Cultural Optimization of Thraustochytrids for Biomass and Fatty Acid Production

Arafiles KHV [1], Alcantara JCO[1], Cordero PRF [2], Batoon JAL [1], Galura FS [1], Leaño EM[3] and Dedeles GR [1,2*]

[1]Department of Biological Sciences, College of Science, University of Santo Tomas, España, Manila, 1015 Philippines
[2]Research Center for the Natural and Applied Sciences, Thomas Aquinas Research Complex, University of Santo Tomas, 1015 Philippines
[3]Network of Aquaculture Centres in Asia-Pacific, Suraswadi Bldg., Kasetsart University Campus, Ladyao, Jatujak, Bangkok 10900, Thailand

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Two thraustochytrids isolated from fallen mangrove leaves in Subic Bay, Philippines were identified based on their cell morphology, sporogenesis and spore release as: *Thraustochytrium* sp. SB04; and *Schizochytrium* sp. SB11. Physiological screening (temperature, seawater concentration, glucose concentration and initial pH) was undertaken on the two isolates for optimization of culture conditions for biomass and total fatty acid production. Both isolates survived and grew well on glucose as their sole source of carbon. Highest biomass production for the two isolates were obtained at lower glucose concentrations (3–5% w/v) in a half-strength natural seawater (50–60% v/v), at near neutral pH (6.0), and incubation temperatures of 20–30°C. Growth curve analysis showed slower growth (1 d lag time) and a shorter stationary phase (less than 1 day) for *Thraustochytrium* sp. compared to *Schizochytrium* sp. Additional extracellular enzyme screening showed that both isolates only produced lipase. Analysis of fatty acid methyl ester results showed that *Thraustochytrium* sp. produced predominantly short chain fatty acids with oleic acid (18:1), a monounsaturated fatty acid (MUFA) making up as much as 71% of the total fatty acids (TFA). *Schizochytrium* sp., on the other hand, produced high amount of docosahexaenoic acid (DHA) and comprised up to 22.5% of TFA. The large amount of MUFA makes this *Thraustochytrium* sp. a viable source of oil for biodiesel production. Just as important is the high amount of DHA produced by *Schizochytrium* sp., a potential source for local large-scale DHA production.

Key words – thraustochytrid/s – MUFA – PUFA – culture optimization – biomass production – fatty acid production

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*Corresponding author: Dedeles GR – e-mail – grdedeles@yahoo.com, grdedeles@mnl.ust.edu.ph

Introduction

Thraustochytrids are a diverse group of marine protists belonging to the Labyrinthulomycetes, Kingdom Straminipila (Raghukumar 2002). There are seven genera (*Althornia*, *Diplophrys*, *Elina*, *Japonochytrium*, *Thraustochytrium*, *Schizochytrium*, *Ulkenia*) (Raghukumar 2002) and about 35 species. They are commonly found in marine and estuarine habitats and have been shown to play important role in these ecosystems.

Thraustochytrids have recently gained attention due to their ability to produce large amounts of fatty acids (Raghukumar 2008).

These organisms produce omega-3 polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), both of which are of importance to human health as well as in aquaculture (Leaño & Liao 2004, 2007, Raghukumar 2008,). Biomass of thraustochytrids is 10–50% total oil and 30–70% of which is DHA (Singh & Ward 2005). Thaustochytrids also produce up to 90% monounsaturated fatty acids (MUFAs) such as palmitoleic acid (C16:1), oleic acid (C18:1), eicosenoic acid (C20:1), and erucic acid (C22:1). This makes these organisms highly significant as a source of fatty acids for biodiesel production (Fisher et al. 2008).

The utilization of PUFA and MUFA from thraustochytrids has been studied extensively (Fan et al. 2000, 2001, 2002, Fan & Kamlangdee, 2003, Perveen et al. 2006) but, only marginally in the Philippines (Leaño, 2001, Leaño et al. 2003, 2009). DHA has been extensively advertised in commercial products (e.g. milk, infant formulas) for it enhances the formation of the grey matter in the brain, retina, and tissue of the heart (Singh & Ward 2005). Recent studies also report its importance in alleviating symptoms of coronary heart disease, stroke, and rheumatoid arthritis (Fan & Kamlangdee 2003). Alternative biofuels, on the other hand, are usually of plant and animal origin where production is expensive, entails input of much energy, and necessitates the shifting of land use from food production (Meng et al. 2009). Focus on thraustochytrids for biofuels could be more economical and environment friendly, as well as help reduce in the long run the spiraling cost of fuel brought about by the ever-increasing demand for a limited and non-renewable supply of oil (Fukuda et al. 2001, Jolley 2006).

The potential application of thraustochytrids, particularly in the prospect of DHA and biodiesel production, makes it more noteworthy to study the different cultural conditions that affect the growth of these microorganisms. In this study, we have characterized thraustochytrids isolated from fallen mangrove leaves in Subic, Philippines. Their biomass and lipid production as a function of different culture conditions were also determined.

Materials and methods

Collection of mangrove leaves

Senescent fallen mangrove leaves from the Triboa Bay Mangrove Park in Subic, Philippines were collected in ziplock plastic bags and transported to the laboratory in ice. The pH (6.0–7.0) and temperature (31–32°C) of the collection site were also noted. The leaves were immediately processed in the laboratory by punching 2.5 mm disks from each leaf sample. The leaf disks were then washed three times for an hour each with 50% sterile natural seawater (NSW) (Fan & Kamlangdee 2003).

Isolation and identification of thraustochytrids

The washed leaf disks were placed on glucose-yeast extract peptone seawater (GYPS) agar (composed of 3.0 g glucose, 1.25 g yeast extract, 1.25 g peptone, 1.0 L 50% natural seawater (NSW) at pH 6.0, 15.0 g agar (Biogel)) supplemented with 300 mg/L streptomycin and penicillin G (Leaño et al. 2003, Perveen et al. 2006). A small amount of sterile 50% NSW was dropped onto the leaf disks to promote primary sporulation of the organisms. The plates were incubated at 25–28°C for 2–3 d. Thraustochytrid cells growing around the leaf disks were then picked-up and sub-cultured successively on fresh GYPS agar until pure cultures were obtained.

The identification of thraustochytrids was based primarily on the morphology of their thallic stage, differences in sporogenesis, and spore release (Raghukumar 2002). Pure cultures of thraustochytrids were flooded with a thin layer of sterile 50% NSW to induce sporulation. This suspension was then observed microscopically to determine the organism's mode of sporulation, hence, affirming their purity and determining their identity to the genus level (Raghukumar 2002).

Culture condition optimization

Inoculum preparation

The inoculum for biomass optimization was prepared by inoculating pure cultures of

thraustochytrids into GYPS broth (Leaño et al. 2003), and incubating at 25–28°C for 2 days After incubation, the density was adjusted to 0.5 McFarland standard (McFarland 1907) with sterile GYPS broth for use in the succeeding experiments.

Parameters for optimization

The growth of thraustochytrid isolates were tested under four different parameters (incubation temperature, seawater concentration, glucose concentration, and pH) using a modified GYPS broth (composed of 10.0 g glucose, 10.0 g yeast extract, 1.0 g peptone, 1.0 L 50% NSW at pH 6.0) to determine their effects on the biomass production. Five levels of each parameter were tested: temperature (20, 25, 30, 35 and 40°C); seawater concentrations (30, 40, 50, 60 and 70%, v/v); glucose concentrations (3, 5, 7, 9, and 11%, w/v); and initial pH of the medium (4.0, 5.0, 6.0, 7.0 and 8.0). Each parameter was tested separately with other parameters kept constant (temperature 30°C; 50% NSW; 3 or 5% glucose; and initial pH 6.0). One milliliter of the prepared inoculum was inoculated into 100 mL of test media (in triplicate) and incubated for 3 d with constant agitation.

Thraustochytrid cells were harvested by centrifugation at 3500 rpm for 10 mins. The supernatant was then discarded and the cells were dried in an oven at 70°C for 2 d (Anbu et al. 2007). Total biomass was expressed as dry weight (g) per 100 mL medium.

The culture conditions with the greatest growth were statistically validated and set as the optimal conditions. These culture conditions were then used for growth analysis and fatty acid profiling.

Growth analysis under optimum conditions

Flasks containing 100 mL of the modified GYPS medium adjusted to optimal pH, glucose and seawater concentrations obtained for each of the two thraustochytrid isolates were inoculated with one mL of inoculum, and incubated under optimal temperature with constant agitation. At day 0, a sample of the culture was taken for spectrophotometric analysis at OD 660 nm; the blank used was sterile modified GYPS medium. Two flasks were then sampled daily for optical density and biomass yield determination. A calibration curve comparing optical density and biomass of the isolates was prepared.

Fatty acid profiling

The two thraustochytrid isolates were mass produced under optimal conditions as described above. Cells were harvested by centrifugation, freeze dried (Millrock Benchtop Manifold Freeze Dryer), weighed and stored at –80°C until use. Fatty acid profiling was done at the Philippine Institute of Pure and Applied Chemistry (PIPAC), Ateneo de Manila University.

Extraction of fatty acid

One hundred and fifty (150) mg of freeze-dried thraustochytrid cells was put into a 50 mL volumetric flask. Five mL of 0.5 N methanolic-NaOH and boiling chips were then added and the sample was heated in a steam bath until the appearance of fat globules. Afterwards, 5 mL mixture of boron trifluoride (BF_3) and methanol was added and the solution was boiled for 4 mins. Saturated sodium chloride (NaCl) solution was then added to the volumetric flask and filled up to the mark. The flask was shaken vigorously after the addition of 1 mL hexane. The hexane upper layer was then collected in a small vial for injection into a gas chromatograph.

Fatty acid profile analysis

The extracted fatty acid methyl esters (FAME) was immediately injected into the gas chromatograph (Shimadzu GC-2010) equipped with a flame ionization detector with an RT-2560 capillary column (100 m × 0.25 mm ID × 0.20 µm) and helium as carrier gas. The detector temperature was 260°C with the injector temperature at 250°C. The temperature programming was at 60°C for 2 mins to 140°C at 20°C/min, then to 240°C at 4°C/min. The fatty acids present in the sample as well as their quantities were identified by correlating their retention times to FAME standards provided by PIPAC.

Screening of exoenzymes

The two thraustochytrid isolates were screened for extracellular enzyme production using different substrates: milk (protease), so-

luble starch (amylase), Tween 80 (with 0.1g/L $CaCl_2$; lipase) and carboxymethyl cellulose (CMC; cellulase). These substrates were incurporated into a basal medium (BM; composed of 0.5 g yeast extract, 0.5 g peptone, 1 L 50% NSW) at 1% concentration.

All substrate media were inoculated with a linear streak of the isolates and incubated at ambient room temperature for 3 to 5 d, before being observed for exoenzyme activity. Amylase was detected by the presence of clear zone around the colony after addition of 5% Logul's iodine for 3 mins. Lipase activity was detected by the appearance of an opaque zone around the colonies. Clear zone in 1% milk, and the presence of yellow halo around the colony after staining with Congo red and counterstaining with 1M NaCl confirmed the presence of protease and cellulose activity, respectively.

Statistical analysis

Biomass production was statistically analyzed using one-way analysis of variance (ANOVA) with Tukey and Scheffe as post-hoc tests using Statistica Software 6.0 (StatSoft, Inc.) to determine the significant differences under different culture conditions in each parameter used in optimization.

Results

Isolation and identification

Two thraustochytrid strains were identified based on their morphological characteristics, and sporulation, and they were designated as *Thraustochytrium* sp. SB04 and *Schizochytrium* sp. SB11, hereafter referred to as *Thraustochytrium* sp. and *Schizochytrium* sp. *Thraustochytrium* sp. (Fig. 1A) has spherical cells and is characterized by the direct release of zoospores from a single mature thallus (Fig. 1B). It does not undergo repeated binary division of vegetative cells but propagates solely by producing zoospores. On culture plates colonies are pale cream in colour. *Schizochytrium* sp. (Fig. 1C) is characterized by the successive bipartition of the cells into tetrads and octads prior to production of zoospores. This organism also produced irregularly shaped amoeboid cells. They appear as light orange colonies on culture plates.

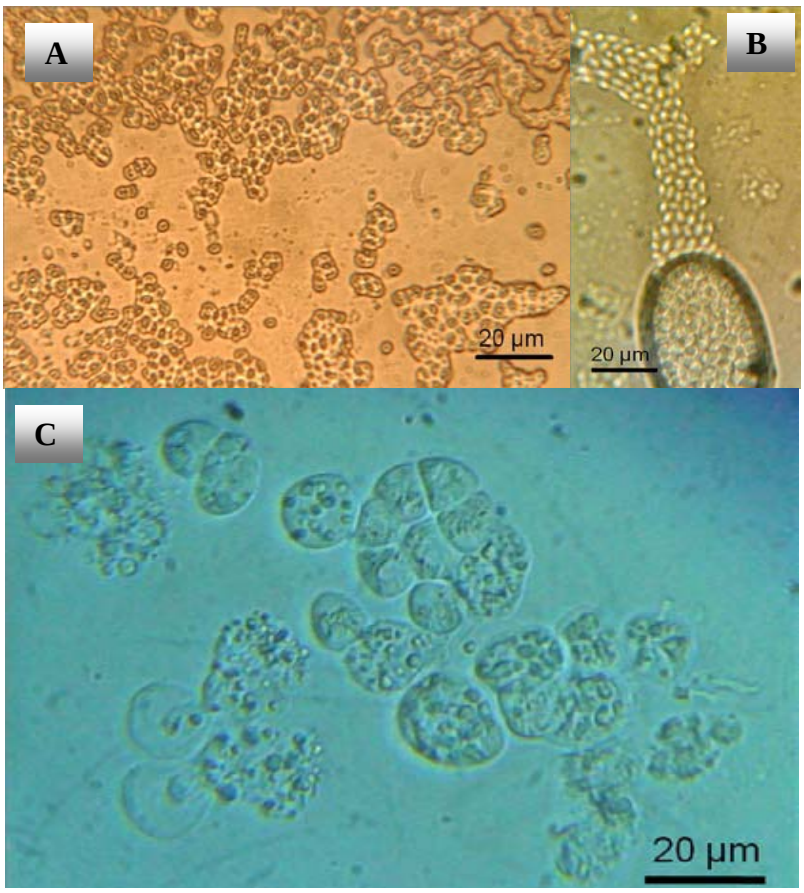
Optimization of culture conditions

Thraustochytrium sp. showed highest biomass after 3 days of culture at an incubation temperature of 30°C, 60% seawater concentration, 3% glucose concentration, and an initial medium pH 6.0 (Fig. 2). Biomass production at these culture conditions ranged from 0.233 to 1.08 g dry weight per 100 mL medium and are statistically significant ($P<0.05$) in three parameters: temperature, seawater concentration and glucose concentration. *Schizochytrium* sp. Produced highest biomass at 20°C, 50% seawater concentration, 5% glucose concentration, and an initial pH of the medium of 6.0 (Fig. 3). Biomass produced under these culture conditions ranged from 0.0200 to 0.0788 g dry weight per 100 mL medium after 3 d of culture. The produced biomass differs significantly ($P<0.05$) in only two parameters, incubation temperature and initial medium pH.

Growth analysis under optimum condition

Biomass production of *Thraustochytrium* sp. was very low (0.028 g/100 mL of medium) with corresponding optical density (OD) at 0.003 after 1 d of incubation (~25.5 hours) indicating the lag phase of growth (Fig. 4). The log phase was observed after 46.5 h of incubation, where a small increase in biomass and OD was already noted. This increase continued until 71.75 h when the highest increase in the OD (a difference of 0.933 absorbance) was observed. On the other hand, highest biomass (0.680 g/100 mL) was only observed at 93.5 h with the corresponding slight increase in OD (by 0.193 absorbance). At 120 h, the organisms growth started to slow indicating the end of the log phase. After this stage, the biomass and OD started to decline indicating stationary phase and start of death phase (144 h).

In contrast, *Schizochytrium* sp. produced a considerable amount of biomass (0.181 g/100 mL) at 25.5 h with corresponding OD of 0.283 (Fig. 5), indicating a shorter lag phase of growth. The highest increase in biomass (difference of 0.265 g/100 mL) and OD (difference of 1.013) was observed at 46.5 h. The increase in biomass and OD was observed until 93.5 h. Thereafter, the biomass started to decrease (by 0.227 g) at 139 h, although a slight increase in OD (by 0.064) was still observed at this stage.



Figs 1 (A–C) – Micrographs of *Thraustochytrium* sp., showing, **A** vegetative cells, **B** direct release of zoospores. Micrograph of *Schizochytrium* sp. and **C** showing octad formation.

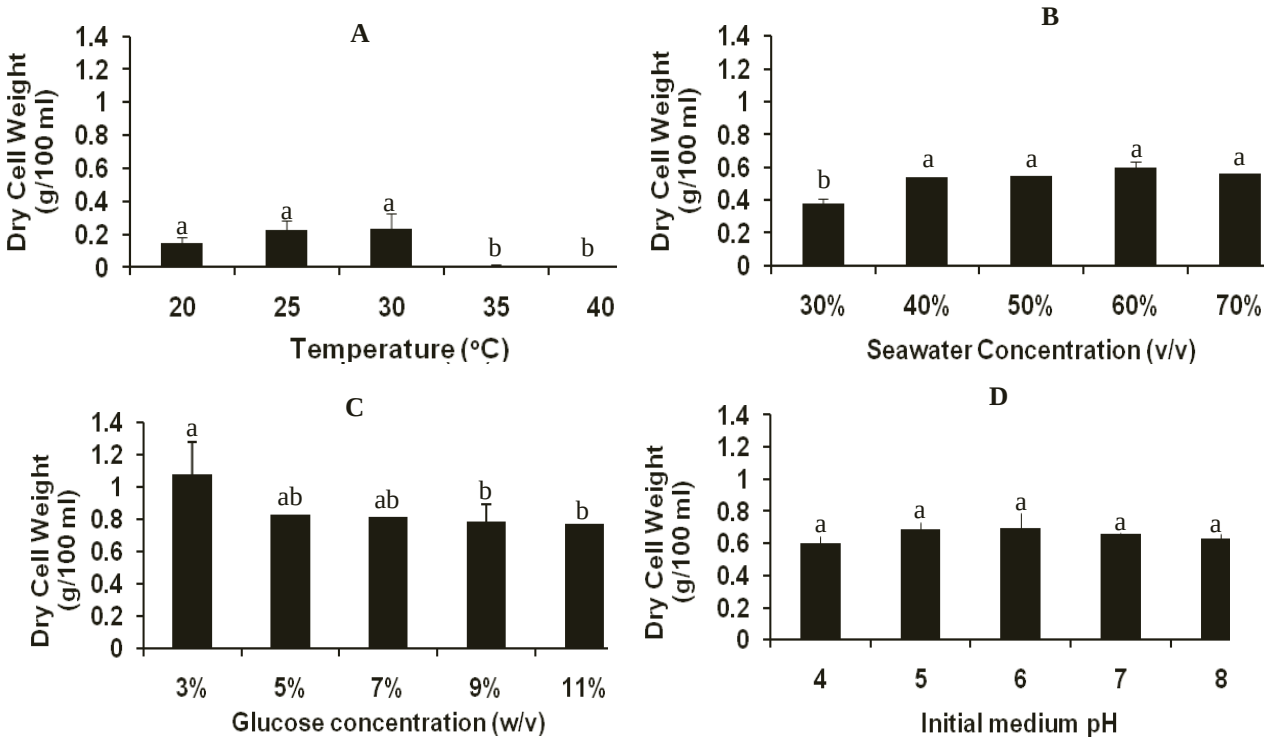


Fig. 2 – Dry biomass produced by *Thraustochytrium* sp. at varying, **A** temperature (ºC), **B** seawater concentration (%,v/v), **C** glucose concentration (%, w/v), and **D** initial pH of medium. The same letter superscript above the bars mean that values are not significantly different ($p>0.05$).

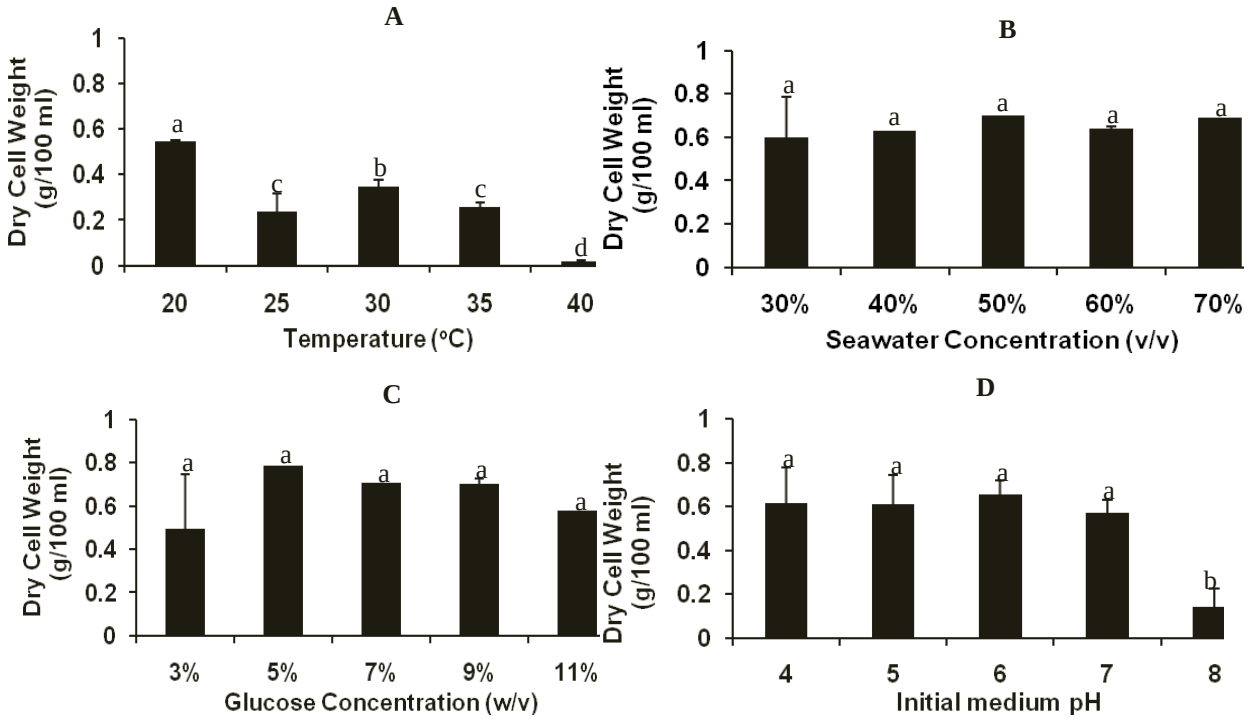


Fig. 3 – Dry biomass produced by *Schizochytrium* sp. at varying **A** temperature (ºC), **B** seawater concentration (%,v/v), **C** glucose concentration (%, w/v), and **D** initial pH of medium The same letter superscript above the bars mean that values are not significantly different ($p>0.05$).

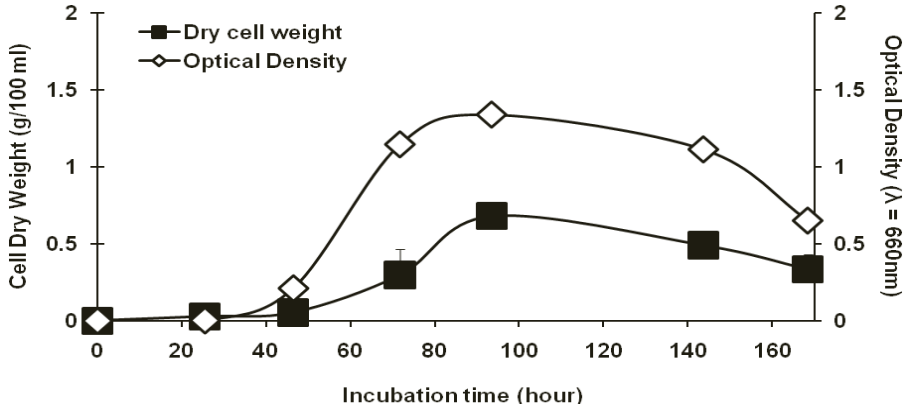


Fig. 4 – Growth curve of *Thraustochytrium* sp. correlating its average cell dry weight (g/100 ml) and average optical density (absorbance at 660 nm) within a 7-day cultivation period.

Fatty acid profile

The predominant fatty acid produced by *Thraustochytrium* sp. was oleic acid (18:1) amounting to 70.6% of total fatty acids (TFA) (Table 1). Other fatty acids produced in smaller amounts were palmitic acid (16:0; 17.7% TFA) and linoleic acid (18:2; 11.7% TFA). *Schizochytrium* sp., on the other hand, produced high amounts of palmitic acid (16:0) and DHA (22:6) at 61.8% and 22.6% of TFA, respectively (Table 1). It also produced other fatty acids including myristic acid (14:0; 3.2% TFA), stearic acid (18:0, 1.6% TFA), and linolenic acid (18:3; 0.5% TFA).

Exoenzyme production

Both isolates produced only lipase and showed negative enzyme activities for protease, amylase and cellulose (Table 2).

Discussion

Mangroves are most commonly selected site for the isolation of thraustochytrids owing to the heterogeneity of the environments and

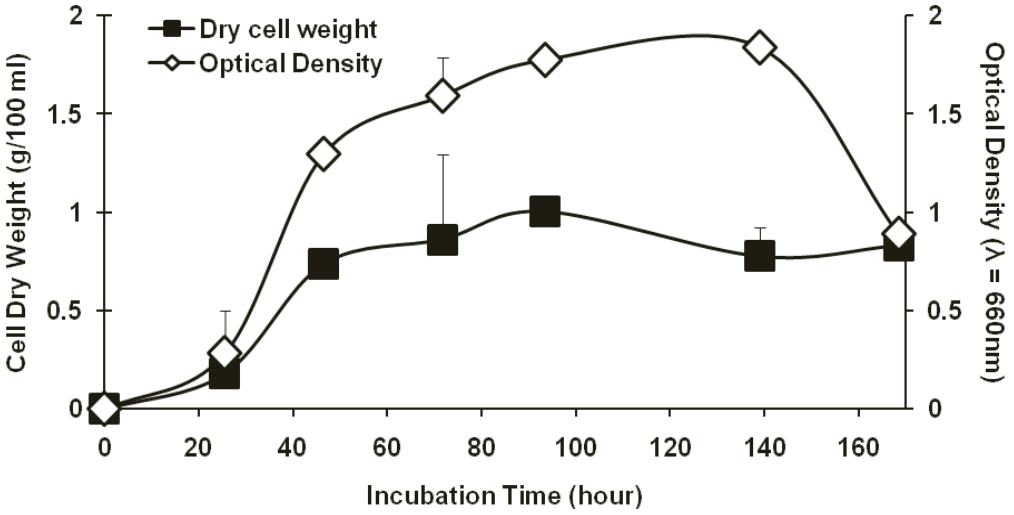


Fig. 5 – Growth curve of *Schizochytrium* sp. correlating with its average cell dry weight (g/100 ml) and average optical density (absorbance at 660 nm) within a 7– day cultivation period.

Table 1 Fatty acid profile of *Thraustochytrium* sp. and *Schizochytrium* sp. under optimized conditions.

Strains	**Fatty acid (% of total fatty acids*)**							
	14:0	**16:0**	**18:0**	**18:1**	**18:2**	**18:3**	**22:6**	**Other**
Thraustochytrium sp.	-	17.7	-	70.6	11.7	-	-	-
Schizochytrium sp.	3.2	61.8	1.6	-	-	0.5	22.6	10.3

* Extracted from 150 mg freeze-dried cells. ** 14:0 (myristic acid), 16:0 (palmitic acid), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic acid), 18:3 (linolenic acid), 22:6 (docosahexaenoic acid, DHA).

Table 2 Extracellular enzyme production and alternative carbon source utilization of *Thraustochytrium* sp. and *Schizochytrium* sp.

Substrate	**Enzyme being tested**	***Thraustochytrium* sp.**	***Schizochytrium* sp.**
Starch	Amylase	-	-
Tween 80	Lipase	+	+
Milk	Protease	-	-
Cellulose	Cellulase	-	-

the abundance of suitable organic matter found in decaying mangrove woods/leaves that may favour for these thraustochytrids' rapid growth. In this study, two strains of thraustochytrids were obtained from fallen senescent mangrove leaves in Philippines.

Although both strains have similar morphological and physiological characteristics, they could be distinguished by colony colour on PYGS agar. *Thraustochytrium* sp. produced pale creamy colour colonies, while *Schizochytrium* sp. produced colonies with light orange colour, perhaps due to the presence of carotenoids. The assumption that this strain may contain pigments such as carotenoids, would warrant for its potential application in the production of another useful product. Carotenoids are known to play crucial roles in human nutrition in which their antioxidative property is believed to be responsible for protecting against free-radical mediated damage from degenerative disorders such as epithelial cancer (Carmona et al. 2003). Carotenoid production by thraustochytrids has been reported by Fan & Chen (2007). Thus, our future endeavor is geared towards optimizing the production of carotenoids in these isolates.

The biomass production of the isolates under four different growth conditions generally agrees with previous reports on the growth of thraustochytrids. The optimum seawater concentration (50–60%) for biomass production concurs with the findings of Perveen et al. (2006). Also, the pH range for growth (pH 5–8)

as reported by Singh & Ward (1996) and Perveen et al. (2006) was confirmed in this study with these isolates able to grow well at these pH levels. The optimal growth temperature of *Schizochytrium* sp. agrees with reports on the best cultivation temperature for *Schizochytrium* sp. by Leaño et al. (2003) and Fan et al. (2002).

Biomass production of thraustochytrids is expected to increase proportionally with the glucose concentration of the culture medium (Bowles et al. 1999). In this study, however, the optimal glucose concentrations for biomass production were observed at the two lowest concentrations tested (3–5%). This may be indicative that the thraustochytrid isolates are inclined towards a lower nutrient environment.

This study showed that the test isolates can grow over wide range of glucose concentrations, seawater concentrations, pH, and incubation temperatures. This capability is expected of thraustochytrids, which thrive in estuarine habitats, such as mangrove forests, where constant fluctuations of physico-chemical parameters (of the aquatic environment) occur. The ability of thraustochytrids to grow at different conditions tested in the study shows their adaptation to the changing conditions present in their natural habitat. Aside from these, the preference of the isolates to thrive on relatively low glucose concentrations (3–5%, w/v) show their heterotrophic nature, preferring lower levels of organic material compared to other organisms which require high amounts of substrate.

The growth curve data obtained for *Thraustochytrium* sp. agrees with the study of Wethered & Jennings (1985) on the growth of *T. aureum*. As in this study, the lag phase of *T. aureum* also lasted for one day followed immediately by log phase until the 3rd day, where maximum amount of biomass was produced. This was also observed by Bajpai et al. (1991a, b) in *T. aureum* ATCC 34304 where growth was observed as early as the first day of cultivation. In comparison, *Schizochytrium* sp. has a shorter lag phase and has achieved stationary phase on day 3 until day 5. Higher biomass was also produced by this isolate. A similar result was obtained by Naganuma et al. (2006) on *S. limacinum* OUC88 using cellullar ATP monitoring. In their study, log phase was observed from 12 h to 48 h, while stationary phase commenced at 72 h.

The fatty acid profiles of the two isolates in this study concur with other documented reports on fatty acid composition of thraustochytrids. Li & Ward (1994) found that *T. striatum* ATCC 24473 was able to produce 33.92% (w/w of TFA) of palmitic acid (16:0) and 48.21% of oleic acid (18:1) but only 0.75% of DHA (22:6). This high yield of MUFA by *Thraustochytrium* spp., as observed in this and other studies, indicates their potential for use in biodiesel production. *Thraustochytrium* spp may provide an inexpensive alternative to common sources of oils such as plants, wherein their utilization would negatively affect food supply. In particular, the high levels of palmitic and oleic acids detected from the *Thraustochytrium* biomass are exceptionally important in the biofuel industry (Fisher et al. 2008).

Schizochytrium sp. on the other hand, being able to produce a significant amount of DHA (22.6% TFA) showed great potential for use in large-scale DHA production. Similar findings were reported by Leaño et al. (2003) where DHA (24–35% TFA) and palmitic acid (39–42% TFA) were the two most abundant fatty acids in their thraustochytrid isolates from Panay Island, Philippines. *Schizochytrium* sp. (ATCC 20888) also produced high amounts of DHA (32.9% TFA) and palmitic acid (25.1% TFA) (Ashford et al. 2000). In another study of Leaño et al. (2009), *S. mangrovei* isolate IAo-1 was found to produce 31.53% DHA (%TFA) and 34.9% palmitic acid. Although the DHA production of *Schizochytrium* sp. SB11 is still not as high as those previously documented (Yamasaki et al. 2006, Chi et al. 2007), its cultivation condition can still be studied so that its PUFA production can be significantly optimized.

The high production of palmitic acid by *Schizochytrium* spp. also shows their potential for biodiesel production. This complements the idea that these organisms could utilize food processing wastes (Fan et al. 2000, 2001, Yamasaki et al. 2006, Chi et al. 2007), making biodiesel production by thraustochytrids more inexpensive and environment friendly.

Besides their capability to produce fatty acids, thraustochytrids can also produce

enzymes that degrade decaying matter in their environment. Most of these decaying matters are composed of complex polysaccharides that may not be assimilated easily, thus production of enzymes aids these microbes to fully utilize the chemically-bound nutrients. In this study both isolates showed negative results for protease and amylase production. This may be due to the nature of the substrates that these organisms have been exposed to in nature. These findings contradict the report by Taoka et al. (2009), where they found that ATCC strains of *Thraustochytrium* sp. and *Schizochytrium* sp. showed protease activity. They also noted amylase activity in *Thraustochytrium* sp. but not in *Schizochytrium* sp. In this study, the two thraustochytrid isolates also showed negative activity for cellulase despite the fact that they were taken from decaying mangrove leaves, which contain cellulose. This result agrees with Taoka et al. (2009) where *Thraustochytrium* sp. and *Schizochytrium* sp. used in their study had negative activity for cellulase.

The negative cellulase activity may be due to the specialized nature of mangrove leaves, which usually only contain cellulose on the deepest layer of leaf tissue beneath a thick epicuticular wax (Killops & Frewin 1994), which prevents the escape of water (Kaplan 1999) especially in saline environments. Being in a tropical climate, the mangrove leaf cuticle is much thicker compared to those found in temperate environment (Killops & Frewin 1994). The wax usually comprises of alkanes ranging from 25 to 33 carbons, and fatty esters of 16–18 carbon alkanoic acids and alkanols (Killops & Frewin 1994), which might explain the positive lipase activity of the thraustochytrid isolates in this study. This lipase activity may allow the thraustochytrids to accumulate precursors to their fatty acid synthesizing steps, then using the desaturase-elongase system to produce PUFA and MUFA. This finding may indicate the role of thraustochytrids in the initial colonization of fallen mangrove leaves to allow for the microbial succession in the degradation of these materials, contributing to the nutrient cycle.

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