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Repeated use of whole-cell biocatalysts immobilized within biomass support particles for biodiesel fuel production

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Abstract

With a view to utilizing *Rhizopus oryzae* cells immobilized within biomass support particles (BSPs) as a whole-cell biocatalyst for biodiesel fuel production, an investigation was made of the effect of cross-linking treatment with glutaraldehyde (GA) on the stability of lipase activity. Although the lipase activity of the BSP-immobilized cells decreased considerably in the presence of the methyl esters produced by methanolysis, the activity of cells treated with 0.1% GA solution showed no significant decrease during six batch cycles, with the methyl ester content of the reaction mixture reaching 70–83% in each cycle. In contrast, without GA treatment, activity decreased gradually with each cycle to give a methyl ester content of only 50% at the sixth batch cycle. These findings indicate that, given the simplicity of the lipase production process and the long-term stability of lipase activity, the use of whole-cell biocatalysts immobilized within BSPs and treated with GA solution offers a promising means of biodiesel fuel production for industrial application.

Keywords: Biodiesel fuel; Methanolysis; Methyl ester; Glutaraldehyde treatment; Whole-cell biocatalyst

1. Introduction

Biodiesel (fatty acid methyl esters), which is produced by transesterification of triglycerides with methanol (i.e. methanolysis), has become increasingly important due to diminishing petroleum reserves and the environmental consequences of exhaust gases from petroleum-fueled engines. Among the attractive features of biodiesel fuel are: (i) it is plant-derived, not petroleum-derived, and as such its combustion does not increase current net atmospheric levels of CO_2, a "greenhouse" gas; (ii) it can be domestically produced, offering the possibility of reducing petroleum imports; (iii) it is biodegradable; (iv) relative to conventional diesel fuel, its combustion products have reduced levels of particulates, carbon monoxide, sulfur oxides, hydrocarbons, soot, and under some conditions, nitrogen oxides.

Although chemical methanolysis using an alkali-catalysis process gives high conversion levels of triglycerides to their corresponding methyl esters in short reaction times, the reaction has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the alkaline catalyst has to be removed from the product, alkaline wastewater requires treatment, and free fatty acids and water interfere with the reaction.

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Recently, enzymatic methanolysis using lipases has become more attractive for biodiesel fuel production, since it can overcome the problems mentioned. In particular, it should be noted that the by-product, glycerol, can be easily recovered without complex processing, and also that free fatty acids contained in waste oils and fats can be completely converted to methyl esters. Effective methanolysis reactions using several extracellular lipases from *Candida* sp. [1–3], *Pseudomonas* sp. [4], and *Rhizopus* sp. [5] have been developed by several researchers. With these lipases, a methyl ester content in the reaction mixture of more than 90% is obtained using either low- or high-water content systems. However, the use of extracellular lipase as a catalyst requires complicated recovery, purification, and immobilization processes for industrial application.

Consequently, there has been considerable interest in the direct use as a whole-cell biocatalyst of intracellular lipase, which, unlike extracellular lipase, requires no purification or immobilization process when prepared using a technique based on porous biomass support particles (BSPs) [6], since immobilization can be achieved spontaneously during batch cultivation. For the industrial interesterification of fats and oils, aceton-dried cells of *Rhizopus chinensis* immobilized within polyurethane foam BSPs can be used directly as a whole-cell biocatalyst [7–9].

Utilizing *R. oryzae* cells immobilized within BSPs as a whole-cell biocatalyst for biodiesel fuel production, the authors [10] found that methanolysis was carried out with stepwise addition of methanol in the presence of 10–20% water in a batch operation produced a methyl ester content of 80–90% in the reaction mixture without organic solvent pre-treatment. This level of methyl ester production is almost the same as that achieved using extracellular lipase [5].

In the present study, in order to stabilize the immobilized *R. oryzae* cells within BSPs for repeated batch operation, a cross-linking treatment with glutaraldehyde (GA) solution was examined.

2. Materials and methods

2.1. Microorganism and media

All experiments were carried out using *R. oryzae* IFO 4697, which has a 1,3-positional specificity lipase. The basal medium used contained, in 1 l of tap water (pH 5.6):polypepton 70 g; $NaNO_3$ 1.0 g; KH_2PO_4 1.0 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; olive oil 30 g.

2.2. Biomass support particles (BSPs) and shake-flask cultivation

Sakaguchi flasks (500 ml) containing 100 ml of the basal medium with BSPs were inoculated by aseptically transferring spores from a fresh agar slant using potato dextrose agar, and incubated at 35 °C for 90 h on a reciprocal shaker (150 oscillations/min, amplitude 70 mm). The *R. oryzae* cells became well immobilized within the BSPs as a natural consequence of their growth during shake-flask cultivation. Immobilization was effected by placing 150 particles inside a flask together with the medium, subjected to prior sterilization. The BSPs used for immobilization were 6 mm cubes of reticulated polyurethane foam (Bridgestone Co. Ltd., Osaka, Japan) with a particle voidage beyond 97% and a pore size of 50 pores per linear inch.

BSP-immobilized cells were separated from the culture broth by filtration. After washing with tap water for 1 min, they were dried at 25 °C for, approximately 48 h and used as a methanolysis catalyst.

2.3. Glutaraldehyde treatment of BSP-immobilized cells

The GA treatment was carried out by adding 0.01–1.0 vol.% of GA solution (0.1 M phosphate buffer, pH 6.8) to BSP-immobilized cells, which were separated from the culture broth, and incubating them at 25 °C for 1 h. After separation of the GA-treated cells from the solution by filtration, they were shaken in phosphate buffer at 4 °C for 5 min, washed with tap water for 1 min, and dried for, approximately 24 h at room temperature. The GA-treated cells thus obtained (approximately 5% water content) were used as a methanolysis catalyst.

2.4. Methanolysis reaction

Methanolysis of soybean oil was carried out at 35 °C in a 50 ml screw-cap bottle with incubation on a reciprocal shaker (150 oscillations/min, amplitude 70 mm). The reaction mixture (soybean oil 9.65 g,

0.1 M phosphate buffer (pH 6.8) 1.5 ml, and methanol 0.175–0.525 g) was dispensed with 50 BSPs into a screw-cap bottle. The molar equivalent of methanol to 9.65 g soybean oil was 0.35 g.

To fully convert the oil to its corresponding methyl esters, at least three molar equivalents of methanol are necessary. Consequently, 0.175, 0.35, or 0.525 g methanol was added stepwise to the reaction mixture (six, three, and two times, respectively). In these cases, the reaction mixture was incubated for 72 h. When the BSP-immobilized cells were used repeatedly for methanolysis, they were dried at room temperature after washing with tap water and methanolysis carried out several times with a fresh reaction mixture. The methanolysis products were analyzed by capillary gas chromatography (cGC) as described next.

2.5. Effect of methyl esters on extracellular and intracellular lipase activities

2.5.1. Extracellular lipase

Lipase powder (0.5 g) from *R. oryzae* (F-AP15), purchased from Amano Pharmaceutical Co. Ltd. (Aichi), was dissolved in 5.0 ml of 0.1 M phosphate buffer (pH 6.8). The mixture was stirred using a magnetic stirrer for 1 h, followed by centrifugation at 3500 × *g* for 10 min, and the supernatant (1.5 ml) pre-incubated with 9.65 g of either methyl oleate or soybean oil in a 30 ml screw-cap bottle at 35 °C for 3–96 h with agitation at 300 rpm. After centrifugation at 12,000 × *g* for 5 min, 0.3 ml of the water layer containing lipase was used for methanolysis with a reaction mixture of soybean oil 1.93 g and methanol 70 mg. The reaction mixture was stirred at 35 °C for 2.5 h with agitation at 300 rpm.

The ratio of methyl ester content in the reaction mixture prepared using the water layer containing lipase pre-treated with methyl oleate or soybean oil to that in a non-pre-treated mixture was determined as relative activity.

2.5.2. Intracellular lipase (whole-cell biocatalyst)

Fifty BSP-immobilized cells were pre-incubated with methyl oleate, methyl linoleate or soybean oil (9.65 or 2.90 g) and 0.1 M phosphate buffer (pH 6.8) 1.5 ml at 35 °C for 6–168 h with shaking at 150 oscillations/min in a reciprocal shaker. After being washed with tap water for 1 min, they were dried for, approximately 24 h at room temperature. Methanolysis using the BSPs thus obtained was carried out at 35 °C for 2.5 h in a reciprocal shaker (150 oscillations/min, amplitude 70 mm). The reaction mixture for methanolysis (soybean oil 9.65 g, 0.1 M phosphate buffer (pH 6.8) and methanol 0.35 g) was dispensed with 50 BSPs into a screw-cap bottle. The ratio of methyl ester content in the reaction mixture prepared using the BSP-immobilized cells pre-treated with methyl oleate, methyl linoleate or soybean oil to that in a non-pre-treated mixture was determined as relative activity.

2.6. Analysis

2.6.1. Capillary gas chromatography

The methyl ester content of the reaction mixture was quantified using a GC-18A gas chromatograph (Shimadzu Corp., Kyoto, Japan) connected to a DB-5 capillary column (0.25 mm × 10 m; J&W Scientific, Folsom, CA, USA). Samples (150 ml) were taken from the reaction mixture at specified times and centrifuged at 12, 000 × *g* for 5 min.

For cGC analysis, 100 μl of the aforementioned mixture and 20 ml of tricaprylin were precisely measured into a 10 ml bottle, to which a specified amount of anhydrous sodium sulfate as a dehydrating agent and 3.0 ml hexane were added. Tricaprylin served as the internal standard for cGC. A 1.0 μl aliquot of the treated sample was injected into a GC-18A gas chromatograph connected to a DB-5 capillary column (0.25 mm × 10 m) to determine the methyl ester content of the reaction mixture. Methyl ester content was expressed as the percentage proportion of converted methyl esters in the reaction mixture exclusive of water and glycerol. The column temperature was held at 150 °C for 0.5 min, raised to 300 °C at 10 °C/min, and maintained at this temperature for 3 min. The temperatures of injector and detector were set at 245 and 250 °C, respectively.

2.6.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis

To examine lipase leakage from the BSP-immobilized cells, the soluble fractions in the pre-treated mixtures were analyzed by SDS–PAGE [11]. Samples taken at 168 h from the pre-treated mixture using

BSP-immobilized cells (see Section 2.5.2) were subjected to SDS–PAGE using a 12.5% gel. Lipase and other proteins were detected by staining the gel with Coomassie brilliant blue with a lipase solution (10 g/l) as control.

3. Results and discussion

3.1. Effect of methanol concentration on lipase activity of BSP-immobilized cells

To examine the effect of the methanol concentration of the reaction mixture on the methanolytic activity of the BSP-immobilized cells, 0.175, 0.35 or 0.525 g methanol was added stepwise to the reaction mixture. Fig. 1 shows the methyl ester content of the reaction mixtures during four batch cycles. In the case of 0.525 g methanol, methyl ester content decreased sharply with each cycle from, approximately 70%, resulting in the cessation of methanolysis at the fourth batch cycle. In contrast, with 0.175 or 0.35 g methanol, methyl ester content reached approximately 80% in the first batch, and persisted at, approximately 60% in the fourth batch cycle. Kaieda et al. [5] found that the extracellular *R. oryzae* lipase efficiently catalyzed methanolysis in the presence of 4–30% water in the starting material, but that the enzyme was nearly inactive in the absence of water. Thus, an insufficient amount of water probably results in the irreversible inactivation of the lipase, which may be due to denaturation of the enzyme by methanol. Shimada et al. [1] also report that immobilized *Candida antarctica* lipase (Novozym 435) is inactivated by shaking in a mixture containing more than 1.5 M equivalents of methanol to oil. Since a high methanol concentration causes irreversible denaturation, complete inactivation occurs with 0.525 g methanol (1.5 M equivalents of methanol to oil) when using BSP-immobilized cells. Given these results, 0.35 g methanol was used in the subsequent experiments.

3.2. Effect of glutaraldehyde concentration on BSP-immobilized cells

Table 1 presents data on the methanolytic activity of the BSP-immobilized cells following GA treatment and on the stability of the GA-treated cells after 7 days incubation at 35 °C. Compared to the control, methanolytic activity following GA treatment increased slightly in the GA concentration range of over 0.01–1.0 vol.%. Residual activity increased with GA concentration up to 0.02 vol.%, but beyond 0.05 vol.% almost no change in activity (74–78%) was observed.

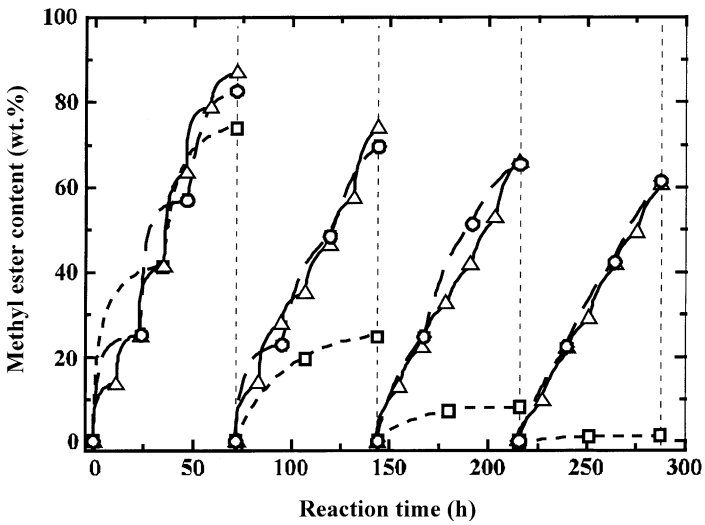

Fig. 1. Effects of concentration of methanol added to reaction mixture on methanolytic activity of BSP-immobilized cells. Methanol (0.175, 0.35, or 0.525 g) was added six, three, or two times, respectively, in one batch cycle: (△) 0.175 g; (○) 0.35 g; (□) 0.525 g methanol.

Table 1
Effect of GA concentration on stabilization of methanolytic activity in BSP-immobilized cells

GA concentration[a] (vol.%)	Relative activity (%)	
	Initial methanolytic activity[b]	Residual activity[c]
0.01	109	27
0.02	111	62
0.05	115	76
0.1	114	76
0.2	117	76
0.5	113	78
1.0	115	74
0 (control)[d]	100	16

[a] Adding GA concentration to BSP-immobilized cells.
[b] Methanolytic activity of BSP-immobilized cells just after GA treatment (percentage of control value).
[c] Methanolytic activity after incubation for 7 days at 35 °C (percentage of initial value).
[d] BSP-immobilized cells without GA treatment.

Without GA treatment, however, activity decreased considerably. Flores et al. [12] also found that a whole-cell biocatalyst (*Kluyveromyces lactis* NRRL) with β-galactosidase activity was significantly stabilized by GA treatment with 0.15–0.20 vol.% solution. To stabilize the lipase activity of the BSP-immobilized cells, 0.1 vol.% GA solution was therefore used in the subsequent experiments.

3.3. Effects of methanolysis products on extracellular and intracellular lipase activity

To examine the effects of methanolysis products on lipase activity, both extracellular and intracellular lipases were pre-treated by incubation with methyl esters before use in methanolysis. Since the major components of the fatty acids commonly found in vegetable oils such as corn, rapeseed, soybean or sunflower oils are oleic and/or linoleic acid [13,14], methyl oleate and linoleate were used as methanolysis products. Soybean oil was used as control.

Fig. 2 shows the effect of incubation time on relative activity in extracellular *R. oryzae* lipase. In the case of soybean oil, there was almost no decrease in relative activity even with 96 h treatment. In contrast, use of methyl oleate resulted in a rapid decrease with incubation time; almost all catalytic activity was lost at 96 h.

Fig. 3a and b show the effect of incubation time on relative activity in the cases of methyl oleate and methyl linoleate using GA-treated and untreated cells with each. In all cases, there was almost no decrease in relative activity with soybean oil, whereas methyl oleate or linoleate resulted in rapid decrease at high concentrations, as in Fig. 2, but gradual decrease at low concentrations, with almost 70% of activity maintained even at 168 h with both substances. The lipase activity of BSP-immobilized cells was thus found to

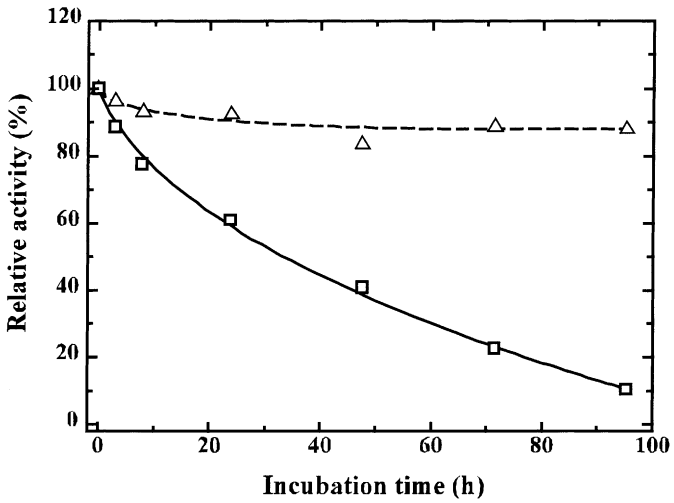

Fig. 2. Effects of methyl oleate and soybean oil on methanolytic activity of extracellular *R. oryzae* lipase: (△) soybean oil; (□) methyl oleate.

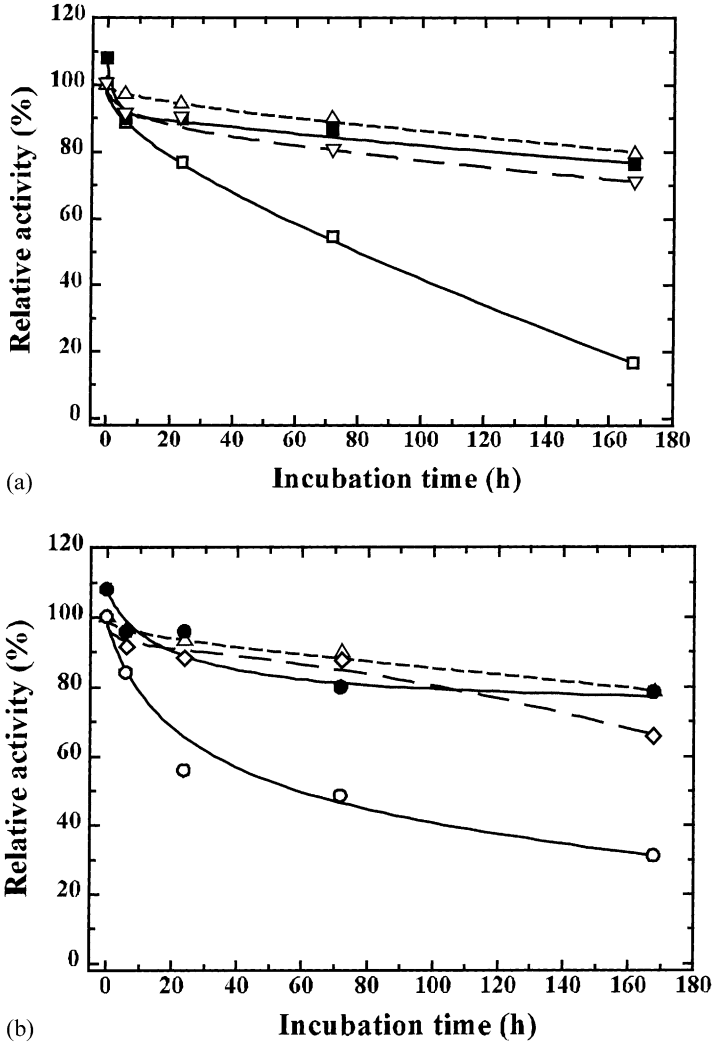

Fig. 3. Effects of methyl oleate (a), methyl linoleate (b), and soybean oil on methanolytic activity of BSP-immobilized cells with and without GA treatment: (□) methyl oleate (9.65 g)/GA-untreated cells; (▽) methyl oleate (2.90 g)/GA-untreated cells; (△) soybean oil/GA-untreated cells; (■) methyl oleate (9.65 g)/GA-treated cells; (○) methyl linoleate (9.65 g)/GA-untreated cells; (◇) methyl linoleate (2.90 g)/GA-untreated cells; (●) methyl linoleate (9.65 g)/GA-treated cells.

be significantly impaired by the high concentration of methyl esters produced by methanolysis.

Interestingly, however, the relative activities of the GA-treated cells were mostly maintained at high values even in the presence of high concentrations of methyl oleate or linoleate, with almost 80% of activity maintained at 168 h with both substances. This indicates that the lipase activity of the BSP-immobilized cells was effectively stabilized by GA treatment.

Fig. 4 shows the results of SDS–PAGE analysis of samples taken at 168 h from the pre-treated mixtures used in Fig. 3a. When the cells were pre-treated with either soybean oil (lane 2) or methyl oleate (lane 3), distinct bands of lipase were observed, but none with GA-treated cells (lane 4). It thus appears that the lipase of the GA-untreated cells leaked into the reaction mixture with both soybean oil and methyl oleate during methanolysis, but that the leakage from the cells was

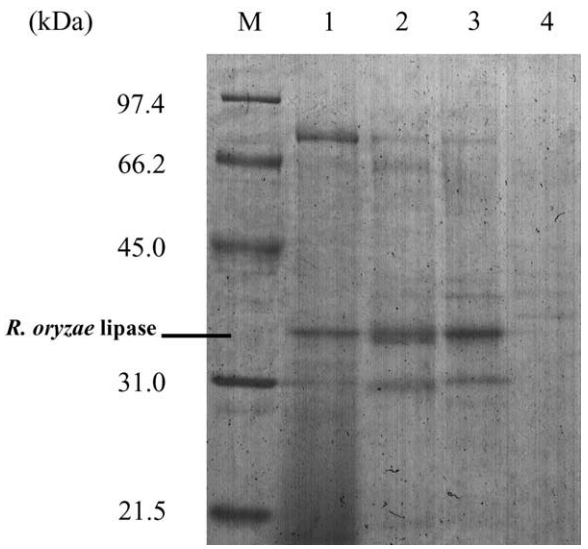

Fig. 4. Results of SDS–PAGE analysis of samples taken at 168 h from the pre-treated mixtures used in Fig. 3a. Lanes contain molecular weight markers (M), solution of extracellular *R. oryzae* lipase (lane 1), soybean oil with GA-untreated cells (lane 2), methyl oleate with GA-untreated cells (lane 3), and methyl oleate with GA-treated cells (lane 4).

prevented effectively by GA treatment. Although the lipase of the GA-untreated cells with pre-treatment by soybean oil leaked certainly into the reaction mixture, almost no decrease in relative activity was observed (see Figs. 3a and 4). Presumably the lipase activity of the BSP-immobilized cells was mainly is susceptible to attack by methyl esters rather than leakage from the cells. Consequently, GA-treatment is effective in protection from the attack by methyl esters as well as the leakage of the lipase from the cells.

3.4. Repeated use of BSP-immobilized cells for methanolysis with and without GA treatment

Fig. 5 shows the time courses of repeated methanolysis using GA-treated and -untreated cells. A mixture of soybean oil and methanol (9.65/0.35, g/g = 1/1, mol/mol) was used for methanolysis in each batch cycle. Almost no difference was found in methyl ester content between GA-treated and -untreated cells during the 11 batch cycles. In addition, almost no decrease in the initial methanolysis reaction rates was observed through the 11 cycles. These results suggest that lipase activity was not impaired by the methyl esters produced, since the methyl ester content of each batch cycle was less than 30% (see Fig. 3a and b).

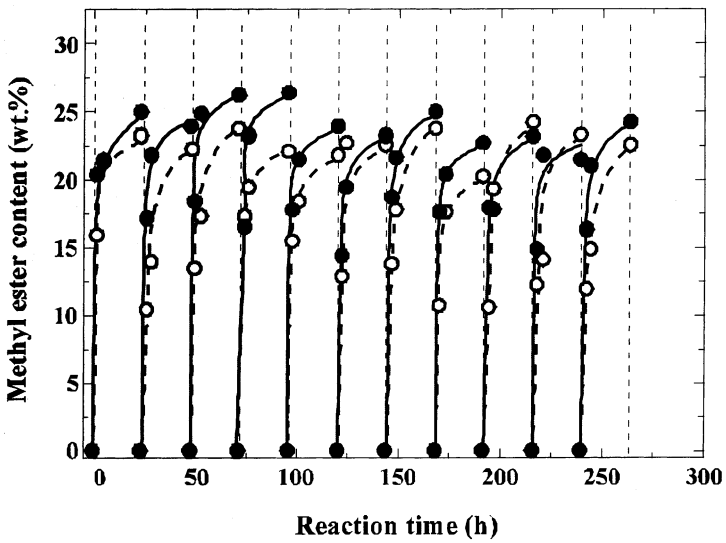

Fig. 5. Time courses of methyl ester contents during repeated use of GA-treated and -untreated cells. Methanolysis was carried out with substrate of oil/methanol (1/1, mol/mol) in each batch cycle: (●) GA-treated cells; (○) GA-untreated cells.

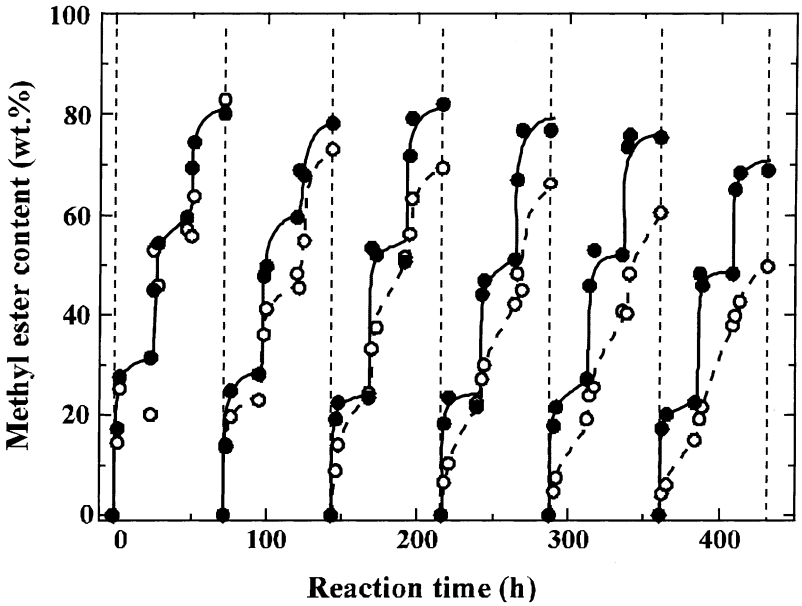

Fig. 6. Time courses of methyl ester contents during repeated use of GA-treated and -untreated cells with three-step addition of methanol. Methanolysis was carried out with oil/methanol (1/3, mol/mol) for 72 h in each batch cycle, where one molar equivalent of methanol was added twice: (●) GA-treated cells; (○) GA-untreated cells.

Although a certain amount of lipase may leak from the GA-untreated cells during the 11 cycles, as shown in Fig. 4, almost no effect on methanolytic activity was observed. The amount of leaked lipase was thus concluded to be insufficient to affect activity.

Fig. 6 shows the time courses of repeated methanolysis with stepwise addition of methanol using GA-treated and -untreated cells. To prevent irreversible denaturation of the lipase by high methanol concentration (see Fig. 1), the methanol molar equivalent of the oil (0.35 g) was added twice in each cycle. As seen in Fig. 6, in the case of GA-untreated cells, both the methyl ester content and the initial rate of methyl ester production decreased gradually with each cycle to give a methyl ester content of only 50% at the end of six batch cycles. From the results shown in Figs. 5 and 6, the activity loss of the GA-untreated cells is concluded to be caused mainly by high methyl ester concentration. In contrast, with the GA-treated cells, lipase activity showed no significant decrease during six batch cycles, with methyl ester content in each cycle reaching 70–83% within 72 h. This was presumably mainly the result of protection against the high methyl ester content of over 70% produced in methanolysis.

These findings indicate that the BSP-immobilized cells were significantly stabilized by cross-linking treatment with GA solution, and could be used as a whole-cell biocatalyst for practical biodiesel fuel production.

4. Conclusions

As a means of stabilizing the lipase activity of BSP-immobilized cells for biodiesel fuel production, a cross-linking treatment with GA was investigated. The major factor in the loss of cellular lipase activity during repeated use is generally thought to be the high methyl ester concentrations produced by methanolysis; in the present study, however, the amount of lipase leakage from the GA-treated cells was small and decreased activity only negligibly. Lipase activity was maintained during six batch cycles without significant decrease due to the protection afforded by GA treatment against attack by methyl esters.

Since the cost of lipase production is the main hurdle to commercialization of the lipase-catalyzed process, several attempts have been made to develop cost-effective systems [1–4,10]. One of these, the use of intracellular lipase as a whole-cell biocatalyst [10], is an effective way of lowering lipase production costs, since complex purification is not necessary [15]. BSP technology using GA-treated cells as a whole-cell biocatalyst is greatly superior to other methods so far proposed because of the ease with which cells can be repeatedly used and the simple immobilization procedure.

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