Docosahexaenoic acid (DHA) Producing Thraustochytrid Strain - SC1

Field of Invention:

The invention relates to the screening of a novel unreported strain of Thraustochytrid – SC1 producing comparable amounts of Docosahexaenoic acid isolated from the backwaters of Goa by the Pollen baiting method. The fatty acid profiles of the isolated organism indicating the production of the omega-3 fatty acids in significant amounts are disclosed. Also revealed is the 18S rRNA sequence of the isolated organisms indicative of their molecular phylogeny.

Background of Invention:

Interest in the nutritional importance of polyunsaturated fatty acids (PUFAs) has increased markedly during the past decade. Polyunsaturated fatty acids are long chain fatty acids containing two or more double bonds. Interest in them arises from their potential in therapeutic, food and nutritional applications. They are produced commercially from the selected seed plants and some marine sources. But the production of the purified PUFAs from the current sources will become inadequate for supplying the expanding market (Gill and Valivety 1997). In order to meet the expected rise in demand and to circumvent the drawbacks of fish oils, alternative production processes for the PUFAs are currently being developed.

PUFAs are grouped in 2 series on the basis of the position of the terminal bond being 3C or 6C from the terminal carbon atom of the fatty acid chain. They are generally classified into 2 main groups, the omega-6 (ω6 or n-6) and omega-3 (ω3 or n-3 series). Of the omega-6 fatty acids, arachidonic acid is gaining particular importance as it is the major precursor of many prostaglandins and eicosanoids and among the omega-3 fatty acids, Docosahexaenoic acid is currently receiving much attention and have been termed as "Essential Fatty Acids".

Docosahexaenoic acid (DHA), the unsaturated, 22-carbon long, omega-3 fatty acid is commonly found in fish and marine plants. It has gained major significance owing to the fact that it is the major building block of the human brain tissue and is the primary structural fatty acid in the gray matter of the brain and in the retina. Recent findings have shown a correlation between low levels of DHA and certain behavioral and neurological conditions associated with aging such as dementia, depression, memory loss and visual problems demonstrating the physiological importance of DHA for humans.

The WHO recently has recommended to cover 1-2 % of the daily intake of energy from the omega-3 fatty acids corresponding to about 2.2 to 4.4 g daily based on the 2000 calorie diet intake. The intake of omega-3 PUFAs via our diet occurs mainly through the consumption of sea food, which is characteristically rich in omega-3 PUFAs. The average intake varies among the populations. This strong demand has resulted in the introduction of large scale marine fish farms and the normal growth and development of several marine fish larvae depend on the supplementation of the omega-3 polyunsaturated fatty acids in the diet especially DHA and eicosapentaenoic acid (Rodriguez et al.1998)

At present selected fish oils and microalgal species are the main industrial sources of DHA. Fish oils with the highest levels of EPA and DHA include mackerel, herring and Salmon. Some fish such as cod, haddock, store most of the fat in the liver. The best sources though are cold-water fish such as tuna, mackerel, sardines, herring and lake trout. But to obtain the maximum benefits of DHA from fish oils, one has to preferably eat raw fish or boiled fish and moreover one should eat the skin behind the gills around the fins and along the belly as these areas are where most of the oil is stored. It must be pertinently noted that light, heat and oxygen all diminish the EPA and DHA in fish oils. Because fish oils are highly polyunsaturated, they become rancid quickly. Rancid fish smell fishy and hence are not very appetizing. The application of PUFAs from fish oils or their inclusion in infant formulas also has many disadvantages. Fish oils generally contain eicosapentanoic acid, an undesirable component in infant formulas because it leads to reduced arachidonic acid levels in infants. This has been correlated with the reduced rates of infant weight gain.

Also, supplies of fish oil may be unreliable due to failure or variability of various fisheries. There is concern that sufficient fish oil will not be available to meet the global demands of DHA.

As an alternative to fish oil, PUFAs can be obtained from microorganisms. In particular, the marine algae are thought to be the primary producers of the omega-3 polyunsaturated fatty acids in the marine food chain. These marine microorganisms represent the greatest percentage of the undescribed marine species (Colwell 1997) of which the marine microalgae form a large potential source of Docosahexaenoic acid.

The potential source of n-3 oils is the group of micro-heterotrophs called Thraustochytrids. These are a group of non-photosynthetic, heterotrophic organisms presently classified under the Stramenophila kingdom, together with oomycetes and labyrinthulids. Members of the genera Schizochytrium and Thraustochytrium have been studied as potential omega-3 producers for industrial use due to their high lipid content and high levels of DHA. These species are sometimes also classified as marine fungi. Thraustochytrids are the common name of the microheterotrophs that feed as saprobes or occasionally as parasites. Thraustochytrids have a wide geographical distribution with strains isolated from Antarctica, North Sea, India, Japan and Australia. (Reviewed by Lewis et al., 1999).They are rarely found on living plants and appear to be inhibhited by plant anti-microbial agents. Members of these groups are often abound on dead autochthonous as well as allochthonous plant material such as macro algae and submerged Mangrove leaves. They are common in the neritic and the oceanic water column and the sediments including the deep sea.

Compared to fish oils, microbes can provide a stable supply of fatty acids that can easily mass produced, have a less fishy smell and highly purified DHA and other PUFAs.

Several recent studies have catalogued the ability of thraustochytrids strains to produce

1. A high biomass in culture
2. A high proportion of lipid as a part of this biomass
3. A high proportion of the PUFAs in the lipid

Most reports concerning the production of PUFAs by Thraustochytrids have dealt almost exclusively with DHA production as this compound is the most abundant PUFA produced by many of the strains of thraustochytrids reported to date.

High levels of DHA are also found in dinoflagellates, such as Crypthecodinium cohni and the Amphidinium species. Data available in the scientific literature demonstrate the large variation in biomass, lipid and maximum DHA yields obtained for different Thraustochytrid strains. For example, Schizochytrium aggregatum produced a biomass of 0.9g per litre after 10days (Vazhappilly and Chen,1998), while a biomass of 48g per litre after 4 days was achieved using Schizochytrium sp. SR21 (yaguchi et al., 1997). It is very clear that development of a microbial PUFA production process requires the selection of the proper microorganism and the optimized culture techniques.

To achieve an increased production of PUFA rich products, the following key strategies need to be negotiated:

1. Further isolation, screening and the maintenance of PUFA-producing strains: Several strains with the potential for the commercial production of DHA-rich oils have already been isolated. However, if thraustochytrids that produce higher PUFA yields and /or more attractive PUFA profiles can be isolated and optimized. Demands for these isolates and the compounds may well increase.
2. Optimisation of efficiency of PUFA production: The types and the amounts of the PUFAs produced by individual strains of thraustochytrids are susceptible to manipulation by varying culture conditions. Enhancement of the PUFA profiles using molecular techniques may also be considered. Different markets will provide demand for strains that produce high levels of PUFAs measured either in terms of the biomass (i.e., PUFA production w/w cell mass) or volume (i.e., Production w/v fermentation medium).
3. Schizochytrium sp. is used to produce DHA by heterotrophic cultivation (OmegaTech, Boulder, Colorado, USA). A major drawback of the Schizochytrium strains is the production of the omega-6 docosapentaenoic acid (DPA) in the microbial oils, in addition to DHA (Nakhara et al.1996: Yokochi et al.1998: Ratledge 2001). The nutraceutical properties of DPA are currently not well known and therefore its presence in oils for food and pharmaceutical applications is undesirable. Separation of DPA from DHA is difficult and expensive.

The present invention relates to the isolation of several strains of thraustochytrids, a few strains of which produce significant amounts of Docosahexaenoic acid (DHA; C22:6, n-3) that were screened from the dead leaves and dendrites of marine vascular plants called Mangroves collected from the backwaters of Goa. These strains will have major significance for the commercial production of Docosahexaenoic Acid.

Prior Art:

Huang et al., have described the grouping of newly isolated docosahexaenoic acid producing thraustochytrids based on their polyunsaturated fatty acid profiles and the comparative analysis of the 18S rRNA genes. Seven strains of marine microbes producing a significant amount of DHA were screened from the seawater collected in coastal areas of Japan and Fiji. They accumulate their intermediate respiratory intermediate fatty acids in addition to DHA. These isolates were proved to be new thraustochytrids by their specific insertion sequences in the 18S rRNA genes. The phylogenetic tree constructed by the molecular analysis of the 18S rRNA genes from the isolates and the typical thraustochytrids shows that the strains with the same PUFA profile form each monophyletic cluster. These results suggest that the C20-22 PUFA profile may be appreciable as an eefective characteristic for grouping thraustochytrids.

Fan et al., have published in the Journal of Industrial Microbiology & Biotechnology (2001) the article describing Eicosapentaenoic acid and Docosahexaenoic acids production by nine thraustochytrids strains and the okara-utilizing potential of the thraustochytrids. These thraustochytrids strains have been isolated from the subtropical mangroves and were screened for the production potential of the above fatty acids in glucose yeast medium. Their ability to utilize okara (Soymilk residue) for growth and EPA and DHA production was also evaluated. The EPA yield was low in most of the strains, while DHA level was high on the glucose yeast ext. medium producing 28.1-41.1% of the total fatty acids, for all strains, with the exception of the Ulkenia sp. KF13. The DHA yield of the Schizochytrium mangrovie ranged from 747.7 to 2778.9 mg/L after 52 hours of fermentation at 25C.

The publication titled "Profiles of polyunsaturated fatty acids produced by Thraustochytrium sp KK17-3 " relates to the isolation of more than 300 strains of microorganisms producing polyunsaturated fatty acids (PUFA) were newly isolated from the coastal seawater in the Seto Inland Sea and around the Iromote Island, Japan, by the baiting method. The profiles of PUFA from the DHA producing strains were classified into four types. A strain named KK17-3 was then chosen for further study owing to its high DHA content.(52.1% of the total fatty acid.) and a wide range of other PUFAs including arachidonic, eicosapentaenoic acid and docosapentaenoic acid as well as DHA. Molecular phylogenetic analysis of the 18S rRNA gene sequences showed KK17-3 to be a thraustochytrids.

Yet another publication by Bowles et al., relates to Long chain n-3 polyunsaturated fatty acid production by members of the marine protistan group of thraustochytrids. Screening of the isolates were undertaken followed by the optimization of DHA production. The isolation program was undertaken from three different locations, 57 isolates were screened for their biomass, oil and docosahexaenoic acid production (DHA). DHA represented 50% of the total fatty acids present in these isolates. Some of the studies have also indicated that a medium of high C:N ratio stimulated DHA production . The optimum DHA production was 2.17 g per litre after 107 hours of cultivation.

The publication titled " Fatty acid composition and the Squalene content of the Marine micro algae Schizochytrium mangrovie ". This publication relates to the identification of the fatty acid composition and the squalene content in the thraustochytrids S.mangrovie that was newly isolated from the decaying Kandelia candel leaves in the Hong Kong mangrove habitat. The major fatty acid constituents in all the three mangrovie strains were tetradecanoic acid, hexadecanoic acid, docosapentanoic acid, and docosahexaenoic acid. DHA was the most predominant polyunsaturated fatty acid and the percentage of DHA (of total fatty acids) in all these strains varied from 32.29 to 39.14%.

The patent No WO9801536 describes the microorganisms belonging to the genus Shewanella or pseudoalteromonas which can produce docosahexaenoic acid within a short culture period.

Our invention relates to identification of 10 unreported strains of Thraustochytrids inhabiting in the dead leaves and the dendrites of marine vascular plants called Mangroves from the backwaters of Goa. Subsequent to isolation, GC-MS analysis for the organisms were carried out to determine their total fatty acid content. The 18S rRNA sequences of these organisms indicate their phylogeny. On futher study, these organisms can be efficient large scale producers of Docosahexaenoic acid.

Figures:

Fig.1 Fatty acid profile of different strains of Thraustochytrids

Sequence Listings:

Seq ID 1: represents the 18S rRNA sequence of SC1
Seq ID 2: represents the 18S ribosomal sequence of AVE1
Seq ID 3: represents the 18S ribosomal sequence of AVE2
Seq ID 4: represents the 18S ribosomal sequence of AVE3
Seq ID 5: represents the 18S ribosomal DNA sequence of AVE4
Seq ID 6: represents the 18S ribosomal DNA sequence of AVE5
Seq ID 7: represents the 18S ribosomal sequence of AVE6
Seq ID 8: represents the 18S ribosomal sequence of AVE7
Seq ID 9: represents the 18S ribosomal DNA sequence of AVE8.
Seq ID 10: represents the 18S ribosomal sequence of AVE9.

Detailed description of the Invention:

Thraustochytrium strains are inhabited in the dead leaves and dendrites of marine vascular plants called Mangroves. Dead leaves from these plants were collected from the backwaters of Goa and isolated by pollen baiting method. The leaves were cultured in sterile seawater with antibiotics (streptomycin and penicillin) and pine pollen grains for three days. These pollens were re-inoculated into fresh sterile seawater with antibiotics. After 3 days of incubation the pollens were examined under the microscope. 10 different strains of thraustochytrids were found and these were plated on MV (Rock salt -3.4%, Peptone - 0.15%, Yeast Extract- 0.1%, Glucose-2%, KH_2PO_4- 0.025%, pH 7) agar plate for 5 days with antibiotics. The thraustochytrids form colony/ layer on the plate. These Thraustochytrids were inoculated in fresh MV media with antibiotics. After 3 days of growth the pure culture were obtained. Thraustochytrids were cultured for a period of 5 days and the cells subjected to GC-MS analysis for determining the total fatty acid content.

Screening of thraustochytrids for identifying strains which produce high amounts of DHA .

Total lipid was extracted from the cultures using Folch method of extracting with 2:1 v/v of chloroform: methanol. For quantification of total lipid as well as individual fatty acids, an internal standard (heptadecanoic or pentadecanoic acid) was added before extraction. Fatty acids were extracted, esterified and GC-MS analysis was carried out with Agilent 6890 N gas chromatograph connected to Agilent 5973 mass spectrometer and GC on HP 6850 Series gas chromatograph equipped with a FID detector. The GC-MS detection was performed at 70 eV (m/z 50-550; source at 230 °C and quadruple at 150 °C) in the EI mode with a capillary column (30 m, HP-5ms, WCOT, i.d. 0.25 mm, film thickness 0.25 μm, oven 2 min at 150 °C, 6 °C min^{-1} to 300 °C, 20 min at 300 °C, Helium carrier gas flow, 1.0 ml/min, split ratio 50:1). For GC-FID, the capillary column DB-23 (30m,

WCOT, i.d. 0.25 mm, film thickness 0.5 μm) was used. The oven temperature was programmed as 2 min at 160 °C, 6 °C min^{-1} to 180 °C, 2 min at 180 °C, 4 °C min^{-1} to 230 °C and 10 min at 230 °C, with N_2 carrier gas flow, 1.5 ml/min, and injector temp at 230 °C and detector temp at 250 °C; split ratio 50:1.
The fatty acid profile of the 10 strains isolated is represented in the **Fig.1**

The fatty acid profile of these strains suggests that the AVE5, AVE7 and SC-1 produce comparable amounts of DHA. AVE1 is also a fair producer of DHA. However, the other strains do not produce large amounts of DHA.

18s rRNA sequencing of the Thraustochytrids

1.7kb 18s rRNA sequence of SC-1, AVE5 and AVE7 which had high DHA content was amplified from the genomes using primers to the conserved regions. The amplified fragment was sequenced. The sequences are represented in the sequence listing. Thus the three strains – SC-1, AVE5 and AVE7 are seen to be producers of DHA These strains have been deposited at the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH) ,Chandigarh, India.

These organisms on further study can prove to be very efficacious in the commercial production of DHA in large amounts.

We claim,

1. The microorganisms producing Docosahexanoic acid (DHA) and other omega-3 fatty acid/ fatty acid intermediates selected from the group comprising the genus Thraustochytrium.

2. The omega-3 fatty acid producing microorganisms of Claim1, comprising the 18S RNA sequence represented by the SEQ ID 1.

3. The omega-3 fatty acid producing microorganisms according to Claim 2, producing greater than 30% of Docosahexaenoic acid.

4. The omega-3 fatty acid producing microorganisms according to Claim 2, producing greater than 25% of Docosahexaenoic acid.

5. The omega-3 fatty acid producing microorganisms according to Claim 2, producing greater than 20% of Docosahexaenoic acid.

6. The omega-3 fatty acid producing microorganisms according to Claim 2, producing greater than 15% of Docosahexaenoic acid.

7. The omega-3 fatty acid producing microorganisms according to Claim 2, producing greater than 10% of Docosahexaenoic acid.