JOURNAL OF OLEO SCIENCE

J. Oleo Sci., Vol. **52**, No. 2, 65-72 (2003)

JOS

Synthesis of Palm Kernel Oil Alkanolamide Using Lipase

M.B. Abdul Rahman*, C.L. Yap, K. Dzulkefly, R.N.Z. Abdul Rahman, A.B. Salleh and M. Basri

Centre for Research in Enzyme and Microbial Technology, Faculty of Science and Environmental Studies (Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, MALAYSIA)

Edited by D. Kitamoto, AIST, Tsukuba, and accepted October 18, 2002 (received for review July 1, 2002)

Abstract: Fatty monoethanolamides were synthesized in organic solvent from palm kernel olein (PKL) and palm kernel stearin (PKS) using a lipase from *Candida rugosa*. The transamidation reactions of PKL and PKS were enhanced in the presence of lipase. The optimal yield was achieved at reaction time 72 hours for both systems with PKL and PKS as the substrates. For PKL as the substrates, the optimal reaction temperature was 40℃, whereas with PKS as the substrate, no optimum temperature was found (in the range of temperature studied) where the relative yield increased with the increasing temperature. Lipase functioned better in hydrophobic solvents compared to hydrophilic solvents. The best solvent for the reactions was isooctane. Increasing the amount of the monoethanolamine used resulted in the increasing solubility of the reactants and products, hence, increasing the yield of the product. For PKL, increasing the mole ratio of PKL:monoethanolamine from 1:1 to 1:15 increased the relative yield to 4.5 fold. However, for PKS, the increase was only 2.5 fold. The optimal ratio of enzyme/PKL (or PKS) was 0.035. In the water activity studies, the preequilibrium and the direct salt hydrate addition methods were used. Generally, PKL always showed the higher relative yield compared to PKS. At the optimum condition at room temperature, the yield of PKL monoethanolamide was 77.0% and the yield of PKS monoethanolamide was 39.0%. Kinetic studies also showed a clear preference to PKL in which its K_m value was 10-fold lower than that of PKS at room temperature.

Key words: monoethanolamide, lipase, palm kernel oil, esterification

1 Introduction

Fatty monoethanolamides have many uses especially for household and personal care uses. Their principal functions are stabilizing foam, increase viscosity and react as emulsifiers (1,2). Commercially, fatty alkanoamides can be produced by transamidation of the fatty methyl ester with monoethanolamide at elevated temperature and pressure (3). This method not only gave a variety of undesirable side reactions but also lead to higher production costs. In recent years, enzymatic catalyzed reaction has been widely understood and able to produce a highly pure product at mild temperature and atmospheric pressure.

Coconut oil based fatty monoalkanoamides is the most popular source of monoethanolamides (4). Palm kernel olein (PKL) is the liquid phase and palm kernel stearin (PKS) is the solid phase from the fractionation palm kernel oil (PKO). These fractions of PKO are excellent sources of fatty acids, fatty alcohols and nitrogen derivatives of 12-18 carbon atoms (**Table 1**) (2). PKL is used in baked goods, hydrogenation and soup

*Correspondence to: M.B. Abdul Rahman, Centre for Research in Enzyme and Microbial Technology, Faculty of Science and Environmental Studies, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, MALAYSIA
E-mail: basya@fsas.upm.edu.my

Table 1 Fatty Acid Compositions of PKL and PKS (PORIM, 1981) (5).

Fatty Acid Compositon	PKL (%)	PKS (%)
C12:0 Lauric	44.7	56.6
C 14:0 Myristic	14.0	22.4
C16:0 Palmitic	8.3	8.0
C18:0 Stearic	2.3	1.8
C18:1 Oleic	19.2	5.6

manufacture, and PKS is generally used as a substitute for cocoa butter. In this work, the synthesis of fatty monoethanolamides using PKO fractions, catalyzed by lipase from *Candida rugosa* were carried out. Parameters, such as reaction time course, temperature, organic solvents, mole ratio of substrate, ratio of enzyme to substrate and a_w were investigated.

2 Experimental Procedures

2·1 Materials

Lipase from *Candida rugosa* (Type VII) was purchased from Sigma Chemical Co. (St. Louis, USA). PKL and PKS were supplied by Southern Edible Oil (M) Sdn. Bhd. (Kuala Lumpur, Malaysia). The mean molecular weights of PKL and PKS based on average saponification values were 704.28 and 678.73, respectively (5). All other chemicals were of analytical grade.

2·2 Determination of Protein

The amount of protein was determined by titration of the amino acids with trinitrobenzene sulfonate (TNBS) following the hydrolysis of the enzyme or its derivatives (6).

2·3 Synthesis of PKL and PKS Monoethanolamides

The reaction mixture consisted of PKL (or PKS) (4.0 mmole), monoethanolamine (4.0 mmole), lipase (0.1 g) and addition of hexane to bring to the total volume to 8.0 mL. The reaction mixture was incubated at 30℃ for 60 h, with continuous shaking at 150 rpm in a horizontal shaker waterbath. All experiments were done in triplicate. The control experiments were carried out without enzyme. The product of PKL (or PKS) monoethanolamide was isolated and then purified by crystallization method at 4℃. The crystals were collected by filtration, washed with 25 ml hexane and dried in a vacuum desicator until constant weight. The pure product of PKL (or PKS) monoethanolamide was quantitated gravimetrically. The percentage yield for each reaction was calculated as :

$$\% \text{ Yield} = \frac{\text{Mole of PKL (or PKS) monoethanolamide obtained}}{3 \times \text{Mole of PKL (or PKS) used}} \times 100$$

2·4 Effect of Reaction Time

The reaction mixture was incubated at various times (12 to 96 h). The relative yield was determined as :

$$\text{Relative Yield (\%)} = \frac{\text{\% Yield with PKL (or PKS) at different time}}{\text{Maximum \% Yield with PKL (or PKS)}} \times 100$$

2·5 Effect of Temperature

The reaction mixture was incubated at various temperatures (30 to 60℃) for 60 h. The relative yield was determined as :

$$\text{Relative Yield (\%)} = \frac{\text{\% Yield with PKL (or PKS) at different temperature}}{\text{Maximum \% Yield with PKL (or PKS)}} \times 100$$

2·6 Effect of Organic Solvent

The effects of various organic solvents were investigated. The solvents used were ethyl acetate (log P= 0.68), benzene (log P=2.00), hexane (log P=3.50), n-heptane (log P=4.00), isooctane (log P=4.20), n-decane (log P=5.60), dodecane (log P=6.60) and n-hexadecane (log P=8.80) (7). The relative yield was determined as :

$$\text{Relative Yield (\%)} = \frac{\text{\% Yield with PKL (or PKS) at different log P}}{\text{Maximum\% Yield with PKL (or PKS)}} \times 100$$

2·7 Effect of Molar Ratio of Substrates

PKL (or PKS) was reacted with different quantity of monoethanolamine, with the mole ratio (PKL (or PKS) : monoethanolamine) of 1:2, 1:3, 1:4, 1:6, 1:8, 1:10, 1:12, 1:15 and 1:20, in 8 ml total volume of reaction mixture. The relative yield was determined as :

Relative Yield (%) =

$$\frac{\text{\% Yield with PKL (or PKS) at different molar ratio}}{\text{Maximum \% Yield with PKL (or PKS)}} \times 100$$

2·8 Effect of Amount of Lipase

The reaction mixtures containing the different quantity of enzyme (25 to 250 mg) were incubated to determine the relative yield as :

Relative Yield (%) =

$$\frac{\text{\% Yield with PKL (or PKS) at different amount of lipase}}{\text{Maximum\% Yield with PKL (or PKS)}} \times 100$$

2·9 Effect of Water Activity (a_w)

In the effect of initial a_w study, enzyme and reaction media were pre-equilibrated with the vapor of saturated salt solutions at ≈ 25℃ in separate containers. Equilibration was performed overnight (at least 16 h). The reaction was started by mixing the substrate and enzyme preparation and incubated at 60 h, 30℃ and 150 rpm in a horizontal shaker waterbath. The salts used were LiCl (a_w=0.12), $MgCl.6H_2O$ (a_w=0.32), $MgNO_3.6H_2O$ (a_w=0.55), NaCl (a_w=0.75), KCl (a_w=0.86) and KNO_3 (a_w=0.90). In the addition of salt hydrate method, the salt hydrates (1.0 g) with various a_w were added to the reaction mixture prior to the incubation of the reaction mixture. The salt hydrates used were $Na_2HPO_4.2H_2O$ (a_w=0.177), $Na_2S_2O_3.5H_2O$ (a_w=0.40), $K_4Fe(CN)_6.3H_2O$ (a_w=0.48), $Na_4P_2O_7.10H_2O$ (a_w=0.52) and $Na_2SO_4.10H_2O$ (a_w=0.83). The relative yield was determined as :

Relative Yield (%) =

$$\frac{\text{\% Yield with PKL (or PKS) at different } a_w}{\text{Maximum \% Yield with PKL (or PKS)}} \times 100$$

2·10 Analysis of the Products

The products of the reactions were examined by thin layer chromatography (TLC) on precoated silica gels plate ($60F_{254}$, Merck, Darmstadt, Germany) and developed in chloroform/methanol (90:10, v/v). Identification was made by comparison with known standards. The analysis of standards and the products formed were carried out on a Shimadzu 8A gas chromatograph (Kyoto, Japan). A non-polar capillary column dimethylpolysilicon (RTX-1, Restek Corporation, Bellefonte, PA) with an internal diameter of 0.32 mm, length of 30 m and film thickness of 0.25 μm was used. Helium was used as a carrier gas with flow rate 1.0 mL/min. The injector and detector were set at 280℃. The column temperature was programmed with an initial temperature 160℃ for 5 min, then heating at 6℃/min to a final temperature of 260℃ for 5 min. Hexadecene was chosen as an internal standard. The samples undergo silylation process before being injected into gas chromatography (8). Infrared (IR) analysis of the products was carried out using Perkin Elmer Fourier-transform Infrared spectrophotometer (Model 1765, Perkin-Elmer Corp., Norwalk, CT).

2·11 Kinetic Studies

Reaction mixtures with different PKL or PKS concentration (0.75 to 0.95 M) and excess monoethanolamine (1:4) and the appropriate volume of hexane to make up to 10 mL were incubated with continuous shaking at different time interval. The time course graphs of the % yield of each enzyme at different PKL or PKS concentration were constructed. The initial reaction rates were determined. Lineweaver-Burk plots of 1/V vs. 1/[S] were constructed and the kinetic constants, K_m and V_{max} were determined from the linear regression of the plots.

3 Results and Discussion

3·1 Time Course

The effect of time course on enzymatic transamidation reaction is shown in **Fig. 1**. Both PKL and PKS enzyme-catalyzed reactions showed higher activity than the control experiment under the same conditions as the reaction proceeds rapidly within 72 h. There after the percentage yield remain constant. This may be due to mass transfer limitations which inevitably arise in a reaction mixture containing a high proportion of solid product or/and the reaction achieve an equilibrium state where the rate of forward reaction was equal to the rate of backward reaction, hence the concentration of the product remain unchanged. Previous studies showed that the product may be increased by disturbing the equilibrium reaction which is easily shifted to the desired direction by removal of the product (9-11). On the other hand, solidification may help in product separation or in recovery and reuse of enzyme. For the control reaction, the reaction still showed a gradual increment even after 72 h. This is because the accumulation of the PKL (or PKS) monoethanolamide was still low,

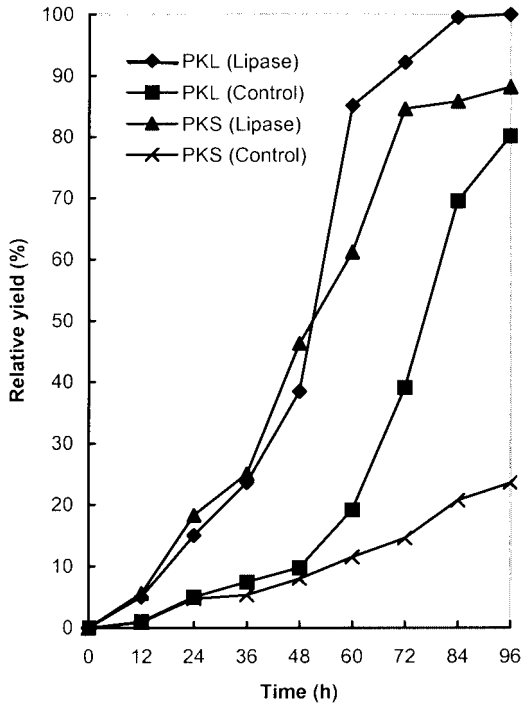

Fig. 1 Effect of Reaction Time on the Relative Yield of Fatty Monoethanolamide Produced (reaction temperature 30℃ and mole ratio 1:1).

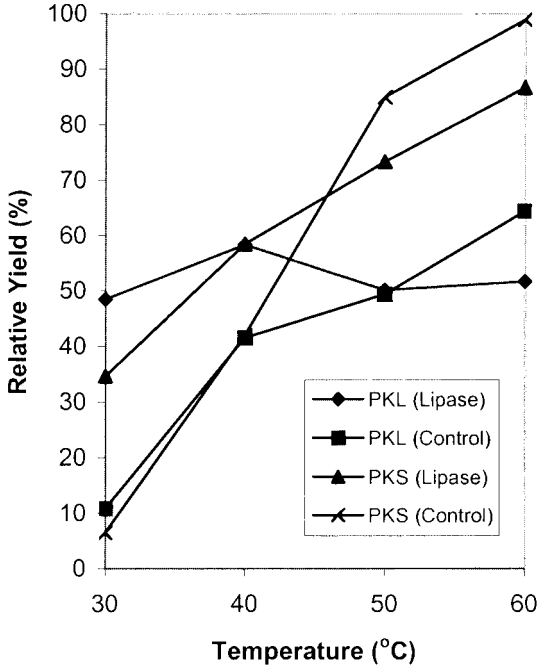

Fig. 2 Effect of Reaction Temperature on the Relative Yield of Fatty Monoethanolamide Produced (reaction time 60 h and mole ratio 1:1).

the solution remains fluid, enabling the reaction to progress without mass transfer limitation.

3·2 Reaction Temperature

The enzymatic reactions showed an increment of yield as the temperature was increased from 30℃ to 40℃ as shown in **Fig. 2**. For PKL as the substrate, the optimal reaction temperature was found at 40℃. At temperature above 50℃, the relative yield was slightly decreased, which may cause by denaturing of the lipase. For PKS as the substrate, the optimal reaction temperature was not found in the range of temperature studied. For the control reaction, the yield increased with increasing temperature and for temperature above 50℃, their relative yield were even higher then the enzymatic reaction. The result is in agreement with Bilyk (3) which showed that monoalkanolamides may be formed in the absence of enzyme and enhanced by increasing temperature.

3·3 Organic Solvent (Log P)

The effect of various organic solvents on the transamidation reaction is shown in **Fig. 3**. The yield was relatively lower with solvents having log P less than 4.0 but higher yield was obtained in organic solvents which are having log P values greater than 4.0 such as heptane and isooctane. The results showed that isooctane (log P=4.20) is the best organic solvent for PKL and n-heptane (log P=4.00) for PKS transamidation reaction. This finding was in agreement with Adlercrutz (12) that in all solvent containing biocatalytic system, the nature of solvent influences the activity and stability of the enzyme to a large extent. Basri (13) reported that lipases function better in the more hydrophobic solvents, which enable the enzyme to retain its native conformation and *C. rugosa* prefers more hydrophobic solvents (log P more than 3.0) compared to the less hydrophobic solvent.

3·4 Mole Ratio of Substrates

The effect of mole ratio of substrates on the transamidation reaction is shown in **Fig. 4**. For PKL as substrates, increasing amount of monoethanolamine to a mole ratio of 1:8 (PKL: monoethanolamine) resulted in higher yield. It gave the highest yield at mole ratio of

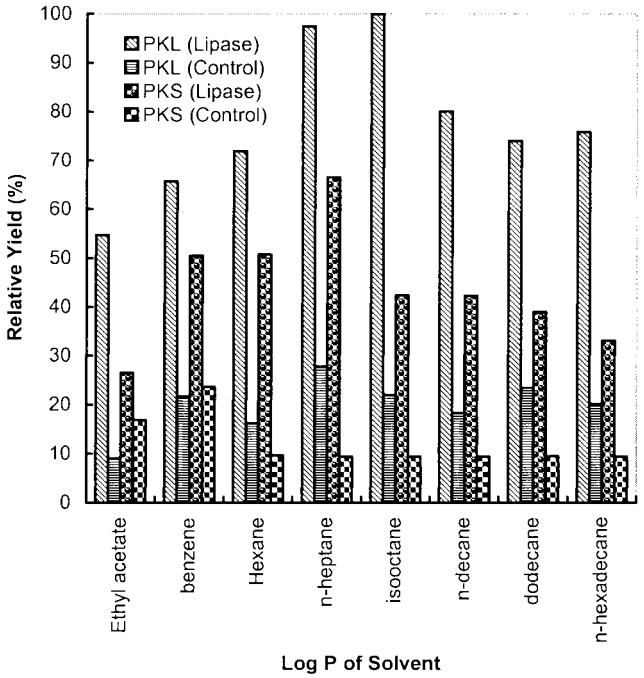

Fig. 3 Effect of Organic Solvent on the Relative Yield of Fatty Monoethanolamide Produced (reaction time 60 h, temperature 30℃ and mole ratio 1:1).

1:15, which is about 4.5-fold that relative yield of 1:1. Increasing of mole ratio higher than 1:15 did not improve the relative yield of PKL monoethanolamide. For PKS as substrate, the relative yield increased from mole ratio (PKS: monoethanolamine) 1:1 to 1:3, after which the relative yield remain constant. The yield at mole ratio 1:15 was 2.5-fold higher than the yield at 1:1. Kitaguchi *et al*. (14) reported that high concentration of monoethanolamine is needed for an effective peptide bond formation.

3·5 Amount of Enzyme

The effect of amount of enzyme on the transamidation reaction is shown in **Fig. 5**. The relative yield increased as the ratio of enzyme:PKL (or PKS) was increased. However, with weight ratio of enzyme:PKL above 0.035 (≈ 0.1 g), there was relatively small increase in relative yield but for PKS, it showed a significant drops in relative yield. An excess of enzyme did not contribute to the increase in the yield of product. This may due to substrate limitation and mass transfer limitation.

3·6 Water Activity (a_w)

The effect of initial a_w was investigated on the transamidation reaction where the pre-equilibrium concept was applied. The result is shown in **Fig. 6**. The enzyme required an optimum a_w of 0.55 ($MgNO_3.6H_2O$) to give the highest activity. In the direct addition of hydrated salt method, the yields of products formed are low for all samples of PKL except in a_w of 0.48 ($K_4Fe(CN)_6.3H_2O$) (**Fig. 7**). Apparently, the enzyme did

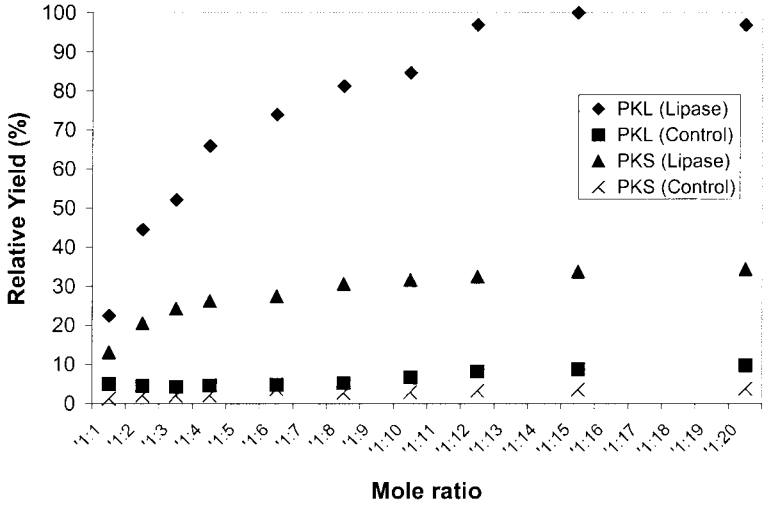

Fig. 4 Effect of Mole Ratio (PKL or (PKS): monoethanolamine) on the Relative Yield of Fatty Monoethanolamide Produced (reaction time 60 h and temperature 30℃).

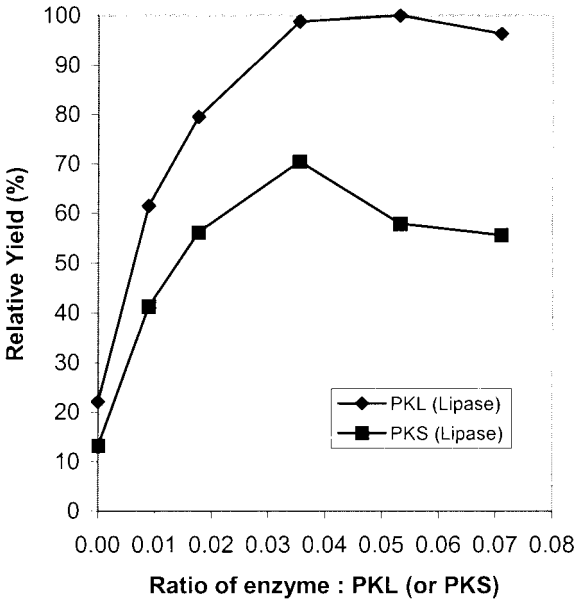

Fig. 5 Effect of Ratio of Enzyme:PKL (or PKS) on the Relative Yield of Fatty Monoethanolamide Produced (reaction time 60 h, temperature 30℃ and mole ratio 1:1).

not fully express its function when it was added to the reaction mixture. Reactions with PKS and control reactions with PKL and PKS gave negative results and thus are not reported. In both methods, the enzyme tend to coagulate and precipitate at the bottom of the reaction mixture after absorbing water from the equilibrium process or from the salt. Tol (15) reported that particle size of the lipase can effect the enzyme activity and may reflect trivial effects such as catalyst aggregation and enzyme inactivation during a_w equilibrium.

3·7 Analysis of Products

The enzymatically synthesized monoethanolamides were ascertained by TLC. The characteristic peaks of IR spectrum bands at 1642.0 cm^{-1} ($\upsilon C=O$) and 1562 cm^{-1} (υN-H) referred to products in the reaction mixture after the incubation period. The optimal studies done in isooctane at 30℃, 60 h, 0.1 g enzyme and 1:1 molar ratio, succesfully gave 77% yields of PKL monoethanolamide and 39% yields of PKL monoethanolamide. The percentage of the major fatty monoethanolamides composition obtained from PKL and PKS monoethanolamides were analyzed by gas chromatography (**Table 2**). It shows a slight difference

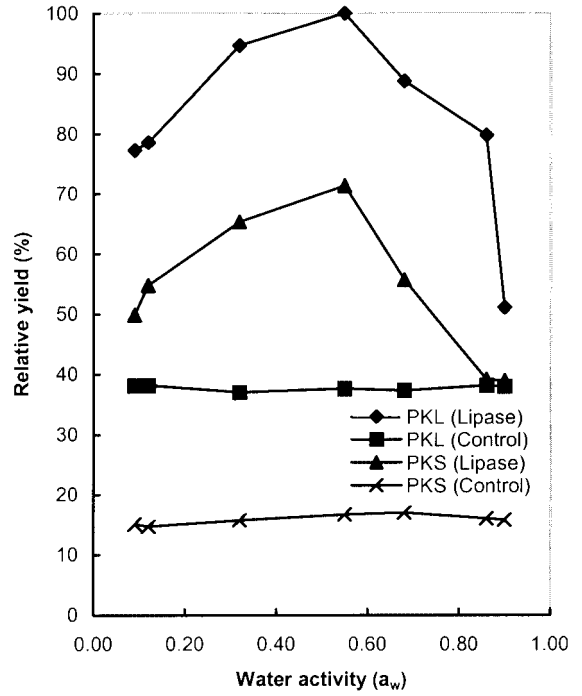

Fig. 6 Effect of Water Activity on the Relative Yield of Fatty Monoethanolamide Produced (preequilibrium method) (reaction time 60 h, temperature 30℃ and mole ratio 1:1).

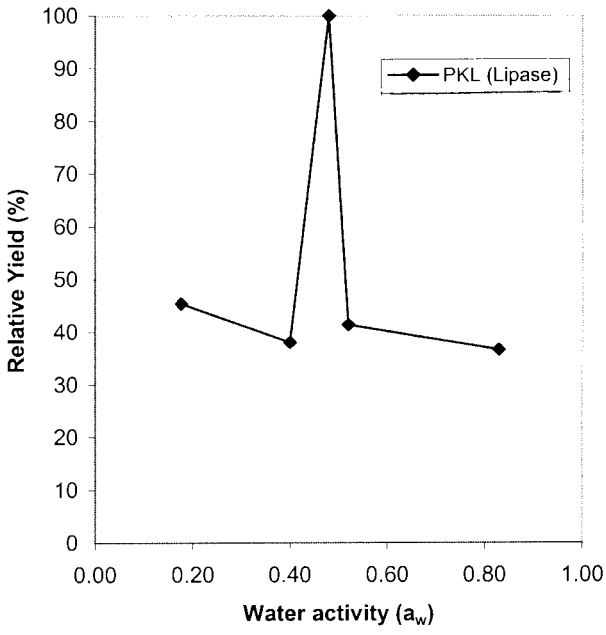

Fig. 7 Effect of Water Activity on the Relative Yield of Fatty Monoethanolamide Produced (direct salt addition method) (reaction time 60 h, temperature 30℃ and mole ratio 1:1).

Table 2 Composition of Purified PKL and PKS Monoethanolamides Determined Using Gas Chromatography, by Internal Standard Method.

Fatty Monoethanolamide Compositon	PKL Monoethanolamide (%)	PKS Monoethanolamide (%)
N-(2-Hydroxyethyl)lauramide	51.3 − 54.3	55.8 − 56.7
N-(2-Hydroxyethyl)myristamide	18.6 − 21.7	20.8 − 22.7
N-(2-Hydroxyethyl)palmitamide	6.8 − 9.1	5.1 − 6.5

Table 3 Kinetic Parameters of Transamidation Reactions.

Substrate (Palm Oil Fractions)	Reaction Temperature (℃)	V_{max} [a]	K_m [b]
PKL	30	0.13	1.25
PKL	40	0.15	0.38
PKS	30	0.25	12.05
PKS	40	0.21	1.21

a Maximum initial rate of conversion of PKL (or PKS) to PKL (or PKS) monoethanolamide, determined from graphs of initial rate vs. concentration of PKL or PKS.

b The apparent Michaelis-Menten constants for PKL or PKS determined from the Lineweaver-Burk plots.

in accordance with their fatty acid composition.

3·8 Kinetic Studies

The Lineweaver-Burk plots for both PKL and PKS as the substrates, showed a decreased of K_m values at higher temperature, with PKL had the lower K_m values compared to PKS. The Michaelis-Menten constant is shown in **Table 3**. It indicated the increase in the affinity of the substrates (PKL or PKS) for the active sites of the enzymes at higher temperature. At 30℃, the K_m value of PKL is 10-fold lower than PKS. It showed that at room temperature, the reaction showed a clear preference to PKL. It may be because PKL have the higher percentage of shorter fatty acid chain compared to PKS. Janssen *et al*. reported that binding of the short chain fatty acids is much better than for the long chain fatty acid (16). The V_{max} for PKS are higher compared to PKL at the particular temperature, which may be due to the slow conversion of substrate to product.

4 Conclusions

The studies proposed that fatty monoethanolamides are easily synthesized from PKL and PKS using lipase as catalyst at mild condition with high percentage of conversion with 77.0% yields of PKL monoethanolamide and 39.0% yields of PKS monoethanolamide. They are easily isolated and purified by extraction and crystallization methods.

Acknowledgement

This project was financed by the Ministry of Science, Technology and Environment, Malaysia.

References

1. S.H. FEAIRHELLER, R.G. BISTLINE, A. BILYK, R.L. DUDLEY, M.F. KOZEMPEL and M.J. HAAS, A Novel Technique for the Preparation of Secondary Fatty Amides III, *J. Am. Oil Chem. Soc*., Vol. **71**, 863-866 (1994).
2. R.A. RECK, Nitrogen Derivatives: Amides, Diamides, Nitriles, Primary Amines and Oxides, *J. Am. Oil Chem. Soc*., Vol. **56**, 796A-801A (1979).
3. A. BILYK, G.J. PIAZZA, R.G. BISTLINE, S.H. FEARHAILLER and M.J. HAAS, A Novel Technique for the Preparation of Secondary Fatty Amides, *J. Am. Oil Chem. Soc*., Vol. **69**, 488-491 (1992).
4. R.A. RECK, Industrial Uses of Palm Kernel and Coconut Oils Nitrogen Derivatives, *J. Am. Oil Chem. Soc*., Vol. **62**, 355-365 (1985).
5. B.K. TAN and C.H.O. OH, *PORIM Technology 16-Malaysia Palm Kernel Sterin, Palm Kernel Olein and Their Hydrogenated Products*, Palm Oil Research Institute of Malaysia, Bangi, pp. 1-19 (1996).
6. A.K. HAZRA, S.P. CHOCK and R.W. ALBERS, Protein Determination with Trinitrobenzene Sulfonate: A Method Relatively Independent of Amino Acid Composition, *Anal. Biochem*., Vol. **137**, 437-443 (1984).
7. C. LAANE, S. BOEREN, K. VOS and C. VEEGER, Rules for Optimazation of Biocatalysis in Organic Solvents, *Biotechnol. Bioeng*., Vol. **30**, 81-87 (1986).
8. B.K. TAN and C.H.O. OH, *PORIM Technology 3-Malaysia Palm Oil Chemical and Physical Characteristics*, Palm Oil

Research Institute of Malaysia, pp. 20-23 (1981).
9. J.M.S. CABRAL, D. BEST, I. BOROSS and J. TRAMPER, *Applied Biocatalysis*, Harwood Academic Publisher, Switzerland, pp. 1-469 (1994).
10. D.E. STEVENSON, A.S. Roger and H.F. Richard, Near-quantitative Production of Fatty Acid Alkyl Ester by Lipase-catalyzed Alcoholysis of Fats and Oils with Adsorption of Glycerol by Silica Gel., *Enz. Microb. Technol*., Vol. **16**, 478-484 (1994).
11. E.N. VULFSON, Industrial Applications of Lipases, *in Lipases: Their Structure, Biochemistry and Application* (P. Woolley and S.B. Peterson, eds), Cambridge University Press, Cambridge, pp. 271-288 (1994).
12. P. ADLERCREUTZ, Modes of Using Enzyme in Organic Media, in *Enzymatic Reactions in Organic Media* (A.M.P. Koskinen and A.M. Klibanov, eds), Blackie Academic and Professional, USA, pp. 9-43 (1996).
13. M. BASRI, K. AMPON, W.M.Z. WAN YUNUS, C.N.A. RAZAK and A.B. SALLEH, Enzymatic Synthesis of Fatty Ester by Hydrophobic Lipase Derivatives Immobilized on Organic Polymer Beads, *J. Am. Oil Chem. Soc*., Vol. **72**, 113-116 (1997).
14. H. KITAGUCHI, D.A. FITZPARTICK, J.E. HUSER and A.M. KLIBANOV, Enzymatic Resolution of Racemic Amines: Crucial Rule of the Solvent, *J. Am. Chem. Soc*., Vol. **111**, 3094-3095 (1989).
15. J.B.V. TOL, R.M. STEVENS and W.J. VELDHUIZEN, Do Organic Solvent Affects the Catalytic Properties of Lipase? Intrinsic Kinetic Parameters of Lipase in Ester Hydrolysis and Formation in Various Organic Solvent, *Biotechnol. Bioeng*., Vol. **47**, 71-81 (1995).
16. A.E. JANSSEN, A.M. VAIDYA and P.J. HALLING, Substrate Specificity and Kinetics of *Candida rugosa* in Organic Media, *Enz. Microb. Technol*., Vol. **18**, 340-346 (1996).