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Optimization of Downstream process parameters for the purification of Docosahexaenoic acid to attain high purity.

Umesh Luthra*[1], Yogesh Patel[2], Aditi Trivedi[3], Mitali Lade[4], Alka Singh[5]
Ipca Laboratories Ltd., Biotech R&D, Kandivali (W), Mumbai- 400067, Maharashtra, India

ABSTRACT
Microalgae have increasingly gained research interest as a source of speciality lipids such as eicosapentanoic acid and docosahexainoic acid which are often reported to provide various health benefits. Extracting and purifying the oil from the algae continues to prove a challenge both in microalgae bioproducts and biofuel. The current study involves the optimization of the purification of Docosahexaenoic acid from fermenter broth. The current study also depicts the role of pH maintenance during the fermentation process. Intermittent feeding of liquid ammonia shows better growth and better yield. The process involves the selection of solvents and optimization of their concentration to attain maximum purity. Ethyl acetate was the most suitable solvent for the final extraction. The initial concentration of ethyl acetate was optimized to be twice the volume of the cake. Sodium hydroxide was the best form of alkali for the maximal removal of free acids.
Keywords: Solvent extraction. Docosahexaenoic acid, Downstream processing, solvent selection

I. INTRODUCTION

Docosahexaenoic acid (DHA) is an omega-3 fatty acid that is a primary structural component of the human brain, cerebral cortex,skin, sperm, testicles and retina. It can be synthesized from alpha-linolenic acid or obtained directly from maternal milk (breast milk), fish oil or algae oil (Guesnet *et al*, 2011).DHA (docosahexaenoic acid) has a wide range of scientifically established health benefits attributed to their consumption. It is believed that the main dietary source of DHA which is fish oil has reached its maximum production, the search is now on for a sustainable alternative. Microorganisms naturally produce this ω-3 fatty acid and in fact they are the primary producers in the food chain. A recent commercial development of this century has been the production of oils rich in DHA from microalgae. Along with being utilized as a part of the infant formula it is recently used in health supplements and enriched food products (Winwood, 2013).

Currently, the most common micro-algae used for the production of DHA rich algal oil and biomass are from the marine members of the families *Thraustochytriaceae* which include the genera *Schizochytrium and Ulkenia.* Efforts are required to improve the growth behaviour of these microbes for the large scale production of DHA.

Microalgae biomass is particularly suitable for extraction and purification of DHA due to its stable and reliable composition. In addition, DHA from cultured microalgae is cholesterol-free, contaminant-free (*e.g.* heavy metals, polychlorobiphenyls (PCBs)) and taste good (Mendes *et al*, 2006). The purification process of DHA purification process microorganism is very attractive and significant particularly for pharmaceutical applications, since the inclusion of a PUFA as a drug component requires its purification to over 95 % (Ratledge *et al,* 2005).

There are several methods of DHA purification but only a few methods are suitable for large scale production. The current study involves the purification of DHA from fermented broth carried out by *Schizochytrium* by selecting the appropriate solvents and optimizing various parameters involved in the process.

II. MATERIAL AND METHODS

The fermentation process for the production of Docosahexaenoic acid was carried out using the microorganism, *Schizochytrium.* The harvested broth was then further processed to get crude DHA

Procedure

Large scale batch fermentation for DHA production was conducted using a 50 L vessel containing 30 L media. Cells were precultured in the basal medium (Dextrose 50g/l, typtone 2.5 g/l, yeast extract 2.5 g/l, salt solution 250ml and trace salt solution 5ml) at 150 rpm and 28°C for 1 day. This preculture was added at a concentration of 10% of seed fermenter volume. The seed fermenter medium contained 50 g/L dextrose, 2.5 g/l of biopeptone, 2.5 g/l of tryptone, salt solution and trace salt solution. The seed fermenter was run at 35 lpm air with a tip speed of 1.57 m/s for 2-3

days. 10 % volume of the production medium of seed was transferred to production fermenter. The production fermenter was run at 36 lpm air with a tip speed of 1.4 m/s throughout the cycle. The sugar was maintained at 1-2 g/l and the pH was maintained at 5.8-6.2 by feeding 50% dextrose and 25 % liquid ammonia respectively. The batch was run for 120 hours. The yield was monitored every 24 hrs by HPLC and on harvesting the broth was further processes to obtain crude DHA.

Crude DHA extraction

The fermented broth was centrifuged at 8000 rpm for 5 mins using the centrifuge. The settled cake was then taken in a round bottom flask and then extracted using a certain volume of ethyl acetate. The mixture was stirred for 2-3 hrs at room temperature. This step was repeated thrice to complete the extraction process. Filtrate was treated with charcoal and then filtered on hyflow bed.The filtrate was concentrated at reduced pressure at 720-740 mm vacuum to give crude oil. The crude oil was further purified by the removal of impurity using solvent crystallization process.

Purification of Crude DHA

The crude DHA was taken in a round bottom flask and a suitable base was added to it under continuous stirring and constant temperature monitoring. The mixture was stirred at room temperature for 3-4 hrs. 2.3 litres of solvent was added to the mixture and stirred for 1 hour. The aqueous layer and emulsion was separated. This process was repeated for twice. The organic layer was washed water till neutral pH. Activated charcoal was added and this was stirred for 4-5 hours at room temperature. The mixture was filtered on hyflow bed. 0.1% of Antioxidant was added to the filtered organic layer and it was concentrated under 700-750 mm vacuum.

Quantification of DHA by HPLC

DHA productivity in the culture broth was analyzed by HPLC method. The extraction of culture broth was done in ethanol. The extracted solution was injected into the HPLC. Hypersil BDS C-18, 100X4.6mm, 5µ column was used for the estimation of DHA. 0.1% ortho-phosphoric acid and acetonitrile was used as a mobile phase. The flow rate was set at 1.3 ml/min. Concentration of DHA was calculated by comparing the obtained peak area with standard area.

Experiments

Maintenance of pH throughout the batch is very essential for optimal growth of the organism, *Schizochytrium* and in turn attain higher yield. Three experimental batches of production fermenter were run where the amount of ammonia feeding were varied. Seed from the same source was inoculated in all the three vessels, A, B and C. Exactly the same process parameters were maintained for all the three vessels.

In vessel A, continuous feeding of ammonia was done at the interval of every two hours up to 120 hrs. In vessel B, ammonia feeding was done up to 50 hours and then stopped and then continued from 100 hours. In vessel C, feeding was done up to 50 hours only.

In order to optimize the purification of DHA various the following experiments were designed.

Selection of solvent

Three different solvents, namely toluene, hexane and ethyl acetate were chosen during the process to obtain pure DHA from crude DHA. They were assessed in terms if solubility and percentage purity.

Volume of ethyl acetate to be used to for the initial extraction step

Different volumes and the number of washes carried out with these volumes were studied and optimized based on the maximum recovery. A 3 times volume of cake with two washes, 6 times volume of cake with single wash, and two times volume of cake with three washes were studied.

Maximize removal of impurity

The quicker and better the removal of impurity, the better and faster is the the purification process. The concentrated mass obtained after removal of ethyl acetate was dissolved in different volumes of hexane that is 0.5, 0.75 and 1 times the volume of the concentrated mass. The assessment was based on the maximal removal of impurity.

Optimizing the type of alkali for the removal of free acids

Free radicals interfere with the purification process. Effective removal of the free acids is very important. Sodium hydroxide, sodium carbonate and sodium bicarbonate were used to check the removal of free acids.

III. RESULTS AND DISCUSSION

Fermentation

Maintenance of pH is very crucial for attaining optimal growth of the culture and also getting high yield.

Table 1: Type of vessel based on feeding and their yield

Vessel	Yield g/l
A	6.584
B	8.741
C	4.512

Table 1 represents the vessel type with varied feeding and the yield obtained. Vessel B shows the maximum yield which is 8.741 g/l. Here the feeding was stopped at 50 hours and then started at 100 hours. The lowest yield is observed in vessel C where feeding is stopped at early age which is 4.512 g/l. Ammonia plays a in maintaining the pH thus supporting the proper growth of the cells. It is well utilized by *Schizochytrium*. The yield was low in the vessel with continuous ammonia feeding as higher concentrations of ammonia have a negative impact on the growth rate (Yaguchi et al, 1997). Feeding of ammonia as per requirement as done in vessel B works in maintaining the pH as well as, lower concentration of ammonia supports faster growth of cells in turn increasing the yield (Ganuza et al, 2008).

Purification of DHA

In microalgal biotechnological processes, the downstream stage can account for 50–80% of total production costs, depending on the biochemical characteristics of the compound and the purity ratio that needs to be achieved. (Molina Grima *et al*., 2003). Hence the proper selection of the solvents and the process parameters is very crucial to ensure higher yield and better purity.

Selection of solvent

The results obtained in terms of recovery are depicted in table 2

Table 2: Extraction recovery of the product with different solvents used.

Solvent	Extraction recovery
Ethyl aceate	95%
Toluene	70%
Hexane	80-85%

As per the table it was observed that maximum extraction was attained with Ethyl Acetate . Use of Toluene led to formation of emulsion making it difficult for physical separation of toluene layer.As ethyl acetate has some solubility in water it penetrates easily and leads to degradation of cell wall and extracts the oil due to its solubility. Since Hexane almost insoluble in water, extraction efficiency is on lower side.

Volume of ethyl acetate to be used to for the initial extraction step

The recovery of the product during the initial step of the extraction along with the number of washes and the volume of the ethyl acetate used of the washes is given in table 3.

Table 3: Volume of wash and number of washes with recovery

Volume of wash	No of washes	Recovery %
3	2	85 to 90
6	1	80 to 85
2	3	92 to 96

Maximum recovery was seen when 2 times the volume of ethyl acetate of the cake was used with three washes. 3 times the volume with 2 washes also gives relatively similar results but is not cost effective. The six times volume was does not give good recovery. Multiple washes gives better recovery.

Maximize removal of impurity

The concentrated mass was treated with a certain volume of hexane to remove the impurities. The amount of hexane was based on the amount of impurities to be removed. Maximum removal of was obtained by 1.0 volume of hexane and at lower temperature 0-5°C . This method is cost effective as well as lesser volume of solvent is consumed.

Optimizing the type of alkali for the removal of free acids

Different types of alkali were used to check the removal of free acids. The maximum permissible limit for free acid is <0.5. Table 4 shows the type of alkali and its removal limit.

Table 4: Alkali along with its removal limit for free acids.

Alkali	Removal Limit
Sodium hydroxide	<0.5
Sodium carbonate	<1.0
Sodium bicarbonate	<1.5

It was observed that maximum removal of free acids is obtained with sodium hydroxide.It is necessary that the removal of free acids is maximum so that the interference with the purification process is prevented to the maximum extent.

IV. CONCLUSION

Maintenance of pH by ammonia was studied in the fermenter. It was observed that intermittent feeding of ammonia gave best results in terms of pH maintenance and productivity. In the current study it was observed that maximum recovery and purity was attained by using acetone as the main solvent for the extraction process. In the initial extraction process 2 times volume of

ethyl acetate with three washes shows maximum recovery. On treating the concentrated mass with hexane, maximum recovery was obtained and sodium hydroxide was the best for the removal of free acids. Process optimization in this manner gave maximum purity. The rising awareness around the globe about the health concerns and well being hold enormous potential in the future for the production of microbial fatty acids. There are only few commercial production process exists which partially fulfills the ever growing demand. In-order to fully harness the natural ability of the microorganisms to produce the fatty acids, rapid progress in bridging the gap between the metabolic engineering and process engineering should be acknowledged. Further, advancements in the knowledge about the physiology of the microorganisms, biochemistry of accumulation, use of renewable sources and sustainable production systems will have to be accomplished.

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