ISOLATION AND CHARACTERIZATION OF NOVEL *AURANTOCHYTRIUM* LIKE ORGANISM FOR PRODUCTION OF BIO OIL

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Abstract: Polyunsaturated fatty acid (PuFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are important components for infant as well as adult nutrition as PuFAs serve as structural elements of cell membranes, and have an influence on retinal and brain development. Polyunsaturated fatty acids though currently are obtained from higher plants and oily fish, it is evident its production is inadequate to supply the expanding market and alternate sources need to be screened.

Anovel microalga was isolated from the mangrove region of Mumbai. Identification was based on microscopic characteristics of the isolate obtained. The isolate when grown on medium containing glucose 1%, yeast extract 0.1% and peptone 0.15% produced 76.8±2.23%% of dry cell mass as lipid, of which 40% of total fatty acid was DHA, while 25% was palmitic acid along with others that occurred in insignificant concentration. This data represents the first study on the isolation of *Aurantochytrium* like organism across the coast of Mumbai yielding DHAat a concentration that is higher than that reported till date.

Keywords: Microalga, Lipids, Docosahexaenoic acid, Palmitic acid.

Introduction: Docosahexaenoic acid isan essential poly unsaturated fatty acid (PUFA), recognized as an importantnutraceutical, due to the many health benefits offered. DHA is an important nutrient responsible for infant brain and retinal development, cardiovascular diseases [1], rheumatoid arthritis, arteriosclerosis etc. It is an essential fatty acid known to be responsible formaintenance of brain function in adults as it is a major component of gray matter.[2] [3]. Currently DHA is extracted from oily fishes. However, due to the over increasing demand of fish and organoleptic characteristics of fish oil, microalgal oil serve as superior alternative source of oil[4]. Palmitic acid on the other hand is a saturated fatty acid mainly used in cosmetic industries and also in bio fuel based industries.

Many dinoflagellates and micro algae have been explored for production of Bio oil, with Labyrinthulomycetes being one of the commercially viable microalgae. Microalgae belonging to class Labyrinthomycota are rich in DHA content and have comparatively simpler PUFA profile. *Thraustochytrids* are ubiquitous found on decaying plant material, such as seaweed and submerged leaves, and are capable of producing extracellular enzymes such as cellulases, amylases, chitinases, proteases, lipases that chemically alter and mineralize detritus via its ectoplasmic net system [5]. Several studies have suggest that heterotrophic cultivation of *Aurantochytrium*results in high production of biomass and accumulation of high lipid content in cells [6] [7]. Due to their fast growth rate and high lipid content, *Thraustochytrids*thus have potential for producing a feedstock for biofuels and omega-3 oils for nutraceuticals, as well as animal feeds. In this study we have isolated and studied an *Aurantochytrium* like organism from

mangrove region of Mumbai, capable of producing high amount of lipids.

Materials and methods:

Isolation of heterotrophic microalgae: Various pneumatophores, leaves and sediment from the coastal mangrove regions of Mumbai were collected in 20-mL vials containing 10 mL of sterile, 0.2-µm filtered, artificial seawater (ASW) supplemented with 300 mg L^{-1} penicillin, and 500 mg L^{-1} streptomycin. The samples were vortexed vigorously and inoculated at 1% concentration into 10 ml of B1 broth (Glucose 1%, yeast extract 0.1% and sea salt 3.4%). The B1 broth vials were incubated under static conditions for 72 hours after which the enriched sample was serially diluted and plated onto B1 agar plates containing 300 mg L^{-1} penicillin, and 500 mg L^{-1} streptomycin. Each colony so obtained was streaked onto fresh B1 agar plate without antibiotic and the process was repeated until axenic heterotrophic microalgae were obtained. Isolates were observed microscopically to examine the size and morphology. Organisms showing similarity to microalgae were selected for further analysis [8].

Lipid staining: Nile red(9-diethylamino-5H-benzo[α]phenoxazine-5-one) is a lipid soluble fluorescent dye that mainly binds with the intracellular neutral lipids. Nile red was therefore used to screen the lipid rich isolates as per Bertozzini, 2011, from the isolates obtained after primary screening process. Fluorescence of Nile red was visualized using Axio Scope.A1 microscope, at 490nm and 525 nm excitation and emission wavelength respectively. [9]

Dry cell weight determination: A single colony of 48 hour old isolate grown on B1 agar was inoculated into 50ml B1 broth, and incubated at 28°C at 120 rpm for 48 hours to be used as 2% inoculum. The inoculated media was incubated at 28°C at 120 rpm for 96 hours. Dry cell weight (DCW) was estimated by harvesting cells by passing through a preweighed filter of 1µ pore size. The culture thus obtained on the filter was washed with phosphate buffer pH7.4 and dried at 90°C overnight in a hot air oven and weighed repeatedly till a constant weight was obtained.

Lipid analysis: Total lipid content was calculated using a modified miniaturized Bligh-Dyer method [10].All experiments were performed in triplicates for statistical analysis.

GLC analysis for fatty acid: PUFA profile was studied by GC after esterification using methanolic sulphuric acid as per Hong 2011. Qualitative analysis of PUFA profile was carried out by GC-MS (GC-HP5 column, Agilent 7890) with manual injection, 1:20 split column. The temperature profile was similar to that used for GC-FID analysis as discussed below. Mass spectrometer model used was AccuTOF GCV and operating conditions were: mass range 10-2000amu, Mass resolution – 6000. Mass spectra were acquired and processed using time of flight analyzer.

Quantitative determination of DHA using GC – FID: Quantitative determination of DHA using GC – FIDwas undertaken using an instrument equipped with flame-ionization detector (FID) and an SP 2300 column having length of 2m and internal diameter of 2mm. The column temperature was raised from 150°C (after 2 min of holding) to 220°C (with a further 2 min of holding) at a rate of 5°C per min. The temperature of injection port was 220°C while that of the detector was used at 230°C. Nitrogen was used as carrier gas. Fatty acids were identified by comparing retention time of standard fatty acid methyl esters purchased from Sigma Aldrich.

Results and Discussion:

Screening: Single, irregular, hyaline colonies made up of spherical or limaciform cells and atypical of either yeast or bacterial colonies were

picked up and subcultured at least three times on B1 plates for purity. Isolate so obtained in axenic form after the screening program based on its morphology was identified as *Aurantiochytrium* like spps.as microscopic analysis of the isolate showed spherical cells occurring as singles or in clusters which showed presence of refractile granules within the cellsby Phase contrast imaging (Fig. 1a) which were confirmed by fluorescent lipid staining using Nile red(Fig. 1b). Nile red when dissolved in organic solvent has high affinity for hydrophobic environment at wavelength ≤570nmand specifically binds to the neutral lipids. Microscopic examination showed presence of lipid granules stained as orange red under fluorescent microscopy. [11]

Lipid analysis: Since lipid accumulates intracellularly, increase in biomass is an important prerequisite. Additionally, accumulation of neutral lipids is governed by available C:N ratio provided during growth . Lipid accumulation occurs during nitrogen starvation and under carbon excess. Therefore, the medium composition was composed to contain higher glucose in ratio to that of nitrogen source.The isolate was found to attain maximum biomass of 6.67±0.18g/L after 72 hours of growth and could accumulate lipidupto76.8±2.23% of their cell mass which is higher than any reported value to our knowledge. The total fatty acid content was found to be5.1g/L

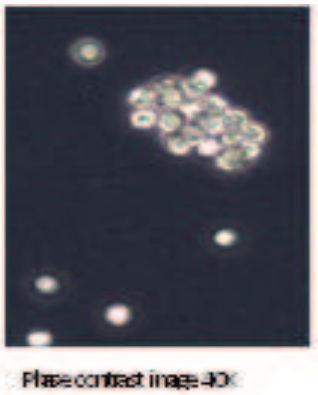

Fig 1a. Phase contrast image (40X)

Fluorescent Lipid staining (40X)

Fig 1b. Fluorescent staining using Nile red (40X)

GC analysis: Qualitative analysis of PUFA profile of the isolate was studied by GC-MS analysis. MS identification showed dominance of odd chain saturated fatty acids like penta decanoic acid(15:0; RT 9.1), hepta decanoic acid(17:0; RT 11.7) and an even chain saturated fatty acid hexa decanoic acid/palmitic acid(16:0; RT 10.7)docosahexaenoic acid(22:6; RT 16.9) along with a small amount of eicosapentaenoic acid (20:5; RT 14.5) and docosa pentaenoic acid (22:5). Linoleic acid (18:2) was completely absent(Fig 2).

Food', is an omega 3 fatty acid and occupies the major section of market of infant formulae. In India, DHA is recently included as fortifying agent in milk supplement that included product like bournvita and horlicks. A part from these products a lot of

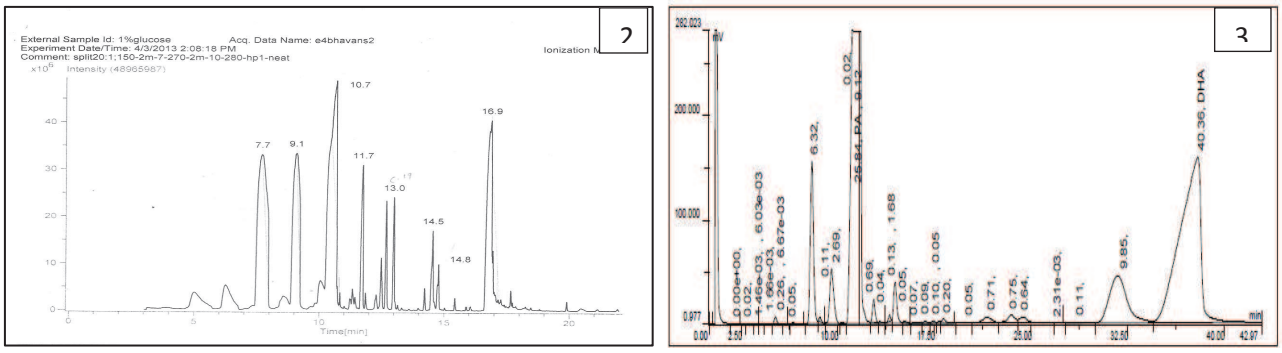

Fig 2: Chromatogram showing PUFA profile of BhP22 generated by GC-MS

Fig 3: PUFA profile of Bhp22 generated by GC-FID key: plamitic acid, DHA:

Total DHA yield was calculated by GC-FID and was found to be 40.36% (Fig. 3) of total fatty acid calculated by area percentage method. The total DHA yield from the isolate BhP22 was calculated to be 1.97g/L Thus DHA was the major fatty acid produced by our isolate , apart from DHA the isolate also produced Palmitic acid as second major fatty acid contributing to 25% (Fig. 3) of the total fatty acid. To add up to the advantage, all the other class of fatty acids were produced in very low quantity , which makes downstream processing easier at the industrial scale.

DHA and palmitic acid both are very important products commercially in the market with DHA having high demand in the field of nutrition while palmitic acid is used extensively in cosmetics and detergents. DHA, also known as 'Brain food', is an omega 3 fatty acid and occupies the major section of market of infant formulae. In India, DHA is recently included as fortifying agent in milk supplements that includes products like Bournvita and Horlicks. Apart from these products a lot of Fig 3: PUFA profile of BhP22 generated by GC-FID. Key: PA: Palmitic acid, DHA: Docosahexaenoic acid

other food products including eggs and fish are being taken under consideration for DHA fortification, as the dried microalgae like *Schizochytrium* has GRAS status and is used as feed to broiler chickens and laying hen feed to enhance DHA in the meat and eggs [12] [3]. It is also recommended to pregnant and nursing women. *Schizochytrium* oil, known as DHA Gold, is marketed by Omega Tech Inc to supplement animal feeds [13].Palmitic acid on the other hand is a saturated fatty acid is an important ingredient in cosmetics and biodiesel industries. Hydrogenation of palmitic acid produces cetyl alcohol which is used in shampoos, moisturizersand lotions as thickening agent. Apart from occupying cosmetic industries, it is also an important component of biodiesel industries [14].

Conclusion: Thus our isolate was isolated from Mangrove region of Mumbai in an axenic form shows very high potential to produceintracellular lipids, with DHA and palmitic acid being the dominant group of fatty acids and thus can find industrial applications.

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