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The effects of culture condition on the growth property and docosahexaenoic acid production from *Thraustochytrium aureum* ATCC 34304

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Abstract−The effect of the composition of artificial sea water (ASW) medium on the growth properties and docosahexaenoic acid (DHA) production from *Thraustochytrium aureum* ATCC 34304 was investigated. A maximum dry cell weight (DCW) of 3.71 g/*l* was obtained when the NaCl and $MgSO_4$ concentration in the ASW media were 15 and 0 g/*l*, respectively. The maximum initial specific growth (ISG) rate of 0.16 was attained at 7.5 g/*l* NaCl and 18 g/*l* $MgSO_4$, while the minimum ISG rate (0.02) was obtained at 5.0 g/*l* NaCl and 4.5 g/*l* $MgSO_4$. The least doubling time required for biomass production was 4.3 h at 7.5 g/*l* NaCl and different $MgSO_4$ concentrations. A maximum of 7.9 g/*l* DCW was obtained on the fourth day of cultivation at 30 g/*l* glucose and 2.5 g/*l* (each) yeast extract (YE) and peptone. The DHA content in the lipids was significantly affected by the concentration of glucose and nitrogen sources (YE and peptone) in the ASW medium. At the lowest glucose (10 g/*l*) and YE/peptone (0.5 g/*l*) concentration and highest glucose (30 g/*l*) and YE/peptone (2.5 g/*l*) concentration, the DHA content was 34.725 and 40.33%, respectively, relative to total lipid content. However, the DHA content in the lipid was not affected by the NaCl and $MgSO_4$ concentration. At the lowest NaCl (2.5 g/*l*) and $MgSO_4$ (4.5 g/*l*) concentration and highest NaCl (60 g/*l*) and $MgSO_4$ (18 g/*l*) concentration the DHA content was 39.62 and 38.48%, respectively. The maximum DHA content in the lipid was 49.01% after four days of cultivation when 7.5 g/*l* NaCl and 4.5 g/*l* $MgSO_4$ were in the ASW medium. The growth properties of *T. aureum* ATCC 34304 for biomass production and DHA yield in the lipid content were found to be affected by NaCl and glucose concentration.

Key words: *Thraustochytrium aureum*, Polyunsaturated Fatty Acids (PUFAs), Salt Concentration, Docosahexaenoic Acid (DHA), Dry Cell Weight

INTRODUCTION

There is increasing demand for polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3) due to their beneficial effects on human health, which range from prevention of cancer and cardiovascular diseases to treatment in mental illnesses [1]. They are important structural components of the membrane phospholipids of nervous, visual and reproductive tissue cells and play important roles in biological functions [2,3] and work as important precursors for eicosanoids in signaling molecules [4]. The EPA and DHA are considered essential fatty acids and must be obtained from the diet [5].

At present, the major source of PUFAs including the DHA is fish oil, which is obtained from ocean fish including herring, tuna, salmon, and sardines [6]. Over the past decades the worldwide consumption of PUFAs as dietary supplements has increased and there is an increasing demand for sustainable and cheaper sources of PUFAs. The current production of PUFAs from fish sources will eventually be curbed due to a decrease in their population, variability in oil components, low purified form of DHA yield, peculiar taste and odor, variations with seasonal changes and accumulation of heavy metals [7,8]. Thus, sources for the production of PUFAs like DHA and EPA have been examined. There are now several reports that have identified and produced PUFAs from sources other than fish [8,9].

Thraustochytrids have the potential on commercial value due to their ability to produce large quantities of lipoidal compounds, especially DHA, and their heterotrophic nature, which is suitable for fermentation technology [10-12]. There is great variability in the biomass and DHA production from using *Thraustochytrium aureum*, which depends on the strains, quality and quantity of nutrient sources and growth conditions [6,13]. Previously, Bajpai et al. [14] and Hur et al. [15] reported that biomass and DHA production increased by manipulating the culture medium and environmental factors such as aeration, pH, temperature, light intensity, culture time and sugar concentrations. There have been a few reports that examined the effects of salt and sugar on growth and biomass as well as PUFAs production on the molecular level [4,6]. Therefore, the present study was carried out to determine the role of carbon, nitrogen and salt sources and the effect of their concentration on biomass and PUFA production in *T. aureum* ATCC 34304. To the best of our knowledge, this is the first study that examines the growth of *T. aureum* ATCC 34304 over this wide range of NaCl concentration.

MATERIALS AND METHODS

1. Microorganisms and Culture Conditions

T. aureum (ATCC 34304) was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The strain was subcultured every two weeks and maintained on artificial sea water (ASW) agar medium. The medium for the inocula and main culture

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was the same ASW, which was composed of 24 g/*l* NaCl, 12 g/*l* $MgSO_4 \cdot 7H_2O$, 0.75 g/*l* KCl, 0.01 g/*l* KH_2PO_4, 1 g/*l* $CaCl_2 \cdot 2H_2O$, 1 g/*l* Tris, 12 mg/*l* Na_2EDTA, 2 mg/*l* $ZnSO_4 \cdot 7H_2O$, 1 mg/*l* $Na_2MoO_4 \cdot 2H_2O$, 40 mg/*l* $NaNO_3$, 10 μg/*l* thiamine-HCl, 0.1 g/L $NaHCO_3$, 1 μg/*l* vitamin B_{12}, 20 μg/*l* aminobenzoate, 10 μg/*l* calcium penthothenate, 4 μg/*l* cyanobalamin, 0.5 mg/L $FeCl_3 \cdot 6H_2O$, 0.2 mg/L $MnCl_2 \cdot 4H_2O$, 2 μg/*l* $CoCl_2 \cdot 6H_2O$, 10 g/*l* glucose, 1 g/*l* yeast extract, 1 g/*l* peptone. The concentrations of glucose, yeast extract (YE), peptone, vitamins and other minerals were fixed and the effects of NaCl and $MgSO_4$ salts at various concentrations (0, 2.5, 5, 7.5, 15, 22.5, 30, 45, 60 and 0, 4.5, 9, 13.5, 18 g/*l*) were evaluated. The effect of carbon source (glucose) and the nitrogen sources (YE/peptone) was investigated at the following concentrations: 10, 15, 20, 25 and 30 g/*l* for D-glucose and 0.5, 1, 1.5, 2 and 2.5 g/*l* for YE/peptone.

2. Cultivation

A single colony of *T. aureum* was inoculated into a 50 ml of liquid medium in a 250-ml Erlenmeyer flask and cultivated for 48 h at 25 °C with shaking at 180 rpm. The culture was then transferred to fresh media containing various concentrations of NaCl and $MgSO_4$ as well as various concentrations of glucose as the carbon source and YE/peptone as the nitrogen sources. The samples were cultivated for seven days under the same conditions. The cells were harvested at 24 h intervals by centrifugation at 3,000 rpm for 15 minutes, and used to determine the dry cell weight (DCW) and fatty acid profiles.

3. DCW Determination

The DCW was determined by washing the cell suspension with distilled water and drying at 60 °C for 6 h.

4. Initial Specific Growth Rate Determination

The initial specific growth (ISG) rate in various salt concentrations was determined by using the following formula:

$$Y=f(x)=a0+a1.t+a2.t2$$

5. Lipid Extraction and Fatty Acid Analysis

The lipid extracted from the dried cells was esterified through treatment with 3 ml of H_2SO_4 and methanol (5 : 100 v/v) followed by drying at 100 °C for 1 h. The fatty acid profiles were determined using the method reported by Lepage and Roy [16]. In this analysis, gas chromatography (Hewlett Packard 6890, USA) equipped with a flame-ionized detector (FID) and a DB23 capillary column (30 m×0.25 mm×0.26 m, Agilent Technologies, USA) was used. The column temperature was increased from 150 to 270 °C (2 min) at a rate of 7 °C min^{-1}. The fatty acid components of the samples were identified by comparing the retention times against known standard fatty acids.

RESULTS AND DISCUSSION

Thraustochytrids are widely distributed in marine habitats and can be subjected to rapid changes in environmental osmolality [6, 17,18]. The strain *T. aureum* ATCC 34304 requires seawater for its growth [12] and their salinity optima and tolerance levels vary among strains [6]. Although salt is used to inhibit growth rather than as a substrate, some non-halophiles require a certain amount of salt to grow. Thus, salt should be considered as a growth substrate and essential component of the marine habitat [19]. Kang et al. [8] reported that the NaCl and $MgSO_4$ were important constituents in ASW medium for the cultivation of *T. aureum* ATCC 34304, and Iida et al. [20] reported that the range of salinity tolerance for this strain was narrow. Thus, we evaluated the effects of different concentrations of NaCl and $MgSO_4$ salts in the medium on growth properties.

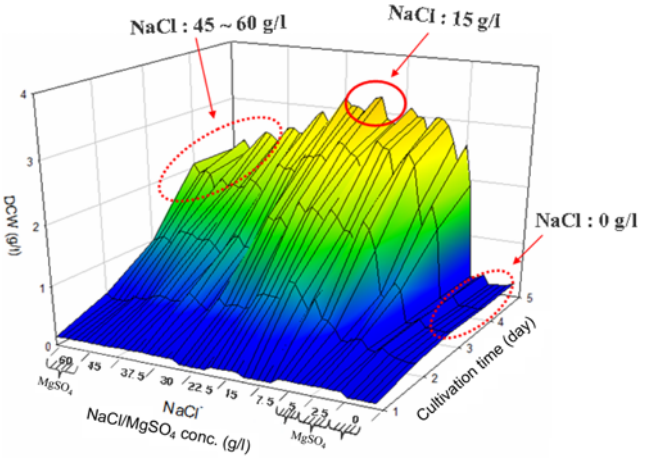

Fig. 1. Effect of different NaCl and $MgSO_4$ concentrations on the growth properties (DCW) of *T. aureum*.

1. Effect of NaCl and $MgSO_4$ on the Growth Properties

Very low growth was observed at 2.5 g/*l* NaCl in the absence of $MgSO_4$. Under these conditions, the DCW was 0.37 g/*l* on the 1st day of cultivation when the initial inoculum was 0.16 g/*l* and growth ceased after four days of cultivation. A higher biomass (0.6 g/*l*) and DCW (1.27 g/*l*) was observed on the second and third day of cultivation when 5 g/*l* of NaCl without $MgSO_4$ was used. When 60 g/*l* NaCl was used in the absence of $MgSO_4$, the DCW was 2.2 g/*l*. A maximum DCW of 3.71 g/*l* was obtained at 15 g/*l* NaCl in the absence of $MgSO_4$ (Fig. 1). No growth was observed when NaCl was omitted from the medium. This finding was in agreement with the results reported in a previous study [15].

The omission of $MgSO_4$ from the ASW medium did not result in any detrimental effects on the growth and survival when NaCl was supplied at concentration of 5 g/*l* or above; however, $MgSO_4$ was important to the initiation and maintenance of the growth at lower NaCl concentrations (Fig. 1). When the same experiment was conducted on another marine Thraustochytrid *Aurantiochytrium limacinum* mh0186, growth was not restricted in the absence of any one salt at a time, but a huge reduction in growth occurred in the absence of $MgSO_4$, which indicates the importance of $MgSO_4$ for the growth of the *A. limacinum* mh0186 strain [21]. The effect of $MgSO_4$ thus seems to be species specific rather than a strict requirement of marine habitat.

According to Iida et al. [20], the highest biomass can be obtained at a salinity of 0.5 times the salinity of sea water when *T. aureum* ATCC 34304 is cultivated in ASW medium. No growth was observed when the salinity was zero and growth was completely inhibited when the salinity was twice the salinity of sea water, which further verifies the narrow tolerance of salt concentrations for this organism. The results presented in this study are similar to those reported by Iida et al. [20] in regards to the lack of growth in the absence of NaCl or at low salinity. However, these results are quite different in terms of salinity tolerance of the cells at high NaCl and $MgSO_4$ salt

concentrations (60 and 18 g/*l*, respectively), which produced a DCW of 2.56 g/*l*. The narrow range of salinity tolerance for this organism in the Iida et al. [20] report may be due to the effect of other chemical components of ASW in the artificial sea salt component of Tropic Marin (Aquarientecnik, Germany) than the direct effect of NaCl and $MgSO_4$ salts present in their ASW medium composition. Kumon et al. [22] also investigated the role of salt concentration on the growth of marine *Labyrunthila* species strain L72 and no growth was also observed at zero salt concentration.

2. Initial Specific Growth (ISG) Rate and Time of Doubling at Different Salt Concentrations

NaCl and $MgSO_4$ concentration in the medium had a direct effect on the ISG rate. The maximum ISG rate of 0.161 was obtained at 7.5 g/*l* NaCl and 18 g/*l* $MgSO_4$, and the minimum ISG rate of 0.02 ISG was obtained at 5.0 g/*l* NaCl and 4.5 g/*l* $MgSO_4$. However, when the NaCl concentration was above 7.5 g/*l*, the ISG rate of 0.118 did not vary at different $MgSO_4$ concentrations, indicating that NaCl was more important than $MgSO_4$ for the growth of this organism (Fig. 2).

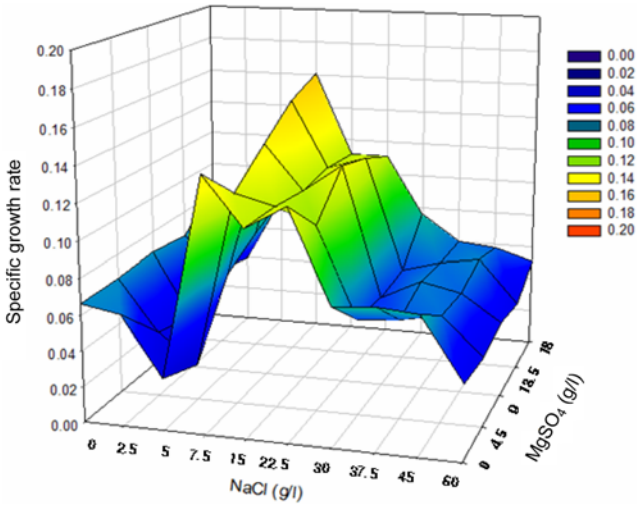

Fig. 2. Effect of different NaCl and $MgSO_4$ concentrations on the initial specific growth rate of *T. aureum*.

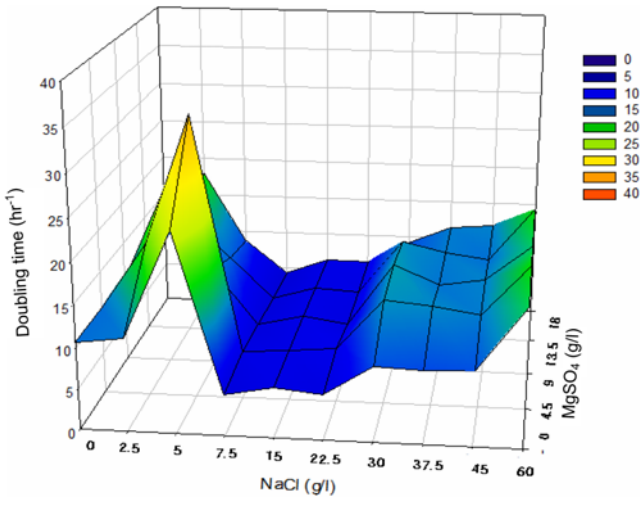

Fig. 3. Initial doubling time (h) of *T. aureum* at different NaCl and $MgSO_4$ concentrations (g/*l*).

Table 1. Mean DHA (%) content in total lipid at different NaCl and $MgSO_4$ concentrations after 4 days of cultivation in ASW medium

Salts (g/*l*)	DHA (%) in total lipid				
$MgSO_4$ / NaCl	0	4.5	9	13.5	18
2.5	39.28	39.6225	39.375	42.495	42.9125
5	45.9875	44.2875	45.1875	45.56487	45.2825
7.5	46.565	49.0125	47.8875	47.1225	46.8525
15	47.245	45.3325	45.62	44.8675	45.0325
22.5	46.2	45.285	44.275	44.715	44.33
30	43.445	42.1675	41.4425	41.1425	41.1075
37.5	40.72	41.93	40.46	41.4125	40.63
45	41.8775	41.2075	40.7425	40.0725	40.3875
60	40.515	39.7	39.64	39.23	38.48

The least doubling time required was 4.3 h for the DCW when the NaCl concentration was 7.5 g/*l* and at different $MgSO_4$ concentrations (Fig. 3). The effects of NaCl concentration on different Thraustochytrids were shown to be dependent on the tolerance level of the different strains [23]. The lowest doubling time was reported to be 5 h at 0.25 M NaCl, which is approximately 15 g/*l* NaCl, and the doubling time increased when the NaCl concentration was above 1 M (about 60 g/*l*), which is consistent with the present results. However, in this previous study, the addition of 4 mM g/lycine betaine was shown to be considerably decreased the doubling time.

3. Effect NaCl and $MgSO_4$ Salt on Biosynthesis of PUFAs

The DHA content in the total fatty acids (FAs) obtained at different NaCl and $MgSO_4$ concentrations was analyzed by GC and the mean value after four days of cultivation is presented in Table 1. At low NaCl (2.5 g/*l*) and $MgSO_4$ (4.5 g/*l*) concentrations, the DHA was 44.94% relative to the total FAs. The maximum DHA (49.013%) was obtained when the concentration of NaCl and $MgSO_4$ in the medium was 7.5 and 4.5 g/*l*, respectively (Table 1). At the lowest salinity, the stimulation and accumulation of PUFAs may be favored, which has been reported in previous studies on *T. aureum* [8] as well as *Thraustochytrium* sp. ONC-T18 [16].

In addition, the cellular contents of oleic acid (C18:1) and linoleic acid (C18:2), were reported to be gradually decreased as the concentrations of both salts decreased [8]. It was reported that by adding glucose and sodium thiosulphate at low concentration to the culture medium of marine *Chlorella* sp., the fatty acid production was enhanced significantly [24]. These results suggest that low salt concentration stimulates the desaturation of fatty acids and PUFAs accumulation in the cell. However, obtaining the maximum possible biomass with a high amount of lipids in the shortest possible time requires media optimization, and the best time to harvest DHA was shown to vary from 4 to 7 days [11,14,25,26]. Thus, there should be an optimum NaCl and $MgSO_4$ concentration that allows for maximum biomass and DHA production. In the present study, after four days of cultivation in ASW containing 7.5 g/*l* NaCl and 4.5 g/*l* $MgSO_4$, the DHA content was determined to be 49.82% of the total lipid

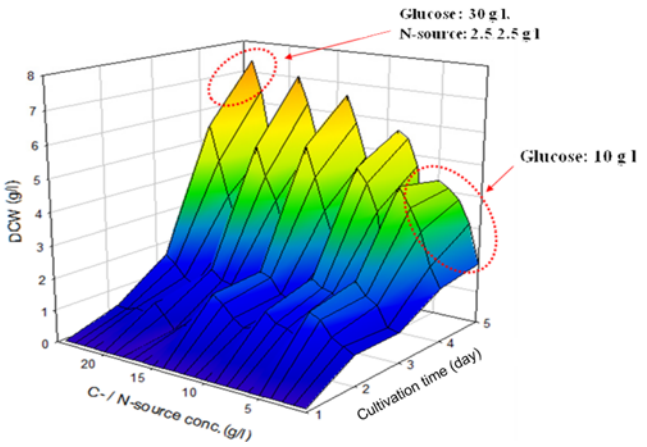

Fig. 4. Growth properties of *T. aureum* (DCW) at different C (glucose g/l) and N (YE/peptone each g/l) concentrations.

obtained from the biomass.

4. Effect of Initial Glucose and Nitrogen (YE and Peptone) Concentration on Growth Properties and Fatty Acid Biosynthesis

The effect of initial glucose and nitrogen (YE and peptone) concentration on biomass production, fatty acid profile and DHA yield was investigated while the concentration of the other components in the medium was fixed. The effect of the initial glucose concentration and different YE and peptone concentrations on the production of biomass is shown in Fig. 4. A maximum DCW (7.9 g/*l*) was obtained on the fourth day of cultivation when the glucose concentration was 30 g/*l* and the YE and peptone concentration was 2.5 g/*l*. The lowest DCW was obtained with 10 g/*l* glucose and 0.5 g/*l* YE and peptone on the fourth day of cultivation. In a previous report, a maximum of 5.7 g/*l* DCW was obtained when glucose, Na-g/lutamate and YE and peptone concentration was 10, 25 and 1 g/*l*, respectively [20] for *T. aureum* ATCC 34304, while a DCW of 13.73 g/*l* was obtained at 40 g/*l* glucose and 10 g/*l* YE/peptone in combination with the other components of ASW [6].

It was reported that the carbon (C) and nitrogen (N) ratio should be based on the cell ratio for maximum biomass production and an excess of C/N ratio is important for lipid accumulation [6,11]. In this study, the DHA composition was shown to be dependent on the concentrations of the C and N sources after four days of cultivation. However, the C and N concentration had no significant effect on DHA yield. A maximum DHA of 41.11% was obtained at 2.0 g/*l* YE/peptone with 20 g/*l* glucose (Table 2). The present study suggests the growth of *T. aureum* ATCC 34304 with highest biomass yield is possible by increasing the initial glucose and nitrogen concentration and lowering the NaCl and $MgSO_4$ concentration. By lowering these salt concentrations to the minimum required level, it would be possible to maximize PUFAs such as DHA and biomass. The growth properties and biosynthesis of PUFAs of *T. aureum* ATCC 34304 were found to be affected by NaCl and glucose concentration.

Table 2. Mean DHA (%) content in total lipid at different glucose and YE/peptone concentrations after 4 days of cultivation in ASW medium

Carbon and nitrogen source (g/*l*)	DHA (%) in lipid			
(YE/peptone) / Glucose	0.5	1.5	2	2.5
10	34.72	40.31	41.11	39.41
15	37.64	35.15	39.68	41.08
20	40.34	36.735	34.70	41.41
25	38.3	35.12	30.14	40.46
30	34.81	33.76	30.15	40.33

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REFERENCES

1. J. P. Bergé and G. Barnathan. *Adv. Biochem. Eng. Biotechnol.*, **96**, 49 (2005).
2. I. Gill and R. Valivety, *Trends Biotechnol.*, **15**, 401 (1997).
3. Z. Perveen, H. Ando, A. Ueno, Y. Ito, Y. Yamamoto, Y. Yamada, T. Takagi, T. Kabeko, K. Kogame and H. Okuyama, *Biotechnol. Lett.*, **28**, 197 (2006).
4. D. H. Kang, P. Anbu, Y. S. Jeong, B. P. Chaulagain, J. W. Seo and B. K. Hur, *Biotechnol. Bioprocess Eng.*, **15**, 261 (2010).
5. A. P. Simopoulos, *Experiment. Biol. Med.*, **233**, 674 (2008).
6. A. M. Burja, H. Radianingtyas, A. Windust and C. J. Barrow, *Appl. Microbiol. Biotechnol.*, **72**, 1161 (2006).
7. R. Yamamura and Y. Shimomura, *J. Am.Oil Chem. Soc.*, **74**, 1435 (1997).
8. D. H. Kang, E. J. Jeh, J. W. Seo, B. H. Chun and B. K. Hur, *Korean J. Chem. Eng.*, **24**, 651 (2007).
9. K. W. Fan, Y. Jiang, L. T. Ho and F. Chen, *J. Agric. Food Chem.*, **57**, 6334 (2009).
10. O. P. Ward and A. Singh, *Process Biochem.*, **40**, 3627 (2005).
11. S. Raghukumar, *Marine Biotechnol.*, **10**, 631 (2008).
12. J. L. Harwood and I. A. Guschina, *Biochimie*, **91**, 679 (2009).
13. P. Anbu, D. U. Kim, E. J. Jeh, Y. S. Jeong and B. K. Hur, *Biotechnol. Bioprocess Eng.*, **12**, 720 (2007).
14. B. K. Bajpai, P. Bajpai and O. P. Ward, *J. Am. Oil Chem. Soc.*, **68**, 509 (1991).
15. B. K. Hur, D. W. Cho, H. J. Kim, C. I. Park and H. J. Suh, *Biotechnol. Bioprocess Eng.*, **7**, 10 (2002).
16. G. Lepage and C. C. Roy, *J. Lipid Res.*, **25**, 1391 (1984).
17. T. Yokochi, D. Honda, T. Higashihara and T. Nakahara, *Appl. Microbiol. Biotechnol.*, **49**, 72 (1998).
18. S. Raghukumar, *Eur. J. Protistol.*, **38**, 127 (2002).
19. A. Dotsch, J. Severin, W. Alt, E. A. Galinski and J. U. Kreft, *Microbiology*, **154**, 2956 (2008).
20. I. Iida, T. Nakahara, T. Yokochi, Y. Kamisaka, H. Yagi, M. Yamaoka and O. Suzuki, *J. Ferment. Bioeng.*, **81**, 76 (1996).
21. N. Nagano, Y. Taoka, D. Honda and M. Hayashi, *J. Oleo Sci.*, **58**, 623 (2009).
22. Y. Kumon, R. Yokoyama, Z. Haque, T. Yokochi, D. Honda and T. Nakahara, *Marine Biotechnol.*, **8**, 170 (2006).
23. A. N. Jacobsen, Doctoral Thesis, Norwegian University of Science

and Technology (2008).
24. F. Y. Feng, W. Yang, G. Z. Jiang, Y. N. Xu and T. Y. Kuang, *Process Biochem.*, **40**, 1315 (2005).
25. A. Singh, S. Wilson and O. P. Ward, *World J. Microbiol. Biotechnol.*, **12**, 76 (1996).
26. T. Yaguchi, S. Tanaka, T. Yokochi, T. Nakahara and T. Higashihara, *J. Am. Oil Chem. Soc.*, **74**, 1431 (1997).