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Isolation and phylogenetic identification of heterotrophic thraustochytrids from mangrove habitats along the southwest coast of India and prospecting their PUFA accumulation

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Abstract

Among several sustainable resources, marine heterotrophic microorganisms have gained much interest for various commercial applications owing to their potential to produce high quantities of polyunsaturated fatty acids. Above 70% of the globally produced fish oil is utilised for aqua feed formulations. Intense research is being carried out to find suitable alternatives to fish oil. Oleaginous protists are being explored as a promising substitute to fish oil and also for various high-value-added products. However, characterisation and utilisation of these unique protists remain underexplored in many countries including India and this prompted us to screen and isolate these heterotrophic microorganisms capable of producing PUFAs. In this study, 11 strains of heterotrophic thraustochytrid capable of producing significant quantities of polyunsaturated fatty acids were successfully isolated from different mangrove habitats along the southwest coast of India. Morphological characterisation along with molecular identification revealed that these strains showed close similarity to *Aurantiochytrium* spp. of the family Thraustochytridae. They are able to produce 7–11 g of dry biomass per litre of culture. Their fatty acid content was up to 12% (wt/wt) of biomass and GC analysis confirmed that these protists can accumulate docosahexaenoic acid as high as 22–60% of the total fatty acids. Their enzymatic activity, tolerance to various abiotic stress factors and antagonistic potential against vibrios are also described. These heterotrophic protists can antagonise aquaculture pathogens and act as an excellent source of docosahexaenoic acid that indicates their potential as a sustainable alternative to fish oil.

Keywords *Aurantiochytrium* spp. · PUFA · Thraustochytrids · Mangrove ecosystem · Fish oil alternative

Introduction

Microalgae play a vital role in marine ecosystems and are an integral part of the marine food web. They are one of the primary producers of long-chain polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Lewis et al. 1999; Lee and Kim 2002; Marchan et al. 2017). Besides the rich varieties of microalgae, heterotrophic protists are promising candidates for the commercial production of long-chain ω-3 fatty acids, unlike their autotrophic counterparts (Lewis et al. 1999; Gupta et al. 2012; Ugalde et al. 2018). LC-PUFAs are an important component in aquaculture and livestock feeds, and are valuable dietary sources for humans especially in infant formula. Thraustochytrids are one of the potent organisms and an emerging source in the PUFA market (Lewis et al. 1999; Martins et al. 2013).

Thraustochytrids are osmoheterotrophic, oleaginous, eukaryotic, unicellular monocentric fungi-like protists. They are classified under kingdom Heterokonta and class Labyrinthulomycetes (Leander et al. 2004; Damare 2009). Several terminologies are used to describe them and difficulties still persist to align these organisms taxonomically. Cavalier-Smith et al. (1994) positioned thraustochytrids as a divergent group of heterokonta by molecular techniques and concluded that apart from structural and phylogenetic similarity, helix 47 in the V9 region of 18S rRNA, thraustochytrids are also characterised by the presence of AU base pairs like other Heterokonta. However, some authors like Leyland et al. (2017) strongly oppose the evolutionary relationship of

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thraustochytrids with heterokont algae and the usage of the term 'microalgae' for thraustochytrids. Thus, the taxonomic position of these non-photosynthetic marine protists is still controversial. Thraustochytrids comprise 10 genera (Yokoyama and Honda 2007; Raghukumar and Damare 2011) with more than 30 species and half of all species are placed in the Thraustochytridae family (15 species) and belong to the genus *Thraustochytrium* (Porter 1990; Mo et al. 2002). They have widespread distribution in both marine and estuarine habitats (Naganuma et al. 1998; Kimura et al. 1999; Raghukumar 2002; Damare 2009; Taoka et al. 2009; Gupta et al. 2013) and also are documented in associations like commensals, symbionts and sometimes pathogens to marine invertebrates, and are one of the few nuisances during marine invertebrate cell culture development (Rabinowitz et al. 2006; Raghukumar and Damare 2011).

Thraustochytrids are known to secrete a wide variety of hydrolytic exoenzymes such as protease, lipase, cellulase, amylase and xylanase (Raghukumar et al. 1994; Damare 2009) to break down complex organic matter and thus play a vital role in nutrient recycling. Some species are capable of degrading even crude oil and tar balls (Raghukumar et al. 1994; Bremer 1995; Bongiorni et al. 2005a, b; Taoka et al. 2009; Nagano et al. 2011). These heterotrophs can accumulate carotenoid pigments and lipids, especially omega 3 fatty acids (DHA and EPA) along with other saturated and unsaturated fatty acids (Gupta et al. 2012, 2013). Dietary intake of LC-PUFA is essential for the normal development and functioning of the brain and central nervous system (Schuchardt et al. 2010) and can lower the risk of cardiovascular diseases (Martins et al. 2013). Cells utilise DHA as an energy source during starvation and its antioxidant properties protect the cells from oxidative stresses (Shene et al. 2010).

Presently, fish oil is being utilised for a variety of commercial applications and current production cannot meet the increasing global demand. Global annual production of fish oil has already exceeded one million tonnes and the major quantity of fish oil (73%) produced is mainly utilised for aqua feed formulation (Lewis et al. 1999; Rahman et al. 2018). Algal oil has a better scope and is considered superior to fish oil, making it an alternative DHA source (Shah et al 2018). Even though the occurrence and distribution of thraustochytrids from oceanic and mangrove ecosystems have been extensively reported, their study in India is still underdeveloped (Raghukumar 2002). The present study is focused on the isolation and characterisation of strains of thraustochytrids from different mangrove habitats of Kerala and to assess their PUFA potential.

Material and methods

Sampling

Partially degraded mangrove leaf litter was collected along with the sediment from selected mangrove habitats of Kerala Coast, India, using a hand-operated grab (van Veen). Details of the collection sites and their GPS co-ordinates are given in Table 1. Samples collected were transported aseptically to the laboratory and processed immediately.

Sample processing, isolation and identification

The leaf samples were washed twice with sterilised seawater to avoid unwanted bacterial load and other debris. Aseptic processing of leaf samples and segments was done with sterile surgical blades. The excised segments were then inoculated on YEPGA (yeast extract 2 g L^{-1}, peptone 1 g L^{-1}, glucose 4 g L^{-1}, agar 10 g L^{-1}) (HiMedia, India) prepared in half-strength (50%) seawater (Yang et al. 2010) supplemented with a cocktail of antibiotics (streptomycin, ampicillin and kanamycin—filter sterilised) prepared at a concentration of 100 mg L^{-1}. The plates were then incubated at 25 °C and observed for growth at every 24 h for 2 to 3 days. Morphologically distinct colonies were selected and purified. The isolates obtained were observed under light microscope and images were documented (Leica DFC 295, Germany). The isolated strains were cryopreserved (− 80 °C) as glycerol (20%) stocks for future use.

Total genomic DNA was extracted from 1- to 2-day-old cultures using the phenol-chloroform method (Wu et al. 2000). The extracted DNA was quantified using a Biophotometer (Eppendorf, Germany). 18S rRNA gene fragment was amplified by polymerase chain reaction (PCR) (Veriti, Applied System, USA) using universal primers 18S Univ F -5′-TGGTTGATCCTGCCAGT-3′ and 18S Univ R - 5′-TAATGATCCTTCCGCAGGTTCACCT-3′. A 25 μL PCR reaction was set comprising of 19.75 μL of ddH_2O, 2.5 μL of 10X buffer, 0.5 μL of dNTPs and 0.5 μL each of forward and reverse primers, and 0.25 μL of *Taq* polymerase to amplify 1 μg of DNA template. The amplification was established with initial denaturation at 95 °C for 5 min, followed by denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s, extension at 72 °C for 1:50 min and final extension at 72 °C for 7 min for 35 cycles. The PCR product was eluted from agarose gel using QIAquick PCR purification kit (Qiagen). 18S rRNA gene was ligated into pJET 1.2 cloning vector (Thermo Scientific) as per manufacturer's instructions. From transformants, recombinant plasmids were isolated (Gene JET plasmid mini prep kit, Thermo Scientific), and the inserts were amplified and sequenced (18S Univ F and 18S Univ R).

Phylogenetic analysis

Relative phylogenetic affiliations were identified using BLAST analysis of 18S rRNA gene sequence. For phylogenetic references, 18S rRNA gene sequences of 19 closely related species were retrieved from the NCBI GenBank database. ClustalW algorithm (MEGA7 software) was used to

Table 1 Details of sampling sites (mangrove stands) along the Kerala Coast

Strain code	Site of origin	Nearest city	GPS co-ordinates	pH	**Salinity (PSU)**
MBTDCMFRIJMVL1	Valapattanam	Kannur	11.90° N 75.37° E	7.18	32
MBTDCMFRIJMVL2	Mundappuram	Kannur	12.01° N 75.27° E	7.2	30
MBTDCMFRIJMVL3	Mangalavanam	Ernakulam	9°54′ N 76° 18′ E	7.1	17
MBTDCMFRIJMVL4	Mangalavanam	Ernakulam	9° 54′ N 76° 18′ E	7.1	17
MBTDCMFRIJMVL5	Kadalundi	Calicut	11° 8′ 0″ N 75° 49′ 0″ E	7.19	25
MBTDCMFRIJMVL6	Kadalundi	Calicut	11° 8′ 0″ N 75° 49′ 0″ E	7.19	25
MBTDCMFRIJMVL7	Payyoli	Vadakara	11° 32′ 0″ N 75° 40′ 0″ E	7.1	32
MBTDCMFRIJMVL8	Puthuvypu	Ernakulam	9° 59′ 11″ N 76° 13′ 50″ E	7.1	30
MBTDCMFRIJMVL9	Puthuvypu	Ernakulam	9° 59′ 11″ N 76° 13′ 50″ E	7.1	30
MBTDCMFRIJMVL10	Puthuvypu	Ernakulam	9° 59′ 11″ N 76° 13′ 50″ E	7.1	30
MBTDCMFRIJMVL11	Puthuvypu	Ernakulam	9° 59′ 11″ N 76° 13′ 50″ E	7.1	30

align the sequences with referral sequences (Thompson et al. 1994). The evolutionary history was inferred by using the maximum likelihood method based on Tamura-Nei model (Tamura and Nei 1993) with 1000 bootstrap value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). The 18S rRNA gene sequences of the isolates were submitted to NCBI GenBank.

Growth characteristics

The growth patterns of isolated thraustochytrid strains were studied by using three referral strains (JMVL1, JMVL3 and JMVL4). Growth was monitored by inoculating 1% of actively growing culture into sterile YEPG broth in triplicates and incubated in a shaker at 121 rpm at room temperature. Growth curve was plotted by measuring the optical density at 600 nm for up to 196 h (Biophotometer, Eppendorf, Germany).

Enzyme assay

The isolates were screened for their ability to produce hydrolytic exoenzymes such as urease, lipase, amylase, cellulase, protease, chitinase and gelatinase in triplicates. Strains were spot inoculated on nutrient agar impregnated with respective substrates (HiMedia, India) and 1% sea salt (Sigma, USA). The substrates include starch for amylolytic activity, skimmed milk for proteolytic activity, tributyrin for lipolytic activity, gelatin for gelatinase activity, carboxymethyl cellulose for cellulase activity and urea for urease activity (Nair et al. 2012). After incubation at 30 °C for 48 h, enzyme production was observed as a clear zone around the colonies with or without the addition of specific reagents. Enzyme indices (EI) were calculated as ratio of halo diameter to colony diameter. EI value of above 1.0 indicates potential producer.

Abiotic stress tolerance assay

The isolates were subjected to different stress conditions such as pH, salinity and temperature to detect their ability to tolerate different stresses by altering a single variable at a time and keeping two variables constant. The isolates were screened for temperature tolerance by growing in YEPGA plates at different temperature (20, 30, 40, 50, 60 °C) and keeping pH and salinity of 7.0 and 15 PSU, respectively, salinity tolerance by growing in different salinity ranges (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 PSU) by amending YEPGA with respective concentration of salt (NaCl) at room temperature and tolerance to pH by growing isolates in media with different pH (4, 5, 6, 7, 8, 9) (adjusted with 0.1 N HCl and 0.1 N NaOH) at room temperature and salinity of 15 PSU.

Antagonistic assay

The isolates were screened for antibacterial activity against major aquaculture pathogens belonging to the genus *Vibrio* by the spot diffusion method as described in Nair et al. (2012). The pathogens used were *Vibrio harveyi* 101, *V. anguillarum* A1 and *V. alginolyticus* 101 (courtesy of Central Institute of Brackishwater Aquaculture, Chennai, India); *V. vulnificus* MTCC1145 and *V. parahaemolyticus* MTCC451 (courtesy of Microbial Type Culture Collection, Chandigrah, India). The test isolates were spotted separately on Mueller Hinton Agar (MHA) plates prepared with 1% NaCl (HiMedia, India) over the spread pathogen (grown overnight). The plates were incubated at 30 °C for 24–48 h. Antagonism could be observed as zone of inhibition around the test organisms.

Fatty acid extraction and GC

Freshly prepared YEPG broth was inoculated with 1% of active culture in triplicates and incubated on a shaker incubator for 30 h

at room temperature. After incubation, the cells were harvested by centrifugation at 5000 rpm for 8 min. The supernatant was discarded and the cells packed as pellets were oven-dried at 58 ± 2 °C for 2 days and weighed. Fatty acid extraction and profiling were done according to Folch et al. (1957). In brief, the cold extraction method with chloroform:methanol mixture in 2:1 ratio was used for extracting total lipid in the samples. Petroleum ether was used to quantitatively determine dry fatty acid methyl esters. The esterified fatty acids were analysed using a gas chromatograph (Perkin Elmer, USA) equipped with a SP 2560 (crossbond 5% diphenyl—95% dimethyl polsiloxane) capillary column (100 m × 0.25 mm i.d., 0.50 μm film thickness, Supelco, USA) using a flame ionisation detector (FID) equipped with a split/splitless injector, which was used in the split (1:15) mode. A temperature programme was used which rises from 140 °C for 1 min to 250 °C at 30 °C min^{-1} where it was held for 1 min followed by an increase of 25 °C min^{-1} to 285 °C, where it was held for 2 min. Peaks were identified using fatty acid methyl ester standards. PUFA production was calculated according to peak area of chromatogram in accordance with the peak area of internal standards. Both quantification and profiling were done in triplicates and mean value was calculated.

Results

Isolation and molecular phylogeny

Isolation of thraustochytrids was carried out by leaf disc method. Even though media were supplemented with a variety of broad-spectrum antibiotics, several antibiotic-resistant bacteria and fungi were also found to grow on the plate. The distinct colony characteristics with typical morphology of thraustochytrids were selected. These colonies were selected and identified microscopically, and pure cultures were obtained after a series of subcultures. A total of 11 morphologically and culturally distinct strains were identified from different mangrove stands of Kerala Coast (Table 1). The strain code, accession numbers and their characteristics of isolates are listed in Table 2. BLAST analysis of 18S rRNA gene sequences revealed that the isolates showed similarity with genus *Aurantiochytrium*, the most predominant strain reported from mangrove habitats. The phylogenetic tree (Fig. 1) constructed using 18S rRNA gene sequences of our isolates with the sequences of similar species retrieved from the GenBank database showed a close phylogenetic relationship with *Aurantiochytrium* sp. The name and accession number of 18S rRNA gene sequences submitted are given in Table 2. Our study resulted in selection of *Aurantiochytrium* sp.

Morphological, cultural and microscopic observations revealed that they were small to medium-sized, opaque, uneven and slimy colonies. Colonies were off-white in colour and became pale orange upon prolonged incubation. On microscopical observation, cells are spherical in shape and ranged from 10 to 20 μm in diameter. Diads and tetrads were also observed in different stages of the life cycle (Fig. 2). Isolates were characterised by underdeveloped ectoplasmic network, occurrence of successive binary division and spherical to ovoid motile biflagellate zoospores. All the strains released 8–32 zoospores by rupturing at one end. The isolates were seen as grains settling at the bottom of the flask when grown in nutrient broth.

Characterisation of *Aurantiochytrium* spp.

All the three referral strains showed rapid growth with an initial lag of 1–3 h followed by exponential growth and attaining maximum growth within 30–33 h (Fig. 3). All the strains produced a significant amount of biomass after 30 h of incubation at room temperature (Fig. 4). Strain JMVL1 possessed the highest biomass of 10.6 g L^{-1} and strain JMVL7 had the least biomass of 7.3 g L^{-1}. All strains showed positive for the lipase and urease activity and strain JMVL7 also showed amylolytic activity (Table 3). The EI of strains specific to lipase activity are presented in Table 4.

Though antagonistic activity was tested for all isolates, only strains JMVL3 and JMVL4 showed potent antagonistic activity against two test pathogens *Vibrio anguillarum* A1 and *V. parahaemolyticus* MTCC451. They showed a halo zone against *V. vulnificus* MTCC1145, but did not show activity against *V. alginolyticus* and *V. harveyi* (Table 5). To the best of our knowledge, antagonistic potential of the thraustochytrids in general and the abovementioned strains in particular against *Vibrio* spp. has not been reported earlier and the remaining nine strains did not show any antagonistic activity against any of the pathogens tested. On evaluating the isolates for tolerance, all isolated strains showed good growth within the pH range from 4 to 9. All the strains except JMVL3 and JMVL4 were grown in the salinity range of 0–100 PSU, whereas JMVL3 and JMVL4 could not grow in zero salinity. A salinity of 20–50 PSU supported confluent growth for all the strains tested. Maximum temperature tolerance was observed within 35 °C (Table 3).

The fatty acid profile of the isolates comprised mainly of saturated fatty acids dominated particularly by palmitic acid, and among long-chain unsaturated fatty acids, DHA was the highest. The ratio of saturated fatty acid corresponding to total fatty acids (TFA) ranged from 39 to 66% and with polyunsaturated fatty acids ranging from 23 to 60%. Palmitic acid was the major saturated fatty acid accumulated and accounted for 39–48% of TFA with JMVL9 being the highest producer (48.3%). Odd-chain saturated fatty acids C15:0 and C17:0 were also accumulated in some strains. C15:0 was present in all strains except JMVL8 and ranged from 6 to 16% with the highest accumulation in JMVL3 (16.8% of TFA). C17:0 was accumulated in all the strains except JMVL8 and JMVL9 with

Table 2 Strains used in molecular phylogenetic analysis of this study and accession numbers of their 18S rRNA gene sequences

Taxon	Strain	Accession number
Schizochytrium sp.	SEK 210	AB290576
Schizochytrium sp.	SEK 345	AB290577
Schizochytrium aggregatum	ATTC28209	AB022106
Thraustochytrium kinnei	KMPB 1694d	L34668
Ulkenia profunda	KMPB N 3077T	L34054
Japonochytrium sp.	ATCC 28207	AB022104
Ulkenia visurgensis	ATCC 28208T	AB022116
Aurantiochytrium sp.	BURABQ 133	DQ023620
Aurantiochytrium mangrovei	RCC893	DQ367049
Aurantiochytrium sp.	MBTDCMFRIJMVL4	
Aurantiochytrium sp.	MBTDCMFRIJMVL3	
Aurantiochytrium limacinum	NIBH SR21T (IFO 32693)	AB022107
Thraustochytrium aggregatum	KMPB N-BA-110T	AB022109
Labyrinthula sp.	AN-1565 (NBRC 33215)	AB022105
Schizochytrium minutum	KMPB N-BA-77T	AB022108
Thraustochytrium multirudimentale	KMPB N-BA-113	AB022111
Aplanochytrium kerguelense	KMPB N-BA-107	AB022103
Aplanochytrium stocchinoi		AJ519935
Ochromonas.danica	n/a	M32704
Aurantiochytrium sp.	MBTDCMFRIJMVL1	MH059480
Aurantiochytrium sp.	MBTDCMFRIJMVL2	MH059481
Aurantiochytrium sp.	MBTDCMFRIJMVL3	KU565329
Aurantiochytrium sp.	MBTDCMFRIJMVL4	KU565330
Aurantiochytrium sp.	MBTDCMFRIJMVL5	MH059482
Aurantiochytrium sp.	MBTDCMFRIJMVL6	MH059483
Aurantiochytrium sp.	MBTDCMFRIJMVL7	MH059484
Aurantiochytrium sp.	MBTDCMFRIJMVL8	MH059485
Aurantiochytrium sp.	MBTDCMFRIJMVL9	MH059486
Aurantiochytrium sp.	MBTDCMFRIJMVL10	MH059487
Aurantiochytrium sp.	MBTDCMFRIJMVL11	MH059488

highest in JMVL3 (4.1% of TFA). EPA (C20:5 *cis*) was present only in JMVL3 and JMVL4 in the range of about 0.5–0.7% of TFA. DHA (C22:6 *cis*) was the major polyunsaturated fatty acid and was present in every strain in significant quantities ranging from 22 to 60% of TFA. Table 6 depicts the average percentage fatty acids of all the isolates. JMVL8 showed maximum production of DHA of about 60.3% of TFA. Even though the percentage accumulation of DHA corresponding to TFA was higher in JMVL8, the quantity of DHA accumulated corresponding to biomass was higher in JMVL1 and was about 3.5%; whereas in JMVL8, it was only 0.6% (Table 7).

Discussion

In the present study, we describe the occurrence of *Aurantiochytrium* sp. from the mangrove ecosystems of Kerala Coast. We obtained 11 strains of heterotrophic protists from different mangrove habitats along the southwest coat of India and detailed characterisation of these microorganisms revealed that they could produce significant quantity of biomass as well as higher levels of PUFA. Morphological, cultural and microscopic observation along with molecular identification based on 18S rRNA gene sequence similarity confirmed that all these strains were of *Aurantiochytrium* species belonging to the family Thraustochytridae. Their fatty acid profile also offered to support our identification. Yokoyama and Honda (2007) suggested that arachidonic acid will be less than 5% in species of *Aurantiochytrium*. As shown in Table 6, most of the isolates did not accumulate arachidonic acid, except for JMVL3 and JMVL4 which produced arachidonic acid at 0.2 to 0.3%. *Aurantiochytrium mangrovei* was first described from mangroves of Goa, India (Raghukumar 1988). The presence of *Aurantiochytrium* spp. also has been reported from Shuidong Bay, China (Gao et al. 2013);

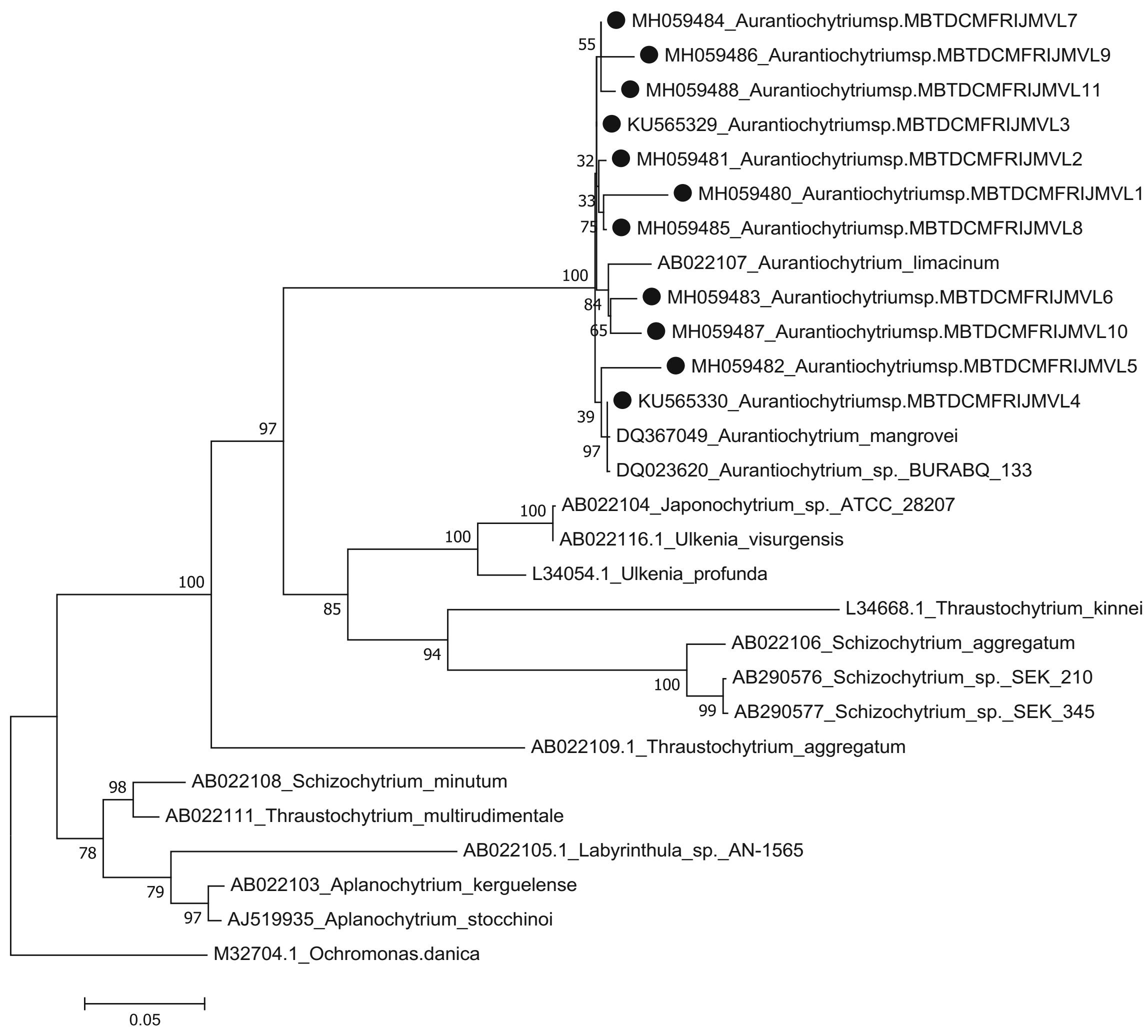

Fig. 1 Molecular phylogenetic analysis by maximum likelihood method showing a close relationship between the isolates and other selected sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches

Okinawa Prefecture, Japan (Nakazawa et al. 2012); Taipei City, Taiwan (Chaung et al. 2012); and Japan (Yokoyama and Honda 2007). However, to the best of our knowledge, this is the first report on isolation of *Aurantiochytrium* sp. from mangroves of Kerala, India. These species of *Aurantiochytrium* can be further explored for the production of a wide variety of bioactive compounds and pharmaceuticals and could be commercially utilised for the production of DHA-rich oil or biodiesel (Chang 2013), squalene (Nakazawa et al. 2012, 2014), exopolysaccharides, extracellular enzymes (Taoka et al. 2009) and carotenoids (Atienza 2012).

The isolated strains were further characterised for their enzyme production potential, antagonism against pathogens, fatty acid production and their ability to tolerate various physiological conditions like temperature, salinity and pH. Thraustochytrids have been reported to secrete a wide variety of hydrolytic exoenzymes such as protease, lipase, cellulase, amylase and xylanase which help break down organic matter (Raghukumar et al. 1994; Damare 2009). However, our isolates are predominant in production of only lipases and urease with few exceptions like JMVL7. At the same time, our observation was in agreement with the findings of Taoka et al. (2009). Even though there are reports for cellulolytic activity by *Aurantiochytrium* sp. (Raghukumar et al. 1994), none of the tested strains showed cellulase activity when carboxy methyl cellulose was used as a substrate which is in total agreement with Nagano et al. (2011). Similar findings of Taoka et al. (2009) also support the absence of exoenzymatic activity like amylase, gelatinase, cellulase and chitinase in *Aurantiochytrium* sp. but with the ability to produce protease. Kanchana et al. (2011) observed lipase activity in *Thraustochytrium* sp. with optimum activity at alkaline pH and established that lipase production is salt dependent. Although Shirodkar et al. (2017) and Bongiorni et al. (2005a, b) have established the ability of thraustochytrids to

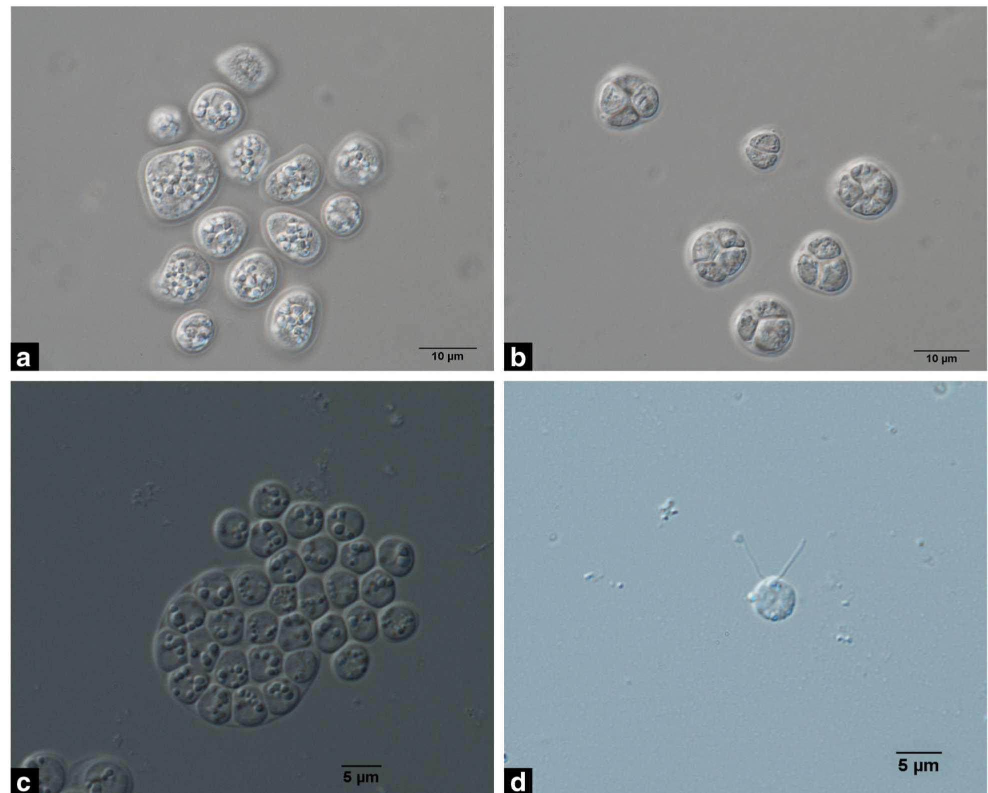

Fig. 2 Microscope images of *Aurantiochytrium* sp. **a** Thallus. **b** Mitotic cell division in thallus. **c** Sporangium and zoospore release. **d** Zoospore

produce multiple degradative enzymes, the inability of our isolates to produce a variety of enzymes might be due to a lack of specific substrates to induce activity. For example, a study by Yokoyama and Honda (2007) found that co-culturing of *Aurantiochytrium* sp. with degraded mangrove leaves induced the cells to release cellulase. Thus, our isolates might also produce different enzymes in the presence of natural substrates or by substituting essential factors.

Organisms inhabiting mangrove ecosystems are capable of surviving harsh environmental conditions. Salinity plays a

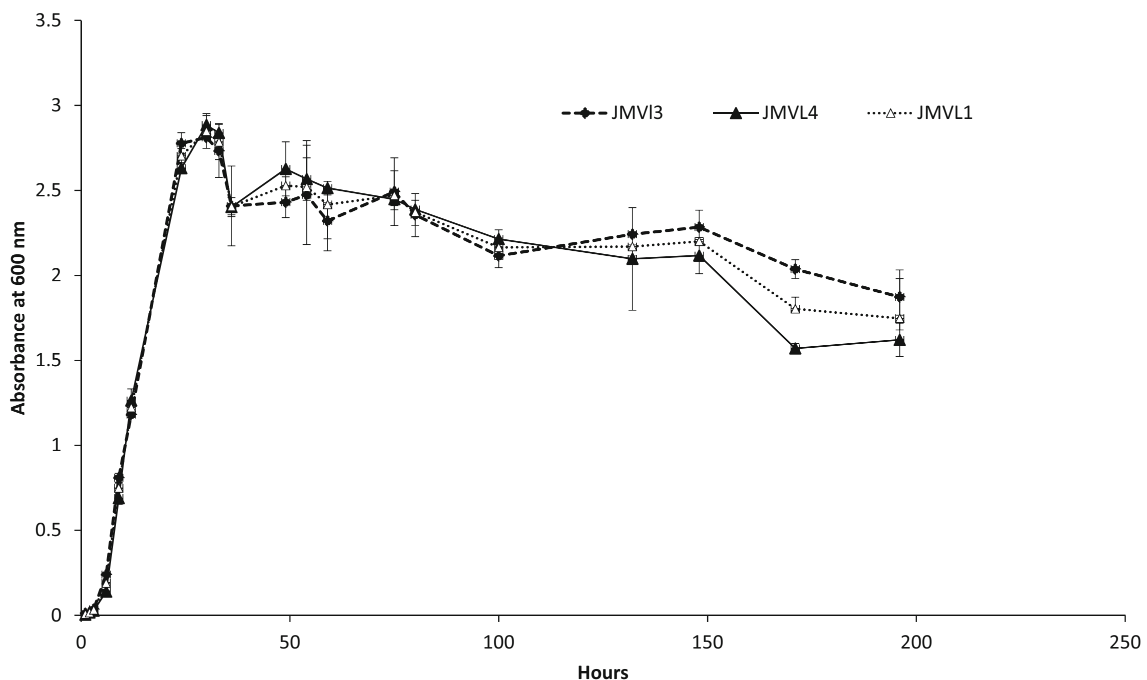

Fig. 3 Growth curve of strains JMVL1, JMVL3 and JMVL4 (each value is mean ± SD, $n = 3$)

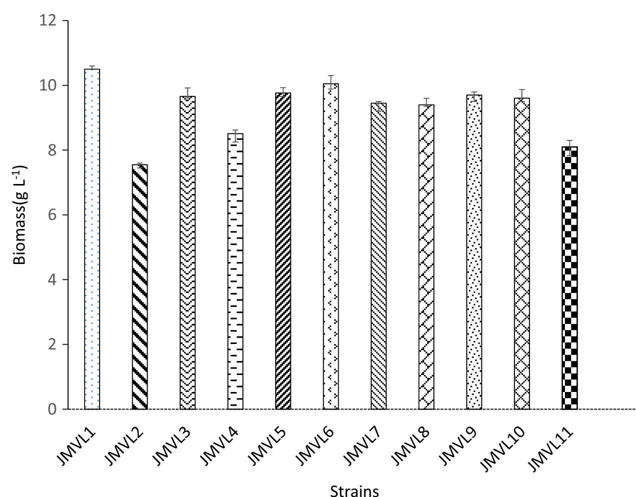

Fig. 4 Biomass of all the isolates (each value is mean ± SD, $n = 3$)

major role in growth and fatty acid accumulation of cells by controlling the cytoplasmic ion gradient and enzyme activity involved in cell wall expansion (Leano et al. 2003; Hakim 2005). Our study on tolerance to salinity showed that most of the isolated strains are euryhaline and can tolerate up to 100 PSU and can grow without NaCl except for JMVL3 and JMVL4 which showed growth only above 10 PSU. The reason behind good growth at higher salinity and poor growth at zero salinity is due to the lack of ions to support the growth of marine origin and also due to requirement of optimal osmotic conditions (Fan et al. 2002; Shabala et al. 2013). From this study, it was evident that salinity of the sampling site does not have any relationship to the salinity tolerance level of isolates in in vitro conditions. Similar tolerance to salinity changes was reported from genera like *Schizochytrium* (Kamlangdee and Fan 2003), *Aurantiochytrium* (Chaung et al. 2012) and *Thraustochytrium* (Hakim 2005).

Temperature also affects the growth of species of *Aurantiochytrium* and the production of metabolites. All our isolates can tolerate only up to 35 °C which is in concordance with the reports by Nakahara et al. (1996), Burja et al. (2006) and Zhu et al. (2008) who found that the optimal DHA production was at 23–28 °C. Slight acidity or variation in initial pH of the medium may also interfere with the growth of microorganisms by affecting cell membrane function, nutrient uptake and biosynthesis of metabolites (Kim et al. 2005). All our isolates could tolerate wide pH range and our results were in agreement with that of Fan et al. (2002) who observed wide tolerance in pH ranging from 4 to 9 by thraustochytrids except *Ulkenia*. Hakim (2005) reported that pH 4 and pH 7 were optimum for biomass and DHA production respectively. Our findings of physiological tolerance indicate that *Auantiochytrium* spp. are capable of adapting to changes in the physical conditions in the mangrove ecosystem.

The antibacterial activity exhibited by our isolates has added advantage to use our strains to control pathogens or as probionts in aquaculture. Thraustochytrids can act as bacterivores or bacterial feeders (Raghukumar 1992; Atienza 2012; Chang 2013) and many previous reports have demonstrated antibacterial activity of several microalgae (e.g., Abedin and Taha 2008; Martins et al. 2008). The potential of antibacterial agent production by microorganisms as a defensive mechanism could be exploited as a source of new bioactive compounds (Mundt et al. 2001). Antagonistic potential can be used in aquaculture sector as these strains can be used as probiont/live agent to protect the host against pathogens and control pathogenic bacterial proliferation in aquaculture systems.

All our isolated strains are oleaginous and can be considered as an alternative source for biodiesel and PUFA. Chang et al. (2014) suggested *Aurantiochytrium* as the best

Table 3 Enzyme profile and stress tolerances of strains ($n = 3$)

Isolate code	Enzyme assay							Abiotic tolerance test		
	A	B	C	D	E	F	G	H	I	J
MBTD CMFRI JMVL1	–	–	+++	–	–	–	++	4–9	20–35	0–100
MBTD CMFRI JMVL2	–	–	++	–	–	–	++	4–9	20–35	0–100
MBTD CMFRI JMVL3	–	–	+++	–	–	–	++	4–9	20–35	10–100
MBTD CMFRI JMVL4	–	–	+++	–	–	–	++	4–9	20–35	10–100
MBTD CMFRI JMVL5	–	–	++	–	–	–	++	4–9	20–35	0–100
MBTD CMFRI JMVL6	–	–	+++	–	–	–	++	4–9	20–35	0–100
MBTD CMFRI JMVL7	+	–	++	–	–	–	++	4–9	20–35	0–100
MBTD CMFRI JMVL8	–	–	++	–	–	–	++	4–9	20–35	0–100
MBTD CMFRI JMVL9	–	–	++	–	–	–	++	4–9	20–35	0–100
MBTD CMFRI JMVL10	–	–	++	–	–	–	++	4–9	20–35	0–100
MBTD CMFRI JMVL11	–	–	+	–	–	–	++	4–9	20–35	0–100

A, amylase; *B*, cellulase; *C*, lipase; *D*, protease; *E*, chitinase; *F*, gelatinase; *G*, urease; *H*, hydrogen ion concentration (pH); *I*, temperature (°C); *J*, salinity (PSU); −, no enzymatic activity; +, zone diameter 10–20 mm; ++, zone diameter 20–30 mm

Table 4 Enzyme index (EI) value (mean ± SD, $n = 3$)

Strain	EI of lipase = halo diameter/culture diameter (Øh/Øc)
MBTDCMFRIJMVL1	5.05 ± 0.07
MBTDCMFRIJMVL2	4.675 ± 0.10
MBTDCMFRIJMVL3	6.05 ± 0.21
MBTDCMFRIMVL4	6.55 ± 1.06
MBTDCMFRIJMVL5	3.655 ± 0.07
MBTDCMFRIJMVL6	4.23 ± 0.10
MBTDCMFRIJMVL7	3.3 ± 0.14
MBTDCMFRIJMVL8	4.35 ± 0.07
MBTDCMFRIJMVL9	3.9 ± 0.14
MBTDCMFRIJMVL10	4.65 ± 0.21
MBTDCMFRIJMVL11	2.6 ± 0.14

candidate for the production of biodiesel and PUFA when compared to the species of *Thraustochytrium*, *Schizochytrium* and *Ulkenia*. Yokoyama and Honda (2007) and Ma et al. (2015) also supported these findings that *Aurantiochytrium* sp. can produce large amount of lipids with significant amounts of PUFA especially DHA due to its fast growth. Saturated fatty acids constitute about 55–60% of total fatty acid. The major fatty acid accumulated was palmitic acid in agreement with the results of Nakazawa et al. (2012) and hence these organisms can be considered as renewable sources for biodiesel production. The strain JMVL9 produces maximum palmitic acids of about 48.3% of TFA. Since omega 3 fatty acids are unstable, strains with high saturated to unsaturated fatty acid ratios may be potentially used in biodiesel production. Thus, with the highest saturated fatty acid percentage of 66%, JMVL4 can be used as a good candidate in biodiesel industry. This study also reports the accumulation of odd-chain saturated fatty acids (OC-FA) which is in agreement with Chang et al. (2011). Varieties of DHA-producing heterotrophic strains of microorganisms have been isolated from different parts of the world (Yang et al. 2010; Gupta et al. 2013). Most of the isolates we obtained were ideal for DHA production as they accumulate DHA in the range of 22–

Table 5 Antagonistic activity of the isolates ($n = 3$)

Isolate code	Identification	Zone of inhibition (mm)				
		A	B	C	D	E
MBTDCMFRIJMVL3	*Aurantiochytrium* sp.	18	14	–	–	12
MBTDCMFRIJMVL4	*Aurantiochytrium* sp.	22	16	–	–	14

Value indicates zone (mm) of inhibition around the test. The assays were carried out in triplicates through spot diffusion method. The values may differ ±1 mm from the size mentioned

A, *V. vulnificus* MTCC1145; *B*, *V. parahaemolyticus* MTCC451; *C*, *V. alginolyticus* 101; *D*, *V. harveyi* 101; *E*, *V. anguillarum*

Table 6 Fatty acid composition of *Aurantiochtrium* sp. (total fatty acids %, each value is mean ± SD, $n = 3$)

Fatty acids	JMVL1	JMVL2	JMVL 3	JMVL 4	JMVL5	JMVL 6	JMVL 7	JMVL8	JMVL9	JMVL10	JMVL11
Lauric acid ($C_{12:0}$)	–	–	0.15 ± 0.00	0.13 ± 0.00	–	–	–	–	–	–	–
Tridecanoic acid ($C_{13:0}$)	–	–	0.18 ± 0.01	0.16 ± 0.02	–	–	–	–	–		–
Myristic acid ($C_{14:0}$)	2.16 ± 0.00	1.90 ± 0.3	2.71 ± .01	2.75 ± 0.09	2.62 ± 0.03	2.62 ± 0.12	2.37 ± 0.09	–	–	2.06 ± 0.03	
Pentadecanoic acid ($C_{15:0}$)	9.5 ± .07	9.35 ± .18	16.86 ± 0.02	14.99 ± 0.80	10.24 ± 0.52	9.40 ± 0.09	6.58 ± 0.3	–	9.7 ± 0.25	8.22 ± 0.59	8.70 ± 0.03
Palmitic acid ($C_{16:0}$)	41.34 ± 0.1	41.82 ± 0.5	40.7 ± 0.85	43.01 ± 0.28	48.04 ± 0.05	44.32 ± 0.58	44.02 ± 0.03	39.67 ± 1.07	48.37 ± 0.21	44.73 ± 0.09	45.39 ± 0.08
Heptadecanoic acid ($C_{17:0}$)	2.27 ± 0.13	2.90 ± 0.15	4.19 ± 0.04	3.72 ± 0.17	2.64 ± 0.05	2.1 ± 0.06	2.25 ± 0.03	–	–	2.04 ± 0.07	2.96 ± 0.31
Stearic acid ($C_{18:0}$)	0.97 ± 0.01		1.16 ± 0.016	1.33 ± 0.34	1.01 ± 0.02	0.94 ± 0.09	1.17 ± 0.032	–	–		
SFA (saturated fatty acid)	56.24 ± 0.11	55.97 ± 0.53	65.96 ± 0.83	66.09 ± 0.1	64.54 ± 0.43	59.38 ± 0.77	56.39 ± 0.21	39.68 ± 1.07	58.06 ± 0.04	57.05 ± 0.66	57.06 ± 0.35
Arachidonic acid ($C_{20:4}$) n-6 or ω-6	–		0.27 ± 0.02	0.35 ± 0.09				–	–		
Eicosapentaenoic acid ($C_{20:5}$ *cis*) n-3 or ω-3	–		0.54 ± 0.0	0.78 ± 0.01				–	–		
Docosahexaenoic acid ($C_{22:6}$) n-3 or ω-3	43.755 ± 0.11	42.03 ± 0.14	22.5 ± 0.2	22.13 ± 0.09	35.46 ± 0.05	40.63 ± 0.44	42.62 ± 0.24	60.33 ± 0.81	41.94 ± 0.95	42.95 ± 0.20	42.95 ± 0.08
PUFA (polyunsaturated fatty acids)	43.755 ± 0.11	42.03 ± 0.14	23.31 ± 0.18	23.27 ± 0.00	35.46 ± 0.05	40.63 ± 0.43	42.62 ± 0.24	60.33 ± 0.805		42.95 ± 0.2	42.95 ± 0.08

Table 7 Quantity of individual fatty acids in grams relative to biomass (wt/wt); each value is mean ± SD, $n = 3$

Fatty acids	JMVL1	JMVL2	JMVL 3	JMVL 4	JMVL5	JMVL 6	JMVL 7	JMVL8	JMVL9	JMVL10	JMVL11
Lauric acid ($C_{12:0}$)			0.01 ± 0.00	0.01 ± 0.00	–						
Tridecanoic acid ($C_{13:0}$)			0.01 ± 0.00	0.01 ± 0.00	–						
Myristic acid ($C_{14:0}$)	0.28 ± 0.01	0.07 ± 0.00	0.12 ± 0.01	0.16 ± 0.01	0.39 ± 0.01	0.39 ± 0.01	0.14 ± 0.01			0.07 ± 0.00	
Pentadecanoic acid ($C_{15:0}$)	1.33 ± 0.08	0.39 ± 0.02	0.75 ± 0.01	0.84 ± 0.03	1.67 ± 0.05	1.50 ± 0.01	0.42 ± 0.03		0.28 ± 0.03	0.29 ± 0.05	0.37 ± 0.00
Palmitic acid ($C_{16:0}$)	4.77 ± 0.19	1.45 ± 0.05	1.82 ± 0.15	2.41 ± 0.17	6.50 ± 0.01	5.9 ± 0.05	2.32 ± 0.03	0.58 ± 0.17	1.23 ± 0.02	1.36 ± 0.01	1.67 ± 0.01
Heptadecanoic acid ($C_{17:0}$)	0.28 ± 0.03	0.10 ± 0.01	0.12 ± 0.11	0.21 ± 0.02	0.37 ± 0.01	0.28 ± 0.01	0.12 ± 0.00			0.06 ± 0.01	0.11 ± 0.03
Stearic acid ($C_{18:0}$)	0.11 ± 0.01		0.06 ± 0.02	0.08 ± 0.01	0.13 ± 0.00	0.11 ± 0.01	0.06 ± 0.00				
SFA (saturated fatty acid)	6.77 ± 0.08	2.01 ± 0.08	2.88 ± 0.01	3.7 ± 0.14	9.06 ± 0.08	8.19 ± 0.08	3.06 ± 0.01	0.58 ± 0.17	1.51 ± 0.01	1.78 ± 0.07	2.15 ± 0.04
Arachidonic acid ($C_{20:4}$) n-6 or ω-6			0.01 ± 0.01	0.02 ± 0.00							
Eicosapentaenoic acid ($C_{20:5}$ *cis*) n-3 or ω-3			0.02 ± 0.00	0.04 ± 0.01							
Docosahexaenoic acid ($C_{22:6}$) n-3 or ω-3	3.54 ± 0.06	1.07 ± 0.01	1.01 ± 0.02	1.19 ± 0.13	3.37 ± 0.01	3.39 ± 0.05	1.61 ± 0.02	0.64 ± 0.08	0.77 ± 0.09	0.94 ± 0.02	1.15 ± 0.01
PUFA (polyunsaturated fatty acids)	3.54 ± 0.06	1.07 ± 0.01	1.04 ± 0.01	1.25 ± 0.13	3.37 ± 0.01	3.39 ± 0.05	1.61 ± 0.02	0.64 ± 0.08	0.77 ± 0.09	0.94 ± 0.02	1.15 ± 0.01

60% of TFA. The strain with the most potential for DHA production in our study was JMVL1 as it accumulated 43.6% of TFA and in terms of biomass it produced about 3.5% DHA even in minimal media, which is much higher than commercial strain *Schizochytrium* sp. SR21 (30% of DHA of TFA; Gupta et al. 2013). Jakobsen et al. (2008) succeeded in producing a biomass of 100 g L^{-1} in *Aurantiochytrium* sp. by feeding it excess carbon under nitrogen limited conditions with 58% TFA of which 30% is DHA (about 15.6 g L^{-1}). Similar reports on the influence of carbon on production of biomass, total fatty acid accumulation and DHA production in different thraustochytrids such as *Aurantiochytrium* sp. and *Schizochytrium* sp. are available (Ren et al. 2010; Qu et al. 2011; Huang et al. 2012; Chang et al. 2014). Another study which regulated intermittent glucose supply of above 15 g L^{-1} in *Schizochytrium* sp. HX 308 achieved a biomass of 92.7 g L^{-1} with 50% TFA of which 42% (17.7 g L^{-1}) was DHA (Qu et al. 2011). Later, Chang et al. (2014) utilised *Aurantiochytrium* sp. TC22 and produced 70.8 g L^{-1} biomass by following fed batch strategy with excess supply of glycerol (40 g L^{-1}) to obtain 52% TFA with 39% DHA (14.3 g L^{-1}). These findings suggest that optimisation of culture conditions and culturing techniques will greatly influence the yield in both biomass and PUFAs. Even though our results are not on par with these findings, further manipulation in media and culture conditions would definitely offer higher yield.

DHA is of interest due to its applications in nutraceuticals, infant formula, fish feed, etc. In aquaculture systems, PUFAs contribute energy, growth, reproductive maturity of the candidate species cultured and their natural immunity (Sargent et al. 1999). At present, fish oil is utilised for PUFA production. Fish oil contains only 7–14% DHA, and has disadvantages due to coastal contaminants in fishes, undesirable fishy taste, odour and oxidative instability (Ma et al. 2015). Fish oil may also be contaminated with mercury, dioxins and polychlorinated biphenyls (Gupta et al. 2012). Global fish catch is relatively static and its utilisation for fish oil production has increased remarkably. In general, about 100 kg fresh fish is exploited to produce 5 kg of fish oil and about 25% of world captured fish is being utilised for this purpose (De Silva et al. 2010). It is well established that (Huang et al. 2012) algal oil is superior when compared to fish oil because of its PUFA profile, stability and lack of odour. Several attempts have been demonstrated to replace fish-based ingredients for commercial application (Huang et al. 2012; Shah et al. 2018). Fatty acid profile of our isolates strongly indicates that they could also be promising candidates for the aforementioned application. DHA production by *Aurantiochytrium* is high even under non-standardised conditions and the levels are comparable with that of other commercial DHA producers.

In summary, the present study resulted in the isolation of 11 strains of oleaginous *Aurantiochytrium* sp. of industrial and pharmaceutical interest from different mangrove habitats of Kerala, India. All strains exhibited a wide range of tolerance to physical factors, potentially allowing large-scale production of these strains for production of important enzymes like lipase and urease. These strains accumulate fatty acids especially palmitic acid and DHA. All these strains produce a much higher level of DHA than the commercial strain *Schizochytrium* SR21. Further studies on these unique protists and standardisation of growth condition for biomass and fatty acids production should allow their utilisation as an alternative source for DHA and thus fish oil replacement in aquaculture and as a sustainable energy source for biodiesel production.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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