Asian Journal of Chemistry; Vol. 24, No. 6 (2012), 2679-2684

ASIAN JOURNAL OF CHEMISTRY

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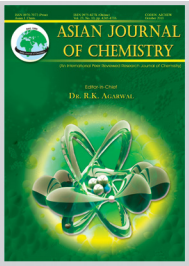

Enzymatic Esterification of River Catfish (*Mystus nemurus*) Fatty Acids to Enrich ω-3 Polyunsaturated Fatty Acids

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(*Received*: 17 June 2011; *Accepted*: 17 January 2012) AJC-10972

The health benefits of ω-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexanoic acid (DHA, 22:6 n-3) are widely recognized and fish oil is an important source of polyunsaturated fatty acids. In this work, the effect of reaction conditions on esterification of river catfish (*M. nemurus*) free fatty acid (FFA) with oleyl alcohol, catalyzed by immobilized *R. meihei* lipase in order to enrich polyunsaturated fatty acids was studied. The fatty acid compositions of the reaction products were analyzed using gas chromatography on conversion to fatty acid methyl ester (FAME). The highest ratio of especially eicosapentaenoic acid and docosahexanoic acid was observed after 4 h and hexane (log P = 3.5) was found to be the best solvent. The highest especially eicosapentaenoic acid and docosahexanoic acid enrichment was achieved when the molar ratio of oleyl alcohol and free fatty acid is 2:1. In conclusion, river catfish-free fatty acid was successfully enriched by enzymatic esterification.

Key Words: PUFAs, Fish oil, Oleyl alcohol, Immobilized lipase, Enrichment, Docosahexaenoic acid, River catfish.

INTRODUCTION

Fish oil is an important source of ω-3 polyunsaturated fatty acids (PUFAs) in particular, the eicosapentaenoic acid (EPA) and the docosahexanoic acid (DHA). The ω-3 PUFAs are beneficial to the human health for example on bone formation and metabolism and in the prevention of cardiovascular disease[1,2]. The ω-3 PUFA's was also shown to be useful in the treatment of mental disorders like schizophrenia and in the combat against cancer[3]. As the human body cannot synthesized its own PUFAs[4], dietary intake of these PUFAs is essential.

Most fish oil supplements contain 18 % of EPA and 12 % of DHA, or a total 30 % of ω-3 fatty acids[5]. As the PUFA content in tropical fish is not high, an enrichment technique is required to increase the PUFA content in the triglycerides (TGs). The best possibility for obtaining PUFA-enriched triglyceridess is by ambient temperature, lipase-catalyze reaction. This is because ω-3 PUFA are highly labile and sensitive to heat and oxidation. Its *cis* ω-3 structure is prone to partial destruction by oxidation, double bond migration, *cis-trans* isomerization and polymerization[6]. In the present investigation, the fatty acids of river catfish, an easy to breed tropical fish, were studied with a view to explore the possibilities of enriching the fish oils with PUFAs of ω-3 series.

EXPERIMENTAL

Chemicals and solvents such as butylated hydroxyl toluene (BHT), sodium sulfate (anhydrous), hexane and chloroform, were obtained from Sigma Chemical Company, USA, BDH Limited, USA and JT Baker, USA. Lipozyme IM and Novozyme 435 were obtained from Novozymes, Denmark.

Preparation of fish sample: The river catfish (*M. nemurus*) was breed by the Biology Department, Universiti Putra Malaysia. The internal organs were removed and the fish was washed to remove the residual blood. Fish fillet were obtained by cutting the fish lengthwise along the backbone to obtain maximum amount of flesh.

Extraction of lipid: Fish oils were extracted according to the method of Bligh and Dyer[7], with some modification by Kinsella *et al.*[8]. Representatives' samples of fish fillets (50 g) were homogenized with a mixture of methanol and chloroform (2:1, vol/vol). The homogenate was filtered under vacuum. BHT at a concentration of 0.2 % was added to the filtrate to prevent oxidation. In a separation funnel, hexane was added

to the filtrate and the aqueous layer was removed. The organic phase was then evaporated using vacuum rotary evaporator at 40 ºC and the fish oil was collected. The fish oil was then stored at -18 ºC prior to further analysis.

Saponification: Saponification was carried out in order to hydrolyze the oils into fatty acids and alcohol. Fish oil (1.0 g) was mixed with 20 mL of 3.75 M NaOH in water:ethanol, 1:1 (v/v) and heated with continuous stirring for 0.5 h at 60 ºC. Distilled water (4.0 mL) was added to the mixture to extract the saponified oil.

Extraction of free fatty acid: Hexane (40 mL) was added to the saponified oil and stirred for 1 h. The saponifiable matter was acidified to pH 1 with concentrated HCl. The lower aqueous phase was discarded and hexane layer containing the fatty acids was collected. Hexane was removed by using a rotary vacuum evaporator at 40 ºC leaving the free fatty acids.

Preparation of fatty acid methyl esters: All the free fatty acids were converted to their constituent fatty acid methyl esters (FAMEs) by refluxing the free fatty acid in methylating reagent consisting of concentrated H_2SO_4/toluene/methanol (1:10:20), v/v/v) for 1 h at 90-100 ºC. The solution was cooled and mixed in a separating funnel with water (3 mL), hexane (2 mL) and internal standard (1 mL), which contained a mixture of methyl pentadecaenoate and methyl nonadecaenoate. The upper organic layer containing FAMEs was collected and dried over sodium sulfate anhydrous and was stored at -18 ºC prior to use. The fatty acid composition of this sample was determined using gas chromatography (GC).

Enzymatic esterification: The reaction mixture consisted of *M. nemurus*-free fatty acid solution (0.05 mol), oleyl alcohol (0.1 mol), immobilized enzymes and hexane which was added to a total volume of 5 mL. The mixture was incubated in a horizontal shaker water bath with a speed of 150 rpm at 30 ºC for 24 h. The reaction was terminated with the addition of 2 mL ethanol. All samples were assayed in triplicate and the experiments were repeated twice. The control experiments were carried out without enzyme.

After the reaction, immobilized enzyme was separated from the mixture. The mixture was titrated to pH 10 by using NaOH. Diethyl ether:water, 1:1 (v/v) was added to the mixture in a separating funnel. The upper organic layer which contained the fatty esters was separated from the lower aqueous layer which contained the unesterified fatty acid. Hexane was added to the aqueous layer and was acidified to pH 1 with concentrated HCl. The lower aqueous phase was discarded and hexane layer containing the fatty acids was collected. Hexane was removed under vacuum at 40 ºC furnishing the free fatty acids which was dried using sodium sulfate anhydrous. The fatty acid composition was analyzed using gas chromatography after being converted to methyl esters.

Gas chromatography analysis: Gas chromatography analysis was carried out using a Hitachi-2500 equipped with a SUPELCOWAX column 30 m × 0.32 mm id and FID detector. The injector and detector temperatures were 250 and 280 ºC, respectively. The column temperature was held at 108 ºC for 2 min and then programmed to 240 ºC at 6 ºC/min. The carrier gas used was helium set at a flow rate of 30 mL/min (psi). The FAMEs were identified by comparing their retention time against those of authentic standard. The content of FAME as g/100 g oil was calculated with methyl pentadecaenoate and methyl nonadecaenoate as the internal standard.

Analysis of methyl esters: Analyses of methyl esters were performed by gas chromatography. The product composition was quantitated by an internal standard method with methyl pentadecaenoate and methyl nonadecaenoate as the internal standards The amounts of unesterified fatty acids methyl esters, m_x, were calculated by equation: $m_x = (RRF \times m_{IS} \times A_x)/(A_{IS})$, where RRF is relative response factor, m_{IS} is amount of internal standard, A_x is peak area of fatty acid and A_{IS} is peak area of internal standard. The amount of unesterified fatty acid, m, was calculated by equation: m = (m_x × molecular weight of fatty acid)/(molecular weight of fatty acid methyl ester). The percentage of unesterified individual fatty acid, P is defined as: P = [m/initial amount of the fatty acid used in the reaction] × 100 %. The DHA concentration can be expressed as the weight percentage of DHA out of total weight of the residual fatty acid obtained from the reaction. This is being expressed as DHA ratio, which is defined as:

$$\text{DHA ratio} = \frac{\text{Weight of DHA}}{\text{Total weight of all identified fatty acids}}$$

Ratio of other fatty acid is defined in the same way.

The esterification extent was calculated by equation: esterification extent = $[(V_i - V_t)/V_i] \times 100$ %, where V_i is a volume of KOH used to titrate the mixture without enzyme and V_t is a volume of KOH used to titrate the mixture after reaction.

Reaction of *M. nemurus* free fatty acid catalyzed by lipozyme IM and Novozym 435

Effect of reaction time: The effect of time on the esterification reaction was studied by varying the reaction time (0, 1, 2, 4, 6, 8, 12 and 24 h). The content of fatty acid in unesterified free fatty acid was determined as described earlier.

Effect of molar ratio of substrate: The reaction mixture was incubated with different molar ratio of substrates, n mole oleyl alcohol/1 mol fish free fatty acid. (n = 1, 2, 3, 4 and 5). The content of fatty acid in unesterified free fatty acid was determined as described earlier.

Effect of various organic solvents: The effect of various organic solvents on the enrichment reaction was investigated. The solvents used were acetonitrile; log P = -0.33, diethyl ether; log P = 0.85, chloroform; log P = 2.00, toluene; log P = 2.50, hexane; log P = 3.50, *n*-heptane; log P = 4.00. The content of fatty acid in unesterified free fatty acid was determined as described earlier.

RESULTS AND DISCUSSION

Compositions of fatty acid in saponified *M. nemurus* oil: In this work, the lipid content of *M. nemurus* was found to be in the range of 7.00-8.00 g/100 g wet tissue in the body. In a study by Varljen *et al.*[9], it was found that the lipid content of Conger conger (sea eel) is 3.7 %, which was three times higher than in *Dipodus vulgaris* (sea bream) and two times lower than in *M. nemurus* in present study. The fatty acid compositions of saponified oil are tabulated in Table-1. The fish oil

TABLE-1
FATTY ACID COMPOSITION OF SAPONIFIED OIL FROM *M. nemurus* 1: FATTY ACID COMPOSITION OF SAPONIFIED OIL FROM *M. nemurus*

Fatty acid	g/100 g of total fatty acid
Saturated	
Myristic (C14:0)	1.95
Palmitic (C16:0)	27.16
Stearic (C18:0)	6.54
Arachidic (C20:0)	0.55
Monounsaturated	
Oleic (C18:1)	24.40
Polyunsaturated	
Linoleic (C18:2)	12.60
Linolenic (C18:3)	0.21
AA (C20:4)	0.79
EPA (C20:5)	0.92
DHA C22:6)	5.39
*AA Ratio	9.8×10^{-3}
*EPA Ratio	0.01
*DHA Ratio	0.06

*AA/EPA/DHA ratio: $\frac{\text{Weight of AA/EPA/DHA}}{\text{Totalweight of all identified fatty acids}}$.

was saponified in order to hydrolyze the lipid into fatty acids and the alcohol fraction. The final product of the saponification was a mixture of free fatty acids (FFAs) isolated from the lipid where all unsaponifiable materials were removed.

The major fatty acids found in *M. nemurus* oil were C16:0, C18:1, C18:2, C20:4, C20:5 and C22:6. Palmitic acid (C16:0) was the major saturated fatty acids present with 27.16 % out of total fatty acid as DHA is the major polyunsaturated fatty acids present at 5.39 % of total fatty acids content. The content of DHA in *M. nemurus* is relatively lower compared to DHA value obtained from tuna oil at 26.6 % of total fatty acids[10]. The difference may be due to the different diet availability of these fish as well as the environment whereby the proportion of the DHA and EPA in lipids depends on the feeding habits of marine animals.

Reaction of *M. nemurus* FFA catalyzed by lipozyme IM and novozyme 435: Two commercially available immobilized enzymes was used to identify the most suitable enzyme in the enrichment of DHA *via* enzymatic esterification of *M. nemurus* oil. Fig. 1 shows esterification extent of *M. nemurus* FFA with oleyl alcohol catalyzed by lipozyme IM and Novozyme 435. The reaction of *M. nemurus* FFA was almost completed in 4 h. The rate of esterification of *M. nemurus* FFA catalyzed by lipozyme IM and novozyme 435 are almost similar at yield (85 and 88 %, respectively). Schmitt-Rozieres *et al.*[11] found that the esterification extent in the reaction of sardine cannery effluent mediated by lipozyme IM with 1-butanol reached over 80 %. However, it was reported that the higher the esterification extent, the lower the enrichment.

The percentage of unesterified fatty acids in the reaction of *M. nemurus* FFA with oleyl alcohol catalyzed by lipozyme IM and novozym 435 is shown in Fig. 2. Lipozyme 435 showed higher percentage of DHA in unesterified fatty acid at 66.6 % of the initial content. Using this immobilized enzyme (lipozyme), the DHA ratio was increased from 0.01-0.13 compared to reaction using Novozym 435, where the final DHA ratio was 0.10 as presented in Fig. 3. This indicated that

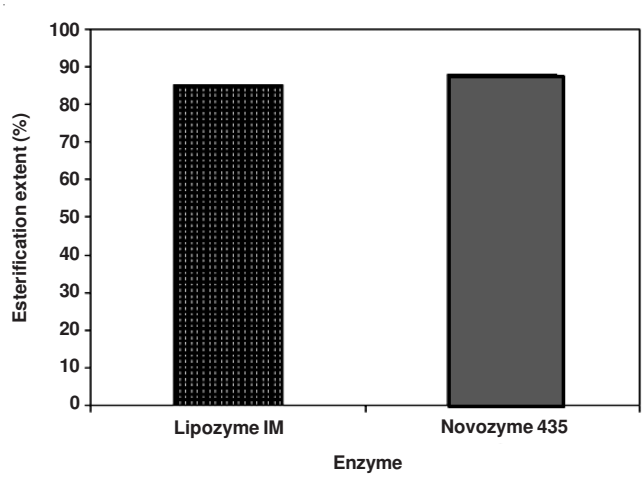

Fig. 1. Esterification extent of *M. nemurus* FFA. The reaction mixture consisted of *M. nemurus* -FFA (2 mol), oleyl alcohol (1 mol), hexane (to a total volume of 5 mL) and Lipozyme IM or novozym 435 (1.0 %), 150 rpm for 4 h

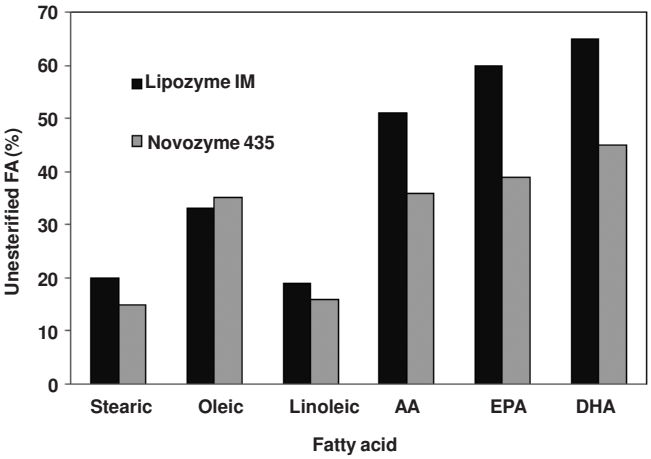

Fig. 2. Percentage of unesterified fatty acids in the reaction of *M. nemurus* FFA with oleyl alcohol catalyzed by lipozyme IM and novozym 435. The reaction mixture consisted of *M. nemurus* -FFA (2 mol), oleyl alcohol (1 mol), hexane (to a total volume of 5 mL) and lipozyme IM or novozym 435 (1.0 %), 150 rpm for 4 h

lipozyme IM exhibited least ability in esterifying ω-3 DHA. Lipozyme IM, thus is the most suitable enzyme for the enzymatic enrichment of ω-3 DHA in *M. nemurus* FFA, as compared to Novozyme 435.

Effect of molar ratio: Fig. 4 shows the percentage of unesterified fatty acid in the reaction of *M. nemurus* FFA with oleyl alcohol catalyzed by lipozyme IM at different molar ratio of the substrates. Stoichiometrically oleyl alcohol reacts with FFA in a molar ratio of 1:1. However, an excess of alcohol is required in order to shift the equilibrium towards esterification. On the other hand, too much alcohol is not economical. At equimolar ratio of oleyl alcohol:*M. nemurus* FFA percentage of AA, EPA and DHA in unesterified fatty acid after 4 h reaction were 60.48, 68.49 and 70.06 % of the initial content, respectively (Fig. 5). As shown in Fig. 6 shows that the AA, EPA and DHA ratios after the reaction were 0.01, 0.01 and 0.09, respectively. When the molar ratio was raised to 2:1 (oleyl alcohol:FFA), there were significant increased in the PUFAs ratio; AA was increased to 0.015, EPA to 0.015 and DHA to

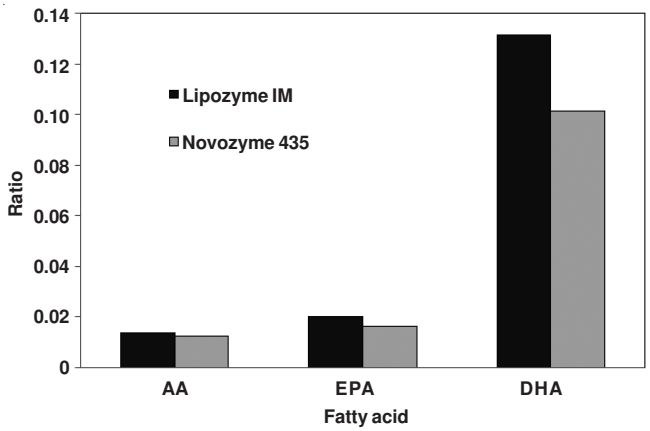

Fig. 3. Ratio of AA, EPA and DHA in the reaction of *M. nemurus* FFA with oleyl alcohol catalyzed by lipozyme IM and novozym 435. The reaction mixture consisted of *M. nemurus* -FFA (2 mol), oleyl alcohol (1 mol), hexane (to a total volume of 5 mL) and lipozyme IM or novozym 435 (1.0 %), 150 rpm for 4 h

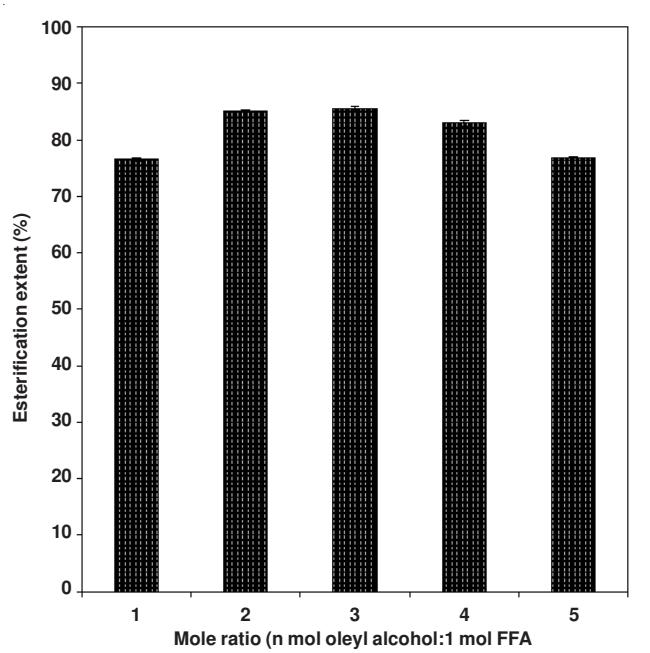

Fig. 4. Effect of mole ratio of substrates (n mol ratio of oleyl alcohol:1 mol ratio FFA) on the Esterification Extent. The reaction mixture consisted of *M. nemurus* -FFA (n mol), oleyl alcohol (1 mol), hexane (to a total volume of 5 mL) and lipozyme IM (1.0 %), 150 rpm for 4 h

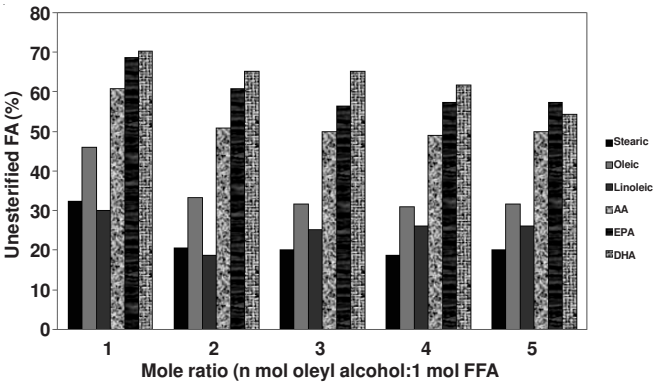

Fig. 5. Effect of mole ratio of substrates (n mol ratio of oleyl alcohol:1 mol ratio FFA) on the percentage of unesterified fatty acids. The reaction mixture consisted of *M. nemurus*-FFA (n mol), oleyl alcohol (1 mol), hexane (to a total volume of 5 mL) and lipozyme IM (1.0 %), 150 rom for 4 h

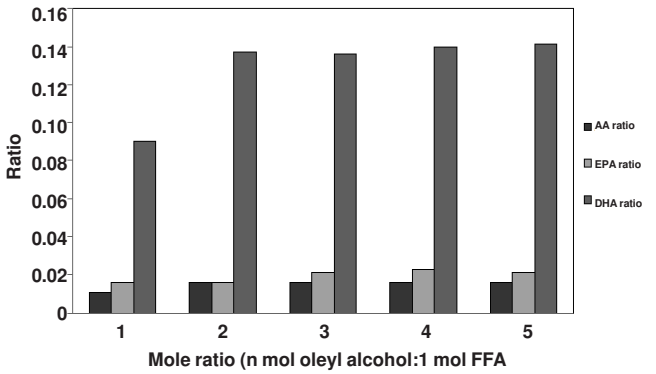

Fig. 6. Effect of mole ratio of substrates (n mol ratio of oleyl alcohol:1 mol ratio FFA) on the ratio of EPA and DHA. The reaction mixture consisted of *M. nemurus* -FFA (n mol), oleyl alcohol (1 mol), hexane (to a total volume of 5 mL) and lipozyme IM (1.0 %), 150 rpm for 4 h

0.14. However, further increase in the alcohol concentration did not result in further increase of PUFA's ratio. This observation is in accordance to finding of Krishna *et al.*[12] in the esterification of isoamyl alcohol and acetic acid[12]. It was suggested that 'at ratios higher than 2:1, the increase in alcohol binding to the enzyme may slightly hamper the equilibrium concentration of the bound acid thereby decreasing the esterification rates[12]. This can be explained by the ability of the excess oleyl alcohol to distort the essential water layer that stabilizes the immobilized enzyme, which could inhibit the activity of enzyme[13]. Therefore, the *M. nemurus* oleyl alcohol:FFA mole ratio was set at 2:1 as the optimum ratio.

Effect of various organic solvents: In order to study the effect of various solvents on the enrichment of ω-3 DHA, several solvents ranging from polar to nonpolar were screened. Fig. 7 shows the effect of various organic solvents on the esterification extent of *M. nemurus* -FFA. log P value of a solvent has been commonly used to describe solvent polarity and their effect on enzyme activity[14], where P is the partition coefficient between octanol and water. A useful solvent for lipase-catalyzed esterifications should be able to dissolve all the substrate, not influence the stability and the activity of biocatalysts and be non-toxic in a wide range of product application[15]. Generally, the percentage of unesterified fatty acid decreases with the increase in log P value of the solvents. Hexane (log P = 3.5) was found to be the best solvent in the enrichment of DHA in *M. nemurus* FFA. The content of DHA in unesterified fatty acid was 66.6 % of the initial content as shown in Fig. 5 and the DHA ratio was 0.14 in the reaction of *M. nemurus* FFA with hexane as solvent. When toluene was used, although the percentage of unesterified DHA was higher than using hexane (80.44 % of the initial content), but it gave a lower ratio of DHA at 0.08 as shown in Fig. 8 due of higher percentage of other unesterified fatty acid. This is also true for other more polar solvents such as chloroform, ether and acetonitrile. This could be due the fact that when the reaction medium is changed from hydrophilic (log P < 3.0) to hydrophobic (log P > 3.0) solvents, the overall efficiency of the

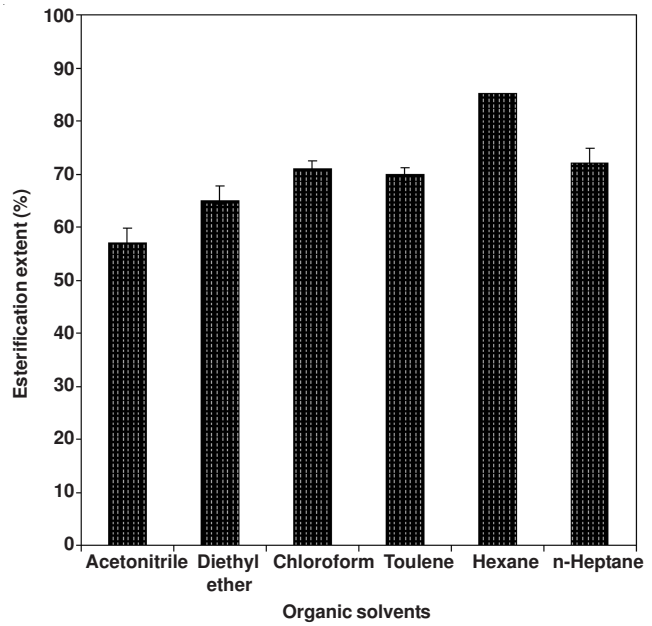

Fig. 7. Effect of various organic solvents on the esterification extent of *M. nemurus*-FFA. The reaction mixture consisted of *M. nemurus* -FFA (2 mol), oleyl alcohol (1 mol), solvents (to a total volume of 5 mL) and lipozyme IM (1.0 %), 150 rpm for 4 h. Solvents used were acetonitrile; log P=-0.33, diethyl ether; log P=0.85, chloroform; log P=2.0, toluene; log P=2.5, hexane; log P=3.5, n-heptane; log P = 4.0

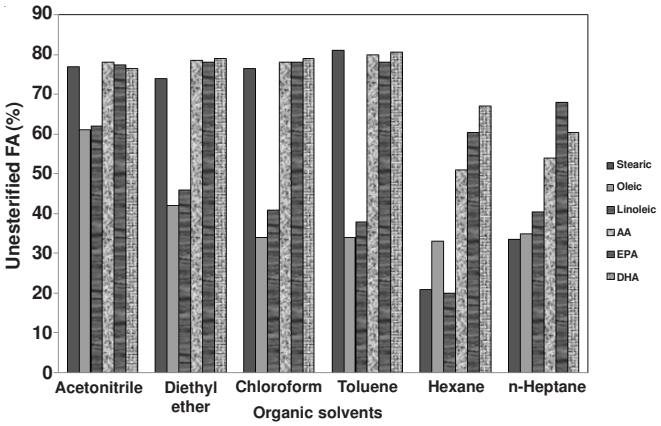

Fig. 8. Effect of various organic solvents on the percentage of unesterified fatty acids. The reaction mixture consisted of *M. nemurus* -FFA (2 mol), oleyl alcohol (1 mol), solvents (to a total volume of 5 mL) and lipozyme IM (1.0 %), 150 rpm for 4 h. Solvents used were acetonitrile; log P = -0.33, diethyl ether; log P = 0.85, chloroform; log P = 2.0, toluene; log P = 2.5, hexane; log P = 3.5, *n*-heptane; log P = 4.0

enzymes changes[16]. On the other hand, hydrophobic solvents such as hexane and *n*-heptane preserve the catalytic activity without disturbing the micro aqueous layer of the enzyme and this mechanism will enrich the ratio of DHA and other long chain fatty acid such as AA and EPA in the unesterified fatty acid as shown in Fig. 9.

Effect of reaction time: Subsequently, the esterification was conducted at 30 ºC in a mixture containing of oleyl alcohol/ *M. nemurus* FFA (2: 1, mol/mol) and 0.05 g of lipozyme. The contents of DHA and EPA in the unesterified FA were decreased with increasing time and reached almost constant after 20 h (Fig. 10). Most of the fatty acids achieved maximum

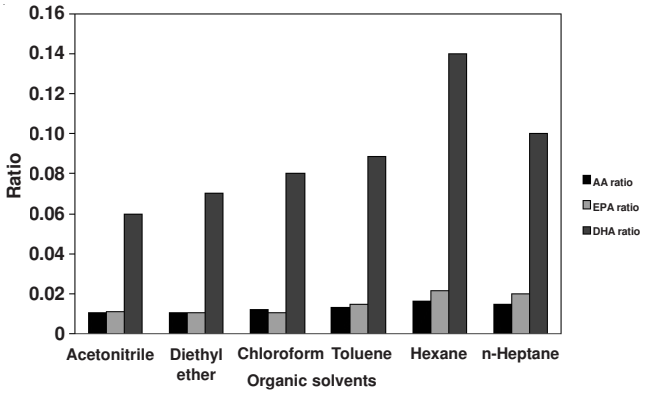

Fig. 9. Effect of various organic solvents on the ratio of AA, EPA, and DHA. The reaction mixture consisted of *M. nemurus* -FFA (2 mol), oleyl alcohol (1 mol), solvents (to a total volume of 5 mL) and lipozyme IM (1.0 %), 150 rpm for 4 h. Solvents used were acetonitrile; log P = -0.33, diethyl ether; log P = 0.85, chloroform; log P = 2.0, toluene; log P = 2.5, hexane; log P = 3.5, *n*-heptane; log P = 4.0

esterification after 10 h of reaction except EPA and DHA. At this time there is still a high amount of unesterified DHA. After 24 h of reaction, 50 % of DHA still remained in the unesterified fraction. A longer reaction time would not be favourable since this would cause more DHA to be esterified. Fig. 11 shows the percentage of unesterified stearic acid, oleic acid, linoleic acid, AA, EPA and DHA as the reaction progressed. These values could be used to compare the absolute concentration of the fatty acids. Stearic acid is chosen because it is the most abundant FA in the *M. nemurus* FFA and its trend of change is typical of other fatty acids except AA, EPA and DHA. DHA ratio, which has the initial value of 0.06, increased throughout the 24 h reaction and seems to reach a maximum value of 0.18 after 24 h as shown in Fig. 12.

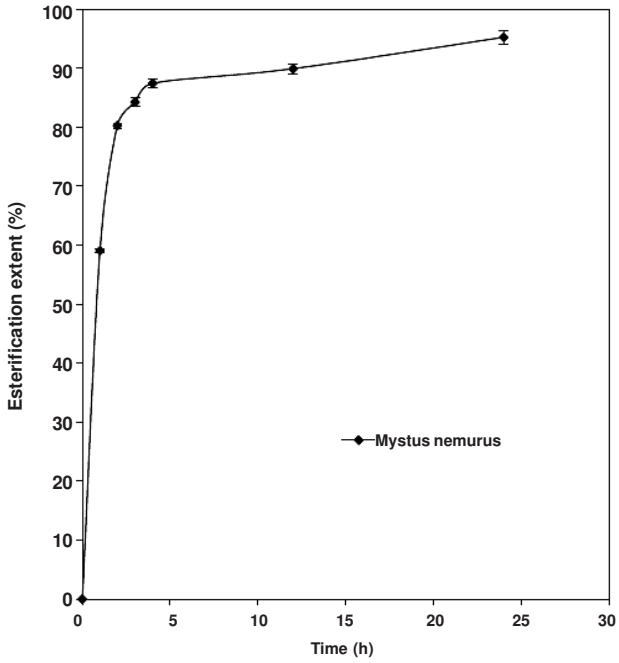

Fig. 10. Effect of time reaction on the esterification extent of *M. nemurus* -FFA. The reaction mixture consisted of *M. nemurus* -FFA (2 mol), oleyl alcohol (1 mol), hexane (to a total volume of 5 mL) and lipozyme IM (1.0 %), 150 rpm

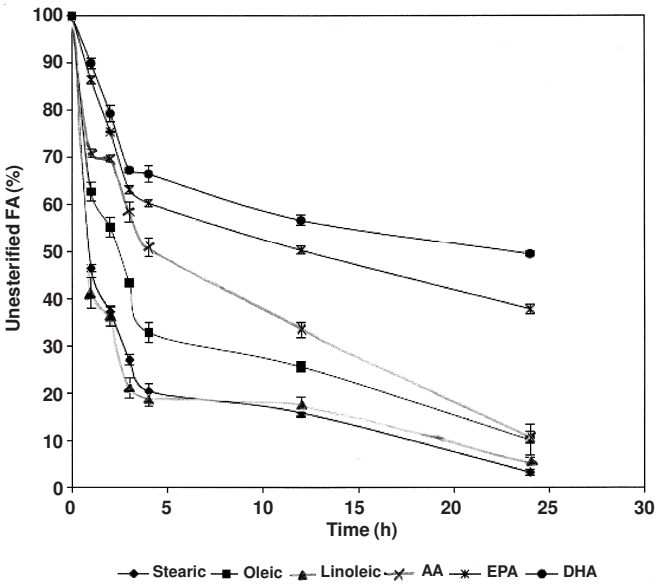

Fig. 11. Effect of time reaction on the percentage of unesterified fatty acids. The reaction mixture consisted of *M. nemurus*-FFA (2 mol), oleyl alcohol (1 mol), hexane (to a total volume of 5 mL) and lipozyme IM (1.0 %), 150 rpm

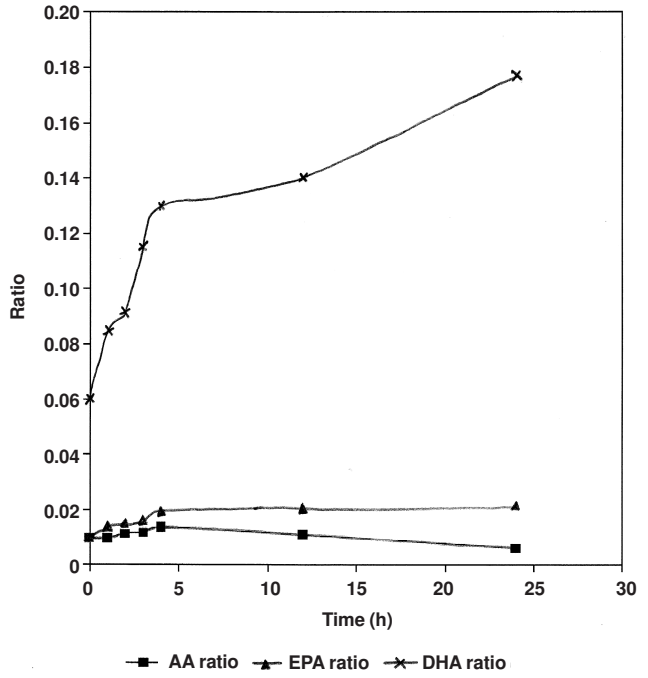

Fig. 12. Effect of time reaction on the ratio of stearic, AA, EPA and DHA. The reaction mixture consisted of *M. nemurus* -FFA (2 mol), oleyl alcohol (1 mol), hexane (to a total volume of 5 mL) and lipozyme IM (1.0 %), 150 rpm

Conclusion

This work suggest that ω-3 fatty acid in fish oil from *M. nemurus* could be enriched *via* enzymatic synthesis using lipozym IM at mole ratio of 2:1 (oleyl alcohol:*M. nemurus* FFA), at 4 h reaction time. Hexane was found to be the best solvent to enrich ω-3 fatty acid in *M. nemurus* oil.

ACKNOWLEDGEMENTS

This project was financed by Universiti Putra Malaysia.

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