemar* (■), *R. japonicus* (□), *R. javanicus* (◆), *R. chinensis* (◇), *R. oligosporus* (▲), *R. stolonifer* (△), Lipozyme IM20 (×), and Lipozyme IM60 (+).

into PC. It is interesting that significant differences were observed in the reactivities of the transesterification of PC among the immobilized lipase preparations used, despite the fact that the same amount of enzyme, equivalent to about 2.5 U transesterification lipase activity, was added into the reaction mixtures. All BSP-immobilized cells other than *R. chinensis* showed a reactivity higher than that of Lipozyme IM20. In particular, the dried cells of *R. niveus, R. delemar,* and *R. javanicus* exhibited good transesterification performances, their reaction rates being equivalent to or greater than that of Lipozyme IM60. A similar tendency was also recognized in the hydrolysis reaction (Fig. 2B). The best conversion of PC was achieved by *R. niveus* immobilized in BSP (Fig. 2C).

Dried cells immobilized on BSP were readily obtained by immobilizing a fungus, the lipase producer, on BSP simultaneously during cultivation; the catalyst then could be prepared merely by drying the cells. In this way, the complicated recovery, purification, and immobilization processes needed when extracellular lipase is used could be dispensed with. It is anticipated that this immobilized fungus system will be economically advantageous over the conventional system that uses extracellular lipase. Therefore, BSP-immobilized cells have potential for industrial transesterification of phospholipids from an economic viewpoint.

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