

Relationship between Morphology and Docosahexaenoic Acid Production by *Schizochytrium* sp. BCC 25505 and 25509 in Batch and Pulsed-Batch Cultivation

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The relationship between morphology of *Schizochytrium* sp. BCC 25505 and 25509 and production of docosahexaenoic (DHA) were studied in batch and pulsed-batch processes in optimized medium. In batch cultivation, the highest production by *Schizochytrium* sp. BCC 25505 (60-100% vegetative cells for 72 h) of biomass, DHA, and total fatty acid were 38.7 ± 0.14 g/L at 72 h, 5.9 ± 1.56 g/L at 63 h, and 13.03 ± 0.09 g/L at 54 h, respectively, whereas that of *Schizochytrium* sp. BCC 25509 (70-100% vegetative cells for 72 h) were 41.35 ± 0.46 g/L at 72 h, 12.09 ± 0.81 g/L at 69 h, and 26.32 ± 2.28 g/L at 66 h. In pulsed-batch cultivation, the highest production by *Schizochytrium* sp. BCC 25505 (57-86% vegetative cells) of biomass, DHA, and total fatty acid were 97.05 ± 1.63 g/L at 166 h, 20.73 ± 1.21 g/L at 166 h, and 35.24 ± 3.27 g/L at 166 h, respectively, whereas that of *Schizochytrium* sp. BCC 25509 (25-94% vegetative cells) were 101.75 ± 2.19 g/L at 166 h, 20.0 ± 1.31 g/L at 143 h, and 36.48 ± 3.97 g/L at 118 h, respectively. Synchronous vegetative growth in high nitrogen content medium and in controlled high glucose supply for lipid accumulation enhanced efficiency of DHA production.

Key words: *Schizochytrium*, Docosahexaenoic acid, High cell density, Oleaginous microorganism, Polyunsaturated fatty acid, *Thaustochytrid*.

Polyunsaturated fatty acid (PUFAs) play an important role in human health. The ω -3-fatty acid, docosahexaenoic acid (DHA), is essential for functional development and maintenance of human brain and retina¹. DHA also is involved in the prevention of cardiovascular diseases and in improving neural and retinal development in infants²⁻⁴. DHA has been added to infant formulae⁵, food and beverage for human consumption⁶ and animal and aquaculture feed⁷⁻⁸.

The largest source of PUFAs, especially DHA, comes from marine fish, but other organisms also can synthesize DHA at high yields, including

*Aurantiochytrium*⁹, *Cryptocodinium cohnii*¹⁰⁻¹², and *thraustochytrids*¹³⁻¹⁸. *Thraustochytrids* are marine protists, whose dominant genera are *Thraustochytrium*, *Aurantiochytrium* and *Schizochytrium* and belong to the kingdom *Chromista*¹⁶. *Schizochytrium* sp. can synthesize PUFAs containing 30-40% DHA¹⁹⁻²² and has become an industrial source of DHA²³.

Cultivation of *Schizochytrium* for industrial production of DHA involves two phases: cell growth (biomass production) and lipid accumulation. The early stage of cell growth is dependent on nitrogen supply and the later stage of cell enlargement for lipid accumulation on carbon supply²⁴⁻²⁵ (Figure 1). In order to achieve high accumulation of lipids and DHA production, synchronous *Schizochytrium* cell morphologies

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have to be controlled during both phases of growth at high cell density and lipid accumulation²⁶.

Although *Schizochytrium* is an established microorganism for the commercial production of long-chain PUFAs including DHA at low cost, there is still a need to develop an optimal fermentation process that can provide increased biomass via high-cell density cultivation^{6, 25}. In the present study, the relationship between *Schizochytrium* sp. morphology and DHA production in batch and pulsed-batch fermentation processes were compared. Morphological control using pulsed-batch cultivation was investigated.

MATERIALS AND METHODS

Microorganism strains

Schizochytrium sp. BCC 25505 and BCC 25509, marine protists, were collected from mangrove leaf in the gulf of Thailand and identified by Dr. Panida Unagul, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Thailand. The microbes were deposited in BIOTEC Culture Collection. *Schizochytrium* sp. BCC 25505 and BCC 25509 were maintained at 22 °C by monthly sub-cultivation on GPY medium (glucose 10 g/L; peptone 1 g/L; yeast extract 1 g/L) supplemented with 15 g/L agar (Difco, Franklin Lakes, USA) and 15 g/L artificial sea salts (Sigma, St. Louis, USA). *Schizochytrium* sp. BCC 25509 had higher growth rate and higher lipid content than BCC 25505.

Inoculum preparation

Schizochytrium sp. BCC 25505 and BCC 25509, stored in 20% (v/v) glycerol at -80 °C, were grown in GYP medium (20 g/L glucose, 10 g/L peptone and 5 g/L yeast extract) at 28 °C for 3 days. Seed cultures were prepared in a basal medium comprising of 40 g/L glucose, 17.57 g/L yeast extract, 2.56 g/L ammonium dihydrogen phosphate, 12.32 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mL/L Ami G (waste from glutamic acid production; Ajinomoto Co., Ltd, Samutprakarn, Thailand), and 1 mL/L multivitamins complex (Blackmores, NSW, Australia), on a rotary shaker (250 rpm) at 28 °C for 24-36 h. A 10% (v/v) inoculum was used as starter culture in each experiment.

Batch fermentation

Medium used in a 5-L batch bioreactor

(B. Braun, Goettingen, Germany) consisted of 40 g/L glucose, 17.57 g/L yeast extract, 2.56 g/L ammonium dihydrogen phosphate, 12.32 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mL/L Ami G, and 1 mL/L vitamins complex, with a working volume of 3.5 L, cultivation temperature of 28 °C, agitation at 300 rpm, aeration rate of 1 vvm and cultivation for 2-3 days. A 1 mL aliquot of fermentation broth was removed every 3 h for measurements of dry weight, residual glucose concentration, *Schizochytrium* sp. morphology, and DHA and TFA contents.

Pulsed-batch fermentation

When 1-5 g/L glucose was left in the fermentation broth of the batch cultivation, fresh medium (800 g/L glucose, 12 g/L sodium nitrate, 0.5 g/L KCl, 2.0 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.65 g/L K_2SO_4 , 3.0 g/L KH_2PO_4 , 10 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.17 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3.0 g/L yeast extract, 10.0 g/L $\text{NH}_4\text{H}_2\text{PO}_4$, 1 mL/L vitamins solution, 0.35 mL/L antifoam (PMX-200, Dow Corning, MI, USA), and 1 mL/L trace solution (4.5 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 3.0 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g/L H_3BO_3 , 0.1 g/L KI, 1.0 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.4 g/L $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 4.5 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) was pulsed into the bioreactor to obtain a medium of 40 g/L glucose.

Dry biomass determination

Cells were harvested by centrifugation at 10,000 g for 5 min and washed twice with distilled water followed by freeze-dried in pre-weighed tubes for 72-96 h. Tubes were weighed and the amounts of dry biomass calculated.

Fatty acid extraction and analysis

Two mL aliquot of 4% sulfuric acid in methanol containing butylated hydroxytoluene (0.1 g/L) (as anti-oxidant) and C17:0 (as internal standard) was added to freeze-dried cell pellet (10-20 mg) in a vial equipped with tightly fitting Teflon-lined cap, and incubated at 90 °C for 1 h. Following rapid cooling, the produced fatty acid methyl esters were extracted twice with 2 mL of hexane:water (1:1) solution and a few grains of sodium sulfate were added to the hexane layer to absorb any remaining water. Then the hexane sample was injected using an auto-injector with a 1:100 split into a Shimadzu GC-17A instrument (Osaka, Japan) equipped with Supelco Omegawax-250 (Supelco, Bellefonte, USA) fused silica capillary column (30 m in length, 0.25 mm internal diameter). Injector and detector temperature was 250 and 260 °C respectively. Carrier

gas was helium with a linear flow of 30 cm/s. Column temperature was kept at 200 °C for 10 min and then increased to 230 °C at a rate of 10 °C/min. After 17 min at 230 °C, the column was cooled to 200 °C and a new cycle initiated. Calibration was performed using a mixture of fatty acid methyl ester (FAME) standards (Sigma no 189–19) as well as DHA-FAME (Sigma). In addition, the procedure was tested using methylated C17:0 and DHA (Sigma) and recovery was determined against FAME standards.

Morphological study

Protist morphologies were visualized under a compound microscope (Leica Microsystems CMS GmbH, Germany) (400x magnification) and counted using a hemacytometer.

Sugar analysis

Supernatant was centrifuged at 10,000 *g* for 10 min and filtered through a 0.22 µm paper filter (Millex, MA, USA). Filtrate was subjected to HPLC analysis using Aminex HPX-87H column (Bio-Rad, Hercules, Calif.) in a (Waters, MA, USA) equipped with a Waters 410 differential refractometer detector (Millipore Corp., Milford, MA, USA) at 60 °C using 5 mM H₂SO₄ as a mobile phase at a flow rate of 0.6 mL/min.

RESULTS AND DISCUSSION

DHA production by *Schizochytrium* sp. BCC 25505 and BCC 25509 in 5-L batch bioreactor

Batch cultivation in a 5-L bioreactor of *Schizochytrium* sp. BCC 25505 and BCC 25509 produced highest biomass of 38.7 ± 0.14 g/L at 72 h and 41.35 ± 0.46 g/L at 72 h (Fig. 2A and 3A), complete glucose consumption after 63 h with highest DHA yield of 5.9 ± 1.56 g/L and after 54 h

with 12.09 ± 0.81 g/L (at 69 h) (Fig. 2B and 3B), and highest total fatty acid content of 13.03 ± 0.09 g/L at 54 h with DHA/TFA ratio ranging from 27.3 to 50.75% and 26.32 ± 2.28 g/L at 66 h with ratio ranging from 24 to 53% (Fig. 2B and 3B), respectively.

For *Schizochytrium* sp. BCC 25505, 67–100% vegetative cells were observed throughout the first 60 h of cultivation but decreased thereafter. When glucose was completely consumed (after 63 h), increasing numbers of zoospores were observed (Fig. 4A). As for *Schizochytrium* sp. BCC 25509, 70–100% vegetative cells were observed throughout 72 h of cultivation (Fig. 4B).

The medium used in this study is optimized for growth and DHA production of *Schizochytrium* sp., in which high concentrations (50 mM) of magnesium salts are included to maintain osmotic pressure in order to allow the protists to grow synchronously as vegetative cells in a medium of high nitrogen content followed by that of high carbon content for lipid production (26). Synchronous vegetative growth of *Schizochytrium* sp. BCC 25505 and BCC 25509 were obtained during the early phase (48 h) in the

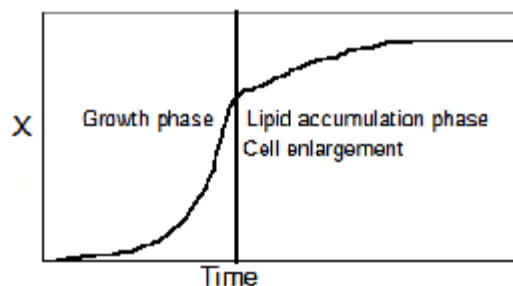


Fig. 1. Growth and lipid accumulation phases of oleaginous microorganisms.

Table 1. Maximum biomass, docosahexaenoic acid (DHA), total fatty acid (TFA) and amount of vegetative cells of *Schizochytrium* sp. BCC 25505 and BCC 25509 in batch and pulsed-batch fermentation processes

Variable	<i>Schizochytrium</i> sp. BCC 25505		<i>Schizochytrium</i> sp. BCC 25509	
	Batch	Pulsed-batch	Batch	Pulsed-batch
Biomass (g/L)	38.70 ± 0.14	97.05 ± 1.63	41.35 ± 0.46	101.75 ± 2.19
DHA (g/L)	5.90 ± 1.56	20.73 ± 1.21	12.09 ± 0.81	20.00 ± 1.31
TFA (g/L)	13.03 ± 0.09	35.24 ± 3.27	26.32 ± 2.28	36.48 ± 3.97
Vegetative cells (%)	60-100	57-86	70-100	72-94
Average vegetative cells (%)	82.10 ± 15.31	71.32 ± 10.12	89.50 ± 8.90	73.17 ± 20.64

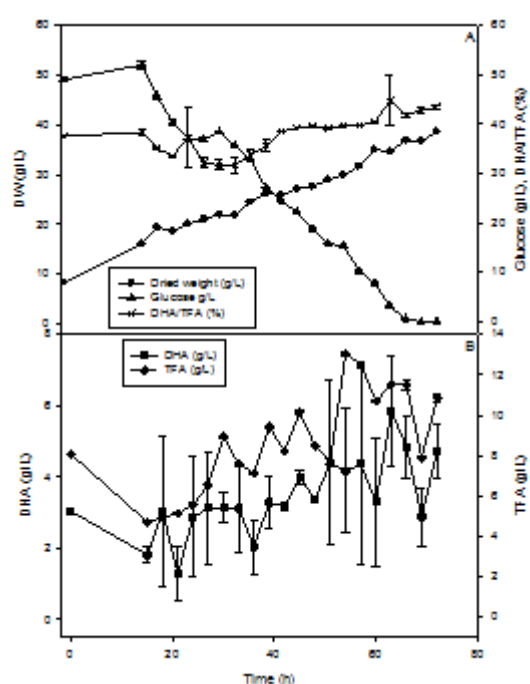


Fig. 2. Time profile of (A) glucose concentration, dry weight (DW), and ratio of docosahexaenoic acid (DHA)/total fatty acid (TFA) and of (B) DHA and TFA of *Schizochytrium* sp. BCC 25505 cultivated in a 5-L batch bioreactor

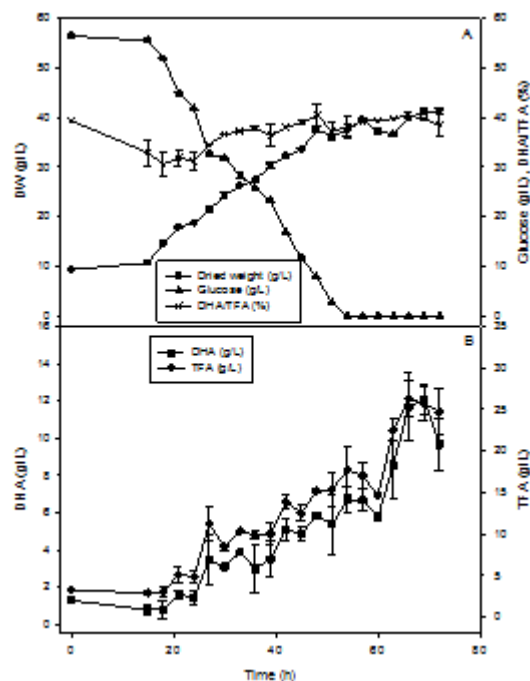


Fig. 3. Time profile of (A) glucose concentration, dry weight (DW), ratio of docosahexaenoic acid (DHA)/total fatty acid (TFA) and of (B) DHA and TFA of *Schizochytrium* sp. BCC 25509 cultivated in 5-L batch bioreactor

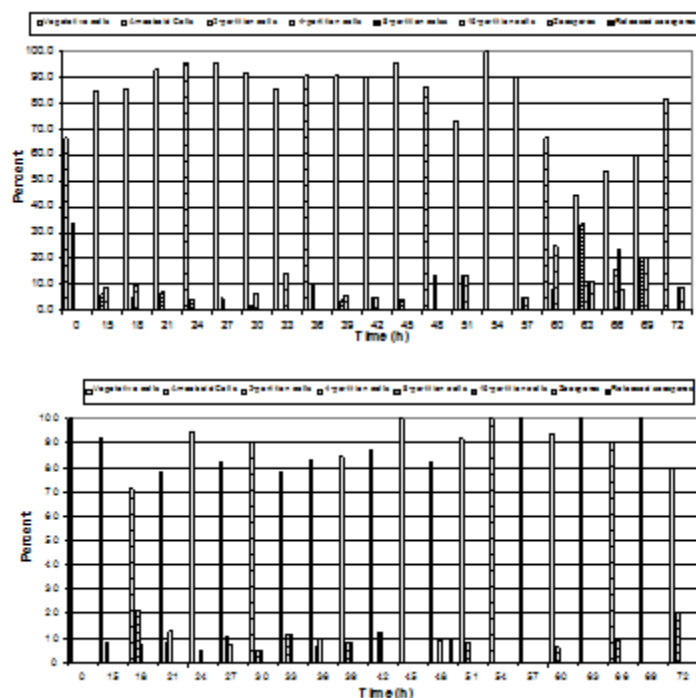


Fig. 4. Time profile of morphology of (A) *Schizochytrium* sp. BCC 25505 and (B) *Schizochytrium* sp. BCC 25509, during batch cultivation in 5-L bioreactor.

condition of nitrogen excess, and low amounts of DHA and TFA were synthesized. During the later phase of excess of carbon source from non-glucose repression medium, lipid accumulation occurred resulting in higher DHA and TFA content. The higher number of synchronous vegetative *Schizochytrium* sp. BCC 25509 cells compared to BCC 25505 accounts for the better performance of the former strain in terms of biomass, DHA and TFA production.

DHA production by *Schizochytrium* sp. BCC 25505 and BCC 25509 in 5-L pulsed-batch bioreactor

Pulsed-batch cultivation in a 5-L bioreactor of *Schizochytrium* sp. BCC 25505 and BCC 25509 produced the highest biomass of 97.05 ± 1.63 g/L at 166 h and 101.75 ± 2.19 g/L at 166 h (Fig. 5A and 6A), highest DHA yield of 20.73 ± 1.21 at 166 h and 20.0 ± 1.31 g/L at 143 h, highest total fatty acid content of 35.24 ± 3.27 at 166 h and 36.48 ± 3.97 g/L at 118 h (Fig. 5B and 6B), and DHA/TFA ratio of 40% and 35-44% (Fig. 5A and 6A),

respectively. For *Schizochytrium* sp. BCC 25505, 57-86% vegetative cell were observed during 166 h of cultivation but amoeboid cells, with 2, 4 and 6 partitions, were found thereafter (Fig. 7A). As for *Schizochytrium* sp. BCC 25509, 72-94% vegetative cells were observed during 166 h of cultivation (Fig. 7B). At 166 h of fed-batch cultivation, *Schizochytrium* sp. BCC 25505 and BCC 25509 had larger lipid-accumulating cells compared to those in the earlier stages of cultivation (Fig. 8). The majority of *Schizochytrium* sp. cells were maintained at the vegetative stage throughout the pulsed-batch cultivation process.

In pulsed-batch cultivation, both strains had similar numbers of vegetative cells and production of DHA, but different TFA content, and this might be due to the non-glucose repression level. Under stress conditions of glucose depletion and glucose repression, protist morphologies changed to other stages (viz. zoospores and released zoospores) resulting in lower DHA production. As *Schizochytrium* is a slow growing

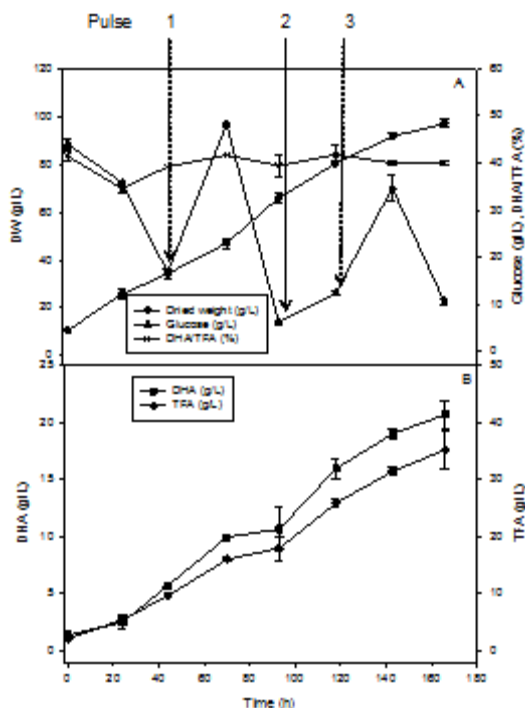


Fig. 5. Time profile of docosahexaenoic acid (DHA), total fatty acid (TFA), ratio of DHA/TFA, glucose concentration and dry weight (DW) of *Schizochytrium* sp. BCC 25505 cultivated in a 5-L pulsed-batch bioreactor

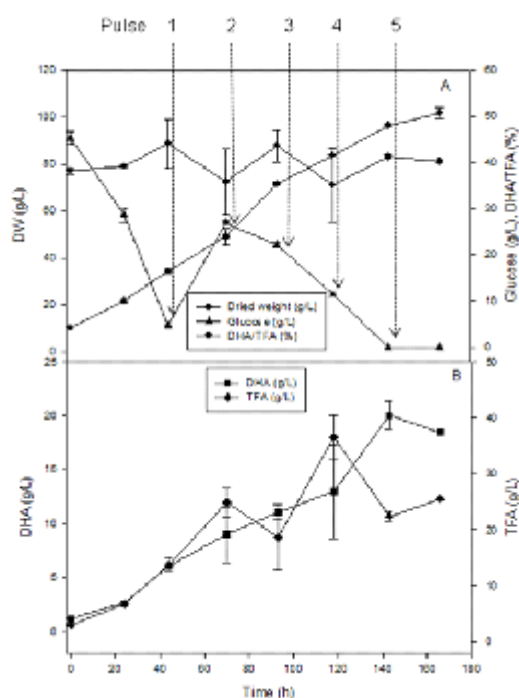


Fig. 6. Time profile of docosahexaenoic acid (DHA), total fatty acid (TFA), ratio of DHA/TFA, glucose concentration and dry weight (DW) of *Schizochytrium* sp. BCC 25509 cultivated in 5-L pulsed-batch bioreactor.

heterotroph consuming glucose at a slow rate, cultivation in a non carbon catabolite repression condition or in an increased acetyl-CoA synthetase state gives rise to higher growth and glucose consumption rates²⁷. Similar findings have been reported regarding excess nitrogen supply for optimal cell growth and excess carbon feeding for cell enlargement to accommodate lipid accumulation²⁴. Synchronous growth of *Schizochytrium limacinum* SR21 affects lipid and fatty acid composition²⁸. Fed-batch process of high

cell density has been reported to be the more efficient in DHA production by *Schizochytrium* sp. compared with batch process²⁵.

Thus, in order to obtain high DHA production by *Schizochytrium* sp., early phase synchronous vegetative growth has to be maintained by supplying high nitrogen content, which is then followed by controlled high glucose supply to allow size enlargement of vegetative cells for lipid accumulation. This strategy is recommended for high cell density cultivation of

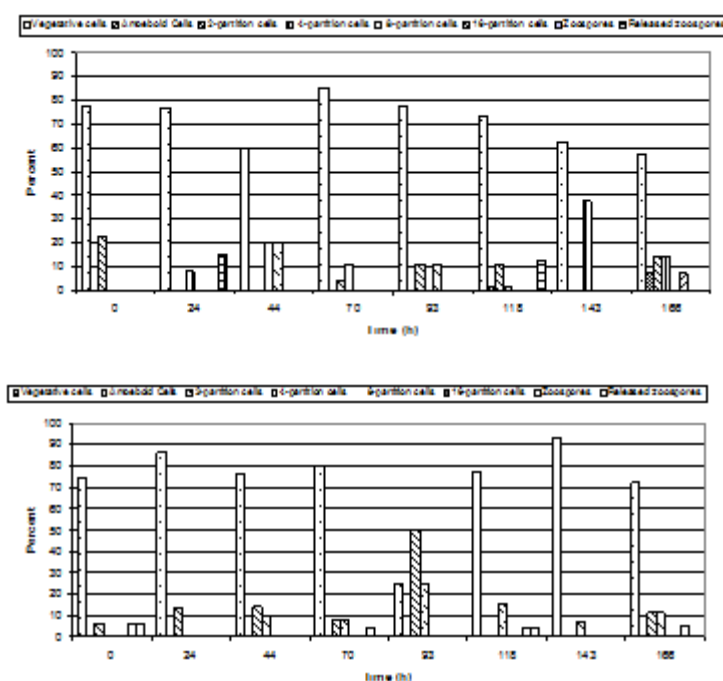


Fig. 7. Time profile of morphology of (A) *Schizochytrium* sp. BCC 25505 and (B) *Schizochytrium* sp. BCC 25509, during batch cultivation in a 5-L bioreactor.

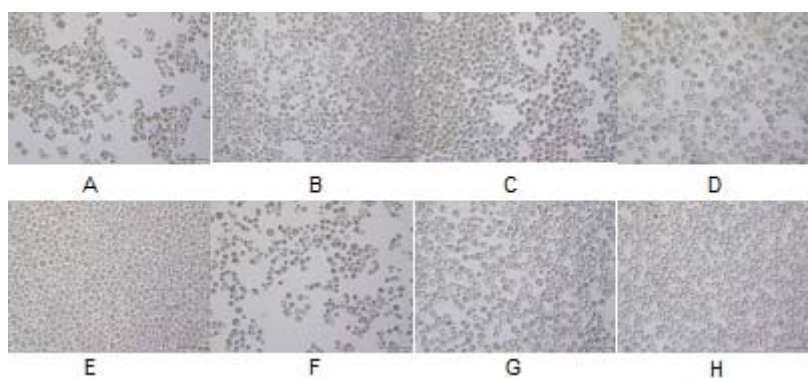


Fig. 8. Cell morphology of *Schizochytrium* sp. BCC 25505 and BCC 25509 during pulsed-batch cultivation visualized under a light microscope (400x). *Schizochytrium* sp. BCC 25505: (A) 0 h, (B) 24 h, (C) 70 h, (D) 166 h. *Schizochytrium* sp. BCC 25509: (E) 0 h, (F) 24 h, (G) 70 h, (H) 166 h

oleaginous protists for industrial production of polyunsaturated fatty acids, especially É-3 fatty acids such as DHA.

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REFERENCES

- Connor, W.E., Neuringer, M., Reisbick, S. Essential fatty acid: the importance of n-3 fatty acids in the retina and brain. *Nutr. Rev.*, 1992, **50**: 21-29.
- Das, U.N. Fams, M.D. Long-chain polyunsaturated fatty acids in the growth and development of the brain and memory. *Nutr.*, 2003; **19**: 62-65.
- Horrocks, L.A., Yeo, Y. K. Health benefits of docosahexanoic acid (DHA). *Pharmacol. Res.*, 1999; **40**: 211-225.
- Nordoy, A.R., Marchioli, R., Amesén, H., Videbaek, J. n-3 polyunsaturated fatty acids and cardiovascular diseases. *Lipids*, 2001; **36**: 127-129.
- Arterburn, L.M. Boswell. K.D., Henwood, S.M., Kyle, D.J. A developmental safety study in rats using DHA- and ARA-rich single cell oils. *Food Chem. Toxicol.*, 2000; **38**: 763-771.
- Ward, O.P., Singh, A., Omega-3/6 fatty acids: Alternative sources of production. *Process Biochem.*, 2005; **40**: 3627-3652.
- Miller, M.R., Nichols, P.D., Carter, C.G. Replacement of fish oil with thraustochytrid *Schizochytrium* sp. L oil in Atlantic salmon parr (*Salmo salar* L) diets. *Comp. Biochem. Physiol.*, 2007; **148**: 382-392.
- Simopoulos, A.P., Leaf, A., Salem, Jr.N. Workshop on the essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. *Food Aust.*, 1999, **5**: 332-333.
- Wong, M.K.M. Tsui, C.K.M., Au, D.W.T., Vrijmoed, L.L.P. Docosahexanoic acid production and ultrastructure of the thraustochytrid *Aurantiochytrium mangrovei* MP2 under high glucose concentrations. *Mycosci.*, 2010; **49**: 266-270.
- Kyle, D.J. Microbial oil mixtures and uses thereof. US Pat 5374657. 20 December, 1994.
- De Swaaf, M.E., de Rijk, T.C., Eggink, G., Sijtsma, L. Optimisation of docosahexanoic acid production in batch cultivations by *Cryptocodiniumcohnii*. *J. Biotechnol.*, 1999; **70**: 185-192.
- De Swaaf, M.E., Pronk, J.T., Sijtsma, L. Fed-batch culture of the docosahexanoic-acid-producing marine alga *Cryptocodinium cihni* on ethanol. *Appl. Microbiol. Biotechnol.*, 2003; **61**: 40-43.
- Bajpai, P., Bajpai, P., Ward, O.P. Production of docosahexanoic acid (DHA) by *T. aureum*. *Appl. Microbiol. Biotechnol.*, 1991; **35**: 706-710.
- Bajpai, P., Bajpai, P., Ward, O.P. Optimisation of production of docosahexanoic acid (DHA) by *Thraustochytrium aureum* ATCC 34304. *J. Am. Oil Chem. Soc.*, 1991; **68**: 509-514.
- Kyle, D.J. Arachidonic acid and methods for the production and use thereof. US Pat 5658767, 19 August, 1997.
- Bowles, R.D. Hunt, A.E. Bremer, G.B. Duchars, M.G., Eaton, R.A. Longchain n-3 polyunsaturated fatty acid production by members of the marine protistan group the *thraustochytrids*: screening of isolates and optimization of docosahexanoic acid production. *J. Biotechnol.* 1999; **70**: 193-202.
- Singh, A., Ward, O.P. Production of high yields of docosahexanoic acid by *Thraustochytrium roseum* ATCC 28210. *J. Ind. Microbiol.*, 1996; **16**: 370-373.
- Singh, A., Ward, O.P. Microbial Production of Docosahexanoic Acid (DHA, C22:6). *Adv. Appl. Microbiol.*, 1997; **45**: 271-312.
- Yokochi, T., Honda, D., Higashihara, T. Nakahara, T. Optimization of docosahexanoic acid production by *Schizochytrium limacinum* SR21. *Appl. Microbiol. Biotechnol.*, 1998; **49**: 72-76.
- Unagul, P., Assantachai, C., Phadungruengluij, S., Suphantharika, M., Tanticharoen, M., Verduyn, C. Coconut water as a medium additive for the production of docosahexanoic acid (C22:6 n3) by *Schizochytrium mangrovei* Sk-02. *Bioresour. Tech.* 2007; **98**: 281-287.
- Fan, K.W. Chen, F. Jones, E.B.G. Vrijmoed, L.L.P. Eicosapentaenoic and docosahexanoic acids production by and okara-utilizing potential of *thraustochytrids*. *J. Ind. Microbiol. Biotechnol.*, 2001; **27**: 199-202.
- Bailey, R. B., DiMasi, D., Hansen, J.M.M., Mirrasoul, P.J., Ruecker, C.M., Veeder, III G.T. Enhanced production of lipids containing polyenoic fatty acid by very high density cultures of eukaryotic microbes in fermenters.

- US Pat 6607900, 25 September, 2003.
23. Aki, T., Hachida, K., Yoshinaga, M., Katai, Y., Yamasaki, T., Kawamoto, S., Kakizono, T., Maoka, T., Shigeta, S., Suzuki, O. Ono, K. Thraustochytrid as a potential source of carotenoids. *J. Am. Oil. Chem. Soc.*, 2003; **80**: 789-794.
24. Kim, K., Kim, E.J., Ryu, B.G., Park, S. Yang, Y.C. A novel fed-batch process based on the biology of *Aurantiochytrium* sp. KRS101 for the production of biodiesel and docosahexaenoic acid. *Bioresour. Tech.*, 2013; **135**: 269-274.
25. Qu, L., Ren, L.J., Sun, G.N., Ji, X.J., Nie, Z.K., Huang, H. Batch, fed-batch and repeated fed-batch fermentation processes of the marine thraustochytrid *Schizochytrium* sp. for producing docosahexaenoic acid. *Bioprocess Biosyst. Eng.*, 2013; **36**: 1-8.
26. Gorain, P.C., Bagchi, S.K., Mallick, N. Effects of calcium, magnesium and sodium chloride in enhancing lipid accumulation in two green microalgae. *Environ. Technol.*, 2013; **34**: 1887-1894.
27. Yan, J. Cheng, R., Lin, X., You, S., Li, K., Rong, H., Ma, Y. Overexpression of acetyl-CoA synthetase increased the biomass and fatty acid proportion in microalga *Schizochytrium*. *Appl. Microbiol. Biotechnol.* 2013; **97**: 1-7.
28. Morita, E., Kumon, Y., Nakahara, T., Kagiwada, S. Noguchi, T. Docosahexaenoic acid production and lipid-body formation in *Schizochytrium limacinum* SR21. *Mar. Biotechnol. (NY)*, 2006; **8**: 319-327.