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Continuous production of monoacylglycerols by glycerolysis of palm olein with immobilized lipase

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Abstract

Nine commercial lipases from *Pseudomonas* sp. (lipase PS), *Pseudomonas fluorescens* (lipase AK), *Candida rugosa* (lipase AY), *Rhizopus delemar* (lipase D), *Mucor javanicus* (lipase M), *Rhizopus oryzae* (lipase F), *C. rugosa* (lipase OF) *Alcaligenes* sp. (lipase PL) and *Chromobacterium viscosum* (lipase LP) were screened for production of monoacylglycerols (MAG). Lipase PS was the most suitable enzyme for glycerolysis of palm olein with glycerol. This lipase had hydrolytic activity 10.42 U/mg and provided a high yield of MAG with 28.05% at 45 °C. Celite, silica gel, $CaCO_3$, Accurel EP100 and activated charcoal were used as supports to immobilize lipase PS. Accurel EP100 (<200 μm) was the best support. The optimum conditions for immobilization included the enzyme concentration of 50 U/ml and immobilization temperature at 30 °C for 30 min. When 5.0 ml enzyme solution was mixed with 0.5 g support the immobilized activity was 0.23 U/mg support and immobilized yield was 45.38%. The immobilized lipase PS (IM-PS) on Accurel had optimal activity at 45–65 °C and more than 90.0% of the activity remained after incubated at 45 °C for 24 h. In batch production 20.74% MAG was obtained at 45 °C for 24 h. The continuous production of MAG was performed with IM-PS (350 U) in the packed-bed reactor (PBR) (0.68 cm ID, 25 cm long) and a continuous stirred-tank reactor (CSTR) (4.5 cm ID, 6.0 cm height) for 96 h at 45 °C. When the flow rate of the substrate mixture was 0.02 ml/min the average yields of MAG were 14.01 and 14.34% in PBR and CSTR, respectively.

Keywords: Glycerolysis; Immobilized lipase; Monoacylglycerols; *Pseudomonas* sp.; Lipase

1. Introduction

Monoacylglycerols (MAG) are the most widely used emulsifiers in food, pharmaceutical, and cosmetic industries [1]. In the pharmaceutical industry, MAG are used as binders in tablets and as emollients for transdermal, slow-release drugs [2]. In the food industry, MAG are the most common food emulsifiers for bakery products, magarines, dairy products, confectionary and sauces, etc. [2]. In the cosmetic industry, they are used as texturizing agents and for improving the consistency of creams and lotions [3]. Monopentadecanoylglycerol is used as a hair care additive [4]. In addition, owing to their excellent lubricant and plasticizing properties, monoglycerides are used in textile processing, production of plastics and formulation of oil for different types of machinery [5].

Currently, MAG are manufactured on an industrial scale by continuous chemical glycerolysis of fats and oils at high temperature (220–250 °C) employing inorganic alkaline catalysts under a nitrogen gas atmosphere [6]. The products produced by this strategy have several drawbacks [4]. A molar excess of glycerol is used and because the reaction temperature is greater than 220 °C, dark-colored by-products with an undesirable flavor are formed. Moreover, the yield of MAG is rather low (30–40%) [7]. Molecular distillation is necessary because MAG need to be highly pure in the food industry, since they have better emulsifying properties than a mixture of different acylglycerols [4]. Recently, many approaches have been investigated in the enzymic synthesis of MAG [4]. These are selective hydrolysis using 1,3-regiospecific lipases [8], esterification of fatty acids or transesterification of fatty esters with glycerol [9], and the glycerolysis of fats or oils [10].

At present, the main disadvantage of the use of lipase in industrial applications is the cost of the enzyme. To over-

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come this problem, the lipase is employed in immobilized form because it would allow the reutilization of the enzyme. By immobilizing the enzyme, it is possible to operate enzymic processes continuously. Enzyme immobilization has been accomplished by chemical and physical attachment to solid surfaces [11]. Many supports, such as calcium carbonate ($CaCO_3$) [12], Celite [13], ion exchange resin [3] and Accurel [14] have been used for immobilized lipase. Several approaches for the synthesis of MAG by immobilized lipase have been reported [15–17]. In particularly, glycerolysis of palm oil with glycerol by immobilized lipase has been used for MAG production [7,18,19]. However, the reactions were performed in a batch stirred-tank reactor (BSTR) using a solid phase system. Thus, continuous production was impossible.

The aims of this research were to select lipase for glycerolysis and optimize immobilization as well as investigate continuous glycerolysis of palm olein for MAG production by immobilized lipase in packed-bed reactor (PBR) and stirred-tank reactor (CSTR).

2. Materials and methods

2.1. Materials

Lipase PS (*Pseudomonas* sp.), lipase AK (*Pseudomonas fluorescens*), lipase AY (*Candida rugosa*), lipase D (*Rhizopus delemar*), lipase M (*Mucor javanicus*), lipase F (*Rhizopus oryzae*) were gifts from Amano Pharmaceutical Co. Ltd., Nagoya, Japan. Lipase OF (*C. rugosa*) and lipase PL (*Alcaligenes* sp.) were gifts from Meito Sangyo Co. Ltd., Japan. Lipase LP (*Chromobacterium viscosum*) was gift from Asahi Chemical Industry Co. Ltd., Japan. The supports were Celite 545 (200 μm) from Wako Pure Chemical Industries, Ltd., Silica gel 60 (200 μm) from Merck Co. Ltd., and $CaCO_3$ (Softon 3200) from Shiraishi Calcium Co. Ltd. Polypropylene powder EP100 (Accurel) was a gift from Akzo Nobel (Obernburg, Germany). Activated charcoal was purchased from Fluka Chemical Co. Ltd. Palm olein was purchased from Morakot Industry Co. Ltd., Thailand. All other chemicals were also obtained from commercial sources.

2.2. Immobilization

The support in powdered form (0.5 g) was added to 5.0 ml lipase solution containing approximately 100 U/ml enzyme and stirred with a magnetic bar at 100 rpm for 1 h. Afterwards, 5.0 ml of 0.1 M phosphate buffer pH 7.0 was added and the suspension was filtered through a Buchner funnel. The immobilized enzyme was washed on the filter paper with another 5.0 ml of 0.1 M phosphate buffer pH 7.0 and dried in a vacuum desiccator for 8 h. For this immobilization study, the immobilized yield was calculated using the following formula:

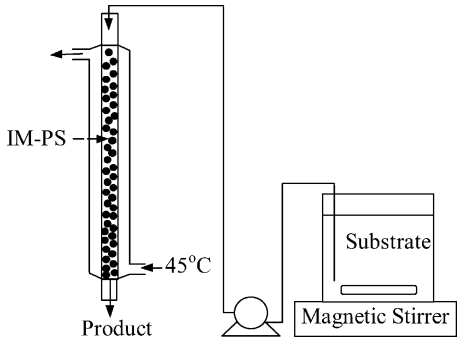

Fig. 1. Schematic diagram for continuous glycerolysis of palm oil by IM-PS in PBR at 45 °C.

$$\text{immobilized yield} = \frac{\text{total immobilized activity (U)}}{\text{total initial soluble enzyme activity (U)}} \times 100$$

2.3. Glycerolysis

Glycerolysis experiments were carried out in batch and continuous systems. The substrate mixture consisted of palm olein and glycerol containing 4.0% (w/w) water. The glycerol to palm olein molar ratio was 2.7 [13]. In the batch system, the substrate mixture was mixed with soluble or immobilized lipase with a magnetic stirrer at 300 rpm. In the continuous system, the substrate mixture was stirred at 300 rpm by magnetic stirrer and introduced into the PBR or CSTR reactor with a peristaltic pump. The reaction was maintained at 45 °C by water circulation. For the PBR, the immobilized lipase (1500 mg) was packed in a jacketed column (0.68 cm ID, 25.0 cm long). The substrate mixture was mixed well and introduced to the top of the column at a flow rate of 0.02 ml/min and the product was removed at the bottom of the column (Fig. 1). For the CSTR, the immobilized lipase (1500 mg) was placed in a jacketed cylindrical vessel (4.5 cm ID, 6.0 cm height) and agitated at 300 rpm. The substrate mixture was introduced to the top of the vessel with 0.02 ml/min of the flow rate and the product was removed at the bottom of the vessel (Fig. 2).

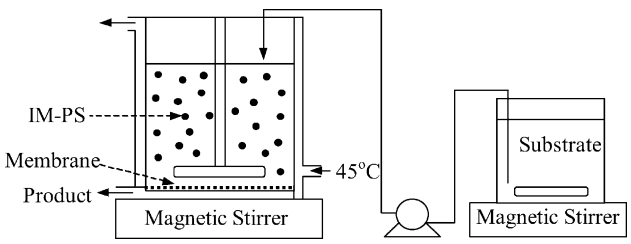

Fig. 2. Schematic diagram for continuous glycerolysis of palm oil by IM-PS in CSTR at 45 °C.

2.4. Analysis

The course of glycerolysis was monitored by intermittent sampling (150 mg) followed by chloroform extraction. The extract was analyzed for triacylglycerol (TAG), 1,3-diacylglycerol (1,3-DAG), 1,2-diacylglycerol (1,2-DAG), MAG and free fatty acid (FFA) using a thin-layer chromatography/flame ionization detection (TLC/FID) (IATROSCAN MK5, Iatron Laboratories Inc., Tokyo) [12]. In this paper, the percentage of peak area was assumed as percentage content of the corresponding compound. Enzyme protein were determined by the Folin–Lowry method [20]. Hydrolytic activity of the lipase was assayed by a modified cupric acetate method [21]. One unit of hydrolytic activity is defined as the amount of the enzyme that liberates 1 μmole equivalent of palmitic acid from palm olein in 1 min at 30 °C.

3. Results and discussion

3.1. Selection of commercial lipase for MAG production

Nine commercial lipases were screened for their ability to produce MAG through glycerolysis of palm olein at 30 °C in batch system. Results are shown in Fig. 3. Lipase LP, lipase PL, lipase D, lipase F and lipase PS gave the high yield of MAG with 49.16, 48.16, 40.99, 35.33 and 32.67%, respectively. On the other hand, lipase OF, lipase AK, lipase AY and lipase M gave a low yield of MAG (<3.0%). According to the other researchers, the lipase from *C. viscosum* [1,18] and *Pseudomonas* sp. [7] gave a high yield of MAG. However, after the product was produced, the reaction mixture become solid and further continuous production was impossible. Therefore, the five commercial lipases that gave a high yield of MAG were screened again at higher temperature (45 °C) and results are shown in Fig. 4. Lipase LP and lipase PS gave a high yield of MAG with 30.15 and 28.05%, respectively. According to the results of heat stability at 45 °C (Fig. 5) lipase LP and lipase PS were suitable for MAG production at 45 °C but the price of lipase LP is more expensive. Thus, lipase PS was chosen for glycerolysis of palm olein in this work.

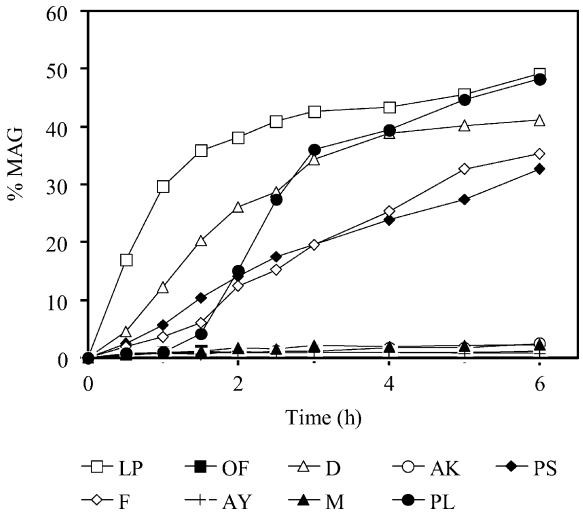

Fig. 3. Glycerolysis of palm olein by lipases at 30 °C.

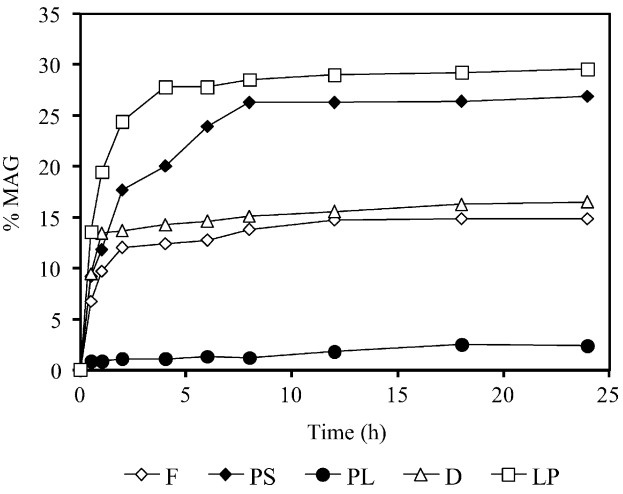

Fig. 4. Glycerolysis of palm olein by lipases at 45 °C.

3.2. Selection of supports to immobilize lipase

Table 1 shows the result of lipase PS immobilized on different solid supports by physical adsorption. Accurel EP100 (<200 μm) displayed the best immobilized activity and the highest immobilized yield. Brady et al. [14] reported that hydrophobic microporous materials such as Accurel provided better performances for immobilized lipases. Furthermore, Kimura et al. [22] immobilized lipases on different inorganic and organic supports and found that the hydrophobic matrices exhibited a higher activity in the hydrolysis

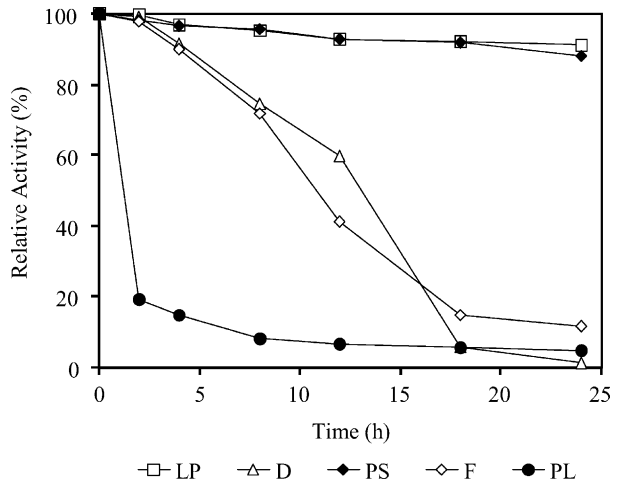

Fig. 5. Stability of soluble lipases at 45 °C.

Table 1
Immobilization of lipase PS on various supports

Support	Immobilized activity (U/mg support)	Immobilized yield (%)
Accurel EP100 (<200 μm)	0.368	37.16
Accurel EP100 (200–400 μm)	0.305	31.10
Calcium carbonate	0.008	0.79
Celite	0.035	3.56
Silica gel	0.064	6.42
Activated charcoal	0.004	0.36

of olive oil. Accurel EP100 (<200 μm) was the most suitable support for immobilization of lipase PS (IM-PS) in this study.

The IM-PS on Accurel EP100 (<200 μm) was tested for MAG production (Fig. 6), producing 20.74% MAG with 25.16% TAG remaining after incubation at 45 °C for 24 h.

3.3. Optimal conditions for enzyme immobilization

Accurel EP100 (<200 μm) was chosen to immobilize lipase PS by adsorption. The parameters that effect immobilization were investigated.

3.3.1. Effect of enzyme loading

The effect of the enzyme loading on immobilization of lipase PS with Accurel was determined. Results are shown in Table 2. The activity of the immobilized enzyme increased while the immobilized yield decreased with increasing enzyme concentration. This could be due to limitation of substrate diffusion toward the surface and into the pores of the support because of its microporous nature. Moreover, the lipase molecules would penetrate and be immobilized to binding sites in the matrix pores, in sites inaccessible to the substrate [23]. When the immobilized activity and immobilized yield were considered, it was found that the concentration of enzyme with 50 U/ml was suitable for lipase PS immobilization with Accurel.

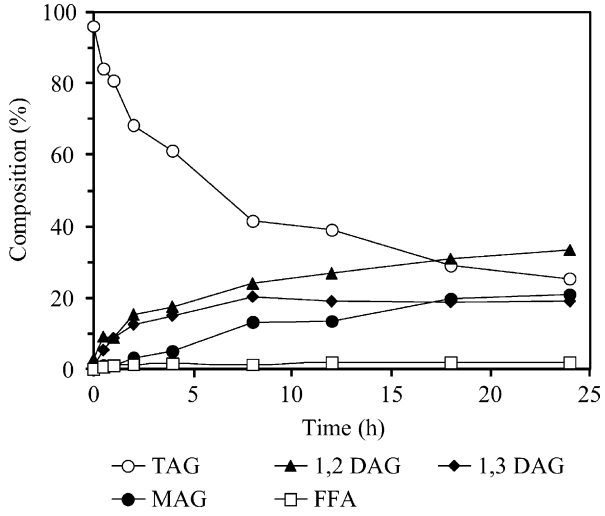

Fig. 6. The compositions of the reaction mixture during glycerolysis of palm olein with glycerol by IM-PS on Accurel EP100 (<200 μm).

Table 2
Effect of enzyme loading on immobilization of lipase PS with Accurel

Enzyme concentration U/ml	Immobilized activity (U/mg support)	Immobilized yield (%)
5	0.049	97.20
10	0.081	80.41
50	0.217	42.92
100	0.353	34.99
150	0.458	28.75

Table 3
Effect of temperature on immobilization of lipase PS with Accurel

Temperature (°C)	Immobilized activity (U/mg support)	Immobilized yield (%)
4	0.234	44.78
25	0.233	44.67
30	0.230	43.94

3.3.2. Effect of temperature

The effect of temperature on immobilization of lipase PS with Accurel was investigated and results are shown in Table 3. Variation of the immobilization temperatures (4, 25 and 30 °C) for 60 min had minimal effect on immobilized activity and yield. In all cases, the immobilized activity and immobilized yield were approximately 0.23 U/mg support and 44.0%, respectively.

3.3.3. Effect of time

The effect of time on immobilization of lipase PS with Accurel was studied and results are shown in Table 4. The immobilized activity and immobilized yield increased when the immobilization time was increased from 5 to 30 min. However, an immobilization time of more than 30 min had no effect on immobilized activity and yield. According to Montero et al. [23], *C. rugosa* lipase was rapidly adsorbed on Accurel and more than 60% of the soluble activity disappeared from the medium after 1 min of incubation. Thus, an immobilization time of 30 min was sufficient to immobilize lipase PS on Accurel.

Table 4
Effect of time on immobilization of lipase PS with Accurel

Immobilization time (min)	Immobilized activity (U/mg support)	Immobilized yield (%)
5	0.223	43.29
10	0.220	43.25
15	0.223	43.29
20	0.228	44.40
30	0.233	45.38
60	0.234	45.53
120	0.234	45.51
240	0.234	45.50

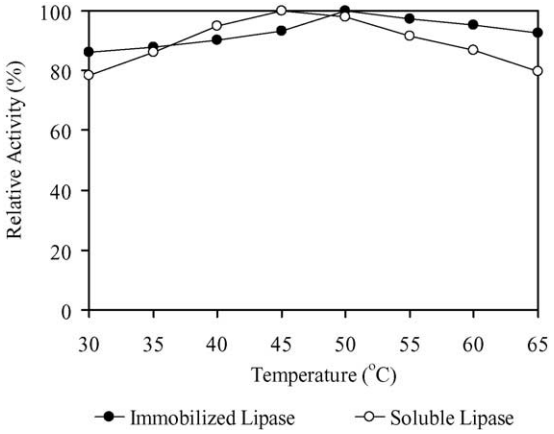

Fig. 7. Effect of temperature on hydrolysis activity of lipase PS.

3.4. Properties of immobilized lipase PS

3.4.1. Optimum temperature

The optimum temperature for IM-PS activity was determined and results are shown in Fig. 7. IM-PS showed maximal activity at 50 °C, while that for the soluble enzyme was 45 °C. Montero et al. [23], working with *C. rugosa* lipase found that immobilization on Accurel promoted a shift in the temperature profiles. This would arise from a lower restriction in the diffusion of the substrate and products at higher reaction temperatures.

3.4.2. Stability

The stability of IM-PS on Accurel was studied at 45 °C (Fig. 8) and more than 90% of the immobilized activity remained after incubation for 24 h. Furthermore, the immobilized lipase was slightly more stable than the soluble enzyme, according to Montero et al. [23]. Furthermore, Brady et al. [14] studied the storage stability of *Candida* lipase immobilized on Accurel powder and found that the immobilized lipase lost less than 10% of its original activity after 120 days of storage at room temperature.

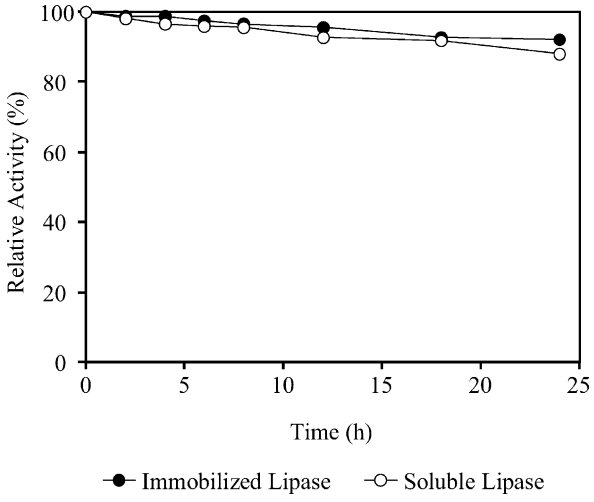

Fig. 8. Stability of lipase PS at 45 °C.

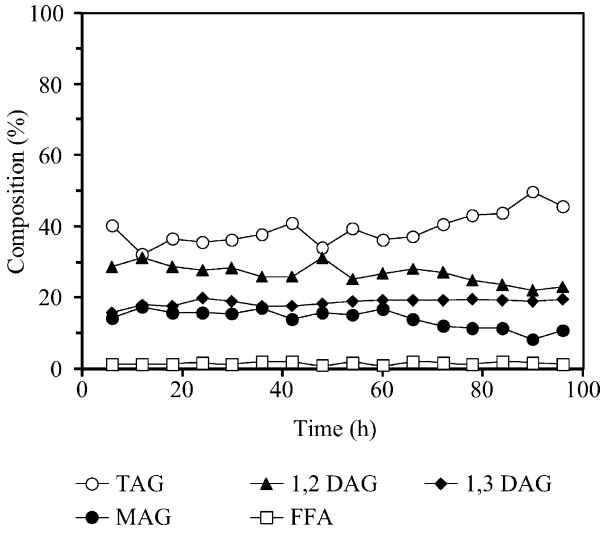

Fig. 9. Glycerolysis of palm olein with glycerol by IM-PS in PBR at 45 °C.

3.5. Continuous glycerolysis in PBR

Continuous MAG production using IM-PS was performed in a PBR reactor. Results are shown in Fig. 9. The average yield of MAG was 14.01% and was slightly decreased on increasing the operation time. A productivity of 1.05×10^{-2} g MAG/U day at 100 h was obtained. Stevenson et al. [3] studied the continuous MAG production by lipozyme in PBR and reported that the final yield of 17–19% MAG were obtained.

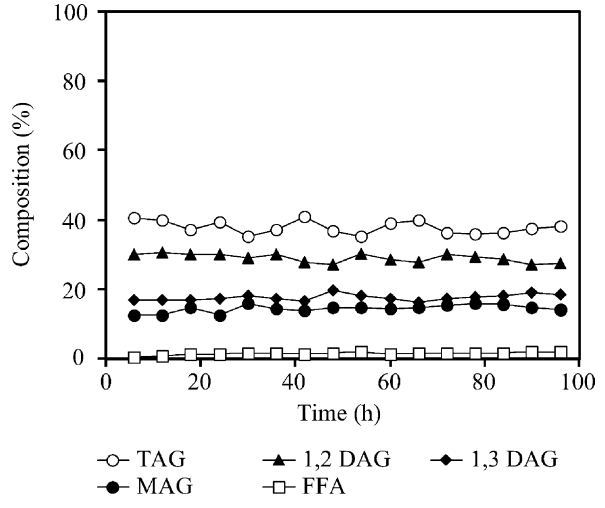

Fig. 10. Glycerolysis of palm olein with glycerol by IM-PS in CSTR at 45 °C.

3.6. Continuous glycerolysis in CSTR

The continuous glycerolysis of palm olein with glycerol was also performed in a CSTR. Results are shown in Fig. 10. The average yield of MAG was 14.34% and a productivity of 1.07×10^{-2} g MAG/U day was obtained. Chang and Rhee studied the continuous glycerolysis of olive oil by *C. viscosum* lipase immobilized on liposomes in reversed micelles in a CSTR with polysulfone membranes at 37 °C [24]. 1-Monoolein of more than 1.25 μmoles/ml outlet was reported while in this study the MAG production was 5.08 μmoles/ml.

4. Conclusion

Lipase PS (*Pseudomonas* sp.) immobilized on Accurel was most suitable for MAG production by glycerolysis of palm olein with glycerol. Immobilization of lipase PS on Accurel gave a higher hydrolytic activity than other supports and was more thermostable than the soluble enzyme. In addition, it could be used for continuous operation in PBR and CSTR. However, further study on the half life of immobilized lipase and long term continuous operating in reactor is necessary.

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