Production of Docosahexaenoic and Docosapentaenoic Acids by *Schizochytrium* sp. Isolated from Yap Islands

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ABSTRACT: A marine microbe (strain SR21) from the coral reef area of the Yap Islands was isolated by a screening test for polyunsaturated fatty acids and was found to accumulate lipid that contained 22:5n-6 docosapentaenoic acid (DPA) as well as 22:6n-3 docosahexaenoic acid (DHA). Strain SR21 was identified as genus *Schizochytrium* in Labyrinthulomycota, owing to its ultrastructural character and life cycle, which is composed of vegetative cell, zoosporangium, and zoospore stages. After cultural optimization, both in flask and fermenter, the highest DHA and DPA productivities of 2.0 and 0.44 g/L per day, respectively, were obtained in a medium of 60 g/L glucose and corn steep liquor/ammonium sulfate in a half salt concentration of seawater in fermenter culture at 28°C and pH 4. This productivity was almost twice that obtained with flask culture, indicating its high resistance to mechanical stirring. The lipid extracted from the cell was about 50% of the dry cell weight and was composed of 93% triacylglycerol (TG). DHA content of the lipid was 34% of total fatty acids. The TG profile was simple, and the content of the most dominant TG, 1,3-dipalmitoyl-2-DHA-TG, was 27%. TG that contained DHA and n-6-DPA amounted to 57 and 17%, respectively, of total TG molecules. Strain SR21 was revealed to be an excellent source of microbial DHA and n-6 DPA.

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KEY WORDS: Docosahexaenoic acid, essential fatty acids, n-6-docosapentaenoic acid, *Schizochytrium*, single-cell oil, Thraustochytrid.

Docosahexaenoic acid (22:6n-3) (DHA) as well as icosapentaenoic acid (20:5n-3) (IPA) have attracted increasing attention since the first epidemiological report on the importance of n-3 essential fatty acids (EFA) (1). Among n-3 EFA, DHA is important due to its specific function in the brain (2) and retina (3–6). In research on single cell oils, microbial DHA production has been an important research area (7–15) because of both the specified function of DHA mentioned above and the difficulty of DHA purification from fish oil. Similarly, the content of docosapentaenoic acid 22:5 (DPA) in most organisms is low, and the DPA contained in most fish oils is at higher levels of the n-3 form than that of the n-6 form (16). A relatively high level of n-6 DPA has been observed in brain/retina tissues (17–19) under conditions of n-3 EFA deficiency. Deficiency of n-3 EFA during lactation results in an increase of n-6 DPA and a decrease of the n-3 DHA level, even in liver/serum lipids (20). On the other hand, n-6 DPA as well as n-3 DHA were known to occur in some *Thraustochytrium* sp. (21).

We isolated a new strain of Thraustochytrids from the coastal seawater of the Yap Islands that contained high levels of DHA and n-6 DPA. In the present study, the microbial isolate was cultivated for the production of n-6 DPA as well as DHA, and the characteristics of the lipid extracted from the cells were studied.

MATERIALS AND METHODS

Microorganisms. About 400 strains of marine fungi were isolated from seawater collected at the coral reef area of the Yap Islands in the Federated States of Micronesia. The isolated strains were then cultured on GPY medium, which contained 20 g glucose, 10 g peptone, 5 g yeast extract, and 20 g agar in 1 L of a half salt concentration of artificial sea water (Tropic Marin, Aquarientechnik, Germany). After 4-day culture, each grown cell on the agar medium was scraped and was directly methyl-esterified with 10% HCl in methanol and applied to high-performance liquid chromatography (HPLC) (LC 5A; Shimadzu, Kyoto, Japan) analysis with an ODS column (Intersil ODS II; GL Science, Tokyo, Japan), acetonitrile/water (97.5:2.5, vol/vol) as mobile phase, and an ultraviolet (UV) (203 nm) analyzer. One strain, named SR21, showed a peak identical to the DHA standard (Fig. 1). This isolate, strain SR21, was identified first as *Thraustochytrium* sp., but is thought later to be a *Schizochytrium* sp. owing to successive binary divisions of its vegetative cells (Fig. 2).

Culture conditions. Flask culture and culture in a stirred tank fermenter (Model LS-5 Oriental Biotechnological Systems, Tokyo, Japan) were carried out in a medium that contained 60 g glucose, 0.7 g corn steep liquor (Wako Pure Chemical Industry Ltd., Osaka, Japan), 2 g $(NH_4)_2SO_4$, 3 g KH_2PO_4 in 1 L of a half salt concentration of artificial sea water.

Lipid analysis. Culture broths at stationary growth phase were harvested and washed with water by centrifugation. The washed cells were dried at 105°C for 3 h and weighed. The

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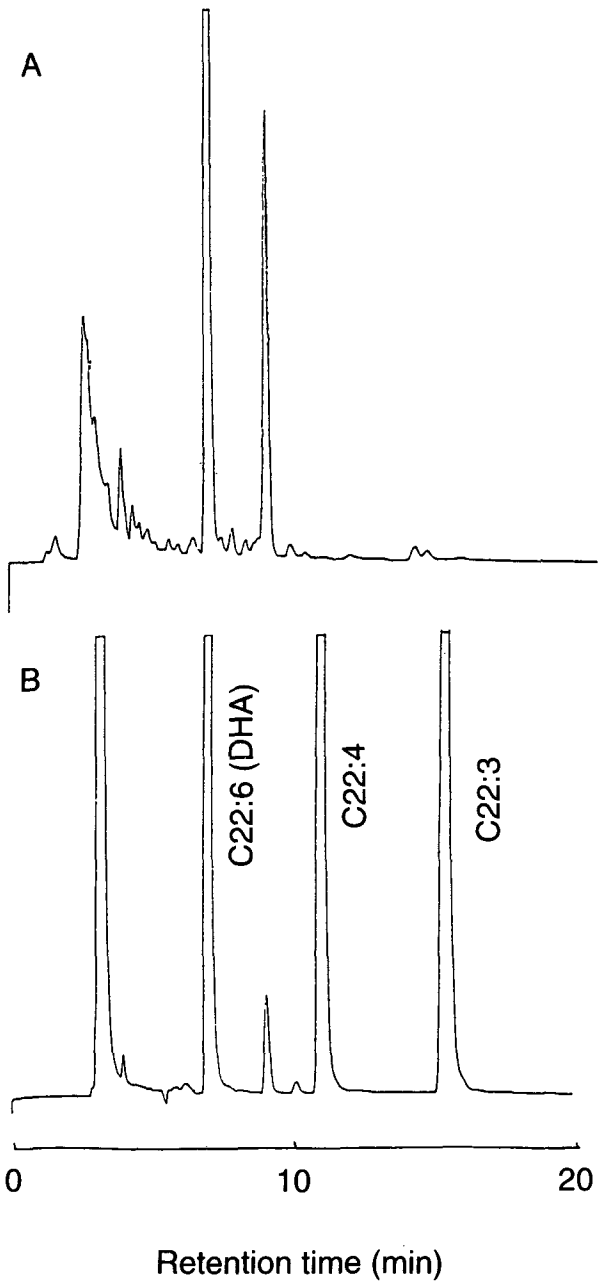

FIG. 1. Reverse-phase high-performance liquid chromatography analysis of the fatty acid methyl esters from SR21 (A) and standard (B). Mobile phase: acetonitrile/water (97.5:2.5, vol/vol); detector: ultraviolet analyzer at 203 nm. Second peak in (A) was identified to be n-6 docosapentaenoic acid. DHA, docosahexaenoic acid.

dried cells then were directly methyl-esterified with 10% HCl in methanol after addition of 1 mg of icosanoic acid (20:0) as the internal standard. The esterified samples were applied to gas chromatography (GC) (Shimadzu), equipped with a TC-70 capillary column (GL Science) under temperature programming (170 to 220°C at a 2°C/min increment). The fatty acid composition and total fatty acid (TFA) were determined from the peak areas relative to the peak area of icosanoic acid.

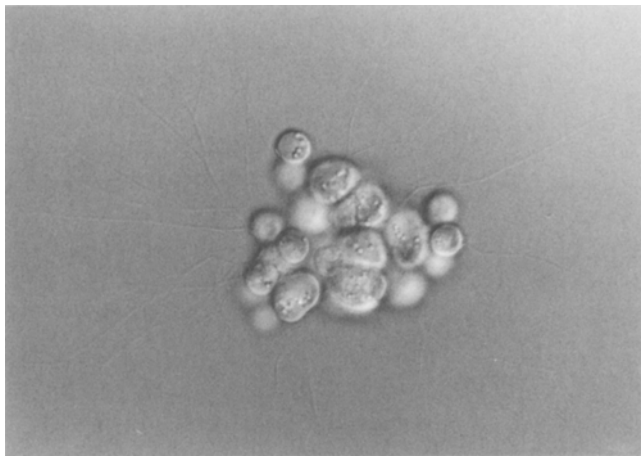

FIG. 2. Microphotograph of vegetative cells of SR21. About 30 cells aggregated with ectoplasmic net element (rhizoid). Cell size: 7—15 μm diameter.

The washed cells were also freeze-dried. From the freeze-dried cells, we extracted lipid by mixing with glass beads at 10,000 rpm for 5 min in chloroform/methanol (1:2, vol/vol) solution in an AM-7 Homogenizer (Nihonseiki, Tokyo, Japan). The extracted lipid, total lipid, was fractionated to polar lipid and neutral lipid by liquid-liquid partitioning in hexane and 90% methanol aqueous solution. The content of each lipid fraction was determined by a thin-layer chromatography/flame-ionization detector (TLC/FID) Analyzer (Iatroscan MK-5; Iatron, Tokyo, Japan). Determination of double-bond positions of polyunsaturated fatty acids (PUFA) was carried out by GC–mass spectrometry (MS) (HP 5971A; Hewlett-Packard, Palo Alto, CA) as picolynyl derivatives of PUFA by Harvey (22). For DPA analysis, whose content was lower than that of DHA, we needed to concentrate the PUFA content by urea fractionation. To 9 g methyl esters, 200 mL methanol and 20 g urea were added, and the mixture was solubilized at 60°C and then crystallized with a gradual cooling to 10°C. The mother liquor filtrate was concentrated by evaporator, and the crystallized fraction was again removed by filtration. The filtrate was used for the determination of double-bond positions of DPA. The phospholipid profile was determined by TLC (Kiesel Gel 60; Merck, Darmstadt, Germany) with chloroform/methanol/water (65:25:4, vol/vol/vol) as mobile phase. The composition of triacylglycerol (TG) was analyzed by HPLC (LC 5A; Shimadzu) with an ODS column (Intersil ODS II; GL Science), acetone/acetonitrile (3:2, vol/vol) as mobile phase, and a refractive index detector (Shodex RI SE-61; Showa Denko, Tokyo, Japan). To determine the positional distribution of DHA in the most abundant TG fraction, dipalmitoyl-DHA-TG, the fractionated TG sample was hydrolyzed by lipase of *Rhizopus japonicus* (Lilipase; Nagase Biochemicals, Fukuchiyama, Japan). To the fractionated TG sample, 4 mL 0.05 M acetate buffer (pH 5.5), 0.1 mL 0.1 M $CaCl_2$, and 1 mL lipase solution (5,000 unit) were

added, and the reaction mixture was shaken and kept at 30°C for 2 h. The hydrolysis product was extracted into diethyl ether and was applied to GC–MS analysis after trimethylsilyl (TMS) ether derivation.

RESULTS

The first peak in Figure 1 was identified as n-3 DHA by GC–MS (Fig. 3A). The molecular weight of the second PUFA methyl ester was 344, that of 22:5 DPA. To determine the position of double bonds, a methyl-esterified sample was adopted to urea fractionation to raise the concentration of the PUFA. From the mother liquor, the PUFA-concentrated fraction with 73.3% of DHA and 17.7% of DPA was collected from the original methyl esters (34.9% of DHA and 8.7% of DPA). The mass spectrum of DPA picolynyl ester is shown in Figure 3B, indicating that DPA is not a n-3 but a n-6 fatty acid.

A culture temperature of 28°C, pH = 4.0 and a half salt concentration of sea water were chosen as the optimum culture conditions for DHA/DPA production. Compared to the culture of *Thraustochytrium aureum*, which grew better in flask culture than in a stirred tank fermenter (15), SR21 grew better in fermenter culture. Glucose as carbon source and corn steep liquor as nitrogen source were selected, and $(NH_4)_2SO_4$ was found to be able to substitute mostly for corn steep liquor. The optimum medium, the components of which were given above, was consequently chosen, and the time course of a culture with this medium in the stirred tank fermenter at 28°C and pH 4 is shown in Figure 4. Addition of $(NH_4)_2SO_4$ was convenient also for pH control in the fermenter culture because consumption of $(NH_4)_2SO_4$ caused a decrease of pH. At 56 h, 60 g/L of glucose was completely consumed, and 21.0 g/L of dry cells and 4.7 g/L of DHA, 1.0 g/L of DPA were obtained. These productivities are equivalent to 9.0 g/L dry cells, 2.0 g/L DHA and 0.44 g/L DPA per day. The fatty acid composition of the total lipid extracted is shown in Figure 5. The three major fatty acids, palmitic acid, DHA and DPA, amounted to nearly 95% of the TFA. The content of other PUFA, such as IPA and arachidonic acid, on the other hand, was less than 0.5%. The lipid content in dry-cell weight was about 50%, and the extracted lipid was composed of 95% neutral lipids and 5% polar lipids. Moreover, the content of TG in neutral lipids was 98%, and the polar lipids were composed of 71% phosphatidylcholine, 11% phosphatidylethanolamine, and 5% phosphatidylinositol. The HPLC chromatogram of the TG fraction (Fig. 6) showed a relatively simple profile of TG molecules, that is, the TG fraction was composed of only seven main peaks. Table 1 shows the

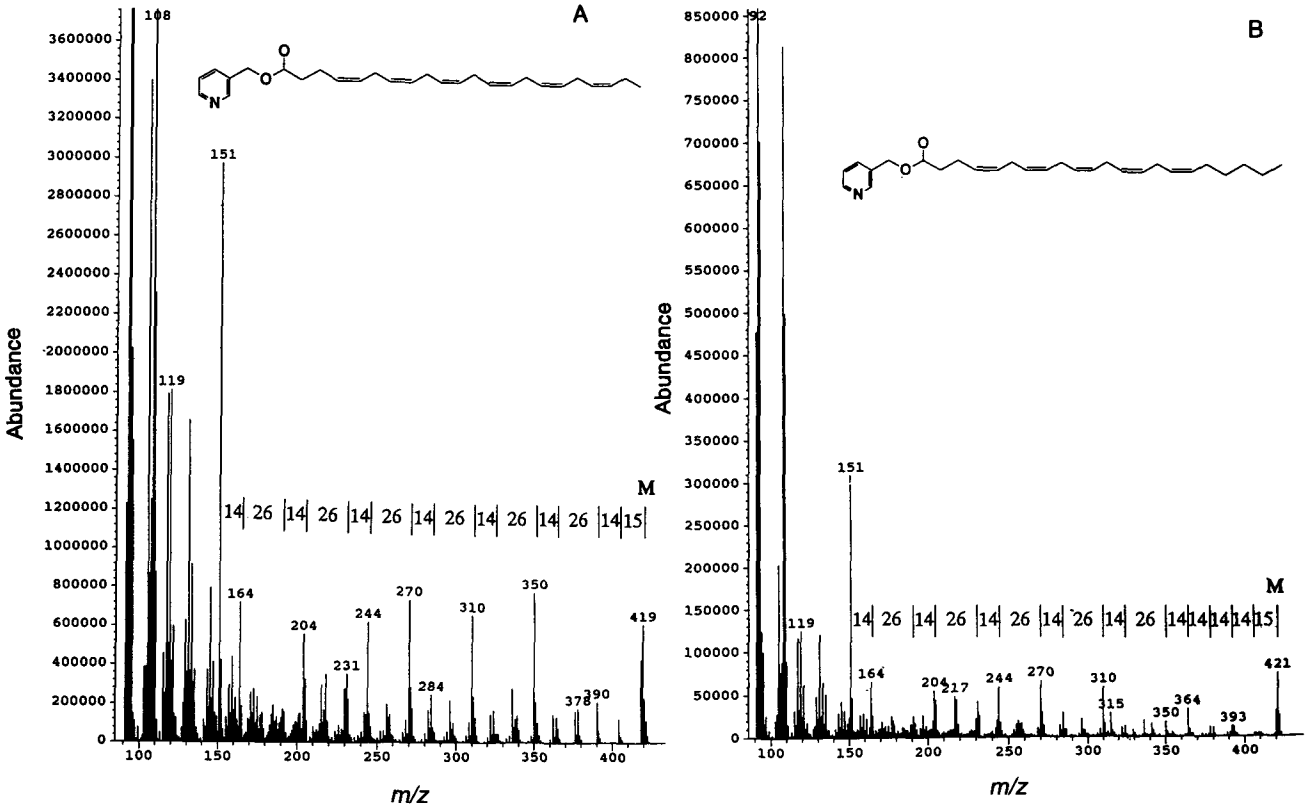

FIG. 3. Mass spectra of polyunsaturated fatty acids picolynyl esters. From the spectra, the major two peaks in Figure 1 were proved to be (A) n-3 docosahexaenoic acid and (B) n-6 docosapentaenoic acid.

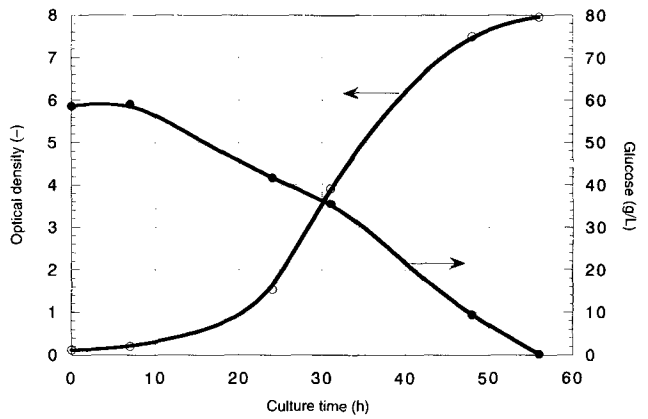

FIG. 4. Time course of a culture in the stirred tank fermenter at 28°C, pH 4. Medium: 60 g glucose, 0.7 g corn steep liquor, 2 g $(NH_4)_2SO_4$, 3 g KH_2PO_4 in 1 L of a half salt concentration of artificial seawater.

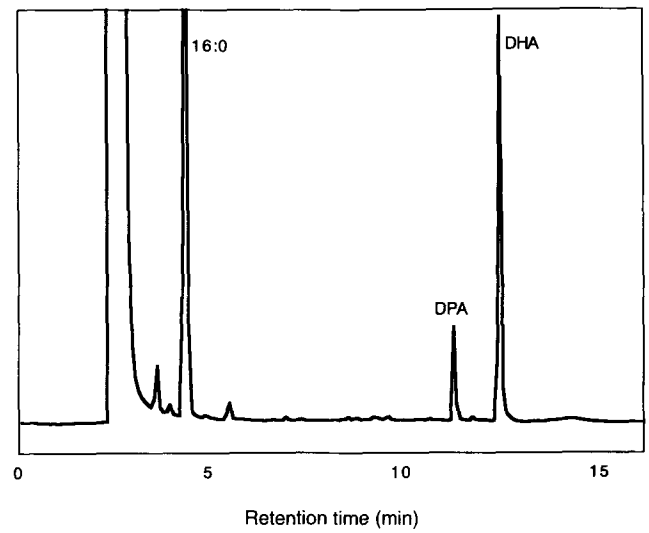

FIG. 5. Gas–liquid chromatography analysis of the fatty acid methyl esters of SR21 total lipid. Contents of the three major peaks were 53.1% 16:0, 33.9% DHA, and 7.4% n-6 docosapentaenoic acid (DPA). Low levels of other polyunsaturated fatty acids were observed. See Figure 1 for other abbreviation.

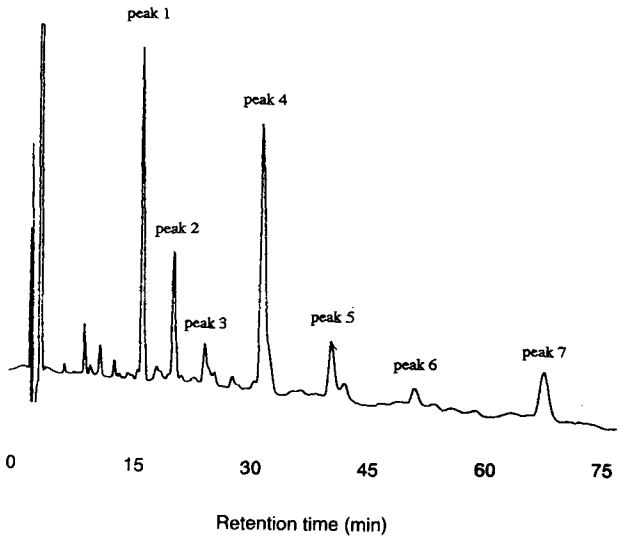

FIG. 6. Reverse-phase high-performance liquid chromatography analysis of triacylglycerols (TG) from SR21. Mobile phase: acetone/acetonitrile (3:2, vol/vol); detector: refractive index detector. Peaks 1 to 7 were identified to be TG shown in Table 1.

TABLE 1
Proportion of Triacylglycerol Species[a]

Peak number	Triacylglycerol species	Content (%)
1	PA–DHA–DHA	16.9
2	PA–DPA–DHA	8.4
3	MA–PA–DHA	4.0
4	PA–PA–DHA	27.4
5	PA–PA–DPA	7.8
6	MA–PA–PA	3.4
7	PA–PA–PA	8.0

[a]PA, palmitic acid, 16:0; MA, myristic acid, 14:0; DHA, n-3 docosahexaenoic acid; DPA, n-6 docosapentaenoic acid.

molecular composition of the seven main peaks. The content of the most dominant molecule, dipalmitoyl-DHA-TG, was 27.4% of the total TG. TG that contained DHA and DPA amounted to 56.7 and 16.2%, respectively, of the total TG. After hydrolysis of the dipalmitoyl-DHA-TG fraction, GC–MS analysis of the TMS derivatives showed three major peaks in the total ion chromatogram (data not shown). The mass spectrum of the biggest peak was composed of ions at *m/z* 117, 73, 132, 75, 313 (M – CH_3), 123, 145, and 328 (M), indicative of the TMS ether of palmitic acid (23). The second peak was for sorbitol, which was included in the enzyme sample as stabilizing reagent. The mass spectrum of the third peak is shown in Figure 7. Ions at *m/z* 219 [-$CH(CH_2OTMS)_2$] and 218 indicated that the peak was 2-monoacylglycerol (24), and the ion at *m/z* 531 was for [M – CH_3], which indicated that it is mono-DHA-acylglycerol. The dipalmitoyl-DHA-TG fraction was identified to be 1,3-dipalmitoyl-2-DHA-TG.

DISCUSSION

Schizochytrium sp. SR21 was isolated from the coral reef area of the Yap Islands. Its lipid was characterized by its fatty acid profile as well as by its high content in the cell. It was mostly composed of TG as the accumulative lipid. The content of n-6 DPA in strain SR21 ranged from 6 to 10%, which seems to be extremely high in consideration of a limited occurrence of n-6 DPA in the biosphere. Similar levels of n-6 DPA were reported by Ellenbogen *et al.* (21) in *T. aureum* (9.5%) and *T. roseum* (6.6%). Owing to its limited occurrence, Findlay *et al.* (25) and Amon (26) suggested that the presence of n-6 DPA in marine organisms/sediments could serve as an index substance of Thraustochytrids. Findlay *et al.* (25) also showed that Thraustochytrids were responsible for the decomposition

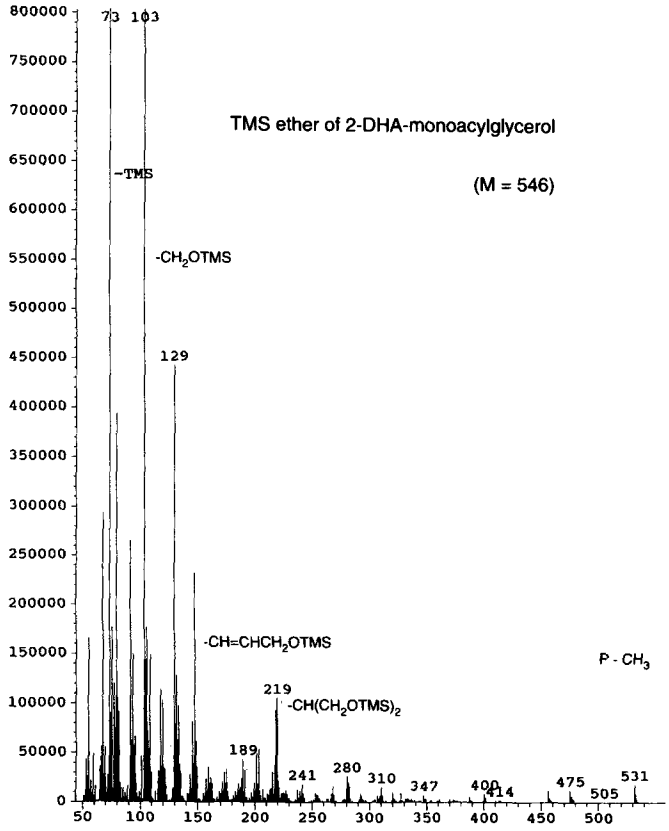

FIG. 7. Mass spectrum of trimethylsilyl (TMS) ether of the hydrolysis product of the dipalmitoyl-DHA-TG fraction. See Figures 1 and 6 for abbreviations.

of fallen mangrove leaves. Moreover, as much as 1.9×10^6 Thraustochytrids/g dry weight of mucus detritus was reported to occur in coral and coral mucus (27). A derivative of n-6 DPA, 14-hydroxy-(n-6)-DPA, was also found to occur in the sponge (*Echinochalina mollis*) of the coral sea (28). These findings suggested that Thraustochytrids play an important role in the ecosystem of the coral reef sea.

Deficiency of n-3 EFA in animal bodies causes a raise of the n-6 DPA level in brain/retina as compensation. The physiological function of n-6 DPA itself has not been clarified so far. Some cases of n-3 EFA deficiency reported so far are shown below. Comparison of sunflower diet for n-3 EFA deficiency and a soybean oil diet showed that sunflower oil decreases the DHA level and increases the n-6 DPA level in brain capillaries and choroid plexus (17). A high level of n-6 DPA was found as compensation for the decrease of the DHA level during pregnancy and lactation of α-linolenic acid-deficient rats, even in liver/plasma lipid, which cannot be found under normal conditions there (29). Although the diet was returned to the normal EFA level, recovery of DHA was slower than that of n-6 DPA (18). Except for the case of n-3 EFA deficiency, zinc deficiency (30), vitamin A deficiency (31), and exposure to perchloroethylene (32) were reported to affect the content of n-6 DPA in rats. DPA has also been proposed as a pharmaceutical that is permeable to the blood–brain barrier (33). Those findings suggested some physiological functions of n-6 DPA in animals.

Research on microbial DHA production is needed for the following reasons: (i) it can serve as an alternative to unstable supplies of fish oil, (ii) it is a product with little fishy smell, (iii) it can provide highly purified DHA and PUFA other than DHA. DeLong and Yayanos (34) showed the occurrence of DHA in a psychrophilic bacterium, *Vibrio marinus*, which was the first finding in procaryotes. Recently, Yano *et al.* (11) found that five bacterial strains (*Vibrio* sp.) from the intestine of deep-sea fish produce DHA, and one of them produced 0.8 mg/L DHA within 6 d. This method is limited by the low rate of bacterial growth and must be improved.

Compared to bacteria, Thraustochytrids have higher potential in DHA production. The first report that focused on DHA productivity by Thraustochytrids was in 1991 by Bajpai *et al.* (8). Cell productivity of 3.8 g/L and DHA productivity of 270 mg/L for 6 d were obtained on glucose and glutamate. Later, they obtained DHA productivity of 510 mg/L for 40 h on soluble starch as carbon source (9). Kendrick and Ratledge (10) obtained cell productivity of 4.0 g/L and lipid productivity of 400 mg/L in 3 d. Li and Ward (7) reported DHA productivity of 850 mg/L under fluorescent light in 5 d with *T. roseum*. We obtained cell productivity of 5.7 g/L and 460 mg/L total lipid in 69 h with *T. aureum* (15). In those previous studies, flask culture resulted mostly in better growth than fermenter culture, indicative of some cell fragility at high content of PUFA. Better growth of strain SR21 in fermenter culture than in flask culture showed that strain SR21 was more tolerant to mechanical stirring than the other Thraustochytrids. Compared to the previous productivities, the values of the present report (9.0 g/L cells, 2.0 g/L DHA and 0.44 g/L DPA per day) were much higher, showing that SR21 is an excellent source of microbial DHA and n-6 DPA.

The DPA/DHA ratio was kept fairly constant under a variety of culture conditions, indicating that n-6 DPA was the direct precursor of DHA. Similar behavior was already observed in *Saprolegnia parasitica* (35) and *Mortierella alpina* (36), in which n-6 arachidonic acid was proved to be the direct precursor of n-3 IPA.

The most dominant TG molecule in SR21 was 1,3-dipalmitoyl-2-DHA-TG. The positional specificity of DHA was completely different from that of DHA-producing microalgae (MK 8805) (12).

In summary, culture in a stirred tank fermenter of strain SR21, *Schizochytrium* sp., isolated from the coral reef area of the Yap Islands, resulted in high DHA/DPA productivity. High DHA/DPA productivity was due to high growth rate, high lipid content, and also high DHA/DPA content of the strain. Strain SR21 is an excellent source of microbial DHA and n-6 DPA.

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