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Prepolymer Synthesis of Polyester by Using Lipase as a Catalyst

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Abstract

For block and graft-copolymer synthesis, two requirements of prepolymers or oligomers are a narrow distribution and a specific molecular weight. These can be easily obtained from bio-catalyst synthesis. Lipase, a biocatalyst, in a low-water content environment, can catalyze both esterification and transesterification reactions. Using Cadida rugosa lipase, solution polymerization gave only di-mer and tri-mer products with a narrow distribution. Immobilization of a sol-gel process to enhance the activity of lipase gave minor products with a higher molecular weight. The major products were still di-mer and tri-mer. Microemulsion polymerization, on the other hand, gave a much better performance in synthesizing prepolymers since the molecular weight obtained was in the range of 2000 with a narrow distribution product.

Keywords : Microemulsion Polymerization, Solution Polymerization, Polyester Synthesis, and Immobilized Lipase

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Introduction

Lipase, a biocatalyst, is a water-soluble and hydrolytic enzyme, which naturally catalyzes the cleavage of an ester bond in ester-hydrolysis reactions, esterification reactions and transesterificaiton reactions, giving high chemo-, regio-and sterio-selective products with mild synthesis of conditions (de Gomez-puyou, *et al.* 1998; and Chaudhary, *et al.* 1998). They are triacyl glycerol and acyl hydrolase, in which the catalytic triases are Asp-His-Ser and are chemically analogous to, but structurally different from that in the serine protease. An active serine is buried under a short helical fragment of a long surface loop (lid) that causes it to be inaccessible to the solvent. Lipase attacks triacyl glycerols and acts at the oil-water interface which probably involves reorientation of the lid. This conformational change includes a large displacement of a lid and stabilizes the interaction with the substrate.

By reducing the amount of water in the system, lipase is also able to catalyze esterification and transesterification (de Gomez-puyou, *et al.* 1998; and Chaudhary, *et al.* 1998). Due to the advantages in operating under mild conditions, which are no toxic solvent generation and environmental friendliness, they are accepted and widely used in many industries. Many techniques were developed for lipase catalysis, such as suspending the enzyme in organic media or encapsulating the enzyme in microemulsion (de Gomez-puyou, *et al.* 1998; and Chaudhary, *et al.* 1998). The enzyme for suspending the system can be both a free and immobilized enzyme. Suspending free enzymes in organic media was mostly used for transesterification synthesis, which gave a highly enantioselective product and low polydispersity (Wang, *et al.* 1995; Wang, *et al.* 1996; Wu, *et al.* 1996; and Jaaskelainen, *et al.* 1997). However, high amounts of enzyme are required due to an aggregation problem. The other type of enzyme used for the suspending system is immobilized lipase, which can be made by adsorption on (Mojovic, *et al.* 1998; Coulon, *et al.* 1998; Goncalves, *et al.* 1998; Cao, *et al.* 1999; and Martin, *et al.* 1999), attachment on (Chen and Chen, 1998), or entrapment into (Reetz, *et al.* 1996; Karlsson, *et al.* 1998; Del-Val, *et al.* 1998; Hedstrom, *et al.* 1998; Nagayama, *et al.* 1998; and Stamatis, *et al.* 1999) the support. The activity of the enzyme is improved and the enantioselectivity can be altered. The other advantages are that it is easy to separate and reuse (Mojovic, *et al.* 1998; Coulon, *et al.* 1998; Goncalves, *et al.* 1998; Cao, *et al.* 1999; Martin, *et al.* 1999; Chen, *et al.* 1998; Reetz, *et al.* 1996; Karlsson, *et al.* 1998; Del-Val, *et al.* 1998; Hedstrom, *et al.* 1998; Nagayama, *et al.* 1998; and Stamatis, *et al.* 1999). However, adsorption on or attachment on or entrapment into a solid support bonds weakly to the enzyme

resulting in a low reusable number, of enzymes while covalent bonding techniques require several processes. Immobilized lipase by a sol-gel process was brought out to overcome those problems. Alkyl substituted silane precursors were used to form modified hydrophobic silica matrices (Reetz, *et al.* 1996). Encapsulated lipase made by this method is a suitable way for making a wide variety of lipases and it gives immobilized lipases with esterification activities an enhancement by a factor of up to 88, compared to the commercial enzyme powder under identical conditions.

Due to the thermodynamically stable, isotropic solution containing water, substrates and a surfactant, lipase is appropriate in microemulsion, since it needs a hydrophobic surface to become active and the interfacial area in a microemulsion is large (de Gomez-puyou, *et al.* 1998; Lindman, *et al.* 1981; Hedström, *et al.* 1992; Hedström, *et al.* 1993; Stamatis, *et al.* 1993; Stamatis, *et al.* 1992; Langrand, *et al.* 1988; Österverg, *et al.* 1988; and Bello, *et al.* 1987).

Suspended enzyme and microemulsion techniques were successfully used for synthesizing both esters and polyesters, starting from a high molecular weight of diacid-derivatives and dialcohols (normally for a trans-esterification reaction). In this work, both techniques were applied and comparatively studied for pre-polyester synthesis from diacids and di-alcohols or tri-alcohols in several organic solvents to obtain an appropriate technique for pre-polyester synthesis.

Materials and Experimental

Materials

Lipase from Candida rugosa (L-1754, 746 units/mg, free of α–amylase and protease) was purchased from Sigma. Succinic acid and ethylene glycol (EG) were purchased from AJAX Chemicals. Adipic acid and 1,4-bis (2-ethyl hexyl) sulpho-succinate sodium salt (AOT) were obtained from Fulka. Sebacic acid, tetraethyl orthosilicate (TEOS) and polyvinyl alcohol (98% hydrolyzed, MW 13,000-23,000) were obtained from Aldrich. Glycerol was from Farmitalia Caro Erba. (γ-Methacryloxy propyl) trimethoxysilane was donated from Asia Gung Num Company. All chemicals were used without purification.

Lipase Immobilization

NaF (100μl, 1 M), polyvinyl alcohol (200μl, 4%wt in water) and water (164μl) were mixed with lipase solution (400μl, 5mg/ml in phosphate buffer, 50mM, pH 7), followed by adding silane (6 mmol, or equal to total water/silane ratio, [R], = 8). The solution mixture was mixed using a vortex mixer for 5 sec followed by shaking by hand for 1 min, resulting in a formation of gelatin. The solution mixture was cooled in an ice-bath for 10 min and then dried. Two methods of

drying were applied, at 37°C in an incubator for 5 days and vacuum drying for overnight. The white gel was crushed into powder form.

Pre-Polyester Synthesis in Organic Media Using Free or Immobilized Lipase as Catalyst

Succinic acid (1.5 mmol, or other diacids) and EG (1.5 mmol, or glycerol) were mixed in 2.25 ml dimethyl sulfoxide (DMSO). Immobilized lipase (125 mg, or free lipase) was added. The solution mixture was stirred at 100 rpm, 37°C for 5 days under vacuum (110 mmHg).

Pre-Polyester Synthesis in Micro-emulsion

Succinic acid (2 mmol, or other diacids) and AOT (12 mmol) were dissolved in 20ml iso-octane followed by adding lipase solution (5.4ml = water/AOT ratio of 10, 3mg/ml in phosphate buffer solution; pH 7). The solution mixture was added to iso-octane to a ~50ml reaction volume. After mixing, EG (8 mmol, or glycerol) was added to start the reaction and incubated at 37°C for a specific time (5 – 7 days). Methanol and acetonitrile in the ratio of 1:2 were used to precipitate the product.

Analytical Method

Molecular Weight Measurement

The weight average molecular weight (Mw) of the obtained polymers was determined by gel permeation chromatography (GPC) using THF as a mobile phase, UV (Water 486 at 254 nm) and RI (Water 410) detectors. GPC analysis was carried out on a Waters 700. Three serially connected styragel columns (Water HR0.5, HR4E and HR5E, having molecular weights in the range of $0 - 4 \times 10^6$ $g.mol^{-1}$) were used. Polystyrenes with known average molar mass from 418 $g.mol^{-1}$ to 4×10^6 $g.mol^{-1}$ were used as standard.

Hydrolysis Activity Measurement

Gum arabic (20 ml, 10% in water) and olive oil (6.5 ml) were homogenized by stirring for 30 min. Buffer (20 ml, 0.1 M sodium phosphate, pH 9) was mixed with substrate emulsion (25 ml) and then the pH of the solution mixture was adjusted to 8.0 by adding 0.1 M sodium hydroxide. The solution mixture was mildly stirred for 30 min. Buffer substrate emulsion (1.9 ml) was incubated at 37°C for 30 min. Immobilized lipase (10 mg) was incubated with 0.1 ml water for 5 min followed by adding the mixture into the buffer substrate emulsion. The mixture was then shaken for 30 min. The reaction was quenched by adding 200μl methanol and then titated with 0.01M potassium hydroxide in water, using phenolphthalein as an indicator.

Results and Discussion

Lipase Immobilization

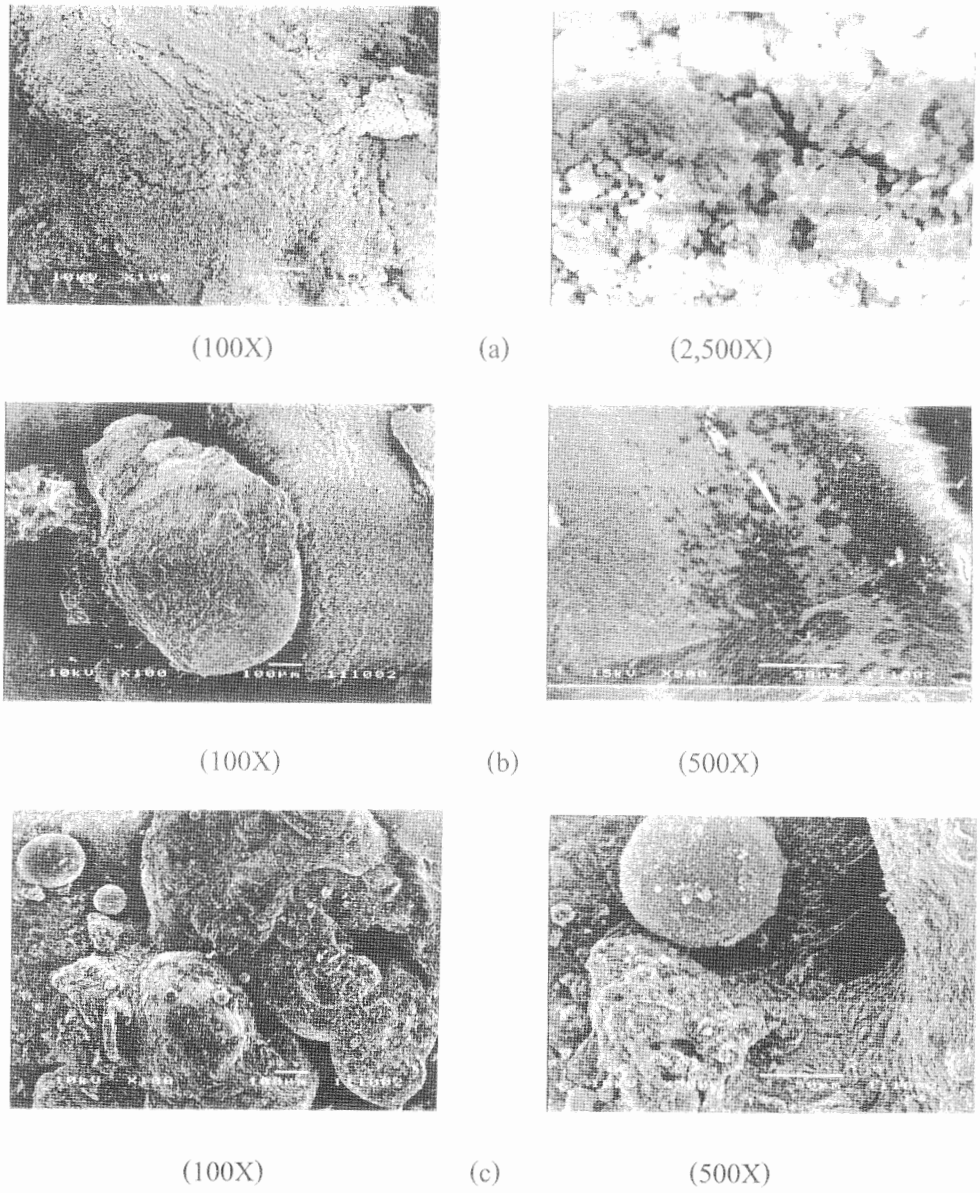
In this work a sol-gel process was selected for entrapping lipase due to its hydrophobic silica matrices providing hydrophobic and hydrophilic

boundaries, which are suitable for lipase in the active form. Two types of silanes were selected to study in this work, (γ-methacryloxy propyl) triethoxy silane (γ-silane) and tetraethoxy silane (TEOS). The hydrolysis activity of immobilized lipase at various conditions is summarized in Table 1. The results showed that a mixture of (γ-methacryloxy propyl) triethoxy silane and TEOS gave the highest hydrolysis activity and the lowest loss of activity after entrapping in hydrophobic silica matrices. This is due to a strong hydrogen bonding of lipase with the acryl group of γ-silane that arranged lipase in the active form. Moreover, the morphology of immobilized lipase obtained from the mixture of γ-silane and TEOS was very fine spherical particles Figure 1, providing a high contact surface area with media solution while TEOS provided denser material causing a lower surface area and entrapping most of the lipase inside the silica network. These might lave on effect on the dramatic lowering of the hydrolysis activity especially for vacuum drying of TEOS.

Table 1 Hydrolysis activity of Candida rugosa lipase in sol-gel matrices derived from various silanes

Condition	**Hydrolysis Activity (μmol.mg protein^{-1}. h^{-1})**	**Relative Activity**
TEOS/vacuum drying	97	0.13
TEOS/dried at 37°C	313	0.43
(γ-methacryloxy propyl) triethoxy silane/ dried at 37°C	442	0.61
(γ-methacryloxy propyl) triethoxy silane : TEOS = 5:1/dried at 37°C	547	0.75

Note : Hydrolysis activity of Candida rugosa = 729 (μmol. mg protein^{-1}. h^{-1})



(100X) (a) (2,500X)

(100X) (b) (500X)

(100X) (c) (500X)

Figure 1 Morphology of immobilized lipase obtained from (a) (γ-methacryloxy propyl) triethoxy silane : TEOS = 5:1/dried at 37°C, from (b) TEOS/dried at 37°C and from (c) TEOS/vacuum dried

Pre-Polyester Synthesis in Organic Media Using Free or Immobilized Lipase as a Catalyst (Solution Polymerization)

Lipase from Candida rugosa has been known as a good hydrolysis agent and hasn't provided good results in polyester synthesis (Wang, *et al.* 1995; Wang, *et al.* 1996; Wu, *et al.* 1996; and Jaaskelainen, *et al.* 1997). Hence, in this work, we selected this lipase to develop its capability of synthesizing pre-polyester between two techniques; solution polymerization using free or immobilized lipase and microemulsion polymerization using free lipase.

Pre-polyester synthesis from various free diacids and ethylene glycol using free lipase as a catalyst in various types of organic solvents, is presented in Table 2. Most products have equal molecular weight, except for isooctane due to its very low polarity which helped to separate the water by product from the system, while the other solvents are capable of adsorbing water, hence inducing the agglomeration of the free lipase.

Table 2 Effect of organic solvents on pre-polyester synthesis by solution polymerization using free lipase as a catalyst

Solvent	Poly(ethylene sevacate)		Poly(ethylene adipate)		Poly(ethylene succinate)	
	Mw	PD	Mw	PD	Mw	PD
Dimethyl sulfoxide	360	1.096	349	1.150	320	1.011
Cyclohexanone	338	1.135	303	1.155	313	1.074
Diphenyl ether	344	1.026	-	-	-	-
Isooctane	574	1.341	-	-	-	-

By using immobilized lipases as catalysts, as seen in Table 3, the results showed low molecular weight components as major products, which were similar to those, obtained from using free lipase as a catalyst. However, using the immobilized lipases obtained from γ-silane and a mixture of γ-silane and TEOS gave higher molecular weight pre-polymers as minor products (3 – 4%). As discussed previously, this might come from the strong bonding between lipase and the acryl group of γ-silane confirming the lipase in the active form. However, a mixture of γ-silane and TEOS gave a higher molecular weight product than γ-silane alone due to the lesser packing of the supporter network in which organic portions of γ-silane inhibited the formation of a silica network.

Using a free or immobilized lipase mostly provided that the molecular weight was in the same range, which were ABA and BAB product structures. Also different polarity organic solvents were employed to improve the capability of the lipase. Only low molecular weight products were obtained due to the water adsorbed in the media and the agglomeration of free lipase or the de-structuring of immobilized lipase.

Table 3 Effect of immobilized lipases obtained from different silane precursors on pre-polyester synthesis by solution polymerization in dimethyl sulfoxide

Silane Precursor	Poly(ethylene sebacate)		Products' Fraction
	Mw	PD	
γ-silane/dried at 37°C	334	1.091	0.9393
	2,124	1.247	0.0407
γ-silane+TEOS/dried at 37°C	359	1.090	0.9669
	3,361	1.294	0.0331
TEOS/dried at 37°C	382	1.091	1
TEOS/vacuum dried	374	1.094	1

Pre-Polyester Synthesis in Microemulsion (Microemulsion Polymerization)

The reaction investigated in this section is the esterification of ethylene glycol and succinic acid in isooctane-based reverse micelle media formed by the surfactant AOT. In this study, the water-AOT molar ratio was fixed at 10, according to many works indicating that the lipase worked well and has the highest activity at this ratio (Wolf, *et al.* 1981; Papadimitriou, *et al.* 1993; and Pileni, *et al.* 1985). Figure 2 showed the phase boundaries of succinic acid-ethylene glycol-AOT's reverse micelle phase in terms of molar fraction. From this figure, microemulsion can be initially formed at a succinic acid-AOT molar ratio of 1:5 and the maximum load of ethylene glycol was ~17 times that of succinic acid. Hence this study was carried out at the succinic acid-AOT molar ratio of 1:6 and the maximum load of ethylene glycol was ~22 times that of the succinic acid.

Haber, *et al.* (1993) studied polyester synthesis in a microemulsion and found that the reaction was very complicated. Normally, the enzyme is in the water pool while the alcohol is in the palisade layer. In this study, diacids do not dissolve in isooctane, therefore, they always stay in the water pool. Due to a very small size of microemulsion, the rate of the reaction depended on the collision rate of the reverse micelles (or the exchange rate of the micelle contents between

substrates and enzyme filled micelles) (Haber, *et al.* 1993) and/or the reaction rate of the enzyme (Bru, *et al.* 1995). In other words, the lipase firstly attached to the carbonyl group of the diacids to produce an ester and form the enzyme-substrate complexes (Holmberg, 1997; Brzozowski, *et al.* 1991; Brady, *et al.* 1990; and Winkler, *et al.* 1990). The hydroxyl group of the alcohol then reacted with the carbonyl group to form the ester bond, consequently loosening out the enzyme. Thus, the first effect studied was the shaking time after adding lipase into reverse micelles (before adding ethylene glycol), as illustrated in Figure 3.

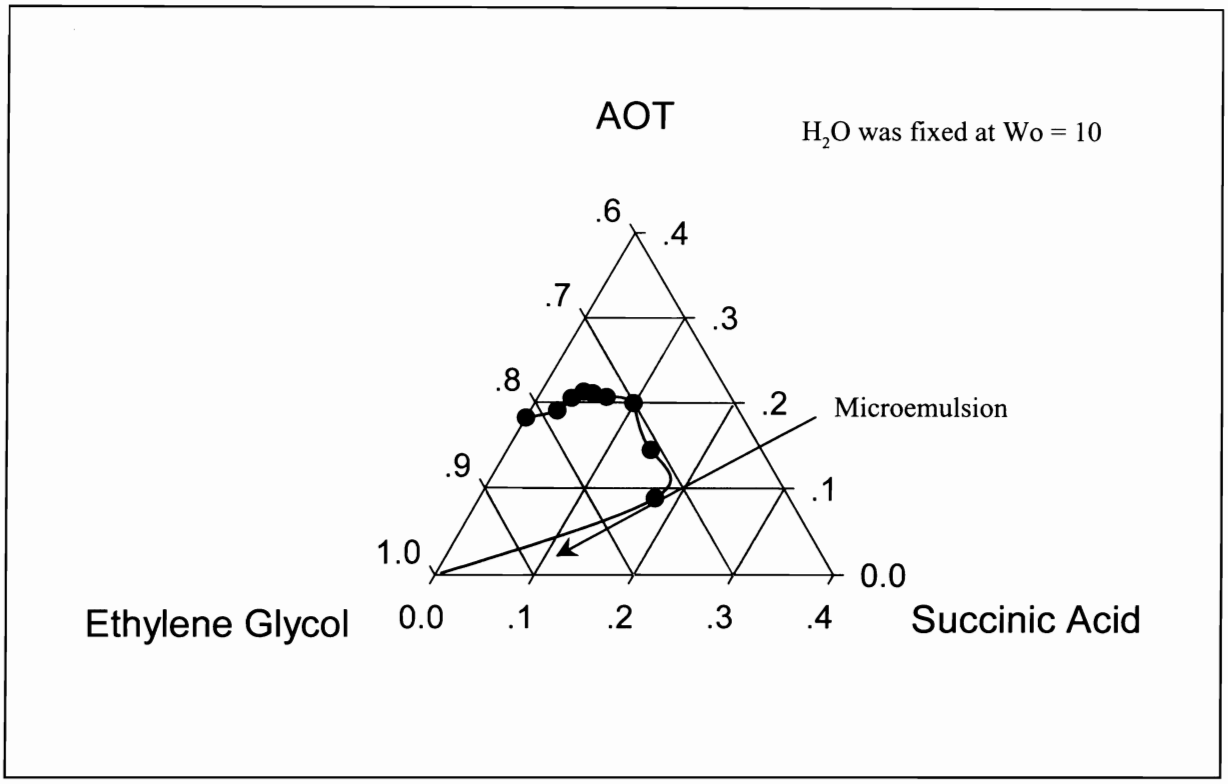


Figure 2 Phase Boundaries for succinic acid-ethylene glycol-AOT's reverse micelle phase at the fix ratio of water-AOT (W_o) at 10 and 24°C

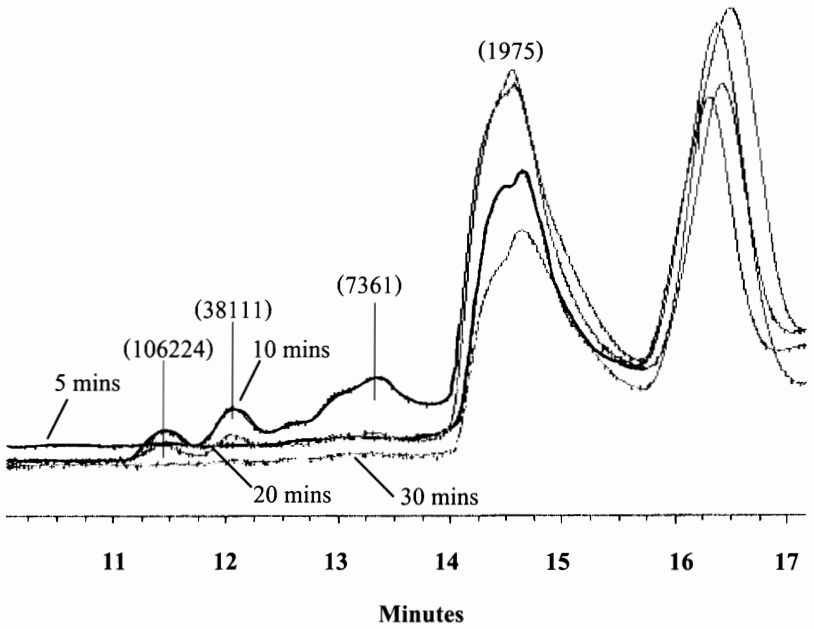


Figure 3 GPC chromatograms of poly(ethylene succinate) synthesis at different shaking times after adding lipase at [succinic acid]:[EG] = 1:12, 25°C and 14 days reaction time

From this GPC chromatogram, it was found that the shaking time after adding lipase into a reverse micelle has a high impact on increasing the molecular weight. In general, the thermal and storage stabilities of the enzymes in organic solvent are higher than those of the enzymes in water (Erdogan, *et al.* 1995; Zaks, *et al.* 1984; and Klibanov, 1989). However, in our case of a microemulsion system, the lipase was dissolved in and stayed inside the water pool. The storage stability of the enzyme inside the micelle would behave like that of an enzyme in water. As a result, the longer the shaking time, the easier the lipase was denatured, as shown in Figure 3. Although shaking for a short time could increase the exchange rate of micelle contents, the major product obtained was ~2000.

For a different lipase concentration, the results are shown in Figure 4. The major product obtained was ~2300, but the product-AOT ratios, which correlate to the production yield at constant AOT, were different. The optimum peak was found at 5 mg/ml of lipase concentration and after this point the product-AOT ratio was reduced possibly due to the aggregation of the lipase.

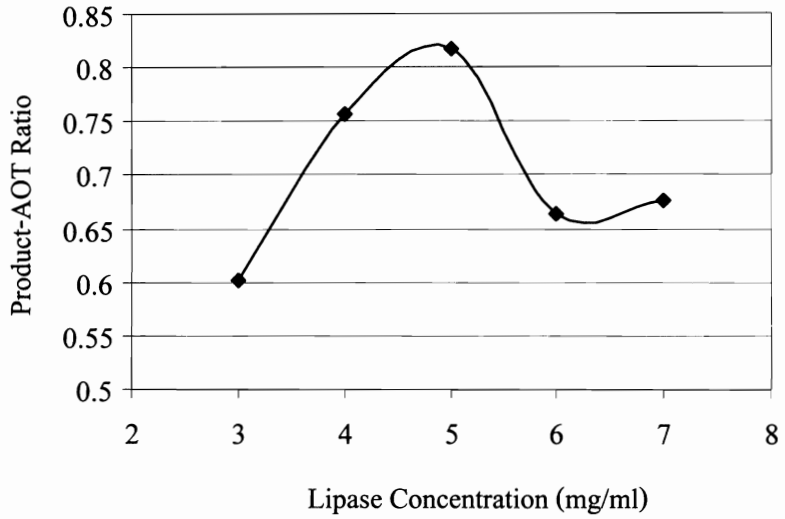


Figure 4 Poly(ethylene succinate)-AOT area ratio at different lipase concentrations at [succinic aicd]: [EG] = 1:12, 10 mins shaking time after adding lipase, 155 rpm shaking speed, 40°C and 7 days reaction time

After adding ethylene glycol, which induced strong attractive interactions and enhanced the micelle solubilization of water (Gupta, 1992), ethylene glycol stabilizes and prevents the micelles from exchanging their contents during a collision. However, it might change the polarity of the water pool and increase the stability of the lipase, as illustrated in Figure 5. The shaking time could go up to 40 mins after adding the glycerol, and more productivity can be produced at longer time. At a shaking time of 30 min with various shaking speeds, a high production was produced at 145 rpm, as shown in Figure 6.

Under all conditions, the major product has a molecular weight of ~2300. Since the system has water, which normally needs to be added to activate the lipase, it influences the reaction in terms of shifting the equilibrium of the reaction backwards. Moreover, succinic acid is more suited to dissolving in water. Water molecules might stay around the succinic acid molecules, thus inhibiting the formation of on enzyme-substrate complex (succinic acid) (Brzozowski, *et al.* 1991; and Del-Val, *et al* 1998). Additionally, the hydrophilicity of succinic acid might strip the essential water molecules from the enzyme, causing a reduction in the lipase activity and changing the enzyme conformation.

Based on the above assumption, various types of diacids were studied at an [acid]:[EG] ratio of 1:12, a 10 min shaking time after adding lipase into reversed micelle at 155 rpm shaking speed, 30 min shaking time after adding dialcohols at 145 rpm shaking speed and 37°C. The reaction mixture was kept for 4 days. The results are shown in Table 4.

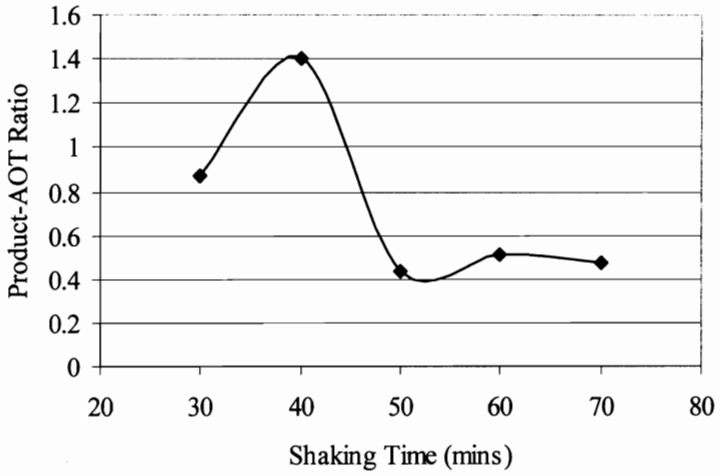


Figure 5 Poly(ethylene succinate)-AOT area ratio at different shaking times (by fixing shaking speed at 125 rpm) after adding the ethylene glycol at [succinic acid]:[EG] = 1:12, 10 mins shaking time after adding lipase, 155 rpm shaking speed, 25°C and 2 days reaction time.

Column type : HR0.5&HR4E&HR5E, Working Range of MW = 0 - 4 x10^6

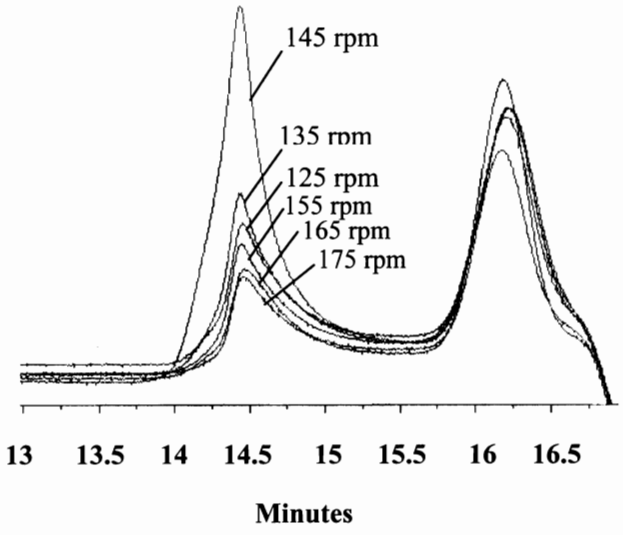


Figure 6 GPC chromatograms of poly(ethylene succinate) synthesis at different shaking speeds (by fixing shaking time at 40 mins) after adding the ethylene glycol at [succinic acid]:[EG] = 1:12, 10 mins shaking time after adding lipase, 155 rpm shaking speed, 25°C and 2 days reaction time.

Table 4 Effect of diacids and dialcohols on pre-polyester synthesis by microemulsion polymerization at a [di-acid]:[poly-alcohol] ratio of 1:12, 10 mins shaking time after adding lipase at 155 rpm shaking speed, 30 mins shaking time after adding poly-alcohol at 145 rpm and 37°C using isooctane as a solvent

Product	MW	"n"	PD
Poly(ethylene succinate)	2343	16.27	1.11
Poly(ethylene adipate)	2284	13.28	1.08
Poly(ethylene sebacate)	2377	10.43	1.10
Poly(glycerol succinate)	2743	15.76	1.11
	1194	6.86	1.04

Again, observed results showed no change in molecular weight of the synthesized product. Generally, diacid dissolved well in a polar solvent. Increasing the hydrophobicity contributed less to the solubility effect inside the micelle and encourages it to stay at the micelle boundary (palisade layer), which was the more favorable condition for the lipase to react with. However, in this work, we found that the polarity of the diacid had a lesser effect on increasing the molecular weight. Changing the polarity of the alcohol also showed less significance in increasing the molecular weight, as illustrated in Table 4. There was probably, not enough hydrophobicity in the starting materials used. The other possibility might come from the effect of microemulsion size. If the synthesized product stayed inside and/or at palisade layer only, the size of micelle limited the molecular weight of the product.

Conclusions

For entrapment by the sol-gel process using Candida rugosa lipase as a catalyst, the mixture of (γ-methacryloxy propyo) triethoxy silane and tetraethoxy silane (TEOS) gave the highest result in hydrolysis activity, with a 25% reduction in immobilized lipase activity. Pre-polyester synthesis by solution polymerization using both a free and immobilized lipase provided only di-mer and tri-mer products (Mw~350), while microemulsion polymerization gave a higher molecular weight (Mw~2300). Both techniques provided narrow

distribution products. Since all studied effects had a lesser effect on increasing the molecular weight, the most dominant factors controlling the molecular weight of the microemulsion polymerization might be the microemulsion size. However, for synthesis of a pre-polyester, it was found that microemulsion polymerization was the most suitable method. This method provided an exact molecular weight and a very narrow molecular weight distribution.

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References

Bello, M., Thomas, D. and Legoy, M. D. 1987. Interesterification and synthesis by Candida Cylindracea lipase in microemulsion. *Biochem. Biophys. Res. Commun.* **146** : 361-367.

Brady, L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J. P., Christiansen, L., Huge-Jensen, B., Norskov, L., Tim, L. and Menge, U. 1990. A Serien Protease Triad Forms the Catalytic Center of a Triacylglycerol Lipase. *Nature.* **343** : 767-770.

Bru, R., Sanchez-Ferrer, A. and Garcia-Carmona, F. 1995. Kinetic models in reverse micelles. *Biochem. J.* **310** : 721-739.

Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., Bjorkling, F., Huge-Jensen, B., Patkar, S. A. and Thim, L. 1991. A Model for Interfacial Activation in Lipase from the Structure of a Fungal Lipase-inhibitor Complex. *Nature.* **351** : 491-494.

Cao, L., Bornscheuer, U. T. and Schmid, R. D. 1999. Lipase-catalyzed solid-phase synthesis of sugar ester. Influence of immobilization on productivity and stability of the ezyme. *J. Mol. Catal. B: Enzym.* **6** : 279-285.

Chaudhary, A. K., Beckman, E. J. and Russell, A. J. 1998. Enzymes for polyester synthesis. Chapter 2. In: *Enzymes in Polymer Synthesis.* Washington, D.C. American Chemical Society : 18-57.

Chen, J. P. and Chen, J. Y. 1998. Preparation and characterization of immobilized phospolipase A2 on Chitosan beads for lowering serum cholesterol concentration. *J. Mol. Catal. B: Enzym.* **5** : 483-490.

Coulon, D., Ismail, A., Girardin, M. and Ghoul, M. 1998. Enzymatic synthesis of alkylglycoside fatty acid esters catalyzed by an immobilized lipase. *J. Mol. Catal. B: Enzym.* **5** : 45-48.

de Gomez-puyou, M. T. and Gomez-Puyou, A. 1998. Enzymes in low water systems. *Crit. Rev. Biochem. Mol. Biol.* **33(1)** : 53-89.

del-Val, M. I. and Otero, C. 1998. Kinetic and spectroscopic behavior of a lipase-microgel derivative in aqueous and micellar media. *J. Mol. Catal. B: Enzym.* **4** :137-147.

Erdogan, G. and Douglas, G. H. 1995. Ethylene glycol and fatty acids have a profound impact on the behavior of water-in-oil microemulsions formed by the surfactant Aerosol-OT. *Langmuir.* **10(13)** : 4695-4702.

Goncalves, A. P. V., Cabral, J. M. S. and Aires-Barros, M. R. 1998. Analysis of a BSTR reactor for triglyceride hydrolysis with an immobilized cutinase. *J. Mol. Catal. B: Enzym.* **5** : 35-38.

Gupta, M. N. 1992. Enzyme function in organic solvents. *Eur. J. Biochem.* **203** : 25-32.

Haber, J., Maslakiewicz, P., Rodakiewicz-Nowak, J. and Walde, P. 1993. Activity and spectroscopic properties of bovine liver catalase in sodium bis(2-ethylhexyl) sulfosuccinate/isooctane reverse micelles. *Eur. J. Biochem.* **217** : 567-573.

Hedström, G., Backlund, M. and Slotte, J. P. 1993. Enantioselective synthesis of Ibuprofen esters in AOT isooctane microemulsions by Candida-cylindracea lipase. *Biotechnol. Bioeng.* **42** : 618.

Hedstrom, G., Backlund, S., Eriksson, F. and Karlsson, S. 1998. Lipase-catalysed stereo-selective esterifications using gelatin-based hydrogels. *Colloids. Surf. B: Biointerfaces.* **10** : 379-384.

Hedström, G., Slotte, J. P., Backlund, M., Molander, O. and Rosenholm, J. B. 1992. Enzyme catalyzed oxidation of cholesterol in a physically characterized water-in-oil microemulsion. *Biocatal.* **6** : 281.

Holmberg, K. 1997. Microemulsions in Biotechnology. In: *Industrial Applications of Microemulsions. (eds.) Conxita Solans and Hiromobee Kunieda.* New York, Marcel Dekker : 65.

Jaaskelainen, S., Linko, S., Raaska, T., Laaksonen, L. and Linko, Y. Y. 1997. Molecular modelling of lipase-catalyzed polyester synthesis. *J. Biotechnol.* **52** : 267-275.

Karlsson, S., Backlund, S., Eriksson, F. I. and Hedstrom, G. 1998. Enzymatic esterifications and transesterification in AOT-base gels with different compositions. *Colloids Surf. B: Biointerfaces.* **10** : 137-147.

Klibanov, A. M. 1989. Why are enzymes less active in organic solvents than in water?. *Trends Biochem. Sci.* **14** : 141.

Langrand, G., Triantaphylides, C. and Baratti, J. 1988. Lipase catalyzed formation of flavor esters. *Biotechnol. Lett.* **10** : 549-554.

Lindman, B., Stilbs, P. and Moseley, M. E. 1981. Fourier transform NMR self-diffusion and microemulsion structure. *J. Colloid Interface. Sci.* **83** : 569-582.

Martin, L. D., Ebert, C., Garau, G., Gardossi, L. and Linda, P. 1999. Pencillin G amidase in low-water media: immobilization and control of water activity by means of celite rods. *J. Mol. Catal. B: Enzym.* **6** : 437-445.

Mojovic, L., Knezevic, Z. Popadic, R. and Jovanovic, S. 1998. Immobilization of lipase from Candida rugosa on a polymer supporty. *Appl. Microbiol. Biotechnol.* **50** : 676-681.

Nagayama, K., Karaiwa, K., Doi, T. and Imai, M. 1998. Esterification activity and stability of Candida rugosa lipase in AOT microemulsion-based organo-gels. *Biochem. Bioeng. J.* **2** : 121-126.

Österverg, E., Ristoff, C. and Holmberg, K. 1988. Enzymic preparation of monoglycerides in a microemulsion. *Tenside Surfactants Deterg.* **25** : 293.

Papadimitriou, V., Xenakis, A. and Evangelopoulos, A. E. 1993. Proteclytic activity in various water-in-oil microemulsions as related to the polarity of the reaction medium. *Colloids Surf. B : Biointerface.* **1(5)** : 295.

Pileni, M.P., Zemb, T. and Petit, V. 1985. Solubilization by reverse micelles : solute localization and structure perturbation. *Chem. Phys. Lett.* **118** : 414-420.

Reetz, M. T., Zonta, A. and Simpelkamp, J. 1996. Efficient immobilization of lipases by entrapment in hydrophobic Sol-gel materials. *Biotechnol. Bioeng.* **49** : 527-534.

Stamatis, H., and Xenakis, A. 1999. Biocatalysis using micoremulsion-base polymer gels containing lipase. *J. Mol. Catal. B: Enzym.* **6** : 399-406.

Stamatis, H., Xenakis, A. and Kolisis, F. N. 1993. Enantiomeric selectivity of a lipase from penicillum-simplicissmum in the esterification of menthol in

microemulsions. *Biotechnol. Lett.* **15** : 471-476.

Stamatis, H., Xenakis, A., Sztajcr, H., Menge, U. and Kolisis, F. N., Tramper, J., Vermüe, M. H., Beeftink, H.M., Stockar, U. V. 1992. Biocatalysis in Nanoconventional Media. *Proceeding of the International Symposium Immobilized Biocatalysis of the European Federation of Biotechnology.* Amsterdam, Noordwijkerhout, Elsevier. : 733.

Wang, Z. L., Linko,Y. Y. and Seppala, J. 1995. Lipase catalyst polytrans-esterification in an organic solvent. *Biotechnol. Tech.* **9(5)** : 349-354.

Wang, Z. L., Hiltunen, K., Orava, P., Seppala, J. and Linko, Y. Y. 1996. Lipase-catalyzed polyester synthesis. *Pure Appl. Chem.* **A33 (5)** : 599-612.

Winkler, F. K., D'Arcy, A. and Huziker, W. 1990. Structure of Human Pancreatic Lipase. *Nature.* **343** : 771-774.

Wolf, X. and Luisi, P. L. 1981. Micellar solubilization of an enzyme in hydrocarbon studies of ribonuclease in n-octane. *Biochem. Biophys. Res. Commu.* **81** : 209-217.

Wu, X. Y., Seppala, J. and Linko, Y. Y. 1996. Lipase-catalyzed polyester synthe-sis. *Biotechnol. Tech.* **10(10)** : 793-798.

Zaks, A. and Klibanov, A. M. 1984. Enzymatic catalysis in organic media at 100°C. *Science.* **224** : 1249-1251.

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