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Facilitatory effect of immobilized lipase-producing *Rhizopus oryzae* cells on acyl migration in biodiesel-fuel production

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Abstract

For biodiesel-fuel production by methanolysis of plant oils, *Rhizopus oryzae* cells producing a 1,3-positional specificity lipase were cultured with polyurethane foam biomass support particles (BSPs) in a 20 l air-lift bioreactor, and the cells immobilized within BSPs were used as whole-cell biocatalyst in repeated batch-cycle methanolysis reaction of soybean oil. The whole-cell biocatalyst had a higher durability in the methanolysis reaction when obtained from air-lift bioreactor cultivation than from shake-flask cultivation. Following repeated methanolysis reaction using the whole-cell biocatalyst, analysis of the reaction mixture composition indicated that monoglycerides (MGs) decreased and free fatty acids (FFAs) increased with increasing water content in the reaction mixture, and that MGs, diglycerides (DGs), and triglycerides (TGs) increased with increasing number of reaction cycles. The isomers of MGs and DGs generated during the 20th methanolysis reaction cycle consisted of 2-MGs and 1,2(2,3)-DGs, respectively. The hydrolytic activity of the whole-cell biocatalyst, on the other hand, was stable regardless of the number of reaction cycles. It was demonstrated thus that the whole cell biocatalyst promotes acyl migration of partial glycerides, and that the facilitatory effect is increased by increase in the water content of the reaction mixture but it is lost gradually with increasing number of reaction cycles.

Keywords: Biodiesel fuel; Methanolysis; *Rhizopus oryzae*; Lipase; Whole-cell biocatalyst; Air-lift bioreactor; Acyl migration

1. Introduction

Biodiesel fuel consists of methyl ester (ME) produced by transesterification of triglycerides (TGs) with methanol (i.e. methanolysis). As a fuel with the environmental advantages of renewability, biodegradability, and non-toxicity, it has attracted considerable attention, and is in current production using plant oil in Europe and the USA and waste oil in Japan.

A number of processes have been developed for biodiesel-fuel production involving chemical or enzyme catalysis or supercritical alcohol treatment [1–4]. Although each process has drawbacks and advantages [1], increasing environmental concerns have led to growing interest in the use of enzyme catalysis. There are many reports on biodiesel-fuel production using enzyme catalysis by free or immobilized lipase [5–12]. Immobilized lipase in particular is suitable for continuous biodiesel-fuel production because of its ease of recovery from the reaction mixture. However, the various processes involved result in high cost and can be a barrier to widespread use of enzymatic processing. The use of lipase-producing microbial cells immobilized within porous biomass support particles (BSPs) as whole-cell biocatalyst is effective in improving cost efficiency since the immobilization can be achieved spontaneously during batch cultivation and no purification of lipase is necessary. In a previous study [13], we found that

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glutaraldehyde (GA)-treated *Rhizopus oryzae* cells immobilized within polyurethane foam BSPs had a high durability in comparison with non-treated cells and were suitable for repeated use. For industrial application of whole-cell biocatalyst to biodiesel-fuel production, a technique for preparing whole-cell biocatalyst in large quantity is needed.

In the present study, *R. oryzae* was cultured in an air-lift bioreactor (20 l), one of whose advantages is the low level of shear stress during cell growth compared to the stirred-tank bioreactor. Immobilized cells from shake-flask and air-lift cultures were compared for their durability in repeated methanolysis reaction. By analyzing the composition of the resultant reaction mixture, the effect of the number of reaction cycles on the methanolysis activity of the immobilized cells was also investigated.

2. Materials and methods

2.1. Microorganism, culture media, and BSPs

All experiments were carried out using the filamentous fungus *R. oryzae* IFO 4697, which has a 1,3-positional specificity lipase [14–16]. The agar slant was made from 4% potato dextrose agar and 2% agar powder. *R. oryzae* was grown in basal medium (polypepton 70 g, $NaNO_3$ 1.0 g, KH_2PO_4 1.0 g, and $MgSO_4 \cdot 7H_2O$ 0.5 g in 1 l of distilled water) with olive oil or glucose as carbon source. The pH of the medium was initially adjusted to 5.6 and then allowed to follow its natural course. Six millimeters cubes of reticulated polyurethane foam (Bridgestone Co. Ltd., Osaka, Japan) with a particle voidage of more than 97% and a pore size of 50 pores per linear inch were used as BSPs.

2.2. Air-lift bioreactor

Fig. 1 is a diagrammatic illustration of the air-lift bioreactor (20 l) used. The bioreactor is divided into an upper part (200 mm in internal diameter and 350 mm in height) and a lower part (150 mm in internal diameter and 415 mm in height), and has an inner draft tube of 80 mm diameter and 350 mm height.

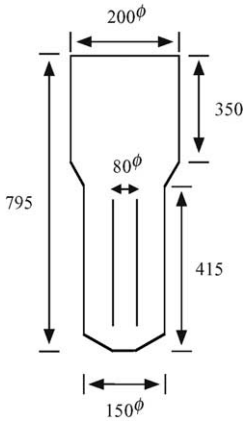

Fig. 1. Dimensions of air-lift bioreactor (mm).

2.3. Shake-flask and air-lift bioreactor cultivation

For shake-flask cultivation, *R. oryzae* hyphae and spores grown for 72 h on an agar slant were aseptically inoculated into a Sakaguchi flask containing 100 ml of basal medium with 30 g/l olive oil and 150 BSPs, and cultivated for 90 h at 35 °C on a reciprocal shaker (150 oscillations/min, amplitude 70 mm).

For air-lift cultivation, meanwhile, the seed culture was processed for 24 h in a Sakaguchi flask containing 100 ml basal medium with 10 g/l glucose. The resultant culture medium and the *R. oryzae* cells were then transferred to the air-lift bioreactor containing 10 l basal medium with 30 g/l olive oil and 12,000 BSPs. The bioreactor was aerated at 2.5 vvm at 35 °C. The *R. oryzae* cells became well immobilized within the BSPs as a natural consequence of their growth during shake-flask and air-lift cultivation.

After cultivation, the BSP-immobilized cells were separated from the culture broth by filtration, washed with tap water, dried at room temperature for around 24 h, and cross-linked with GA.

2.4. GA treatment of BSP-immobilized cells

The GA treatment was carried out by adding 0.1 vol.% GA solution to BSP-immobilized cells, which were separated from the culture broth, and incubating them at 25 °C for 1 h [13]. In the case of BSP-immobilized cells obtained in air-lift cultivation, 10 l of the GA solution was added into the air-lift bioreactor without the draft tube, which was aerated at 1.0 vvm at 25 °C for 1 h. After separation of the GA-treated cells from the solution by filtration, they were washed with tap water at 4 °C for a few minutes, dried for approximately 24 h at room temperature, and used as whole-cell biocatalyst.

2.5. Measurement of weight of cells immobilized within BSPs

The cell concentration immobilized within one BSP was measured as follows: 10 BSPs containing immobilized cells were taken, washed with acetone for 10 min to remove adherent olive oil, and dried for 24 h at 80 °C. The particles plus dried cells were then weighed and treated with an aqueous solution of sodium hypochlorite (approximately 10 vol.%) to remove the immobilized cells. The cell weight was estimated from the difference between the weights.

2.6. Methanolysis reaction

Methanolysis reaction was carried out at 30 °C for 72 h in a 50 ml screw-capped bottle placed inside a reciprocal shaker (150 oscillations/min, amplitude 70 mm). The reaction mix-