



Original article

Fatty acid production of thraustochytrids from Saudi Arabian mangroves

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ABSTRACT

This is the first report of thraustochytrids from Saudi Arabia. A total of 108 isolates of thraustochytrid were cultured from Syhat mangroves, Arabian Gulf, Saudi Arabia. Isolated thraustochytrids belonged to five genera: *Aplanochytrium*, *Aurantiochytrium*, *Schizochytrium*, *Thraustochytrium* and *Ulkenia*. Cultured thraustochytrids isolated from decaying leaves of *Avicennia marina* (77 isolates), sediment (15), seawater (10) and decaying thalli of *Sargassum* (6). Of the 108 isolates, three strains (SY25, SY38 and SY52) were selected based on their high biomass productivity and high percentages of PUFAs. Phylogenetic analyses based on 18S rDNA placed the three strains within the *Aurantiochytrium* clade with high statistical support. Species of *Aurantiochytrium* formed six separate clades, the two strains (SY38 and SY52) formed a separate clade that is a sister clade to the one that contains the type species *A. limacinum*, while SY25 grouped with *Aurantiochytrium* sp. TA4, that is also isolated from mangroves in Iran, Arabian Gulf. The strains (SY38 and SY52) shared the phylogenetic placement, their morphology and fatty acid profile. The strain SY25 have different shape of sporangia that divide to give zoospores directly, sporogenous cells are surrounded by thick gelatinous sheath and produce high levels of Linoleic and Oleic essential unsaturated fatty acids. The three studied strain produced high levels of Palmitic acid (ranged between 31.1 and 65.3 % of total fatty acids) that can be further optimized for biofuel production.

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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 Thraustochytrida (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 Labyrinthulea (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, Amphifilidae Cavalier-Smith, Aplanochytridae Leander ex Cavalier-Smith, Diplophryidae Cavalier-Smith, Labyrinthulidae Cienkowski, Oblongichytridae Cavalier-Smith, Sorodiplophryidae Cavalier-Smith and Thraustochytridae Sparrow ex Cej (Anderson and Cavalier-Smith, 2012). The family Thraustochytridae contains 31 described species belong to nine genera: *Aurantiochytrium* R. Yokoy. & D. Honda (3 species), *Botryochytrium* R. Yokoy., Salleh & D. Honda (1), *Japono-chytrium* Kobayasi & M. Ôkubo (1), *Monorhizochytrium* K. Doi & D. Honda (1), *Parietichytrium* R. Yokoy., Salleh & D. Honda (1), *Schizochytrium* S. Goldst. & Belsky ex Raghuk. (4), *Sicyoidochytrium* R.

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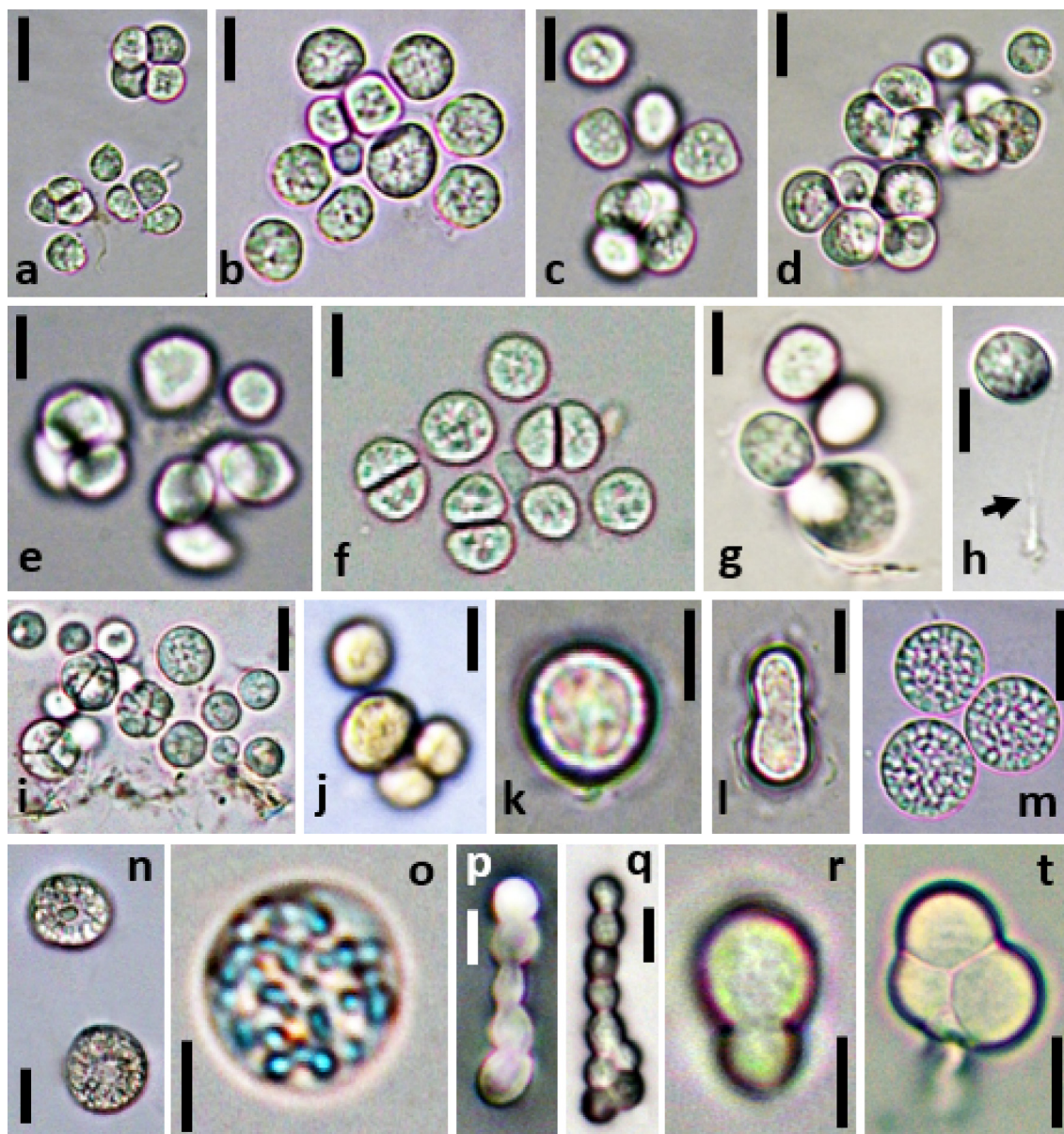


Fig. 1. Thraustochytrids isolated from decaying leaves of *Avicennia marina*, Syhat mangroves, Dammam city, Arabian Gulf, Saudi Arabia. **a–b** *Aurantiocytrium* sp. (SY-03). **c** *Thraustochytrium* sp. (SY-04). **d** *Schizochytrium* sp. (SY-05). **e** *Aurantiocytrium* sp. (SY-06). **f** *Schizochytrium* sp. (SY-09). **g–h** *Thraustochytrium* sp. (SY-10, ectoplasmic net is arrowed in **h**). **i** *Schizochytrium* sp. (SY-11). **j** *Aurantiocytrium* 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 μ m.

Yokoy., Salleh & D. Honda (1), *Thraustochytrium* Sparrow (16, type genus) and *Ulkenia* A. Gaertn. ex M.W. Dick (3).

Yokoyama and Honda (2007) studied *Schizochytrium* species based on 18S rDNA gene analysis, morphological characteristics, and PUFA and carotenoid profiles and established the genus *Aurantiocytrium* 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 *Aurantiocytrium* 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 *Aurantiocytrium* 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;

Ganuza 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 *Aurantiocytrium* 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).

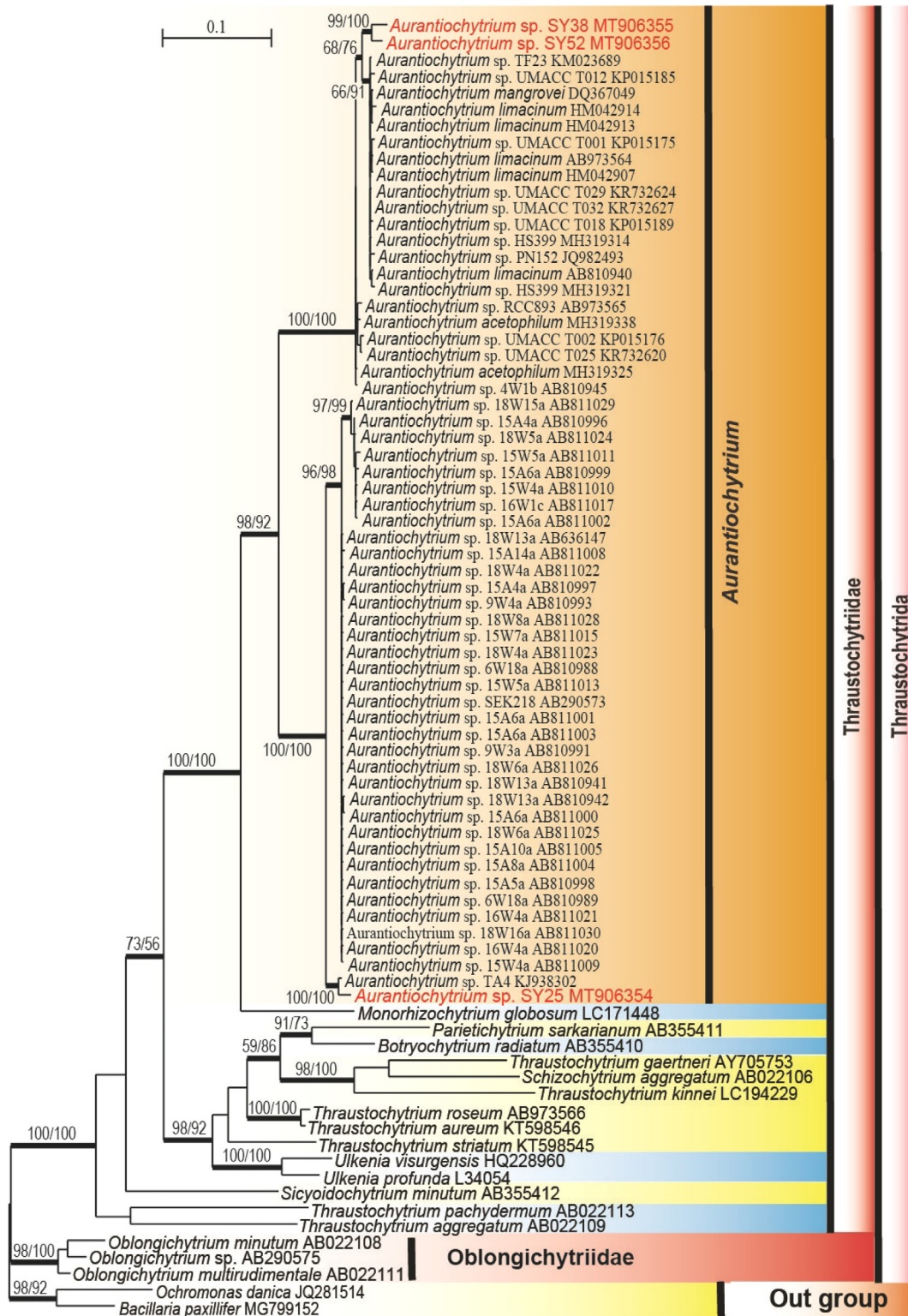


Fig. 2. Bayesian phylogenetic tree based on 18S rDNA of the three strains of *Aurantiochytrium* with other species and strains of the genus, other genera of the family Thraustochytriidae and representatives of Oblongichytriidae. The tree is rooted with *Bacillaria paxillifer* and *Ochromonas danica*. Bootstrap support on the nodes represents ML and MP $\geq 50\%$. Branches with a BYPP of $\geq 95\%$ are in bold. The three sequences of *Aurantiochytrium* strains generated in this study are in red.

Table 1

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

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

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 Thraustochytriaceae and the outgroup taxa: *Bacillaria paxillifer* 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

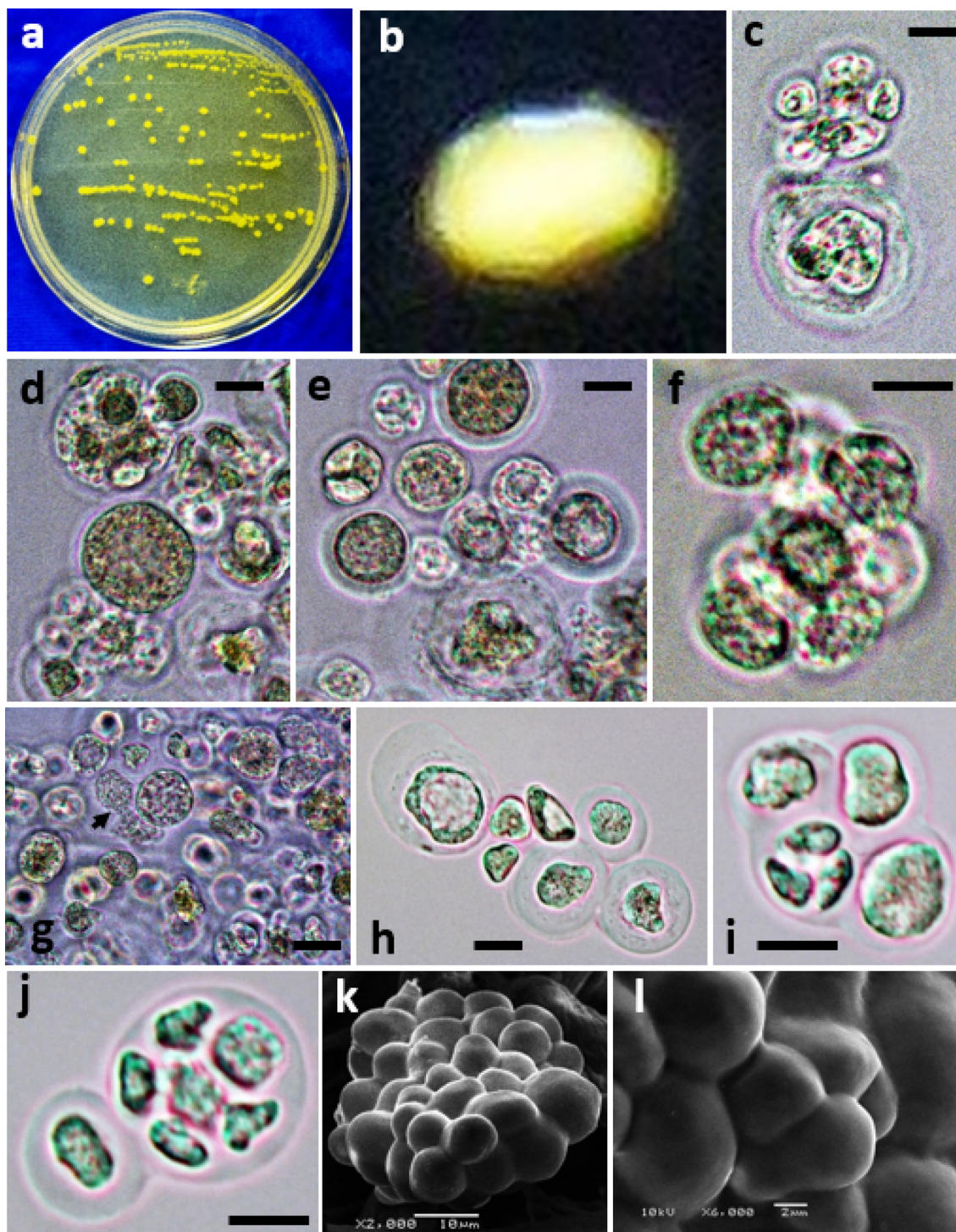


Fig. 3. *Aurantiochytrium* sp. (SY25) isolated from decaying leaves of *Avicennia marina*. **a** Pure culture of GYP medium. **b** Single colony. **c-e** Actively dividing sporogenous cells. **f** Released zoospores. **g** Amoeboid cells (arrowed). **c,h-j** Sporogenous cells that produced thick stratified cell walls. **k-l** Scanning Electron Micrographs (SEM) show the smooth-walled sporangia and zoospores. Bars: c-j = 10 μm.

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 isolates 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).

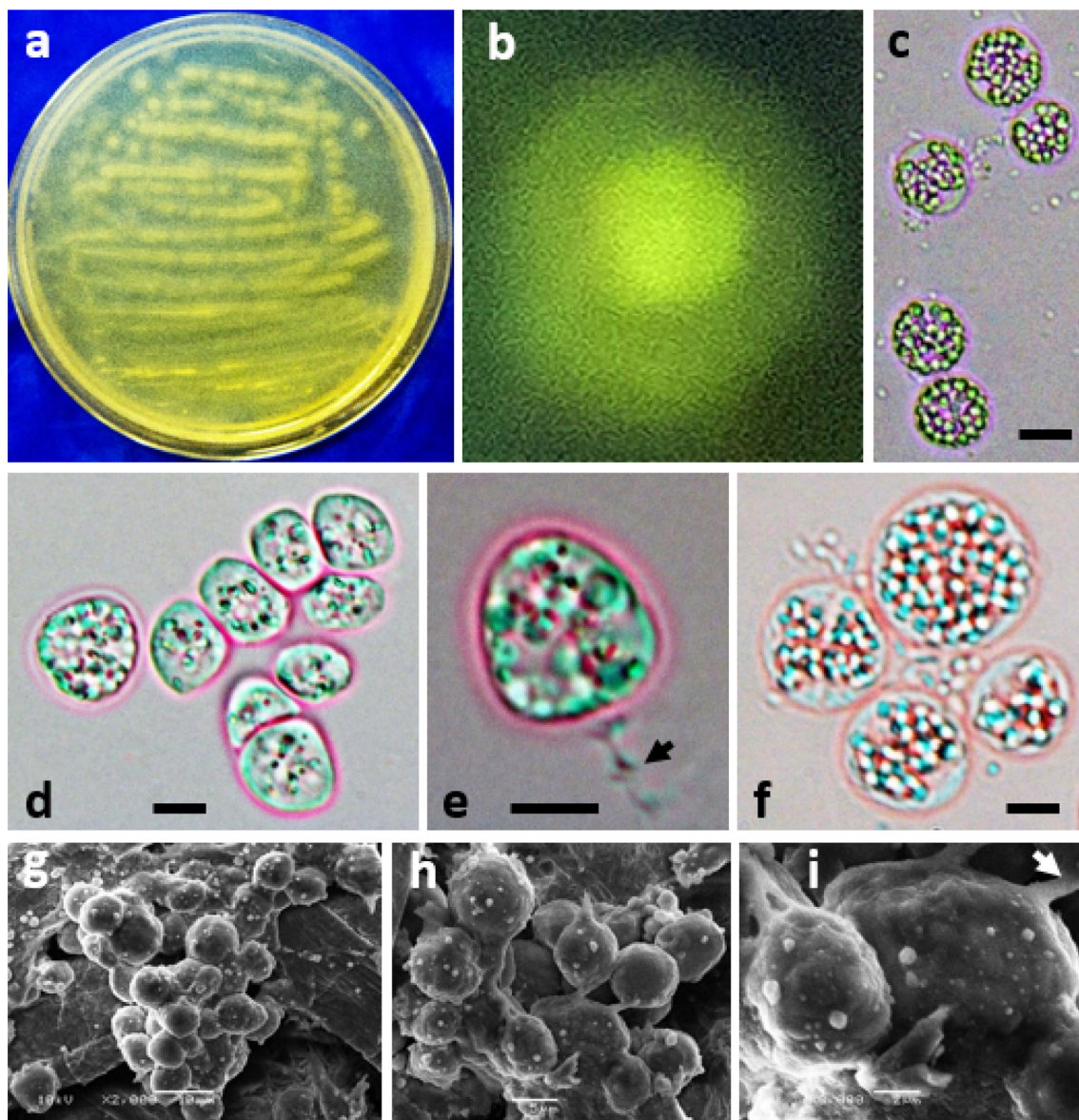


Fig. 4. *Aurantiochytrium* sp. (SY38) isolated from seawater. **a** Pure culture on GYP medium. **b** Single colony. **c-f** Sporangia at different stages of development, released zoospores visible in **c,f**. Ectoplasmic net is arrowed in **e,i**. **g-i** Scanning Electron Micrographs (SEM) show the rough surface wall of sporangia and the inter ectoplasmic net. Bars: a-f = 10 μ m.

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 (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

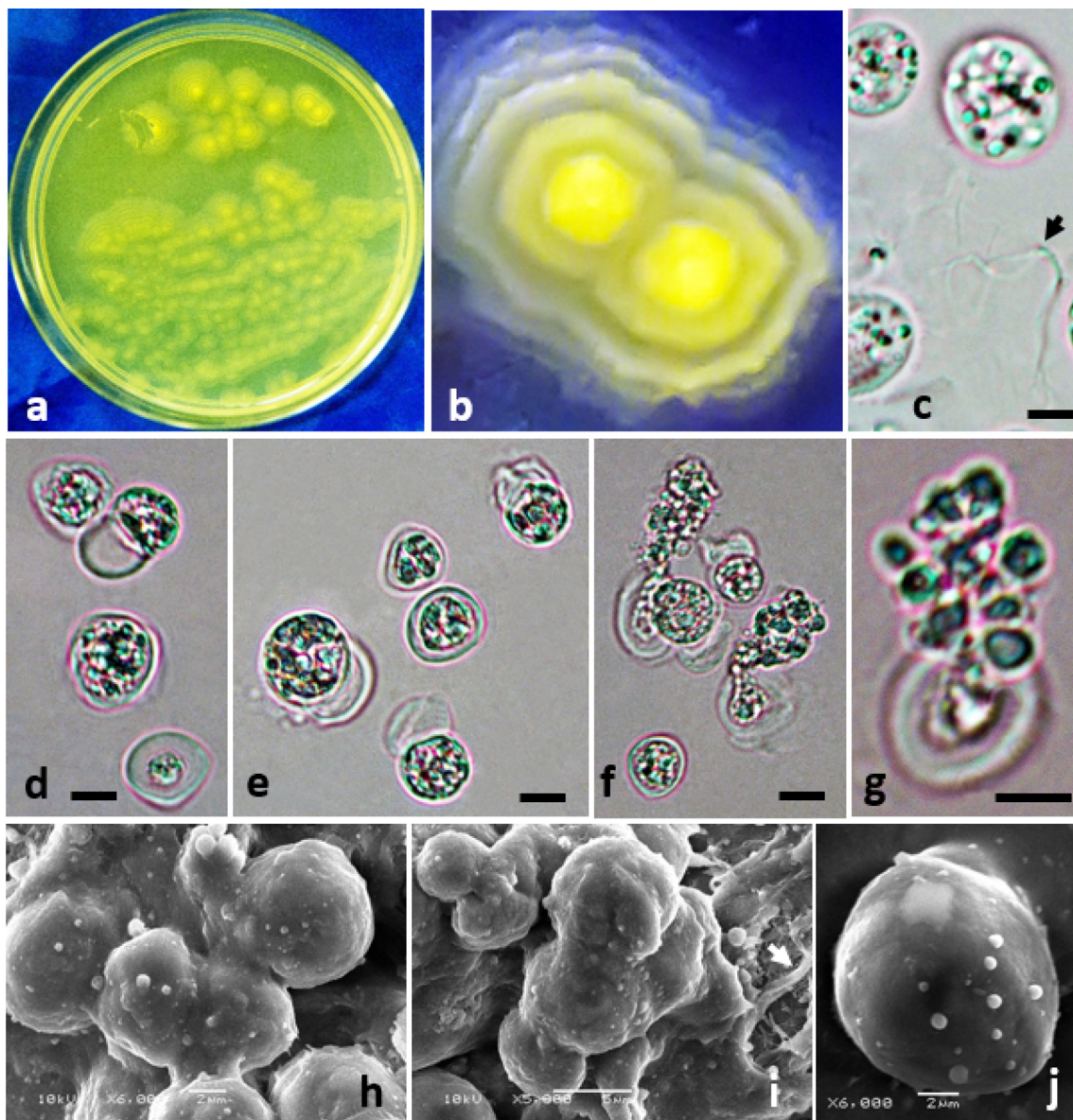


Fig. 5. *Aurantiochytrium* sp. (SY52) isolated from decaying leaves of *Avicennia marina*. **a** Pure culture on GYP medium. **b** Single colony. **c-g** Sporangia at different stages of development, released zoospores in a mucilaginous sac visible in f,g. Ectoplasmic net is arrowed in c. **h-j** Scanning Electron Micrographs (SEM) show the rough surface wall of sporangia and the inter-ectoplasmic net, arrowed in i. Bars: c-g = 10 μ m.

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 ω 6 (31.18%) and Oleic acid, C18:1 ω 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. The three strains produced high level of

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

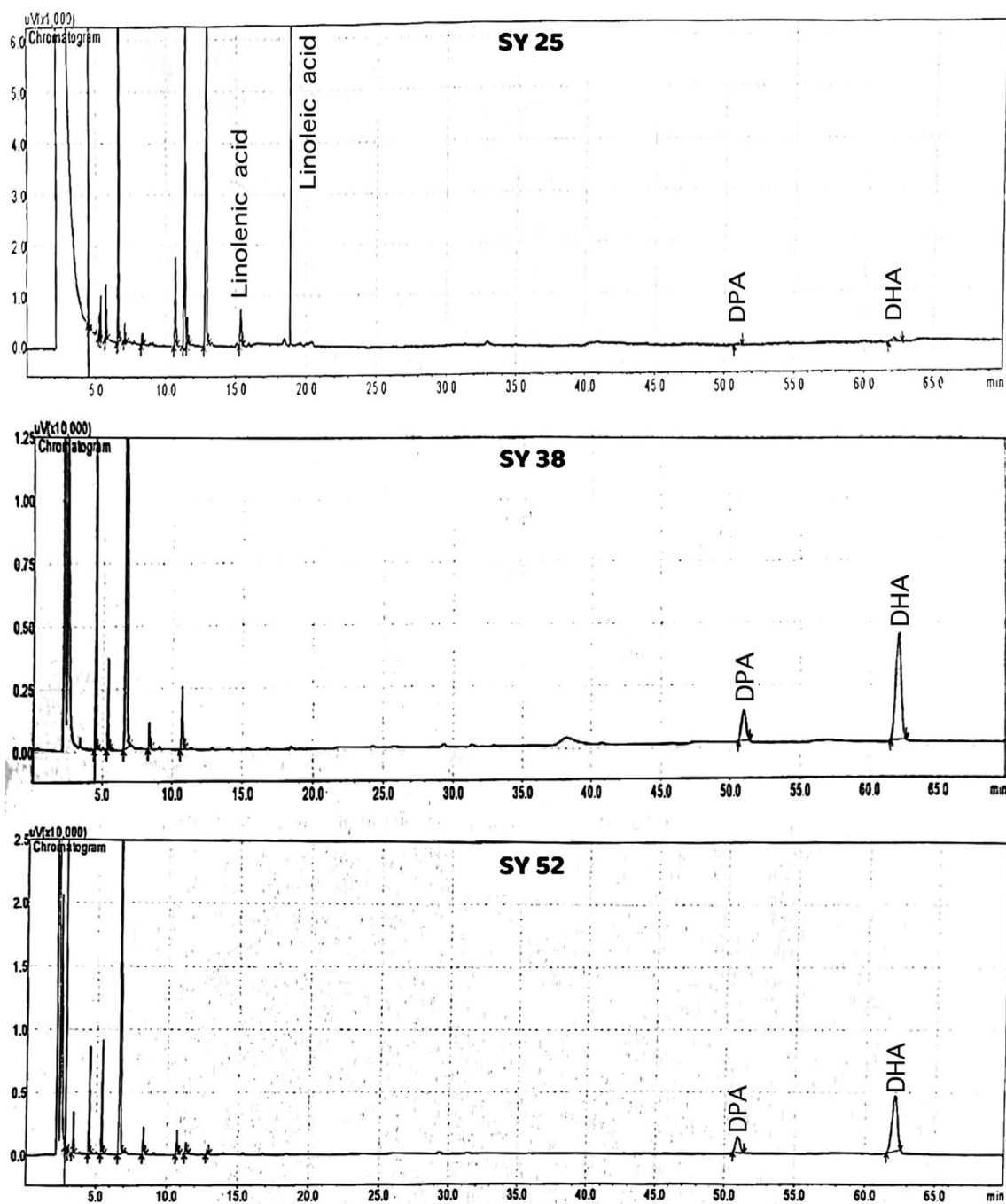


Fig. 6. GC-MS graph of the fatty acids profile of the three strain of *Aurantiochytrium* (SY25, SY38 and SY52).

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 pro-

file 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

Table 2
Fatty acid composition (% of total FA) of the *Aurantiochytrium* species isolated from Syhat mangroves:

Fatty acids	Name	<i>Aurantiochytrium</i> sp. SY25	<i>Aurantiochytrium</i> sp. SY38	<i>Aurantiochytrium</i> sp. SY52
C10:0	Capric acid	–	–	1.53
C11:0	Undecanoic acid	–	–	0.26
C12:0	Lauric acid	–	–	1.43
C14:0	Myristic acid	0.91	4.11	4.08
C15:0	Pentadecanoic acid	2.13	1.42	4.95
C16:0	Palmitic acid	31.1	75.36	59.78
C16:1 ω 7	Palmitoleic acid	0.73	–	–
C17:0	Heptadecanoic acid	0.54	0.59	1.64
C18:0	Stearic acid	5.34	1.76	1.8
C18:1 ω 9	Oleic acid	21.59	–	0.87
C18:1 ω 7	Vaccinic acid	1.89	–	–
C18:2 ω 6	Linoleic acid	31.18	–	0.3
C18:3 ω 3	Linolenic acid	3.01	–	–
C22:5 ω 6	Docosapentaenoic acid (DPA)	0.34	3.15	4.39
C22:6 ω 3	Docosahexaenoic acid (DHA)	1.25	13.61	18.97
Total saturated fatty acids (SFA)		40.02	83.24	75.47
Total polyunsaturated fatty acids (PUFA)		59.98	16.76	24.53

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 productions of PUFAs especially DHA and for biodiesel production.

Acknowledgments

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