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Pollen baiting facilitates the isolation of marine thraustochytrids with potential in omega-3 and biodiesel production

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Abstract Marine heterotrophic microbes are capable of accumulating large amounts of lipids, omega-3 fatty acids, carotenoids, and have potential for biodiesel production. Pollen baiting using *Pinus radiata* pollen grain along with direct plating techniques were used in this study as techniques for the isolation of oil-producing marine thraustochytrid species from Queenscliff, Victoria, Australia. Thirteen isolates were obtained using either direct plating or using pine pollen, with pine pollen acting as a specific substrate for the surface attachment of thraustochytrids. The isolates obtained from the pollen baiting technique showed a wide range of docosahexaenoic acid (DHA) accumulation, from 11 to 41 % of total fatty acid content (TFA). Direct plating isolates showed a moderate range of DHA accumulation, from 19 to 25 % of TFA. Seven isolates were identified on the basis of 18S rRNA sequencing technique as *Thraustochytrium* species, *Schizochytrium* species, and *Ulkenia* species. Although both methods appear to result in the isolation of similar strains, pollen baiting proved to be a simpler method for the isolation of these relatively slow-growing organisms.

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Introduction

Thraustochytrids are monocentric protists and are ubiquitous in marine habitats and mangrove areas [30]. These marine protists are known for accumulating high levels of the omega-3 fatty acid, docosahexaenoic acid (DHA) [14, 31]. Despite their abundance in marine ecosystems, they are difficult to isolate since they are relatively slow growing compared to other marine microorganisms such as bacteria, yeast, and fungi. Faster-growing bacteria, fungi, and yeast tend to contaminate samples during the isolation process when traditional serial dilution and spread plating methods are used [37, 41]. Despite these contamination issues, microorganisms of the genus *Thraustochytrium* have been isolated from a variety of locations, from as early as the 1950s [40]. Thraustochytrids have been isolated from coastal environments such as cold temperate littoral, cool temperate littoral, temperate environment and sub-tropical mangrove areas and from different samples such as brackish and marine water, moist sediments, algae, shells and degrading mangrove leaves [4, 23, 29, 32].

We found traditional methods of isolation difficult to apply due to rapid contamination by faster-growing microbes [39]. Thraustochytrid growth can be selected for, based on their ability to metabolize lignocellulosic substrates such as pine pollen grains. Here we describe the isolation and identification of thraustochytrids isolated using a modified pine pollen baiting method [5]. We compare the use of direct plating and a pollen-baiting assisted technique for the isolation of thraustochytrids.

Direct plating involves spread-plating of sea water or inoculating the sediment onto an agar plate containing a combination of antibiotics and antifungal agents. Baiting techniques using hairs (with *Geodermatophilus obscurus*) and pollen grains (with *Actinoplanes* sp.) have been reported for the isolation of zoosporic actinomycetes [12, 17]. *Actinoplanes* sp. has been selectively isolated from pollen grains of *Pinus* sp. with the application of a chemotactic method [12]. In addition, 30 species of zoosporic fungi related to Kingdom Chromista and Kingdom Fungi have recently been isolated using different types of baits that included onion skin, corn leaves, cellophane, shrimp exoskeleton, snake skin, *Sorghum* sp. seed, some strands of blond hair from children and pine pollen grains [28]. Previously, pollen-baiting methods have been used by researchers for quantitative study of zoosporic fungi from soil and water samples [9, 24]. The pollen-baiting technique involves the use of pollen grains, such as pine pollen, which are interspersed on the surface of the water sample, again with antibiotics and antifungal agents.

Materials and methods

All chemicals used in this study were of analytical grade and obtained from Sigma-Aldrich, (Castle Hill, NSW, Australia) and Merck Chemicals (Kilsyth, VIC, Australia). For 18S rRNA sequencing of thraustochytrids, molecular biology-grade chemicals were used. The PureLink Genomic DNA Mini Kit was obtained from Invitrogen (Melbourne, VIC, Australia). The PCR clean-up kit was procured from Promega (Sydney, NSW, Australia) and the DNA sequencing kit from Applied Biosystems (Melbourne, VIC, Australia).

Location of the sampling site

Seawater samples and sediments containing degraded leaf material were collected from the temperate inter-tidal zone at Queenscliff, Victoria, Australia, during February 2011. The GPS position (S 38°16.202′, E 144°38.212′) and the temperature of the location (18–20 °C) were recorded. The samples were placed in sterile 50-ml Falcon tubes containing 500 mg l^{-1} of penicillin and streptomycin. The tubes were moderately agitated for uniform dispersal of the antibiotics. Sample tubes were placed in an ice box and transported to the laboratory and stored at 4 °C until further use.

Isolation procedure for thraustochytrids

The isolation procedure was performed within 24 h of sample collection. The collected sea water samples were processed according to different isolation techniques described below.

Direct plating technique

Seawater samples (3 ml) and sediments (1 g) were suspended in 20 ml of YP medium with the following components; yeast extract 1 g, peptone 1 g, penicillin 500 mg, streptomycin 500 mg, rifampicin 50 mg, and nystatin 10 mg in 1 l of sterile seawater. The antibiotics and antifungal agent were pre-filtered using a 0.22-µm sterile filter and immediately added to the autoclaved and cooled medium just before pouring the agar medium onto the Petri plates. A portion of the suspension culture, with appropriate antibiotic dilutions, was spread plated on YP plates containing the above-mentioned antibiotic concentration and incubated at 20 °C for 5 days. The leaf samples were washed with sterile sea water to remove any particulates, which can be a source of contaminants. The leaf sample was then inoculated onto the YP plates containing the same antibiotic concentrations. Growth on the plates was monitored at 24-h intervals. Different colonies identified by their morphology as thraustochytrid-like were taken, using a sterile loop, from the Petri dish and re-grown in a 50-ml Erlenmeyer flask with 10 ml of fresh medium. Spherical, uneven, and slimy colonies along with some translucent, orange color producing colonies were selected for further study. Pure colonies were obtained by the streak plate method.

Pollen baiting technique

The Falcon tubes containing the seawater samples were pollen baited with sterile pine pollen grains (Allife, Silverwater, NSW, Australia). The same concentrations of antibiotic and antifungal agents were added to the tubes as in the direct plating method. The tubes were incubated at 20 °C for 2 weeks. Sub-samples (10 µl) were removed from the water surface and observed under the microscope at 24-h intervals, to check for the appearance of thraustochytrid colonies growing on the pollen grains. Tubes showing a thin film over the water level were checked for contamination under a microscope and when microorganisms other than thraustochytrids were observed the tubes were discarded. Twenty-µl subsamples of thraustochytrid cells and pine pollen were spread plated on YP agar medium containing yeast extract 1 g, peptone 1 g, agar 10 g, and antibiotic and antifungal agents in 1 l of sterile seawater and incubated at 20 °C for 1 week. Thraustochytrid colonies were sub-cultured on YP agar medium containing antibiotics to obtain pure isolates.

Microscopy studies

The isolates were observed using differential interference contrast (DIC) and Nomarski microscopy (Axio-imager, Zeiss, Germany). Thraustochytrid cells were fixed on a slide and air-dried before observation under a microscope.

Fatty acid production

To determine the fatty acid profile, all isolates were cultured in sterile (autoclaved) GYP medium containing glucose 5 g, yeast extract 2 g, peptone 2 g in 1 l of 70 % artificial seawater (Instant Ocean Sea Salts, Aquarium Systems, Inc., USA). Fifty milliliters of liquid medium was inoculated with the isolates from agar plates and incubated at 20 °C with a shaking speed of 150 rpm. The cell suspension was centrifuged at 10,000 × *g* for 10 min to obtain a pellet. The thraustochytrid cell pellet was freeze-dried and stored at −20 °C before proceeding with fatty acid extraction. Results are presented as mean ± SD of samples prepared in duplicate and analyzed twice.

Fatty acid extraction and GC analysis

Lipids were extracted from freeze-dried cells (10 mg) with chloroform and methanol (2:1) [15, 16]. The sample was vortexed and centrifuged, the supernatant collected, and the solvent evaporated under a stream of nitrogen. Lipid content (% dry weight) was determined gravimetrically. Fatty acids were converted to methyl esters by acid-catalyzed trans-esterification following the method of Christie and Han [8] with minor modifications. Briefly, the lipid extract was dissolved in 1 ml of toluene, and 200 µl of internal standard (methyl nonadecanoate, 5 mg ml^{-1}) and 200 µl of butylated hydroxytoluene (BHT, 1 mg ml^{-1}) were added. To this, 2 ml of hydrogen chloride in methanol (prepared by adding 5 ml acetyl chloride dropwise to 50 ml methanol on ice) was added, the solutions mixed and incubated overnight at 50 °C. Fatty acid methyl esters (FAMEs) were extracted into hexane. The hexane layer was collected and dried over sodium sulphate. Solvent was evaporated under a stream of nitrogen. The samples were analyzed using an Agilent 6890 gas chromatograph with flame ionization detector (Agilent Technologies, Australia). The GC was equipped with a capillary column (Suplecowax 10, 30 m × 0.25 mm, 0.25-µm film thickness). Helium was used as the carrier gas at a flow rate of 1.5 ml min^{-1} (constant flow). The injector was maintained at 250 °C and a sample volume of 1 µl was injected with a 50:1 split ratio. The oven was programmed from 140 (5-min hold) to 240 °C (10-min hold) at a rate of 4 °C min^{-1}. Fatty acids peaks were identified on comparison of retention time data with external standards (Sigma-Aldrich, Australia). Peaks were quantified with ChemStation chromatography software (Agilent Technologies) and corrected using theoretical relative FID response factors [2]. Results are presented as mean ± SD of samples prepared in duplicate and analyzed twice.

DNA extraction and 18S rRNA sequencing

A 3-ml subsample of 7-day thraustochytrid culture in YP medium was centrifuged at 8,000 × *g* for 10 min at 4 °C to obtain a cell pellet. The total genomic DNA of the isolates was isolated and purified using PureLink Genomic DNA Mini Kit (Invitrogen, Australia) following the manufacturer's instructions. The extracted DNA was dissolved in 50 µl of sterile distilled water and kept at −20 °C for further use. DNA was amplified by polymerase chain reaction (PCR) on a Thermal cycler (Eppendorf Master Cycler, PA, USA). The PCR strategy was modified from Mo and coworkers [26]. The amplification of 18S rRNA gene from extracted DNA of isolate was performed with following primers: F-5′-CAACCTGGTTGATCCTGC CAGTA-3′ and R-5′-TCACTACGGAAACCTTGTTAC GAC-3′ [6]. A 25-µl PCR reaction mixture contained 200 µM of master mix (ready to use solution containing *Taq* DNA polymerase, dNTPs, $MgCl_2$, Promega) and 1.25 µM of forward and reverse primers to amplify 200 ng of DNA template. The conditions for the PCR amplification were as follows: initial denaturation (3 min at 94 °C), final denaturation (45 s at 94 °C) with annealing (30 s at 64 °C), extension at 72 °C for 2 min followed by final extension at 72 °C for 10 min. PCR was run for 30 cycles. The amplified DNA was purified from agarose gel using the Wizard PCR purification kit (Promega). DNA concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, FL, USA). The purified DNA of the isolates was sequenced using a DNA sequencer (Applied Biosystems, CA, USA).

Phylogenetic analysis

Seven potential thraustochytrids isolated in the lab were selected for molecular characterization followed by evolutionary analysis. PCR amplified 18S rRNA were sequenced and validated after BLAST analysis [3] and found to be closely related to *thraustochytrids* species. All sequences were submitted to GenBank with accession numbers JX993839–JX993845. Further 18S rRNA sequences from 22 related species were outsourced/retrieved from GenBank databases and used as reference sequences for evolutionary studies. The phylogenetic tree

was constructed using MEGA5 software [36]. The evolutionary relationship was concluded by using the maximum likelihood method based on the Kimura 2-parameter model [21]. The bootstrap consensus tree established from 1,000 replicates is taken to represent the evolutionary history of the analyzed taxa [11]. Branches analogous to partitions reproduced in less than 50 % bootstrap replicates are collapsed. Initial tree(s) for the experimental search were obtained as follows. When the number of common sites was <100 or less than one-fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch distances measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1,150 positions in the final dataset.

Effect of different carbon sources on the fatty acid profile of *Thraustochytrium* sp. BAITING3

Various carbon sources such as sucrose, galactose, fructose, β-lactose, xylose, starch, maltose, and glycerol were used to grow the BAITING3 strain, and the fatty acid profiles were analyzed. Based on having the highest DHA-to-total fatty acid (TFA) ratio, the strain BAITING3 was selected for further study. The medium composition was based on GYP medium (autoclaved); 0.5 % w/v of the carbon sources were used in 50 ml of the medium contained in a 250-ml flask. Fatty acid extraction and GC analysis was performed as detailed in the section above. Results are presented as mean ± SD of samples prepared in duplicate and analyzed twice.

Results

Thirteen thraustochytrid strains were isolated from the low-tide region of Queenscliff, Victoria using two methods of isolation; direct plating and pollen baiting. Although the media was supplemented with several broad-spectrum antibiotics and an antifungal, some bacteria and fungi were also found to grow on the agar plates. Pure thraustochytrid strains identified microscopically were obtained after serial subculturing. The selection of oleaginous strains was based on results of GC PUFA profiling.

Variously sized thraustochytrids spores (10–50 μm) were observed under the microscope, with the zoospores moving erratically in the medium. Thraustochytrid cells of varying sizes (10–50 μm) were found to be attached to the pollen surface (Fig. 1). At low magnifications (5× and 10×), these cells appeared highly reflective, indicating the presence of oils. At high magnification, a clear sporangium was observed, with progressive cleavage demonstrating that the cell was on the verge of releasing zoospores (Fig. 1c). Zoospore release is a characteristic feature of thraustochytrid cell division [38]. Direct plating resulted in the isolation of five strains (PLATING1, PLATING2, PLATING3, PLATING4, and PLATING5), whereas 8 strains (BAITING1, BAITING2, BAITING3, BAITING4, BAITING5, BAITING6, BAITING7, and BAITING8) were isolated using the pollen baiting technique.

All isolates exhibited a fatty acid profile composed primarily of saturated fatty acids (C16:0, C17:0, and C18:0), monounsaturated fatty acids (C18:1n9) and medium and long-chain PUFAs (C18:2n6, C20:4n6, C20:5n3, and C22:6n3). EPA and DHA as percentage of TFA ranged from 5.6 to 12.8 % and 11 to 41.2 % of TFA, with the coefficient of variation ranging from 0.002 to 0.4 (Fig. 2). Of all the isolates, BAITING3 (isolated from pollen baiting) was found to have the highest amount of DHA as a percentage of total oil, comprising 41.2 % of TFA. The present study is consistent with that of Burja and coworkers [6] and Huang and coworkers [19], who showed that thraustochytrids from cold water environments may accumulate DHA up to 35–50 % of TFA. BAITING3 accumulated EPA at an amount of 8.7 % of TFA. However, the highest EPA levels were recorded in PLATING5 (direct plating isolate) at 12.8 % of TFA. A recent study observed the accumulation of long chain omega-3 PUFAs in some related strains, including *Schizochytrium* sp. (29 % TFA), *Thraustochytrium* sp. (40.5–47 % TFA), *Ulkenia* sp. (52.6 % TFA), and *Aurantiochytrium* sp. (37.8–69.1 % TFA) [23].

In the current study, the fatty acid profile for omega-3 PUFAs was between 20 and 50 % TFA depending upon the strain. BAITING3 accumulated C16:0 and C18:0 at about 40 % of TFA, considerably more than the commercial strain *Schizochytrium* sp. SR21 [42]. Furthermore, BAITING7 accumulated 74.7 % of TFA as even-chain saturated fatty acids (C16:0 and C18:0). This is a relatively high amount when compared to other strains, such as the 52 % of TFA in *Aurantiochytrium* sp. [23]. DPA (C22:5n6) was found to be in the range of 6.8–17.9 % with PLATING3 accumulating the highest quantity. The odd-chain saturated fatty acid (C17:0) was found to be present in two of the isolates, with the highest in PLATING3 (4.5 % of TFA). C18:1n9 and C20:4n6 were also detected in some isolates in the range of 2.7–4.1 and 2.5–5.3 %, respectively, mainly in strains obtained from the direct plating method. Direct plating isolates accumulated moderate levels of DHA in TFA (18.7–25.1 %). The pollen-based isolates (except BAITING7 and BAITING8) showed higher DHA-to-TFA ratios when compared to the direct plating isolates (Fig. 3). A summary of the properties of these isolates is given in Table 1.

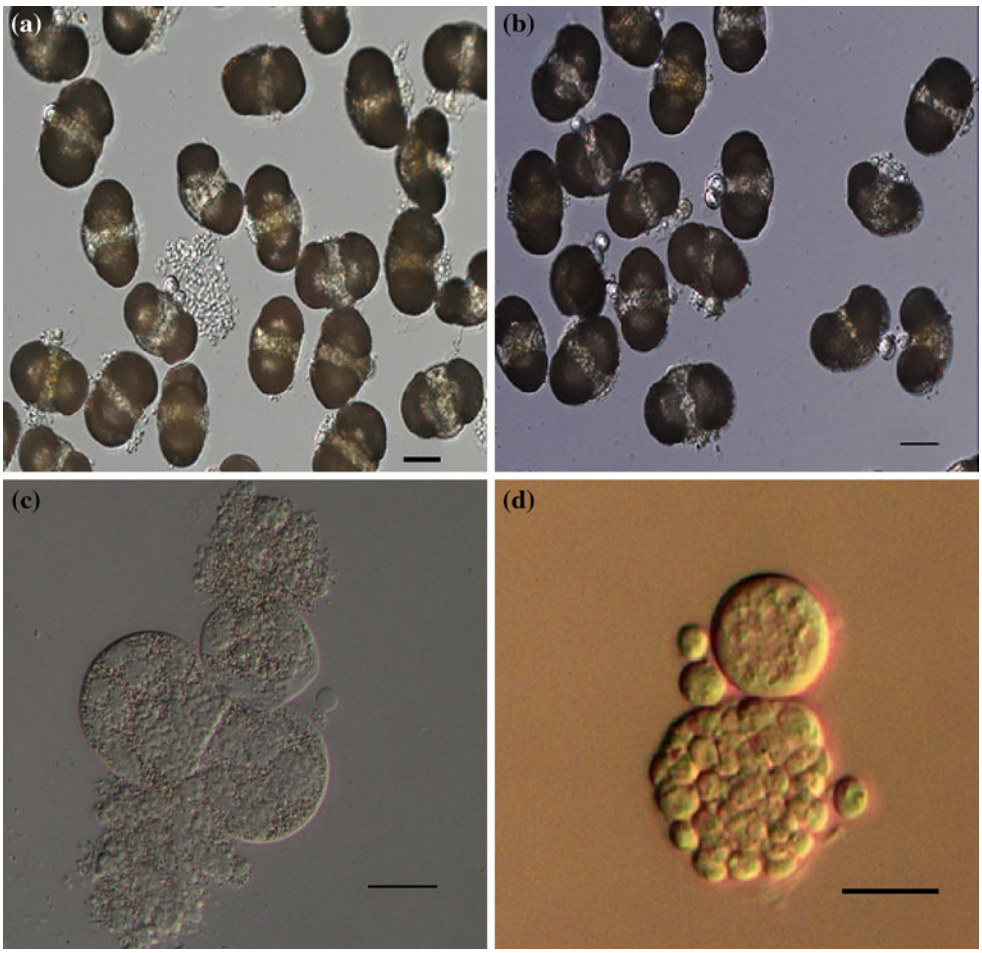

Fig. 1 Microscopic images of various marine isolates. **a**, **b** Thraustochytrid cells attached to the surface of pine pollen with released zoo spores (*scale bar* 20 μm), **c** PLATING5 mature sporangium ruptured to release zoo spores (*scale bar* 20 μm), and **d** BAITING3 (*scale bar* 20 μm)

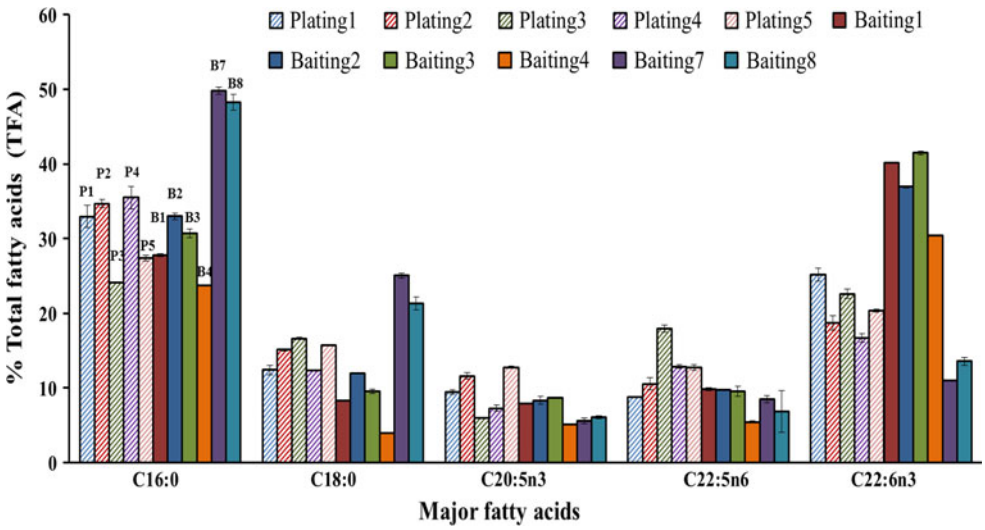

Fig. 2 Fatty acid profiles of the collected isolates. *C16:0* palmitic acid, *C18:0* stearic acid, *C20:5n3* eicosapentaenoic acid, *C22:5n6* docosapentaenoic acid, *C22:6n3* docosahexaenoic acid. The *hashed bar* indicates plating isolates and *solid bars* indicate baiting isolates

Seven isolates were identified based on 18S rRNA gene sequences and found to be closely related to other 18S rRNA sequence data from thraustochytrid strains available in the GenBank database. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Fig. 4). The isolates BAITING1, BAITING2, BAITING3, and PLATING5 were found to be closely

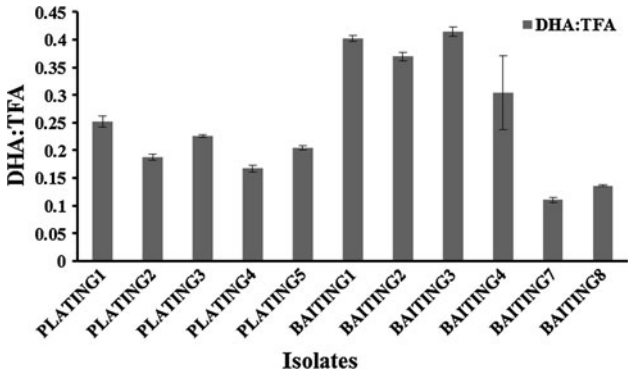

Fig. 3 Relative DHA-to-TFA productivity for each strain. Four highest DHA to TFA-producing strains were collected using the baiting technique. PLATING strains showed less DHA-to-TFA productivity when compared to BAITING strains

related to the sequence of *Thraustochytrium aureum* (GenBank accession number AB022110). These isolates were designated as *Thraustochytrium* sp. (Fig. 4). Two isolates BAITING4 and PLATING4 were evolutionarily related to *Ulkenia* sp. (GenBank accession number HQ228976 and HQ228958, respectively) and BAITING7 was found to be closely related to *Schizochytrium* sp. (GenBank accession number DQ367050) (*Schizochytrium* sp. are renamed as *Aurantiochytrium* sp.) [44].

In order to explore the potential of one of the new thraustochytrid isolates to grow on carbon sources other than glucose, BAITING3 was selected for further study. The isolate showed promising lipid content (33.3–50 %) and fatty acid profiles with the different carbon sources used, which included monosaccharides (galactose and fructose), disaccharides (sucrose, maltose, and lactose), polysaccharide (starch) and glycerol (Fig. 5). DHA was found to be in the range of 25.6–39 % of TFA with the best accumulation in medium containing glycerol. The pentose sugar xylose was also found to achieve about 30 % of DHA in TFAs. It will be interesting to expand this result in future studies. The monosaccharide and polysaccharide sugars showed about 26 % of DHA in TFA with more contribution towards accumulation of palmitic acid (~34–35 %).

Discussion

The direct plating technique involves spreading a seawater sample containing thraustochytrids on agar plates. This method may increase the level of contamination by other yeast cells and antibiotic resistant bacteria or fungi. In this research, pollen baiting acted as a selective medium predominantly for thraustochytrids. We observed that the pollen baiting method is better suited to the isolation of thraustochytrids from seawater or sediment samples [5]. It has already been observed that pollen grains from coniferous plants such as pine trees are more prone to attract thraustochytrids than those of other plants [13]. We found

Table 1 Summary of the collected strains from the Queenscliff region, Victoria, Australia, at a glance

Newly isolated strains	Colony description		TFA (% of dry weight)	% TFA		DHA:EPA
	Sporulation/morphology	Cell size (μm)		C20:5n3	C22:6n3	
Pollen baiting						
B1*	Vegetative, spherical	~30	27.8 ± 1.8	7.9 ± 0.06	40.2 ± 0.05	2.7
B2*	Vegetative, spherical	~30	28.6 ± 1.9	8.3 ± 0.6	36.9 ± 0.2	1.6
B3*	Vegetative, spherical	~30	29.3 ± 2.1	8.7 ± 0.05	41.5 ± 0.3	3.8
B4*	Vegetative, spherical	~20	24.4 ± 2.2	5.1 ± 0.01	30.4 ± 0.2	2.3
B7*	Cluster of zoospores released, spherical	~25	23 ± 0.2	5.6 ± 0.4	11.03 ± 0.1	1.6
B8	Cluster of zoospores released, spherical	~25	19.9 ± 0.3	6.1 ± 0.2	13.6 ± 0.6	5.1
Plating						
P1	Vegetative, spherical	~20	20.5 ± 0.3	11.6 ± 0.45	18.8 ± 0.97	4.4
P2	Vegetative, spherical	~20	19.7 ± 1.7	9.5 ± 0.28	25.2 ± 0.8	4.8
P3	Vegetative, spherical	~50	23.5 ± 3.2	5.9 ± 0.07	22.6 ± 0.6	6.0
P4*	Vegetative, spherical	~20	18.1 ± 1.7	7.3 ± 0.4	16.7 ± 0.6	2.0
P5*	Vegetative, spherical	~25	24.9 ± 1.9	12.8 ± 0.1	20.4 ± 0.2	2.2

Thirteen strains were isolated, out of which 11 strains were selected for fatty acid production and seven potential strains (*) were identified through 18S rRNA sequencing with GenBank accession numbers as mentioned in the text. The strains were grown in a medium containing glucose 5 g, yeast extract 2 g, peptone 2 g in 1 l of 70 % artificial seawater. Where *B* BAITING, *P* PLATING, *C20:5n3* EPA, *C22:6n3* DHA

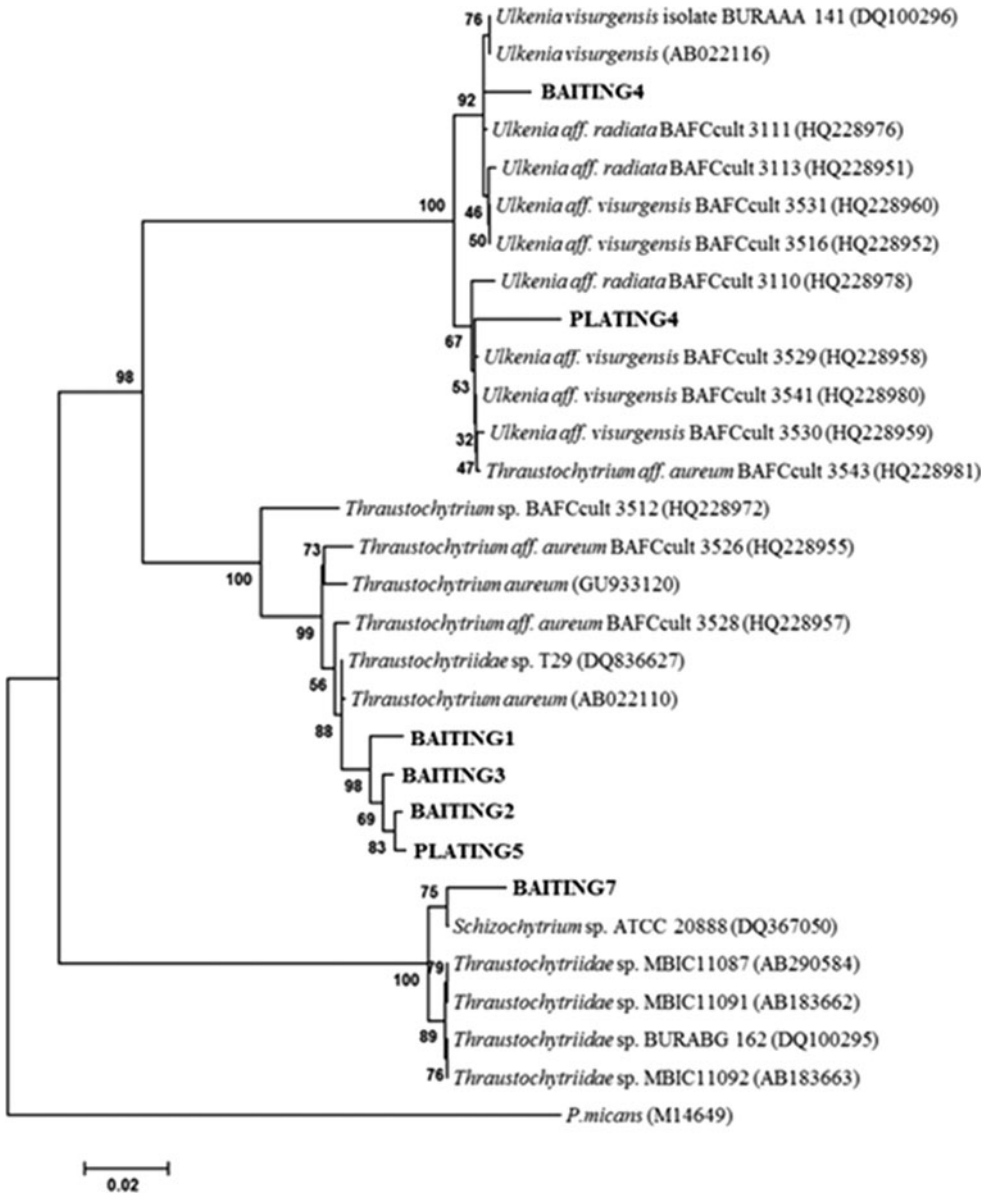

Fig. 4 Molecular phylogenetic analysis by maximum likelihood method showing a close relationship between the isolates and other selected sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches

that pine pollen grains acted as specific substrate where thraustochytrids were found to readily attach.

Pine pollen is a rich source of nutrients and includes carbohydrates in the form of starch (44 %) and sugars (4–10 %), protein (6–28 %), amino acids (14–22 %), lipids (1–20 %), flavonoids (2.5 %), and trace amounts of vitamins and sterols [20]. Specific major components include stearic acid, palmitic acid, ursolic acid, flavonoids (isorhamnetin glycoside, narcissin, and luteolin), pentacosane, sitosterol, and 6-amino purine. The reason for the effectiveness of pollen baiting has been proposed by Hayakawa and coworkers [17], who postulated that the pollen grains are responsible for the release of saccharides and proteins that may attract the zoospores of actinomycetes. A similar theory may be applied to the attraction of thraustochytrid zoospores towards the pollen grains. Recently, a revised method using pollen grains from sweet gum (*Liquidambar* sp.) and brine shrimp larvae as bait has also been reported for the isolation of thraustochytrids [32].

DHA levels ranged from 11 to 41.2 % for the pollen-based isolates. A commercial strain, *Schizochytrium limacinum* SR21, exhibited 30 % DHA of TFAs as reported [27, 43]. Also, the saturated fatty acids, especially C16:0 (49.7 %) and C18:0 (25.1 %), were produced at their highest quantity by one of the pollen-based isolates. The findings of this study are in agreement with the observations of Bowles and coworkers [4], in which isolates from temperate regions produced a high level of DHA (as percentage of TFA) with relatively low biomass production. Low growth rates in new strains of thraustochytrids were also observed in isolation studies from marine samples in Argentina [32].

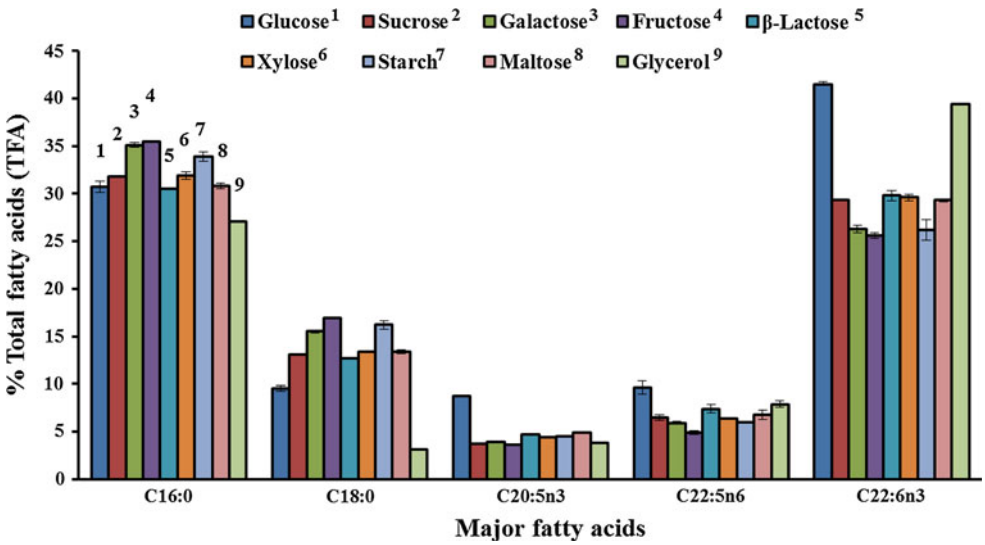

Fig. 5 Effect of carbon sources on the fatty acid profile of *Thraustochytrium* sp. BAITING3

The lipid-rich biomass produced in the current study can be considered as an alternative substrate for biodiesel production in oleaginous microorganisms such as microalgae, yeast, bacteria, and fungi [7, 25] and also for aquaculture (as feed for fish and aquatic invertebrates) [34]. Strains that produce low levels of omega-3 fatty acids and higher levels of monounsaturated and saturated fatty acids are potential candidates for use in biodiesel production. The oxidative instability of omega-3 fatty acids make them unsuitable for biodiesel. Specifically, the presence of about 75 % of TFA in two isolates, BAITING7 and BAITING8, as saturated fatty acids (C16:0 and C18:0) indicates that these strains may have a good fatty acid profile for use in biodiesel generation. PUFAs, due to their propensity to undergo oxidation, are not suitable for use in biodiesel, whereas, saturated fatty acids are better for this application [10, 22]. Strains accumulating larger amounts of DHA are potentially useful for the production of this PUFA for food and nutritional supplement applications, including infant formula [35]. Strains that produce oil with a higher EPA-to-DHA ratio have potential for addition to food, where both EPA and DHA are desirable. If the EPA and DHA can be selectively concentrated from the oil, then these PUFA can constitute a value-added co-product, with the remaining oil being useful for biodiesel. Carotenoids could also be selectively isolated as a value-added co-product, further reducing the cost of biodiesel production using these organisms.

The presence of arachidonic acid (C20:4n6) was found to be significant in identifying any strain as closely related to *Schizochytrium aggregatum* [19]. Arachidonic acid accumulation was observed in isolates BAITING1, BAITING2, and BAITING3. However, in developing a chemotaxonomic grouping method (A to H) based on fatty acid and sterol profiles of 36 strains in order to minimize the difficulty of distributing the strains based solely on fatty acid profiles, Lee Chang and coworkers [23] found a close relationship between chemotaxonomic and phylogenetic grouping of most of their 36 thraustochytrid strains. Though there was always a disagreement with the classification of thraustochytrid strains, with recent developments in genetic identification techniques and chemotaxonomy, an evolutionary relationship can be established [18, 44].

The evaluation of the new isolate, BAITING3 to grow on other carbon sources such as glycerol based on oil content and fatty acid profile was an interesting outcome. Thraustochytrids have been found to grow on different carbon sources such as glucose, fructose, galactose, sucrose, and others [33]. Glycerol has recently been used in the fermentation medium for exhibiting thraustochytrids growth [1, 15]. Although thraustochytrids can utilize monosaccharides resulting in a good DHA yield [6], xylose (pentose sugar) was found to act better than fructose, galactose (monosaccharide), and starch (polysaccharide) in terms of DHA profile in the TFAs produced. Fructose, galactose, and starch were found to enhance the palmitic acid in the fatty acid profile (Fig. 5). The utilization of a pentose sugar by one of the isolates in this study is an important observation that may lead to the metabolism of lignocellulosic biomass for DHA production.

In summary, pollen baiting and direct plating techniques were both found to be effective in isolating thraustochytrids from marine samples. However, pollen baiting provided a simpler method than direct plating for the isolation of thraustochytrids from complex samples.

Highlights

1. New thraustochytrid strains have been isolated from Queenscliff, Victoria, Australia.

2. Pine pollen baiting provides a simpler method than direct plating for the isolation of thraustochytrids.
3. One of the isolates (BAITING3) can grow on a variety of carbon sources.

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