Research Article

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Employing marine invertebrate cell culture media for isolation and cultivation of thraustochytrids

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published online October 20, 2021**Keywords:** Ascidiars; cultivation; Labyrinthulomycetes; marine invertebrates; PUFA.

Abstract: Thraustochytrids, a common group of marine eukaryotic protists, have drawn considerable scientific and industrial interest due to their ability to synthesize high levels of bioactive compounds, including polyunsaturated fatty acids, docosahexaenoic acid, squalene and carotenoids, and their new applications for biofuels. The pharmaceutical and industrial potential of thraustochytrids necessitate effective isolation of new strains and establishment of axenic cultures. To date, existing isolation protocols have used baiting and direct plating methods to generate axenic cultures with varied media compositions that contain peptone and yeast extracts as nitrogen sources, glucose as carbon source, seawater and antibiotics. Here we reveal a new approach for the isolation of thraustochytrids from tissues of marine invertebrates using (a) primary cell cultures in a liquid medium containing basal medium, 50% artificial seawater, vitamins, proteins and antibiotics, and (b) cultivation in the same cell culture medium. Using the colonial tunicate *Botryllus schlosseri* as a model system, thraustochytrid cells thrived in the medium from the day of extraction, grew and proliferated for the next five weeks (five-passages, up to 1.9×10^6 cells ml^{-1} in passage 5; 1.45-fold multiplication week^{-1}). This new approach for isolation and cultivation of axenic thraustochytrid cultures enables the isolation of new species with promising bioactive compounds.

1 Introduction

Polyunsaturated fatty acids (PUFAs) have been recognized as highly valuable for a wide range of health benefits in adults and infants (Calder and Yaqoob 2009; Huang et al. 2001; Morabito et al. 2019; Zárte et al. 2017). The current primary source of essential dietary PUFAs is through the consumption of oily fish, such as salmon, sardines, tuna, and herring, and the ingestion of their processed oils (Calder and Yaqoob 2009), which are ultimately derived throughout the food chain from various microorganisms (Burja et al. 2007; Calder and Yaqoob 2009; Nham Tran et al. 2020). One group of such microorganisms is the thraustochytrids, marine protists that produce large quantities of PUFA, docosahexaenoic acid (DHA), squalene and carotenoids (Burja et al. 2006; Huang et al. 2003; Jakobsen et al. 2007, 2008; Nham Tran et al. 2020). The thraustochytrids have been further explored as new source materials for biofuels and for lipid biofactories, two emerging fields of industrial biotechnology (Gupta et al. 2012; Nham Tran et al. 2020).

Thraustochytrids represent an eukaryotic group of unicellular protists within the class Labyrinthulomycetes of the kingdom Chromista, and include >12 genera of which the most studied are *Aplanochytrium*, *Schizochytrium*, *Ulkenia*, *Japonochytrium*, and *Thraustochytrium* (Bongiorni 2012; Lyu et al. 2021; Nham Tran et al. 2020; Raghukumar 2002). They are found throughout the world in estuarine and marine habitats, and feed as saprobes, as parasites or as bacterivores, while generally found in association with organic detritus, decaying algal and plant material, and in sediments (Lyu et al. 2021; Nham Tran et al. 2020). Traditionally, thraustochytrids were isolated from a variety of marine sources such as sediments, fallen mangrove leaves, seagrasses, algae and coastal waters (Jain et al. 2005; Lyu et al. 2021; Yokochi et al. 2001). Porter (1990) described the baiting isolation approach performed by using sterile pollen grains (from pine or maple)

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suspended in a basic liquid nutrient culture medium for several days, followed by the removal of single pollen particles with their attached thraustochytrids and their subsequent inoculation onto an agar medium. The second common approach is the direct isolation of thraustochytrids from substrata such as decaying animal and plant materials, animal mucus, sediments, and water, usually directly plated on agar, and then inoculation of emerged thraustochytrid colonies into various media (Bockelmann et al. 2012; Burgaud et al. 2009; Hinzpeter et al. 2009; Lyu et al. 2021). In parallel, research efforts have focused on the development of basal media for thraustochytrids in solid or liquid forms (Burja et al. 2006; Jakobsen et al. 2007; Lyu et al. 2021). The basic components in all media generally consist of polypeptone (P), and yeast extract (Y) as nitrogen sources, and agar (A) or glucose (G) as the carbon source. Any combination of these basal media (PYA or GPY) is supplemented with antibiotics (most commonly streptomycin sulfate, penicillin, ampicillin, kanamycin sulfate and rifampicin), and diluted with sterile water, sterile natural or artificial seawater (ASW) with up to ~50% salinity (Lyu et al. 2021).

Many yet undescribed strains of thraustochytrids were found on and within tissues of marine organisms such as sponges, corals, hydroids, bivalves, octopus, squids, nudibranchs, echinoids and tunicates (Bower 1995; Cousserans et al. 1974; Mo et al. 2002; Nham Tran et al. 2020; Porter 1990; Raghukumar 1988; Raghukumar and Balasbramanian 1991). These strains are generally missed by the baiting and direct plating isolation approaches used, and to date culturing conditions have not been optimized for their continuous growth (Rinkevich 1999). However, some marine invertebrate primary cell cultures have supported the proliferation of thraustochytrids originating from sponges, cnidarians, crustaceans, mollusks, echinoderms and tunicates (Awaji 1997; Blisko 1998; Frank et al. 1994; Ilan et al. 1996; Rabinowitz et al. 2006). In primary cultures, these strains thrived up to one month from inoculation, following the appearance of opportunistic forms like yeast and bacteria (Awaji 1997; Blisko 1998; Ellis and Bishop 1989; Ermak and Odintsova 1996; Frank et al. 1994; Hsu et al. 1995; Ilan et al. 1996; Kawamura and Fujiwara 1995; Kaneko et al. 1995; Rinkevich and Rabinowitz 1993, 1994, 1997; Rabinowitz et al. 2006), results that point to the necessity of developing aseptic conditions in such primary cultures for improved thraustochytrid isolation and cultivation. Here, we present a new approach for the isolation and cultivation of thraustochytrids from marine invertebrate tissues using the model colonial tunicate *Botryllus schlosseri* (Pallas 1766).

2 Materials and methods

2.1 Washing solution

Washing solution was used to reduce bacterial contaminants during cell isolation. Washing solution was made with artificial seawater (ASW) that was prepared as described in Rabinowitz and Rinkevich (2003), autoclaved, sterilized by 0.2 µm filter membrane (Millipore) and stored at room temperature. For each 50 ml washing solution, we used 44 ml of ASW, supplemented with 3 ml of PSA (Biological Industries; Penicillin 10,000 units ml⁻¹, Streptomycin sulphate 10 mg ml⁻¹ and Amphotericin B 25 µg ml⁻¹; Cat. 03-033-1B) and 3 ml of Gentamycin Sulfate (Biological Industries; 50 mg ml⁻¹; Cat. 03-035-1).

2.2 Isolation of thraustochytrids

Thraustochytrids were collected aseptically from nine colonies of the colonial ascidian *Botryllus schlosseri*, all at blastogenic stage C (detailed description on asexual stages in Voskoboinik et al. 2007 and Manni et al. 2019). Colonies originated from laboratory stocks at the National Institute of Oceanography (Haifa, Israel) and were maintained in aquaria with a running seawater system in a cooled room of 20 °C (12:12 light:dark regimen) as specified (Rinkevich and Shapira 1998). The blood vessels of each colony were punctured under aseptic conditions and the circulating blood cells were collected into 15 ml tubes with large volumes (~5 ml) of washing solution. Prior to culture incubation, the collected *B. schlosseri* blood cells were washed 5 times with washing solution followed by centrifugation (Eppendorf, Hamburg, Germany) of 10 min at 1000 g and incubated at 20 °C.

2.3 Medium

Thraustochytrid cultures were maintained in a liquid growth medium containing 21.25 ml DMEM/F-12[HAM] 1:1 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, Cat. 01-170-1A), 21.25 ml ASW, 5 ml Fetal Bovine Serum (European grade; Cat. 04-007-1A), 0.5 ml L-Glutamine (Cat. 03-020-1B), 0.5 ml HEPES buffer solution 1 M (Cat. 03-025-1B), 0.5 ml PSA, 0.5 ml Gentamycin Sulfate and 0.5 ml Sodium Pyruvate solution (Cat. 03-042-1B). In addition to sterilized conditions during preparation, the medium was filtered through 0.2 µm membrane (Millipore) and stored at 4 °C. All components, except ASW, were purchased from Biological Industries, Kibbutz Beit-HaEmek, Israel.

2.4 Culture conditions

Attempts were made to maintain pathogen-free conditions during cultivation. In addition to sterilization by filtering (0.2 µm) of the medium before use, glassware was autoclaved and only sterilized plasticware was used. Three experiments were performed and held for 42 days, each containing isolated *B. schlosseri* blood cells originating from three colonies (total nine plates from nine colonies). Each of the isolated *B. schlosseri* blood cell cultures was maintained in a 35 mm Petri dish (Greiner bio-one, CELLSTARR, Petri-dish 35 × 10 mm) containing 3 ml of liquid medium and incubated

(Incubator-Leec; Colwick Industrial Estate, Nottingham NG42AJ, England) at 20 °C under normal atmosphere conditions. The medium was changed every other day. Once an axenic thraustochyrid culture was established, the cells were sub-cultured (passaging), divided into new plates and fed with fresh medium. The contents of each plate were collected into a 15 ml tube, washed 3 times with fresh medium, followed by centrifugation (at 1000 g for 10 min) and seeded to new plates with fresh medium.

2.5 Observations, cell counting and fluorescence staining

All cultures were observed once every other day where cells were counted using a hemocytometer and photographed under the microscope (Olympus inverted system microscope, model Ix70, equipped with DP73 camera). Cell viability was determined using Trypan Blue Solution (Biological Industries; Cat. 03-102-1B). In order to confirm the presence of thraustochytrids, cultures were sampled and cells were stained with fluorescent dye Hoechst 33342 (cat: 62249, Thermo Fisher) for nuclei staining. The stained cells were observed and photographed under an epifluorescence microscope with UV excitation filter (360–370 nm). Viable cell fold increase was calculated at the end of each specific passage (average of three plates $\pm$ SD) divided by the viable cell number at onset of that passage (average of three plates $\pm$ SD).

2.6 DNA extraction and PCR conditions

Cell cultures were centrifuged at 1000 g for 10 min at room temperature and DNA extracted as described by Mo and Rinkevich (2001). DNA samples were subjected to a polymerase chain reaction (PCR) protocol optimized for the recovery of thraustochyrid 18S rDNA profiles using three sets of primers (A1, A2 and A3) following Mo et al. (2002). PCR amplifications were carried out using an Eppendorf Mastercycler gradient (Germany) and products visualized on 1.5% agarose gel.

3 Results

3.1 Characteristics of cell culture initiation

Blood cells were collected from nine *Botryllus schlosseri* colonies (Figure 1a and b). At onset (day 0), heterogeneous cell populations ($1.16 \times 10^6 \pm 0.18$ cells ml⁻¹) were obtained, revealing *B. schlosseri* and clearly visible thraustochytrid cells growing side by side in suspension with *Botryllus* cells (Figure 1c and d), followed by an increase in thraustochytrid cell numbers by 24 h from onset. At 8 d from onset, thraustochytrid cells dominated the cultures (Figure 1d and f), leaving debris of dead *B. schlosseri* cells. The thraustochytrid cell types revealed both vegetative and amoeboid cell forms (Lyu et al. 2021; Morabito et al. 2019). The vegetative cells included

mononucleated cells (5–10 μ m; Figure 2a and b), multinucleated cells (2 up to 30 nuclei cell⁻¹ 10–50 μ m; Figure 2c and d) and sporangia (20–200 μ m; Figure 2e and f), while the amoeboid mononucleated cells appeared as cell clusters, each composed of at least 3 cells, reaching sizes of up to 200 μ m cluster⁻¹ (Figure 2a). Zoospores or gametes were not observed. During this period *B. schlosseri* cells decreased by >95%, from $1.16 \times 10^6 \pm 0.2$ cells ml⁻¹ at onset to $0.05 \times 10^6 \pm 0.04$ cells ml⁻¹ at day 8 (Figure 3a), and the number of thraustochytrid aggregates that could be accurately counted increased from 7.2 ± 3.9 at onset to 72.8 ± 9.1 at day 8 (Figure 3b). At day 8, all cultures contained thraustochytrid aggregates and single cells including mononucleated, multinucleated and sporangia (Figure 1f).

Figure 1: Primary cell cultures from *Botryllus schlosseri* circulating cells. a, b: Anterior and posterior views, respectively, of a *B. schlosseri* colony from which the cells were obtained. Peripheral blood vessels (white arrows) were punctured and the cells were collected aseptically for cultivation. Scale bars = 1 mm. c, d: Cultures at onset; heterogeneous and mixed cell suspensions with *B. schlosseri* blood cells (white arrows) and few thraustochytrid cells (blue arrows). e: 24 h later, cultures show debris of dead *B. schlosseri* cells (black arrows) and thraustochytrid cells (blue arrows). f: Eight days from initiation, thraustochytrid cells (singles and aggregates) outnumbered the *Botryllus* cells, taking over the cultures. Scale bars for c–f = 100 μ m.

Figure 2: Thraustochytrid cell morphologies in developing cultures. a: A typical cell aggregation with 12 mononucleated cells, surrounded by two single cells and a single bi-cell aggregation. b: Single mononucleated cell stained with Hoechst for nucleus staining. c: A single multinucleated cell containing four nuclei. d: A single multinucleated cell stained with Hoechst, revealing 30 nuclei. e: Small and large sporangia. f: A single sporangium cell stained with Hoechst. Scale bars = 20 µm.

Figure 3: *Botryllus schlosseri* and thraustochytrid cells in primary cultures during the first 8 days from initiation. a: *B. schlosseri* viable cells ($\times 10^6$ cells ml^{-1}). Points are averages ($\pm$ SD) of three experiments. Trend line has equation $y = -0.1278 \times 1.1493$ with $R^2 = 0.9491$. b: Thraustochytrid aggregates per plate for three experiments. Each column is average of three plates ($\pm$ SD).

3.2 Molecular identification

PCR amplifications of 18S rDNA of the thraustochytrid axenic cultures (day 18; $n = 3$) were performed using three sets of primers. The amplifications revealed the existence of the undescribed strain Thraustochytriidae sp. BS2 (100% identity; GenBank accession number AF257315.2) (Mo et al. 2002).

3.3 Thraustochytrid axenic subculture

Axenic cultures of thraustochytrid cells ($n = 9$) were established (91.4–97.1% viable cells) after 8 days post cell

extraction. Then, the cultures were split on a weekly basis, while the medium was replaced every other day. During the subsequent 5 weeks, five passages (P1–P5) were performed. Starting from P1 (9–14 days from onset) the thraustochytrid cells grew in suspensions as single cells or aggregates, each 10–150 µm in size (Figure 4a and b). The aggregates were composed of sporangia and mononucleated cells and the single cells were composed of sporangia, mononucleated and multinucleated cells (Figure 4b). Thraustochytrid cells for P1 plates ($0.12 \times 10^6 \pm 0.12$ cells ml^{-1} ; Figure 4k) were counted on days 10, 12 and 14, revealing $0.05 \times 10^6 \pm 0.01$, $0.07 \times 10^6 \pm 0.01$ and $0.25 \times 10^6 \pm 0.13$ cells ml^{-1} , respectively (Figure 4l). At P2 (15–21 days from onset; $0.64 \times 10^6 \pm 0.17$ cells ml^{-1} ; Figure 4k), the thraustochytrid

suspension was composed of single cells (mononucleated, multinucleated and sporangia) and aggregates (varied 10–300 μm ; Figure 4c) of sporangia and mononucleated cells (Figure 4c and d). The thraustochytrid cells for P2 plates were counted on days 16, 18 and 20, revealing $0.43 \times 10^6 \pm 0.09$, $0.72 \times 10^6 \pm 0.06$ and $0.76 \times 10^6 \pm 0.08$ cells ml^{-1} , respectively (Figure 4l). At P3 (22–28 days from onset; $0.92 \times 10^6 \pm 0.11$ cells ml^{-1} ; Figure 4k), the suspensions were composed of single cells and aggregates (different sizes that varied between 100 to about 500 μm ; Figure 4e) of sporangia and mononucleated cells (Figure 4e and f). The single sporangia reached sizes of up to 200 μm (Figure 4f). Thraustochytrid cells for P3 plates were counted on days 22, 24, 26 and 28, revealing $0.84 \times 10^6 \pm 0.09$, $0.89 \times 10^6 \pm 0.05$, $0.89 \times 10^6 \pm 0.05$ and $1.06 \times 10^6 \pm 0.12$ cells ml^{-1} , respectively (Figure 4l). At P4 (29–35 days from onset; $1.28 \times 10^6 \pm 0.2$ cells ml^{-1} ;

Figure 4k), the suspensions were made of single cells (mononucleated, multinucleated and sporangia) and few aggregates (Figure 4g and h). The pentagonal, hexagonal, heptagonal and octagonal single cell shapes (Figure 4h) were probably the results of high cell densities. Thraustochytrid cells for P4 plates were counted on days 30, 32 and 34, revealing $1.06 \times 10^6 \pm 0.15$, $1.3 \times 10^6 \pm 0.03$ and $1.5 \times 10^6 \pm 0.06$ cells ml^{-1} , respectively (Figure 4l). At P5 (36–42 days from onset; $1.67 \times 10^6 \pm 0.12$ cells ml^{-1} ; Figure 4k), the suspensions were made of single cells (mononucleated and sporangia; Figure 4i) and few aggregates (Figure 4i and j). The single cells appeared as pentagonal, hexagonal, heptagonal and octagonal shapes, as in P4 (Figure 4j). Thraustochytrid cells for P5 plates were counted on days 36, 38, 40 and 42, revealing $1.48 \times 10^6 \pm 0.02$, $1.62 \times 10^6 \pm 0.02$, $1.67 \times 10^6 \pm 0.03$ and $1.90 \times 10^6 \pm 0.01$ cells ml^{-1} , respectively (Figure 4l).

Figure 4: Five weeks cultivation of thraustochytrid axenic cultures, including five passages (P1–P5). a, b: Cell cultures at day 12 from initiation, P1. Thraustochytrid cells phenotypes appear as single cells and aggregates. c, d: Day 18, P2. Single cells and aggregates. e, f: Day 26, P3. Single cells and aggregates. g, h: Day 32, P4. Pentagonal, hexagonal, heptagonal and octagonal shapes of single cells. Aggregates observed as well but not counted. h1: Enlargement of Figure 4h, showing details of cell structures. i, j: Day 40, P5. Single cells with circular, pentagonal, hexagonal, heptagonal and octagonal shapes. Aggregates observed as well but not counted. k, l: Five weeks growth pattern of thraustochytrids under the current conditions. k: Thraustochytrid cells ($\times 10^6$ cells ml^{-1}) at the five weekly passages. Data are averages $\pm$ SD for a specific passage (3–4 counts per passage). l: Cell numbers ($\times 10^6$ cells ml^{-1}) for thraustochytrid cultures, 10–42 days from initiation. P1–P5 represent passage numbers. Data are averages of three experiments $\pm$ SD Scale bars for a–j = 100 μm .

3.4 Long term cultivation

Fold increase of thraustochytrid cells was evaluated for the five passages (P1–P5) to establish preferred long-term culture conditions for thraustochytrid growth. Fold increase values were: 4.75 ± 2.25 for P1 and 1.82 ± 0.5 , 1.28 ± 0.3 , 1.41 ± 0.2 and 1.3 ± 0.01 , for P2, P3, P4 and P5, respectively, summarizing a weekly average increase of 1.45 fold.

4 Discussion

Over the past two decades, isolation methods such as direct plating and pollen baiting (Mariana Rosa et al. 2011) have helped to identify marine thraustochytrids that are capable of synthesizing large quantities of PUFAs and other compounds, making them of considerable industrial and scientific interest (Burja et al. 2006; FioRito et al. 2016; Huang et al. 2003; Jain et al. 2005; Jakobsen et al. 2007, 2008; Lyu et al. 2021; Nham Tran et al. 2020; Yokochi et al. 2001). The direct plating method for thraustochytrid isolation has been successfully applied on sediment samples (Gupta et al. 2012), seawater (Ueda et al. 2015) and decaying mangrove leaves and debris (Fan et al. 2002), and is based on direct plating of thraustochytrids onto solid nutrient media, supplemented with antibiotics (Lyu et al. 2021; Mariana Rosa et al. 2011). These solid media contain mostly glucose (carbon source), yeast extract and peptone (nitrogen sources), agar and seawater. The pollen baiting methodology was used for isolation of thraustochytrids from seaweeds, mangroves and plant debris (Damare 2015; Gao et al. 2013; Mariana Rosa et al. 2011; Wang et al. 2019; Wilkens and Maas 2012) and is based on collections of the pollen baits with the attached thraustochytrids (one of the few marine microorganisms that is able to feed on pollen nutrients) all inserted in nutrient free seawater, transplanting them into media for cultivation.

Here we developed and tested a new approach for the isolation and cultivation of thraustochytrids from tissues of marine invertebrates via the establishment of primary host cell cultures. This approach is further based on previous studies revealing the spontaneous proliferation of thraustochytrids in primary cell cultures from a wide range of marine organisms (sponges, corals and tunicates), where many long-term cultures were contaminated by thraustochytrids (Grasela et al. 2012; Rabinowitz et al. 2006; Rinkevich 1999). These primary cell cultures were supported by a medium containing a basic medium supplemented with vitamins, proteins and antibiotics. Working

on primary cultures from extirpated buds of *B. schlosseri*, Rabinowitz et al. (2006) recorded a lag period of 1–4 weeks from culture initiation until thraustochytrids became discernable in cultures, a significantly longer period than observed in this study. While in the former study the media were replaced once a week, and the initial density of animal cells was approximately 10^4 cells well⁻¹, we show here that, by changing the medium every other day and by initiating the cultures with about two orders of magnitude higher numbers of extracted cells (10^5 – 10^6 cells ml⁻¹), thraustochytrids were observed as of onset and multiplied by 1.45 folds/week, at least for the first 5 weeks following initiation.

The current study thus demonstrates that improved cell culture media can be used for thraustochytrid isolation from tissues of marine organisms and thereafter, can be used to sustain proliferating thraustochytrid cultures. This new approach, tested on *B. schlosseri* primary cell cultures may increase the thraustochytrid strains/species available for research into various industrial and pharmaceutical applications.

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