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**Isolation and characterization of a novel thraustochytrid-like microorganism that efficiently produces docosahexaenoic acid**

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## **Abstract**

A thraustochytrid-like microorganism (strain 12B) was isolated from the mangrove area of Okinawa, Japan. On basis of its ectoplasmic net structure and biflagellate zoospores we determined strain 12B as a novel member of the phylum Labyrinthulomycota in the kingdom Protoctista. When grown on glucose/seawater at 28 °C, it had a lipid content of 57.8% with DHA of 43.1% of the total fatty acids. It had a growth rate of 0.38 h<sup>-1</sup>. The DHA production rate of 2.8 ± 0.7 g l<sup>-1</sup>day<sup>-1</sup> is the highest value reported for any microorganism.

## **Introduction**

Interest in n-3 polyunsaturated fatty acids (n-3 PUFAs) began some 30 years ago and it is now known that n-3 PUFAs are important for human nutrition. n-3 PUFAs such as docosahexaenoic acid (DHA; 22:6n-3) are the building structures of membrane phospholipids of nervous, visual, and reproductive tissues cells and have physiologically important functions in humans (reviewed by Kroes *et al.* 2003, Gill & Valivety 1997) and animals (Bezard *et al.* 1994). Marine fish-oil is the major source of DHA and is often used in pharmaceuticals and/or food supplements. Oils that are extracted from cultured phototrophic microalgae have also been marketed in recent years (Lewis *et al.* 1999, Sijtsma & Swaaf 2004). However, fish and micro-algal oils have complex PUFA profiles which make the cost of preparation of highly pure individual PUFA oils very high. Oils produced by fermentation of thraustochytrids and *Cryptocodinium cohnii* as a dinoflagellate which are another source of PUFAs, preferably contain one specific PUFA especially DHA (Sijtsma & Swaaf 2004) and are now commercially available as poultry feed and as a feed for aquaculture. Moreover, the use of DHA-containing oils in adult nutritional supplement will start in near future (Ratledge 2004) and those from *C. cohnii* are used extensively in infant formulas (Kyle *et al.* 1995).

Thraustochytrids are a marine microheterotroph and are commonly isolated from

various samples such as seawater, estuarine water, sediments and fallen leaves of mangrove forests (Lewis *et al.* 1999). There is large variation in biomass, lipid, and DHA yields of thraustochytrid strains, and the yields depend on the strains used and their cultivation conditions (Singh & Ward 1996, Vazhappilly & Chen 1998, Aki *et al.* 2003, Sijtsma & Swaaf 2004). *Schizochytrium limacinum* SR21 produced 4.2 g DHA l<sup>-1</sup> in 5 days in shake-flask cultures (Yokochi *et al.* 1998). The commercially used *C. cohnii* produced 11.7 g DHA l<sup>-1</sup> after 9.2 days in fed-batch culture (Swaaf *et al.* 2003). To our knowledge *S. limacinum* SR21 is in general the best DHA-producing strain. It is therefore unfortunate, however, that *S. limacinum* SR21 has never been commercially utilized as a source of DHA-containing oils.

The main focus of the present study is to isolate microorganisms of which the DHA productivity is superior to that of *S. limacinum* SR21. Such microorganisms are expected to be of interest as a source organism for the commercial production of DHA.

## **Materials and methods**

### *Microorganisms and cultivation*

To isolate DHA-producing microorganisms, fallen leaves from the mangrove area in Okinawa Prefecture in Japan were collected. These leaves were directly placed on agar plates comprising of 0.5% (w/v) glucose, 0.1% (w/v) yeast extract, 0.1% (w/v) peptone and 50% (v/v) seawater, 1% (w/v) agar, and streptomycin and penicillin G ( $0.3 \text{ g l}^{-1}$  each) (designated as By+ medium) and incubated at 28 °C for several days. Colonies were taken from plate and re-grown in 50 ml flasks with By+ medium. After cultivation for 2–3 days at 28 °C a small portion of the liquid cultures was spread on agar plates of By+ medium and cultivated for several days. Pure isolates were obtained by the streak plate technique. The liquid cultures were then centrifuged at  $14,000 \times g$  for 20 min and cells were directly transmethylation (see below) after washing twice with 1% (v/v) NaCl and once with distilled water. Natural seawater was utilized after filtration throughout this work.

#### *Optimization of the cultivation of strain 12B*

For optimization strain 12B was cultured in basal F medium consisting of 5% (w/v) glucose, 1% (w/v) yeast extract, 1% (w/v) peptone and 50% (v/v) seawater. The pH of the basal F medium, which was originally around 6, was not adjusted unless otherwise stated. Strain

12B was pre-cultured at 28 °C with shaking at 180 rpm for 1 day and then approx. 0.1 ml of the pre-culture was transferred to 10 ml fresh medium in a 50 ml flask to give an OD value of 0.1 at 600 nm. Thus, the inoculum size was 1% (v/v) or less. Cultivation was performed for 2–3 days under the same conditions. For optimizing biomass, total fatty acid (TFA), and DHA yields glucose and seawater concentrations in medium F were changed but the pH was not adjusted. When necessary, pH of medium F was adjusted with NaOH or HCl. The biomass (cells) from each culture was harvested and washed twice with 1% (v/v) NaCl, and finally washed with distilled water. The weight of pellet was measured after each pellet was lyophilized for more than 1 day. The growth of isolates in liquid medium was measured at OD<sub>600</sub>.

#### *Lipid extraction and fatty acid analysis*

Total lipids were extracted from lyophilized cells by the method of Bligh & Dyer (1959) and weighed. Ten mg of total lipid and dry cells were directly transmethylated with aqueous 2 M hydrochloric acid in methanol at 100 °C for 1 h (Gaver & Sweeley 1965, Takakuwa *et al.* 2002). As an internal standard, heneicosanoic acid (21:0) was added to the reaction mixture. Resultant fatty acid methyl esters were extracted with *n*-hexane and then analyzed by gas

chromatography and gas chromatography/mass spectrometry as described previously (Orikasa *et al.* 2004). The yield of lipid was expressed as the amount of TFA or total lipid per liter of culture.

#### *Morphological observation of strain 12B*

The strain was morphologically studied by light microscopy and transmission electron microscopy.

### **Results and discussion**

#### *Isolation of DHA-producing microorganisms*

Only one DHA-producing microorganism out of 26 was obtained from leaf samples. It was a thraustochytrid-like microorganism (see below). Since this strain, named as 12B, had a high content of DHA up to 40% of the total fatty acids, it was utilized for further characterization.

### *Microscopic observations of strain 12B*

Figure 1A shows a cluster of vegetative cells of strain 12B. The cells formed ectoplasmic net elements. The heterokont biflagellate zoospore of strain 12B was clearly observed and the anterior long flagellum possessed tubular hairs (Figures 1B and C). From these results strain 12B should be classified into the class Labyrinthulea of the phylum Labyrinthulomycota in the kingdom Protoctista (Porter 1989).

### *Effects of growth temperature on DHA yield of strain 12B*

Strain 12B had the ability to grow at range temperatures varying between 15.0 °C–32.9 °C. The yield of biomass ( $\text{g l}^{-1}$ ) slightly increased with increasing temperature (Figure 2A). In contrast, however, the amount of TFA ( $\text{g l}^{-1}$ ) decreased with increased temperature. The profile of fatty acids scarcely changed in a range between 25 °C–30 °C. The highest yield of 3.7 g DHA  $\text{l}^{-1}$  was obtained at 28 °C.

### *Effects of glucose concentration on DHA yield of strain 12B*

When cultivated at 28 °C for 2 and 3 days strain 12B maximally produced 4.6 and 6.8 g DHA l<sup>-1</sup>, respectively, with 8% (v/w) glucose (Table 1). The content of DHA varied between 40–48%, depending on the concentration of glucose and the duration of cultivation.

### *Effects of seawater concentration on DHA yield of strain 12B*

The biomass yield at 25% (v/v) and 50% (v/v) seawater was approx. 24 g l<sup>-1</sup> and it was significantly decreased to approx. 8 g l<sup>-1</sup> at 0% seawater (Figure 2B). The proportion of DHA was 41–42% at 25–100% (v/v) seawater and it was decreased to 35% in medium containing no seawater. The maximal yield of 3.2 g DHA l<sup>-1</sup> was obtained at 50% seawater.

### *Effects of pH on DHA yield of strain 12B*

Strain 12B was cultivated at 28 °C for 2 days in media containing 8% (w/v) glucose, 1% (w/v) yeast extract, 1% (w/v) peptone, and 50% (v/v) of seawater of which the pH was adjusted to a range between 2–9. The strain could completely not grow at pH 2 and 9. The

levels of biomass increased with increasing pH and maximized at pH 8. The fatty acid composition of dry cell from the culture (pH 8) is given in Table 2. The maximal yield of 5.6 g DHA l<sup>-1</sup> was obtained (Figure 2C).

#### *Growth rate and lipid content of strain 12B under optimal conditions*

When grown under the optimal conditions in F medium strain 12B reached the stationary phase within 2 days (Figure 3). The maximum growth rate of strain 12B was 0.38 h<sup>-1</sup> (13.2 doublings day<sup>-1</sup>). This growth rate is notably much higher than those (less than 10 doublings day<sup>-1</sup>) of some ATTC *Schizochytrium* and *Thraustochytrium* strains and DHA-producing microorganisms screened and isolated by Barclay (1992).

The content of total lipid was 12.4 ± 0.4 g l<sup>-1</sup> and the total lipid was 57.8 ± 5.5% (w/w) of the biomass after 2 days. The proportion of DHA in total lipid was 43.1 ± 0.3%.

When the DHA yield of 5.6 g l<sup>-1</sup> after 2 days was reached, the DHA production rate a day of strain 12B under optimal conditions was thus calculated as 2.8 g l<sup>-1</sup> day<sup>-1</sup>. This value is to our knowledge the highest ever reported for flask-cultured DHA-producing microorganisms (Sijtsma & Swaaf 2004).

Some microorganisms grow less efficiently in flasks than in bioreactors (e.g.

fermenters) where aeration and agitation speed triggers the growth of cells (see Yaguchi *et al.* 1997 and Yokochi *et al.* 1998). One of the most notable characteristics of strain 12B is that it has a very high growth rate. Optimizing cultivation conditions for strain 12B using bioreactors would probably result in even higher yields of biomass and DHA per time. Another characteristic of this strain is that its fast growth-rate is made possible by a relatively small inoculum, which would be of great benefit for industrial exploitation of this strain.

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*Table 1.* Effects of glucose concentration on DHA yield of strain 12B.

Table 2. Fatty acid composition of strain 12B grown under optimal conditions.

## Figure legends

*Fig. 1.* Micrographs of vegetative cells of strain 12B with ectoplasmic net elements (A), a zoospore with two heterokont flagella laterally inserted (B), and a high magnification image of tubular hairs on the anterior long flagellum (C). (A), Nomarski interference contrast image. (B) and (C), negative stained images. Arrows indicate tubular hairs. Scale bar; 20  $\mu\text{m}$  (A), 2  $\mu\text{m}$  (B), and 50 nm (C). Living cells with ectoplasmic nets, which were grown in autoclaved seawater on a slide glass for 17–18 h at room temperature, were observed with a light microscope (type Eclipse E600; Nikon Corp., Tokyo, Japan). For fine-structural studies, the strain was grown in By+ medium for 7–8 h at 25 °C. Specimens for negative staining were fixed with 2% (v/v) glutaraldehyde. After stained with 4% (w/v) uranyl acetate as described (Haschemeyer & Meysers 1972) they were observed with a transmission electron microscope JEM1200EXS (JEOL Ltd., Tokyo).

*Fig. 2.* Effects of growth temperature (A), seawater concentration (B), and pH on biomass, TFA, and DHA yields of strain 12B. Cells were grown at 28 °C (B and C) for 2 days with shaking at 180 rpm in F medium consisting of 5% (w/v) glucose (A), 1%

(w/v) yeast extract, 1% (w/v) peptone, and 50% (v/v) seawater (A and C). In (A) and (B) pH was not adjusted. In (B) and (C) glucose concentration was 8%. Hatched and dotted bars represent dry biomass and TFA, respectively. Solid line shows DHA yield. Values represent the averages of triplicate determinations.

*Fig. 3.* Growth profile of strain 12B under optimal conditions.

*Table 1.* Effects of glucose concentration on DHA yield of strain 12B.

Time (day)	Glucose (% [w/v])	Dry biomass (g l <sup>-1</sup> )	TFA <sup>a</sup> (g l <sup>-1</sup> )	DHA content of TFA (%)	DHA yield (g l <sup>-1</sup> )
2	5	21.2±2.0 <sup>b</sup>	9.8±0.3	40.1±1.1	3.9±0.2
	8	22.5±0.6	11.4±0.9	40.1±1.2	4.6±0.3
	10	20.5±0.2	8.3±0.7	44.6±1.8	3.7±0.2
	12	17.4±3.1	7.4±2.6	44.3±2.4	3.2±1.0
	15	15.4±1.1	5.0±0.9	42.9±1.0	2.1±0.4
3	5	20.1±0.5	7.6±0.2	43.4±1.7	3.3±0.1
	8	30.9±0.5	16.0±1.9	42.4±3.8	6.8±0.2
	10	27.5±1.5	13.7±0.5	42.4±2.9	5.8±0.6
	12	27.6±1.0	13.6±0.7	43.9±1.3	6.0±0.4
	15	24.3±0.4	12.3±1.2	47.5±0.4	5.9±0.6

<sup>a</sup>Total fatty acids

<sup>b</sup>Values represent the averages of triplicate determinations.

Table 2. Fatty acid composition of strain 12B grown under optimal conditions.

Fatty acid, % (w/w)										
14:0 <sup>a</sup>	15:0	16:0	17:0	18:0	20:0	20:4	20:5n-3	22:5n-6	22:6n-3	Others <sup>b</sup>
2.5±0.2 <sup>c</sup>	5.2±1.8	34.5±1.4	1.5±0.3	1.3±0.3	1.0±0.5	0.6±0.4	0.9±0.2	8.7±0.2	40.1±0.1	3.4±1.9

<sup>a</sup>For fatty acid, the numbers before and after colon indicate those of carbon atom and double bond, respectively.

<sup>b</sup>Others include 12:0, 13:0, 18:1, 18:2, 18:3, and unknowns.

<sup>c</sup>Values represent the averages of triplicate determinations.





