Carotenoid analysis of locally isolated Thraustochytrids and their potential as an alternative fish feed for *Oreochromis niloticus* (Nile tilapia)

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Thraustochytrids are marine heterotrophic straminipilans that have recently gained attention due to their capability to produce large amounts of lipids and especially to their utility as an alternative source of the omega-3 polyunsaturated fatty acids (PUFAs).Two identified thraustochytrids (*Thraustochytrium* sp. SB04 and *Schizochytrium* sp. SB11) isolated from senescent fallen mangrove leaves in Subic Bay, Philippines were further studied for their carotenoid contents and potential as alternative fish feed for *Oreochromisniloticus* (Nile tilapia). Their optimized culture conditions for biomass and total fatty acid production have been reported recently. In this present study, the carotenoid contents of these two isolates were analyzed via Thin Layer Chromatography (TLC) of acetone extracts of fresh and oven-dried cells. Their pigments present were identified through their Rf values. *Thraustochytrium* sp. SB04 showed astaxanthin monoesters, astaxanthindiesters, astaxanthin free, and echinenone; whereas *Schizochytrium* sp. SB11showed echinenone, lutein, astaxanthin monoesters, and astaxanthindiesters. These identified compounds were recently reported to have antioxidant properties, anticancer, and immunomodulatory effects to humans. Further, astaxanthin is a red pigment common to many marine animals and are used as food coloring and feed additive for poultry and aquaculture industry. Further, when dried cells of the mass-produced isolates were used as feed supplements for the growth of *O. niloticus*, the growth yield of the fish showed a significant difference ($p<0.05$) when compared to commercial fish feeds. Results of this study showed that thraustochytridcells can be used as source of naturally occurring carotenoids and as promising alternative fish feed to *O. niloticus.*

Key words – Carotenoids – *Oreochromis niloticus* – *Schizochytrium* sp. – Thraustochytrids – *Thraustochytrium* sp.

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Introduction

Thraustochytrids are ubiquitous marine straminipilans renowned in the aquaculture and food supplement industry due to their ability to produce polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Fan & Kamlangdee 2003, Raghukumar 2008). This group of straminipilan marine organisms comprises species

belonging to the genera, *Althornia, Diplophrys, Elina, Japonochytrium, Thraustochytrium, Schizochytrium* and *Ulkenia* (Raghukumar 2002). In the environment, they act as commensals of other organisms and as saprobes feeding on organic material. In mangrove communities, these organisms are found associated with decaying mangrove leaves, actively playing a dual role in nutrient cycling as bacterial feeders when in amoeboid form and as organic material degraders when in thallic form (Fan et al. 2002).

Recently, Arafileset al.(2011) reported the optimum conditions (temperature, seawater concentration, glucose concentration, and initial pH) of the two species of thraustochytrids designated as *Thraustochytrium* sp. SB04 and *Schizochytrium* sp. SB11 isolated from mangrove leaves in Subic Bay, the Philippines. These two strains can actively produce high yields of myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), and docosahexaenoic acid (22:6).In addition, *Thraustochytrium* sp. SB04 produced pale creamy colour colonies which turn orange after several days of incubation while *Schizochytrium* sp. SB11 produced pale colonies with light orange colour, perhaps due to the presence of carotenoids. Since lipids are important in fish nutrition by providing the essential PUFA required for their normal cell membrane function (Sargent et al. 1995), in this study, we attempted to investigate further the carotenoid contents of the two isolates and tested their effect on the growth of tilapia fingerlings.

Several investigators have studied the utilization of DHA and EPA as essential fatty acids for marine species because they do not have or have only a very low Δ5-desaturase activity, which is necessary for the conversion of C-18 PUFA to long chain highly unsaturated fatty acids (HUFA) (Barclay & Zeller 1996, Coutteau & Sorgeloos 1995,Ganuza et al. 2006, Metz et al. 2009, Sargent et al. 1993). According to Sargent et al.(1993) and Shields et al. (1999), the (n-3) HUFA requirement of juvenile marine fish is ~0.5 -1.0% of the dry weight of their diet although in the early developmental stage of their larvae, a greater amount of HUFA is needed due to their rapid growth and the critical early development of their specialized cells and tissues.Therefore, the aim of this study was to determinewhether the carotenoids present in these two thraustochytrid species along with their PUFA contents can be used as an alternative feed for *O.niloticus* (Nile tilapia).

Materials and methods

Isolation and maintenance of thraustochytrids

Fallen senescent mangrove leaves were randomly collected from Triboa Bay Mangrove Park in Subic, Philippines. Thraustochytrids were isolated from leaf samples and identified to genus level based on the morphology of their thallic stage, differences in sporogenesis, and spore release (Raghukumar 2002).Two thraustochytrid strains: *Thraustochytrium* sp. SB04 and *Schizochytrium* sp. SB11, identified by the group of Arafileset al. (2011) were utilized in this study. These thraustochytrid strains were maintained in agar plates consisting (per Liter) 0.3g glucose, 1.25g yeast extract, 1.25g peptone, 15g agar, and 50% NSW supplemented with 300 mg/L streptomycin and penicillin G following the method described by Leañoet al.(2003) and sub cultured weekly.

Inoculum preparation and biomass production

Pure cultures of thraustochytridswere inoculated onto pre-culture GYPS tubes and incubated for 2 d at 25-28ºC. One milliliter aliquot of the pre-culture medium was added to 100mL GYPS flasks. This was further incubated for 3 d at 25-28ºC with constant agitation. Cells were harvested through centrifugation at 3500 rpm for 10 minsand dried at 70ºC for 2 d (Anbuet al. 2007). The resulting dried biomass was then ground using mortar and pestle.

Carotenoid analysis by thin layer chromatography

Ten to 15 (10-15) mg of fresh or oven-driedthraustochytrid cells were placed in a 15mL centrifuge tube, added with 5g of glass beads to dislodge the cells, and 2mL of acetone. This was vigorously vortexed for 5

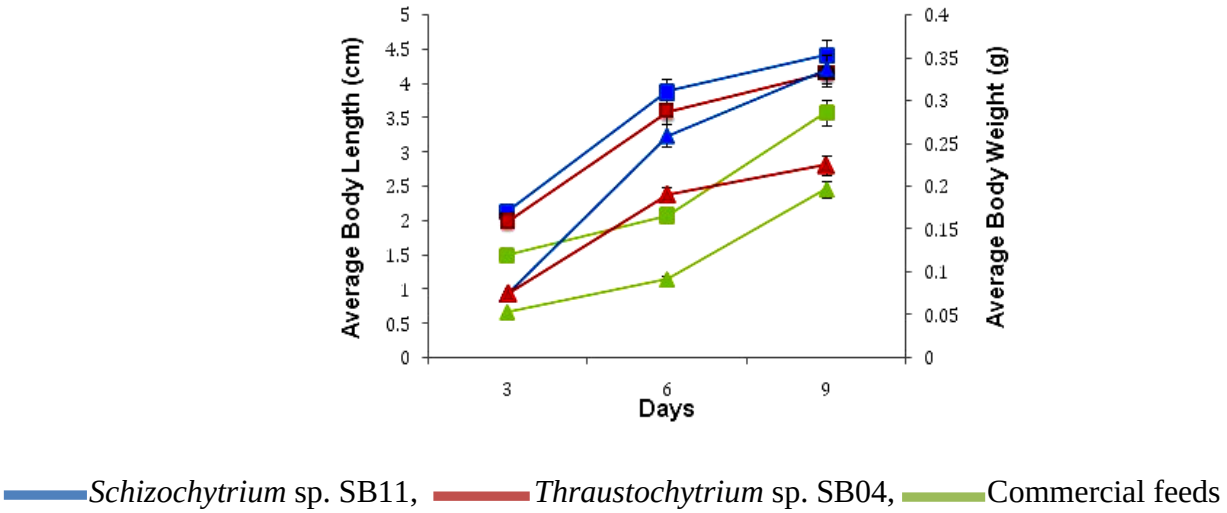

Fig.1–Comparison of the average body lengths (Δ) and weights (□) of *O. niloticus* fed with diets ofthraustochytrids versus commercial feeds.

mins. An additional 5mL of acetone was placed in the centrifuge tube and were vortexed briefly. Then, it was centrifuged for 10 minsat 3500 rpm. The supernatant was pipetted off and was transferred into a clean vial. The acetone extract was dried using rotary evaporator at 0°C and was re-suspended in 0.5mL of petroleum ether. The extract was carefully spotted onto the TLC plate and dried. The dried TLC plate was developed in a fresh mixture of 25% acetone/hexane and was allowed to develop for about 30 mins. The TLC plate was removed from the chamber and was exposed to UV light. The center of the carotenoid spot was marked with a pencil. Pigments were identified using the standard Rf values of carotenoids based on the study of Lorenz (1998, Table 1).

Evaluation of the growth promoting capabilities of thraustochytrids for *O.niloticus*

Experimental set-up of *O.niloticus*

The Nile tilapia fishes (*O.niloticus*) were obtained from Southeast Asian Fisheries Development Center (SEAFDEC), Binangonan, Rizal. Fingerlings (mean weight 0.02g) were stocked in 12L aquarium (16 per aquarium). Two replicate aquaria for each treatment and the control were used. A total of 144 fingerlings were divided into nine (9) aquaria with 10 × 15 ×15 dimensionhoused at the Biology and Biopharmacy Laboratory of the Thomas Aquinas Research Complex, University of Santo Tomas. The fish were acclimatized for 3 d before experimentation and the water quality (pH, dissolved oxygen, ammonia, salinity and temperature) was monitored consistently using GLXXplorer (Pasco). The water was changed every 3 d and 3drops of anti-chlorine and methylene blue solution were added every 24 h to assure the reliability of results. Uneaten feeds and feces were siphoned-out daily (Leaño et al. 2007).

Feed preparation and feeding

Experimental feeds were composed of *Thraustochytrium* sp. SB04 and *Schizochytrium* sp. SB11 dried biomass prepared into small pellets. The control feed was a commercial pellet. All set-ups were assayed for 9 d simultaneously for the experimental and control groups, with measurements taken every 3 d. This experiment was performed twice having a total of 18 d observation on the growth effect of thraustochytrid biomass as alternative feeds for *O.niloticus*. Five (5) fishes were randomly sampled from each aquarium then their mean weight and size were calculated. The amount of feedsgiven was also determined according to the following calculations:

Average body weight (ABW) =Weight of fish sampled / no. of fishes weighed× 100

Ave rage body length (ABL)= Length of fish sampled / no. of fishes measured × 100

Table 1 Carotenoid standard Rfvalues (Lorenz 1998)

Carotenoid	Typical Rf Value
β-carotene	Rf = 0.99
Echinone	Rf = 0.87
AstaxanthinDiesters	Rf = 0.75
Astaxanthin Monoesters	Rf = 0.50
Cantaxanthin	Rf = 0.40
Astaxanthin Free	Rf = 0.33
Lutein	Rf = 0.25

Amount of feeds = (Weight of fish sampled / No. of fish sampled) × 0.05 × no. of live fish

After 9 d of observation, the final weight and length of the surviving fish were noted. Gathered data were analyzed using Analysis of Variance (ANOVA) to assess the efficiency of the use of thraustochytrids biomass over commercial feeds in the promotion of fish growth.

Results

Maintenance and characteristics of thraustochytrids

Two thraustochytrids species were isolated and microscopically identified at the genus level as *Thraustochytrium*sp. And *Schizochytrium*sp., formerly designated as *Thraustochytrium*sp. SB04 and *Schizochytrium*sp. SB11 by Arafiles et al. (2011). These isolates were first cultivated for investigation of their heterotrophic growth characteristics and optimized culture conditions for biomass and fatty acid production. Further, both species produce orange-colored pigments, thus, we attempted to analyze their carotenoid contents and test their effect on the growth of tilapia fingerlings.

In this study, *Thraustochytrium* sp. SB04 and *Schizochytrium* sp. SB11 grew rapidly, and their highest biomass (2.13g/L) was reached at 72h and (2.4g/L) at 24h, respectively. Their growth characteristics were similar to those studied by Arafiles et al. (2011), suggesting the purity of the organisms and the absence of genetic alterations in their physiological properties.

The biomass was harvested continuously by centrifugation at 3500rpm for 10mins.and dried at 70ºC for 2 d. The resulting dried biomass was used as feed pellets for the *O. niloticus.*

Carotenoid analysis by thin layer chromatography

The analysis of carotenoid content of thraustochytrid isolates was determined via thin layer chromatography (TLC). To identify the carotenoids present in thraustochytrid cells, extracts of fresh and oven-dried cells were carefully spotted into their respective TLC plates. Further, for result accuracy, TLC-spotting was carried out in low light and temperatures since carotenoids are sensitive to light, oxygen, and heat. It was then followed by exposing TLC to UV light. Data shown in Tables 2 & 3 present the summary of carotenoids obtained from fresh and oven-dried cells of *Thraustochytrium*sp. SB04 and *Schizochytrium*sp. SB11, respectively.

As shown in Table 2, fresh cells of *Thraustochytrium* sp. SB04 displayed two pigments in Trial 1 withRf values of 0.61and 0.79, respectively. Trial 2, on the other hand, showed four pigments: A, B, C, and D which gave Rf values of 0.39, 0.46, 0.60 and 0.81, respectively. These carotenoids present based on their Rf values are astaxanthin monoesters, astaxanthindiesters, astaxanthin free and echinenone.

For oven-dried cells of *Thraustochytrium* sp. SB04 as similarly depicted in Table 2, two pigments have been shown for both Trials 1 and 2. Identified pigments for Trial 1 gave Rf values of 0.67 and 0.84, respectively; while for Trial 2, 0.66 and 0.82 were the identified Rf values, respectively. These carotenoids present are identified as astaxanthindiesters and echinenone.

As shown in Table 3 for fresh cells of *Schizochytrium*sp. SB11, only one pigment was identified for Trial 1 with Rf value of 0.87.

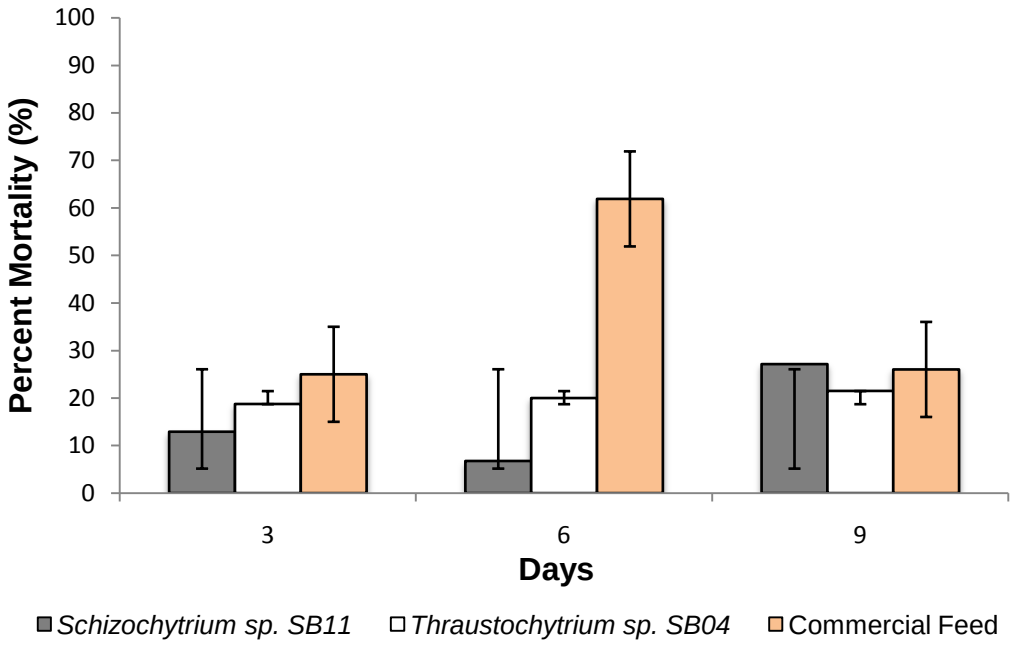

Fig. 2–Mortality rates of *O.niloticus*fed with diets of thraustochytrids and commercial feeds.

For trial 2, however, it exhibited two pigments with Rf values of 0.71 and 0.85. These carotenoidpigments are identified as echinenone and astaxanthindiesters.

For the oven-dried cells of *Schizochytrium* sp. SB11 in Table 3, it showed three pigments in Trial 1, giving the corresponding Rf values of 0.26, 0.54 and 0.91, respectively. In Trial 2, however, no pigment has been identified which implies that the use of fresh cells is more effective than the oven dried-cells in the identification of carotenoids. The said pigments for Trial 1 are identified as lutein, astaxanthin monoesters and echinenone.

Growth promoting capabilities of thraustochytrids for *O.niloticus* growth

After observing the growth of the Nile tilapia for 9 d, the results showed the efficacy of using thraustochytrid biomass to promote increase in the body weight and length of the fish. As shown in Fig. 1, *Schizochytrium*sp. SB11 was better at increasing fish weight and length compared to *Thraustochytrium* sp. SB04. It was construed that a significant difference (*p<0.05)* occurredon the body weight and length of fishes fed with *Thraustochytrid*sp. SB04, *Schizochytrium*sp. SB11 and commercial feeds. This strongly suggests the potential of thraustochytrid cells as alternative feeds to *O.niloticus*. Comparing the effect of different feeds in fish survival descriptively, it was found that commercial fish feeds wereconsistently the feed with the highest mortality rate as observed (Fig. 2). However, no significant difference (*p>0.05)*was construed based on analysis of variance, suggesting that mortality rates could probablybe attributed to external factors applied in the set-up and not on the differences of effects of thraustochytrid cells and commercial feeds. Water quality was maintained at a range of 7.56 – 7.81 for pH, 5.2 – 5.8 for dissolved oxygen, 0 – 0.25 for ammonia, 0 for salinity and 26.57 – 28.99°C for temperature. The specified physico-chemical culture conditions were within the normal range as described by Mjoun&Rosentrater(2010).

Discussion

Thraustochytrids have been reported as producers of carotenoids by Aki et al.(2003). This was the first report on the carotenoid production in *Schizochytrium* sp. Their pigments were extracted using chloroform or acetone resolved on a thin-layer silicaplate. According to their report, this organism produces carotenoid-like pigments due to its fatty acid profile and molecular phylogenetics

Table 2 Rf values of carotenoids from fresh and oven-dried cells of *Thraustochytrium* sp. SB04

Fresh Cells				Dried Cells			
Trial	**Pigments**	**Rf values**	**Carotenoids present**	**Trial**	**Pigments**	**Rf values**	**Carotenoids present**
1	A	0.61	Astaxanthin monoesters	1	A	0.67	Astaxanthindiesters
	B	0.79	Astaxanthindiesters		B	0.84	Echinenone
2	A	0.39	Astaxanthin free	2	A	0.66	Astaxanthindiesters
	B	0.46	Astaxanthin monoesters		B	0.82	Echinenone
	C	0.60	Astaxanthin monoesters				
	D	0.81	Echinenone				

allocated based on the nucleotide sequence of its 18S rRNA analysis. Their isolate,*Schizochytrium sp.* KH105, showed a muddy orange to yellow ochre liquid culture similar to the characteristics observed in our own isolate, *Schizochytrium*sp. SB11. Aki et al. (2003) pointed out that carotenoids in microorganisms were used to scavenge oxygen radicals generated, at least partly, byphotochemical reactions. Carotenoids, being secondarymetabolites, are not essential for the growth of the organism; however, environmental factors may or may not lead to the production of these carotenoids.

On the other hand, according to Carmona et al.(2003) and Yokoyama & Honda (2007), the main carotenoid found in *Thraustochytrium*sp. is astaxanthin. Then, β-carotene, ketocarotenoid, echinenone and canthaxanthin are the inferred carotenoids present in *Schizochytrium* sp. In our study, astaxanthin was found in both the oven-dried and fresh cells of *Thraustochytrium* sp. SB04.Higher efficiency was even obtained by using fresh cells that showed more pigments than the oven-dried cells. The presence of carotenoids in our thraustochytrid isolates makes these organisms economically important. According to some investigators, β-carotene and lutein have several health benefits (Carmona et al. 2003, Aki et al. 2003, Ganuza et al. 2008). β-carotene has long been known to have antioxidant activity while lutein is being extensively studied in relation to eye health for the prevention of cataracts and age- related macular degeneration.Other carotenoids like canthaxanthin display similar or slightly reduced levels of antioxidant effect compared to astaxanthin. This carotenoid exhibits anti-cancer and immunomodulating activities.Astaxanthin, however, is used as food colorants in eggs and farmed fish (Akiet al.2003). Recently, there has been a growing interest in the use of astaxanthin as a food-coloring agent and as a natural feed additive for the poultry industry. The use of astaxanthin as feed supplement in the culture of salmon, trout, and shrimp are currently being explored. Likewise, in medicine, its antioxidant capacityis also currently being studied. In our study, the orange-pigmented *Thraustochytrium* sp. SB04 was found to contain astaxanthin as the main carotenoid pigment. This result conformedwiththe report made by Carmona etal. (2003). According to their report,the conversioncontent of β-carotene declines on the 8th day of culture while astaxanthin content increases. The increase of glucose concentration was accompanied by the increase in astaxanthin content and a decrease in β-carotene content. Aki et al. (2003) added that on lowof β-carotene to astaxanthin occurs via ketocarotenoids like cantaxathin and echinenone. However, the effect of variation in a number of parameters such as the physicalfactors (pH and temperature) and nutritional factors (carbon and nitrogen sources) duringthe culture can alter the relative quantities ofthe carotenoid produced. It is said that the totalconcentration of nitrogen sources, increase in astaxanthin content and decline in cantaxanthin content can be observed. Table 4 shows thesummary of carotenoids inferred from the two thraustochytrid isolates with great significance in the field of aquaculture and medicine.

The data presented in Table 4 shows that both species have astaxanthin monoesters, astaxanthindiesters, and echinenone. However, astaxanthin free was shown only in *Thrasutochytrium* sp. SB04, and lutein in *Schizochytrium* sp. SB11.

Table 3 Rf values of carotenoids from fresh and oven-dried cells of *Schizochytrium*sp.SB11

Fresh Cells				Dried Cells			
Trial	**Pigments**	**Rf value**	**Carotenoids present**	**Trial**	**Pigments**	**Rf value**	**Carotenoids present**
1	A	0.87	Echinenone	1	A	0.26	Lutein
					B	0.54	Astaxanthin monoesters
					C	0.91	Echinenone
2	A	0.71	Astaxanthindiesters	2	*	*	*
	B	0.85	Echinenone				

* no identified carotenoid pigments

Table4 Summary of Carotenoids present in Thraustochytrid species

Carotenoids present in *Thraustochytrium* sp. SB04	**Carotenoids present in *Schizochytrium* sp. SB11**
Astaxanthin monoesters	Echinenone
Astaxanthindiesters	Astaxanthindiesters
Astaxanthin free	Lutein
Echinenone	Astaxanthin monoesters

In the Philippines, *O. niloticus*has become increasingly important in fish culture and represents an important source of cheap protein in the country. Further, this species of bony fishes are capable of growing fast, able to adapt to a wide range of culture conditions, and most specially to their high consumer acceptability. The Food and Agriculture Organization (FAO) cited the Philippines as one the of the world's leading producers of Nile tilapia in Asia along with Malaysia, Vietnam, China, and Sri Lanka (Josupeit 2005). Thus, production of *O. niloticus* is important and an alternative organic-nutritious feeds are needed to supply the demands of the growing market of *O. niloticus*.In addition to the carotenoid contents in thraustochytrid species, there exists the high concentration of the polyunsaturated fatty acids in this organism. Lipids are particularly important in fish nutrition not only for supplying calorific energy but also for providing the essential PUFA such as the highly unsaturated fatty acids (HUFA) of the n-3 series, eicosapentaenoic acid [20:5(n-3); EPA] and docosahexaenoic acid [22:6(n-3); DHA] (Sargent et al. 1995).

In contrast, aquaculture feeds are usually composed of proteins (18-50%), lipids (10-25%) and carbohydrates (15-20%) (Craig &Helfrich2009).It can be inferred that thraustochytrids' advantage of growth promotion was attributed to their protein accumulation as well as their ability to synthesize valuable lipids.Along with this, thethraustochytrids' inherent production of lipids (specifically PUFA) as part of their natural metabolism (Jain et al.2007) allows for the supplementation of the cells and ultimately supplying the fish diet with essential fatty acids like the PUFA for marine fish and linolenic acid (18:3) for freshwater fish (Craig &Helfrich 2009).Further, our result on the efficacy of *Schizochytrium* sp. SB11 as a better promoter of *O. Niloticus* growth conformed to the results obtained by Yamasaki et al.(2007), in which their isolate, *Schizochytrium*KH105,produced high levels of DHA and xanthophylls thereby showing a positive growth on Artemiaspecies. On the basisof these foregoing findings, it can be inferred that our isolates possess a promising application in the field of aquaculture industry as an effective alternative feeds to marine and freshwater organisms.

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