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Enhancement of harvesting efficiency and polyunsaturated fatty acid-rich lipid production of *Aurantiochytrium* sp. SW1 by co-cultivation with oleaginous fungus *Cunninghamella bainieri* 2A1

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ABSTRACT

Aims: Thraustochytrids have been shown to be excellent lipid producers due to their ability to accumulate over 50% lipid (g/g biomass) containing up to 50% docosahexaenoic acid (DHA). However, efficient and cost-effective cell recovery of lipid-rich biomass has become a significant challenge at the industrial scale. In this study, we attempted to enhance the harvesting efficiency (HE) and the DHA content of *Aurantiochytrium* sp. through co-cultivation with a γ-linolenic acid (GLA)-producing oleaginous filamentous fungus, *Cunninghamella bainieri* 2A1.
Methodology and results: A 72 h old *C. bainieri* 2A1 culture in the form of loose mycelia or pellets of various sizes was added into 72 h old *Aurantiochytrium* sp. cultures and further incubated for 48 h. The HE of *Aurantiochytrium* sp. was then determined by comparing the remaining OD values of the supernatant with and without minimal centrifugation at 4000× *g*. Results showed that 63.23% of HE was achieved without centrifugation from co-cultivation with dispersed mycelia. Higher HE between 96.71-99.55% was achieved when centrifugation was implemented, with the highest value resulting from co-cultivation with dispersed mycelia. These are higher than HE of centrifuged control cultures (80%) consisting of *Aurantiochytrium* sp. monocultures, suggesting that co-cultivation with *C. bainieri* 2A1 facilitates the recovery of *Aurantiochytrium* sp. cells. Moreover, the co-cultivation also resulted in a 28% increase in DHA compared to non-optimized cultures.
Conclusion, significance and impact of study: This study provides the first evidence of enhancement in harvesting and DHA content of oleaginous thraustochytrids that could be achieved through co-cultivation with oleaginous fungi.

Keywords: Aurantiochytrium, bioflocculation, co-cultivation, *Cunninghamella*, harvesting

INTRODUCTION

The application of oleaginous microalgae and thraustochytrids has attracted researchers and industries, especially those related to producing highly valued microbial lipids rich in polyunsaturated fatty acids. Docosahexaenoic acid (DHA, C22:6n3), an ω-3 polyunsaturated fatty acid, is essential for the proper physiological functions and development of humans, most notably for the brain and eye development of infants (Fedorova-Dahms *et al.*, 2011). It is also crucial in the treatment and prevention of various human diseases such as cardiovascular risk, blood pressure and psychiatric disorders (Kris-Etherton and Hill, 2008). Hence, the inclusion of 300 mg DHA/day has been recommended by Wong (2020). Thus, nutritional supplementation in the form of fish oil, widely known as the most abundant source of DHA, becomes essential. However, there are some issues associated with fish oil, such as environmental toxins due to seawater pollution with mercury and arsenic, odour and stability problems (Mahaffey *et al.*, 2008; Shah *et al.*, 2009). Furthermore, overfishing activities to meet the expanding demand for DHA have also been reported to lead to the extinction of wild fatty fishes. Moreover, Colombo *et al.* (2020) predict that by 2100, 96% of the world's population will lose DHA resources due to rising seawater temperatures caused by global warming. Therefore, the quest for alternative sources of DHA becomes essential and studies have

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shown that thraustochytrids, such as *Aurantiochytrium* sp. being one of the most potential.

Aurantiochytrium sp. has been shown to have the ability to accumulate over 50% (w/w biomass) of lipid-containing 50% DHA (of total fatty acids) (Manikan *et al.*, 2015). However, the high lipid content of *Aurantiochytrium* sp. has become one of the major issues for efficient cell recovery, which will directly influence the cost of DHA production. Several studies have reported that the harvesting process accounts for almost 20% to 30% of the total production cost (Danquah *et al.*, 2009; Christenson and Sims, 2011; Rawat *et al.*, 2011; Salim *et al.*, 2011). Various approaches have been utilized in the cell recovery of thraustochytrids, such as centrifugation, filtration, gravity sedimentation, flotation and flocculation. However, none of these approaches combines the techno-economic feasibility factors such as harvesting efficiency (HE), costs and energy efficiency. Although highly effective, centrifugation is not energy efficient and expensive, whereas filtration is suitable for larger cells but still suffers issues of clogging and blocking of the pores (Branyikova *et al.*, 2018). Thus, a potential chemical-free alternative for efficient recovery of cells and consuming less energy is co-cultivation (Yuan and Xie, 2012; Wrede *et al.*, 2014; Kim *et al.*, 2015).

Co-cultivation without chemical aids is the most cost-effective and economical method because of lesser-needed substances. Zhou *et al.* (2012) even demonstrated that this method eliminates the usage of chemical flocculants so that the culture media can be reused. Furthermore, this method does not require different cultural conditions, resulting in double savings. Fungal-assisted microalgal flocculation is effective for both heterotrophic and autotrophic microalgal species, and little information is available regarding its effectiveness on thraustochytrids. Prajapati *et al.* (2014) and Srinuanpan *et al.* (2018) found that the capture of microalgae by *Aspergillus* sp. hyphae for biofuel production reaches almost 100%. Filamentous fungi have been employed intensively in co-cultivation involving microalgal cells because of their ability to self-pelletize and achieve high efficiency in microalgae trapping/adsorption.

This paper investigates the possibility of co-cultivation of DHA-producing thraustochytrids, *Aurantiochytrium* sp. for increased HE with a gamma-linolenic producing oleaginous filamentous fungus, *C. bainieri* 2A1 for the first time. *Cunninghamella bainieri* 2A1, a zygomycete, has the competency of producing up to 30% lipid (g/g biomass) containing 10-15% GLA (Hamid *et al.*, 2001; Muhid *et al.*, 2010). Therefore, co-cultivation of both organisms would produce lipids rich in DHA and GLA.

MATERIALS AND METHODS

Microorganisms

Aurantiochytrium sp. and *C. bainieri* 2A1 cultures were obtained from the Microbial Physiology Laboratory, Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. *Aurantiochytrium* sp. was maintained on seawater nutrient agar (SNA) slants which contained 28 g/L nutrient agar and 17.5 g/L artificial sea salt accounting for 50% salinity at room temperature. *Cunninghamella bainieri* 2A1 was subcultured every three weeks by transferring 1 cm^2 of culture to Potato Dextrose Agar (PDA). The plates were incubated at 30 °C for 3 days for storage culture and 7 days for spore harvesting preparation before being kept at 4 °C.

Preparation of spore suspension of *C. bainieri* 2A1

Preparation of *C. bainieri* 2A1 spore suspension was performed by adding 15 mL of sterile distilled water into agar plates containing 7-day-old *C. bainieri* 2A1 cultures. The surface of the fungal colony was scraped using an inoculation loop to obtain spore suspension. These suspensions were collected into sterile universal bottles, and the concentration of the spore suspension was determined using a Brightline hemocytometer (Hausser Scientific, USA), and spore enumeration was done using a formula as shown below:

$$5 \times n \times (1/10 - x) \times 10^4$$

where n is the number of spores in the hemocytometer grid and x is the dilution factor.

Preparation of seed cultures

Cunninghamella bainieri 2A1 seed cultures were prepared by transferring spore suspensions into a nitrogen-limited medium as described by Kendrick and Ratledge (1992), containing the following medium constituents (g/L): ammonium tartarate, 1.0; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2$, 0.1; $Co(NO_3) \cdot 6H_2O$, 0.0001; $FeCl_3 \cdot 6H_2O$, 0.008; $ZnSO_4 \cdot 7H_2O$, 0.0001; $CuSO_4 \cdot 5H_2O$, 0.0001; $MnSO_4 \cdot 5H_2O$, 0.0001 and High Fructose Corn Syrup (HFCS), 30.0 (added to the basal medium after being sterilized separately). To generate different mycelial forms, different final concentrations of spores (10^2, 10^3, 10^4 and 10^5 spores/mL) were inoculated into 500 mL conical flasks containing 200 mL of nitrogen-limited medium and incubated at 30 °C with 200 rpm agitation for 48 h.

Aurantiochytrium sp. seed cultures were prepared in Burja medium composed of (g/L): yeast extract, 2; MSG, 8; artificial sea salt, 6; and HFCS, 60. HFCS was added to the basal medium after being sterilized separately (Burja *et al.*, 2006). A strip of SNA slant agar containing approximately 20 colonies of 48 h old cultures was inoculated into 200 mL of a fresh seed broth medium in a 500 mL Erlenmeyer flask and cultivated on a rotary shaker at 200 rpm and 30 °C for 48 h.

Co-cultivation experimental scheme

Antagonistic test between Aurantiochytrium sp. and *C.*

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*bainieri 2*A1.

A preliminary study was performed before the co-cultivation experiment was conducted to ensure no antagonistic activity between *Aurantiochytrium* sp. and *C. bainieri* 2A1. Antagonistic activity between *C. bainieri* 2A1 and *Aurantiochytrium* sp. was performed on SNA plates using a modified Fokkema (1978) method. *Aurantiochytrium* sp. culture was streaked on the entire SNA plates before a small filter paper that had been soaked into *C. bainieri* 2A1 spore suspension of 1×10^5 spores/mL was placed at the center of the plates. Controls were performed using similar procedures but omitting either one of the organisms. Both plates were incubated at 30 °C for 48 h. The antagonistic activity was determined by observing the presence of inhibition zones.

Tolerance test of C. bainieri 2A1 to sea salt

Concentrated spores (10^2, 10^3, 10^4 and 10^5 spores/mL) were inoculated into the Burja medium and cultured for 72 h to study the tolerance of *C. bainieri* 2A1 to sea salt in the Burja medium. Biomass concentrations obtained were compared to the fungus cultured in Kendrick and Ratledge (1992) medium.

Harvesting efficiency of Aurantiochytrium sp. cells by co-cultivation using different mycelial forms of C. bainieri 2A1

Experiments were conducted by cultivating *Aurantiochytrium* sp. with *C. bainieri* 2A1 of different pellet sizes and dispersed mycelia. All cultivation was conducted in 500 mL conical flasks containing 200 mL of growth media. Different fungal pellets (FP) sizes were prepared by inoculating different spore concentrations (10^2, 10^3, 10^4 spores/mL) into Kendrick medium. Homogeneous fungal mycelium (FM) was prepared by inoculation of the medium with spore concentration at 10^5 spores/mL. All cultures were incubated at 30 °C with 200 rpm agitation speed for 72 h. The FP and FM were then filtered using sterile cotton gauze and rinsed twice with 200 mL of distilled water, followed by a 200 mL Burja medium.

The FP and FM were then transferred into 72 h old *Aurantiochytrium* sp. cultures and further incubated for 48 h at 30 °C with 200 rpm agitation speed. Samples were then collected and HE (%) of *Aurantiochytrium* sp. was determined. Mono-cultures of both organisms were also performed as controls.

Harvesting efficiency of Aurantiochytrium sp. through co-cultivation at a 5 L scale

Co-cultivation at a 5 L scale was performed using a mycelial form of *C. bainieri* 2A1 resulting in the best HE ie dispersed mycelia. *Aurantiochytrium* sp. was cultivated in a 5 L bioreactor (Minifors-Infors HT) with a working volume of 3 L. The temperature and agitation were set at 30 °C and 600 rpm, respectively. Aeration was provided at a rate of 1 v/v/m. A total of 3 L of 72 h old dispersed mycelial cultures of *C. bainieri* 2A1 was filtered using sterilized cotton gauze, and recovered mycelia were rinsed twice with 1 L of sterile distilled water followed by 1 L of Burja medium. The recovered mycelia were then transferred into the bioreactor containing the 3 L of 72 h old *Aurantiochytrium* sp. cultures. The co-culture was then incubated for a further 48 h. Samples were collected at the end of the fermentation for the determination of HE (%).

Scanning electron microscopy

Scanning electron microscopy was performed to investigate the morphology and pore size of *C. bainieri* 2A1 using an LEO VPSEM 1450 (Zeiss, 1450) scanning electron microscope (SEM). The surface and interior pore diameters of pellets were then determined. The size of the pores inside the pellets was measured by cutting them in half and fixed in 2.5% (v/v) glutaraldehyde solution overnight. Then, the samples were rinsed three times with Phosphate Buffered Solution (PBS) pH 7.5 before being dehydrated in 30-100% graded ethanol for 10 min each, followed by drying in a critical point dryer (LEICA) for 2 h.

Sample analysis

Determination of dry cell weight

Cultures of *C. bainieri* 2A1 were harvested and 200 mL was filtered through preweighed Whatman No.1 filter papers and rinsed twice with two volumes of distilled water. Samples were oven-dried at 80 °C for 24-48 h until they reached a constant weight. The biomass was expressed as gram biomass per litre growth medium.

Lipid extraction

Lipid was extracted using chloroform/methanol solution (2:1, v/v) as described by Folch *et al.* (1957). Extracted samples were placed in a vacuum desiccator for 24 h until a constant weight was reached. Lipid content was expressed as % (g/g biomass).

Harvesting efficiency (HE)

HE of *Aurantiochytrium* sp. cells after 48 h of co-cultivation was carried out by determining the % differences of the OD of uncaptured *Aurantiochytrium* sp. cells in the co-cultivation media against the OD of controls consisting of *Aurantiochytrium* sp. monocultures. A sampling of 1 mL of homogenous cultures was done immediately after stopping the agitation. The HE was then calculated based on the following formula:

$$HE\ (\%) = 100 \times [(OD_{control} - OD_{sample})/OD_{control}]$$

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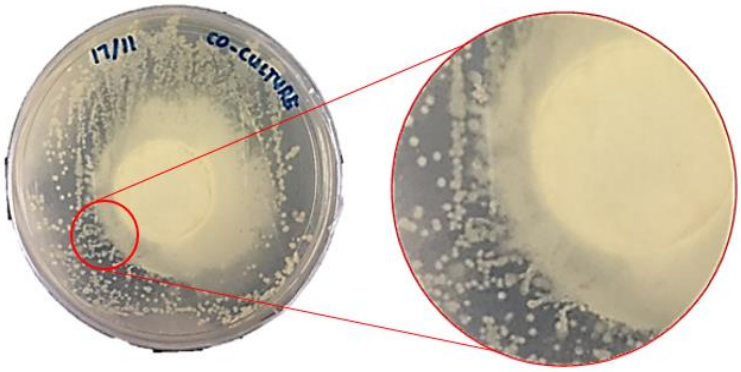

Figure 1: Observation of co-culture antagonist zone between *Aurantiochytrium* sp. and *C. bainieri* 2A1 on SNA agar after 48 h.

Table 1: Differences of *C. bainieri* 2A1 pellet diameter and pore size on the surface and in the pellets.

Spore concentration (spores/mL)	Pellet diameter (mm)	Pore size on the surface (µm)	Pore size inside the pellet (µm)
1×10^2	1.30 ± 0.07 (large)	5.0 - 14.0	9.0 - 52.0
1×10^3	0.8 ± 0.03 (medium)	6.0 - 25.0	20.0 - 78.0
1×10^4	0.51 ± 0.10 (small)	8.0 - 27.0	-
1×10^5	dispersed mycelium	-	-

RESULTS AND DISCUSSION

Aurantiochytrium sp. and *C. bainieri* 2A1 antagonistic test

An antagonistic test between *Aurantiochytrium* sp. and *C. bainieri* 2A1 was carried out to determine the compatibility of the microorganisms for co-cultivation. SNA plates were streaked with *Aurantiochytrium* sp. before inoculating *C. bainieri* 2A1 at the centre of the agar. The presence of inhibitory zones would indicate positive antagonism activity of one or both microorganisms (Prasad and Babu, 2017). In this study, as shown in Figure 1, no inhibitory zones were present after 48 h of incubation, indicating that both microorganisms are suitable for co-cultivation.

To the best of the authors' knowledge, no reports regarding antagonistic activities between fungi and thraustochytrids are available. Nevertheless, inhibitory activities of bacteria against microalgae have been reported, such as *Pseudomonas protegens* which inhibits biflagellated green alga *Chlamydomonas reinhardtii* (Aiyar *et al.*, 2017).

Effects of different spore concentrations on morphology, growth and lipid production of *C. bainieri* 2A1

As the influence of different mycelial forms of *C. bainieri* 2A1 on HE of *Aurantiochytrium* sp. were to be investigated, attempts to generate various mycelial forms of *C. bainieri* 2A1 grown in aqueous culture were carried out by inoculating *C. bainieri* 2A1 in Kendrick medium with different spore concentrations. Figure 2 shows that inoculation with a concentration of 1×10^5 resulted in dispersed mycelial growth, while small pellets were formed when the spore concentration was decreased to 1×10^4. A further decrease in the spore concentration from 10^4 to 10^2 resulted in the formation of medium and large pellets representing a 2.5-fold increase in the pellet size (Table 1). The large and medium-sized pellets were also more compact and denser, with distinctive smooth edges compared to the small pellets, which were more loosely packed and with protruding hyphae around the edges (Figure 3).

These are consistent with the findings of Zhou *et al.* (2013) and Chen *et al.* (2018), who reported that pellet forms of *Penicillium* sp. and *Aspergillus oryzae* were generated by spore concentrations of 1.1×10^2 to 1.1×10^4 and 1.1×10^2 to 1.1×10^5, respectively, where dispersed mycelia were produced when more than 10^5 spore/mL concentration was employed.

Similar responses to increasing spore concentration were also reported on *Aspergillus niger* (Papagianni and Mattey, 2006). However, the study also reported that FP formation is also influenced by other cultivation factors such as pH, dissolved oxygen level, shaking rate, trace element, temperature and reactor type. Fungal pellets are reported to form due to interactive activities, which are specific and nonspecific interactions (Zhang and Zhang, 2015). Nonspecific interactions are electrostatic (Van der Waals force and electrostatic repulsion) and hydrophobic interactions, while specific interactions are the interactions of spore wall components. The forces inherent at the beginning of inoculation between fungal spores are electrostatic and hydrophobic interactions. The hydrophobic interactions decrease during the swelling of

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Figure 2: Morphology of *C. bainieri* 2A1 cultivated using different spore concentrations (a) 1 × 10^2 spores/mL, (b) 1 × 10^3 spores/mL, (c) 1 × 10^4 spores/mL and (d) 1 × 10^5 spores/mL. Cultivation was carried out in 500 mL conical flasks containing 200 mL medium and incubated for 48 h at 30 °C with an agitation speed of 200 rpm.

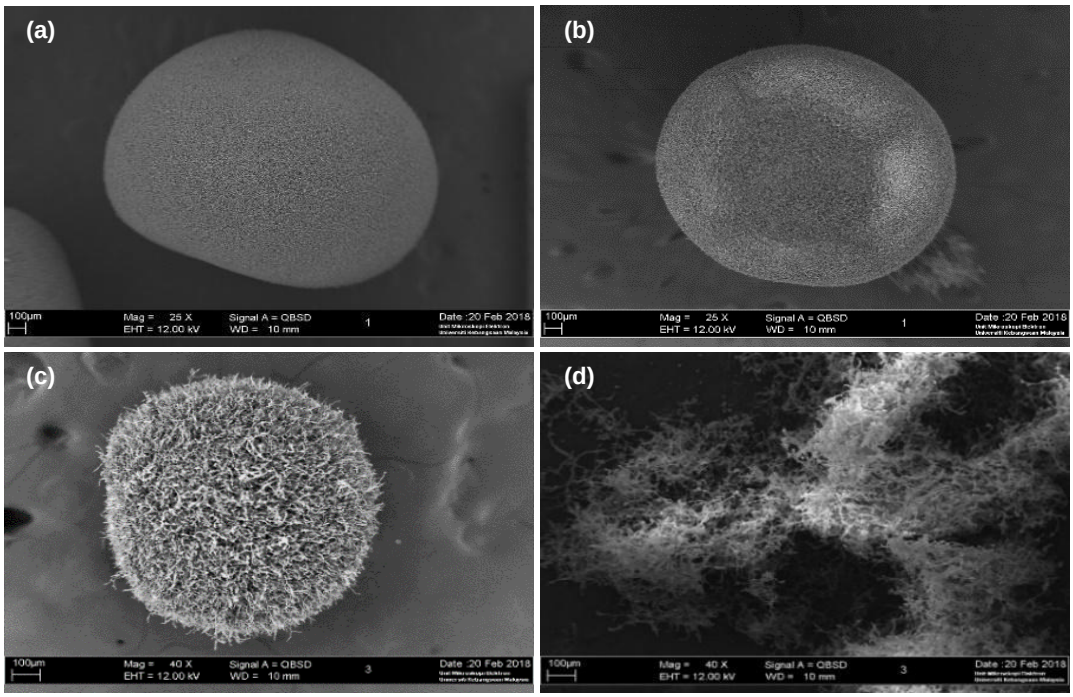

Figure 3: SEM observation of different mycelial forms of *C. bainieri* 2A1, inoculated with spore suspensions (spore/mL) of a) 1 × 10^2, (b) 1 × 10^3, (c) 1 × 10^4 and (d) 1 × 10^5, producing large, medium, small pellets and dispersed mycelia, respectively.

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Table 2: Biomass, lipid dan lipidless biomass of *C. bainieri* 2A1 cultivated using different spore concentrations after 48 h of cultivation in Kendrick medium at 30 °C.

Spore concentration (spores/mL)	Biomass (g/L)	Lipid (%)	Lipidless biomass (g/L)
1×10^2	6.09 ± 0.03	22.33 ± 0.02	4.73 ± 0.03
1×10^3	6.97 ± 0.02	16.50 ± 0.01	5.82 ± 0.02
1×10^4	7.91 ± 0.02	17.83 ± 0.01	6.50 ± 0.02
1×10^5	10.50 ± 0.01	21.52 ± 0.01	8.24 ± 0.01

the spores resulting in the increase in the specific interactions of spore wall components, which then contribute to the formation of pellets observed in different fungal species (Veiter *et al.*, 2018).

Determination of pellet pore sizes on the surface as well as within the pellets was then carried out using SEM. The pore size within the pellets was determined by slicing the pellets in half before SEM observation. Table 1 shows that increasing spore concentrations from 1×10^2 to 1×10^3 spores/mL resulted in an increase in the pore sizes on the surface and in the pellets. Denser pellets were produced using 1×10^2 spores/mL, displaying tightly packed mycelia.

As shown in Table 2, the biomass and lipidless biomass concentration increased in parallel with the increasing spore concentration, where cultures inoculated with 1×10^5 spores/mL produced the highest biomass concentration of 10.50 g/L. This was expected due to the direct effects of increased spore concentration employed as well as increased efficiency of nutrient and oxygen transfer at exposed hyphae of dispersed mycelia generated (Zhang and Zhang, 2015; Veiter *et al.*, 2018). Krull *et al.* (2010) reported that differences in the size and density of *A. niger* pellets affect the effectiveness of oxygen absorption and penetration into the pellets where larger and denser pellet sizes require higher oxygen consumption.

Differences in the lipid content for each different form of *C. bainieri* 2A1 were also observed, where high lipid content is produced in cultures consisting of large-sized pellets and dispersed mycelia (22.33% and 21.52%, respectively) compared with cultures with medium and small-sized pellets (16.50% and 17.83%, respectively) (Table 2).

Effects of different mycelial forms of *C. bainieri* 2A1 on HE of *Aurantiochytrium* sp.

HE of *Aurantiochytrium* sp. without centrifugation step and when co-cultivated with various mycelial forms of *C. bainieri* 2A1 was firstly determined. Spores of *C. bainieri* 2A1 at concentrations of 1×10^2, 1×10^3, 1×10^4 and 1×10^5 spores/mL were inoculated in Kendrick medium and incubated for 72 h. The resulting mycelia were then transferred aseptically into 72 h cultures of *Aurantiochytrium* sp. and further incubated for 48 h.

Figure 4 shows that co-cultivation with dispersed mycelia of *C. bainieri* 2A1 generated through inoculation of 1×10^5 spores/mL resulted in 63% HE of *Aurantiochytrium* sp. cells. This was similar as reported by Luo *et al.* (2019) that showed up to 64.86% HE of *Chlorella* sp. cells were achieved when co-cultivation with medium-sized pellets of *Pleurotus ostreatus* was performed. Whereas, with the exception of co-cultivation performed with small pellets, a decreasing trend in HE was observed as co-cultivation was performed with increasing pellet size. As shown in Figure 4, co-cultivation performed with large pellets of *C. bainieri* 2A1 resulted in a 39% reduction in HE compared to when the dispersed mycelial form was employed. These indicate that the morphological differences of the mycelial forms of *C. bainieri* 2A1 affect the HE of *Aurantiochytrium* sp. These were probably due to the decreasing pore sizes with the increasingly packed mycelial network formed as the pellet size increased, leading to inefficient entrapment of *Aurantiochytrium* sp. cells (Figure 1, Table 1).

Based on Luo *et al.* (2019), the medium-sized pellets' rougher surface and loose internal structure, which allowed for internal and external adsorptions of cells, led to their higher adsorption capability. Therefore the large, compact and dense pellet had a very low harvest efficiency because it primarily captured *Aurantiochytrium* sp. cells through surface adsorption. The cause of the sudden drop in HE observed when smaller pellets were employed, although it has been shown to be reproducible, was not pursued.

Effects of co-cultivation of *C. bainieri* 2A1 and minimal centrifugation on HE of *Aurantiochytrium* sp.

As only 63% HE was achieved through co-cultivation alone, further enhancement attempts were carried out by incorporating minimal centrifugation steps. Effects of centrifugation at various speeds on *Aurantiochytrium* sp. monocultures were firstly determined. Figure 5 shows that HE increased from 75% to 83% as the g force was increased from 2000 to 6000× *g* and a lower HE increment was achieved, although further increment of the g force to 10 000× *g* was carried out. Therefore, minimal centrifugation at 4000× *g* was selected to determine the combinatory effects of co-cultivation and centrifugation to enhance *Aurantiochytrium* sp. HE.

Figure 6 shows the percentage of HE of *Aurantiochytrium* sp. co-cultivated with different mycelial forms of *C. bainieri* 2A1 with additional minimal centrifugation at 4000× *g*. All showed higher values of more than 95% of HE achieved compared to when monocultures of *Aurantiochytrium* sp. were either centrifuged at 4000 or 10 000× *g* (80% and 90% HE, respectively) (Figure 5), with co-cultivation using dispersed mycelia achieving the highest value of 99.55% HE. Reproducible results with 99% HE were achieved wh-

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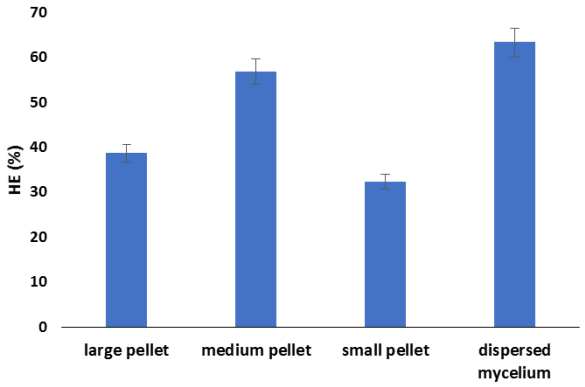

Figure 4: Percentage of HE of *Aurantiochytrium* sp. from co-culture samples cultivated at 30 °C for 48 h in 500 mL conical flasks.

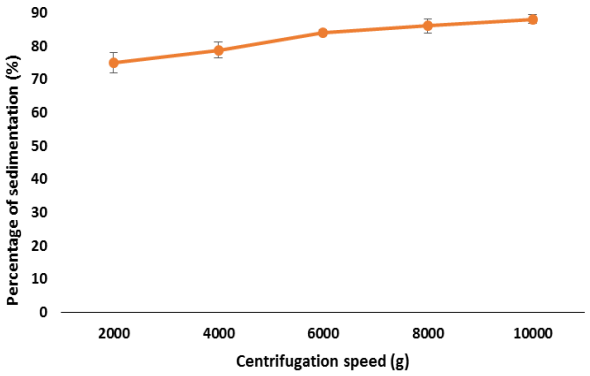

Figure 5: Effect of centrifugation speed on biomass sedimentation of *Aurantiochytrium* sp. monoculture.

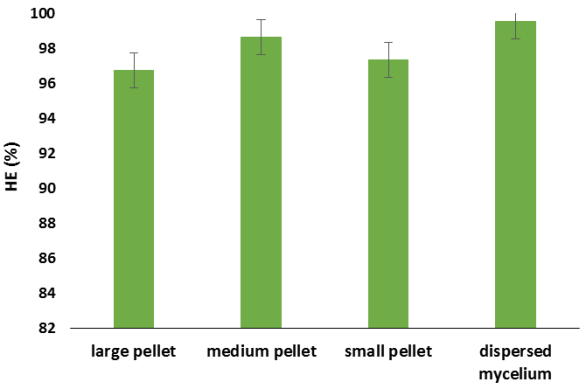

Figure 6: Percentage of HE of *Aurantiochytrium* sp. from co-culture samples cultivated at 30 °C for 48 h in 500 mL conical flasks after centrifuging at 4000× *g*.

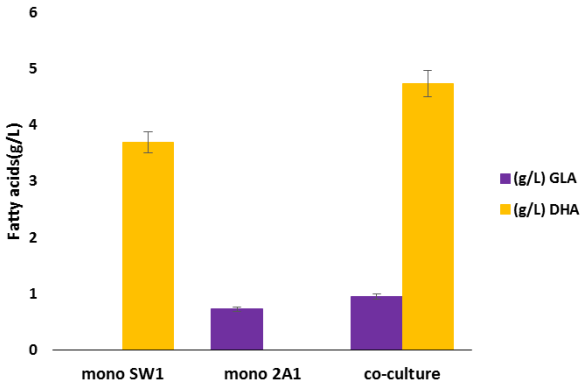

Figure 7: DHA and GLA production (g/L) by monoculture *Aurantiochytrium* sp., *C. bainieri* 2A1 and co-culture in 500 mL shake flask at 30 °C, 200 rpm agitation speed for 120 h.

-en upscaled experiments at 3 L fermentation volumes were performed.

This shows that the one-step direct centrifugation of the co-cultures facilitates the most efficient recovery of *Aurantiochytrium* sp. cells. Generally, microalgal harvesting involving co-cultivation is performed in two separate steps where a concentrated biomass slurry is recovered after cultivation and further centrifuged (Vandamme *et al.*, 2013).

Effects of co-cultivation on lipid and fatty acid production

Figure 7 shows the production of GLA and DHA (g/L) when *C. bainieri* 2A1 and *Aurantiochytrium* sp. are co-cultured in comparison to the monocultures. Results showed that the co-culture produced a 28% higher volumetric DHA yield (total lipid of 7.27 g/L) than the monoculture (total lipid of 6.19 g/L) while no significant increase was observed in the level of GLA production. The increase of volumetric DHA yield in co-cultivation is parallel to reports by Du *et al.* (2018) that showed a 5.5-fold increase in arachidonic acid yield in co-culture of *Nannochloropsis oceanica* with oleaginous fungus *Mortierella gamsii* GBAus compared to monoculture, from 3.1 mg/g to 17.5 mg/g (total dry weight). In the meantime, Wrede *et al.* (2014) reported a two-fold increase in volumetric palmitic acid and palmitoleic acid yield when *A. fumigatus* was cultured with *Nannochloropsis oculata* and *Scenedesmus quadricauda*. Therefore, in addition to facilitating efficient harvesting of *Aurantiochytrium* sp., co-cultivation also results in the increased production of DHA yield by 28%.

CONCLUSION

This work was the first to demonstrate the enhancement of harvesting efficiency of oleaginous thraustochytrids through co-cultivation with filamentous fungi. The HE of *Aurantiochytrium* sp. cells increased with a decrease in the *C. bainieri* 2A1 pellet sizes, with the highest HE value (63%) achieved when dispersed mycelial form was

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employed. The HE was further successfully enhanced to 99.6% by incorporating a minimal centrifugation step at 4000× *g*. Furthermore, a 28% increase in volumetric DHA yield from 3.69 g/L in the monoculture to 4.73 g/L was also achieved through co-cultivation.

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