Production of High Yields of Docosahexaenoic Acid by *Schizochytrium* sp. Strain SR21

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ABSTRACT: The culture conditions for high-yield production of docosahexaenoic acid (DHA) by *Schizochytrium* sp. strain SR21 were investigated in a fermenter. With increasing carbon (glucose) and nitrogen (corn steep liquor and ammonium sulfate) sources (up to 12% glucose) in the medium, DHA productivity increased without a decrease in growth rate, i.e., 2.0, 2.7, and 3.3 g DHA/L/d with 6, 10, and 12% glucose, respectively. Eventually, 48.1 g dry cells/L and 13.3 g DHA/L were produced in 4 d with 12% glucose. DHA productivity was decreased with 15% glucose, i.e., 3.1 g/L/d. With 12% glucose, the lipid content was 77.5% of dry cells, and DHA content was 35.6% of total fatty acids. The lipid was composed of about 95% neutral lipid and 5% polar lipid. In polar lipids, the contents of phosphatidylcholine (PC), phosphatidylethanolamine, and phosphatidylinositol were 74, 11, and 5%, respectively. The PC profile was simple, 70% of PC molecules were 1-palmitoyl-2-DHA-PC and 1,2-di-DHA-PC. These results indicate that *Schizochytrium* sp. strain 21 is an excellent source for microbial DHA production, including not only the acid form of DHA but also 2-DHA-PC. *JAOCS 74,* 1431–1434 (1997).

KEY WORDS: DHA production, docosahexaenoic acid, docosapentaenoic acid and n-6 DPA, DPA, *Schizochytrium.*

Long-chain polyunsaturated fatty acids (PUFA) are important dietary constituents. Their beneficial effects on human health are widely accepted and hence have led to extensive nutritional and clinical studies on their effects on human physiology (1). Docosahexaenoic acid (DHA, 22:6n-3) has attracted great interest recently owing to its specific function in the brain (2,3) and retina (4–8).

The largest commercial source of DHA is fish oil, but fish oil has an undesirable fishy smell, which prevents extensive utilization of fish oil. Recently, microbial production of γ-linolenic acid (9), arachidonic acid (10,11), and DHA (12) has been industrialized. Microbial DHA production has been an important research area (12–21) because of the difficulty of DHA purification from fish oil.

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We isolated a new Thraustochytrid strain from seawater and reported its ability as to high-level DHA and DPA (docosapentaenoic acid, 22:5n-6) production (18). In this paper, we describe a method for polar lipid analysis and the conditions for high-level DHA production by strain SR21.

MATERIALS AND METHODS

Microorganism. Schizochytrium sp. strain SR21 was used in the present study. This strain was isolated from seawater in a coral reef area (18).

Cultures were grown in a medium with 60 g/L glucose, 0.7 g/L corn steep liquor (Wako Pure Chemical Industry Ltd., Osaka, Japan), 2 g/L $(NH_4)_2SO_4$, and 3 g/L KH_2PO_4 in artificial seawater at a half salt concentration. The effects of agitation speed and impeller shape on growth in a fermenter (Model LS-5; Oriental Biotechnological Systems, Tokyo, Japan) were also examined. To obtain higher DHA productivity per unit volume of medium, the optimum amounts of carbon (glucose) and nitrogen [corn steep liquor and $(NH_4)_2SO_4$] sources were studied. The various culture media tested are listed in Table 1.

Lipid analysis. Culture broth was harvested every 12 h, until the stationary growth phase, and the cells were washed with water by centrifugation. The washed cells were dried at 105°C for 3 h and weighed to obtain the dry cell weight (DCW). The dried cells were then suspended in 5 mL of methylene chloride/10% methanolic HCl (1:1, vol/vol) for 3 h at 50°C. As an internal standard, 1 mg of eicosanoic acid (20:0) was added to the methanolysis solution. After extraction with 10 mL *n*-hexane, followed by evaporation, the fatty

TABLE 1
Culture Media[a]

Medium	1	2	3	4	5
Glucose (g/L)	60	80	100	120	150
Corn steep liquor (g/L)	0.7	0.9	1.2	1.4	1.75
$(NH_4)_2SO_4$ (g/L)	2.0	2.7	3.3	4.0	5.0
KH_2PO_4 (g/L)	3.0	3.0	3.0	3.0	3.0
1/2 Artificial sea water (L)	1	1	1	1	1

[a]Cells were cultured in a fermenter with 3 L of each medium, under the conditions of 300 rpm, aerated 3 L/min, 28°C, and pH 4.0.

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acid methyl esters were dissolved in 0.5–1 mL acetonitrile and then subjected to gas–liquid chromatography (GLC) (Shimadzu, Kyoto, Japan) on a TC-70 capillary column (GL Sciences, Tokyo, Japan) with temperature programming (170 to 220°C at 2°C/min). The fatty acid composition and total fatty acid (TFA) content were determined from the peak areas relative to the peak area of eicosanoic acid.

Lipids were extracted from freeze-dried cells as described previously (18). The extracted lipids were fractionated to polar and neutral lipids by liquid–liquid partitioning in hexane and a 90% methanol aqueous solution. The content of each lipid fraction was determined with a thin-layer chromatography/flame-ionization detector (TLC/FID) analyzer (Iatroscan MK-5; Iatron, Tokyo, Japan). The phospholipid profile was determined by TLC (Kieselgel 60; Merck, Darmstadt, Germany) with acetonitrile/methanol/phosphate (900:95:5, vol/vol/vol) as the mobile phase. The phospholipid fraction was then subjected to high-performance liquid chromatography (HPLC) (LC 5A; Shimadzu, Kyoto, Japan) on a TSK-gel Silica 60 column (Merck) with a refractive index (RI) detector (Shodex RI SE-61; Showa Denko, Tokyo, Japan) by using the same mobile phase. The phosphatidylcholine (PC) fraction was collected. Five mg of PC was treated with 50 units phospholipase A2 (Sigma, St. Louis, MO) at 25°C for 3 h in the reaction solution (5 mL of ethyl ether/methanol (98:2), 0.5 mL of 0.1 M Tris-HCl, pH8.0, and 0.01 mM $CaCl_2$). After the reaction, 5 mL water was added and mixed by vortexing. The ether layer was collected. From the resultant solution, all lipid fractions were extracted with 5 mL chloroform/methanol (2:1) solution. The extracted solution was then charged onto a TLC plate and developed with a chloroform/methanol/water (65:25:4, vol/vol/vol) solvent mixture. Both free fatty acid and lysophosphatidylcholine (lyso-PC) spots were scraped from the TLC plate. The free fatty acids and lyso-PC were then methylated and analyzed by GLC as described previously.

RESULTS

Growth of strain SR21 was studied in medium 1. With an increase in the agitation speed in the fermenter from 100 rotations per min (rpm) to 300 rpm with a six-blade turbine impeller, growth rate increased (Fig. 1). At 500 rpm with a turbine impeller, growth was depressed. In contrast, at 500 rpm with a propeller-shaped impeller, the growth rate was not depressed (Fig. 2).

Strain SR21 was cultured in media 1–5 (Table 1), with a turbine-shaped impeller at 300 rpm to study its DHA productivity. When the amounts of carbon and nitrogen were increased, both DCW and DHA productivity increased (Fig. 3, Table 2). With medium 1, strain SR21 produced 21.9 g/L DCW and 5.1 g/L DHA at 65 h. With medium 4 (with twice the amounts of carbon and nitrogen as in medium 1), it produced 48.1 g/L DCW and 13.3 g/L DHA at 96 h. And with medium 5, it produced 59.2 g/L DCW and 15.5 g/L DHA, but under this glucose condition, the growth was somewhat delayed compared with that in other media. TFA in cells and the DHA content of TFA were also measured. With medium 1, strain SR21 produced 14.8 g/L TFA, 67.6% TFA/DCW, and 34.5% DHA/TFA. With medium 4, it produced 37.3 g/L TFA, 77.5% TFA/DCW, and 35.6% DHA/TFA.

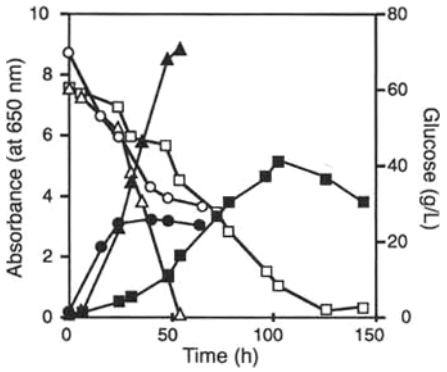

FIG. 1. Effect of agitation on the growth of *Schizochytrium* sp. strain SR21: □, 100 rpm; △, 300 rpm; ○, 500 rpm. Solid data points show the absorbance at 650 nm. Open data points show the glucose concentration in the culture medium.

The lipid content of strain SR21 increased with cell growth (Fig. 4). In the early growth phase, neutral and polar lipids were produced in about equal amounts, and then neutral lipids increased with cell growth. The lipid profile became eventually 95% neutral lipids and 5% polar lipids in the stationary growth phase. Neutral lipids were composed of 35% DHA and 6% DPA. On the other hand, polar lipid was composed of 63.5% DHA and 24.7% DPA. The HPLC chromatogram of the polar lipids fraction showed five main peaks (Fig. 5). Peaks 4 and 5 were identified as PC by nuclear magnetic resonance (data not shown). Analysis of phospholipase A2-treated PC showed that there were 74.4% DHA, 13.9% DPA, and 8% palmitic acid in the free fatty acid spots, and 42% DHA, 3.0% DPA and 50% palmitic acid in the lyso-PC spots (Fig. 6). These results indicate that the main PC molecular

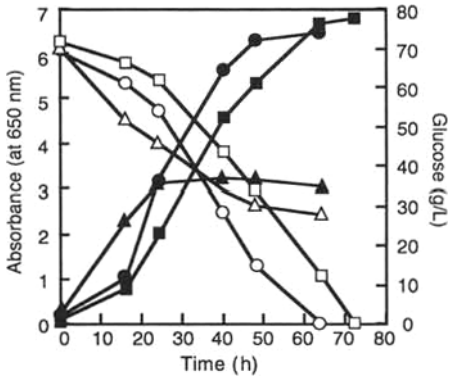

FIG. 2. Effects of agitation and impeller shape on growth: □, propeller-shaped impeller at 300 rpm; △, six-blade turbine-shaped impeller at 500 rpm; ○, six-blade turbine-shaped impeller at 300 rpm. Solid data points show the absorbance at 650 nm. Open data points show the glucose concentration in the culture medium.

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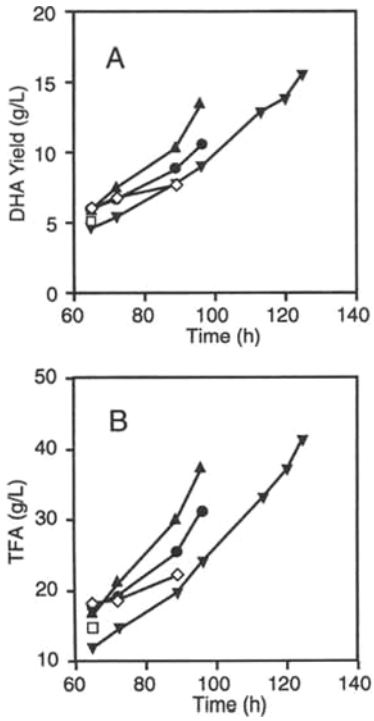

FIG. 3. Effects of glucose concentration on (A) docosahexaenoic acid (DHA) yield, (B) total fatty acid (TFA) produced. The amounts of carbon and nitrogen were increased in proportion, as listed in Table 1: □, medium No. 1; ◇, No. 2; ●, No. 3; ▲, No. 4; ▼, No. 5.

species are 1-palmitoyl-2-docosahexaenoyl-PC and 1,2-didocosahexaenoyl-PC.

DISCUSSION

DeLong and Yayanos (22) detected DHA in a psychrophilic bacterium, *Vibrio marinus*. Yano *et al.* (21) also found that bacterial strains (*Vibrio* sp.) from the intestines of a deep-sea fish produce DHA, one of them producing 0.8 mg/L DHA. Kyle *et al.* (12,16) reported that marine microalgae produce DHA.

Thraustochytrids are known as DHA producers. Bajpai *et al.* (13,14) obtained 510 mg/L DHA in 40 h with soluble starch as the carbon source. Kendrick and Ratledge (15) obtained 400 mg/L DHA in 3 d. Li and Ward (17) reported the production of 850 mg/L DHA under fluorescent light in 5 d by *Thraustochytrium roseum*. Singh and Ward (20) obtained 1433 mg/L DHA in 8 d by supplying starch during the growth

TABLE 2
Analytical Results

Culture time (h)	62	72	92	92	125
Dry cell weight (g/L)	21.9	32.0	37.7	48.1	59.2
Lipid content (%)[a]	67.6	68.6	82.6	77.5	70.3
DHA content (%)[b]	34.5	34.7	33.3	35.6	37.3
DHA yield (g/L)	5.1	7.6	10.4	13.3	15.5

[a]Total fatty acid/dry cell weight.
[b]DHA/total fatty acid. Abbreviation: DHA, docosahexaenoic acid.

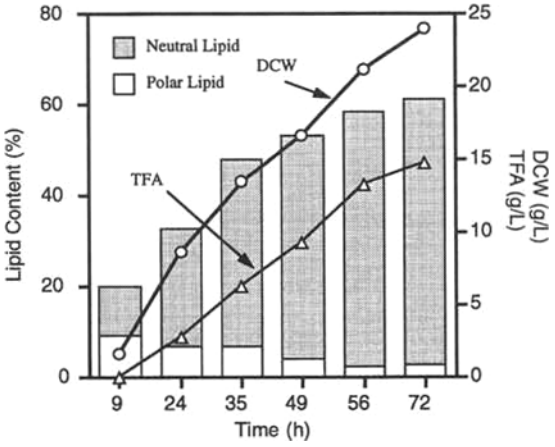

FIG. 4. TFA and lipid content at various growth phases of strain SR21. DCW, dry cell weight. See Figure 3 for other abbreviation.

of *T. roseum*. Singh *et al.* (19) reported production of 707 mg/L DHA by *Thraustochytrium* sp. ATCC 20892. All of these DHA production trials were performed in flask cultures because of the fragility of the cell membrane with a high content of PUFA. Previously, we also tried cultivation of *T. aureum* in a fermenter, but production of DHA in a fermenter was lower than that in a flask culture.

We reported previously that strain SR21 produced 2.0 g/L DHA per day in a fermenter (18). This productivity was the highest ever reported, and we concluded that strain SR21 was a superior source for microbial DHA production. In the present study, we found that strain SR21 grew well with a high concentration of glucose and produced 15.5 g/L DHA in 5 d. DHA was accumulated mainly as triacylglycerol (TG) or storage lipid. Fish oil also contains DHA in the TG form. Therefore, the lipids extracted from SR21 are in the same form as in fish oil.

Increasing the agitation speed from 100 rpm to 300 rpm resulted in a higher growth rate, indicating that oxygen transport

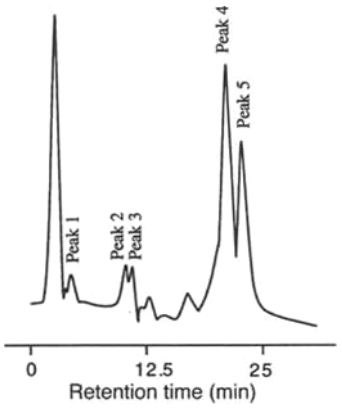

FIG. 5. High-performance liquid chromatography (HPLC) chromatogram of polar lipids. Polar lipids were charged onto a TSK-gel Silica-60 (Merck, Darmstadt, Germany) HPLC column, with acetonitrile/methanol/phosphate (900:95:5) as the solvent, and detected with a refractive index detector. Peak 1 is phosphatidylinositol; Peaks 2 and 3, phosphatidylethanolamine; Peaks 4 and 5, phosphatidylcholine.

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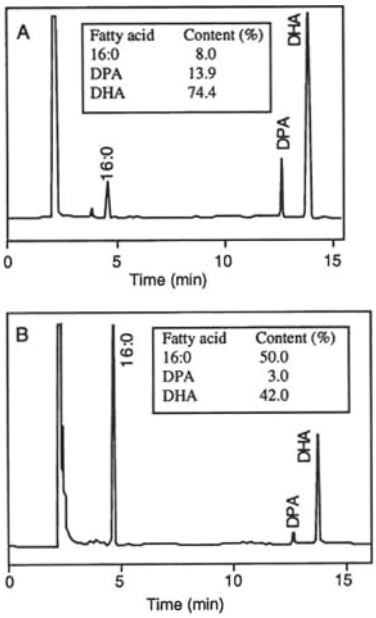

FIG. 6. Gas–liquid chromatogram of phospholipase A2-treated phosphatidylcholine (PC). (A) Free fatty acid fraction; (B) lysophosphatidylcholine fraction. DPA: docosapentaenoic acid. For other abbreviation see Figure 3.

is a limiting factor. In terms of the impeller's shape, the propeller type subjects the cells to less shear stress than the turbine impeller (23). At 500 rpm, the turbine impeller, but not the propeller-shaped impeller, depressed the cell growth.

Strain SR21 mainly accumulates PC in its polar lipids. Over 70% of total PC was 1-palmitoyl-2-docosahexaenoyl-PC and 1,2-didocosahexaenoyl-PC. DHA exists in the human brain as phospholipid (2). Therefore, strain SR21 is a good source for DHA production not only in TG but also in PC form.

We are currently investigating larger-scale fermentation for the production of DHA.

Compared to other Thraustochytrids such as *T. aureum*, SR21 seems to have higher tolerance to shear stress but is not as strong as yeasts or bacteria, for which speeds above 500 rpm generally give high growth. Considering the high content of PUFA in its PC, the main component of the cell membrane, SR21 was revealed to be an excellent source for microbial DHA production.

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[Received May 30, 1997; accepted July 24, 1997]

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