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Optimizing an alginate immobilized lipase for monoacylglycerol production by the glycerolysis reaction

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ABSTRACT

Lipase from *Pseudomonas* sp. was immobilized in alginate gel beads. The optimum condition for lipase entrapment was a 2% (w/v) alginate concentration, 100 mM $CaCl_2$ concentration, 30 U/mL enzyme concentration and a 2.03 mm mean bead size. Under these conditions, 8.11 U/mL of immobilized lipase was obtained with 22.2% of retained activity. Palm oil was used as the starting material to produce monoacylglycerol (MAG) in the glycerolysis reaction. The addition of glycerol in the immobilization step improved the yield of MAG. In order to prevent enzyme from leaking out of the gel beads, beads were coated with silicate. The silicate coated beads showed a higher reusability in the glycerolysis reaction compared to non-coated beads. The production of MAG by coated alginate gel beads was optimized. A 10:1 molar ratio of glycerol to palm oil without water addition, 27 U of immobilized enzyme and 50% (w/v) of palm oil in 2-methyl-2-butanol gave the highest MAG production rate of 0.34 M/h while 10% (w/v) of palm oil in 2-methyl-2-butanol gave the highest overall conversion of triacylglycerol 100% to 54% monoacylglycerol, 9% diacylglycerol and 37% free fatty acid after 4 h.

1. Introduction

Monoacylglycerols (MAGs) are the most widely used emulsifiers in the food, pharmaceutical and cosmetic industries. They have excellent emulsifying properties, low odor and taste. At present, they are manufactured by continuous chemical glycerolysis of fats and oils at high temperatures (220–250 °C) employing inorganic alkaline catalysts under a nitrogen gas atmosphere. The result is a crude mixture of mono- and di-acylglycerols (roughly equal amounts) and some unreacted triacylglycerol (overall conversion 90%). Due to the very high temperatures applied during glycerolysis, decomposition and oxidation reactions take place and often result in a dark-colored product with a burnt-flavor and in this case a low yield of MAG [1]. This then requires extensive purification and downstream processing. Because biocatalysts require ambient reaction temperatures they can potentially yield a higher quality product with lower energy consumption in a more "natural" type process.

Lipases (EC 3.1.1.3) were originally employed as biocatalysts for the hydrolysis of ester bonds of triacylglycerols (TAGs) to produce free fatty acids (FFAs), glycerol and partial acylglycerols (e.g. MAG and DAG, diacylglycerols). Enzymatic syntheses have the advantage of catalysis at lower temperatures and this prevents the discoloration and alteration of unsaturated fatty acids that is common at elevated temperatures [2]. In most cases of lipase-catalyzed glycerolysis reactions have been conducted in non-aqueous or microaqueous systems consisting of an apolar solvent [3,4]. Unfortunately, the application of lipase-catalyzed reactions is more or less limited to aqueous reaction systems due to its poor activity in the presence of organic solvents. Several immobilization techniques have been studied and utilized to contribute to the development of continuous processes, and immobilized enzymes are adaptable to a variety of configurations and specific processes carried out in reactors. Alginates are one of the most frequently used chemicals for immobilization due to their mild gelling properties and nontoxicity [5,6]. The aim of this research was to optimize the glycerolysis reaction of palm oil for MAG production by an alginate immobilized lipase. Even though lipases have been immobilized in alginate gel beads for hydrolysis reactions [7–9], the application of alginate immobilized lipase for MAG production by glycerolysis reaction has not been previously reported. In this study, the effects of alginate, $CaCl_2$, enzyme concentrations and bead size on the activity of an immobilized lipase and its retained activity were investigated. Glycerol, the precursor of MAG was also incorporated into the immobilization step to enhance MAG yield. In addition, a silicate coating technique was also used to prevent the leakage of enzyme from the beads. Finally, the optimization of MAG production by the alginate immobilized lipase in the glycerolysis reaction including the effects of water, molar ratio of glycerol to palm oil, palm

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oil concentrations and immobilized enzyme loading were investigated.

2. Materials and methods

2.1. Materials

Lipase PS (*Pseudomonas* sp.) was a gift from Amano Pharmaceutical Co. Ltd., Japan. Sodium alginate was obtained from Fluka Chemika, Japan. Tetraethyl orthosilicate (TEOS) was obtained from Sigma–Aldrich, Germany. All other chemicals were of analytical grade.

2.2. Entrapment of lipase in alginate gel beads

Five mL of lipase solution was mixed with 5 mL of sodium alginate solution to obtain a final concentration of 10–50 U/mL for lipase and sodium alginate at 1.5–2.5% (w/v). The mixture was then stirred thoroughly to ensure complete mixing. As soon as the mixed solution was dripped into 150 mL of $CaCl_2$ solution (50–200 mM) with a syringe, Ca–alginate beads were formed. After 20 min of hardening, the beads were separated from the $CaCl_2$ solution by vacuum filtration. They were washed on a filter twice with 50 mM Tris–HCl buffer (pH 7). The immobilization efficiency (%) and retained activity (%) were calculated using the following equations:

$$\text{immobilization efficiency}\,(\%) = \frac{C_0V_0 - C_fV_f}{C_0V_0} \times 100$$

$$\text{retained activity}\,(\%) = \frac{C_iV_i}{C_0V_0 - C_fV_f} \times 100$$

where C_i, C_0 and C_f are the hydrolytic activities of immobilized lipase, lipase solution and filtrate, respectively, V_i, V_0 and V_f are the volumes of immobilized lipase, lipase solution and filtrate, respectively.

The bead size was changed by using syringes with different needle diameters (18–24 gauge). The size of the beads was measured using a vernier caliper. The diameter of each bead was measured at three different angles and the average size of 10 beads was calculated, to give an average bead size.

2.3. Coating of alginate gel beads with silicate

Two grams of alginate gel beads was added to enough hexane to cover the beads. Six mL of tetraethyl orthosilicate (TEOS) was added and the mixture was left overnight at room temperature to complete the polymerization process. Finally, the beads were filtered from the solution [9].

2.4. Glycerolysis reaction

The glycerolysis experiments were carried out in a batch system. The reaction mixture consisted of 9–36 U immobilized lipases, 10–60% (w/v) palm oil in an organic solvent (2-methyl-2-butanol) and glycerol at the molar ratio to palm oil of 4:1–12:1 with 0–10% of water addition in glycerol. The reaction was performed at room temperature ($30 \pm 2\,^{\circ}C$) and mixed by shaker at 500 rpm until the concentration of substrates and products had no change over time.

2.5. Reusability of alginate immobilized lipase

In order to evaluate the reusability of immobilized lipase, the beads with and without coating were used several times for MAG production in a glycerolysis reaction. Each run lasted 6 h after which the beads were separated and washed with 50 mM Tris–HCl buffer (pH 7). The reaction medium was then replaced with fresh medium. For comparison the MAG production by freshly prepared beads in the first run was defined as 100%.

2.6. Analytical methods

Hydrolytic activities of immobilized lipase, lipase solution and filtrate were assayed by a modified cupric acetate method [4]. One unit of hydrolytic activity is defined as the amount of the enzyme that liberates 1 μmole equivalent of palmitic acid from palm oil in 1 min at 30 °C.

The components of the oil phase were analyzed for TAG, DAG, MAG and FFA using thin-layer chromatography with a flame ionization detector (TLC/FID) (IATROSCAN MK5, Iatron Laboratories Inc., Tokyo). In this experiment, the percentage of peak area was assumed to be the percentage content of the corresponding compound [10].

All experiments were performed in triplicate. Analysis of variance was performed to calculate significant differences in treatment means, and the least significant difference ($p < 0.05$) was used to separate means, using the SPSS software version 11.

3. Results and discussion

3.1. Optimization of alginate immobilization

On the addition of an alginate solution to a $CaCl_2$ solution, instantaneous interfacial cross-linking takes place with precipitation of Ca–alginate. The concentrations of alginate and $CaCl_2$ are major variable parameters for enzyme gel entrapment. The effects of alginate and $CaCl_2$ concentrations on immobilized lipase activity and retained activity were first investigated (Table 1). The enzyme concentration and the needle size were kept constant at 10 U/mL and 24 gauge, respectively. Immobilization efficiencies of each experiment were not significantly different and in the range of 97.8–99.6% and dependent only slightly on the alginate concentration (data not shown). This indicated that the range of alginate and $CaCl_2$ concentrations used in this study were sufficient to hold the enzyme inside the gel network during the immobilization step. The highest immobilized lipase activity (2.78 U/mL) and retained activity (24.9%) were obtained at a 2% alginate and 100 mM $CaCl_2$ concentration. At any alginate concentration, a $CaCl_2$ concentration of 100 mM gave the highest immobilized lipase activity and also its retained activity. The beads prepared using lower concentrations of alginate and $CaCl_2$ at 1.5% and 50 mM, respectively, were fragile and the enzyme leakage into the substrate solution was high. An explanation might be that at these lower concentrations there was inadequate or incomplete gelation of the alginate beads resulting in a non-uniform enzyme distribution. When the alginate concentration was increased from 1.5 to 2% at any $CaCl_2$ concentrations, the immobilized lipase activity and retained activity was increased. At higher alginate and $CaCl_2$ concentrations of 2.5% and 200 mM, respectively, the bead rigidity was improved, but the immobilized lipase activity and retained activity decreased. This might be due to a limitation of substrate transfer from the bulk phase into the alginate bead to access the lipase [7,9].

The effects of enzyme concentrations on the immobilized lipase activity and retained activity were determined by varying enzyme concentrations while keeping the alginate and $CaCl_2$ concentrations at the optimum level of 2% and 100 mM, respectively (Table 1). With increasing enzyme concentrations, the immobilized lipase activity also increased but retained activity was decreased. This could be again due to the limitation of substrate diffusion into the lipase entrapped in the beads, namely at high concentration of enzyme in the gel matrix some of them would be inaccessible to substrate. Considering the high immobilized lipase activity and acceptable

Table 1
Effect of alginate and $CaCl_2$ concentrations, enzyme concentration, and bead size on immobilized lipase activity and retained activity.

Immobilization parameter		Immobilized lipase activity (U/mL)	Retained activity (%)
Alginate concentration (%)	$CaCl_2$ concentration (mM)		
1.5	50	$2.13 \pm 0.23^{ab*}$	21.2 ± 1.82^{bc}
	100	2.41 ± 0.07^{ab}	23.5 ± 1.28^{ab}
	200	1.85 ± 0.23^{b}	21.7 ± 0.82^{bc}
2.0	50	2.36 ± 0.01^{ab}	21.8 ± 0.01^{bc}
	100	2.78 ± 0.28^{a}	24.9 ± 0.72^{a}
	200	2.46 ± 0.31^{ab}	19.9 ± 0.62^{cd}
2.5	50	1.99 ± 0.31^{ab}	17.1 ± 0.96^{e}
	100	2.24 ± 0.29^{ab}	18.5 ± 0.48^{de}
	200	1.72 ± 0.37^{b}	13.4 ± 0.40^{f}
Enzyme concentration (U/mL)			
10		2.78 ± 0.28^{b}	24.9 ± 0.72^{a}
30		8.11 ± 0.77^{a}	22.2 ± 0.43^{a}
50		8.92 ± 0.83^{a}	17.8 ± 0.16^{b}
Bead size (mm)			
2.03 ± 0.12		8.11 ± 0.77^{a}	22.2 ± 0.43^{a}
2.53 ± 0.09		5.19 ± 0.71^{b}	12.3 ± 0.21^{b}
3.02 ± 0.09		5.27 ± 0.03^{b}	13.3 ± 0.14^{b}

* Different letters in the same column and section are significantly different ($p < 0.05$).

retained activity, the concentration of the enzyme at 30 U/mL was deemed to be suitable for lipase PS immobilization with a 2% alginate and 100 mM $CaCl_2$ concentrations.

Since one of the mass transfer limitations generally found in immobilized enzymes, is the size of the beads in which the enzyme is entrapped, it is also one of the important parameters to evaluate for immobilization. Three different sizes of alginate gel beads were reproducibly generated by changing the size of the needle. Mean bead sizes of 2.03, 2.53 and 3.02 mm were obtained. It was found that bead size did affect the immobilized lipase activity and retained activity (Table 1). The immobilized lipase activity and retained activity decreased when the bead size increased. The bead size will affect the surface area/volume ratio and the internal fluid volume. Large beads have a larger internal volume of fluid and therefore the enzyme and substrate solutions will be relatively more dilute than in smaller beads and thus the activity will be expected to decrease. Also the relatively smaller surface area of the larger beads probably means that the uptake of substrate into the beads is less efficient than into smaller beads. This result was consistent with the results of Knezevic et al. [7] and Won et al. [9]. They also found that a decrease of bead size enhanced the activity of the immobilized enzyme and retained activity. In this study, a mean bead size of 2.03 mm was considered to be suitable for immobilization of lipase in alginate. Under the determined optimal conditions for lipase entrapment, a value of 8.11 U/mL of immobilized lipase activity was obtained with 22.2% of retained activity.

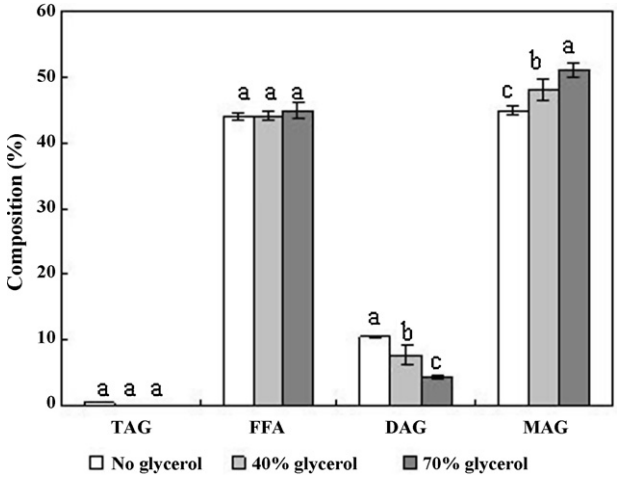

Fig. 1. Effect of including glycerol in the alginate gel beads on the glycerolysis reaction. The reaction contained 30% of palm oil in 2-methyl-2-butanol, 10% of water content in glycerol and glycerol at molar ratio to palm oil of 8:1. The amount of immobilized lipase was 9 U. a, b and c within the same composition indicate significant differences ($p < 0.05$).

3.2. Improvement of alginate immobilized lipase for MAG production

It is thought that the substrate or substrate analogue could induce and stabilize the catalytically active structure of the enzyme [11]. In this study, in order to ensure the stability of the immobilized lipase for MAG production in the glycerolysis reaction, glycerol, the precursor of MAG, was included in the immobilization step. Glycerol concentrations of 0, 40 and 70% were used to dissolve the alginate and entrap lipase. As expected, the entrapment of glycerol with enzyme in the gel beads did enhance the MAG yield (Fig. 1). It was possible that the glycerolysis reaction of DAG to MAG would proceed more rapidly when glycerol was included in the immobilization step. One explanation could be that glycerol helps to maintain the enzyme in its active conformation. In addition, Berger and Schneider [12,13] reported that glycerol is immiscible with oil substrate and with their solutions in hydrophobic organic solvents so that enzymatic syntheses in these media have only limited success. However, this problem could be reduced by the prior adsorption of glycerol into the solid support to enhance the interfacial surface area and facilitate interaction. In this study, it was also thought that a larger interface between glycerol and the hydrophobic organic solvent, in which the palm oil was dissolved, might be created within the bead and the yield of MAG was therefore improved. However, as the alginate could not dissolve in glycerol concentration higher than 70%, a concentration of glycerol of 70% was chosen for inclusion in the immobilization process.

Since the major disadvantages of alginate gel beads are the sensitivity to calcium chelators and larger matrix pores, the prevention of the leakage of enzyme was attempted by coating the beads with silicate [9]. Fig. 2 shows the result of the reusability study of coated and non-coated alginate gel beads for MAG production in the glycerolysis reaction. The yield of MAG in the first run was defined as 100% and there was no difference between coated and non-coated bead, but the yield of MAG catalyzed by the coated bead was maintained over a longer period than that of non-coated beads after the second, third and fourth run. The higher loss of activity for the reuse

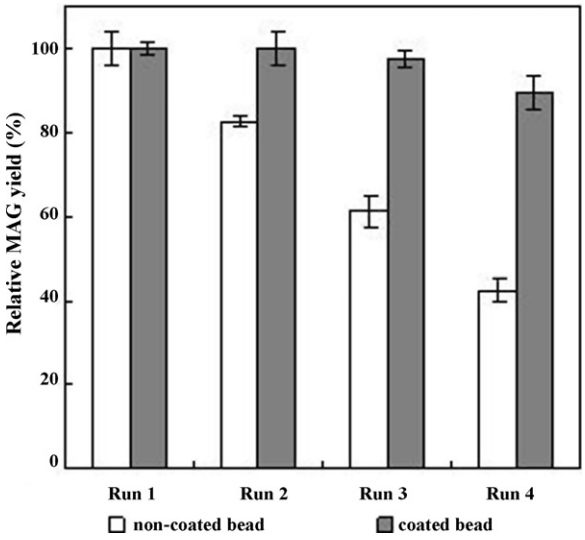

Fig. 2. Effect of silicate coating on the reusability of alginate immobilized lipase in the glycerolysis reaction. The reaction contained 30% of palm oil in 2-methyl-2-butanol, 10% of water in glycerol and glycerol at molar ratio to palm oil of 8:1. The amount of immobilized lipase was 9 U.

of non-coated alginate gel beads in all runs might be mainly due to enzyme leakage from the alginate gel beads or damage of the alginate beads during repeated use [7]. Therefore, the silicate coated alginate gel beads were used for optimization of MAG production in the glycerolysis reaction.

3.3. Optimization of MAG production by alginate immobilized lipase in glycerolysis reaction

The use of alginate immobilized lipase for MAG production in the glycerolysis reaction was tested. It is known that a suitable water content is necessary to maintain enzyme structure and stability. Moreover, in a lipase-catalyzed glycerolysis reaction water is involved in both the hydrolysis and esterification step as reactant and product, respectively. Thus to maximize MAG production in the glycerolysis reaction a trace amount of water is necessary to maintain the hydration layer around lipase molecules and the enzyme activity. In this study, the effect of water addition on MAG production by alginate immobilized lipase was investigated by varying the addition of water in the range of 0–10% (w/w) of the glycerol weight (Fig. 3). It was found that addition of water to the glycerolysis reaction yielded lower MAG concentrations. This could be explained by the water catalyzing a more rapid hydrolysis reaction rather than the glycerolysis reaction. Consequently, the FFA content at equilibrium increased when the water content increased. Yamane et al. [14] also reported that the FFA content at equilibrium depended on the water content in the glycerol phase. Therefore, the aqueous alginate immobilized lipase could be used in the glycerolysis reaction without any addition of water, namely the amount of water already present in the alginate gel beads was sufficient to facilitate the lipase activity. This is a different result from those previously described for lipase immobilized in a microaqueous or hydrophobic support. Yang and Parkin [15] found that a 6% water content in glycerol was suitable for the enzymatic glycerolysis reaction of butter oil by entrapped lipase in a microaqueous media. Kaewthong and H-Kittikun [16] found that a 10% water content in glycerol gave the highest yield of MAG in a glycerolysis reaction of palm oil by lipase immobilized on a hydrophobic support of Accurel EP-100.

One of the main factors affecting the MAG yield in a glycerolysis reaction is the molar ratio of glycerol to palm oil. Yamane et al. [14] reported that the main product of a glycerolysis reaction was

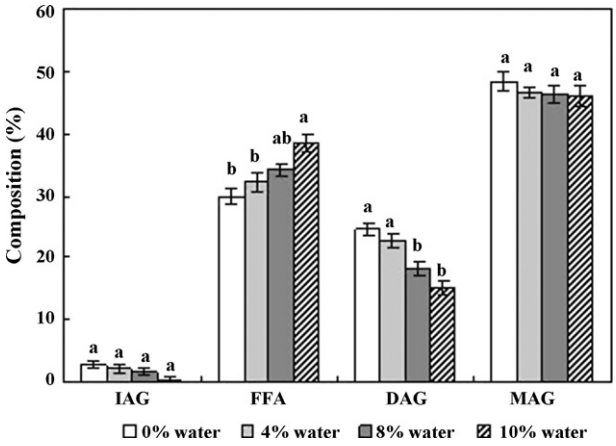

Fig. 3. Effect of water addition on the glycerolysis reaction. The reaction contained 30% of palm oil in 2-methyl-2-butanol, various amounts of water added to the glycerol and glycerol at molar ratio to palm oil of 8:1. The amount of immobilized lipase was 9 U. a and b within the same composition indicate significant differences ($p < 0.05$).

DAG when using a low molar ratio of glycerol to oil (1:2). In addition, Yang and Rhee [17] also suggested that glycerol acted as an effective stabilizer against thermal and solvent denaturation. In this study, the effect of the molar ratio of glycerol to palm oil for MAG production was investigated. The molar ratio of glycerol to palm oil was varied at 4:1, 8:1, 10:1 and 12:1. Higher initial concentrations of glycerol lead to a faster initial rate of MAG production (Fig. 4). When the molar ratio of glycerol to palm oil was increased to 12:1, the highest yield of MAG was obtained. However, the increased yields of MAG at molar ratios of glycerol to palm oil higher than 8:1 were small. Even though the initial rate of MAG production increased with increasing concentrations of glycerol, at a molar ratio of glycerol to palm oil of 12:1 the initial rate of MAG production dropped dramatically. Such a drop might be due to the high glycerol concentration having a lower miscibility with palm oil. Therefore, the highest initial rate and comparative high yield of MAG production was obtained at a molar ratio of glycerol to palm oil of 10:1. Thus, the reaction having excess glycerol with a molar ratio of glycerol to palm oil at 10:1 was chosen for further studies.

To evaluate the optimal amount of immobilized lipase, the MAG production was performed with various immobilized lipase load-

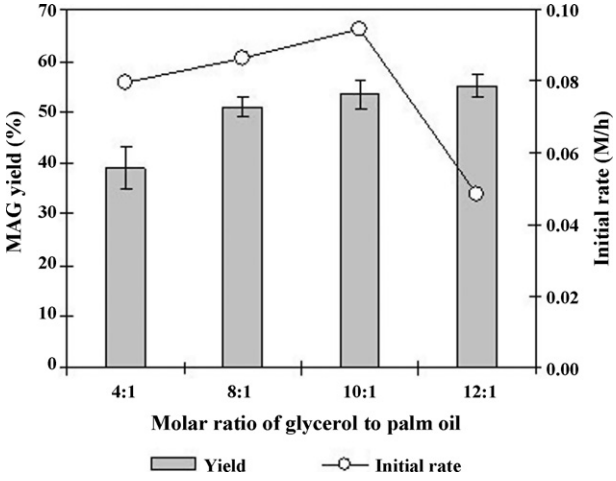

Fig. 4. Effect of molar ratio of glycerol to palm oil on the yield and initial rate of MAG production. The reaction contained 30% of palm oil in 2-methyl-2-butanol and glycerol at various molar ratios to palm oil without water addition. The amount of immobilized lipase was 9 U.

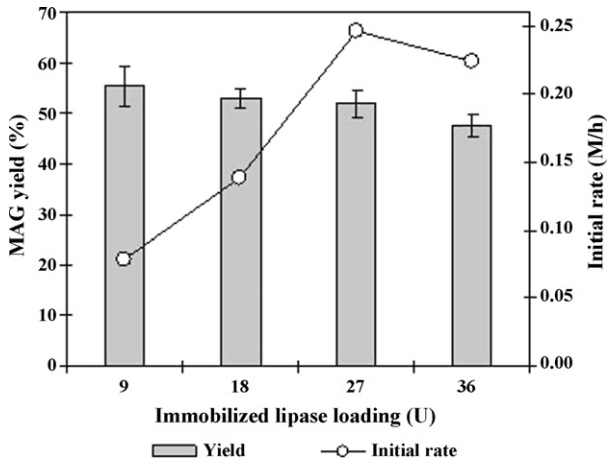

Fig. 5. Effect of immobilized lipase loading on the yield and initial rate of MAG production. The reaction contained 30% of palm oil in 2-methyl-2-butanol and glycerol at molar ratio to palm oil of 10:1 without water addition.

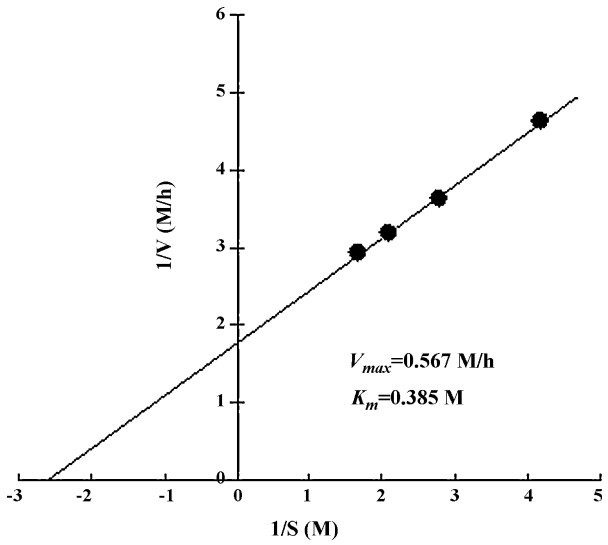

Fig. 7. Lineweaver–Burk plot of alginate immobilized lipase for MAG production in glycerolysis reaction.

ings while keeping the molar ratio of glycerol to palm oil at 10:1. The amount of immobilized lipase was varied at 9, 18, 27 and 36 U for glycerolysis of 30% (w/v) palm oil in 2-methyl-2-butanol (Fig. 5). Increasing the amount of the immobilized lipase in the reaction mixture decreased the MAG yield. This might be due to a faster hydrolysis reaction occurring with a higher immobilized lipase loading. Consequently, FFA increased due to the progress of the hydrolysis reaction rather than the glycerolysis reaction (data not shown). The initial rate of MAG production did increase with increasing amounts of immobilized lipase loading. However, immobilized lipase loading at 27 U was the best for MAG production because a high yield and high initial rate of MAG production was obtained.

In an enzymatic reaction, the concentration of substrate will eventually affect the reaction rate based on Michaelis–Menten kinetics. In order to select the optimum initial substrate concentration for the glycerolysis reaction, the effect of palm oil concentrations in 2-methyl-2-butanol on MAG production was investigated. The palm oil concentration was varied at 10, 20, 30, 40, 50 and 60% in 2-methyl-2-butanol (Fig. 6). The MAG yield decreased with increasing concentrations of palm oil. This may be due to a decreased amount of solvent resulting in a decreasing availability of substrate at the interface between solvent and glycerol. On the other hand, when the palm oil concentration was increased the initial rate of MAG production also increased due to the fact that a higher concentration of palm oil should result in a faster production rate of MAG. However, at a palm oil concentration higher than 60% the initial rate of MAG production dramatically decreased. This might be due to the high viscosity of palm oil resulting in low homogeneity. The concentration of 50% palm oil in 2-methyl-2-butanol gave the highest initial rate of MAG production of 0.34 M/h while 10% palm oil in 2-methyl-2-butanol gave the highest MAG yield of 54%. The kinetic constants for the maximum reaction rate (V_{max}) and Michaelis constant (K_m) for glycerolysis of palm oil by the alginate immobilized lipase were determined by Lineweaver–Burk plot (Fig. 7). V_{max} and K_m were 0.57 M/h (9.45 mM/min) and 0.39 M, respectively. There has been a previous report of a V_{max} of 0.4 mM/min for a lipase PS immobilized on the hydrophobic Accurel EP-100 [16]. Compared to that report, the value obtained for the hydrophilic alginate immobilized

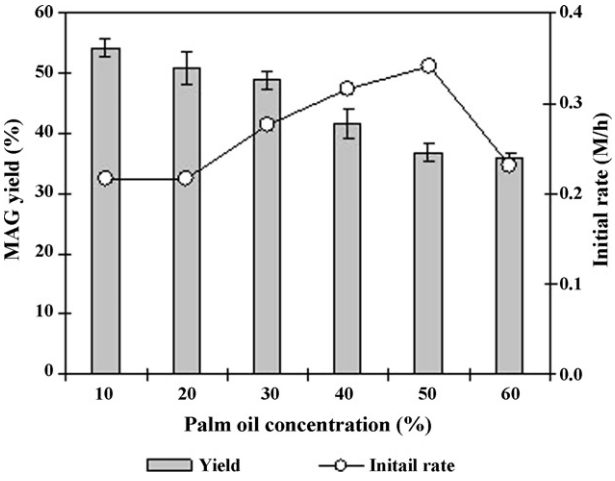

Fig. 6. Effect of palm oil concentration on the yield and initial rate of MAG production. The concentrations of palm oil in 2-methyl-2-butanol was varied, the molar ratio of glycerol to palm oil was 10:1 without water addition. The amount of immobilized lipase was 27 U.

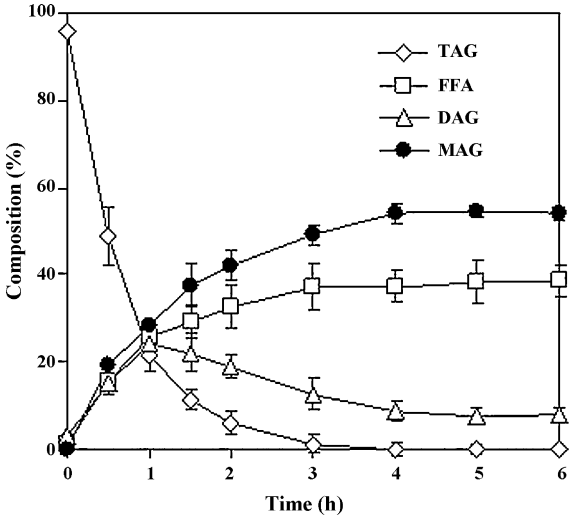

Fig. 8. Time course of the glycerolysis reaction for the highest yield of MAG. The concentration of palm oil in 2-methyl-2-butanol was 10%, the molar ratio of glycerol to palm oil was 10:1 without water addition. The amount of immobilized lipase was 27 U.

lipase in this study was much higher. The time courses of the glycerolysis reaction by alginate immobilized lipase under the optimal condition with a highest yield of MAG are shown in Fig. 8. Under these conditions of 10% palm oil in 2-methyl-2-butanol, 10:1 molar ratio of glycerol to palm oil without water addition, and 27 U of immobilized lipase, the highest conversion of 100% palm oil to 54% monoacylglycerol, 9% diacylglycerol and 37% free fatty acid were obtained at 4 h. It should be noted that when an aqueous support was used for immobilization of the lipase, the production rate of MAG in the glycerolysis reaction was greatly improved compared to that with the hydrophobic support which a maximum yield of MAG of 56% was obtained at 24 h [16].

4. Conclusion

Lipase entrapment in alginate gel beads was attempted for MAG production in the glycerolysis reaction. The highest immobilized lipase activity and retained activity was obtained at 2% of alginate and 100 mM of $CaCl_2$ concentrations. The optimal enzyme concentration and mean bead size for immobilization were 30 U/mL and 2.03 mm, respectively. The inclusion of 70% glycerol in the alginate polymerization solution enhanced the MAG yield. The reusability of the silicate coated beads was higher than that of non-coated beads. The optimum condition for maximizing MAG production by the glycerolysis reaction was 10:1 molar ratio of glycerol to palm oil without water addition, 27 U of alginate immobilized lipase and 10% of palm oil in 2-methyl-2-butanol.

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