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Optimization of Lipase Mediated Enrichment of n-3 PUFA Glycerides in Indian Sardine Oil

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Abstract:*Indian Sardine oil is a rich source of n-3 polyunsaturated fatty acids (n-3 PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which exists in the form of glycerides. The current work aims to optimize a process to enhance n-3 PUFA esters in the refined sardine oil by optimizing the enzymatic hydrolysis of glycerides having saturated fatty acid esters, using Pseudomonas cepacia lipase (PCL). Degree of hydrolysis (DOH) of PCL dissolved in solvent (polypropylene glycol) in the presence of surfactants like tween 80 and SDS was observed to be the highest at 40°C, 4 mg/mL enzyme load, surfactant to enzyme ratio of 2:1 (v/v), oil to water ratio of 1:1 (w/w), hydrolyzed for 15 minutes. After hydrolysis, oil was deacidified using methanolic extraction. Upon analysis of oil by using Gas Chromatography with FID, an increase in n-3 PUFA content from 17.91% (w/w) to 22.63% (w/w) was evidenced. RP-HPLC equipped with ELSD showed that most of the fatty acids exist in the form of monoglycerides.*

Keywords: *Degree of hydrolysis, Docosahexaenoic acid, Eicosapentaenoic acid, Indian Sardine oil, hydrolysis, Pseudomonas cepacia lipase*

1. Introduction

The beneficial effects of n-3 polyunsaturated fatty acids (n-3 PUFA) in the prevention and treatment of coronary, neuromuscular, immunological disorders and allergic conditions are well documented [1]. Oil extracted from several pelagic fishes are found to be the rich source of n-3 PUFA. In order to meet the increasing demands of PUFA concentrates, industries have employed various techniques which lead to the production of PUFA concentrates in the form of free fatty acids which is nutritionally unfavourable and acts as the precursors for the initiation of oxidation in the oil [2].These techniques are known to involve extremes of pH and temperature which may destroy the natural n-3 PUFA by oxidation, cis-trans isomerization and double bond migrations [3]. Use of lipases for hydrolysis has been a reliable technique to concentrate important n-3 PUFA like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) due to the mildness it offers during the course of reaction in terms of temperature, pH and pressure which most certainly prevents the n-3 PUFA from destruction. Also, few lipases exhibit strong preferences for EPA and DHA. Much has been published on hydrolysis by using lipases to concentrate EPA and DHA in the fish oil [2,3]. Nevertheless, not many literatures are available on hydrolysis of Indian Sardine oil using *Pseudomonas cepecia* (PCL).

In the current study, n-3 PUFA enriched glycerols from Indian Sardine oil via hydrolysis using PCL was attempted. Further, HPLC was performed to find out the form of concentrates in the oil.

2. Experimentation

2.1 Materials

Crude fish oil acquired from Mukka Fish Oil Industries (Mangaluru, India) was centrifuged at 5000 rpm using Remi benchtop centrifuge (R- 8C BL) and stored at -21°C in the dark. Orthophosphoric acid (OPA), methanol, activated charcoal, iso propanol, acetonitrile, n-hexane, ethanol, diethyl ether, sodium hydroxide, hydrochloric acid, boron trifluoride in methanol (10%). *Pseudomonas cepacia* lipase (PCL) and glyceride standard for HPLC was purchased from Sigma Aldrich, India. All the reagents (analytical grade) and solvents (chromatographic grade) were purchased from Merck, India and used without further purification.

2.2 Methods

Refining of Crude fish oil, obtained from Mukka Fish Oil Industries (Mangaluru, India) was taken for the study. Different quantities of PCL (2mg, 4mg, 6mg, 8mg, 10mg) were dissolved in 1 mL of various solvents (hydrophobic and hydrophilic), followed by the addition of various surfactants (non-ionic, anionic and cationic) to the enzyme solution. Various parameters like dilution of surfactants (2%, 4% ,6%, 8%, 10%) and quantity of surfactants in enzyme solution (2:1, 4:1, 6:1, 8:1, 10:1) (v/v) were optimized.

2.2.1 Effect of mixed surfactants

This experiment was performed according to Kawase et al. (1985) [4] with slight modifications. This was basically done to inspect the influence of the order of contact of the two surfactants with lipase on the

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amount of protection offered by this mixture against inhibition. The degree of hydrolysis (DOH) was observed by contacting the lipase with non-ionic surfactant initially for 10 minutes with the subsequent addition of anionic surfactant for the next ten minutes.

2.2.2 Lipase-catalysed hydrolysis of sardine oil

The reaction system containing 1 g of fish oil and 1 g of water (1:1 w/w) were placed in the conical flask under constant stirring at 300 rpm after which lipase preparations were added to these homogenized substrates to start hydrolysis reaction. The degree of hydrolysis was studied by subjecting the system to the variations in the parameters like temperature (30°C, 40°C, 50°C, 60°C), oil-water ratio (1:1, 1:2, 1:3, 1:4, 1:5), concentration (2mg/mL, 4mg/mL, 6mg/mL, 8mg/mL, 10mg/mL) of lipase and time of hydrolysis (15 minutes, 30 minutes, 45 minutes and 60 minutes).

2.2.3 Analysis

The DOH was determined by measuring the acid value of both hydrolyzed and unhydrolyzed oil as well as the saponification value of the unhydrolyzed oil. The hydrolysis was performed under the optimized reaction conditions of PCL for 15 minutes and the reaction was stopped by adding 10 ml of ethanol. The hydrolyzed oil was subjected to methanol extraction in the ratio (1:1 w/w) oil to methanol, to eliminate the FFA released during the process. The solvent extracted oil (hydrolyzed) was studied for its fatty acid composition through GC (Trace 3330 GC Ultra, Thermo Electron Corporation) which was compared with the fatty acid composition of the refined oil measured before hydrolysis. Hydrolysis was done under vacuum conditions in order to prevent oxidation of oil. The acid value of oil was calculated according to the official method of American Oil Chemist's Society (AOCS) (2009) methodologies, (Cd3d-63) [5].

2.2.4 Determination of fatty acid composition in oil

After the solvent was evaporated and dried, the residue was esterified with borontrifluoride methanol agent and analyzed by gas chromatography using the guidelines from Ichihara and Fukubayashi (2010) [6].

2.2.5 Determination of the form of glycerides in the hydrolysed oil

To analyze the contents of TG, DG, MG after the hydrolysis reactions, the sardine oil sample (8µL) was mixed well with 4 mL of iso-propanol of chromatographic grade and analysed by HPLC with a pump (LC-20AD, Shimadzu Co, Kyoto, Japan) in the presence of RPC18 column using ELSD (Gilson) in accordance with Aoki et al. (2004) [7].

3. Results and discussions

The competence of lipase for the fatty acid selectivity such as EPA and DHA has allowed the separation of these fatty acids from the remaining fatty acids in fish oils, resulting in the increased yields of n-3 PUFA rich concentrates and hence, in the present study, the DOH was studied under various conditions with the aim of obtaining elevated amounts of EPA and DHA in the hydrolysed oil with the minimum production of unwanted impurities that may develop during hydrolysis. The hydrolysis was performed with the refined sardine oil with the composition as mentioned in Table 1.

Table 1: *Composition of refined Indian Sardine oil*

[1]	Parameters	[2]	Values
[3]	Free fatty acid (% oleic acid)	[4]	0.56%
[5]	Phospholipid (ppm)	[6]	5.66
[7]	Iron (ppm)	[8]	BDL*
[9]	Copper (ppm)	[10]	0.1
[11]	Mercury (ppm)	[12]	BDL*
[13]	Fatty acids (% w/w)	[14]	
[15]	$C_{14:0}$	[16]	26.18
[17]	$C_{16:0}$	[18]	46.87
[19]	$C_{18:1}$	[20]	5.06
[21]	$C_{18:2}$	[22]	5.76
[23]	$C_{20:5}$ (EPA)	[24]	11.81
[25]	$C_{22:6}$ (DHA)	[26]	6.1

BDL-Below detection limit

3.1 Modification of lipases

PCL was treated with a concoction of solvents and surfactants with the purpose of increasing the DOH of the enzyme in the oil.

From Fig.1 it is clear, when PCL (10mg/mL) was treated with 1 mL of various solvents there was an increase in DOH in the presence of polypropylene glycol (PPG) as compared to the other solvents. The reason for the increased hydrolysis by PCL dissolved in PPG is probably because of the neutral pH of this solvent in which PCL remains active due to the presence of residues of amino acids at the active site of PCL which result in maximum binding of the substrate. Studies by Iyer and Ananthanarayan (2008) [8], explained that the addition of polyols like PPG to the enzyme strengthens the hydrophobic interactions among the non-polar amino acid residues which lead to protein rigidification and resistance to thermal deactivation. Pencreach (1997) [9] reported that PCL is optimally active at pH 7.0 (neutral) which is in concurrence with the present study.

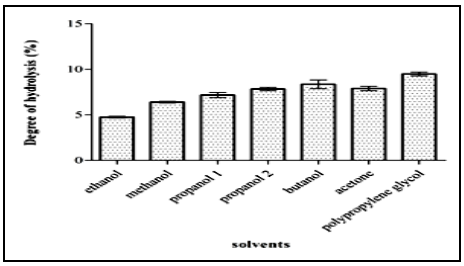

Figure 1: *Effect of solvents on PCL and its DOH in Sardine oil at 30°C.*

3.2 Effect of PCL concentration on DOH

It was noticeable that the DOH at 4mg/mL was recorded to be the highest after which the trend slowed down with the increase in enzyme concentration (Fig.2). It could be reasoned out that beyond 4mg/mL, the oil-lipase solution interface generated under these conditions gets saturated with lipase and there is a formation of monolayer at the interface and accumulated intermediates [10]. Therefore, further studies were carried out using a 4 mg/mL of PCL.

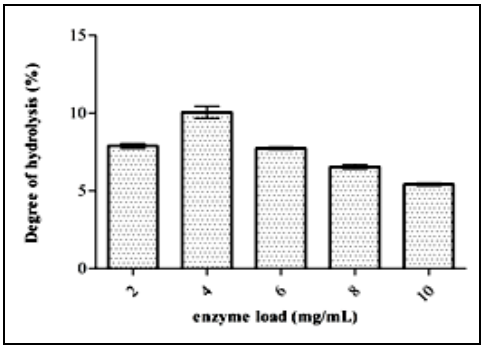

Figure 2: *Effect of PCL load on DOH at 30°C*

3.3 Protective effect of surfactant mixture

In order to protect the lipase from a huge decrease in the hydrolysis ability, the effects of mixture of non-ionic surfactants and anionic surfactants were studied. When the oil was treated with PCL, in presence of tween 80 and SDS, the DOH was 10.93% and 10.1% respectively. It is evident that DOH in oil by tween 80 is higher than SDS which could be because of the slight inactivation of lipase by SDS as it tends to act upon the disulphide linkages of the lipase. Similar effects were noticed in the case of *P. aeruginosa* KKA-5 strain and *P. Pseudoalcaligenes* F-111. When tween 80 was added first and SDS after ten minutes to PCL solution, the DOH was found to have increased to 11.04%. This increase in the hydrolysis efficiency proves not only the combined positive effect of the surfactants on the activity of PCL, but also the protective effect of tween 80 as opposed to SDS. Non-ionic surfactants in general are considered as mild detergents and they do not extensively interact with the protein structure [11].Hence a mixture of tween 80 and SDS were used for the further studies.

3.4 Effect of dilution of surfactants

It's evident from Fig.3 that at 8% of mixed surfactants (4% tween 80 and 4% SDS) with PCL showed a maximum DOH of 10.92% respectively. Beyond 8% of mixed surfactants, a drastic reduction in DOH was observed which is due to the increased concentration of tween 80 and SDS in the system causing the partial or complete unfolding of the tertiary structure of the protein due to the additional hydrophobic interactions [12]. Mogensen et al. (2005) [13] contends the deactivation of lipase in the presence of nonionic and zwitterionic surfactants at high concentrations.

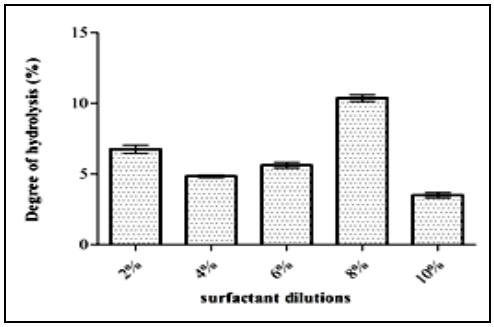

Figure 3: *Effect of dilutions of mixed surfactants on PCL and its DOH at 30°C*

3.5 Effect of surfactant to enzyme ratio (v/v)

It is seen that 2:1 (v/v) of mixed surfactants to PCL, promoted the highest DOH in the oil (Fig.4). Beyond this ratio, the DOH tends to decrease, which is attributed to the inhibitory effects of surfactants by impairing the lipase adsorption at liquid- water interfaces [14].

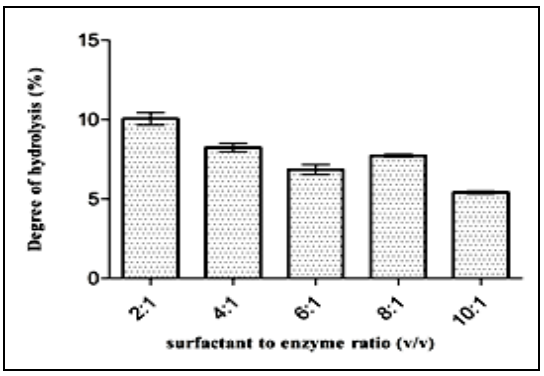

Figure 4: *Effect of quantity of surfactants to PCL (v/v) and its DOH at 30°C*

3.6 Effect of temperature

Trials to determine the maximum DOH for PCL were done at a temperature range of 30°C to 60°C, and it was perceived that PCL showed a maximum DOH of 10.9% at 40°C. However, a rapid decrease in the DOH was observed at the rest of the temperatures (Fig.5). This is due to the denaturation of the enzyme structure at these temperatures. The reaction rate of PCL increased from 25°C to 45°C, and remained constant at 50°C and decreased at higher temperatures [9].

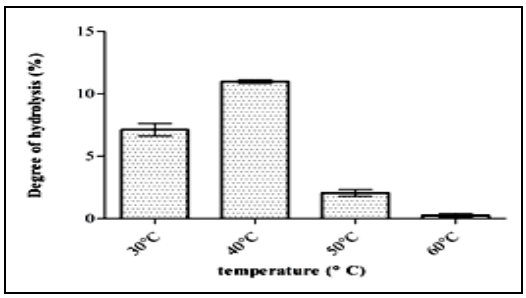

Figure 5: *Effect of temperature on DOH at 2:1 (v/v) mixed surfactants to PCL*

3.7 Effect of amount of water for hydrolysis on DOH

Since lipases are known to cleave the ester bonds of triglycerides with the utilization of water molecules, it becomes essential to study the amount of water required for hydrolysis. From the Fig.6 it was apparent that the DOH was highest at the oil to water ratio of 1:1 (w/w). The ratios above 1:1 (w/w) showed lesser activities because higher quantity of water leads to a thicker water layer around PCL which increases the flexibility of the lipases, causing denaturation [15].

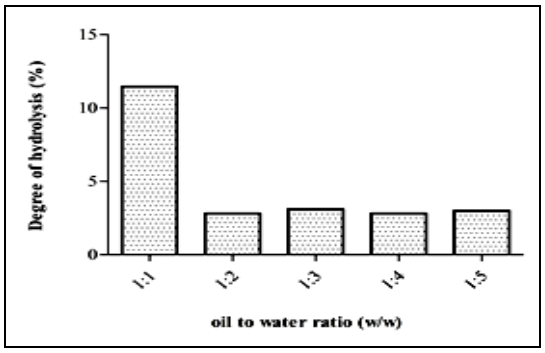

Figure 6: *Effect of quantity of water on DOH at 40°C and 2:1 (v/v) mixed surfactants to PCL*

3.8 Effect of time on DOH

Figure. 7 show the time course of hydrolysis of PCL. PCL were shown to give maximum DOH at 15 minutes. It is clear from the trend in figure that initially with increase in time the product formation was increased up to 15 min. A further increase in time up to 1 hour did not lead to any improvement in the product formation. This may be due to the attainment of equilibrium for PCL in terms of the conversion rates [3]. The maximum was achieved in a shorter reaction time with high EPA and DHA content, maybe because of the lower amounts of fatty acids in the oil to be removed (Table 1).

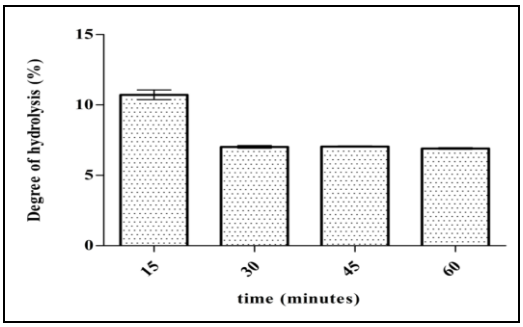

Figure 7: *Effect of time on DOH at 40°C and 2:1 (v/v) mixed surfactants to PCL*

3.9 Enhancement of n-3 PUFA in oil

After hydrolysis with PCL there was an increase in the EPA (15.23%) and DHA (7.4%) from EPA (11.81%) and DHA (6.1%) in the refined oil. It was observed that PCL resulted in the concentration of EPA almost in the same rate as DHA. These results concur with the observations made by Halldorsson et al. (2004) [3] in concentration of EPA and DHA in sardine oil.

3.10 Analysis of the hydrolyzed oil by HPLC

Having studied the EPA and DHA content in the hydrolysed and unhydrolyzed oil, we were also interested in testing these oil samples to have an idea of the form of fatty acid glycerides. Figure.8 reveals the differences among the hydrolyzed oil, unhydrolyzed refined oil and the HPLC standards. Interestingly, it was observed that the hydrolyzed oil contained pronounced quantities of monoglycerides with the drastic reduction in the diglycerides and the triglyceride forms of fatty acids.

It can be derived from the GC and HPLC results that the DOH exhibited by PCL in sardine oil having C16:0 as the most predominant fatty acid proved to be a good substrate for hydrolysis. The high DOH of PCL can be explained because of the non-specificity of the enzyme to a particular fatty acid and secondly, the anatomy of the fatty acid binding sites. PCL is known to have a crevice-funnel shaped substrate binding sites which accommodates bulky substrates and results in the hydrolysis of triglycerides in sardine oil [16]. The efficiency of the hydrolysis can be visualized in the HPLC chromatograms where the reduction in the concentrations of triglycerides and diglycerides to the respective monoglycerides were evident.

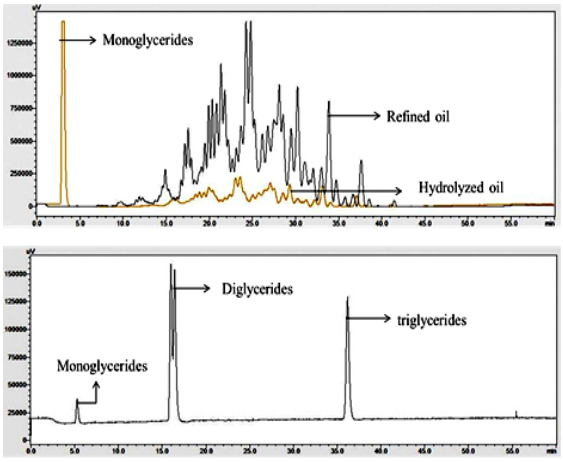

Figure 8: *HPLC chromatograms of oil before and after hydrolysis and HPLC glyceride standards*

4. Conclusions

The following conclusions are deduced from this study:

- There was a dramatic increase in the n-3 PUFA content (1.26 times) in hydrolysed oil compared to the unhydrolysed oil.
- DOH carried out by PCL is significantly influenced by a combination of solvents and surfactants

• Hydrolysis of the oil for 15 minutes led to significant increase in the n-3 PUFA concentrate for the use in nutraceuticals.

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