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# SALMON AND HERRING FISH OIL HYDROLYSIS WITH IMMOBILIZED *CANDIDA ANTARCTICA* LIPASE-B

***Aditi Sharma[1], Ajay K. Dalai[2] & Satyendra P. Chaurasia[3]***

*[1]Department of Chemical Engineering, Banasthali University, Tonk, Rajasthan, India*

*[2]Department of Chemical & Biological Engineering, College of Engineering,*

*University of Saskatchewan, Saskatoon, Canada*

*[1,3]Department of Chemical Engineering, Malaviya National Institute of Technology, Jaipur, Rajasthan, India*

## *ABSTRACT*

*The present paper investigates the comparative hydrolysis of salmon and herring fish oils with immobilized Candida antarctica lipase-B (CAL-B) to release free fatty acids. The physiochemical characterization of two fish oils has been studied using High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), $^1$H Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared Spectroscopy to compare their chemical composition. The kinetic parameters have been determined using short term and long term hydrolysis to fit experimental data assuming no inhibition and product inhibition respectively in form of different kinetic models. The reaction inhibition due to formation of products was found to be negligible for hydrolysis of fish oils with immobilized CAL-B lipase. The activation energy was investigated and results indicate that the reaction was kinetically controlled and effect of diffusion resistances was negligible. The activity retention for immobilized CAL-B after five cycles of repeated use for hydrolysis of salmon and herring fish oils was found to be 22.3 and 18.6 % respectively.*

***KEYWORDS:*** *Activation Energy, Free Fatty Acids, Kinetics, Non-Selective Hydrolysis, Reusability Study*

***Article History***

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## INTRODUCTION

In the world of depleting resources, the betterment of human health and nutrition is a major challenge for researchers. Therefore, the need is to focus on the synthesis of nutritionally valuable components which not only prevent the onset of disease but also provide the means of treatment. Such nutritionally important food ingredients include various long chain polyunsaturated fatty acids (PUFAs) in triglycerides form which are easy to consume in diet (Grimsgaard et al., 1998; Shahidi & Hamam, 2006 and Uauy & Dangour, 2006). Some important omega-3 PUFAs include alpha linolenic acid (ALA, $C_{22:6n3}$); arachidonic acid (AA, $C_{22:6n3}$); eicosapentaenoic acid (EPA, $C_{20:5n3}$); docosapentaenoic acid (DPA, $C_{22:5n3}$) and docosahexaenoic acid (DHA, $C_{22:6n3}$). Fish oils such as tuna, salmon, cod liver, herring etc., are rich source of these omega-3 fatty acids but also contain other saturated fatty acids (Karlovec et al., 2012; Liou et al., 2007 and Rupp et al., 2004). The extraction of omega-3 PUFAs from fish oil have been studied by researchers with various methods through simultaneous hydrolysis and esterification (Espinosa et al., 2007; Fraser et al., 2007 and Guil-Guerrero et al., 2007).

The application of lipases is reported as a promising method because they are highly specific in the reactions involving cleavage and formation of ester bonds (Joseph et al., 2008 and Kourist et al., 2010). Lipases are regio-selective, stereo-specific or chemo-selective in nature depending upon the type and position of substrate. Most known lipases are reported as 1,3-position specific and very few are able to identify sn-2 position in their action on fats and lipids. Lipases work optimally at the water/alcohol-substrate interface for hydrolysis/esterification reactions. The activity of lipase increases many fold when used with the solvent system to provide the interfacial activation. In the presence of solvent, the amphipathic lid structure of lipase opens and leads to the formation of larger hydrophobic surface area for the binding of the substrate at the interface (Kapoor & Gupta, 2012 and Knezevic et al., 2004).

The present study aims to compare chemical composition, hydrolysis kinetics and activation energy for salmon and herring fish oils. The reusability of immobilized CAL-B was examined for these two fish oils to investigate the effect of substrate on percentage activity retention of lipase after repeated use of the immobilized enzyme.

## MATERIALS AND METHODS

### Fish Oil and Lipase

Salmon (blend; Lot: 029/11) and Herring (veterinary grade; Lot: 802-6980) fish oils were purchased from Jedwards International, Inc., Quincy, MA 02169, USA in a packing of 3 liters each. Lipase-B *Candida antarctica* (CAL-B) immobilized on immobead-150 (0.15-0.3 mm; <10% loss on drying), recombinant from yeast (activity >2000 U/mg) was purchased from Sigma-Aldrich, Saskatoon, Canada.

### Chemicals

Analytical grade chemicals such as methanol ($CH_3OH$), potassium hydroxide (KOH), oxalic acid, phenolphthalein indicator, anhydrous sodium sulfate, sodium chloride, hydrochloric acid, $BF_3$/methanol (~1.3 M) 10% solution were purchased from Sigma-Aldrich, Canada and used in the experimental work. Potassium di-basic & mono-basic were used for preparing phosphate buffer of pH 7. All the chemicals used were of AR grade. Industrial grade $N_2$ gas cylinder was supplied by Pyrex for maintaining inert atmosphere during the reaction. Supelco 37 component standard FAME mix 10 mg/ml in methylene chloride ($CH_2Cl_2$) was purchased from Sigma-Aldrich, Saskatoon, Canada for calibration of gas chromatography instrument.

### Experimental Procedure

The kinetics for the hydrolysis of both fish oils (salmon and herring) were studied at the optimized conditions determined with tuna fish oil such as 1:1 (w/w) solvent to oil ratio with iso-octane, 3:1 (w/w) water to oil ratio, and 500 U (0.133 g) enzyme at constant pH 7.0, temperature of 35 °C and 400 rpm (Sharma et al., 2013[a]). For studying the kinetics of hydrolysis reaction, fish oils were dissolved in solvent and immobilized CAL-B lipase was added along with the distilled water to prepare the reaction mixture in a 125 ml Erlenmeyer flask. The percentage hydrolysis (% H) of fish oils were studied at constant speed of agitation (400 rpm in orbital shaker supplied by VWR, Canada; model 1570) using iso-octane as a solvent in biphasic solvent system up to 60 h. The kinetics of fish oil hydrolysis have been studied using conventional Michaelis-Menten model (Sharma et al., 2013[b]) for short term hydrolysis as well as with a nonlinear product inhibition model proposed by Prazeres et al., 1993 for long term hydrolysis at four different substrate concentrations. The study of activation energy (E) for immobilized CAL-B was also performed at various temperatures with salmon and herring fish oils using Arrhenius activation energy model.

## Characterization and Analysis

The physiochemical characterization of salmon and herring fish oils were conducted. The glycerides composition of fish oils have been evaluated by diluting oil sample with THF (tetrahydrofuran) solvent in a ratio of 1:19 (50 µl sample and 950 µl THF solvent). The fish oil samples were analyzed with HPLC (Agilent 1100 Series model) using refractive index detector (model G1362A) with operating conditions 35 °C, 3.7 MPa for 30 min. at a 1 ml/min. flow rate of mobile phase. A Phenogel column (model G1316A; dimensions: 100 Å, 300 x 7.8 mm 5 µm) protected with guard column and equipped with Chemstation for LC3D was used for HPLC analysis to determine glyceride composition. An Agilent Gas Chromatography system (model 7890A) has been used, equipped with flame ionization detector (FID, 260 °C) and capillary column DB-23 (dimensions: 60 m length, 0.25 mm ID, 0.25 µm film) for analysis of fatty acids in methyl ester form (FAMEs). Throughout the experimentation, the GC was operated at constant conditions (Carrier: hydrogen gas with flow rate 20 cm/min & 23.148 psi pressure; Oven temperature: 140 to 240 °C at 4 °C/min. and Injection volume: 1 µl sample, 260 °C & split: 20:1).The calibration of both the analytical instruments has been done with authentic standards.

The FT-IR spectra for fish oils was obtained by KBr pelleting method using Perkin Elmer, FT-IR spectrum GX in the IR range of 400–4000 cm-1 (Naik, S., 2010[a,b]). For the analysis of liquid sample in the same FT-IR, two pellets of KBr were prepared and a drop of oil was placed between the two pellets. Then, FT-IR spectrum was recorded with the method normally used for solid samples. Proton ($^1$H) Nuclear Magnetic Resonance (NMR) of fish oils was performed with Bruker UltraShield™ 500 MHz NMR. The samples of fish oils were dissolved in Cadmium chloride ($CDCl_3$) solvent within a thin walled NMR tubes in an approximate sample to solvent ratio of (1:3). The results for $^1$H spectra were recorded at ambient temperature in parts per million (PPM) scale and analyzed with Spinworks software. NMR analysis for fish oils was conducted to study the differences in structure and composition of molecules. Standard AOCS and ASTM test method were used for estimating the acid value (AOCS, 1997 and ASTM, 2004) of collected samples at different reaction time to determine amount of free fatty acids (FFAs) formed. The percentage hydrolysis (%H) was calculated with equation (1) as given below:

$$\%\text{Hydrolysis} = \frac{[(\text{Acid value at time t}) - (\text{Acid value at time t} = 0)]}{\text{Saponification Value}} \text{ X } 100 \quad (1)$$

The activity of lipase is expressed as µmoles of FFAs formed per ml of reaction mixture in reaction time of one hour. The activity retention is a measure of the ability of immobilized CAL-B remaining to hydrolyze triglycerides in the fish oil after repeated uses.

It is defined as the percentage conversion of triglycerides achieved after reuse of immobilized CAL-B with respect to percentage conversion achieved after first round of its use. According to equation (2), the activity retention is defined as ratio's of percentage conversion. All the results were repeated in 3 sets of experiments for performing the reproducibility analysis and observed to be varied in the range of <± 2 %.

$$\%\text{Activity retention for } (n+1)\text{cycle} = \frac{\%\text{Conversion after } (n+1)\text{time use of immobilized CAL-}}{\%\text{Conversion after one time use of immobilized CAL-B}} \quad (2)$$

## RESULTS AND DISCUSSIONS

### Characterization of Fish Oil

The salmon fish oil has characteristics i.e. color: 5 (Gardner units); acid value: 0.1 (mg KOH/g oil); free fatty acids: 0.05%; saponification value: 181 (mg KOH/g oil); iodine value: 197 (g $I_2$/100 g oil); peroxide value: 2.3 (meq/kg oil); anisidine value: 10.7; refractive index at 20°C: 1.47 and total omega-3 fatty acids: 38.1 (%area).

Similarly, the characteristics of herring fish oil were as color: 5 (Gardner units); acid value: 1.6 (mg KOH/g oil); free fatty acids: 0.8%; saponification value: 179 (mg KOH/g oil); iodine value: 102 (g $I_2$/100 g oil); peroxide value: 3.01 (meq/kg oil); anisidine value: 11.3; refractive index at 20 °C: 1.32 and total omega-3 fatty acids: 37.5 (%area).

According to the HPLC analysis, a total of 98.9 and 96.2 wt% triglycerides with 1.1 and 3.8 wt% ester content were found in salmon and herring fish oil samples respectively. The fatty acid profile of fish oils was analyzed using base catalyzed hydrolysis to release free fatty acids (FFAs). These FFAs were further converted into fatty acids methyl esters (FAMEs) using AOCS standard method (AOCS, 1997). The prepared FAMEs were diluted with methylene chloride solvent and directly injected into GC at specified conditions.

The presence of various fatty acid groups ranging from $C_{14:0}$ to $C_{24:0}$ including both saturated and polyunsaturated fatty acids have been observed in fish oils after GC analysis and mentioned in Table 1. Myristic acid ($C_{14:0}$), Palmitic acid ($C_{16:0}$), Palmitoleic acid ($C_{16:1}$), Oleic acid ($C_{18:1n9c}$), cis-5, 8, 11, 14, 17-Eicosapentaenoic acid ($C_{20:5n3}$), Nervonic acid ($C_{24:1}$) and cis-4, 7, 10, 13, 16, 19-Docosahexaenoic acid ($C_{22:6n3}$) were found in considerable amount compared to other fatty acids in fish oils.

**Table 1: Fatty Acid Composition of Salmon and Herring Fish Oils with Gas Chromatography**

| Type of Fatty Acids | Salmon Oil (wt%) | Herring Oil (wt%) |
|---|---|---|
| Myristic acid ($C_{14:0}$) | 6.7 | 10.4 |
| Palmitic acid ($C_{16:0}$) | 30.4 | 28.4 |
| Palmitoleic acid ($C_{16:1}$) | 7.1 | 9.5 |
| Oleic acid ($C_{18:1n9c}$) | 4.9 | - |
| cis-5, 8, 11, 14, 17-Eicosapentaenoic acid ($C_{20:5n3}$) | 3.9 | 5.9 |
| Nervonic acid ($C_{24:1}$) | 11.7 | 7.5 |
| cis-4, 7, 10, 13, 16, 19-Docosahexaenoic acid ($C_{22:6n3}$) | 25.1 | 20.9 |
| **Others** | **10.1** | **17.4** |

The Fourier Transform-Infrared Spectroscopy (FT-IR) has been carried out to identify the major functional groups in both fish oils as shown in Figure 1. The studies conducted by Guillen and Cabo, 1998 and Richard, 1999 for the IR spectra of fatty acids and esters in oils and fats have been referred to identify major characteristic FT-IR bands in the present case. The FT-IR analysis of the fish oils indicates that although the spectra for all the fish oils appear to be similar, they are different in the intensity of peaks because of variation in the nature, composition and origin of fish oil samples under study (Zhang, 2009).

According to Figure 1, peaks from 3032 to 2852 $cm^{-1}$ (A to D) indicate the presence of multiple double bonds in the fish oils and their height ratio may be used as a marker for the analysis of ester of EPA and DHA specifically. Therefore, the overlapped FT-IR spectrums for salmon and herring fish oils indicate that the ratio of band heights at 3032 and 2921 $cm^{-1}$ is higher for salmon fish oil than herring fish oil.

This qualitatively analysis indicates that salmon fish oil is richer in total EPA and DHA esters of triglycerides. The carbonyl band spectrum at 1743 $cm^{-1}$ indicate that these fish oils contain EPA, DHA in triglyceride form whereas band at 1653 $cm^{-1}$ is indicative for the presence of other unsaturated fatty acid groups in the ester form.

The qualitative characterization of fish oils rich in omega-3 fatty acids have been performed with ($^1$H) proton NMR to understand their molecular composition and structure. The $^1$H NMR chromatograms of salmon and herring fish oils are shown in Figure 2. These $^1$H proton NMR chromatograms have been compared with the fish oil chromatogram developed by PNA, process NMR associates to identify all the peaks present in fish oil chromatograms.

According to this, the height of peak F which corresponds to the presence of DHA ($C_{22:6}$) was found to be greater for salmon fish oil when compared with respective peak in herring fish oils. The $^1$H NMR of lipid molecules is very rapid and quantitative in nature but it generates the spectrum with reduced resolution (Sacchi et al., 2006).

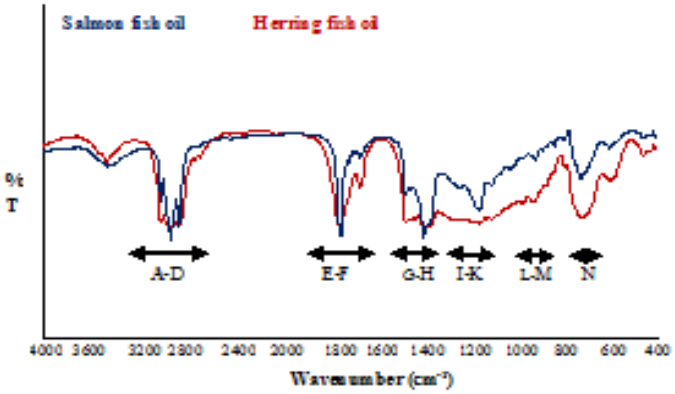


**Figure 1: Overlapped FT-IR Spectrum for Salmon and Herring Fish Oils to Compare the Height and Intensityof Peaks [Where Assignments to Peaks are A = =C-H (CIS, Stretching); B = -C-H ($CH_3$ Groups, Asym Stretching); C = -C-H ($CH_2$ Groups, Asym Stretching); D = -C-H ($CH_2$ and $CH_3$ Groups, Sym Stretching); E = -C=O (Ester Stretching); F = -C=C- (CIS Double Bond Stretching); G = -C-H ($CH_2$ Scissoring Bending); H = -C-H ($CH_3$ Sys Bending); I = -C-O, $CH_2$ (Stretching, Bending); J = -C-O (Stretching); K = -C-O (Stretching); L = =C-H (Trans, Bending Out of Plane); M = =C-H (CIS, Bending Out of Plane) and N = -$(CH_2)_n$=C-H CIS (Rocking, Bending Out)]**

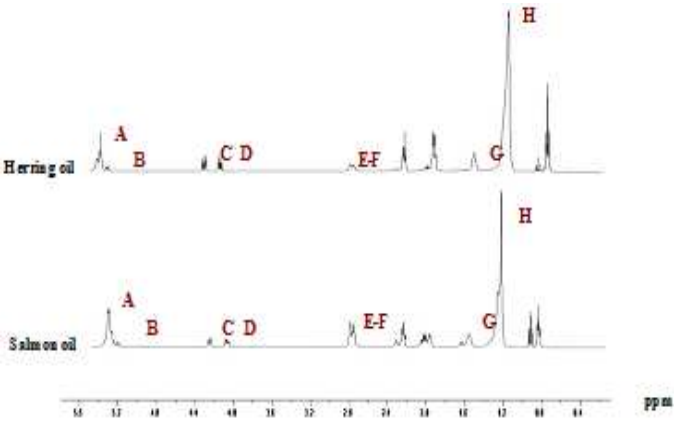


**Figure 2: Proton ($^1$H) NMR Spectra for Salmon and Herring Fish Oils [Where Assignment to Peaks are A = Unsaturated Fatty Acid Proton; B = Glycerol $C_2$ Proton; C-D = Glycerol $C_{1,3}$ Protons; E = Polyunsaturated Fatty Acid Proton; F = $C_{22:6}$ Fatty Acid; G = All Fatty Acid except $C_{22:6}$; H = All Fatty Acid except $C_{20:5}$ and $C_{22:6}$]**

## Determination of Kinetic Parameters

For the hydrolysis of oil's triglycerides in the presence of excess water with lipases, a three step mechanism have been suggested by various researchers in literature (Alenezi et al., 2009) which can be compared with the overall mechanism of enzymatic hydrolysis (Al-Zuhair et al., 2004 and Tsai & Chang 1993) as given below. The fish oil triglycerides (TG) act as a substrate (S) for enzyme (E) action to liberate free fatty acids as product (P) with the formation of glycerol (G) after complete hydrolysis. Moreover, the immobilized lipase enzymes can be recovered from enzyme-substrate complex (ES) for its recycling after every run (Cheong et al., 2012).

| | |
|---|---|
| Step-I | $TG + E \leftrightarrow DG + P_1$ |
| Step-II | $DG + E \leftrightarrow MG + P_2$ |
| Step-III | $MG + E \leftrightarrow E + G + P_3$ |
| Overall Step | $S + E \leftrightarrow ES \rightarrow E + P$ |

According to Michaelis-Menten, the rate of reaction ($r_M$) was determined using equation (3) assuming pseudo first order, steady state kinetic model without substrate or product inhibition.

$$r_M = \frac{V_{max}.[S]}{K_M + [S]} \tag{3}$$

Where, $r_M$ = initial rate of reaction (µmoles of FFAs formed/ml.min); $V_{max}$ = maximum rate of reaction (µmoles of FFAs formed/ml.min) and $K_M$ = Michaelis-Menten rate constant (µmoles of FFAs formed/ml). A plot between $1/r_M$ vs. 1/[S], known as a Line weaver-Burk plot has been drawn to find out the values of kinetic parameters such as $V_{max}$ and $K_M$, and represented by equation (4). According to Lineweaver–Burk plot, $K_M/V_{max}$ corresponds to the slope and $1/V_{max}$ corresponds to the intercept of the straight line.

$$\frac{1}{r_M} = \frac{K_M}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \tag{4}$$

The initial rate of reaction ($r_M$) for hydrolysis of fish oils was determined by plotting free fatty acids formed with respect to time for short intervals of reaction up to 1 h at four different substrate concentration shown in Figures 3 and 4. The plots have been drawn for four different substrate concentrations [S] such as 153.6, 292.3 537.7 and 744.3 total µmoles of FFAs presents in oil per ml of reaction mixture. Therefore, the values obtained for initial rate of reaction from Figures 3 and 4 have been presented in Table 2 for both fish oils.

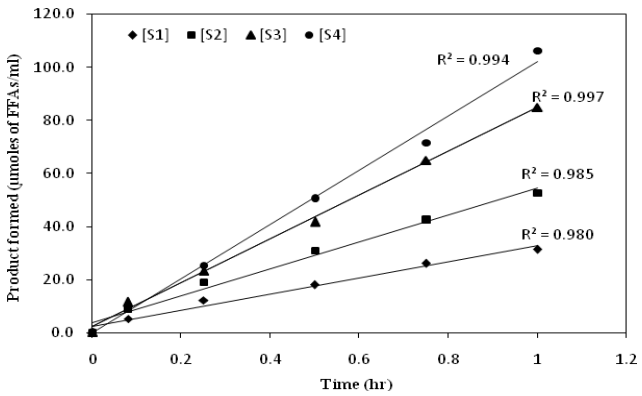


**Figure 3: Rate of Reaction (µmoles of FFAs Formed per ml of Reaction Mixture) with Time (hr), for Hydrolysis of Salmon Fish Oil Using Immobilized CAL-B**

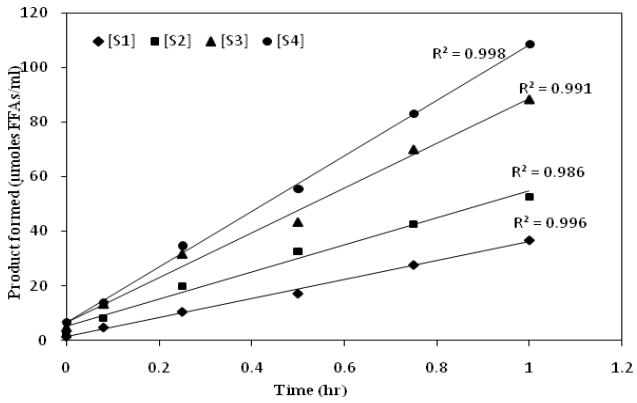


**Figure 4: Rate of Reaction (µmoles of FFAs Formed per ml of Reaction Mixture) with Time (hr), for Hydrolysis of Herring Fish Oil Using Immobilized CAL-B**

**Table 2: Initial Reaction Velocity Obtained at Different Substrate Concentration after Short Term Kinetic Study for Hydrolysis of Fish Oils with Immobilized CAL-B Using Michaelis-Menten Model**

| Substrate Concentration (µmoles FFAs/ml) | Initial Velocity, $r_M$ (µmoles FFAs/min.ml) | | 1/[S] (µmoles FFAs/ml)$^{-1}$ | 1/[$r_M$] (µmoles FFAs/min.ml)$^{-1}$ | |
|---|---|---|---|---|---|
| | Salmon Oil | Herring Oil | | Salmon Oil | Herring Oil |
| [$S_1$] = 153.6 | 30.4 | 34.71 | 0.0065 | 0.033 | 0.029 |
| [$S_2$] = 293.3 | 50.8 | 49.8 | 0.0034 | 0.019 | 0.02 |
| [$S_3$] = 537.7 | 82.6 | 82.1 | 0.0019 | 0.012 | 0.012 |
| [$S_4$] = 744.3 | 102.3 | 101.5 | 0.0013 | 0.011 | 0.009 |

Further, reciprocal of initial rate of reaction (1/$r_M$) was drawn against reciprocal of substrate concentration (1/[S]) to determine Michaelis-Menten kinetic parameters (see Figure 5). The magnitude of maximum rate of reaction ($V_{max}$) was calculated from the intercept whereas Michaelis-Menten kinetic constants ($K_M$) were obtained from the slope of the Figure 5 using equation (4).

The results from the Michaelis-Menten kinetic model have been summarized in Table 3. The kinetic parameters $V_{max}$ and $K_M$ have been found as 333.3 (µmoles FFAs/hr.ml) and 1499.9 (µmoles FFAs/ml) respectively for salmon fish oil, whereas 200 (µmoles FFAs/hr.ml) and 730.4 (µmoles FFAs/ml) for herring fish oil respectively. For the both the fish oils, the magnitude of maximum rate of reaction ($V_{max}$) was found comparatively lower than the magnitude of kinetic constant ($K_M$). This indicates that the hydrolysis of fish oils with immobilized CAL-B is slow but lipase is highly specific for hydrolyzing triglycerides in fish oils.

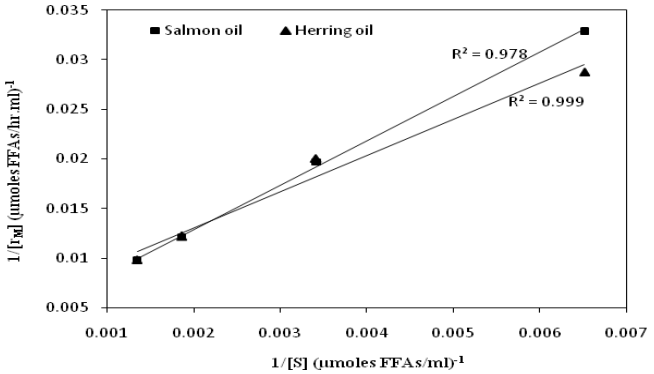


**Figure 5: Lineweaver-Burk Plot for Hydrolysis of Fish Oils Using Immobilized CAL-B**

**Table 3: Summary of Results Obtained after Kinetic Study for Hydrolysis of Fish Oils with Immobilized CAL-B Using Michaelis-Menten Kinetic Model**

| Fish Oil | Maximum Velocity, $V_{max}$ | | Michaelis-Menten Constant, $K_M$ |
|---|---|---|---|
| | (µmoles FFAs/hr.ml) | (µmoles FFAs/min.ml) | (µmoles FFAs/ml) |
| Salmon oil | 333.3 | 4.5 | 1499.9 |
| Herring oil | 200.0 | 3.3 | 730.4 |

According to a kinetic model proposed by Prazeres et al., 1993, the rate of reaction ($r_P$) was determined by equation (5) assuming a multi order nonlinear product inhibition with a maximum three molecules of product binding to the enzyme.

$$r_p = \frac{K_2 E_t S}{K_M(1 + K_{i1}P + K_{i2}P^2 + K_{i3}P^3) + S} \quad (5)$$

Where, $r_P$ = rate of reaction (µmoles of FFAs formed/ml.min); $E_t$ = amount of immobilized CAL-B used in reaction (mg); S = substrate concentration (total FFAs present in oils, µmoles of FFAs formed/ml); P = product formed (free fatty acids/ml); $K_M = 1/K_1$ ($K_1$ is rate constant for first stage hydrolysis; µmoles of FFAs formed/ml); $K_{i1}$ = rate constant for product inhibition after first stage product formation (µmoles of FFAs formed/ml); $K_{i2}$ = rate constant for product inhibition after second stage product formation (µmoles of FFAs formed/ml) and $K_{i3}$ = rate constant for product inhibition after third stage product formation (µmoles of FFAs formed/ml).

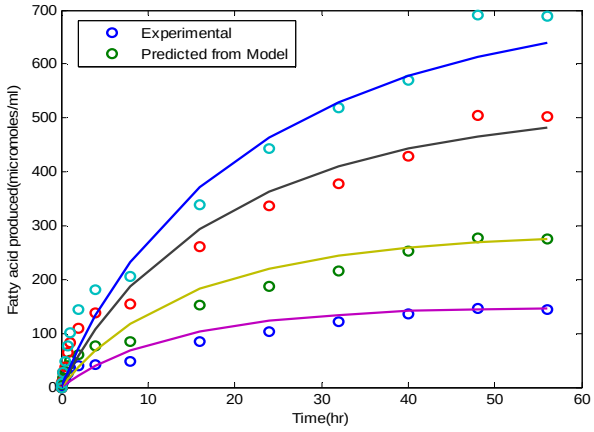


**Figure 6: Curve Fitting of Experimental Data with Predicted Data for Hydrolysis of Salmon Fish Oil Using Immobilized CAL-B [Where Marker 'o' Represents Experimental Data Set with Color Indicating Different Substrate Concentration Such as Blue 'o' = [$S_1$]; Green 'o' = [$S_2$]; Red 'o'= [$S_3$] & Teel 'o'= [$S_4$] and Marker '-' Represents Simulated Data Set with Color Indicating Different Substrate Concentration Such as Purple '-' = [$S_1$]; Yellow '-' = [$S_2$]; Brown '-'= [$S_3$] & Blue '-'= [$S_4$]]**

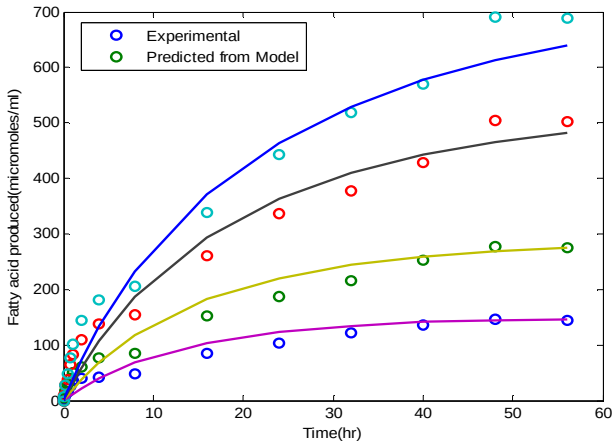


**Figure 7: Curve Fitting of Experimental Data with Predicted Data for Hydrolysis of Herring Fish Oil Using Immobilized CAL-B [Where Marker 'o' Represents Experimental Data Set with Color Indicating Different Substrate Concentration Such as Blue 'o' = [$S_1$]; Green 'o' =[$S_2$]; Red 'o'=[$S_3$] & Teel 'o'= [$S_4$] and Marker '-' Represents Simulated Data Set with Color Indicating Different Substrate Concentration Such as Purple '-' = [$S_1$]; Yellow '-' = [$S_2$]; Brown '-'= [$S_3$] & Blue '-'= [$S_4$]]**

The kinetic parameters such as $K_M$, $k_2$, $K_{i1}$, $K_{i2}$ and $K_{i3}$ along with $R^2$ (regression coefficient) and Root Mean Square Error (RMSE) have been calculated from this model using the applications of MATLAB® software to solve the nonlinear rate equation (5). The experimental data for product formation with respect to time (up to 56 h) at four different initial substrate concentration was fitted to the simulated data obtained by using a nonlinear least squares regression analysis MATLAB® program. The simultaneous fitting of rate equation for the experimental data and the predicted data generated through a MATLAB® program, leads to the development of plots as shown in Figures 6 and 7 for salmon and herring fish oils respectively at substrate concentrations [S] such as 153.6, 292.3 537.7 and 744.3 total µmoles of FFAs initially present in oil per ml of reaction mixture.

The results obtained for set of kinetic parameters using kinetic model suggested by Prazeres et al., 1993 have been summarized in Table 4, indicating that as the value of regression coefficient ($R^2$) is 0.98 for both salmon and herring fish oils, it corresponds to a good fitting of experimental data with predicted data. The results in Table 4 also indicate that the inhibition of hydrolysis reaction due to the formation of products is found to be almost negligible in all the stages because the magnitude of rate constants for product inhibition ($K_{i1}$ and $K_{i2}$) in first two stages only were obtained in the order of $10^{-3}$ and $10^{-14}$ for both fish oils. Moreover, the value of Michaelis-Menten rate constant ($K_M$) through this kinetic model also was found to be 940.9 and 1000 (µmoles FFAs formed/ml) for salmon and herring fish oils respectively, indicating a very good affinity of immobilized CAL-B lipase for hydrolyzing fish oils.

**Table 4: Summary of Results Obtained from Fitting of Experimental Data with Predicted Data Generated Using Kinetic Model Proposed by Prazeres for Long Term Hydrolysis**

| Kinetic Constants | Salmon Oil | Herring Oil |
|---|---|---|
| Michaelis-Mentenrate constant, $K_M$ (µmoles FFAs formed/ml) | 940.9 | 1000 |
| Rate constant for first stage hydrolysis, $K_1$ (µmoles FFAs formed/ml) | 0.0012 | 0.001 |
| Rate constant for second stage hydrolysis, $K_2$ (µmoles FFAs formed/ml) | 28.5 | 23.4 |
| Rate constant due to first stage product inhibition, $K_{i1}$ (µmoles FFAs formed/ml) | 0.004 | 0.0028 |
| Rate constant due to second stage product inhibition, $K_{i2}$ | 2.34E-14 | 2.34E-14 |

| | | |
|---|---|---|
| (µmoles FFAs formed/ml) | | |
| Regression coefficient ($R^2$) | 0.98 | 0.98 |
| Root mean square error (RMSE) | 233.1 | 223.1 |

## Determination of Activation Energy

The activation energy (E) for the hydrolysis reaction of fish oils with immobilized CAL-B enzyme has been determined by studying Arrhenius plot as given below in equation (6).

$$k = A_0 \cdot e^{-E/RT} \quad (6)$$

Where, k = rate constant; $A_o$ = pre-exponential factor; E = activation energy (KJ/mol); R = gas constant and T = temperature (Kelvin).The linearized form of Arrhenius law is given in equation (7).

$$\ln k = \ln A_0 + \left(-\frac{E}{RT}\right) \quad (7)$$

The effect of change in temperature for hydrolysis of salmon and herring fish oils with immobilized CAL-B has been studied at three different temperatures such as 25, 30 and 35 ºC to determine the rate constant (k) respectively. Further, Arrhenius plot (see Figure 8) has been drawn between the rate constant and temperature according to equation (7) using values given in Table 5 to calculate the activation energy. The Arrhenius plot indicates that the slope of the linear equation is equivalent to the value of (-E/R) and intercept gives the pre-exponential factor ($A_0$). Hence, the activation energy (E) equivalent to 16.1 ($E_S$) and 32.1 ($E_H$) KJ/mol were calculated from the Figure 8 for salmon and herring fish oils respectively. The magnitude of activation energy (E) is a mathematical measure to represent the ease of reaction in presence of catalyst. In the present case, the lower magnitude of activation energy for salmon fish oil ($E_S < E_H$) indicates that the hydrolysis of salmon fish oil is comparatively more favorable with immobilized CAL-B than herring fish oil because less energy is required to breakdown the reactants and to form the products. In the other words, the molecules of salmon fish requires less amount of initial energy to proceed the hydrolysis in presence of immobilized CAL-B and therefore, they impose lesser energy barrier to the forward reaction.

**Table 5: Effects of Different Temperature on Rate Constant, k (µmoles of FFAs per ml) with Immobilized CAL-B Enzyme**

| Tempera-tureT (K) | Rate Constant k (µmoles of FFAs per ml) | | 1/T (K-1) | ln k | |
|---|---|---|---|---|---|
| | Salmon Oil | Herring Oil | | Salmon Oil | Herring Oil |
| 298 | 0.064 | 0.031 | 0.0034 | -2.8 | -3.5 |
| 303 | 0.072 | 0.063 | 0.0033 | -2.6 | -3.2 |
| 308 | 0.086 | 0.089 | 0.00325 | -2.5 | -2.7 |

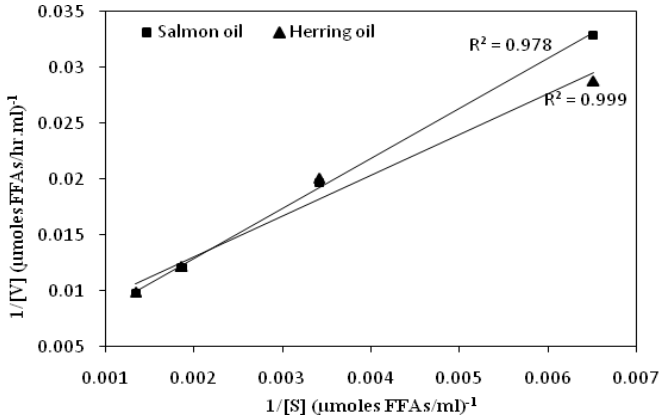


**Figure 8: Arrhenius Plot for Studying Activation Energy with Respect to Temperature**

## Reusability Study of Immobilized CAL-B

The cost effectiveness of a process is an important parameter for enzymatic processes because the cost of enzyme itself contributes to a large extent towards overall processing cost. Therefore, the ability of an enzyme to be reused after several cycles is beneficial for reducing the initial cost of an enzymatic process, even at comparatively lower activity. The reusability study for immobilized CAL-B enzyme was performed up to five cycles at optimized reaction conditions with 1 g of fish oils and 0.133 g of lipase. The percentage conversion of fish oil triglycerides and percentage activity retention of immobilized CAL-B were calculated as shown in Table 6. This study indicates that immobilized CAL-B retains 48.5 and 45.6 of % activity even after third cycle of reuse whereas after the fifth cycle, the activity retention (see Figure 9) falls sharply to 22.6 % and 18.5 % for salmon and herring fish oils respectively. The appreciable drop in activity retention of immobilized CAL-B after third cycle of repeated use was reported in the study is mainly because of the reduction in the surface area available for binding of substrate to the lipase in terms of higher blockage of active sites.

**Table 6: Composition of Salmon and Herring Fish Oils after Hydrolysis to Study the Results of Reusability Experiments with CAL-B Enzyme**

| Runs | % Conversion of Triglycerides | | % Activity Retention of Immobilized CAL-B | |
|---|---|---|---|---|
| | Salmon Oil | Herring Oil | Salmon Oil | Herring Oil |
| 1 | 95.4 | 93.3 | 100 | 100 |
| 2 | 59.9 | 55.2 | 62.8 | 59.2 |
| 3 | 46.3 | 42.5 | 48.5 | 45.6 |
| 4 | 27.1 | 19.4 | 28.4 | 20.7 |
| 5 | 21.6 | 17.3 | 22.6 | 18.5 |

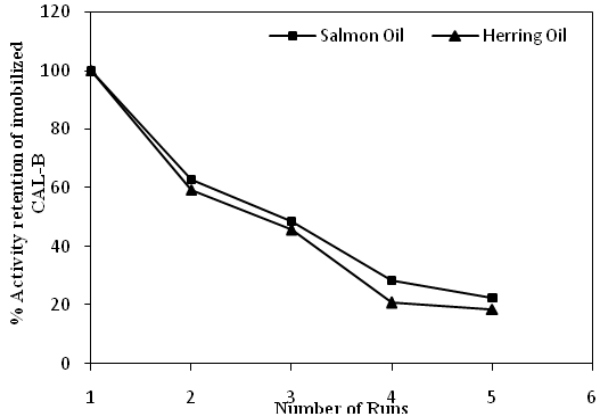


**Figure 9: Reusability Study of Immobilized CAL-B for % Triglycerides Remaining after Hydrolysis of Fish Oils**

## CONCLUSIONS

The physiochemical characterization of salmon and herring fish oils using quantitative methods such as HPLC, GC and AOCS standard tests; and qualitative methods such as FT-IR and NMR, were found efficient for evaluating the structural and chemical composition of oils. The Michaelis-Menten constant for salmon and herring fish oils were found to be 1499 & 730 µmoles FFAs/ml respectively for short term Michaelis-Menten kinetic model and 940 and 1000 µmoles FFAs formed per ml of reaction mixture with long term Prazeres kinetic model.

The hydrolysis of both fish oils with immobilized CAL-B was found to be satisfactory in terms of Michaelis-Menten constants ($K_M$) for both kinetic models. The lower value of activation energy for salmon oil (16.1 KJ/mol) than herring oil (32.1 KJ/mol) corresponds to ease of salmon oil hydrolysis with immobilized CAL-B lipase. The reusability study indicates that activity retention for immobilized CAL-B after third cycle of repeated use was 48.5 % and 45.6 % with salmon and herring fish oils respectively. The present study concluded that the hydrolysis of the both fish oils with immobilized CAL-B, were free from any mass transfer limitations.

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