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A new strain of docosahexaenoic acid producing microalga from Malaysian coastal waters

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ABSTRACT

Thraustochytrids are marine protists belonging to the class Labyrinthula of the kingdom Chromista. This group of microbes is considered a promising alternative source of high-valued omega-3 oils, especially docosahexaenoic acid (DHA, C22:6*n*3), replacing fish oil which is the current major source of DHA and eicosapentaenoic acid (EPA, C20:5*n*3). Our new isolate, SW1, was considered to be an *Aurantiochytrium* based on its morphology, fatty acid profile and molecular phylogenetic analysis. Microscopic observations revealed that SW1 has high similarity to *Aurantiochytrium limacinum* ATCC MYA-1381 (SR21), possessing spherical vegetative cells which undergo repeated bipartition to form diads, tetrads, octads and zoosporangia that release motile zoospores. When cultivated in four different media, SW1 produced the highest biomass (13.17 g/L) and DHA (3.6 g/L) in a medium composed of glucose, sea salt, monosodium glutamate (MSG) and yeast extract. Visualization of lipid droplet development in SW1 using Sudan Black B dye revealed that lipid droplets enlarge to occupy almost the entire cell volume within 72 to 96 h of cultivation. This strain was also found to be able to utilize various saccharides as carbon source. The results of this study show that *Aurantiochytrium* sp. SW1 is a potential candidate to be developed as commercial microbial DHA producer.

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1. Introduction

The human body is capable of synthesising saturated and monounsaturated fatty acids but, polyunsaturated fatty acids (PUFAs) must be acquired from dietary sources [1]. Linoleic acid (LA, C18:2*n*6) and α-linoleic acid (ALA, C18:3*n*3) are PUFAs that can be transformed into arachidonic acid (AA, C20:4*n*6), docosapentaenoic acid (DPA, C22:5*n*6), docosahexaenoic acid (DHA, C22:6*n*3) and eicosapentaenoic acid (EPA, C20:5*n*3). However, the conversion rates of EPA and DHA are very low and therefore these two PUFAs are regarded as essential themselves [2].

Docosahexaenoic acid, one of the essential long chain PUFAs, plays a vital role in human health [3]. It occurs naturally in breast milk and is essential for normal infant brain and eye development [4]. Dietary DHA has positive effects on several diseases such as hypertension, arthritis, atherosclerosis, depression, adult-onset diabetes mellitus, myocardial infarction, thrombosis and some cancers [5]. Due to its significant role in enhancement of human health, DHA is widely used as a nutraceutical component in the food and feed market [3].

The present largest and richest commercial source of DHA is cold water fatty fish, such as salmon, sardine, anchovy, tuna and cod which can accumulate up to 30% of its fat as DHA and EPA [6]. In fish oils, both the composition and quantity of PUFA depend on the species and other external factors [5]. There are a number of problems with fish oil such as the presence of highly saturated fatty acids, low DHA yield, contamination by hazardous substances such as heavy metals and polychlorinated biphenol, odour, lack of oxidative stability and complications in purification processes [7,8]. Since fish obtain *n*-3 fatty acids from zooplankton, which consumes algae, current studies are being diverted on coming forward with a commercially feasible technology to produce PUFA directly from microalgae [6].

Microbial oil, being produced in a controlled environment, is one of the current topics of massive research because it has many advantages compared to fish oil [9]. Marine fungoid protists (thraustochytrids) such as *Aurantiochytrium* and marine microalgae such as *Crypthecodinium cohnii* have been shown to be excellent DHA producers. DHA from *Aurantiochytrium* has also been proven to be safe for human consumption, being free from the common algal toxins such as domoic acid and prymnesin produced by some members of its kingdom, Chromista [10]. Recently, we have isolated a strain of *Aurantiochytrium* from Malaysian

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Table 1
Viability of SR21 and SW1 in different temperatures (✓: viable, ●: not viable).

	SR21					SW1				
	Temperature (°C)									
	4	25	30	35	40	4	25	30	35	40
Day 1	●	✓	✓	✓	●	●	✓	✓	✓	●
Day 2	●	✓	✓	✓	●	●	✓	✓	✓	●
Day 3	●	✓	✓	✓	●	●	✓	✓	●	●
Day 4	●	✓	✓	●	●	●	✓	✓	●	●
Day 5	●	✓	✓	●	●	●	✓	✓	●	●
Day 6	●	✓	✓	●	●	●	✓	✓	●	●
Day 7	●	✓	✓	●	●	●	✓	✓	●	●

seawater. This paper reports the basic characteristics of the aforesaid isolate and its DHA production capacity in different cultivation media and various carbon sources.

2. Materials & methods

2.1. Organisms and culture conditions

Aurantiochytrium limacinum ATCC MYA-1381 strain (SR21) was obtained from American Type Culture Collection (ATCC), while SW1 was isolated from seawater sample from Port Dickson, Malaysia and deposited in UNiCC UPM (WDCM 988) under the accession number of [UPMC 963]. Both organisms were maintained on seawater nutrient agar (SNA) slant prepared with half-strength (50%) artificial seawater (ASW) at 30 °C and subcultured every eight months. Seed cultures were prepared by inoculating 100 mL seeding broth (contained in 500 mL Erlenmeyer flasks) with an SNA strip containing approximately ten colonies of 48 h old cells and incubated at 30 °C for 48 h with 200 rpm agitation. A 10% v/v inoculum was inoculated into 100 mL of four different media: yeast-extract–peptone (YEP) medium [11] containing glucose (20 g/L), yeast extract (1 g/L) and natural seawater (NSW) (35%), 790 By + medium (ATCC) containing glucose (5 g/L), yeast extract (1 g/L), peptone (1 g/L) and NSW (100%), medium B [12] containing glucose (60 g/L), yeast extract (2 g/L), MSG (8 g/L) and NSW (15%) and medium S [7] containing glucose (20 g/L), yeast extract (4 g/L), NaCl (25 g/L), KH_2PO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (5 g/L), $CaCl_2$ (0.2 g/L), KCl (1 g/L), ddH_2O (1 L) and 5 mL/L trace mineral solution: in (g/L) Na_2EDTA 6.0; $FeCl_3 \cdot 6H_2O$ 0.29; H_3BO_3 6.84; $MnCl_2 \cdot 4H_2O$ 0.86; $ZnCl_2$ 0.06; $CoCl_2 \cdot 6H_2O$ 0.026 and $CuSO_4 \cdot 5H_2O$ 0.002. The cultures were incubated for 96 h at 30 °C with 200 rpm agitation.

2.2. Culture maintenance

Stock cultures of SR21 and SW1 were prepared by streaking inoculum on SNA slant containing 17.5 g/L sea salt (half strength ASW) in screw capped 50 mL centrifuge tubes. Cultures were grown at 30 °C for 48 h after which 1 mL of growth suspending medium (GSM), consisting of medium B (refer to Section 2.1) and 10% (*v*/*v*) glycerol was added to the slant of SNA containing cells. The tubes were capped tightly, sealed with parafilm and stored at ambient temperature, protected from sunlight. Viability of the cells were examined every three months by transferring one loopful of cells (dipped in GSM at the bottom of slant before picking a single colony on slant surface) to SNA plates by the streaking method. The plates were then incubated at ambient temperature for 24–96 h.

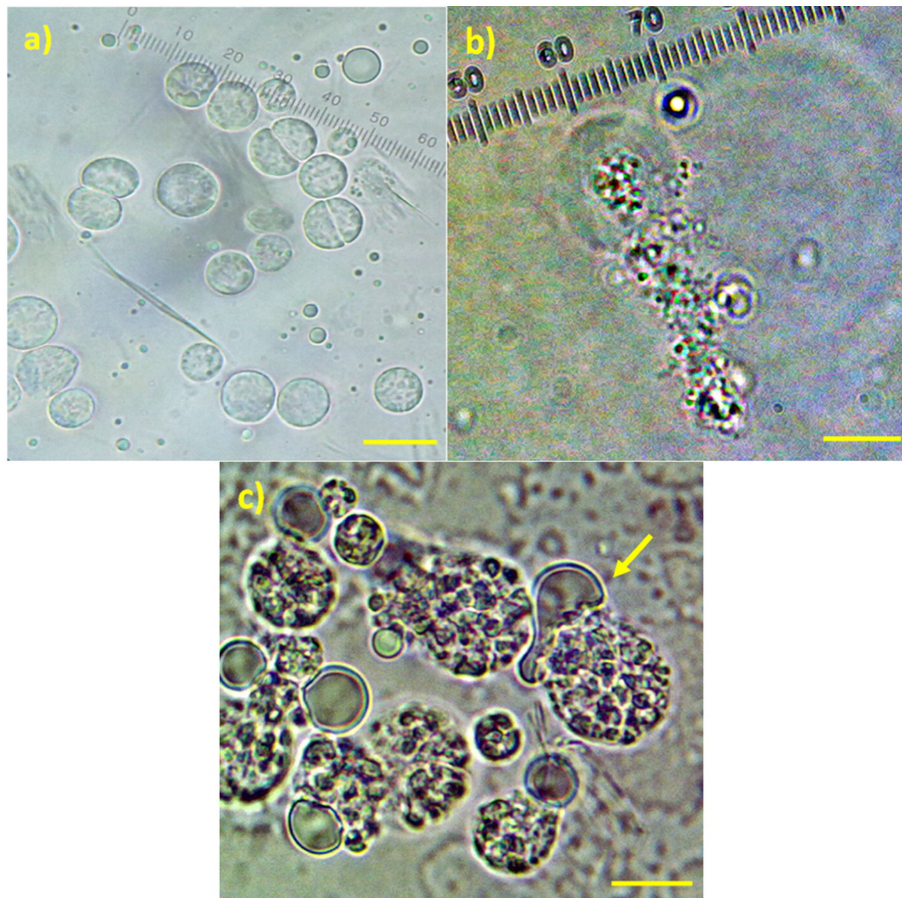

Fig. 1. Micrographs of SW1, a) vegetative cells at various stages, release of zoospores via b) small opening in zoosporangial wall and c) via rupture of zoosporangium (arrow). Scale bar = 10 μm.

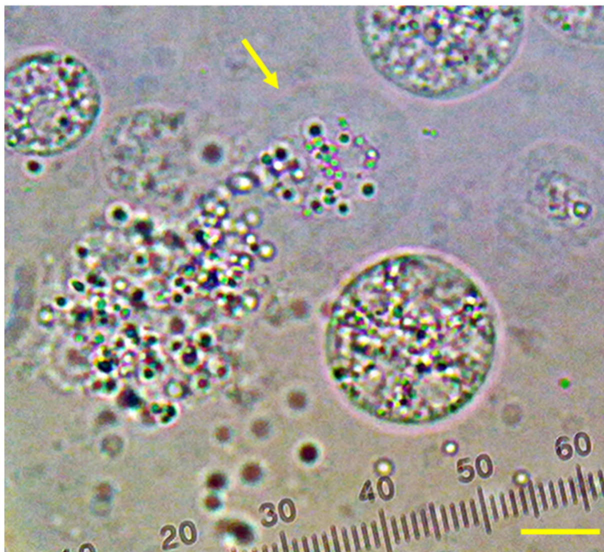

Fig. 2. Micrograph of SR21. Release of zoospores from zoosporangium (48 h old). Scale bar = 10 μm.

2.3. Viability test

Broth cultures of SR21 and SW1 were prepared by adding 1 mL of 48 h inoculum into 9 mL of nutrient broth (NB) in 20 mL universal bottles. The cultures were then incubated in an oven at different temperatures (25–40 °C), meanwhile some were stored in a refrigerator (±4 °C). Viability of the cultures were examined every 24 h by transferring 100 μL of broth to nutrient agar (NA) plates (the spread plate method) and incubating at ambient temperature for 48 h.

2.4. Microscopic characterization

Cell morphology was observed under a light microscope (Olympus CX22LED) at 100 × 10 magnification (without staining) using 24 and 48 h old colonies from SNA. A stock solution of Sudan Black B was prepared in ethanol (0.3 g of powdered stain in 100 mL 70% ethanol) and kept protected from light. Staining of lipid droplets was done using heat fixed cells taken from 24, 48, 72 and 96 h old liquid culture. A smear of cells was prepared and dried thoroughly in the air before brief heat fixation. Fixed cells were stained with Sudan Black B solution for 15 min at room temperature. Excess stain was drained and the cells were counterstained with safranin dye for 30 s after which the slide was washed briefly with running tap water and blotted dry. Cells were observed at 100 × 10 magnification.

2.5. Molecular identification of SW1

The genomic DNA of SW1 was extracted by the DNA extraction kit (MBS-500×-Proviso) provided by the 1st Base Malaysia according to the manufacturer's protocol. Universal eukaryotic 18S rRNA [13] was used to amplify 18S rRNA of SW1. The PCR product was extracted out using 0.5% tris–borate–EDTA agarose gel, purified and cloned into pJET1.2/blunt vector to be sequenced. The obtained sequences were deposited into the GenBank Nucleotide Database [GenBank: KF500513] and compared to the aligned sequences in GenBank using BLAST. A phylogenetic tree was constructed using the neighbour joining method [14], with reliability set at a bootstrap value of 1000 replications.

2.6. Carbon utilization

SW1 was grown in 500 mL Erlenmeyer flasks containing 100 mL of medium consisting of 0.2% yeast extract, 0.8% MSG and 0.6% sea salt with 3% various carbon sources (glucose, fructose, galactose, arabinose, xylose, mannose, lactose, maltose, sucrose, starch, cellulose and chitosan). The negative control contained yeast extract, MSG and sea salt as mentioned above, but with the omission of carbon source. Cultures were grown for 96 h at 30 °C with 200 rpm agitation. Results are presented as the average values of duplicative runs.

2.7. Determination of dry cell weight

Cells were harvested by centrifuging at 3500 rpm for 15 min using Eppendorf Centrifuge 5810 R followed by rinsing twice with 50 mL sterile distilled water. Samples were oven-dried at 95 °C to constant weight. Biomass was expressed as oven-dried weight in gram per litre of growth medium, presented as the average value of duplicative runs.

2.8. Lipid extraction and fatty acid analysis

Lipid extraction was done using the chloroform/methanol method as described by Folch et al. [15]. The extract was vaporized at room temperature and dried in a vacuum desiccator until the weight was constant. Fatty acid compositions of the samples (with maximum standard error <5%) were determined as fatty acid methyl ester (FAME) by gas chromatography (HP 5890) equipped with a capillary column (BPX 70, 30 cm, 0.32 μm). FAME was prepared by dissolving 0.05 g of the sample in 0.95 mL hexane and the mixture was added to 0.05 ml of 1 M sodium methoxide. The injector was maintained at 200 °C. 1 μL of sample was injected (split mode injector) using helium as a carrier gas with

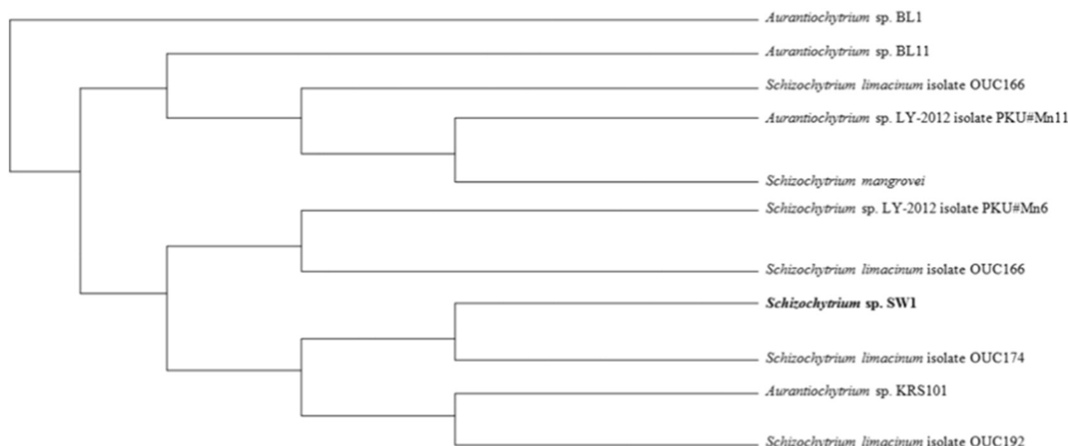

Fig. 3. Phylogenetic tree of *Aurantiochytrium* sp. SW1.

Table 2
Amounts of biomass, TFA and DHA produced by SW1 in various media.

Production medium	Glucose concentration (g/L)	Biomass (g/L)	TFA (% g/g biomass)	DHA (% g/g lipid)	DHA yield Y'_{PS} (g/g^{-1})
YEP broth	20.0	5.21 ± 0.01	44.72 ± 0.03	50.1 ± 1.98	0.06 ± 0.00
790 By +	5.0	3.32 ± 0.14	14.76 ± 0.27	64.1 ± 0.99	0.07 ± 0.01
Medium B	60.0	13.17 ± 0.04	48.90 ± 1.24	56.1 ± 1.13	0.06 ± 0.00
Medium S	20.0	10.04 ± 0.06	9.66 ± 0.08	55.9 ± 1.56	0.03 ± 0.00

flow rate of 40 cm^3 min^{-1}. The temperature of the GC column was gradually increased at 7 °C min^{-1} from 50 (5 min hold) to 200 °C (10 min hold). Fatty acids peaks were identified using Chromeleon chromatography software (Dionex, Sunnyvale, California, USA). FAMEs were identified and quantified by comparison with the retention time and peak areas of SUPELCO (Bellefonte, Pennsylvania, USA). Results presented are the average values of duplicative runs.

2.9. Statistical analysis

The effect of studied factors was analysed statistically by one-way analysis of variance (ANOVA) using SPSS software (SPSS Inc., Version 16.0).

3. Results

3.1. Viability and culture maintenance

Both *A. limacinum* SR21 (cited SR21 henceforth) and SW1 lost viability upon storage at 4 °C for 24 h or more. Growth of SW1 declined markedly at temperatures above 35 °C and eventually ceased after 48 h of cultivation. Table 1 shows the viability of SR21 and SW1 at several levels of ambient temperature and refrigerated temperature of 4 °C for one week.

The cells could be stored at −80 °C for more than sixty days by addition of glycerol (40%) into 48 h old culture broth in screw capped glass vials. The cells were also found to be viable for approximately twelve months at room temperature when grown on SNA slant with the addition of 1 mL GSM composed of medium B and 10% (*v*/*v*) glycerol. However, culturability (ability to yield a visible colony) of the cells upon resuscitation was found to be slower as the stock culture aged. A 1- to 3-month-old stock yielded visible colonies within 24 to 48 h, whereas a 9- to 12-month-old stock took 72 to 96 h to yield visible colonies. Similarly, older stock cultures yielded fewer colonies upon resuscitation.

3.2. Macroscopic and microscopic characteristics

On SNA medium, colonies of SW1 were orangey cream in colour which is similar to colonies of SR21. A one week old colony of SW1 measured up to 5 mm in diameter, whereas those of SR21 measured approximately 2 mm. The vegetative cells were sphere in shape and their size ranges from 8 to 14 μm in diameter (Fig. 1a). In both cases, vegetative cells undergo repeated binary division to form diads, tetrads and octads, leading to formation of clusters of cells preceding production of zoospores.

Vegetative cells increased in size before developing into zoosporangia. The size of zoosporangia of SW1 was slightly smaller (12–18 μm diam.) than that of SR21, which measured up to 25 μm in diameter. Motile amoeboid cells, measuring up to 22 μm were observed in SR21. These cells matured into small zoosporangia (8–12 μm diam.) that release few small ovoid zoospores, measuring approximately 5 μm in length.

In both microorganisms, zoospores released by zoosporangia which originated from vegetative cells were ovoid in shape and up to 9 μm in length. Opening of the zoosporangium in SR21 was unclear and the sporangium appeared loose with a very thin layer of sporangial wall (Fig. 2). The zoosporangial wall of SW1 was thicker compared to that of SR21 and clearly visible, even after the complete release of zoospores. Mechanism of zoospore dispersion appeared to be pressure-associated, based on observation of extremely rapid dispersal of zoospores resembling a burst (Fig. 1b). At some circumstances, the zoosporangium bursts open to release zoospores (Fig. 1c).

The 18S rRNA gene sequence composed of 1782 bp was deposited in GenBank with accession number [GenBank: KF500513]. Through BLAST results, SW1 was positively identified as a member of the genus *Aurantiochytrium*/*Schizochytrium*. Phylogenetic analysis indicated that this strain is closely related to *Schizochytrium limacinum* OUC174, *Aurantiochytrium* sp. KRS101 and *S. limacinum* OUC192 (Fig. 3).

3.3. Biomass, lipid and DHA production

Screening the potential of this isolate was carried out by cultivating in four types of media (YEP medium, 790 By + medium, medium B and medium S) previously reported to be favourable for DHA accumulation by thraustochytrids. Out of these four media, it was observed that SW1 produces the highest biomass, that is 13.2 g/L when cultured in medium B (Table 2), which was composed of 60 g/L glucose, 8 g/L monosodium glutamate, 2 g/L yeast extract and 15% NSW. Table 2 shows the glucose content of the four media and DHA yield (Y'_{PS}) in each medium.

SW1 was found to have a simple profile of PUFA, where DHA is the major PUFA, while EPA and AA are trivial constituents. The predominant fatty acid produced by this strain was DHA, amounting to 56.1% of total fatty acids (TFA) in medium B. The absolute amount of DHA produced by SW1 in this medium was 3.6 g/L. The second most abundant fatty acid was palmitic acid (C16:0, hexadecanoic acid) amounting to 36.3% of TFA. This is a distinctive characteristic of the genus *Aurantiochytrium*, where DHA and palmitic acid are the two predominant fatty acids produced by this genus. SW1 also produced trace amounts of EPA,

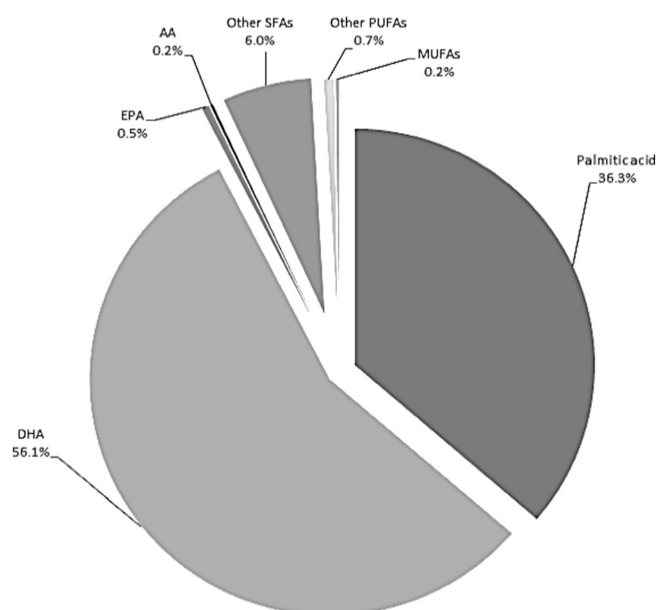

Fig. 4. Fatty acid composition of SW1.

Table 3
Results of one-way ANOVA for the effect of different media on biomass production, lipid and DHA contents, as well as DHA yield.

Parameter	Sum of squares	DF	Mean^2	F-value	P > F
Biomass (g/L)					
Between media	121.120	3	40.373	2.863^{E3}	0.000
Within media	0.056	4	0.014		
TFA (% g/g biomass)					
Between media	2437.802	3	812.601	1.005^{E3}	0.000
Within media	3.236	4	0.809		
DHA (% g/g TFA)					
Between media	198.460	3	66.153	30.769	0.003
Within media	8.600	4	2.150		
DHA yield (Y'_{PS} g/g^{-1})					
Between media	0.002	3	0.001	41.000	0.002
Within media	0.000	4	0.000		

P < 0.05 is significant.

Table 4
Biomass, lipid and DHA accumulation in various carbon sources.

Carbon source	Biomass (g/L)	Lipid (g/L)	Lipid/biomass (%)	DHA (%)	DHA (g/L)
Glucose	5.8 ± 0.21	2.12 ± 0.04	36.50	50.30	1.12 ± 0.04
Fructose	9.2 ± 0.08	3.59 ± 0.09	39.02	46.85	1.68 ± 0.06
Galactose	4.6 ± 0.03	1.53 ± 0.08	32.90	52.00	0.80 ± 0.06
Arabinose	4.0 ± 0.12	1.41 ± 0.10	35.30	51.23	0.72 ± 0.07
Xylose	3.8 ± 0.11	1.22 ± 0.17	32.10	38.50	0.47 ± 0.10
Mannose	6.3 ± 0.10	2.33 ± 0.15	36.90	47.82	1.11 ± 0.11
Lactose	3.9 ± 0.03	0.84 ± 0.05	21.50	43.28	0.36 ± 0.04
Maltose	3.6 ± 0.06	1.24 ± 0.13	34.40	52.73	0.65 ± 0.09
Sucrose	4.3 ± 0.06	1.34 ± 0.06	31.16	50.22	0.67 ± 0.04
Starch	4.4 ± 0.11	1.12 ± 0.08	25.45	56.06	0.63 ± 0.06
Cellulose	3.1 ± 0.11	0.80 ± 0.08	25.80	32.24	0.26 ± 0.04
Chitosan	3.7 ± 0.08	0.96 ± 0.07	26.00	19.26	0.18 ± 0.02
Negative control	3.2 ± 0.02	0.75 ± 0.07	23.40	32.47	0.24 ± 0.04

accounting for approximately 0.5% of total fatty acids. Fig. 4 shows the fatty acid compositions of SW1 in medium B.

Upon analysing the data presented in Table 2 using SPSS software, it was found that all the four responses, namely amount of biomass produced, lipid and DHA contents, as well as DHA yield are significantly different (P < 0.05) in each of the medium tested. This clearly shows that selection of medium plays a key role in affecting the overall production of DHA. Table 3 summarizes the results of ANOVA for this experiment.

3.4. Intracellular lipid body development

The development of lipid droplets in SW1 was observed at fixed time intervals as shown in Fig. 5. The lipids are stained blue–black to light purple/blue by lysochrome, Sudan Black B, whereas the non-lipid cellular materials are stained red/pink by safranin. Generally, the lipid droplets are sphere in shape and the size varies from 1 to 20 μm, depending on the developmental stage of the cells. At the beginning of cultivation, staining of the cells using Sudan Black B mainly highlighted the cell membranes, represented as purplish regions at the periphery of the cells (Fig. 5a). Occasionally, minute blue–black lipid droplets were observed in the cytoplasm of the cells. After 48 h of cultivation, lipid droplets were clearly observable scattered throughout the cells as blue–black spots (Fig. 5b). As the culture progressed, substantially larger lipid droplets which filled almost half of the cytoplasm were observed as light blue/purple patches (Fig. 5c). On the fourth day of cultivation, huge, lipid droplets were observed, filling up almost the entire cell (Fig. 5d).

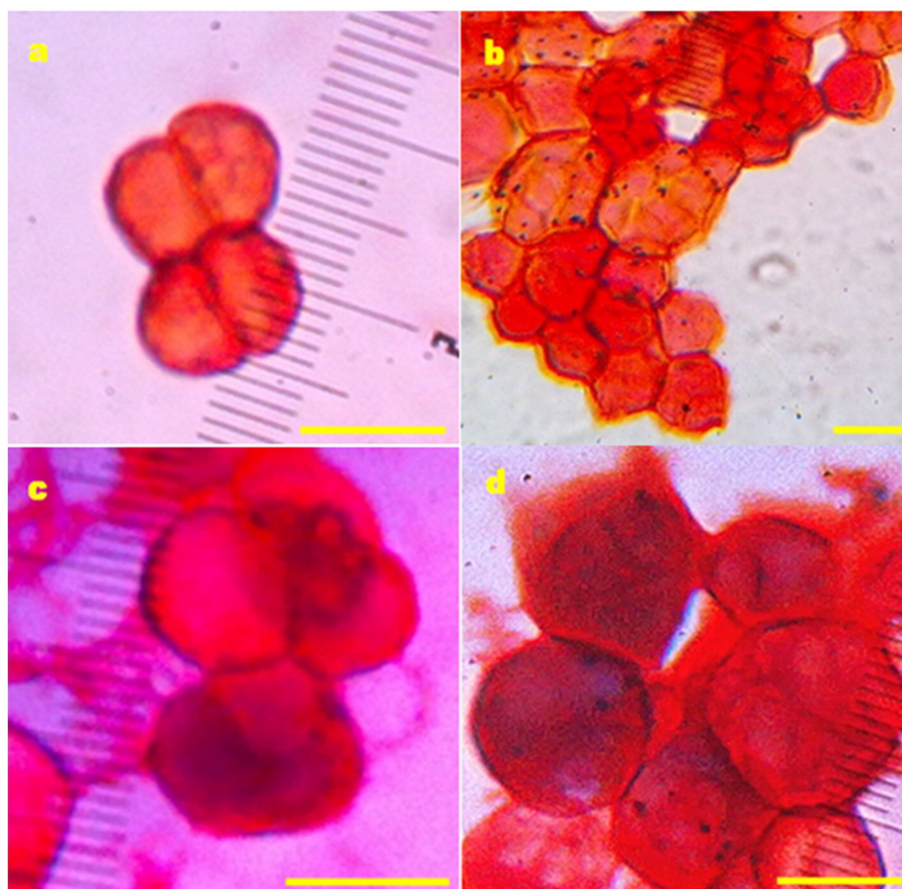

Fig. 5. Staining of lipids by Sudan Black B. a) 24 h, b) 48 h, c) 72 and d) 96 h. Scale bar = 10 μm.

Table 5
Results of one-way ANOVA for the effect of different carbon sources on biomass, lipid and DHA production.

Parameter	Sum of squares	DF	Mean^2	F-value	P > F
Biomass (g/L)					
Between carbon sources	66.22	12	5.518	283.71	0.000
Within carbon sources	0.25	13	0.019		
TFA (g/L)					
Between carbon sources	15.08	12	1.257	66.37	0.000
Within carbon sources	0.25	13	0.019		
DHA (g/L)					
Between carbon sources	4.24	12	0.353	87.25	0.000
Within carbon sources	0.05	13	0.004		

$P < 0.05$ is significant.

3.5. Utilization of carbon sources

Biomass production as well as lipid and DHA accumulation by SW1 in media containing various carbon sources are shown in Table 4. As the basal medium consists of complex nitrogen sources that could also be a source of carbon, biomass concentrations were compared to a negative control, run with lacking major carbon source. Glucose, fructose and mannose were found to be very favourable for growth (biomass considerably higher than that of the negative control, 3.2 g/L). However, most of the carbon sources, except cellulose resulted in higher biomass compared to the negative control, indicating that SW1 is able to utilize a wide variety of carbon sources.

The lipid contents of all cultures were varied to some extent, ranging from 20 to 40% of the biomass, meanwhile DHA content varied widely, ranging from 20 to 56% of TFA. Overall, among the three preferred carbon sources mentioned above, fructose resulted in the highest DHA production, which is 1.68 g/L. Notably, the highest biomass and lipid content were obtained when fructose was used (Table 4) compared to glucose and mannose. Table 5 summarizes the results of ANOVA for this experiment. Overall, each culture supplied with different carbon sources was found to yield significantly different ($P < 0.05$) amounts of biomass, lipid and DHA compared to the other cultures. This signifies that the utilization of the most suitable carbon source can directly help to enhance the total DHA production.

4. Discussion

Aurantiochytrium is ubiquitous in marine water and surrounding environments [16] such as mangroves and mudflats, acting as commensals of other organisms and as saprobes feeding on organic materials [17]. Living in such area, largely covered by water, these microbes are less exposed to temperature fluctuations due to high specific heat capacity of marine environments, thus having limited level of temperature adaptability. This could possibly explain why the cultures of both SR21 and SW1 showed declined growth at temperatures above 35 °C and also ceased growth at refrigerating temperatures. This observation is in agreement with that reported by Chodchoey and Verduyn [18] where both growth and lipid accumulation in *Aurantiochytrium mangrovei* Sk-02 were found to be decreased significantly at 35 °C compared to 30 °C, which was found to be most suitable for growth and lipid accumulation. Being sensitive to refrigerating temperatures, a convenient method of storing stock cultures at 4 °C cannot be applied for successful long term preservation of the members of this genus.

In our study, a relatively simple method has been employed to preserve the cells of SW1 and SR21, where the cells are grown to exponential phase on conventional slant agar (SNA) and thence left to remain dormant by the addition of GSM. Maintenance of thraustochytrids in this manner for long term preservation, especially at ambient temperature, has not been reported previously. Cox et al. [19] successfully developed a cryopreservation protocol using a combination of 10% DMSO, 30% horse serum, 50% By-medium and 10% cell suspension, showing that this protocol is suitable for all three thraustochytrids used in their study. However, the method employed in our study is considered to be advantageous to some extent over the more sophisticated freezing methods because it does not involve freezing and thawing processes, which cause cellular damage to the culture.

The appearance of faint orange colour in the colonies and liquid cultures of SR21 and SW1 most probably indicates the presence of carotenoids, which act as antioxidants in the cells of *Aurantiochytrium* [20,12,21]. According to Aki et al. [20] microorganisms produce carotenoids to scavenge reactive oxygen species (ROS) that are largely generated by cellular metabolism. Among the carotenoids often found in *Aurantiochytrium* are astaxanthin, echinenone and lutein [17]. Such pigments are often considered valuable and can be used as a nutraceutical in food and feeds as well as a bioactive compound for use in cosmetics due to its high antioxidant properties [22,23]. Thus, the presence of carotenoids in SW1 is an added advantage in addition to being a promising DHA producing microbe.

Successive bipartition of vegetative cells resulting in formation of cell clusters that develop into zoosporangia is distinctive characteristics of the genus *Aurantiochytrium*/*Schizochytrium* [24,25]. Exhibiting such cell reproduction mechanisms, SW1 was identified as an *Aurantiochytrium*. The cellular morphology of SW1 closely resembles that of SR21 in terms of cell size, shape and division. However, it was concluded that SW1 does not belong to the species *limacinum*, given that no amoeboid cells could be observed in the cultures of SW1. Amoeboid cells are distinctive characteristic of the species *A. limacinum*. The species name 'limacinum' originated from a Latin word, which refers to slug-like limaciform amoeboid cells [26]. Therefore, the absence of amoeboid cells in SW1 shows that this isolate does not belong to the species.

Another prominent difference between SW1 and SR21 is the structure of the zoosporangium, where they differ in size and sporangial wall thickness. Mechanism of zoospore release from the thick walled zoosporangium of SW1 is likely to be pressure-assisted. The phenomena of rapid zoospore dispersal and rupture of zoosporangium as shown in Fig. 1b and c are most probably caused by the pressure generated to initiate zoospore dispersal from the thick walled zoosporangium. To the best of authors' knowledge, such pressure-assisted spore dispersal mechanism has not been reported previously. The members of *Aurantiochytrium* are differentiated from each other by observing the mechanism of zoospore formation, the size of zoosporangia and

Table 6
Summary of biomass production and DHA content of various thraustochytrid strains in comparison to SW1 (YE: yeast extract, MSG: monosodium glutamate).

Strain	Carbon source	Nitrogen source	% DHA/TFA	Biomass (g/L)	TFA (g/L)	Reference
Schizochytrium sp. F26-b	Glucose	YE	31.8	3.5	0.915	Abe et al. [30]
ONC-18	Glucose	YE, glutamic acid	16.2–31.6	7.5–26.1	0.15–21.32	Burja et al. [12]
A. limacinum SR21	Crude glycerol	YE, Ammonium acetate	33.6	22.1	14.6	Chi et al. [31]
S. mangrovei Sk-02	Coconut water Glucose	YE	20.0	28.0	14.4	Unagul et al. [32]
A. limacinum SR21	Crude glycerol	YE, Peptone	18.3–53.1	2.5–8.0	1.1–2.5	Pyle et al. [33]
A. mangrovei MP2	Glucose	YE	0.8 (g/L)	25.4	–	Wong et al. [34]
Thraustochytriidae sp., M12-X1	Beer residues	YE, MSG	39.5–61.7	0.8–2.3	0.4–0.7	Quilodran et al. [35]
A. limacinum SR21	Sweet sorghum juice	–	34.3	9.4	6.9	Liang et al. [36]
Aurantiochytrium sp. SW1	Glucose	YE, MSG	50.1–64.1	3.32–13.17	0.97–6.44	This study

zoospores as well as the number of zoospores from each zoosporangium [26]. However, since we could not count them accurately due to rapid dispersal and scattering movement of zoospores, we were unable to identify the species of SW1.

The amount of DHA produced by SW1 in the four media could not be directly correlated to the glucose content of the medium. This can be seen by examining the Y'_{PS} value for each medium as shown in Table 2. It can be seen that DHA yield values in YEP broth and medium S are noticeably different although glucose concentration of the two media is the same. Similarly, DHA yield values of YEP broth, 790 By + and medium B are alike, despite containing considerably different amounts of glucose. This clearly shows that DHA yield does not solely depend on the amount of glucose provided, but is influenced by other medium components, such as nitrogen, vitamin and mineral sources, as well as the interaction among the components as suggested by Manikan et al. [27].

Incorporation of monosodium glutamate (MSG) as a simple organic nitrogen source is believed to encourage biomass production and lipid content of thraustochytrids. Glutamic acid is a predominant non-essential amino acid found in the marine environment in sodium salt form ($C_5H_8NNaO_4$). Chen et al. [28] reported that biomass production of *Aurantiochytrium* sp. (a thraustochytrid) is influenced by the interaction between monosodium glutamate, yeast extract and tryptone. Therefore, alteration of MSG and other nitrogen source(s) concentrations could possibly lead to higher overall DHA productivity in *Aurantiochytrium*, as optimization of nutrient conditions is vital to achieve significant levels of DHA productivity by any new isolate [5]. Table 6, reproduced and modified from Gupta et al. [29], summarizes biomass production and DHA content of various thraustochytrid strains cultivated in shake flasks from relatively latest reports (2006 onwards) in comparison to SW1.

Visualization of lipid droplet formation using Sudan dye in this study showed that in cells of SW1, initiation of lipid particle formation occurs in cytoplasm. These lipid droplets occur as free inclusions in the cytoplasm since the early stage of lipid biogenesis, in contrast to prokaryotic cells, in which formation of lipid body is speculated to be initiated at the cytoplasmic membrane as described by Wältermann et al. [37]. This is in accordance with the eukaryotic lipid biogenesis model proposed by Napier et al. [38]. The model suggests that storage lipids are accumulated between two phospholipid leaflets of the endoplasmic reticulum membrane, resulting in a budding lipid body, which is surrounded by a phospholipid monolayer directly derived from the outer membrane of endoplasmic reticulum. In prokaryotes, lipid droplets are synthesized in direct association with the cytoplasmic membrane and free cytoplasmic lipid droplets occur only at a later stage. At the early stage of storage lipid formation, small lipid domains are formed mainly at the peripheral areas of the cells [37]. This provides a comprehensive model that differentiates prokaryotic lipid body formation from eukaryotes.

SW1 was able to utilize a wide variety of carbon sources to produce considerable amounts of biomass, lipid and DHA, with glucose, fructose and mannose resulting in excellent productions compared to other sources. These results are in good agreement with that reported for many strains of *Schizochytrium*/*Aurantiochytrium*, including that by Yokochi et al. [39] for *A. limacinum* SR21, Wu et al. [40] for *Schizochytrium* sp. S31, Nagano et al. [41] for *A. limacinum* mh0186 and Gao et al. [42] for *Aurantiochytrium* sp. SD116. These reports state that hexoses are well utilized for cell growth, meanwhile pentoses, disaccharides and polysaccharides are less favourable. The simultaneous high biomass and lipid obtained using fructose indicates that utilization of this carbon source could subdue the problem of decreased lipid content at higher biomass production when glucose is utilized, as such described by Manikan et al. [27] Although, the percentage of DHA obtained using fructose is slightly lower than that obtained with glucose and mannose, high biomass and lipid content would well-compensate this, resulting in higher overall DHA productivity.

5. Conclusions

In summary, the results of this study show that *Aurantiochytrium* sp. SW1 is a potential candidate to be developed as commercial microbial DHA producer, possessing the ability to produce considerable amounts of DHA and to utilize a wide variety of carbon sources. Glucose, fructose and mannose were found to be the most favourable sugars for biomass production as well as lipid and DHA accumulation. Utilization of customized medium and culture conditions would enhance growth, lipid and DHA production of this new Malaysian strain, further revealing its potential for commercial use.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VM carried out morphological and molecular characterization, sequence alignment, fermentation and profiling, comparison and data interpretations, lipid droplet visualization studies and drafted the manuscript, MYMN: carried out experiment of the utilization of different carbon sources, MSK, MHMI and AJAK conceived of the study, and participated in its design and helped to draft the manuscript, WMWY participated in the isolation of SW1, AAH participated in the isolation of SW1, conceived of the study, participated in its design and coordination, supervised all experimental procedures and helped to draft the manuscript.

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