Asian Biomedicine Vol. 6 No. 5 October 2012; 693-701 DOI: 10.5372/1905-7415.0605.109

**Original article**

# Effect of sodium sulphate salinity for production of docosahexaenoic acid (DHA) by *Thraustochytrids aureum* RAK-21

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***Background:*** The demand for DHAs in human nutrition, fish feeds, and pharmaceutical applications is rapidly growing and it will become inadequate for supplying the expanding market. In order to meet the expected rise in the demand and to circumvent the drawbacks of the fish oil, it is important to develop new sources of this important nutrient, which leads the need for alternative production processes for DHA.
***Objectives:*** The effect of sodium sulphate salinity on Docasahexaenoic acid (DHA) synthesis in *Thraustochytrids aureum* RAK-21 was studied with media constitution as well as conditions in batch fermentation.
***Methods:*** *Thraustochytrids aureum* RAK-21 resuspended cell system using shake ask fermentation was deployed to study the production of DHA synthesis. The effects of key parameters such as salinity, different types of carbon sources, nitrogen sources, temperature, and pH on DHA production were also investigated.
***Results:*** The sodium sulphate salinity was much influenced in growth as well as DHA production as compared with sodium chloride in medium. The results showed that sodium sulphate (30g/L) in the presence of peptone (15g/L) and sucrose (20g/L) were the most effective medium for higher DHA production at pH 7 and 35 C.
***Conclusions:*** The present study reveals that sulphate ions are an important element for effective DHA synthesis along with sodium ions. It may influence the primary or co-metabolism pathways of DHA and other Long-chain polyunsaturated fatty acid (LCPUFA) production.

***Keywords:*** Docasahexaenoic acid (DHA), long-chain polyunsaturated fatty acid (LCPUFA), sodium sulphate, *Thraustochytrids aureum* RAK-21

Docasahexaenoic acid (DHA) is a major component of fish oil and is a long-chain polyunsaturated fatty acid (LCPUFA) of the n-3 or omega-3 type. It is considered as an essential fatty acid in that humans cannot synthesize it, and it must be obtained through diet [1]. LCPUFAs control the expression of specific genes [2], which affect processes in the body such as cholesterol transport and fatty acid biosynthesis. In addition, omega-3 fatty acids regulate the structure, permeability, and various other aspects of the cell membrane. The multi-potential of LCPUFA in regulation of cellular metabolism makes it seem to be required in every organ in order to keep the organ functioning normally. Moreover, LCPUFA deficiencies are generally considered as contributing to abnormalities in various systems in our human body such as nervous, immune, skin, inflammatory, cardiovascular, endocrine, kidneys, respiratory, and reproductive systems [3]. The ω-3 and ω-6 series of polyunsaturated fatty acids (PUFA) have shown tremendous potential for use in food additives and pharmaceuticals for heart and circulatory disorders and cancer as well as inflammatory diseases [4, 5].

Moreover, DHA is naturally transferred to the fetus during pregnancy through the placenta. Rapid brain development takes place during initial childhood (12 months of life). Infants who are breastfed have significantly higher DHA level in their brains as compared to infants fed non-DHA supplemented infant formula. The best sources of DHA are seafood, algae, and especially cold-water fish. Omega-3 fatty acids are natural antifreeze. In cold water, the percentage

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of the omega-3 oil is higher in the popular fish sources such as salmon, sardines, and tuna. Eggs and organ meats also have a small amount of DHA, but the healthiest source of dietary DHA is seafood. Two 4-ounce servings of omega-3-rich fish per week should yield a sufficient amount of omega-3 fatty acids, especially DHA. However, there are some problems in natural fish oil such as contamination of the fish by environmental pollution, fishy smell, unpleasant taste, and less oxidative stability in fish DHA.

Beside the *Thraustochytrids*, the other microorganisms including bacteria (*Bacillus megaterium, Bacillus pumilus, and Pseudomonas aeruginosa)*, yeasts (*Candida curvata, Rhodotorula glutinis, Lipomyces lipofer)*, and fungi (*Aspergillus terreus, Claviceps purpurea, Mucor ramannianus, Tolyposporium ehrenbergii*, and *Mortierella alpina)* are producing the DHA and other LCPUFA [6, 7].

In addition, microalgae also produce omega-3 fatty acids and this source eliminates many of the undesirable factors of traditional sources. In addition, microalgae can supply omega-3 fatty acids at high concentrations. Microalgae can also be grown on low to no-cost nutrients, which make them an economically viable source of omega-3 fatty acids [1]. Transgenic plants have also been considered as an alternative source of omega-3 fatty acids. Robert *et al.* [8] were able to express a set of genes related to the elongation and desaturation of LCPUFA fatty acids in *Arabidopsis thaliana.*

The demand for DHAs in human nutrition, fish feeds, and pharmaceutical applications is rapidly growing. It is therefore expected that, in the near future, the production of purified DHA from current sources will become inadequate for supplying the expanding market. In order to meet the expected rise in demand and to circumvent the drawbacks of the fish oil, it is important to develop new sources of this important nutrient, which leads the need for alternative production processes for DHA. Microbial oil or single cell oil (SCO) production is a relatively new concept, first proposed in the twentieth century [9]. In SCO processes, microorganisms that are able to produce the desired oil can be cultivated in a bioreactor. Microorganisms that are able to produce more essential lipids because of their high growth rate on simple media and the simplicity of their manipulation are a valuable alternative to more traditional sources of nutrient lipids.

The present study was undertaken to investigate the effect of different types of carbon source, nitrogen sources, and salinity on the production of DHA by *Thraustochytrids aureum* RAK-21 resuspended cell system using shake ask fermentation. The effects of several other parameters such as temperature and pH have been also studied.

## Material and methods

### *Microorganism and media*

The strain, *Thraustochytrids aureum* RAK-21 was used throughout this study. *Thraustochytrids aureum* RAK-21 is marine protists, whose dominant genera are *Thraustochytrium* and *Schizochytrium* [10], belongs to the Kingdom Chromista and Class Labyrinthulomycetes. This strain was isolated at Department of Biotechnology, Rajiv Ghandhi Technological University, India. The strain was maintained in the form of dry powder spores stocked at -20 °C. The strain was transferred into Liquid Broth (LB) agar slant for 48 hours at 37°C.

### *Fermentation*

The seed stock cell suspension was prepared from single colony with the following media compositions (glucose 10 gm/L, yeast extract 10 gm/L, NaCl 10 gm/L, and $KH_2PO_4$ 1.0 gm/L). Batch fermentation carried out in 500 mL shake flask containing 200 mL medium. The sterile flask and medium inoculated with seed suspension ($1x10^5$ cells/mL) was used to start the fermentation. The flask was incubated at 30 °C on a rotary shaker agitated at 250 rev/min. The experiment flow of this study is shown in **Figure 1**.

### *Separation of cell pellets and dry weight determination*

The various intervals of the fermented broth were used for separation of cell pellets for DHA extraction. The fermented broths were centrifuged in 10956 RCF for 10 min at 27 °C (Avanti J-20 I, Beckman Coulter, USA, Rotor No. JLA 8.1000). The cell pellets were washed with phosphate buffer (pH7; 0.1 M) for three times and it subsequently centrifuged in above conditions. The wet cell pellets were divided in to two parts about 30 grams each for extraction of DHA and dry biomass analysis respectively. The 30 grams of pellets (wet cell weight) were transferred to a pre-weighed container and it was incubated in 80 °C for 24 hours for dry weight analysis. The other 30 grams of well cell pellets were used for extraction of DHA.

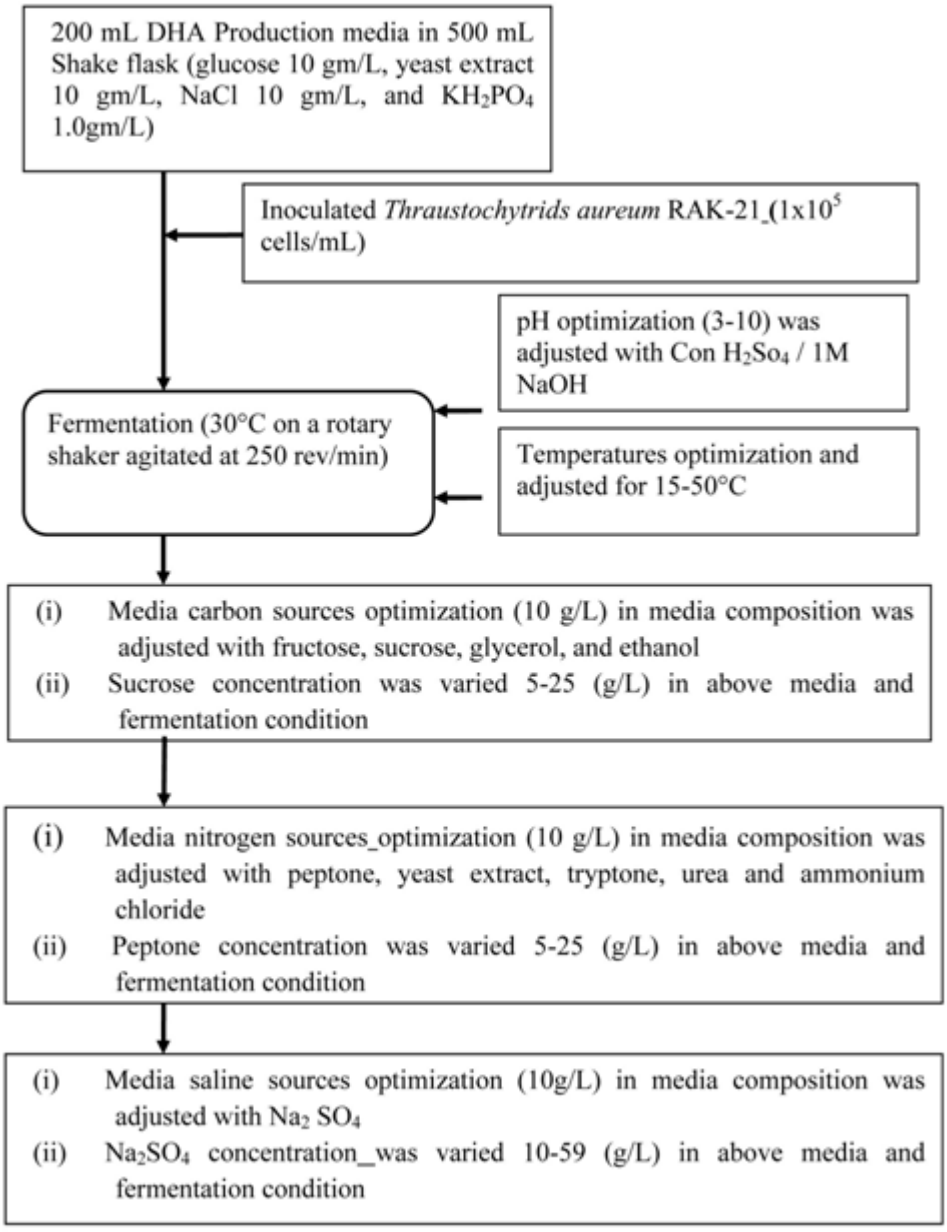


**Figure 1.** The experimental flow

***Extraction of PUFA and DHA***

The 30 grams wet cell pellets were treated with methanol 1:2 ratio (g/g) and allowed to incubate for one hour with while being stirred using a magnetic stir bar. The cells were considered as being lysed after treatment with methanol and the periplasmic oil was released. The released oil was washed with distilled ethyl acetate (5 mL of $CH_3$-$COOC_2H_5$ for one gram of pellet) for an hour on the hotplate magnetic stirrer (60 °C) (Schott, SLR, Nr 00998058, Germany) and then ethyl acetate was separated from the mixture by fractional distillation (55-60 °C) (Rotavac Senso, Labo rota 4003, Heidolph). Moreover, the precipitant oil contains the small amount of aqueous portion and it was treated with hexane. The nonpolar compound fatty acid (PUFA) was mixed with hexane and separated by separation funnel. The collected product was allowed to dry to remove the excess of solvent (Hexane). The sample was reweighed during the drying process and drying was continued until the extracted lipids achieved a constant value.

***Analytical methods***

The cells were counted using a Thoma haemacytometer. The sample was withdrawn at various time intervals during the fermentation under the variable culture conditions described above. The cell suspension samples were centrifuged (Avanti J-20 I, Beckman Coulter, USA, Rotor No. JLA 8.1000) at 10956, RCF for 10 minutes at 27 °C and the supernatants were used for glucose determination. The wet pellet was used for extraction of the PUFA. Glucose concentrations were determined by the dinitrosalicylic acid (DNS) method [11]. Residual oil was analyzed for PUFA composition using gas chromatography [7] with comparison to a standard mixture of fatty acids containing linoleic acid, linolenic acid, EPA and DHA. The given oil treated with methanol and KOH (3N) for methylation and it produced methyl ester derivatives of PUFA (FAME). The condition for estimation of PUFA in GC was used [7] and retention time of DHA was 55.8 minutes.

**Results and discussion**

***DHA production in Thraustochytrids aureum* RAK-21**

***The effect of pH***

**Figure 2** shows the synthesis of DHA in *Thraustochytrids aureum* RAK-21 in batch fermentation. These results suggested that pH 7.0 with media compositions (glucose 15 gm/L, yeast extract 15 gm/L, NaCl 10 gm/L, and $KH_2PO_4$ 1.0gm/L) were able to produce more DHA (0.14 g/g) compared with acidic and basic medium. Similarly, the biomass was also increased in neutral pH and that lead to the production of the maximum DHA in fermentation.

Maximum DHA were produced at the end of the exponential or the early stationary phase. To obtain the maximum biomass with a high amount of total lipids in the shortest possible time represent one of the strategies in media development for DHA production [12].

***The effect of temperature***

The same media was used for this study with temperature variances between 15-50 °C. **Figure 3** shows that maximum biomass as well as DHA (0.15 g/g) production was obtained at 35 °C. Normally, the temperatures of 25-30 °C generally favor the optimal growth in *labyrinthulids* [13-16]. However, the low temperatures also stimulate DHA production, but negatively affect growth, thus resulting in low overall DHA yields. There were two phases of temperature used in DHA production such as initially being grown at a higher temperature (e.g., 25°C) to stimulate growth and later under cold conditions (15 °C) to enhance DHA yield [17, 18]). Moreover, Jain et al. [19] stored harvested biomass at 10 C for 24 to 48 hours to increase absolute levels of DHA, thus circumventing the expense involved in large-scale growth at below-ambient temperatures.

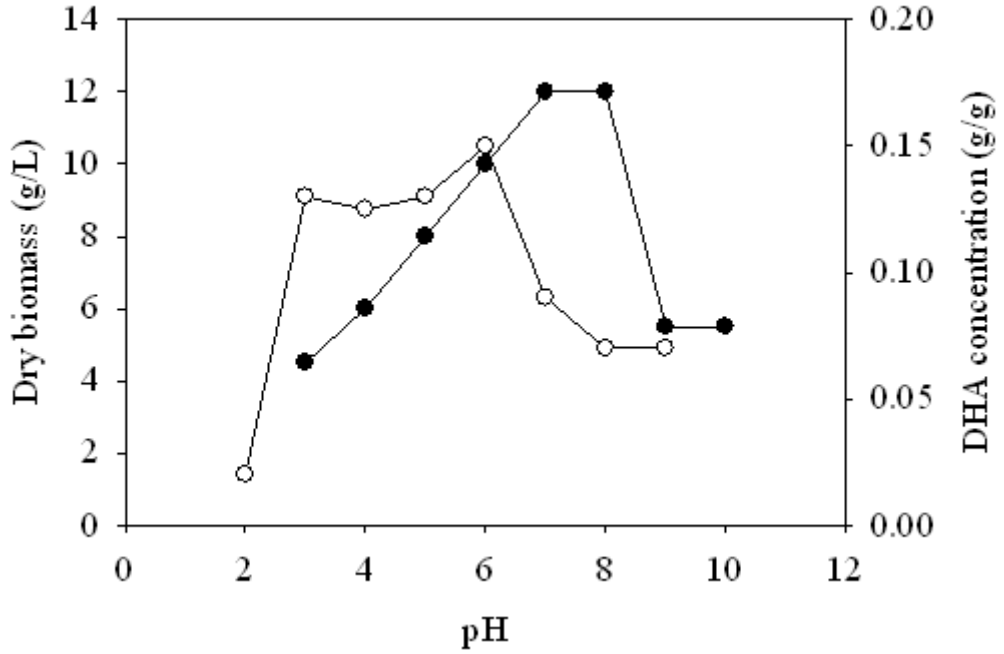


**Figure 2.** Effect of various pH on the DHA production in the fermentation by *Thraustochytrids.* • represents dry biomass and % refers to DHA

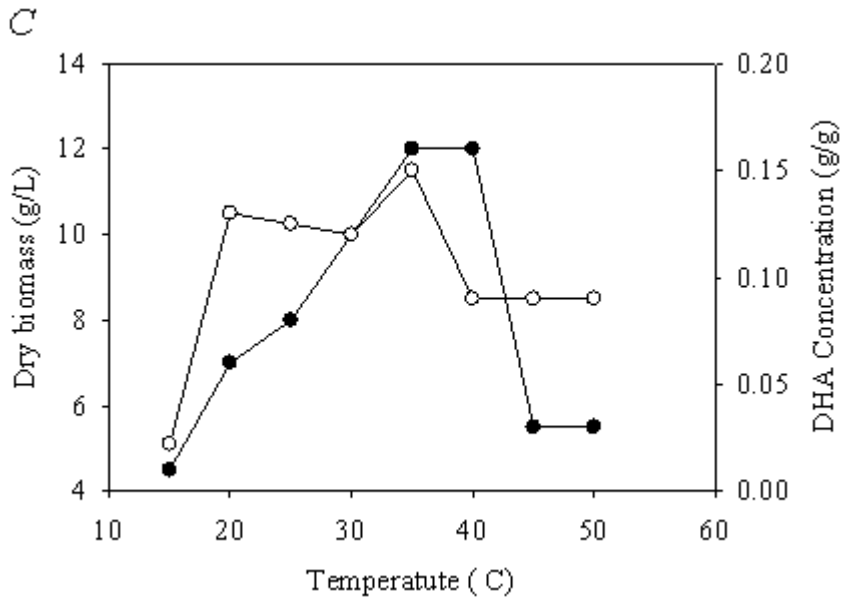


**Figure 3.** The effect of various temperatures on DHA production in during the fermentation by *Thraustochytrids.* • represents dry biomass and % refers to DHA

***The effects of carbon sources***

The right amount of C/N ratio is necessary for optimal biomass production as well as any metabolic product formation. The various carbon sources such glucose, fructose, sucrose, glycerol, and ethanol were used in this study, and the data are shown in **Figure 4.** Only glucose and sucrose were able to produce more DHA as compared with fructose, glycerol, and ethanol.

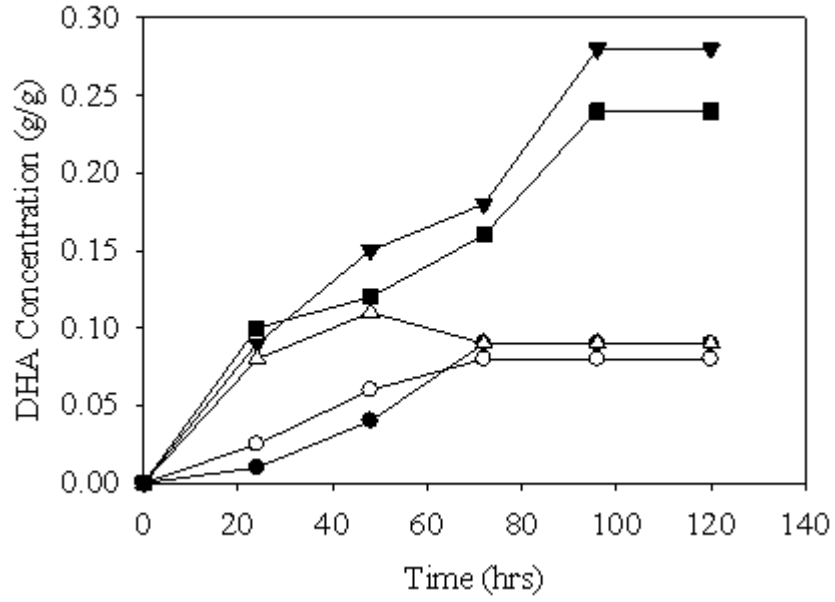


**Figure 4.** The effect of various carbon sources on DHA production during the fermentation by *Thraustochytrids.* In the figure, • represents ethanol, = glycerol, Δ = fructose, ▼ = sucrose, and ■ = glucose as sources of carbon

The maximum DHA production (0.28 g/g) was in sucrose media followed by glucose media (0.24 g/g) after 100 hours of incubation. The fructose was not able to produce high amounts of DHA due to the poor enzyme activities of xylose isomerase in the *Thraustochytrids* [20]. The other carbon sources such as glycerol and ethanol, which also can produce the DHA, provided for low yields of both biomass and DHA. They were not competitive with sucrose and glucose as sources of carbon.

Similarly, the sucrose concentration was varied on DHA production. The sucrose concentration was varied (5-25 g/L) with media in pH 7, 35 °C, and DHA production showed in **Figure 5**. The result revealed that 20 g/L sucrose was optimal concentration of sucrose for the production of DHA.

***The effects of nitrogen sources***

The various nitrogen sources such as peptone, yeast extract, tryptone, and inorganic nitrogen such as urea and ammonium chloride were studied for production of DHA. However, only peptone was producing high amounts of DHA (**Figure 6**) when compared with other organic nitrogen as well as inorganic nitrogen sources.

The inorganic nitrogen sources produced the least amounts of biomass as compared with organic nitrogen sources (data not shown). Moreover, among all the organic nitrogen sources, peptone was the only one to produce the maximum DHA concentrations (0.36 g/g) which was 4 fold higher than inorganic nitrogen sources such as urea and ammonium chloride. In addition, the peptone 15 g/L in medium, which was produced in maximum DHA concentration (0.36 g/g), compared the other peptone concentration in fermentation (**Figure 7**).

***The effects of salinity***

*Thraustochytrids* has an obligate requirement for $Na^+$ ions that cannot be replaced by $K^+$. They are ubiquitous in both coastal and an oceanic habitat where they often attain substantial biomass and possibly play an important role in the marine ecosystem [12].The sodium ion is very important for phosphate ion uptake in sodium phosphate ion channel. This sodium can be supplied in the form of sodium chloride or sea salt. However, half strength seawater was more effective for the production of DHA as well as biomass production of *T.roseum* [21].

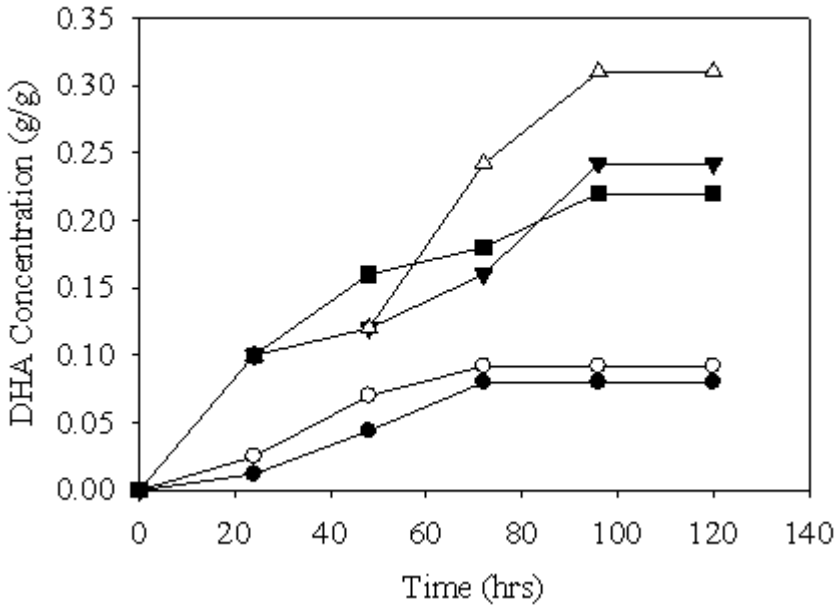


**Figure 5.** The effect of various concentrations of sucrose for DHA production in during the fermentation by *Thraustochytrids.* In the figure, • represents 5 (g/L), ○ = 10 (g/L), ▼ = 15 (g/L), Δ = 20 (g/L), and ■ = 25 (g/L) concentrations

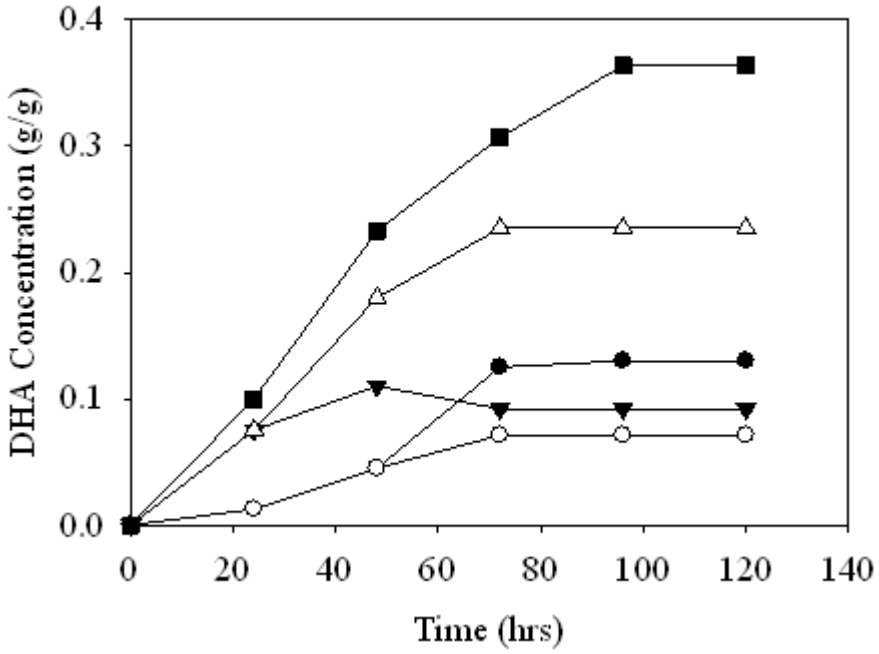


**Figure 6.** The effect of various nitrogen sources on DHA production in during the fermentation by *Thraustochytrids.* In the figure, • represents trypton, = urea, Δ = yeast extract, ▼ = ammonium chloride, and ■ = peptone as sources of nitrogen

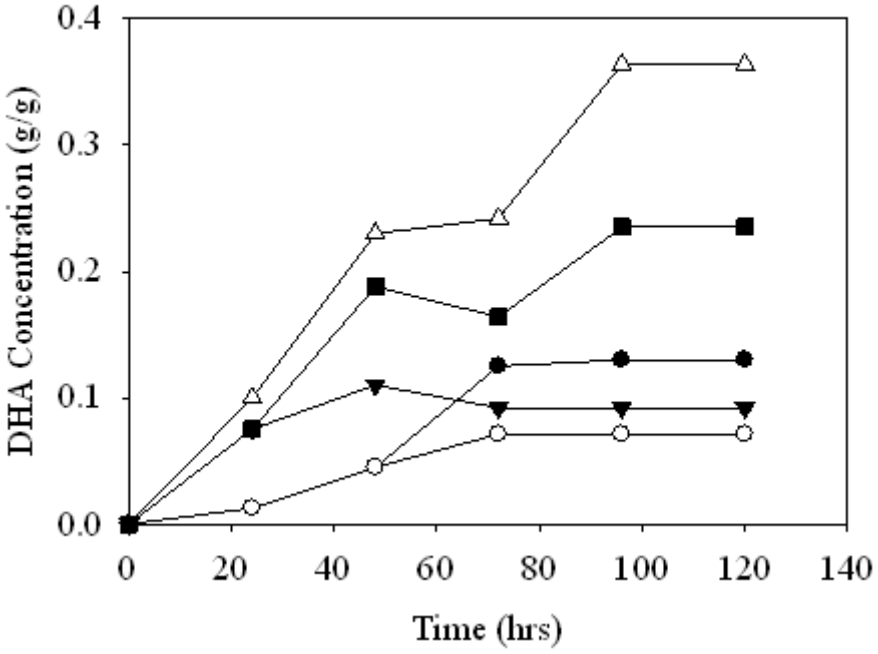


**Figure 7.** The effect of various concentrations of peptone on DHA production during the fermentation by *Thraustochytrids.* In the figure, represents 5 (g/L), • = 10 (g/L), Δ = 15 (g/L), ■ = 20 (g/L) and ▼ = 25 (g/L) concentrations

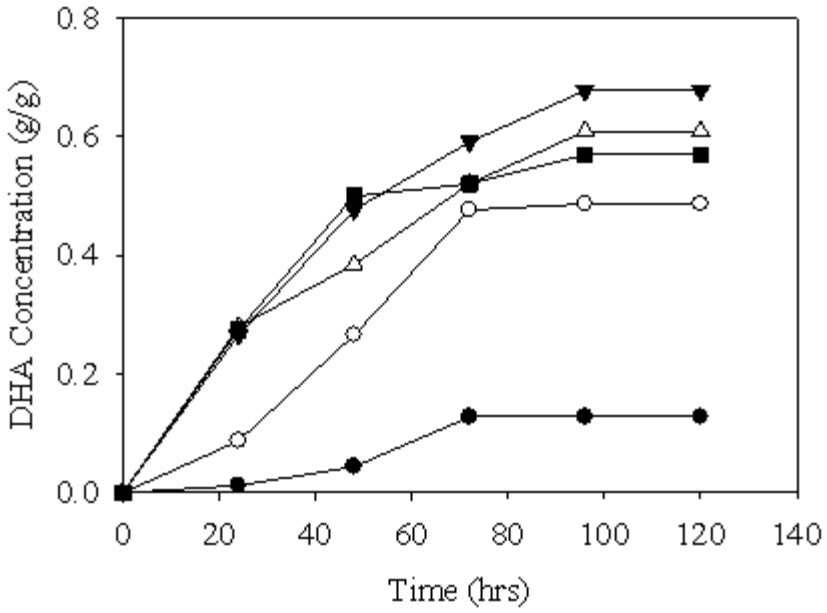


**Figure 8.** The effect of various concentrations of sodium sulphate for DHA production in during the fermentation by *Thraustochytrids*. In the figure, • represents 10 (g/L), = 20 (g/L), ▼ = 30 g/L, Δ = 40 (g/L), and ■ = 50 (g/L) concentrations of sodium sulfate

*Thraustochytrids* did not increase the DHA production in above optimized media in presence of sodium chloride (data are not shown). However, the *Thraustochytrids* produced more DHA in presence of $Na_2$ $SO_4$ with similar biomass production. There were no changes in different concentrations of sodium chloride for DHA production even it has slight difference in biomass (13-14 g/L). However, the $Na_2$ $SO_4$ concentration had much influence on DHA production. **Figure 8** suggests that maximum amount of DHA production (0.68 g/g) was obtained in 30 g/L of sodium sulphite in the medium containing peptone as well as sucrose. It was observed that changes in DHA production in saline medium such as sodium chloride as well as sodium sulphate (which have common $Na^+$ ions) are present. Moreover, the $SO_4^-$ ions are also much important for DHA production along with the $Na^+$ ions. The above results revealed that sulphate ions in combination with sodium ions are important element for DHA synthesis and it may have influences in the primary or co metabolism pathways.

## Conclusion

The strain *Thraustochytrids* showed a greater potentiality to produce DHA in presence of sodium sulphate salinity in fermentation. The results showed that sodium sulphate (30 g/L) in the presence of peptone (15 g/L) and sucrose (20 g/L) were the most effective medium for higher DHA production at pH 7 and 35 °C. The increase of DHA production will greatly reduce the production cost that leads to economic viability for healthy humans.

All authors have no conflict of interest to declare.

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