Original article

Fatty acid production of thraustochytrids from Saudi Arabian mangroves

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1. Introduction

Thraustochytrids are unicellular, eukaryotic protists, found mainly in marine habitats, ubiquitous in their distribution and were isolated from Antarctica (Bahnweg and Sparrow, 1974), the North Sea (Raghukumar and Gaertner, 1980), India (Raghukumar, 1988), Micronesia (Honda et al., 1998), Japan (Naganuma et al., 1998), Australia (Lewis et al., 1998), Hong Kong (Fan et al., 2002; Li et al., 2009), Canada (Lee Chang et al., 2012), Malaysia (Manikan et al., 2015), England (Marchan et al., 2018) and USA (Ganuza et al., 2019). However there are no published articles reporting thraustochytrids from Saudi Arabia. Thraustochytrids were classified as primitive fungi, however, the phylogenetic analyses of their genes sequences assigned them in the subclass Thraustochytriidae (Kingdom: Chromista), closely related with the heterokont algae (e.g., brown algae and diatoms) (Cavalier-Smith et al., 1994; Lewis et al., 1999).

Members of the class Labyrinthulaea (Lister) Olive ex Cavalier-Smith are mostly marine heterotrophs that are characterized by their heterokont, biflagellate zoospores and contains two orders: Labyrinthulida Doflein and Thraustochytrida Sparrow, one superfamily: Amphifiloidea Cavalier-Smith and eight families: Althorniidae Cavalier-Smith, Amphiphilidae Cavalier-Smith, Aplanochytriidae Leander ex Cavalier-Smith, Diplophyridae Cavalier-Smith, Labyrinthulidae Cienkowski, Oblongichytriidae Cavalier-Smith, Sorodiplophyridae Cavalier-Smith and Thraus-

thochytriidae Sparrow ex Cejp (Anderson and Cavalier-Smith, 2012). The family Thraustochytriidae contains 31 described species belong to nine genera: Aurantiochytrium R. Yokoy., & D. Honda (3 species), Botryochytrium R. Yokoy., Salleh & D. Honda (1), Japonochytrium Kobayasi & M. Okubo (1), Monorhizochytrium K. Doi & D. Honda (1), Parietichytrium R. Yokoy., Salleh & D. Honda (1), Schizochytrium S. Goldst. & Belsky ex Raghuk. (4), Sicyoidochytrium R.
Yokoyama and Honda (2007) studied Schizochytrium species based on 18S rDNA gene analysis, morphological characteristics, and PUFA and carotenoid profiles and established the genus Aurantiochytrium to accommodate A. limacinum (D. Honda & Yokochi) R. Yokoy. & D. Honda and A. mangrovei (Raghuk.) R. Yokoy. & D. Honda and the genus Oblongichytrium to accommodate O. octosporum (Raghuk.) R. Yokoy. & D. Honda. Described species of Aurantiochytrium differ from Schizochytrium species by having small colonies on different media and no or poorly developed ectoplasmic net. Of around 50 production candidate-strains of Aurantiochytrium are reported in the literature, only three species has been described and characterized with molecular phylogenetic techniques, namely A. acetophilum E. Ganuza & R.A. Andersen and A. limacinum and A. mangrovei (Yokoyama and Honda, 2007; Gauza et al., 2019). Only two reports of thraustochytrids from the Middle East. Ulken (1986) estimated thraustochytrids propagules from a mangrove stand of Avicennia marina on the Red Sea coast of Egypt. She recorded 22,800 to 65,700 infective units per liter of sediment. Reported species belonged to the genera: Thraustochytrium and Schizochytrium. Farzaneh and Shahryar (2015) isolated 20 strains of thraustochytrids from mangroves in the Arabian Gulf and Oman Sea. They characterized the PUFAs profile of Aurantiochytrium strain (GenBank accession no. KJ938302) of whose DHA represented 16% of total fatty acids. Saudi Arabia has a long coastline spanning the Red Sea and the Arabian Gulf (around 2400 km), however, up until now there is no record of thraustochytrids from the kingdom. This study aims to explore marine environment in Saudi Arabia aiming to discover thraustochytrids capable of producing high levels of polyunsaturated fatty acids (PUFAs).

Fig. 1. Thraustochytrids isolated from decaying leaves of Avicennia marina, Syhat mangroves, Dammam city, Arabian Gulf, Saudi Arabia. a-b Aurantiochytrium sp. (SY-03). c Thraustochytrium sp. (SY-04). d Schizochytrium sp. (SY-05). e Aurantiochytrium sp. (SY-06). f Schizochytrium sp. (SY-09). g-h Thraustochytrium sp. (SY-10, ectoplasmic net is arrowed in h). i Schizochytrium sp. (SY-11). j Aurantiochytrium sp. (SY-13). k-l Unknown (SY-14). m Thraustochytrium sp. (SY-17). n Thraustochytrium sp. (SY-26). o Thraustochytrium sp. (SY-28), isolated from sediments. p-t Unknown (SY-59). Bars: a-t = 10 μm.
Fig. 2. Bayesian phylogenetic tree based on 18S rDNA of the three strains of *Aurantiochytrium* with other species and strains of the genus, other genera of the family Thraustochytriidae and representatives of Oblongichytriidae. The tree is rooted with *Bacillaria paxillifer* and *Ochromonas danica*. Bootstrap support on the nodes represents ML and MP ≥ 50%. Branches with a BYPP of ≥ 95% are in bold. The three sequences of *Aurantiochytrium* strains generated in this study are in red.
were placed in sterile Falcon tubes containing 20 ml of sterile seawater, 23 seawater and 21 decaying algae thalli. Collected samples are included: 32 are decaying leaves (26

Table 1
Thraustochytrids isolated from Syhat mangroves, Dammam city, Arabian Gulf, Saudi Arabia:

| Strain No. | Thraustochytrid name | No. of isolates | Substrate |
|------------|----------------------|----------------|-----------|
| SY-03      | Aurantiochytrium sp. | 1              | Decaying leaves of A. marina |
| SY-04      | Thraustochytrium sp. | 1              | Decaying leaves of A. marina |
| SY-05      | Schizochytrium sp.  | 1              | Decaying leaves of A. marina |
| SY-06      | Aurantiochytrium sp. | 1              | Decaying leaves of A. marina |
| SY-09      | Schizochytrium sp.  | 1              | Decaying leaves of A. marina |
| SY-10      | Thraustochytrium sp. | 26             | Decaying leaves of A. marina |
| SY-11      | Schizochytrium sp.  | 2              | Decaying leaves of A. marina |
| SY-13      | Aurantiochytrium sp. | 2              | Decaying leaves of A. marina |
| SY-14      | Unknown              | 1              | Decaying leaves of A. marina |
| SY-17      | Thraustochytrium sp. | 1              | Decaying leaves of A. marina |
| SY-18      | Thraustochytrium sp. | 14             | Decaying leaves of A. marina |
| SY-20      | Ulkenia sp.          | 2              | Decaying leaves of A. marina |
| SY-22      | Aplanochytrium sp.  | 3              | Decaying leaves of A. marina |
| SY-24      | Unknown              | 1              | Decaying leaves of A. marina |
| #SY-25     | Aurantiochytrium sp. | 6              | Decaying leaves of A. marina |
| SY-26      | Thraustochytrium sp. | 1              | Decaying leaves of A. marina |
| SY-28      | Thraustochytrium sp. | 1              | Sediment |
| SY-30      | Ulkenia sp.          | 7              | Sediment |
| SY-34      | Unknown              | 7              | Sediment |
| #SY-38     | Aurantiochytrium sp. | 3              | Sea water |
| SY-41      | Thraustochytrium sp. | 5              | Sea water |
| SY-42      | Thraustochytrium sp. | 2              | Sea water |
| SY-46      | Aurantiochytrium sp. | 4              | Decaying thallus of Sargassum |
| SY-47      | Schizochytrium sp.  | 1              | Decaying thallus of Sargassum |
| SY-50      | Thraustochytrium sp. | 1              | Decaying thallus of Sargassum |
| #SY-52     | Aurantiochytrium sp. | 5              | Decaying leaves of A. marina |
| SY-54      | Schizochytrium sp.  | 1              | Decaying leaves of A. marina |
| SY-55      | Aurantiochytrium sp. | 1              | Decaying leaves of A. marina |
| SY-56      | Thraustochytrium sp. | 1              | Decaying leaves of A. marina |
| SY-59      | Unknown              | 4              | Decaying leaves of A. marina |
| SY-66      | Thraustochytrium sp. | 1              | Decaying leaves of A. marina |

# Supported by molecular data.

2. Materials and methods

2.1. Isolation of thraustochytrids

A total of 103 samples were collected from Syhat mangroves (26° 29' 32" N 50° 02' 46" E), Dammam city, Arabian Gulf, Saudi Arabia, on 15 January 2020. Samples included: 32 are decaying leaves of Avicennia marina at different stages of deterioration, 27 sediments, 23 seawater and 21 decaying algae thalli. Collected samples were placed in sterile Falcon tubes containing 20 ml of sterile seawater that contained chloramphenicol at 0.5 g l⁻¹ and heat sterilized pine pollen grains. Samples were transferred to the laboratory in an icebox and processed on the second day. In the laboratory, each leaf was cut into five segments 1 cm in length and placed in a Petri dish containing GYP medium (1 g glucose, 1 g yeast extract, 1 g polypeptone, 1 g tween 80. 0.2 g KH₂PO₄, 10 g tomato juice, 0.5 g chloramphenicol, 15 g agar in 1 L of 50% natural seawater). Plates were incubated at 25 °C for 7 days. The colonies that formed were transferred to new plates and further purified by streaking methods until we obtained pure cultures. Colonies were grouped into colony morphological types and examined under stereo- and compound microscopes. Each morphotype was grown in sterile 50% aged natural seawater supplemented with chloramphenicol at 0.5 g l⁻¹ and heat sterilized pine pollen grains and incubated for a few hours prior to the examination under an inverted microscope. Characters used to differentiate between isolates include: sporangia, ectoplasmic net, zoospores, amoeboid cells and successive binary divisions (Yokoyama and Honda, 2007). Photographs were taken using an Olympus BX51 differential interference contrast light microscope (Olympus) and Optika view version 7.3.1.7 (Optika) digital imaging system. Thraustochytrids isolates were preserved in GYP slants and subcultured every two months. Materials for scanning electron microscopy (SEM) were prepared as described by Wong et al. (2003).

2.2. DNA sequencing and phylogenetic analysis

Thraustochytrids strains (SY25, SY38 and SY52) were grown in GYP broth (20 g glucose, 5 g yeast extract, 5 g polypeptone in 1 L of 50% seawater) and the resulting cells were centrifuged twice in sterile distilled water. DNA was extracted using the Microbial DNA extraction kit (MOBIO; Mo Bio Laboratories) according to the manufacturer's instructions. The sequences of partial SSU rRNA gene was determined from genomic DNA using primers 18S001 and 18S13 (Honda et al., 1999). PCR amplification and DNA sequencing were carried out by Macrogen Inc., South Korea. The obtained sequences of the three strains were deposited in GenBank (Fig. 1). Sequences were aligned with other sequences of Aurantiochytrium, other genera of Thraustochytriaeae and the outgroup taxa: Bacillaria pavillifer and Ochromonas danica using ClustalX (Thompson et al., 1997). Maximum-parsimony (MP) and maximum-likelihood (ML) phylogenetic analyses were carried out using MEGA X (Kumar et al., 2018). ML analysis (Felsenstein, 1985) was performed using the Tamura–Nei model. Bayesian phylogenetic analysis was performed using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with the GTR model that was determined using MrModeltest 2.2 (Nylander, 2004). Five million generations were run in four chains with sampling every 100 generations, yielding 50,000 trees, of which the first 12,500 were discarded as “burn-in.” The phylogenetic tree in Fig. 2 was visualized using Njplot (Perrière and Gouy, 1996) and edited using Adobe Illustrator CS6.

2.3. Lipid extraction and the composition of fatty acid

A loopful of each isolate from a pure plate culture was inoculated into 50 ml of GYP broth in 250 ml flask and cultured for 4 days in a shaking incubator (160 rpm) at 25 °C. The wet cells harvested from culture suspension by centrifugation at 3000 × g were washed two times with deionized water and freeze-dried. Lipids were extracted from freeze-dried cells with a mixture of chloroform/methanol (2:1, vol/vol) (Folch et al., 1957). Saturated, unsaturated and total fatty acids were determined in the lipid by using methyl esters boron tri fluoride method (AOAC, 2012). The lipid is saponified with sodium hydroxide in methanol. The fatty acids are methylated with boron tri fluoride in methanol, extracted with heptane and determined on a gas chromatograph with FID detector (PE auto system XL) with auto sampler and Ezchrom integration.
system. Carrier gas (He); ca. 25 Psi – air 450 ml/min – Hydrogen 45 ml – split 100 ml/min.

3. Results and discussion

3.1. Diversity of thraustochytrids

A hundred and eight isolates were obtained from Syhat mangroves that include: 77 from decaying leaves of *Avicennia marina*, 15 from sediment samples, 10 from sea water and 6 from decaying thalli of *Sargassum*. Obtained isolates were grouped into morphological types, examined under stereo- and compound microscopes and one isolate from each morphotype was selected for growing in liquid media for fatty acids, carotenoids and other secondary metabolites determination. Thirty-one strains were identified from the 108 isolates and belonged to the genera: *Aplanochytrium* (1 strain), *Aurantiochytrium* (9), *Thraustochytrium* (11), *Schizochytrium* (5), *Ulkenia* (2) and unknown (4) (Fig. 1). Three strains (SY25, SY38 and SY52) were selected based on their high biomass productivity and high percentage of PUFAs (Table 1, Fig. 1).
3.2. Phylogenetic results of the three strains of *Aurantiochytrium* (SY25, SY38 and SY52)

The SSU rDNA dataset comprised of 79 sequences: 60 *Aurantiochytrium*, 14 from other genera in Thraustochytridae, three from Oblongichytridae and *Ochromonas danica* E. G. Pringsheim and *Bacillaria paxillifer* (O. F. Müller) T. Marsson were used as outgroup taxa. (Fig. 2). The maximum parsimony dataset consisted of a total of 969 characters, of which 396 were constant, 139 variable and parsimony-uninformative, and 434 were counted as parsimony-informative. The most parsimonious tree with length of 2003 steps, a consistency index of 0.468493, a retention index of 0.810436, and the composite index is 0.417767. The MP tree was obtained using the Subtree-Pruning-Redrafting (SPR) algorithm (Nei and Kumar, 2000) with initial trees were obtained by the random addition of sequences (10 replicates), bootstrap analysis was done with 1000 replicates. Maximum likelihood analysis yielded one tree (–ln likelihood = 10698.54), and Bayesian analysis yielded two trees of which one is shown in Fig. 2. The three strains SY25, SY38 and SY52 nested within *Aurantiochytrium* clade with high statistical support (100/100/100 for ML/MP/BYPP respectively) (Fig. 2). Species of *Aurantiochytrium* formed six separate clades, the two strains (SY38 and SY52) formed a separate clade related to the clade contains the type species *A. limacinum* and *A. mangrovei* and nine unidentified species of *Aurantiochytrium*, while SY25 grouped with *Aurantiochytrium* sp. TA4, KJ938302 that is also isolated from mangroves in Iran in the same basin, the Arabian Gulf (Fig. 3) (Farzaneh and Shahryar, 2015).

The strains (SY38 and SY52) shared the phylogenetic placement, their morphology and fatty acid profile (Figs. 4-6 and Table 2). Both strains have rough walled sporangia at SEM level. The strain SY25 have different shape of sporangia that divide to give zoospores directly, sporangia surrounded by thick gelatinous sheath and produce high levels of Linoleic and Oleic essential
unsaturated fatty acids and has smooth walls at SEM level (Fig. 3 and Table 2).

3.3. Lipid profile of Aurantiochytrium spp. (SY25, SY38 and SY52)

The three strains produced high percentage of PUFAs ranged between 26 and 60% of the total fatty acids (Table 2). *Aurantiochytrium* sp. (SY25) produced high percentage of Linoleic acid, C18:2\(\alpha\)6 (31.18%) and Oleic acid, C18:1\(\alpha\)9 (21.59%) and this is the first record of these two essential fatty acids from Thraustochytrids. This strain produced low percentage of DHA (1.25%). Linoleic acid (LA) is one of the essential fatty acids in humans that must be supplied into the diet because it is the precursors of long chain PUFA. The lack of linoleic acid in the diet would affect learning and visual acuity and increase the risk for cardiovascular disease (Williams 2000). The other two strains of *Aurantiochytrium* (SY38 and SY52) produced higher percentages of DHA: 13.61% and 18.97% respectively. Palmitic acid (PA): 31.1%, 65.36% and 49.78% for SY25, SY38 and SY52 respectively. Palmitic acid can be used for biofuel production as it has a high cetane number, high stability and low iodine content.

The three strains of *Aurantiochytrium* isolated from Syhat mangrove in the Arabian Gulf have morphological characters and fatty acids profile that are different from the formally described species. Also the phylogenetic analyses of the 18S rDNA placed them as distinct new species within the *Aurantiochytrium* clade. We will work further to characterize these strains at morphology levels and improve their production levels of PUFAs and screen them for their abilities to produce value added products like squalene and astaxanthin.

Previous studies showed that *Aurantiochytrium* species have the ability to produced value-added products that can be produced at commercial scale with at least 50 production candidate-strains of *Aurantiochytrium* are reported in the literature (Ganuza et al., 2019). *Aurantiochytrium* sp. strain 18 W-13a produced 171 mg/g unsaturated fatty acids and has smooth walls at SEM level (Fig. 3 and Table 2).

![Fig. 5. *Aurantiochytrium* sp. (SY52) isolated from decaying leaves of *Avicennia marina*. a Pure culture on GYP medium. b Single colony. c-g Sporangia at different stages of development, released zoospores in a mucilaginous sac visible in f,g. Ectoplasmic net is arrowed in c. h-j Scanning Electron Micrographs (SEM) show the rough surface wall of sporangia and the inter ectoplasmic net, arrowed in l. Bars: c-g = 10 μm.](image-url)
The dry weight of squalene that much higher than that previously reported from other thraustochytrids, plants and yeasts and the strain can be used as a commercial source of squalene (Nakazawa et al., 2012).

Yokochi et al. (1998) reported the production of DHA yields of > 4 g/L from Aurantiochytrium limacinum SR21 growing on 9% glucose and 12% glycerol, corn steep liquor and with salt at concentrations between 50 and 200% of the seawater salt content. Species of Aurantiochytrium are abundant in marine water, and able to grow on various carbon sources (Yu et al., 2015). Recent studies have demonstrated that Aurantiochytrium spp. can produce high biomass that contain up to 70% lipids, and up to 70% of the lipids may be DHA (Aasen et al., 2016). Lipid content and fatty acid profile can be improved by changing the growth conditions. Abundant carbon and low nitrogen in the fermentation medium will increase oil accumulation in thraustochytrids (Jakobsen et al., 2008). Minimal levels of nitrogen will limit the synthesis of protein and nucleic acid and carbon will be stored as oil. The higher C: N ratio was found to support higher DHA production in thraustochytrids (Yokochi et al., 1998; Bowles et al., 1999; Burja et al., 2006).

The three studied strains (SY25, SY38 and SY52) produced Palmitic acid between 31 and 66% of the total fatty acids. The acid was previously reported in high levels from Aurantiochytrium species (Nagano et al., 2009, Ramos et al., 2009). Palmitic acid produced by thraustochytrids can be used to produce high quality biodiesel due to its high octane number, low iodine content and high...
oxidation stability. Therefore, *Aurantiochytrium* species can be grown to produce both biodiesel and value-added products (Nagano et al., 2009; Ramos et al., 2009).

4. Conclusions

This preliminary study reveals high diversity of thraustochytrids from mangroves in Saudi Arabia. The 180 isolates encountered in this study belonged to five genera, of which three isolates produced considerable amounts of saturated and polyunsaturated fatty acids that can improved for commercial production of PUFA's especially DHA and for biodiesel production.

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