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SHORT COMMUNICATION

Michelle K.M. Wong · Clement K.M. Tsui
Doris W.T. Au · Lilian L.P. Vrijmoed

Docosahexaenoic acid production and ultrastructure of the thraustochytrid *Aurantiochytrium mangrovei* MP2 under high glucose concentrations

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Abstract The effect of high glucose concentrations on the ultrastructure and the production of docosahexaenoic acid (DHA) by *Aurantiochytrium mangrovei* MP2 was investigated at 25°C with orbital shaking. The cultured cells were separated into a floating and a bottom layer after centrifugation during harvest; therefore, the ultrastructure and DHA level were also analyzed separately. Cell size generally increased with glucose concentrations, whereas the overall DHA production (mg/l) increased 19% when the glucose concentration was raised from 6% to 10% w/v. Biomass and DHA production increased significantly, but not linearly, at the floating layer and decreased at the bottom layer in elevated glucose concentrations. Also, the lipid bodies of the cells in the floating layer were more heavily stained in osmium tetroxide than those in the bottom, suggesting that the cells in the floating layer may contain greater amount of unsaturated fatty acids.

Key words Docosahexaenoic acid · Labyrinthulomycetes · Lipids · Polyunsaturated fatty acids · *Schizochytrium*

Docosahexaenoic acid [DHA; 22:6 (n-3)] and eicosapentaenoic acid [EPA; 20:5 (n-3)], two of the omega-3 long-chain polyunsaturated fatty acids (PUFA), have received much attention because of their beneficial effects for humans (Nettleton 1995). The PUFAs are vital for the normal development of infant eyes and brain (Takahata et al. 1998), and they may be crucial in the prevention of diseases such as cancer and stroke (Nettleton 1995; Sanders et al. 2006). Marine fish such as salmon, mackerel, and tuna have been the major commercial sources of DHA. However, there are drawbacks: the DHA levels in their tissues are variable, and they are not sustainable long-term resources as a result of pollution and global climate change (Lewis et al. 1999). To meet the increasing demand for consumption of PUFA, a wide range of autotrophic and heterotrophic microbes, including bacteria, marine algae, fungi, and protists, has been screened as alternative sources of EPA and DHA (Barclay et al. 1994; Lewis et al. 1999; De Swaaf et al. 2003). Microalgae (*Crypthecodinium cohnii* Seligo) and various isolates of thraustochytrids have emerged as promising sources for DHA production (Lewis et al. 1999; Barclay et al. 2005).

Thraustochytrids are fungus-like microbes, but they are closely related to heterokont algae, and they are classified under the Labyrinthulomycetes (Honda et al. 1999). They have a rapid growth rate and are capable of producing significant amounts of PUFA, including DHA. Presently, two isolates, *Schizochytrium* sp. 20888 and *Ulkenia* sp., are being exploited for commercial DHA production (Barclay et al. 2005; Kiy et al. 2005). These strains serve as livestock feed supplements and as poultry feed additives for the production of DHA-enhanced eggs (Franklin et al. 1999; Abril et al. 2000).

We have been studying the physiological characteristics and the DHA production from thraustochytrids, including representatives of *Schizochytrium*, *Thraustochytrium*, and *Ulkenia*, in Hong Kong (Fan et al. 2001, 2002). *Aurantiochytrium mangrovei* (Raghuk.) R. Yokoyama & D. Honda (formerly *Schizochytrium mangrovei* Raghuk.) has received much attention because the species accumulates significant amounts of PUFA in their lipids in medium containing 6% glucose at 25°C (Fan et al. 2002; Yokoyama and Honda 2007). The aim of the present study was to investigate the DHA production of *A. mangrovei* under high glucose concentrations. Because the growth of thraustochytrids was retarded at 12% w/v glucose (Yokochi et al. 1998), we maintained the glucose levels below 10% w/v. We also investigated lipid body formation in *A. mangrovei* by electron microscopy. While we were harvesting the cells for DHA

M.K.M. Wong · C.K.M. Tsui[1] (✉) · D.W.T. Au · L.L.P. Vrijmoed (✉)
Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon Tong, Hong Kong Special Administrative Region, China
Tel. +852-2788-9966; Fax +852-2788-9922
e-mail: clementsui@gmail.com, bhlilian@cityu.edu.hk

[1]*Present address*:
Department of Forest Science, Faculty of Forestry, The University of British Columbia, 2424 Main Mall, Vancouver, V6T 1Z4, Canada

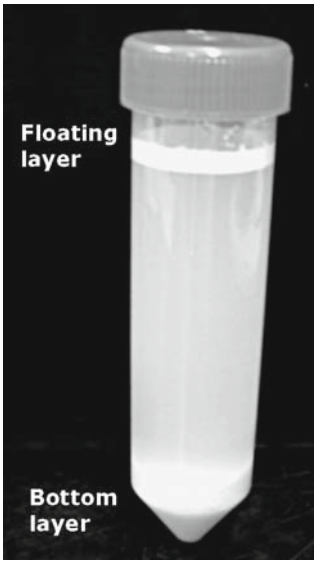

Fig. 1. The cells of *Aurantiochytrium mangrovei* MP2 were separated into two layers after centrifugation at 3500 rpm for 10 min (grown at 8% glucose concentration)

analysis, the cells separated into a floating layer and a pellet at the bottom of the centrifuge tube (Fig. 1). Therefore, we analyzed and studied the DHA levels and the ultrastructure of the cells from the two layers separately in an attempt to test any difference in the morphological and biochemical characteristics between these two layers.

Aurantiochytrium mangrovei MP2 was isolated from the decaying leaves of *Kandelia candel* (L.) Druce in the Mai Po Nature Reserve in Hong Kong. Leaf samples were taken to the laboratory and cut into leaf disks (~1.5 cm in diameter). The leaf disks were washed three times in sterile seawater (SW) (15‰), supplemented with penicillin G and streptomycin sulfate (both 1 mg/ml; Sigma). The disks were then homogenized in 5 ml SW at 9500 rpm for 2 min by an ultra-turrax homogenizer (IKA-Labortechnik; Janke & Kunel, Staufen, Germany) and plated onto yeast extract-peptone (YEP) agar [1 g/l yeast extract (Oxoid), 1 g/l mycological peptone (Difco), 13 g/l agar technical (Difco), 15 g/l artificial sea salt (ASS) (Sigma)]. The agar plates were incubated at 25°C for 24 h with 1 ml sterile SW.

A seed culture was prepared by transferring cells from the YEP agar plates into a 250-ml flask containing 50 ml glucose-yeast extract (GYE) medium (10 g/l glucose; 10 g/l yeast extract; 15 g/l ASS). The flask was incubated at 25°C at 200 rpm for 48 h. An aliquot (2 ml) of the seed cultures was transferred into flasks in triplicates containing 50 ml GYE medium with 6%, 8%, or 10% w/v glucose and 1% (w/v) YE, and incubated on an orbital shaker at 200 rpm at 25°C for 4 days. Cells were harvested by centrifugation at 3500 rpm for 10 min, and the bottom pellets were washed twice with sterile water. The floating layer was transferred into another tube, followed by a second centrifugation, and the medium underneath was removed by a syringe needle. The cells were freeze dried and weighed.

DHA was determined using a modified procedure of Lepage and Roy (1984). Briefly, freeze-dried cells (approximately 100 mg) were methylated in 1 ml 4 % (v/v) sulfuric acid in methanol with the addition of an internal standard (3 mg heptadecaenoic acid, C17:0; Sigma). The mixture was heated at 100°C for 1 h. After cooling, the fatty acid methyl esters (FAMEs) were extracted twice by 1 ml water and hexane (1:1). The FAMEs (1 µl) in the hexane layer were subjected to gas chromatography equipped with a flame ionization detector (Agilent 6890 GC-FID) with a split injector, an autosampler, and a DB-225 capillary column (30 mm × 0.25 mm diameter). Injector was held at 220°C; initial temperature was 90°C for 3 min, then increased from 90°C to 210°C at 20°C/min, and finally the temperature was raised to 230°C at 10°C/min. The detector was held at 230°C, helium was used as carrier gas, and the column flow rate was 1 ml/min. DHA was identified and quantified by a comparison of retention time for laboratory standard and internal standard.

Thraustochytrid cells culturing at different glucose levels were examined under an interference light microscope. Sudan Black B stain (0.7% w/v in ethylene glycerol) was also applied to observe the abundance of lipid bodies in the cells. Transmission electron microscopy (TEM) was carried out according to Honda et al. (1998). An aliquot (400 µl) of thraustochytrid cells was mixed quickly with 400 µl fixative solution (10% w/v glutaraldehyde, 0.8% v/v OsO_4, 0.4 M sucrose in 0.2 M cacodylate buffer). After incubation on ice for 35 min, fixed cells were centrifuged at 3500 rpm for 10 min and rinsed once with 0.1 M ice-cold cacodylate buffer. They were redissolved in 0.2 M cacodylate buffer before postfixation in an equal volume of 4% v/v OsO_4 (the resulting suspension was in 2% OsO_4 in 0.1 M cacodylate buffer). After washing in 0.1 M ice-cold cacodylate buffer, fixed cells (preembedded in 1% agarose gel) were dehydrated in a gradual ethanol series from 10% to 90% (in 20-min-interval steps), and finally three washes of 100% (15 min each). Dehydrated specimens were infiltrated and embedded in Spurr's resin. Ultrathin sections (80–90 nm) prepared on a ultramicrotome (Leica Ultracut UCT) were stained with lead citrate for 20 min and uranyl acetate solution for 20 min before examination under a transmission electron microscope (TEM) (FEI/Philips; Tecnai 12) at 80 kV.

Statistics were performed by one-way analysis of variance (one-way ANOVA) using the statistical software SigmaStat 2.03 (SPSS, Chicago, IL, USA). Where significant differences were identified ($P < 0.05$), all pairwise multiple comparisons between each treatment were carried out using a Tukey test. Data were $\log_{10}$ transformed before the test if they were not normally distributed.

The cells of *A. mangrovei* MP2 are spherical and filled with numerous lipid bodies (Fig. 2a). The cells ruptured easily under freshwater, and the cytoplasm was released readily from the cells (Fig. 2b). The variations of cell size increased with glucose concentration. The greatest variations were observed at 10% glucose, in which the cell size ranged from 10.6 to 45.1 µm (mean = 15.6 ± 6.1 µm; $n = 35$). The TEM image revealed the lipid bodies were also abundant. Some lipid bodies had fine structures consisting of alternating light- and dark-staining bands (Fig. 2c). These fine structures have been reported previously, and probably the patterns were related to the ratio of saturated and monounsaturated fatty acids to highly unsaturated fatty

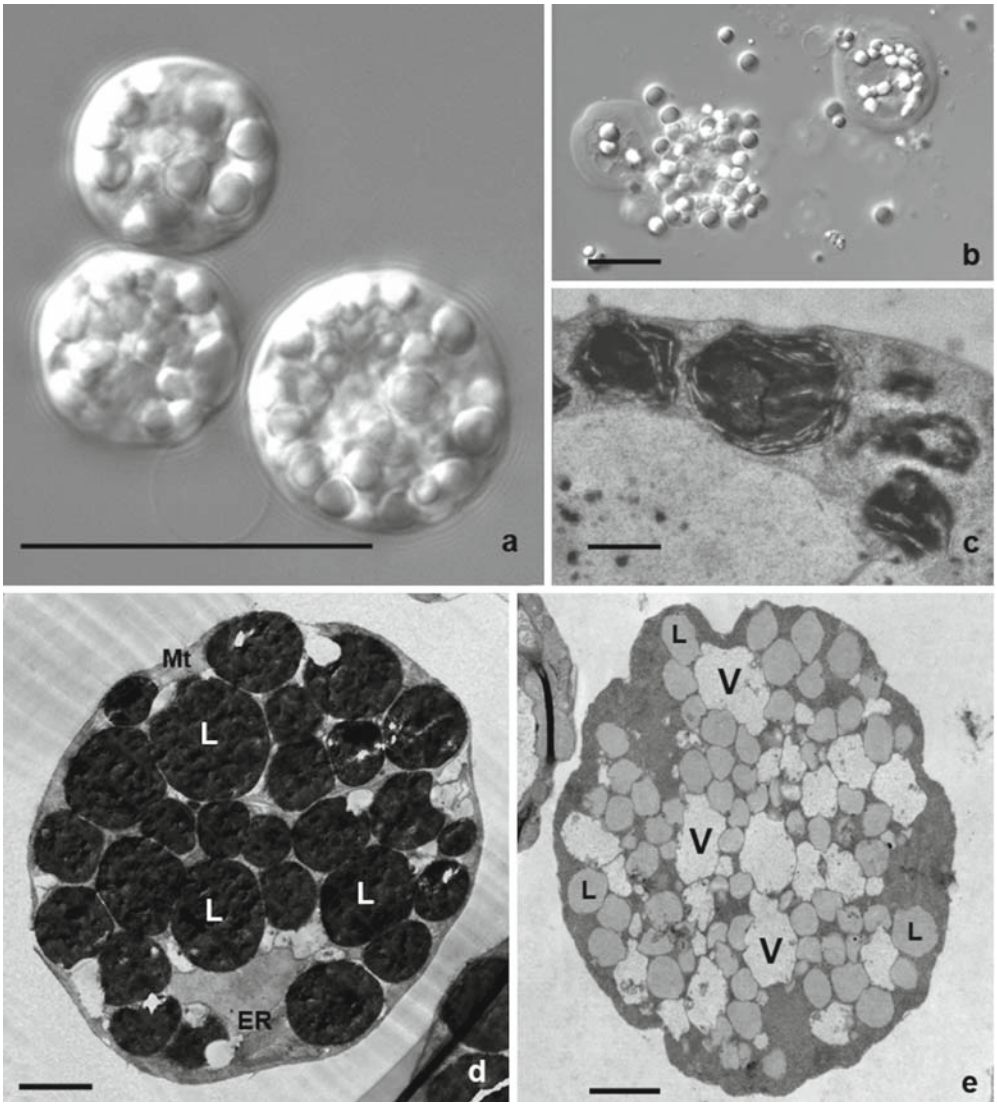

Fig. 2. Interference and transmission electron micrographs of *Aurantiochytrium mangrovei* MP2 cells. **a** Vegetative cells full of lipid bodies mounted in 15‰ seawater in starter medium. **b** Ruptured cells released lipid bodies in freshwater mount. **c** TEM micrographs of the lipid bodies containing alternating light- and dark-staining bands. **d** TEM micrograph of cells grown at 6% (w/v) glucose collected in the floating layer. Lipid bodies were larger and electron dense. **e** TEM micrograph cells at the bottom layer were smaller and electron translucent (also grown at 6% (w/v) glucose). Cells contained vacuole (*V*), mitochondrion (*Mt*), endoplasmic reticulum (*ER*) and lipid (*L*). *Bars* **a** 30 μm; **b** 20 μm; **c** 0.8 μm; **d, e** 1.5 μm

acids (Weete et al. 1997; Ashford et al. 2000; Morita et al. 2006). These structures may also be indicative of secondary structure in the triacylglycerols (Ashford et al. 2000), but they became indistinct during lipid body enlargement (Weete et al. 1997).

The lipid bodies of *Aurantiochytrium* cells from the floating layers of various medium glucose levels were found to be more electron dense than those from the bottom layers (Fig. 2d,e), suggesting the lipid bodies in the floating layer have higher levels of unsaturated fatty acids. As osmium tetroxide binds at double bonds of unsaturated fatty acid chains (Bozzola and Russell 1999), lipids that conferred much electron-dense signal contained a considerable level of unsaturated rather than saturated fatty acids. The reason for "floating cells having greater unsaturated fatty acids" is currently unclear and requires further investigation. Presumably, the presence of *cis* double bonds may interrupt the fully extended conformation of the fatty chain and prevent individual PUFAs from packing with each other (Bell et al. 1986). Normally lipids have higher compressibility and thermal expansion (Yayanos et al. 1978), and therefore cells having greater amount of lipids achieve positive buoyancy and float to the top. Cells with more unsaturated fatty acids are gravitationally less dense than those with more saturated fatty acids, hence making the former float to the top.

The biomass increased gradually with glucose concentration but reached a plateau at 10%. The biomass production was 22.14, 27.35, and 25.40 g/l ($P < 0.05$) at 6%, 8%, and 10% glucose, respectively. The increase of biomass with increasing glucose concentrations agrees well with previous findings (Yokochi et al. 1998; Bowles et al. 1999; Wu et al. 2005). In fact, the cells in the floating layer increased significantly, from 3.80 to 11.23 to 18.38 g/l ($P < 0.05$), compared with those in the bottom layer, which decreased significantly from 18.34 to 16.12 to 7.03 g/l ($P < 0.05$), with increasing glucose concentrations from 6% to 10% (Fig. 3a).

The DHA concentrations (mg/g) of cells at the floating layer also increased significantly, from 20 to 26 to 37 mg/g ($P < 0.05$), compared with those in the bottom layer, which decreased from 34 to 28 to 24 mg/g ($P < 0.05$) (Fig. 3b). The

Table 1. A comparison of biomass and docosahexaenoic acid (DHA) production among different strains of *Aurantiochytrium mangrovei* and *A. limacinum*

Organism	Medium ($g\ l^{-1}$)	Biomass ($g\ l^{-1}$)	DHA (% w/w)	DHA ($g\ l^{-1}$)	Reference
A. mangrovei MP2	Glucose 90, YE 10, ASS 15	25.4	3	0.8	This study
A. mangrovei KF6	Glucose 60, YE 10, ASS 15	15.2	20.3	3.1	Fan et al. 2001
A. mangrovei SK2	Glucose 75, YE 10, ASS 15	22.5	22.5	4.7	Unagul et al. 2005
A. limacinum SR21	Glucose 90, corn steep liquor 20, ASS 15	36	11.7	4.2	Yokochi et al. 1998

ASS, artificial sea salt; YE, yeast extract

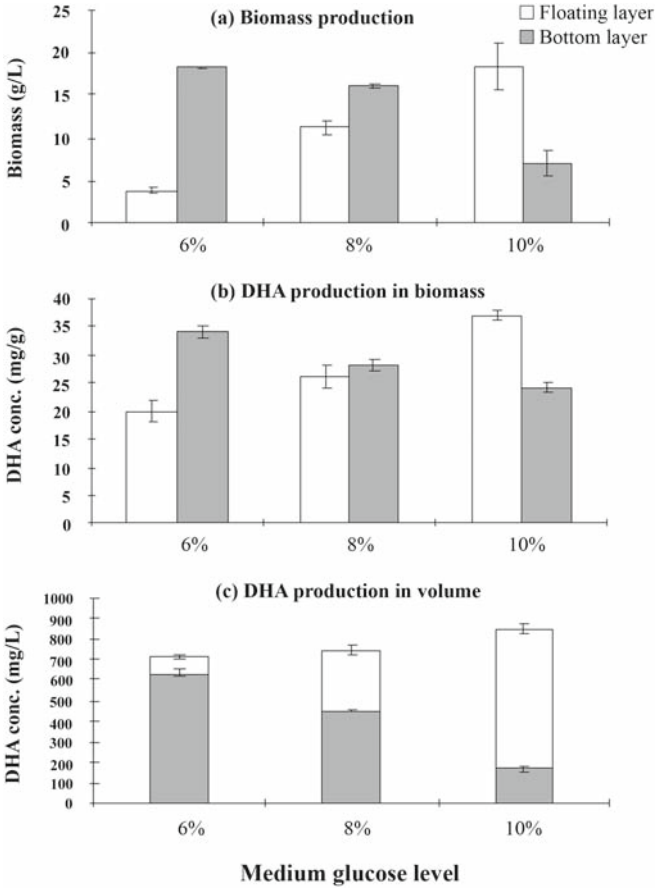

Fig. 3. Biomass and docosahexaenoic acid (DHA) production of *Aurantiochytrium mangrovei* MP2 under various glucose concentrations

overall DHA production (mg/l) of *A. mangrovei* increased significantly, from 708 mg l^{-1}, to 744 mg l^{-1}, to 845 mg l^{-1} ($P < 0.05$) (Fig. 3c) when culturing at glucose concentrations 6%, 8%, and 10% (w/v), respectively, because of the continued biosynthesis of fatty acids in the presence of glucose (Yokochi et al. 1998).

Weete et al. (1997) reported a separation of layers during the fermentation of *Thraustochytrium* sp. but they pooled the two layers together for analysis. Our study illustrated that the thraustochytrid cells are not homogeneous in morphology and lipid composition when being fermented but can only be differentiated into two layers when they are subjected to centrifugation.

High glucose concentration did not necessarily improve the DHA production yield in linear scale. In our study, a 66.7% increase in glucose amount (from 6% to 10% w/v) resulted in only a 19% increase in DHA production (from 708 to 845 mg/l). Unagul et al. (2005) estimated that 58% of the glucose in the medium was not fully converted into fatty acids, suggesting the presence of other limiting factors in the biosynthetic pathway. *Aurantiochytrium mangrovei* is a close relative of *Aurantiochytrium limacinum* (D. Honda & Yokochi) R. Yokoyama & D. Honda (Yokoyama and Honda 2007). The DHA production capacity of MP2 was lower than conspecific or closely related strains reported elsewhere (Fan et al. 2001; Unagul et al. 2005) (Table 1); this may be attributed to natural variation in strain physiology. However, the relatively low productivity at bench scale does not completely rule out the potential for commercial application because scaled-up fermentation can greatly improve the yield of DHA production. The productivity of *Schizochytrium* sp. ATCC 20888 was improved from 20% to 46% after scaling up in an industrial fermentor (Barclay et al. 2005).

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