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HYDROLYSIS OF TUNA FISH OIL USING *CANDIDA RUGOSA* LIPASE FOR PRODUCING FATTY ACIDS CONTAINING DHA

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ABSTRACT

The present paper investigates the enzymatic hydrolysis of tuna fish oil with *Candida rugosa* lipase (CRL) in biphasic solvent system for the production of free fatty acids (FFAs). Effect of reaction parameters were studied by varying suitable reaction conditions such as pH, temperature, agitation speed, water and solvent concentrations. When the reaction was carried out for 24 h, 86.5% hydrolysis was achieved. For hydrolysis kinetics, the model given by Prazeres et al., [1] with second product inhibition was used. The kinetic model was fitted using MATLAB® to determine the best kinetic parameters. R^2 and root mean square error (RMSE) were found to be 0.961 and 56.7, respectively. The average value of kinetic constants using the Prazeres model were estimated as K_M = 4.26 µ moles FFA/ml, K_{i1} = 6.0 X 10^{-6} µ moles FFA/ mg enzyme·h, K_{i2} = 0.042 µ moles FFA/ mg enzyme·h and K_2 = 122.3 µ moles FFA/ mg enzyme·h.

KEYWORDS: Biphasic Solvent, *Candida rugosa* Lipase, Hydrolysis, Tuna Fish Oil

INTRODUCTION

The importance of polyunsaturated fatty acids (PUFA) in human nutrition and disease prevention was scientifically recognized three decades ago. In today's perspective, the social demand is to focus on the synthesis of compounds which can improves the immunity against several chronic and degenerative diseases like dyslexia, dyspraxia, alzheimer , schizophrenia and heart diseases [2-4]. Omega-3 long chain PUFA such as DHA (docosahexaenoic acid) is widely reported to have pharmaceutical and neutraceutical properties. DHA is not only essential for the development of human brain but also used in the prevention and control of various human diseases and disorders such as cardiovascular disease, inflammation, allergy, cancer, immune response, diabetes, hypertension and renal disorders [5]. Tuna fish oil is one of the richest natural sources of DHA with its concentration in the range of 25-30 (wt %). The extraction of DHA from fish oil has been reported with both chemical and enzymatic hydrolysis [6-8]. Enzymatic hydrolysis occurs with high specificity at ambient temperature and pressure in comparison with the conventional fat splitting process [9] and therefore preferred over conventional methods from last few decades [10, 11]. The advantages of enzymatic processes over conventional chemical processes include mild reaction conditions of pH, temperature, reduced energy cost, low amount of enzyme consumption, high catalytic efficiency and wide range of fatty acid selectivity of lipases which leads to specific and pure products [12, 13]. This makes the enzymatic process much cleaner and energy efficient than the conventional thermal fat splitting.

Lipases (commonly known as triacylglycerol acylhydrolyase and classified as EC 3.1.1.3) are used industrially [14-17] and commercially in the breakdown of the acyl glycerol bond in the presence of the sufficient quantity of the water and lead to the formation of the monoglycerides, diglycerides, free fatty acids and the glycerol as by-products [18,19]. Lipases have gained the attention of the researchers worldwide because of their applications in various streams such as synthesis of oleo-chemicals, fine chemicals, pharmaceuticals, fragrances and flavor, value added food products with

medicinal values etc. [2, 3]. Lipases are interfacial enzymes remained activated at the oil-water interface due to the open lid conformation of lipases at the interface of biphasic system [20, 21]. The mechanism involves the attachment of acyl group ($RCOO^-$) on the positive side chain (NH_3^+) and hydrogen ion (H^+) on the negative side chain (COO^-) on the lipase active site. This causes the release of free fatty acids (RCOOH) in the solvent biphasic system and lipase can also be recovered after the extraction of solvent phase.

The kinetics of enzymatic hydrolysis using lipases has been studied primarily in monolayer by Verger et al. [22] assuming that all the products of reactions were soluble in the water phase, diffuse rapidly away from the interface and induce no change in substrate with time. The above assumptions are not true for practical cases such as hydrolysis of oils/fats containing long chain fatty acids. Martinez et al. [23] applied the kinetic model of Verger et al. [22] for hydrolysis of tributyrin by *Candida cylindracea* lipase to derive a rate equation as a function of volume fraction of dispersed phase, Φ, and the speed of agitation, N. They used the equation of Tavlarides and Bapat [24] to calculate the mean drop diameter of dispersed phase. They verified the model as adequate and found the influence of agitation speed low and limited to the domain of low Φ (Φ < 10%). Tsai et al. [25] studied the kinetics of enzymatic hydrolysis of olive oil in biphasic organic-aqueous systems considering the reaction as single substrate reaction. The model does not talk about substrate and product inhibition and hence the validity of this model in long term hydrolysis is doubtful. Hermansyah et al. [26] gave Ping Pong Bi Bi model for the triolein hydrolysis using *Candida rugosa* lipase in the biphasic oil–water system. The model described the effect of the initial enzyme concentration, the interfacial area and the initial concentrations of triolein and a fatty acid on the entire process of the stepwise triolein hydrolysis.

Brijwani and Vadlani [27] proposed a kinetic model for hydrolysis of corn distillers dried grain with solubles (DDGS) oil in emulsion system which uses the effect of interfacial area and product inhibition terms, both simultaneously at higher agitation speed and high enzyme concentration and have also reported that at low enzyme concentration and low speed of agitation, no inhibition was observed.

Knezevic et al. [28] reported lipase catalyzed hydrolysis of palm oil in a lecithin/iso-octane reverse micellar system followed one substrate first-order reversible Michaelis-Menten kinetics, when the initial substrate concentration is less than 0.325 mol/dm^3. Yao et al. [29] studied the kinetics of lipase-catalyzed hydrolysis of olive oil in AOT/isooctane reversed micellar system and proposed a model incorporating product inhibition, enzyme deactivation and influence of both substrate and product on enzyme stability in reverse micellar system.

Prazeres et al. [1] proposed a kinetic model with nonlinear third order product inhibition and found it to be most adequate for the description of long term reaction kinetics during batch hydrolysis of olive oil with *Chromobacterium viscosum* lipase in AOT/Isooctane reverse micellar system. The model proposed by Prazeres et al. [1] was single substrate model as the water content used in their reaction system was very high. It can be seen that the various kinetic models given in the literature are based on assumption of one substrate reaction and some of them also incorporated product inhibition and effect of other reaction conditions like interfacial area and agitation speed etc. However, it is well known that in the hydrolysis reaction, two substrates take part in reaction, and therefore the reaction may be considered as second order reaction. When large excess of one reactant is taken, then the reaction may be considered as pseudo first order reaction.

In the present work, the fitting of experimental data with the kinetic model given by Prazeres et al. [1], incorporating second order product inhibition has been studied. Also, the agitation speed during the reaction has been kept sufficiently high where the effect of interfacial area at the given concentration of enzyme becomes negligible and the water concentration has been kept sufficiently high.

MATERIALS AND METHODS

Materials

Tuna fish oil of refined grade was purchased from Jedwards International Inc., MA, U.S.A. Lipase enzyme such as *Candida rugosa* has random positional specificity with 4.01 U/mg activity was supplied by Sigma Aldrich, Canada. Iso-octane, sodium methoxide, 10% solution of BF_3 in methanol, DHA, EPA, fatty acid methyl ester standards used for GC calibration and Triolein, Diolein, Monolein used for HPLC calibration were purchased from Sigma Aldrich, Ontario, Canada. All other chemicals like potassium hydroxide (KOH), ethanol, hexane, anhydrous sodium sulfate, hydrogen chloride (HCl) and methanol were obtained from VWR, Saskatoon, Canada.

Experimental Procedure

The suitable reaction conditions for hydrolysis of tuna fish oil with CRL was studied at different pH, temperature, agitation speed (rpm), oil to water and oil to solvent ratio. Experiments were carried out in triplicates at different reaction parameters by varying only one reaction parameter at a time and keeping other parameters constant. The reaction mixture consisted of 1g fish oil with 1ml of isooctane, 40 mg lipase enzyme dissolved in 10 ml distilled water was kept in a 50 ml conical flask and agitation was carried out using a magnetic stirrer. Methanol was added to stop the reaction and acid value of the reaction mixture was measured by titration with 0.5N KOH to determine the formation of FFAs and the percentage hydrolysis was calculated by the following formula,

$$\% \text{ Hydrolysis} = \frac{\text{Acid Value (hydrolysed oil} - \text{Blank)}}{\text{Saponification Value (original oil)} - \text{ Acid value (original oil)}} \text{x100\%}$$

Standard AOCS test method was used for estimating the acid value [30] of collected samples at different reaction time to determine amount of free fatty acids (FFAs) formed. The activity of lipase has been expressed as µmoles of FFAs formed per ml of reaction mixture. At the suitable conditions, reaction was carried out for 24 h and the kinetic study was done by changing the substrate concentrations.

The amount of oil was varied in the range of 0.8 g to 1.5 g (0.8 g, 1.0 g. 1.25 g, 1.5 g). Maintaining the batch volume constant i.e. 12 ml, 40 mg CRL was dissolved in 10 ml water while changing the iso-octane amount from 0.5 to 1.2 ml. For the kinetic study, individual batch of experiments have been carried out at initial substrate (oil) concentrations of 214 (S_{01}), 267 (S_{02}), 334 (S_{03}) and 401 (S_{04}) µmoles/ml, for different reaction time and FFAs produced have been determined. Further, MATLAB® software was used to determine the kinetic parameters. R^2 and Root mean square error (RMSE) were evaluated for determining the goodness of fitting.

Analysis: The fatty acid composition of tuna fish oil was determined by using an Agilent make Gas Chromatography system (model 5890A), equipped with flame ionization detector (FID, 250°C) and capillary column DB-23 (dimensions: 60 m length, 0.25 mm ID, 0.25 µm film).

Throughout the experimentation, the GC was operated at constant conditions (carrier: helium gas with flow rate 24 cm/sec; oven: 140-240°C at 4°C/min and injector: 250°C). To determine glyceride composition, HPLC (Agilent 1100 Series model) equipped with refractive index detector (model G1362A), Phenogel 100 A°, 300 × 7.8 mm 0.5 µm column (model G1316A) was used. The calibration of both the analytical instruments has been done with authentic standards.

RESULTS AND DISCUSSIONS

Characterization of Fish Oil

The different characteristics of tuna fish oil were found as density: 928 kg/m^3; acid value: 0.507 mg KOH/g oil;

saponification value: 180.0 mg KOH/g oil and iodine value: 163.0 g I_2/100 g oil. The analysis with HPLC indicated that tuna fish oil contains in total 90 wt% of triglycerides. According to the GC analysis, the tuna fish oil was found to contain mainly fatty acids such as 2.7 wt% myristic acid (C14:0), 16.1 wt% palmitic acid (C16:0), 3.6 wt% palmitoleic acid (C16:1), 4.03 wt% stearic acid (C18:0), 13.1 wt% oleic acid (C18:1), 1.3 wt% linoleic acid (C18:1), 5.2 wt% eicosapentaenoic acid (C20:5), 26.0 wt% docosahexaenoic acid (C22:6) and 29.97 wt% other fatty acids.

Characteristics of *Candida rugosa* Lipase

The *Candida rugosa* lipase used has got the following characteristics. Pore diameter is 48.7 (A°), single point total pore volume is 0.09 (cc/g) and BET surface area of liapse is 7.8 (m^2/g) [31]. The higher surface area of lipase represents the better chances of substrate collision to the binding sites present on the surface of the lipase. Therefore increasing the number of collisions per second increases the rate of the reaction [32]. The shape or conformation of lipase active site plays an important role in the specificity of lipase for its substrate.

Therefore, the perfect binding of substrate depends upon the uniformity in the shape and size of the lipases because the uniform particles of lipase could only provide better binding of substrate in the open lid structure of lipase in their active sites. The K_M constant by Michaelis–Menten kinetics is a mathematical measure to represents the specificity of lipases. According to the Paiva et al. [32] the composition of the lid that covers the active site, the geometry of the catalytic triad, structure and the dynamics of the active site lid opening are the features that impart lipases their unique structure-function characteristics.

Effect of Reaction Parameters

The effect of parameters such as pH, temperature, speed of agitation, water concentration and solvent amount on the activity of CRL for hydrolysis of tuna fish oil in biphasic solvent system are shown in Figures 1-5. According to Figure 1, CRL has shown maximum activity of 140.6 µmoles FFAs formed/ml at pH 6.5 while the activity remains 129 µmoles FFAs formed/ml at pH 7.

It shows that the hydrolysis reaction can be carried out in the neutral range of pH 6.5–7.0 because lipases normally contain amino acid residue as an active site and it generates maximum binding affinity for the substrate in the neutral pH range. Therefore, for the rest of the study it has been selected as optimal pH 6.5 for CRL. Han and Rhee [33] have reported optimum range of pH value i.e. 6.5 to 7.1 for hydrolytic reaction of olive oil catalyzed by CRL enzyme. Similarly, Knezevic [28] and Okada [34] have reported pH 7.0 as optimum for hydrolysis of palm oil and sardine oil catalyzed by CRL enzyme.

Maximum activity of 137.5 µmoles FFAs formed/ml have been obtained for CRL at the temperature of 35°C in 1 h as shown in Figure 2. CRL lipase is vulnerable at the higher temperature (>50°C) indicating a sharp reduction in the enzyme activity with increase in the temperature because it results in the denaturing of the lipase proteins. Han and Rhee [33] have reported optimum temperature in the range of 30 to 35°C for hydrolysis reaction of olive oil catalyzed by CRL enzyme. Shimada et al. [35] have used 35°C temperature for hydrolysis of tuna oil by CRL enzyme. Kahveci et al. [36] have also reported 37°C as optimum temperature for hydrolysis of salmon oil catalyzed by CRL enzyme.

The effect of speed of agitation was studied to examine the mass transfer and diffusion resistances on the hydrolysis reaction of oil as shown in Figure 3. CRL was shown to give maximum product formation at 800 rpm equivalent to 141 µmoles FFAs formed/ml in 1 h. It is clear from the trend observed in Figure 3, that initially with increase in speed; the product formation was increased up to 800 rpm. Further increase in the speed up to 1000 rpm did not lead to

any significant improvement in the product formation, indicating that reaction is proceeding without mass transfer resistance at and beyond 800 rpm. According to Figure 4, at 10:1 (w/w) water to oil ratio, CRL was found to give maximum activity i.e. 138 µmoles FFAs formed per ml reaction mixture in 1 h. Therefore, hydrolysis reaction of tuna fish oil with CRL was carried out at 10:1 (w/w) water to oil ratio to obtain best results. The amount of water used plays a crucial role in hydrolysis reaction. In case of oil hydrolysis, water molecules act on the ester bond of triglycerides and provide free fatty acids and glycerol derivatives as product. Similarly, the excess amount of water could also inhibit the hydrolysis reaction by competitive inhibition with substrate for binding to enzyme. CRL enzyme showed maximum productivity equivalent to 136.6 µmoles FFAs formed per ml in 1 h at 1:1 (w/w) solvent to oil ratio as shown in Figure 5.

It shows that the hydrolysis activity of lipase was greatly affected by the concentration of iso-octane as solvent. Solvent acts as an organic phase in the biphasic reaction system and provides the ease to separate product from the reaction mixture. Product formed after the reaction has tendency to be soluble in solvent and therefore are easily separable due to the weak interaction with enzymes [37] but the excess amount of solvent reduces the accessibility of enzyme active sites to substrate. Therefore, the most suitable reaction conditions for hydrolysis of tuna fish oil with CRL in solvent system were obtained as pH 7.0, temperature 35°C, oil to water ratio of 1:10 (w/v) and oil to solvent ratio of 1:1 (w/v). The activity of lipase for optimized parameters was found to vary from 137.5 to 141.2 for CRL in µmoles of FFAs formed per ml, with results reproducible within <±3%. At the most suitable reaction conditions, 86.5% hydrolysis was achieved with CRL in 24 h. Han and Rhee [33] have also reported similar suitable reaction conditions for the hydrolysis of olive oil catalyzed by CRL enzyme in the range of pH 6.5–7.1, temperature 30-35°C and 0.72–9.78 (v/v) oil to water ratio.

Reaction Mechanism

The lipase catalyzed hydrolysis of triglycerides is a complex reaction that involves numerous species (mono-, di-, and triglycerides) and takes place by consecutive and reversible steps. This complexity makes it difficult to obtain a suitable rate equation for the description of this phenomenon. Usually, the several steps (hydrolysis of mono-, di-, and triglycerides) can be lumped together in order to simplify the kinetic analysis:

$$\text{Triglyceride} + 3H_20 \leftrightarrow \text{glycerol} + 3 \text{ fatty acid} \quad (1)$$

Additionally, inhibition phenomena by fatty acids may appear as the reaction proceeds. The enzyme will bind to this interface changing its geometrical conformation, thus exposing the active center and making it accessible to the substrate [39]. The structure of the active center of lipases, whose high-resolution three-dimensional structures have been determined, revealed a catalytic triad of subcenters Ser-His-Glu for *Candida rugosa* lipase**,** which are involved in the binding of the substrate and in the scission of the ester bonds. The existence of these three subcenters may also justify the appearance of higher order inhibition in the case where the inhibitor attaches itself directly onto the active site of the activated enzyme. To explain the results obtained in this work and taking into account the relatively high water content, a model given by Prazeres et al. [1], including nonlinear product inhibition was used, considering that the water is present in excess and assuming a competitive inhibition of lipase by the fatty acids produced upon hydrolysis of the triglycerides. The lipolysis mechanism can then be expressed by the following set of equations:

$$E + S_1 \overset{K_1}{\leftrightarrow} ES_1 \overset{K_2}{\rightarrow} E + P \quad (2)$$

$$E + P \overset{K_{e1}}{\leftrightarrow} EP \quad (3)$$

$$EP + P \overset{K_{e2}}{\leftrightarrow} EP_2 \quad (4)$$

$$EP_2 + P \overset{K_{es}}{\longleftrightarrow} EP_3 \quad (5)$$

The number of product molecules that we assumed could be bound to the enzyme was arbitrarily taken as 3. The value of 3 corresponds to the number of subsites of the active site, derivation of the rate equation from the steps in equations (2) to (5), assuming all other steps to be in equilibrium, yields

$$V = \frac{K_2 E_t S}{K_M(1+K_{i1}P+K_{i2}P^2+K_{i3}P^3)+S} \quad (6)$$

$$V_M = K_2 E_t \quad (7)$$

where V = initial reaction velocity (µmoles FFA/ml·h), V_M = maximum reaction velocity (µmoles FFA/ml·h), K_2 = enzymatic rate constant (µmoles FFA/mg enzyme.h), K_M = Michaelis Menten constant (µmoles FFA/ml), K_{i1} =K_{e1}, K_{i2} = $K_{e1}K_{e2}$ and K_{i3} = $K_{e1}K_{e2}K_{e3}$, K_{i1}, K_{i2} , K_{i3} = first order, second order and third order inhibition constants (µ moles FFA/mg enzyme·h), S = substrate concentration (µmoles FFA/ml), P = product concentration (µmoles FFA/ml), E_t = total enzyme concentration (mg/ml).

Application of Prazeres Model for Hydrolysis Kinetics of Tuna Fish Oil Using CRL

The model equation given by Prazeres et al. [1] as shown in Equation 6 was used in the present work considering second order product inhibition for which the rate equation is as follows:

$$V = \frac{dP}{dt} = \frac{K_2 E_t S}{K_M(1 + K_{i1}P + K_{i2}P^2) + S} \quad (8)$$

The above model was used to fit the experimental data to determine the kinetic constants and also the values of product (FFAs) formed with reaction time by conducting MATLAB® simulation for four different initial substrate concentrations independently. The comparison of predicted and experimental data of product formation with respect to time of reaction is shown in Figure 6. The kinetic constants obtained by fitting model for different initial substrate concentrations independently using MATLAB® are given in Table 1. The order of the values of K_M (2.31 to 6.62 µmoles FFA/ml), V_M (297.5 to 675.2 µmoles FFA/ml·h), K_{i1} (2.5 X 10^{-6} to 9.5 X 10^{-7} µmoles FFA/mg enzyme·h), K_{i2} (0.034 to 0.06 µmoles FFA/mg enzyme·h) and K_2 (74.4 to 168.8 µmoles FFA/mg enzyme·h) obtained by using Prazeres model is almost similar for different initial substrate concentrations.

The comparison of experimental and predicted values of product formed with respect to reaction time obtained by MATLAB® simulation taking all the four sets of data together, are shown in Figure 7 and the value of kinetic constants were obtained as shown in Table 1. The values of R^2 has been found in the range of 0.952 to 0.973 and the values of RMSE has been found in the range of 43.05 to 126.7, indicating a close fitting between the predicted and the experimental data.

CONCLUSIONS

The most suitable reaction conditions for hydrolysis of tuna oil with CRL in biphasic solvent system have been determined. The lipase was observed to work optimally at pH 7, temperature 35°C, 1:1(w/w) oil to solvent ratio and 1:10 (w/w) oil to water ratio. For hydrolysis, the kinetic model given by Prazeres et al. [1], incorporating second order product inhibition was used. The values of kinetic constants have been evaluated. Two statistical parameters R^2 and RMSE were estimated to check the goodness of fitting between the predicted and experimental values of product formation with respect to time of reaction.

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NOMENCLATURE

CRL= *Candida rugosa* lipase

DDGS = Distillers dried grain with solubles

DHA = Docosahexaenoic acid

FFA = Free Fatty acid

E_t = Total enzyme concentration (mg/ml)

K_2 = Enzymatic rate constant (μ moles FFA/mg enzyme·h)

K_M = Michaelis Menten constant (μ moles FFA/ml)

K_{i1}, K_{i2}, K_{i3} = first order, second order and third order inhibition constants (μmoles FFA/mg enzyme·h)

P = Product concentration (μ moles FFA/ml)

PUFA = Poly Unsaturated Fatty acid

S = Substrate concentration (μ moles FFA/ml)

t = time (h)

V = initial reaction velocity (μmoles FFA/ml·h)

V_M = maximum reaction velocity (μmoles FFA/ml·h)

REFERENCES

1. Prazeres, D.M.F., F. Lernos, F.A.P. Garcia and J.M.S. Cabral, Modelling lipolysis in a reversed micellar system: part I- conventional batch reactor, Biotechnol. Bioeng., 1993; 42, 759-764.
2. Kartal, F., M.H.A. Janssen, F. Hollmann, R.A. Sheldon and A. Kilin, Improved esterification activity of Candida *rugosa* lipase in organic solvent by immobilization as Cross-linked enzyme aggregates (CLEAs), J. Mol. Catal B: Enzym., 2011; 71, 85–89.
3. Manzano, M.F.G. and C.I.A. Igarzabal, Immobilization of lipase from Candida *rugosa* on synthesized hydrogel for hydrolysis reaction, J. Mol. Catal B: Enzym., 2011; 72, 28– 35.
4. Villeneuve, P., J.M. Muderhwa, J. Graille and M.J. Haas, Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches, J. Mol. Catal B: Enzym., 2000; 9, 113–148.
5. Uauy, R. and A. Valenzuela, Marine Oils: The health benefits of n-3 fatty acids, Nutrition, 2000; 6(7/8), 680-684.
6. Ackman, R.G. and R.D. Burgher, Component fatty acids of the milk of the gray (Atlantic) seal, J. Biochem. Physiol.,1963; 41, 2501-2505.

7. DeWitt, K.W., Seasonal variations in cod liver oil, J. Sci. Fd. Agric., 1963; 14, 92-98.

8. Kingsbury, K.J., T.D. Heyes., D.M. Morgan, C. Aylott, P.A. Burton, R. Emmersen and P.J.A. Robinson, The effect of dietary changes on the fatty acid composition of normal human depot fat, Biochem. J., 1962; 84, 124-133.

9. Guil-Guerrero, J.L. and E.H. Belarbi, Purification process for cod liver oil polyunsaturated fatty acids, J. Am. Oil Chem. Soc., 2001; 78, 477–484.

10. Lee, K. and C.C. Akoh, Characterization of enzymatically synthesized structured lipids containing eicosapentaenoic, docosahexaenoic, and caprylic acid, J. Am. Oil Chem. Soc., 1998; 75, 495–499.

11. Soumanou, M.M., U. T. Bornscheuer and R.D. Schmid, Two-step enzymatic reaction for the synthesis of pure structured triacylglycerides, J. Am. Oil Chem. Soc., 1998; 75, 703-710.

12. Shimada, Y., A. Sugihara and Y. Tominaga, Enzymatic purification of PUFA, J. Biosci. Bioeng., 2001; 91, 529-538.

13. Antczak, A., T. Kubiak, S. Antczak and Bielecki, Enzymatic biodiesel synthesis - Key factors affecting efficiency of the process, Renewable Energy, 2009; 34, 1185-1194.

14. Lepage, G. and C. Roy, Improved recovery of fatty acid through direct transesterification without prior extraction or purification, J. Lipid Res., 1984; 25, 1391-1396.

15. Neena, N.G., Application of lipase, J. Am. Oil Chem. Soc., 1997; 74, 621-634.

16. Yadav, G.D. and I.V. Borkar, Synthesis of n-butyl acetamide over immobilized lipase, J. Chem.Tech. Biotechnol., 2009; 84, 420-426.

17. Yadav, G.D. and K.M. Devi, Immobilized lipase-catalysed esterification and transesterification reactions in non-aqueous media for the synthesis of tetrahy-drofurfurylbutyrate: comparison and kinetic modeling, Chem. Eng. Sci., 2004; 59, 373-383.

18. Villeneuve, P., J.M. Muderhwa, J. Graille and M.J. Haas, Customizing lipases for bio-catalysis: a survey of chemical, physical and molecular biological approaches, J. Mol. Catal B: Enzym., 2000; 9, 113-148.

19. Hasan, F. A.A. Shah and A. Hameed, Methods for detection and characterization of lipases: a comprehensive review, Biotechnol. Adv., 2009; 27, 782-798.

20. Yan, J., S. Liu, J. Hu, X. Gui, G. Wang and Y. Yan, Enzymatic enrichment of polyunsaturated fatty acids using novel lipase preparations modified by combination of immobilization and fish oil treatment, Bioresour. Technol., 2011; 102, 7154-7158.

21. Maruyama, T., M. Nakajima, S. Ichikawa, H. Nabetani, S. Furusaki and M. Seki, Oil-water interfacial activation of lipase for interesterification of triglyceride and fatty acid, J. Am. Oil Chem. Soc., 2000; 77, 1121-1127.

22. Verger, R. and M.C.E. Mieras and G.H. Haas, Action of phospholipase A at interfaces, J. Biol. Chem., 1973; 248, 4023.

23. Martinez, O., A.M. Wilhelm and J.P. Riba, Kinetic study of an enzymatic liquid-liquid reaction: The hydrolysis of tributyrin by *Candida cylindracea* lipase, J. Chem. Tech. Biotechnol., 1992; 53, 373-378.

24. Tavlarides, L.L. and B.M. Bapat, Models for scale-up of dispersed phase liquid-liquid reactors, American Institute of Chemical Engineers Symosium Series, 1984; 80, 12-46.

25. Tsai, S.W., G.H. Wu and C.L. Chiang, Kinetics of enzymatic hydrolysis of olive oil in biphasic organic-aqueous systems, Biotechnol. Bioeng., 1991; 38, 761-766.

26. Hermansyah, H., M. Kubo, N. Shibasaki-Kitakawa and T. Yonemoto, Mathematical model for stepwise hydrolysis of triolein using *Candida rugosa* lipase in biphasic oil–water system, Biochem. Eng. J., 2006; 31, 125–132.

27. Brijwani, K. and P.V. Vadlani, Lipase-mediated hydrolysis of corn DDGS oil: Kinetics of linoleic acid production, Biochem. Eng. J., 2010; 52, 289-295.

28. Knezevic, Z.D., S.S.S. Marinkovic and L.V. Mojovic, Kinetics of lipase-catalyzed hydrolysis of palm oil in lecithin/isooctane reversed micelles, Applied Microbiol. Biotechnol., 1998; 49, 267-271.

29. Yao, C., S. Tang, Z. Heb and X. Dengc, Kinetics of lipase-catalyzed hydrolysis of olive oil in AOT/isooctane reversed micelles, J. Mol. Catal. B: Enzym., 2005; 35, 108–112.

30. AOCS, Sampling and Analysis of Commercial Fats and Oil.1997, Cd 3b-63.

31. Sharma A., S. Chaurasia and A. Dalai, Enzymatic hydrolysis of cod liver oil for the fatty acids production, Catal. Today, 2013; 207, 93 – 100.

32. A.L. Paiva, V.M. Balcao and F.X. Malcata, Kinetics and mechanisms of reactions catalyzed by immobilized lipases, Enzyme Microb. Technol., 2000; 27, 187–204.

33. Han, D. and J.S. Rhee, Lipase-catalyzed hydrolysis of milk fat in lecithin reverse micelles, Biotechnol. Bioeng., 1986; 28, 1250–1255

34. Okada, T. and M.T. Morrissey, Production of n-3 polyunsaturated fatty acid concentrate from sardine oil by lipase-catalyzed hydrolysis, Food Chem., (2007); 103, 1411–1419.

35. Shimada, Y., A. Sugiha, H. Nakano, T. Kuramoto, T. Nagao, M. Gemba, M. and Y. Tominaga, Purification of docosahexenoic acid by selective esterification of fatty acids from tuna oil with *Rhizopus delemar* lipase, J. Am. Oil Chem. Soc., 1997; 74, 97-101.

36. Kahveci, D., M. Falkeborg, S. Gregersen and X. Xu, Upgrading of farmed salmon oil through lipase-catalyzed hydrolysis, The Open Biotechnol. J., 2010; 4, 47-55.

37. Yadav, G.D. and K. Manjula Devi, Kinetics of hydrolysis of tetrahydrofurfuryl butyrate in a three phase system containing immobilized lipase from *Candida antarctica*, Biochem. Eng. J., 2004; 17, 57–63.

38. Tsai, S.W. and C.L. Chiang, Kinetics, mechanism, and time course analysis of lipase-catalyzed hydrolysis of high concentration olive oil in AOT-lsooctane reversed micelles, Biotechnol. Bioeng., 1991; 38, 206- 211.

39. Brzozowski, A.M., U. Derewenda, Z.S. Derewenda, G.G. Dodson, D.M. Lawson, J.P. Turkenburg, F. Bjorkling, B. Huge-Jensen, S.A. Patkar and L. Thim, A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex, Nature, 1991; 351, 491-494.

APPENDICES

Table 1: Kinetic Constant at Individual and Combined Substrate Concentrations during Hydrolysis of Tuna Oil with CRL Using Prazeres Model

Substrate Concentrations (µmoles/ml)	K_M (µmoles FFA/ml)	V_M (µmoles FFA/ml·h)	K_{i1} (µmoles FFA/mg enzyme·h)	K_{i2} (µmoles FFA/mg enzyme·h)	K_2 (µmoles FFA/mg enzyme·h)	R^2	RMSE
S_{01} 214	2.31	297.5	9.5×10^{-7}	0.060	74.4	0.961	43.0
S_{02} 267	2.44	318.0	2.5×10^{-6}	0.039	79.5	0.973	45.6
S_{03} 334	5.69	666.8	1.0×10^{-7}	0.036	166.7	0.952	68.5
S_{04} 401	6.62	675.2	3.3×10^{-6}	0.034	168.8	0.960	69.5
Average	4.26	489.4	6.0×10^{-6}	0.042	122.3	0.961	56.7
Using all the four sets of data together	9.90	503.6	6.0×10^{-4}	0.017	125.9	0.957	126.7

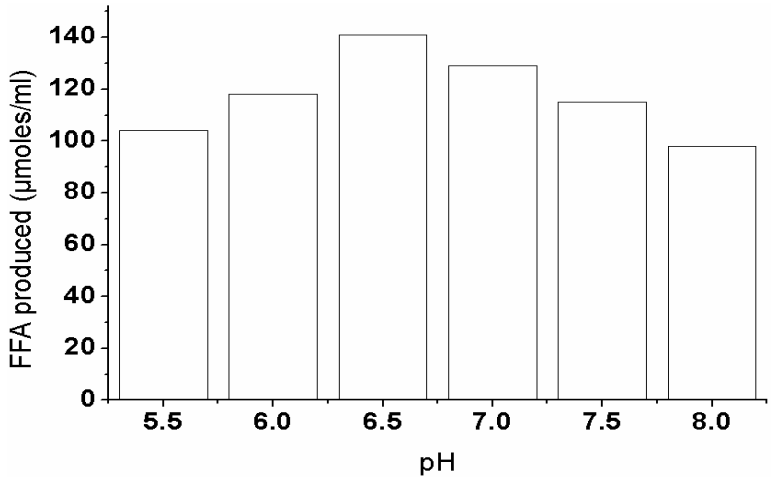

Figure 1: Effect of pH on Activity of CRL during Hydrolysis of Tuna Fish Oil {Experimental Conditions: 1 g Tuna Oil, 1 ml Iso-Octane, 10 ml Water, Temperature 35°C, Agitation Speed 800 rpm, 40 mg Enzyme, Reaction Time 1 h}

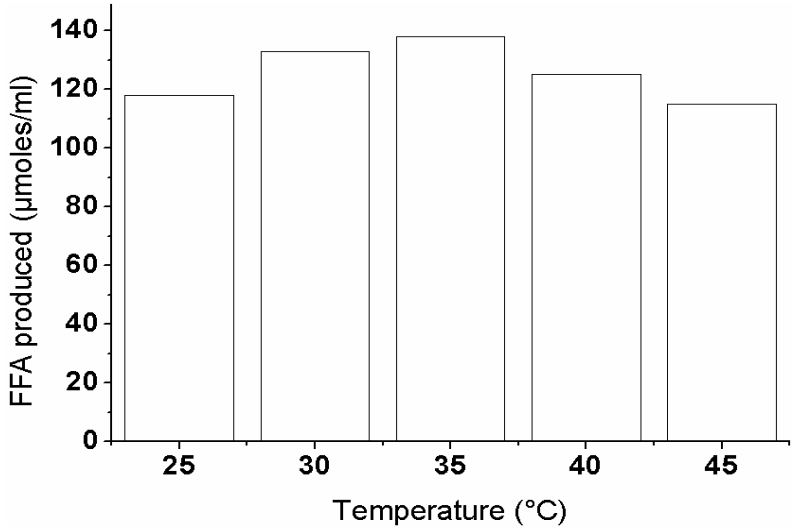

Figure 2: Effect of Temperature on Activity of CRL during Hydrolysis of Tuna Fish Oil {Experimental Conditions: 1 g Tuna Oil, 1 ml Iso-Octane, 10 ml Water, pH 6.5, Agitation Speed 800 rpm, 40 mg Enzyme, Reaction Time 1 h}

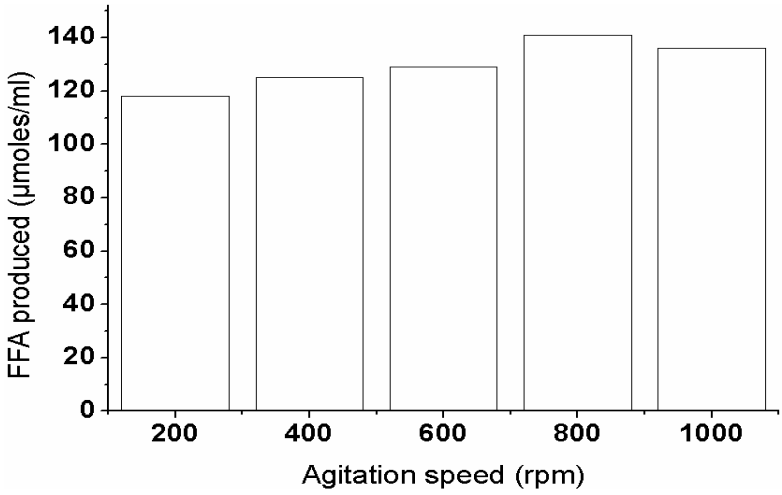

Figure 3: Effect of Agitation Speed (rpm) on Activity of CRL during Hydrolysis of Tuna Fish Oil {Experimental Conditions: 1 g Tuna Oil, 1 ml Iso-Octane, 10 ml Water, pH 6.5, Temperature 35°C, 40 mg Enzyme, Reaction Time 1 h}

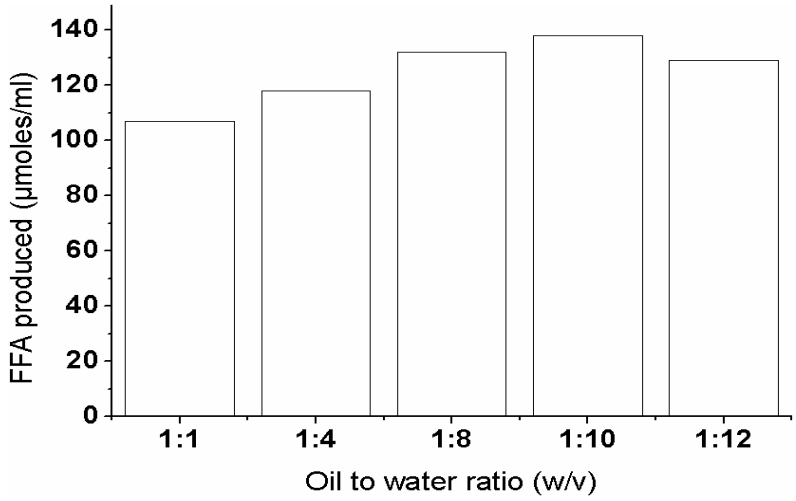

Figure 4: Effect of Oil to Water Ratio (w/v) on Activity of CRL during Hydrolysis of Tuna Fish Oil {Experimental Conditions: 1 g Tuna Oil, 1 ml Iso-Octane, pH 6.5, Temperature 35°C, Agitation Speed 800 rpm, 40 mg Enzyme, Reaction Time 1 h}

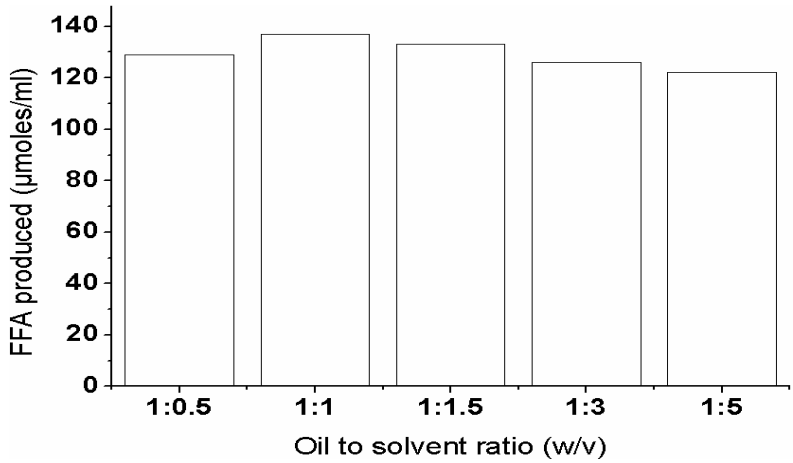

Figure 5: Effect of Oil to Solvent Ratio (w/v) on Activity of CRL during Hydrolysis of Tuna Fish Oil {Experimental Conditions: 1 g Tuna Oil, 10 ml Water, pH 6.5, Temperature 35°C, Agitation Speed 800 rpm, 40 mg Enzyme, Reaction Time 1 h}

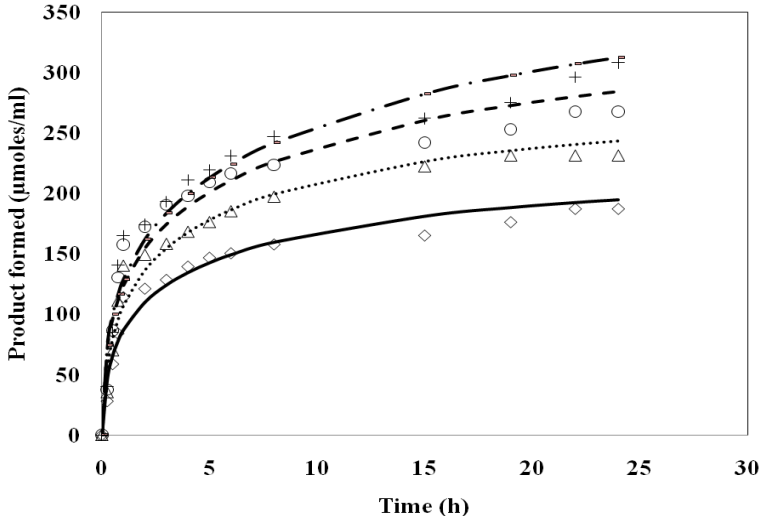

Figure 6: Comparison of Experimental (1→◊, 2→Δ, 3→○, 4→+) and Predicted (1→ ——, 2→····, 3→ — — —, 4→ — · — · —) Data of Product Formation at Initial Substrate Concentration S_{01}, S_{02}, S_{03} and S_{04} Using Prazeres Model

{Experimental Conditions: 1 g Tuna Oil, 10 ml Water, Isooctane in Suitable Amount, pH 6.5, Temperature 35°C, Agitation Speed 800 rpm, 40 mg Enzyme, Batch Volume 12 ml}

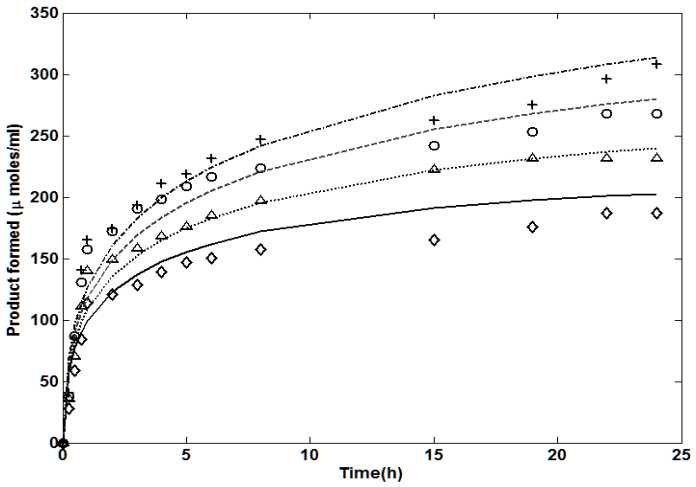

Figure 7: Comparison of Experimental (1→◊, 2→Δ, 3→○, 4→+) and Predicted (1→ ——, 2→····, 3→ — — —, 4→ — · — · —) Data of Product Formation after Combining Four Data sets S_{01}, S_{02}, S_{03} and S_{04} Together Using Prazeres Model {Experimental Conditions: 1 g Tuna Oil, 10 ml Water, Isooctane in Suitable Amount, pH 6.5,

Temperature 35°C, Agitation Speed 800 rpm, 40 mg Enzyme, Batch Volume 12 ml}