SHORT COMMUNICATION

Polyunsaturated fatty acids production by *Schizochytrium* sp. isolated from mangrove

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Abstract

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Polyunsaturated fatty acids production by *Schizochytrium* sp. isolated from mangrove
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Five *Schizochytrium* strains (N-1, N-2, N-5, N-6, and N-9) were isolated from fallen, senescent leaves of mangrove tree (*Kandelia candel*) in Hong Kong. The fungi were cultivated in glucose yeast extract medium containing 60 g of glucose, 10 g of yeast extract and 1 L of 15‰ artificial seawater, initial pH 6.0, with shaking for 52 hr at 25ºC. Biomass yields of 5 isolates ranged from 10.8 to 13.2 g/l. Isolate N-2 yielding the highest dried cell mass at 13.2 g/l and isolate N-9 grew poorly with 10.8 g/l of biomass. EPA (Eicosapentaenoic acid, 20:5n-3) yield was low in most strains, while DHA (Docosahexaenoic acid, 22:6n-3) was high on the same medium. The contents of DHA in biomass varied: 174.9, 203.6, 186.1, 171.3 and 157.9 mg/g of dried-biomass for *Schizochytrium* isolate N-1, N-2, N-5, N-6, and N-9, respectively. Isolate N-2 had the highest proportion of DHA in fatty acid profile with 15:0, 28.7%; 16:0, 21.3%; 18:0, 0.9%; 18:3, 0.2%; 20:4, 0.3%; 20:5, 0.9%; 22:4, 6.7%; 22:6, 36.1%; and others, 9.3%. The salinity range for growth of *Schizochytrium* isolates was from 0-30‰ with optimum salinity for growth between 20-30‰.

Key words : *Schizochytrium*, docosahexaenoic acid (DHA), polyunsaturated fatty acid, Thraustochytrids

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บทคัดย่อ

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การผลิตกรดไขมันไม่อิ่มตัวชนิดโพลี จากเชื้อ *Schizochytrium* sp. ที่คัดแยกจากป่าชายเลน

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เชื้อรา *Schizochytrium* จำนวน 5 สายพันธุ์ (strains N-1, N-2, N-5, N-6, and N-9) แยกได้จากใบ *Kandelia candel* ที่หล่นจมน้ำในป่าชายเลนบริเวณเกาะฮ่องกง ถูกนำมาเพาะเลี้ยงในอาหารแข็ง glucose yeast extract ที่ประกอบด้วยกลูโคส 60 กรัม ยีสต์สกัด 10 กรัม และปรับปริมาตรเป็น 1 ลิตร ด้วยน้ำทะเลสังเคราะห์ความเข้มข้น 15‰ ปรับพีเอชเริ่มต้นเท่ากับ 6.0 ให้อากาศโดยการเขย่าเป็นเวลา 52 ชั่วโมง ที่อุณหภูมิ 25°C มวลชีวภาพ (น้ำหนักแห้ง) ทั้ง 5 สายพันธุ์ เมื่อสิ้นสุดการเพาะเลี้ยงมีค่าตั้งแต่ 10.8 ถึง 13.2 กรัม/ลิตร สายพันธุ์ N-2 มีค่ามวลชีวภาพสูงสุดในรูปของเซลล์แห้งเท่ากับ 13.2 กรัม/ลิตร สายพันธุ์ N-9 เจริญได้น้อยที่สุดและมีมวลชีวภาพ (น้ำหนักแห้ง) เท่ากับ 10.8 กรัม/ลิตร เชื้อ *Schizochytrium* ทั้ง 5 สายพันธุ์สะสมกรดไขมันอีพีเอ (Eicosapentaenoic acid, 20:5n-3) ปริมาณต่ำในเซลล์ ขณะที่มีการสะสมกรดไขมันดีเอชเอ (Docosahexaenoic acid, 22:6n-3) ในปริมาณที่สูง ปริมาณกรดไขมันดีเอชเอในเซลล์ทั้ง 5 สายพันธุ์ (N-1, N-2, N-5, N-6, และ N-9) มีค่าเป็น 174.9, 203.6, 186.1, 171.3 และ 157.9 มิลลิกรัม/กรัมเซลล์แห้ง ตามลำดับ สายพันธุ์ N-2 มีสัดส่วนของกรดไขมันดีเอชเอสูงสุด และมีสัดส่วนของกรดไขมันชนิดต่าง ๆ เป็นดังนี้ C15:0 เท่ากับ 28.7%; C16:0 เท่ากับ 21.3%; C18:0 เท่ากับ 0.9%; C18:3 เท่ากับ 0.2%; C20:4 เท่ากับ 0.3%; C20:5 เท่ากับ 0.9%; C22:4 เท่ากับ 6.7%; C22:6 เท่ากับ 36.1% และกรดไขมันชนิดอื่น ๆ 9.3% เชื้อ *Schizochytrium* ทั้ง 5 สายพันธุ์ เจริญได้ที่ระดับความเค็ม (salinity) ตั้งแต่ 0-30‰ และมีค่าความเค็มที่เหมาะสมในการเจริญระหว่าง 20-30‰.

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Schizochytrium is a group of marine thraustochytrids protists with mono-centric thalli. They possess the following characteristic features: a multi-layered wall composed predominately of L-galactose (Darley *et al*., 1973), an organelle termed a sagenogenetosome from which the ectoplasmic nets arise (Perkins, 1972) and bi-flagellate heterokont zoospores in many of described genera (Moss, 1991). They are ubiquitously distributed and can be found on the algal surface, in estuarine habitats, seawater and saline soil (Miller and Jones, 1983; Ulken, 1981; Gartner, 1981; Booth, 1971). Many of these ecological studies, however, were undertaken in temperate and Antarctic regions. Studies in the tropical and subtropical regions are few, though investigations are increasing especially in mangrove areas (Honda *et al*., 1998; Raghukumar, 1988). These micro-organisms play a potential role in enriching the nutritionally poor mangrove leaves and act as decomposers of mangrove litter (Bremer, 1995). Recent research on thraustochytrids has focused on DHA (docosahexaenoic acid; C22:6n-3) production (Fan *et al*., 2000; 2001; 2002; Nakahara *et al*., 1996) as they are known to produce a wide range of lipids, particularly polyunsaturated fatty acid of the omega-3 series (Weete *et al*., 1997). The long-chain omega-3 polyunsaturated fatty acids (PUFAs) have been shown to have positive effects on the prevention or treatment of arteriosclerosis, cancer and rheumatoid arthritis. The importance of DHA in human and animal nutrition has received a great deal of research attention during the past decade (Simopoulos, 1989; Takahara *et al*., 1998). In particular, docosahexaenoic acid is absolutely essential to humans owing to its functions in the brain and retina (Horrocks and Yeo, 1999). The current commercial source of DHA is fish and fish oils, which contain 7-14% DHA, EPA (eicosapentaenoic acid, C20:5n-3), and other

saturated fatty acids. Because of the relatively low proportion of DHA in fish oil and the problems encountered in extraction and purification of omega-3 fatty acids, and also large scale production of DHA is difficult. Therefore, efforts have been made to seek eukaryotic marine microorganisms as the alternative sources for omega-3 fatty acid production (Yokochi *et al*., 1998).

In the present study, we report levels of polyunsaturated fatty acids, especially for omega-3 PUFAs; DHA and EPA, produced by *Schizochytrium* sp. isolated from Three Fathom Cove mangrove in Hong Kong and the effect of medium-salinity on the growth of the selected isolates.

Materials and Methods

Isolation of *Schizochytrium*

Fallen leaves of mangrove trees (*Kandelia candel*) were collected from the floor of intertidal zone in Three Fathom Cove mangrove, Hong Kong. These leaf samples were put into sterile plastic bags and the salinity and temperature of the collection locations were measured. The leaf samples were taken back to the laboratory for isolation of thraustochytrids within a day. The sample leaves were cut into leaf discs (1.5 cm. diameter) and washed 5 times with 15‰ (ppt) sterile natural sea water (NSW) added with 0.25 mg/l each of penicillin G (Sigma) and streptomycin sulfate (Sigma) to suppress the bacterial growth. Washed leaf-discs were plated onto yeast extract peptone (YEP) agar medium composed of 1 g of yeast extract (Oxoid), 1 g of mycological peptone (Difco), 13 g of agar (Difco) and 1 L of 15‰ NSW at pH 6.0. Antibiotics and 0.5 ml of 15‰ sterile NSW were added before the inoculated plate was wrapped with parafilm prior to incubation at 25ºC for 1-2 days. During the incubation period, plates were periodically observed for *Schizochytrium* colonies. The selected colonies were aseptically transferred to fresh YEP agar plate containing antibiotics and repeatedly subcultured to fresh medium until axenic cultures were obtained. Stock cultures were maintained on YEP agar slants with 0.5 ml of 15‰ sterile NSW at 4ºC and subcultured every 2 months (Fan *et al*., 2002).

Inoculum preparation and heterotrophic growth

For preparation of inoculum, YEP agar cultures were used. Two strips of *Schizochytrium* colony on the YEP agar (3 mm × 10 mm.) were cut and transferred into 50 ml of the seeding broth (containing 1 g of yeast extract (Oxoid), 1 g of mycological peptone (Difco), 10 g of glucose (Sigma), and 1 L of 15‰ NSW) in a 250 ml flask. Flask were plugged with cotton wool and wrapped with aluminum foil. The culture was incubated at 25ºC and shaken at 200 rpm for 48 h. A 5% (v/v) inoculum of the 48-hour old culture broth was used as initial inoculum for biomass production.

The heterotrophic culture of each *Schizochytrium* isolate was grown in a 250 ml flask that contained 50 ml of glucose yeast extract medium. This medium was composed of 60 g of glucose (Sigma), 10 g of yeast extract (Oxoid) and 1 L of 15‰ artificial sea water (ASW) prepared from sea salt (Sigma) at pH 6.0. For each strain, triplicate flasks were incubated and shaken at 200 rpm at 25ºC for 52 hr before being harvested (Fan *et al*., 2000). This medium and incubation condition was used for all subsequent experiments. The culture broth was prepared in one litter of distilled water or ASW with difference salinities (0‰, 5‰, 10‰, 15‰, 20‰, 25‰ and 30‰) by using a salinity refractometer at pH 6.0 for the salinity experiments.

Biomass determination

For dry weight biomass determination, the entire content (50 ml) of the thraustochytrid cells in 250 ml flask was transferred to a pre-weight centrifuge tube and harvested by centrifugation at 3,500 rpm for 10 min. The supernatant was discarded and the cell washed with 50 ml sterile distilled water. The selected samples were examined microscopically to check for cell integrity and 10 ml of distilled water was then added to the washed cells before re-suspension by hand agitation. The washed cells were freeze-dried overnight and quickly weighed after being removed from the freeze-drier (Fan *et al*., 2000). Biomass was

expressed as freeze-dried weight in milligram per 50 ml of growth medium.

Extraction and determination of fatty acids

The fatty acid composition was determined according to a modified procedure of Lapage and Roy (1984). Freeze-dried cells were weighed (20-30 mg) in Teflon-lined screw test tubes of 10 ml capacity and were methylated by a direct acid-catalyzed tranesterification in 2 ml of 4% sulfuric acid in methanol (80ºC for 1 h.) without prior extraction of the total lipids. An internal standard of 3 mg of hepatodecaenoic acid (C17:0) and a magnetic stirring bar were added into each tube before methylation. After the mixtures had cooled, 1 ml of water and 1 ml of hexane were added. The fatty acid methylesters (FAMEs) in the hexane layer were mixed, centrifuged and collected. The esters were then stored at 4ºC prior to injection into a gas-liquid chromatograph for analysis with a Hewlett Packard HP-6890 GC (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector and a HP-Innowax™ capillary column (30 m × 250 µm). Nitrogen was used as the carrier gas and the flow rate was kept at 2 ml/min. A volume of 5 µl was injected under splitless injection mode. The injection port temperature was 250ºC; the detector was at 260ºC; and the column temperature was held at 235ºC for 50 min. Fatty acids were identified by comparison of the relative retention times with standard fatty acids (hepatodecaenoic acid; C17:0) (Sigma, St Louis, MO). The fatty acid contents were quantified by comparing their peak areas with that of the internal standard.

Results and Discussion

Heterotrophic growth

All thraustochytrids were isolated from submerged *Kandelia candel* leaves at various stages of decay in the intertidal zone of the mangrove. Some ecological characteristics of the isolation site are shown in Table 1. The morphological character of the five isolates can be assigned to genus *Schizochytrium* because they produced biflagellate zoospore and the mature cells divided by repeated binary division to form diads, tetrads and clusters (Figure 1). Each *Schizochytrium* cell could develop into a sporangium producing several zoospores. Even though the number of zoospores released from each zoosporangium is one of the most important characteristics in species delimitation within the genus *Schizochytrium* (Raghukumar, 1988), zoospore could not be accurately determined in zoosporangium of those isolates. All *Schizochytrium* strains were observed to process a life cycle with vegetative cells (9-14 µm in diameter), zoosporangium and zoospore stage. The vegetative cell of all strains lacks motility and absence of amoeboid cell. These strains produce biflagellate zoospore inside the zoosporangium. Therefore, five *Schizochytrium* isolates are here-

Table 1. *Schizochytrium* strains isolated from Three Fathoms Cove Mangrove in Hong Kong

Strain	Date of isolation	Substrate[a]	Salinity (‰)[b]
Schizochytrium sp. N-1	22/05/02	Brown	5
Schizochytrium sp. N-2	22/05/02	Black	7
Schizochytrium sp. N-5	22/05/02	Brown	5
Schizochytrium sp. N-6	04/06/02	Brown	8
Schizochytrium sp. N-9	04/06/02	Yellow	8

[a] **All the *Schizochytriums* were isolated from submerged *Kandelia candel* leaves in various stages of decay (yellow, brown or black) in mangrove.**
[b] **Salinity of water in which the mangrove leaves were collected and measured with hand refractometer.**

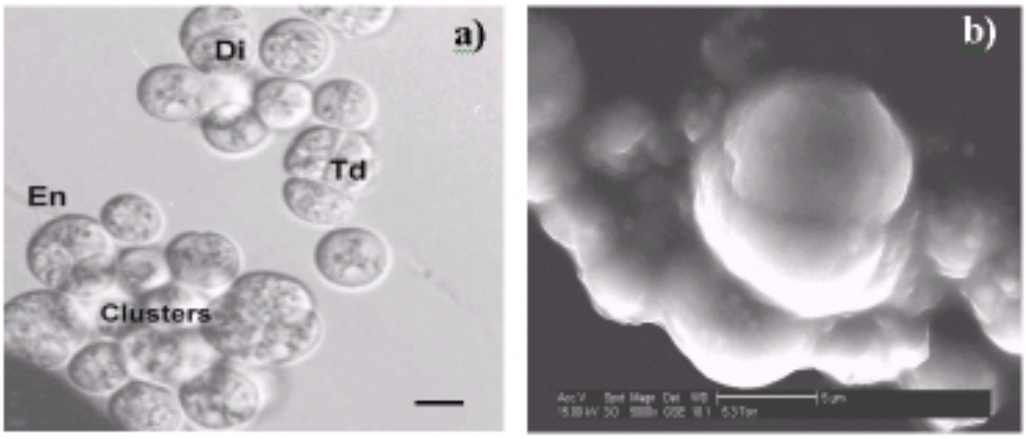

Figure 1. Microphotographs of *Schizochytrium* sp. N-2 under light microscope (a) show diads (Di), tetrads (Td), the extoplasmic net (En) and clusters of the vegetative cell on the glucose yeast extract medium, and under scanning electron microscope (b) show the oily diads cells. The bar in (a) equals 10 μm.

after referred to as *Schizochytrium* strain N-1, N-2, N-5, N-6, and N-9. A further assignment of species identity is ongoing. All *Schizochytrium* isolates showed growth in the glucose yeast extract medium after 52 h of incubation. *Schizochytrium* isolate N-2 grew better than other isolates. These isolates produced cell biomass ranging from 10.8 to 13.2 g/l, with *Schizochytrium* N-2 yielding the highest dried cell mass at 13.2 g/l. *Schizochytrium* N-9 grew poorly with 10.8 g/l of biomass in glucose yeast extract medium (Table 2).

Polyunsaturated fatty acids production

All isolates grew well in glucose yeast extract medium and accumulated DHA intracellularly. Biomass production, fatty acid profiles, and DHA yields obtained from five *Schizochytrium* isolates grown on that medium are summarized in Table 2. Pentadecylic acid (15:0), palmitic acid (16:0) and DHA were the dominant fatty acids present. These strains also produced significant quantities of docosatetraenoic acid (22:4) and trace amounts of unsaturated fatty acids of 18 carbon was observed.

Table 2. Biomass production, fatty acid compositions and docosahexaenoic acid (DHA) yields of freeze-dried *Schizochytrium* strains after culturing for 52 h. at 25ºC in glucose yeast extract mediuma.

Strains	Fatty acid (% of total fatty acids)[b]											DHA	Biomass
	15:0[c]	16:0	18:0	18:1	18:2	18:3	20:4	20:5	22:4	22:6	other	(mg/g)	Production (g/l)
Schizochytrium sp. N-1	28.2	19.7	0.7	0.0	0.0	0.4	0.8	1.1	10.2	31.6	7.6	174.9	11.3
Schizochytrium sp. N-2	28.7	21.3	0.9	0.0	0.0	0.2	0.3	0.9	6.7	36.1	4.9	203.6	13.2
Schizochytrium sp. N-5	24.1	29.4	1.1	0.0	0.0	0.2	0.4	0.9	6.3	33.7	3.9	186.1	12.5
Schizochytrium sp. N-6	28.5	21.7	1.3	0.0	0.0	0.1	0.9	0.6	7.8	30.3	8.8	171.3	11.6
Schizochytrium sp. N-9	23.9	28.3	0.8	0.0	0.0	0.1	0.7	0.5	7.4	31.4	6.9	157.9	10.8

[a] **Medium composed of 60 g of glucose and 10 g of yeast extract in 1 L of 15‰ ASW at pH 6.0**
[b] **Data are expressed as mean of triplicate flasks.**
[c] **15:0, pentadecyclic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1,oleic acid; 18:2(n-6), linoleic acid; 18:3(n-3), α-linolenic acid; 20:4(n-6), arachidonic acid; 20:5(n-3), ecosapentaenoic acid (EPA); 22:4, docosatetraenoic acid; 22:6 (n-3), docosahexaenoic acid (DHA).**

Total fatty acid profiles of five *Schizochytrium* isolates are characterized as follows: (1) most strains had a relatively high level (as a percentage of total fatty acids) of odd-chain saturated fatty acids, mainly pentadecyclic acid (C15:0), 23.9-28.7%); (2) palmitic acid (16:0, 19.7-29.4%), an even-chain saturated fatty acid, was found in all strains; (3) 18-carbon fatty acids, especially oleic and linoleic acids, were absent in all strains; and (4) PUFA level was high in most strains with DHA accounting for 30.3-36.1%.

Schizochytrium strain N-2 in this study was a good source for DHA producers, yielding 203.6 mg/g freeze-dried cell mass in glucose yeast extract medium (Table 2). Only *S. limacinum* SR21 produced a comparable quantity of DHA, resulting in 276.5 mg/g of biomass under optimized condition (Yokochi *et al*., 1998). *S. limacinum* SR21 was isolated from subtropical mangrove in the same way as *Schizochytrium* strains in this study. Bowles *et al*. (1999) screened 57 thraustochytrids isolated from temperate and subtropical areas. Their results indicated that subtropical strains were high biomass producers with moderate DHA levels (4-34%), while their temperate strains produced higher DHA level (17-47%) but low biomass production. In general, this study agrees with the observations of Bowles *et al*. (1999) in which subtropical strains produced high biomass with moderate DHA production.

Table 3. Salinity requirement for vegetative growth of selected thraustochytrids from Antarctic, temperate and subtropical regions compared with strains from the present study.

Species	Vegetative growth		Water salinity (‰) or substrate of isolation	References
	Salinity range (‰)	Optimum salinity (‰)		
Antarctic species				
Thraustochytrium antarcticum Bahnweg *et* Sparrow	5-35	15-30	sea water	Bahnweg, 1979
Thraustochytrium kerguelensis Bahnweg *et* Sparrow	15-35	20-30	sea water	Bahnweg, 1979
Thraustochytrium rossii Bahnweg *et* Sparrow	15-35	15-20	sea water	Bahnweg, 1979
Temperate species				
Althornia crouchii E.B.G. Jones *et* Alderman	15-35	30-35	sea water	Alderman and Jones, 1971
Labyrinthuloides haliotidis Bower	15-45	30	abalone tissue	Bower, 1987
Thraustochytrium aureum S. Goldstein	5-35	20-35	littoral water	Goldstein, 1963b
Thraustochytrium motivum S. Goldstein	5-35	25-35	littoral water	Goldstein, 1963a
Thraustochytrium roseum S. Goldstein	10-35	25-35	littoral water	Goldstein, 1963a
Subtropical species				
Schizochytrium limacinum Honda *et* Yokochi	0-30	15-30	mangrove water	Honda *et al*., 1998
Strains from this study				
Schizochytrium sp. N-1	5-30	15-25	5/ mangrove leaves	this study
Schizochytrium sp. N-2	0-30	20-30	7/ mangrove leaves	this study
Schizochytrium sp. N-5	5-30	20-30	5/ mangrove leaves	this study
Schizochytrium sp. N-6	5-30	25-30	8/ mangrove leaves	this study
Schizochytrium sp. N-9	0-30	25-30	8/ mangrove leaves	this study

Effect of salinity on vegetative growth

The salinity requirements of the selected thraustochytrids from various geographical regions (Antarctic, temperate and subtropical) and this study are summarized in Table 3. The isolates from Hong Kong (N-1, N-2, N-5, N-6, and N-9) demonstrated that thraustochytrids could be isolated from low salinity waters while the remaining strains listed were isolated from environments with salinity levels close to full strength seawater. However, all strains examined here could grow over a wide range of salinity (5-35‰). Isolate N-2 showed a slight resistance to high salinity, up to 30‰ of NSW, which was similar to the excellent DHA-producer, *Schizochytrium limacinum* SR21 (Yokochi *et al*., 1998). Salinity optima for five *Schizochytrium* strains were observed at 25-30‰. The isolates from Hong Kong demonstrated that thraustochytrids could grow over a wide range of salinity from 0-35‰ with strong growth at the 25-30‰. This indicates that the salinity of the isolation site has no effect on the salinity tolerance for vegetative growth under laboratory conditions.

Bahnweg (1979) reported *Thraustochytrium aureum* and *Schizochytrium aggregatum* Golds et Belsky 3401 could grow even with a salinity as low as 1‰, but no growth was observed in a medium prepared from distilled water. Absence of growth or reduced growth in medium with a minimum quantity of sea salts may be largely due to ion deficiencies. Seawater contains major ions of Na, Ca, K and Mg which are essential for the growth of marine fungi (Jennings, 1983). The absence of potassium ions caused reduced growth in thraustochytrids (Bahnweg, 1979). Sodium ions were required in macro-quantities for phosphate uptake and a strong growth of thraustochytrids. In addition, sodium could not be replaced by potassium for the growth of thraustochytrids (Garrill *et al*., 1992).

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