Growth and fatty acid production of thraustochytrids from Panay mangroves, Philippines

Eduardo M. Leaño[1*], Rolando S.J. Gapasin[1], Bernice Polohan[1] and Lilian L.P. Vrijmoed[2]

[1]Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan, 5021 Iloilo, Philippines
[2]Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong SAR, PR China

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Optimization of culture conditions with regard to the combined effects of salinity and temperature on biomass and fatty acid production of four thraustochytrid isolates were undertaken. Two strains of *Schizochytrium mangrovei* (IAo-1 and IXm-6), and one isolate each of *Schizochytrium* sp. (BSn-1) and *Thraustochytrium* sp. (IRa-8), isolated from fallen mangrove leaves, were used in this study. Results of the physiological study show that the best growth condition for *Schizochytrium* isolates was at a salinity range of 15-30 ‰ at 20-30°C, while that for *Thraustochytrium* sp. was at 22.5-30 ‰ at 25°C. Highest biomass production was 350 mg 50 mL^{-1} for *Schizochytrium* spp., and 133 mg 50 mL^{-1} for *Thraustochytrium* sp. Total lipid content (% freeze-dried biomass) ranged from 16.0-33.2% for *S. mangrovei*, 13.0-39.1% for *Schizochytrium* sp., and 11.4-37.5% for *Thraustochytrium* sp. Highest lipid production was observed at 15-22.5 ‰ salinity (25°C) for *S. mangrovei*, and at 15 ‰ (25°C) for *Schizochytrium* sp. and *Thraustochytrium* sp. Palmitic acid (16:0) and docosahexaenoic acid (DHA; 22:6n3) were the major components of the total fatty acid (TFA) content, comprising about 39-42% and 24-35%, repectively.

Key words: biomass, DHA, docosahexaenoic acid, lipid, *Schizochytrium*, *Thraustochytrium*.

Introduction

Thraustochytrids are ubiquitous in marine and estuarine environments, in both tropical and sub-tropical areas. They are reported to be associated with mangrove swamps (Ulken, 1981), seawater (Honda *et al.*, 1998), marine sediments (Raghukumar, 1988), and littoral algae and seaweeds (Miller and Jones, 1983). They are also associated with fallen leaves of many mangrove species (Raghukumar, 1988; Leaño, 2001; Fan *et al.*, 2002). Thraustochytrids

* Corresponding author: E.M. Leaño; e-mail: edleano@yahoo.com

are reported to play an important role in the aquatic food web through their degradative activities (Ulken, 1981). Raghukumar *et al.* (1994) reported that *Schizochytrium mangrovei* was capable of secreting high levels of degradative enzymes including cellulase and polygalacturonase. In more recent studies, many species of *Schizochytrium* and *Thraustochytrium* are also reported to be efficient producers of polyunsaturated fatty acids (PUFA) including the n-3 series, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Nakahara *et al.*, 1996; Singh *et al.*, 1996; Yaguchi *et al.*, 1997; Yokochi *et al.*, 1998; Fan *et al.*, 2001). In this study, we assess the optimal conditions with regard to salinity and temperature on the biomass and fatty acid production of selected thraustochytrid species from Panay mangroves, Philippines.

Materials and methods

Isolates used

Two strains of *Schizochytrium mangrovei* (IAo-1 and IXm-6), one *Schizochytrium* sp. BSn-1, and one *Thraustochytrium* sp. IRa-8 were used in this study. These isolates have been characterized by Leaño (2001). They are maintained in axenic culture in yeast-extract-peptone slants {YEP; composed of 1 g yeast extract (BBL), 1 g mycological peptone (Oxoid), 15 g agar (BBL), 1 L 50% artificial seawater [ASW; using sea salts (Sigma)]} kept at 4°C and sub-cultured every three weeks. Back-up cultures are maintained in 10% glycerol (50% ASW) stored at -80°C, and sub-cultured every three months.

Physiological study

The isolates were sub-cultured on YEP agar and incubated for 2 days at room temperature. When sufficient cell growth is obtained, the agar plate is flooded with a thin layer of sterile 50% ASW to induce sporulation. After 2 hours, a zoospore suspension is then collected and the inoculum was prepared following the procedure of Fan *et al.* (2002) with slight modification (cultures were shaken for 24 hours instead of 40 hours). The zoospore suspension (1 mL) was inoculated into 50 mL of peptone-yeast-extract-glucose (PYG) broth (composed of 1 g peptone, 1 g yeast extract, 10 g glucose, 1 L 50% ASW) and shaken at 200 rpm for 24 hours at 25°C.

Modified PYG broth (composed of 1 g mycological peptone, 10 g yeast extract, 10 g glucose (Ajax), 1 L ASW) were prepared at different salinity levels using sea salts: 7.5, 15, 22.5 and 30 ‰. Fifty mL of the broth were dispensed in 125-mL flasks and autoclaved. The flasks were then inoculated with 1 mL of the inoculum and incubated at different temperature levels (20, 25, 30 and 35°C) in a shaking incubator for 4 days at 200 rpm. Each treatment

has three replicates. Cells were then harvested by centrifugation (1000 rpm), washed in sterile 50% ASW twice, and freeze-dried for 24 hours. Biomass is expressed as mg freeze-dried weight (FDW) per 50 mL broth.

Lipid production and fatty acid level

Biomass production. The isolates were cultured in modified PYG (using 20 g glucose) at two salinity levels (15 and 22.5 ‰) and three temperature levels (20, 25 and 30°C), under shaking at 200 rpm for 4 days. Each salinity and temperature combinations has three replicates. Cells were harvested by centrifugation, washed in 50% ASW, freeze-dried for 24 hours and stored in -80°C until used for lipid extraction and fatty acid analysis.

Lipid extraction. Total lipids (TL) were extracted from approximately 100-200 mg of freeze-dried thraustochytrid samples (2-3 replicates) by homogenization in chloroform/methanol (2:1, v/v) following the ICES method (Coutteau and Sorgeloos, 1995 modified after Ways and Hanahan, 1964). Lipid extracts were evaporated under vacuum (Eyela, Japan) at 38°C and subsequently dried under a stream of nitrogen. Lipid weight was determined gravimetrically. TL samples were stored in -80°C until further analysis.

Fatty acid analysis. Lipids were transesterified with acetylchloride/methanol mixture (5:100, v/v) as described by Coutteau and Sorgeloos, 1995 (modified from Lepage and Roy, 1984). For quantitation purposes, an internal standard heneicosanoic acid methyl ester, 21:0 (Sigma) was added equivalent to 3-5% of the total weight of the fatty acid methyl esters (FAMEs). FAME extracts were evaporated under vacuum, dried under a stream of nitrogen and weighed. Dried FAMEs were resuspended in isooctane (LiChroSolv, Merck) prior to analysis with a GC-17A gas chromatograph (Shimadzu, Kyoto, Japan), equipped with a flame ionization detector (FID), and fitted with a 30 m (long) × 0.32 mm (i.d.) × 0.2 μm (film thickness) SPB PUFA column (Supelco, PA, USA). Helium was used as carrier gas (linear velocity: 25 cm sec^{-1} at 185°C). Injector and detector temperatures were at 250°C and 260°C, respectively. The column temperature was programmed to rise from 185°C to a final temperature of 210°C at a rate of 3°C min^{-1}. FAMEs were identified by comparing the relative retention times of the fatty acids (from the thraustochytrid samples) with that of a known reference standard, PUFA No. 3 from menhaden oil (Supelco, PA, USA) and quantified by a Chromatopac C-R7A integrator (Shimadzu, Kyoto, Japan). The amounts of FAMEs were calculated using the internal standard method and expressed in mg g^{-1} FDW sample.

Table 1. Total lipid production of thraustochytrid isolates at different salinity and temperature levels.

Isolate	Salinity (‰)	Total lipid (% freeze dried weight)		
		20°C	25°C	30°C
S. mangrovei IAo-1	15	19.9[b]*	33.2[a]	18.3[b]
	22.5	18.3[b]	30.2[a]	29.0[a]
S. mangrovei IXm-6	15	21.0[b]	23.7[ab]	17.9[bc]
	22.5	24.0[ab]	29.8a	16.0[c]
Schizochytrium sp. BSn-1	15	16.1[b]	39.1[a]	16.0[b]
	22.5	nt	24.2[b]	13.0[c]
Thraustochytrium sp. IRa-8	15	16.7[b]	37.5[a]	nt**
	22.5	11.4[b]	11.8[b]	nt

* Means with the same letter superscripts (per isolate) are not significantly different ($p > 0.05$).
** nt: not tested.

Statistical analysis

Data were analyzed using two-way analysis of variance (ANOVA) and significant differences among treatment means were compared using Duncan's multiple range test (DMRT) (Zar, 1996).

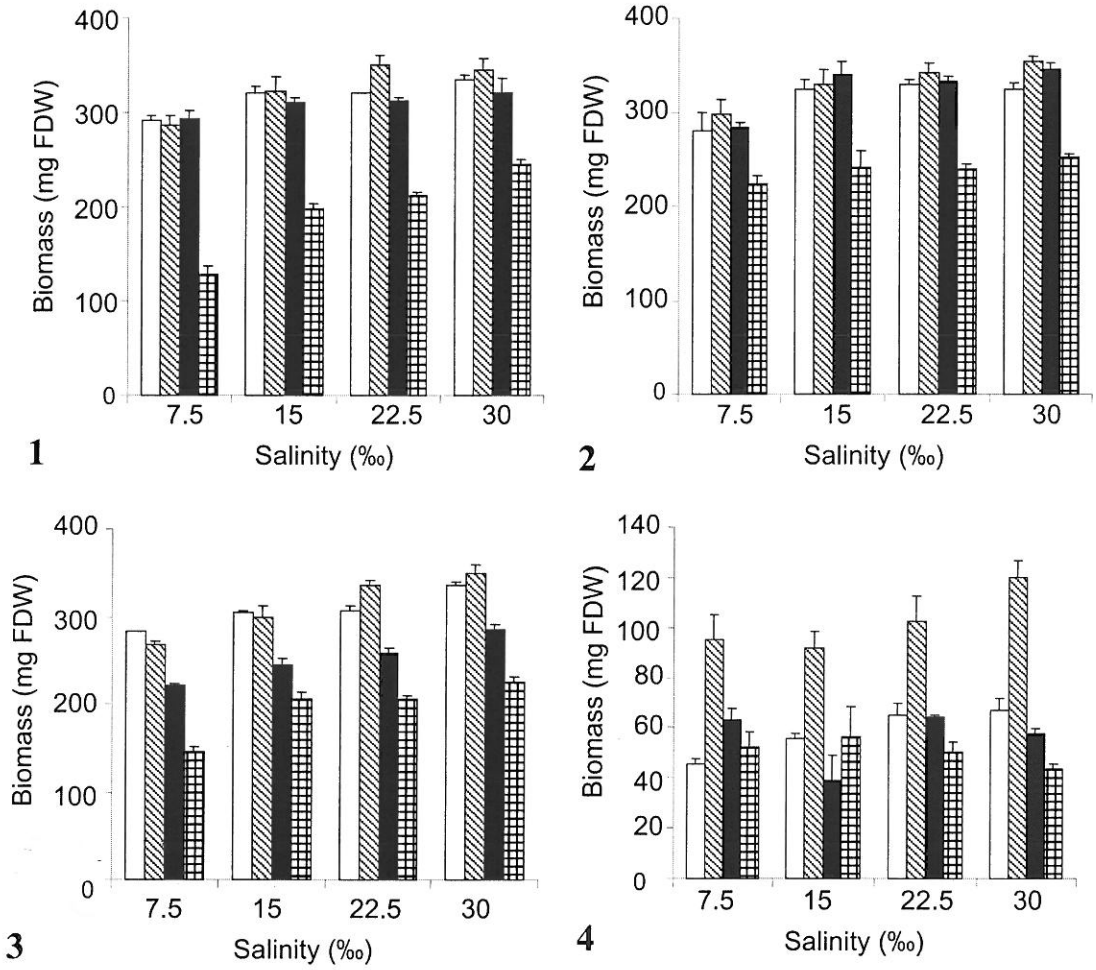
Results

Physiological study

Biomass production of the different thraustochytrid isolates at different salinity and temperature levels are shown in Figs. 1-4. In general, higher biomass was obtained from *Schizochytrium* isolates compared with *Thraustochytrium* sp. Optimum salinity and temperature requirements for biomass production were: 15-30‰ at 20-30°C for *S. mangrovei*; 15-30‰ at 20-25°C for *Schizochytrium* sp.; and, 22.5-30‰ at 25°C for *Thraustochytrium* sp. Biomass production at these salinity and temperature combinations were significantly different ($p < 0.01$) from other salinity-temperature combinations tested. The highest biomass obtained for *S. mangrovei* was 350 mg 50 mL^{-1} (~7g L^{-1}), 346 mg 50 mL^{-1} (~7 g L^{-1}) for *Schizochytrium* sp., and 133 mg 50 mL^{-1} (~2.7g L^{-1}) for *Thraustochytrium* sp.

Lipid production and fatty acid levels

Using PYG with elevated glucose level (20 g), higher biomass were obtained for all the isolates (~22 g L^{-1} for *Schizochytrium* spp. and ~6 g L^{-1} for *Thraustochytrium* sp.), at optimum salinity and temperature levels. Total lipid



Figs. 1-4. Biomass production (per 50 mL broth medium) of the thrasutochytrid isolates at different salinity and temperature levels after 4 days incubation at 200 rpm. **1.** *Schizochytrium mangrovei* IAo-1. **2.** *S. mangrovei* IXm-6. **3.** *Schizochytrium* sp. BSn-1. **4.** *Thraustochytrium* sp. IRa-8. (□ - 20°C; ▨ - 25°C; ■ - 30°C; ▦ - 35°C)

content (% freeze-dried biomass) ranged from 16.0-33.2% for *S. mangrovei* isolates, 13.0-39.1% for *Schizochytrium* sp., and 11.4-37.5% for *Thraustochytrium* sp. (Table 1). Optimum lipid production was observed at 15-22.5‰ salinity (25°C) for *S. mangrovei* isolates, and 15‰ (25°C) for *Schizochytrium* sp. and *Thraustochytrium* sp., which were significantly different ($p < 0.01$) from the other salinity-temperature treatments.

Fatty acid production of the thraustochytrid isolates at different salinity and temperature levels are shown in Tables 2-4. *Schizochytrium* spp. produced

Table 2. Fatty acid production of two strains of *Schizochytrium mangrovei* cultured at different salinity and temperature levels.

Isolate	Salinity (‰)	Fatty acids*	Production, mg g^{-1} (%)		
			20°C	25°C	30°C
IAo-1	15	14:0	2.3 (1.5)	1.2 (0.6)	1.8 (1.4)
		16:0	59.0 (38.9)	74.9 (39.7)	53.1 (40.3)
		16:3n4	1.5 (1.0)	1.5 (0.8)	1.2 (0.9)
		20:4n6	1.0 (0.7)	0.8 (0.4)	0.7 (0.5)
		20:5n3	0.8 (0.6)	1.4 (0.7)	1.0 (0.8)
		22:6n3	43.8 (28.9)	69.9 (37.1)	37.5 (28.0)
		Others	43.1 (28.4)	38.8 (20.6)	37.1 (28.1)
		Total	**151.6 [b]****	**188.6 [a]**	**131.9 [b]**
	22.5	14:0	1.0 (0.9)	4.0 (2.7)	2.9 (1.7)
		16:0	46.5 (44.1)	57.3 (38.3)	65.2 (42.2)
		16:3n4	1.0 (0.9)	2.2 (1.5)	2.4 (1.5)
		20:4n6	0.4 (0.4)	0.9 (0.6)	1.0 (0.6)
		20:5n3	0.9 (0.8)	1.0 (0.7)	1.3 (0.8)
		22:6n3	23.4 (22.2)	49.0 (32.8)	45.5 (29.3)
		Others	32.3 (30.6)	30.6 (34.8)	37.1 (23.9)
		Total	**105.5 [c]**	**149.6 [b]**	**155.2 [b]**
IXm-6	15	14:0	3.0 (1.9)	5.6 (2.8)	4.6 (2.5)
		16:0	61.9 (40.1)	80.0 (39.3)	74.6 (40.0)
		16:3n4	1.8 (1.2)	5.9 (2.9)	4.0 (2.1)
		20:4n6	1.2 (0.8)	1.4 (0.7)	1.1 (0.6)
		20:5n3	1.0 (0.6)	1.5 (0.7)	1.3 (0.7)
		22:6n3	51.6 (33.4)	67.8 (33.3)	61.0 (32.7)
		Others	33.8 (21.9)	41.4 (20.3)	39.9 (21.4)
		Total	**154.3 [a]**	**203.6 [a]**	**186.5 [a]**
	22.5	14:0	3.5 (2.3)	4.6 (2.6)	2.5 (2.2)
		16:0	56.6 (37.3)	69.3 (39.0)	36.1 (31.5)
		16:3n4	2.3 (1.5)	4.3 (2.4)	1.3 (1.1)
		20:4n6	0.9 (0.6)	1.0 (0.6)	1.0 (0.9)
		20:5n3	0.9 (0.6)	1.0 (0.6)	0.6 (0.5)
		22:6n3	49.7 (32.7)	58.7 (33.1)	26.9 (23.4)
		Others	37.9 (25.0)	38.6 (21.7)	46.2 (40.3)
		Total	**151.8 [b]**	**177.5 [a]**	**114.7 [b]**

* 14:0: myristic acid; 16:0: palmitic acid; 16:3n4:16-carbon triene PUFA; 20:4n6: arachidonic acid; 20:5n3: eicosapentaenoic acid (EPA); 22:6n3: docosahexaenoic acid (DHA).

** Total fatty acid values (per isolate) with the same letter superscript are not significantly different ($p > 0.05$).

higher levels of fatty acids compared to *Thraustochytrium* sp. Palmitic acid (16:0) and docosahexaenoic acid (DHA; 22:6n3) were the major components comprising about 39-42% and 24-35%, respectively, of the total fatty acids (TFA). Four other fatty acids were identified but in minimal amounts, including myristic acid (14:0), arachidonic acid (20:4n6), eicosapentaenoic

Table 3. Fatty acid production of *Schizochytrium* sp. BSn-1 cultured at different salinity and temperature levels.

Salinity (‰)	Fatty acid*	Production, mg g^{-1} (%)		
		20°C	**25°C**	**30°C**
15	14:0	3.0 (1.9)	6.2 (3.1)	1.3 (0.9)
	16:0	60.7 (39.3)	83.4 (42.0)	52.8 (37.6)
	16:3n4	2.8 (1.8)	5.7 (2.9)	2.2 (1.6)
	20:4n6	1.3 (0.8)	1.2 (0.6)	0.8 (0.6)
	20:5n3	1.2 (0.8)	1.5 (0.8)	1.1 (0.8)
	22:6n3	51.3 (33.2)	58.3 (29.4)	41.8 (29.8)
	Others	34.2 (22.1)	42.3 (21.2)	40.3 (28.7)
	Total	**154.5** ^{b}**	**198.6** ^{a}	**140.3** ^{bc}
22.5	14:0	-	3.8 (2.7)	1.7 (1.4)
	16:0	-	53.2 (37.3)	54.7 (44.5)
	16:3n4	-	3.1 (2.2)	3.1 (2.5)
	20:4n6	-	0.7 (0.5)	0.6 (0.5)
	20:5n3	-	0.9 (0.6)	0.9 (0.7)
	22:6n3	-	43.1 (30.2)	31.1 (25.3)
	Others	-	37.8 (26.4)	30.9 (25.1)
	Total	**-**	**142.6** ^{b}	**123.0** ^{c}

* As in Table 2.

** Total fatty acid values with the same letter superscript are not significantly different ($p > 0.05$).

Table 4. Fatty acid production of *Thraustochytrium* sp. IRa-8 cultured at different salinity and temperature levels.

Salinity (‰)	Fatty acid*	Production, mg g^{-1} (%)	
		20°C	**25°C**
15	14:0	1.6 (1.4)	1.1 (1.0)
	16:0	46.0 (39.6)	48.5 (42.1)
	16:3n4	0.3 (0.3)	0.6 (0.5)
	20:4n6	2.4 (2.1)	3.0 (2.6)
	20:5n3	1.4 (1.2)	1.4 (1.2)
	22:6n3	28.2 (24.3)	21.7 (18.8)
	Others	36.2 (31.2)	38.9 (33.8)
	Total	**116.1** ^{a}**	**115.1** ^{a}
22.5	14:0	0.3 (0.3)	1.5 (1.7)
	16:0	47.3 (46.1)	32.3 (37.3)
	16:3n4	0.3 (0.3)	0.5 (0.6)
	20:4n6	1.8 (1.8)	5.5 (6.4)
	20:5n3	1.5 (1.5)	1.1 (1.3)
	22:6n3	22.9 (22.3)	15.8 (18.3)
	Others	28.5 (27.8)	29.8 (34.4)
	Total	**102.6** ^{a}	**86.5** ^{a}

* As in Table 2

**Total fatty acid values with the same letter superscript are not significantly different ($p > 0.05$).

acid (20:5n3) and a 16-carbon triene PUFA (16:3n4). Six other fatty acids were unidentified which comprised 20-30% TFA. These unindentified fatty acids might be of plant origin as they cannot be identified using a reference standard coming from an animal source, i.e. menhaden oil. Optimum salinity and temperature levels for TFA production are 15‰ at 25°C for *Schizochytrium* isolates and 15‰ at 20-25°C for *Thraustochytrium* isolate.

Discussion

Marine straminipilous organisms are commonly associated with decaying mangrove leaves in both tropical and sub-tropical mangrove habitats. The most commonly associated species are the halophytophthorans (Leaño *et al.*, 2000, Nakagiri, 2000) and thraustochytrids (Raghukumar, 1988; Bremer, 1995; Leaño, 2001; Fan *et al.*, 2002). These organisms are reported to play an active role in altering the chemistry of mangrove detritus (Raghukumar *et al.*, 1994) and therefore enriching the nutrient contents with their biomass (Nakagiri, 1998). Thus, several studies were undertaken to determine the salinity and temperature tolerance of these organisms in order to understand their wide distribution among mangrove habitats in both marine and estuarine environments (Nakagiri, 1993; Leaño *et al.*, 1998; Yokochi *et al.*, 1998; Fan *et al.*, 2002).

Thraustochytrids, are reported to be a rich source of polyunsaturated fatty acids (PUFAs) specifically docosahexaenoic acid (DHA) (Yaguchi *et al.*, 1997). These organisms are considered as one of the potential alternative sources of PUFA for both commercial and industrial exploitation. Optimization of culture conditions is important for maximum biomass and high yield of the n-3 series fatty acid of potential thraustochytrid isolates. This is usually done either through modification of the culture media (e.g. carbon and nitrogen sources) (Bajpaj *et al.*, 1991; Iida *et al.*, 1996; Yokochi *et al.*, 1998), or manipulating the physico-chemical parameters such as salinity and temperature of the culture conditions (Singh *et al.*, 1996; Yokochi *et al.*, 1998; Fan *et al.*, 2002). In this study, optimization of salinity and temperature levels for maximum biomass production of the thraustochytrid isolates was undertaken. Results show that optimum salinity and temperature levels ranged between 15-22.5‰ salinity and 20-25°C temperature. These salinity and temperature levels are similar to other reports on thraustochytrid isolates including *Schizochytrium limacinum* (Yokochi *et al.*, 1998), *Thraustochytrium aureum* (Iida *et al.*, 1996), *S. mangrovei* (Fan *et al.*, 2002), and other *Schizochytrium* and *Thraustochytrium* species (Nakahara *et al.*, 1996; Singh, *et al.*, 1996; Fan *et al.*, 2002).

Lipid production of the thraustochytrid isolates used in this study was also highest at optimum salinity and temperature levels obtained for biomass production. Maximum lipid yield was 30% of freeze-dried weight (FDW) biomass for *S. mangrovei*, 39% FDW biomass for *Schizochytrium* sp. and 38% FDW biomass for *Thraustochytrium* sp. (Table 2). The total lipid content obtained from this study are comparable with the reported lipid production of other thraustochytrids, ranging between 16 to 50% of the dry weight biomass (Bajpaj, *et al.*, 1991; Iida *et al.*, 1996; Nakahara *et al.*, 1996; Weete *et al.*, 1997)

Total fatty acid (TFA) content of the lipid extracts from thraustochytrid cells are mainly composed of palmitic acid (PA) and DHA. Under optimum culture conditions in this study, PA accounted for a mean of 39% of TFA while DHA was 33% of TFA for *Schizochytrium* species. For *Thraustochytrium* sp., PA was 42% of TFA while DHA was only 23% of TFA. PA and DHA are also the dominant fatty acids produced by *S. limacinum* (Yokochi *et al.*, 1998), *S. mangrovei* (Fan *et al.*, 2001), *T. aureum* (Bajpaj *et al.*, 1991; Iida *et al.*, 1996), and *Thraustochytrium* sp. (Singh *et al.*, 1996).

Glucose was reported as one of the best carbon sources for biomass production as well as DHA yield of thraustochytrids (Yokochi *et al.*, 1998). In this study, the increase in glucose concentration from 10 g L^{-1} to 20 g L^{-1} resulted in a three fold increase in biomass yield for *Schizochytrium* spp. Singh *et al.* (1996) also reported that highest biomass and DHA yield of *Thraustochytrium* sp. was obtained in glucose as carbon source. Bajpaj *et al.* (1991) found that lipid content of the biomass of *T. aureum* increased from 2.7% to 16.5% when the glucose concentration was increased from 5 to 20 g L^{-1}. Consequently, DHA yield also increased from 26 to 270 mg g^{-1}. Extrapolating from the data obtained in this study on DHA production based on optimum biomass production, *S. mangrovei* can produce approximately 1,400 mg L^{-1} DHA in a medium containing 20 g L^{-1} glucose. Bowles *et al.* (1999) reported DHA production of *S. mangrovei* to be 2,170 mg L^{-1} using 40 g glucose, while Fan *et al.* (2001) reported a high of 3,094 mg L^{-1} using 60 g L^{-1} glucose. On the other hand, *S. limacinum* can produce 13,300 mg L^{-1} DHA at a very high glucose concentration of 120 g L^{-1} (Yaguchi *et al.*, 1997). However, as demonstrated by these previous studies, glucose is not the only component in the growth medium which contributes to biomass production. The nature and concentration of nitrogen source as well as other compounds with respect to glucose concentraton is equally important.

From the results obtained from this study, and other studies on DHA production of thraustochytrids, *Schizochytrium* and *Thraustochytrium* species from marine and estuarine habitats are potential alternative sources of PUFA to

fish oil, which is currently the largest commercial source of DHA. Fish oils contain 7-14% DHA (Singh and Ward, 1996). However, the main barriers to substantial utilization of fish oils as source of omega-3 fatty acids (DHA and EPA) relate to the undesirable fishy flavor of such products, the oxidative instability of fish oils, and difficulties in producing concentrates of the individual omega-3 fatty acids (Ward, 1995). Thus, alternative sources such as those from microbes, have been tapped for the following reasons (Nakahara *et al.*, 1996): it can provide stable supply of fatty acids; it is a product with less fishy smell; and it can provide highly purified DHA and other PUFAs. The high proportion of DHA in the total lipids of thraustochytrids and the relatively lower amounts of structurally related PUFAs, could simplify the downstream processing of DHA from this source (Singh *et al.*, 1996). Therefore, screening of different species and strains of thraustochytrids with high lipid and DHA yields should be continued.

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