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Research Article

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Production of PUFA Concentrates from Trash Fish *Sufflaman capistratus* Liver Oil through *Bacillus cereus* MSU AS Lipase Catalysis

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ABSTRACT

In order to find an alternative source for the highly fatty acids oil with lower cost, a marine trash fish Sufflaman capistratus has been analyzed and characterized. The experimental oil sample was extracted from the liver sample by using Bligh and Dyer method. The yield of the oil content was estimated as 58.46 ± 0.32%. Then this crude oil was purified. The physicochemical properties such as acid value, free fatty acid, peroxide value and a saponification value of the crude and purified oils were determined. Fatty acid profile of both crude and purified oil samples was varied much, for instance, the PUFA content of crude oil was 33.83%, whereas it was observed as 31.32% in purified oil. Further, the purified oil was enriched with different concentrations (0.25, 0.50 and 0.75%) of the lipase of B. cereus MSU AS through hydrolysis process at 37ºC for 24h of incubation. From the lipase - mediated hydrolysed liver oil, the PUFA concentrate especially DHA and EPA were separated by preparative HPLC. Detailed results on the production of PUFA concentrates from trash fish Sufflaman capistratus liver oil through Bacillus cereus MSU AS lipase catalysis are discussed here with.

Keywords: *Sufflaman capistratus; Bacillus cereus* MSU AS lipase; PUFA; Preparative HPLC

INTRODUCTION

Fish oils are a rich source of polyunsaturated fatty acids (PUFA) such as γ-linolenic acid, linoleic acid, arachidonic acid, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) etc. These two fatty acids are linked to the presence of two long chain PUFA's such as cis-5,8,11,14,17–EPA and cis-4,7,10,13,16,19–DHA. Now EPA has been used specifically for treatment of atherosclerosis, hyperlipemia, schizophrenia and certain cancers. γ- linolenic acid is used to prevent topic eczemia, rheumatoid arthritis, multiple sclerosis and premenstrual tension [1].
In some fish oils, the concentration of PUFA especially DHA and EPA were found in lower percentage and they can be increased through lipase catalyzed reaction. Lipases are nature's gift for the hydrolysis and synthesis of ester bonds found in fatty acids. Enzymatic enrichment of PUFA has shown potential in producing high quality due to the mild condition (eg: neutral pH and low temperature) of the process [2]. Used purified *Pseudomonas fluorescence* lipase for selective enrichment of n3- PUFA from sardine oil [3]. The PUFA concentrate from Pacific sardine (*Sardinops sagase*) oil by using lipase catalyzed hydrolysis [4]. Considering the importance of the above, in the present study also we have an objective to produce PUFA concentrate from an underutilized fish *sufflaman capistratus* liver oil using *B. cereus* MSU AS lipase.

MATERIALS AND METHODS

Sample collection

In the present study, the liver oil was extracted from the liver mass of marine trash fish *S. capistratus* collected from the local fisheries harbor of Kanyakumari, Tamil Nadu. In the laboratory, the liver was separated from the fish by

dissection. The removed liver samples were cleaned well and stored at –20ºC in a deep freezer until further use. The oil present in the liver was extracted by the method described by [5].

Extraction of liver oil

100g of thawed liver sample was weighed in a 2L beaker, to this 100 ml of distilled water was added and mixed well. Then 100 ml of chloroform: methanol (2:1) were added and homogenized well for 2 min by keeping it in ice. To this mixture, 200 ml of distilled water was added and finally homogenized for 30 seconds. The mixture was centrifuged at 5000 rpm for 30 min. The aqueous layer was removed by suction completely, the solvent layer was recovered, and passed on to anhydrous sodium sulphate. Then the solvent was evaporated using a rotator to remove the chloroform completely. Finally, the yield of oil extracted was calculated.

Purification of liver oil

Usually hydrocarbons, free fatty acids, pigments, sterols etc. are present in crude oil. These materials may degrade the quality of extracted oil. For pharmaceutical purposes, these materials should be removed from the oil [6].
100ml of crude fish oil was heated in water bath at 40ºC and washed with distilled water for 2 minutes. Then heat at 40ºC with 20ml of 0.25% of 50% ortho-phpsphoric acid. After that, separate oil from the ortho-phpsphoric acid mixture. Next, wash oil with water and heat with 20 ml of 0.1N NaOH. Then transfer to the separating funnel for separation of semi-purified oil. This semi-purified oil was heated with 4ml of 6N sodium carbonate and wash this mixture then remove water from the mixture. Again the oil was heated with 4ml of 30% sodium silicate and wash remove water with the help of the separating funnel. Then add pure charcoal and heated at 90ºC. After that, the removal of charcoal from the mixture with the help of separating funnel. Finally, add a little bit of anhydrodrous sodium sulfate in an oil sample. It completely observed excess water from the oil sample. Finally, we get purified oil.

Chemical properties of extracted crude and purified oils

Determination of acid and free fatty acid (FFA) values:

10 g each of crude and purified oil samples were taken individually in 250 ml conical flasks. To this 50 to 100 ml of freshly neutralized hot 95% of ethyl alcohol and about one ml of phenolphthalein (1%) indicator solution were added. The mixture was boiled for about five minutes and titrated against standard alkali solution by shaking vigorously during the titration. The weight of the oil taken for the estimation and the strength of the alkali used for titration shall be such that the volume of alkali required for the titration does not exceed 10 ml.

Calculation:

$$Acid\ value = \frac{56.1\ VN}{W}$$

Where (V = Volume in ml of standard potassium hydroxide or sodium hydroxide used
N = Normality of the potassium hydroxide or sodium hydroxide, W = Weight of oil sample

$$Free\ fatty\ acids\ as\ oleic\ acid\ present\ by\ weight\ = \frac{56.1\ VN}{W}$$

Acid value = percentage of fatty acid (as oleic acid) x 1.99

Determination of saponification value:

To determine the saponification value of oil sample, indicator method was used as specified by [7]. 2 g each of crude and purified oil samples were taken in two conical flasks individually, 25 ml of 0.5N ethanolic potassium hydroxide was then added. The content was constantly stirred and allowed to boil gently for 60 min. A reflux condenser was placed on the flask containing the mixture. Few drops of phenolphthalein indicator were added to the warm solution and then titrated with 0.5N HCl to the end point until the pink colour of the indicator just disappeared. The same procedure was used for blank also.

Determination of peroxide value:

5g each of crude and purified oil samples were taken from 250 ml glass stoppered Erlenmeyer flasks individually and then 30 ml of the acetic acid – chloroform solution was added. The flasks were swirled until the sample was dissolved in the solution. 0.5 ml of saturated potassium iodide was added. The solution was allowed to stand after occasional shaking for exactly 1 min and then 30 ml of distilled water was added. The sample was then titrated with

0.1N sodium thiosulfate by adding it gradually and with constant and vigorous shaking. The titration was continued until the yellow colour gets disappeared. Then 0.5 ml of starch indicator solution was added and the titration was continued by shaking the flask vigorously near the end point to liberate all the iodine from the chloroform layer. Then thuiosulfate has added drop wise until the blue colour gets disappeared. The blank titration must not exceed 0.1 ml of the 0.1N sodium thiosulfate solution.

Calculation:

$$Peroxide\ value\ \left(meq\ of\ peroxide/1000g\ of\ sample\right) = \frac{S \times N \times 1000}{W}$$

Determination of the fatty acid profile of oil samples by Gas chromatography (GC)
Preparation of fish oil fatty acid Methyl esters [8]:
The oil samples were (1g each of crude and purified oils) individually evaporated to dryness under nitrogen in a block heater at 60ºC. The esters were extracted from the oil sample by adding 2ml hexane and 2 ml distilled water. After 15 sec, the extracted oil was centrifuged for 1min at 3000 rpm at room temperature. The hexane phase was collected, and subsequently 2ml of this solvent was added to the extract solution. Then the solution was shaken for 30sec and centrifuged for 1 min at 3000 rpm at room temperature, again the hexane phase was collected and added with the previously collected 2 ml hexane phase. The 4 ml hexane solution containing lipid was stored at -20ºC until the GC analysis.

Fatty acid analysis:
The fatty acid profile of the hexane mixed crude and purified lipids were individually analyzed by Gas chromatography (GC) by following the four steps (Saponification, Methylation, Extraction and Base wash) [9].

Hydrolysis of purified *S. capistratus* liver oil by *B. cereus* MSU AS lipase [6]:
0.5g of purified oil was taken in a 100 ml Erlenmeyer flask. To this 4ml of 0.1 M Tris HCL buffer (pH 8.4) and 1ml of *B. cereus* MSU AS purified lipase solution (1000 U/ml) were added. Air in the flask was replaced by nitrogen gas. Then the flask was incubated with reciprocal shaking at 37ºC for different time intervals (6, 12, 24, 48 and 96h). In each time interval, the reaction was stopped by adding 20ml of ethanol and the free fatty acid liberated was titrated with 0.1N KOH solution. A control was carried out simultaneously, except that the enzyme solution was added after the addition of ethanol. The hydrolysis reaction was calculated by the following equation.

Calculation:

Hydrolysis ratio (%) = Acid value / Saponification value x 100

Where, Saponification value = Original oil value (unhdrolyzed oil)

Enrichment of PUFA concentrate (EPA and DHA) of purified *S. Capistratus* liver oil
12 ml each of purified *S. capistratus* liver oil sample was taken in three individual 50 ml capacity glass bottles and they were treated with 0.01ml of 0.05% w/w TBHQ and 0.8ml of 0.7% v/v of Triton X 100. To start the reaction, the different concentrations (0.25, 0.50 and 0.75%) of lipase preparations were added individually in treated oil sample. The bottles were flushed with nitrogen, sealed with rubber caps and parafilm, and placed in an incubator at 40ºC with magnetic stirring. Reaction mixture samples (0.5ml) were withdrawn periodically, glycerides were extracted with 10ml of n-hexane after adding 3.5ml of 0.5N KOH (30% ethanol solution) to neutralize the PUFA released during hydrolysis. PUFA in the water layer was extracted with 10ml of n-hexane after returning to acidic pH 1.0 with 4N HCl. The PUFA concentrate of the purified oil samples was analyzed by GC. The percentage of hydrolysis of EPA and DHA released from their original content in the oil was then computed.

Separation of free PUFA by preparative HPLC [10]
After GC analysis, the EPA and DHA of PUFA concentrate were recovered individually by using preparative HPLC. The PUFA concentrate was dissolved in 1000 µl of acetic acid / methanol/ water in the ratio of 0.1: 65:35, then 500µl of this sample was injected and filtrated by using preparative HPLC column – 5C18 at a flow rate of 4ml /min. The fractions containing EPA and DHA and their geometrical isomers were collected separately to recover enough material (seven times, at around 10mg per run). The separated EPA and DHA were confirmed based on their retention time and their peaks were analyzed by using Peak detector of UV.

Statistical analysis

The data obtained in the present study were expressed as Mean ± SD and were analyzed using Students 't' test with a *Post hoc* multiple comparision with SNK test as a significant level of 5% using a computer software STATISTICA 06 (Statosaft, Bedford, UK).

RESULTS AND DISCUSSION

Fish oils have been recognized as good sources of PUFA, which are widely used for the pharmaceutical purpose and also as food supplements [11]. In the present study, the percentage of oil yielded from the trash fish *S. capistratus* liver mass was 58.46 ± 0.32% (Table 1). The extracted liver oil was purified and the chemical and nutritional properties of crude and purified oils were estimated. The result on the chemical properties of crude and purified oils of *S. capistratus* are given Table 2.

Table 1: Percentage of oil yield (w/w) from the liver mass of *S. capistratus*

Experiment	Oil yield (%)
1	57.88 ± 0.20
2	58.36 ± 0.31
3	59.14 ± 0.83
Mean value	58.46 ± 0.32

Each value is the Mean ± SD of triplicate analysis

Table 2: Chemical properties of crude and purified liver oil samples of *S. capistratus*

Type of oil	Acid value (mg KOH/g)	Free fatty acid value (%)	Saponification value (mg KOH/g)	Peroxide value (meq/kg)
Crude oil	3.46 ± 0.140	1.54 ± 0.060	198.12 ± 4.204	2.94 ± 0.120
Purified oil	0.12 ± 0.002	0.22 ± 0.004	122.25 ± 2.98	0.52 ± 0.006

Each value is the Mean ± SD of triplicate analysis

Table 3: Fatty acid composition of crude, purified and different concentration of *B. cereus* MSU AS lipase hydrolysed *S. capistratus* liver oils

Fatty acids PUFA	Carbon no.	Crude oil	Purified oil	Lipase hydrolyzed oils		
				0.25	0.5	0.75
Eicosapentaenoic acid (EPA)	C20:5n6	14.61 ± 1.270	12.60 ± 1.440a	18.03 ± 1.680 b	5.07 ± 0.432 c	4.09 ± 0.280d
Docosahexanoic acid (DHA)	C22:6n3	11.05 ± 1.150	10.88 ± 1.186 a	19.40 ± 1.824 b	10.62 ± 1.214 a c	7.46 ± 0.882d
ΣPUFA		33.83	31.32	47.51	25.19	20.35
n3 PUFA		11.77	11.51	20.48	11.57	8.24
n6 PUFA		22.06	19.81	27.03	13.62	12.11

ΣPUFA: total polyunsaturated fatty acids; Fatty acid content is expressed as area for present FAME and % by weight. Data presented as mean values of three samples (Mean ± SD). Within each row, means with different superscript letters are statistically significant ('t'- test; $p< 0.05$ and subsequently *Post hoc* multiple comparision with SNK test). These values do not total 100%, because minor fatty acids are not reported; During hydrolysis process with higher concentration of lipase, all the fatty acids were degraded, therefore the values do not total 100%. nd implies not detectable.

The result indicated that all the chemical properties of purified oil have significantly ($p<0.005$) reduced than crude oil. Various authors have reported that the PUFA concentrate especially the EPA and DHA level of fish oils are varied much depends on the species, food habit of the fish, seasonal availability etc. [12].

Furthermore, during purification process of crude oil, the level of PUFA content which will be reduced considerably [13]. In the present study, also, the same trend was observed, i.e. the EPA and DHA level of crude oil were 14.61 ±1.270 and 11.05 ± 1.150%, whereas these two acids were reduced to 12.60 ±1.440 and 10.88 ± 1.186%, respectively in purified oil (Table 3). The concentrations of PUFA products especially EPA and DHA can be increased through lipase catalyzed reactions. Several authors have reported that the enrichment of PUFA concentrates of fish oils was hydrolyzed by the treatment of microbial lipase to release the amount of free fatty acids [14]. For instance the *Pseudomonas cepacia* lipase (PS-CI) for the production of PUFA through hydrolysis process [15]. In the present study, PUFA enrichment was carried out with the help of lipase catalyzed reaction in *S.*

capistratus purified oil. The initial step for this enrichment process was hydrolysis of purified oil sample with lipase enzyme of *B. cereus* MAU AS. In the present study, the degree of hydrolysis of *S. capistratus* liver oil indicated that the maximum degree of hydrolysis (75.71%) occurred during 24h of incubation with *B. cereus* MSU AS lipase (Figure 1). In accordance with this, evidenced that 60% degree of hydrolysis was recorded in salmon oil after 24h of incubation at 37ºC by using *A. niger* lipase [16].

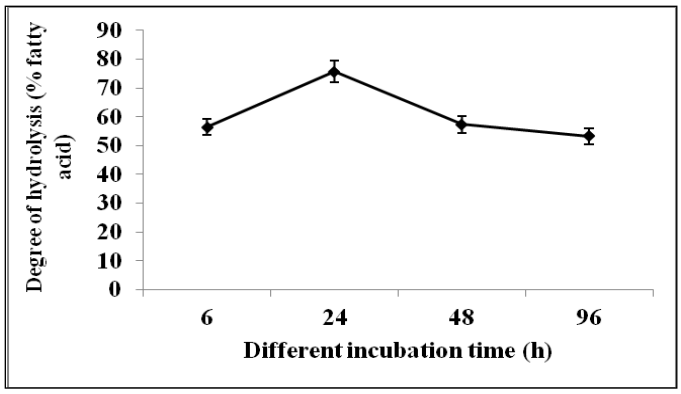

Figure 1: Degree of hydrolysis of purified *S. capistratus* liver oil by *B. cereus* MSU AS lipase during different incubation period

In the present study, purified oil of *S. capistratus* was treated with three different concentrations (0.25, 0.50 and 0.75%) of *B. cereus* MSU AS lipase. The result indicated that both the PUFA components like EPA and DHA levels were considerably increased to 18.03 ±1.680 and 19.40 ±1.824%, respectively in 0.25% *B. cereus* MSU AS lipase treated fish oil after 24h of incubation. When the concentration of lipase supplementation increased (0.50 & 0.75%), the subsequent production of EPA and DHA was significantly ($P<0.05$) reduced (Table 3). These findings were similar to the report of the earlier studies carried out on the fatty acid composition of *O. niger* [17] liver oils. But after hydrolysis with 0.25% lipase of *B. cereus* MSU AS, the composition of PUFA was increased to 47.51%. This showed that the lipase hydrolysed to enrich the PUFA. In accordance with the present study, used purified lipase produced by *Pseudomonas fluorescens* MTCC 2421 for the PUFA enrichment in sardine oil and it resulted that, the enrichment of PUFA from 32.46% to 52.73% [3]. The free PUFA components as well as their oxidative metabolites can be separated by using HPLC or preparative HPLC based on their retention time. The separated 11 kinds of fatty acids including 6 type of PUFA through HPLC after esterification of seafood lipids [18]. In the present study, also the two major components of PUFA concentrate such as EPA (18.20%) and DHA (19.69%) were separated by preparative HPLC (Figure 2).

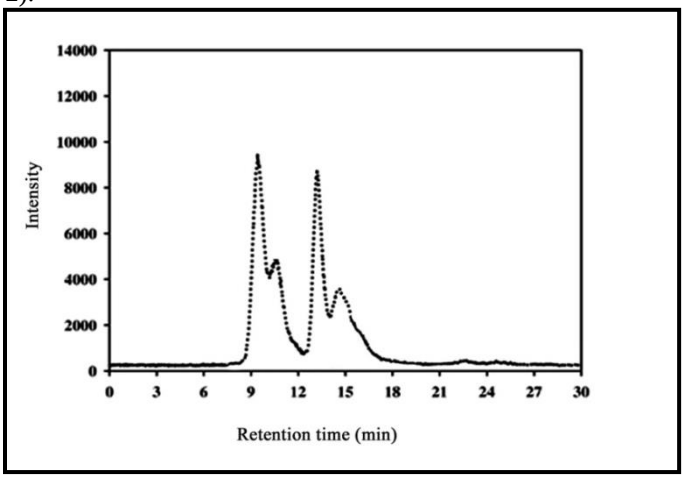

Figure 2: Preparative HPLC analysis of EPA and DHA

1- EPA = RT-9.60, Peak area -18.20% and 2- DHA=RT-13.80, Peak area- 19.69%

CONCLUSION

The pharmacological applications of lipase - mediated PUFA products were studied. For this, the liver sample of an underutilized trash fish- *S. capistratus.* The physicochemical properties of the crude and purified liver oils such as acid value (3.46 ± 0.140 and 0.12 ± 0.002 mgKOH/g), free fatty acid (1.54 ± 0.060 and 0.22 ± 0.004%), peroxide value (2.94 ± 0.120 and 0.52 ± 0.006 meq/kg) and saponification value (198.12 ± 4.204 and 122.25 ± 2.98 mg KOH/g) respectively. Fatty acid profile of both crude and purified oil samples was varied much, for instance, The DHA level of crude and purified oils was 11.05 ± 1.150 and 10.88 ± 1.186% and the EPA level of crude and purified oils was recorded as 14.61 ± 1.270 and 12.60 ± 1.440%, respectively. Further, the purified oil was enriched with 0.25% concentration of *B. cereus* MSU AS lipase through hydrolysis process, the maximum level of enrichment of EPA (18.03 ± 1.680%) and DHA (19.40 ± 1.824%) were recorded. Finally, the PUFA concentrate especially DHA (19.69%) and EPA (18.20%) were separated by preparative HPLC. These PUFA concentrates of both EPA and DHA were used for pharmaceutical industrials.

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