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Effect of Culture Conditions on Growth and Production of Docosahexaenoic Acid (DHA) using *Thraustochytrium aureum* ATCC 34304

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Abstract Environmental and medium factors were investigated as basic data for optimizing DHA production when using *Thraustochytrium aureum*. To study the effect of environmental conditions, the rotation speed and culture temperature were changed. Plus the trend of the growth characteristics, lipid content in the biomass, and DHA content in lipids were evaluated according to various initial glucose concentrations. The biomass, lipid, and DHA analyses showed that the physiological characteristics of *T. aureum* were closely related with the environmental and medium conditions, as in the case of other marine microorganisms. For example, a low rotation speed of 50 rpm lowered the cell growth rate as well as the DHA content in the lipids. A low temperature had a negative effect on the cell growth, yet a positive effect on the lipid content in the biomass. Different initial glucose concentrations had no effect on the lipid content in the biomass or DHA content in the lipids, yet did affect the cell growth. Accordingly, these results show that environmental and medium factors must be synthetically considered in order to optimize DHA production when using *T. aureum*.

Keywords: DHA production, *Thraustochytrium aureum*, environmental factors, glucose concentration effect

INTRODUCTION

The omega-3 fatty acids, eicosapentaenoic acids (EPA) and docosahexaenoic acid (DHA), are long-chain polyunsaturated fatty acids (PUFAs) that contain 20 carbon atoms with five double bonds (20:5) and 22 with six double bonds (22:6), respectively. These fatty acids are widely known to provide beneficial effects in the prevention and treatment of heart disease, high blood pressure, inflammation, and certain cancers [1,2]. Furthermore, DHA has been reported to have important function in the brain and retina [3,4]. DHA is essential for the normal growth and functional development of the brain and makes up about 60% of the structural lipid in the gray meter of the brain [5]. In several neurological disorders, including Alzheimers disease, DHA levels are depleted [1].

Accordingly, the production of DHA has drawn increasing attention due to its beneficial effects on human health. The current major commercial source of DHA is fish oil, which contains 7–14% DHA in addition to EPA and other more saturated fatty acids. However, the production of DHA from fish oil has encountered many barriers, for example, the DHA content is too low to obtain a purified form, the undesirable fishy flavor, the oxidation instability of fish oil, difficulties in concentrating DHA from fish oil, and the many purification steps involved [2,6]. Even though their growth rates are very low, marine microorganisms, such as algae [7-9], fungi [10,11], and bacteria [12,13] have also been found to produce microbial DHA. Plus, certain species that belong to a fungus *Thraustochytrium* would appear to be promising alternative sources for DHA production because they include a high proportion of DHA in their total lipids and a low content of polyunsaturated fatty acids that are structurally similar to DHA [14]. PUFAs are classified into n-6 and n-3 families. They are synthesized through an alternating series of chain elongation and desaturation by the same enzyme mechanisms. The synthesis of the n-6 family begins with linoleic acid (LA, 9,12-18:2) and ends at arachidonic acid (AA, 5,8,11,14-20:4). The reaction of the n-3 family starts from α-linolenic acid (ALA, 9,12,15-18:3) and ends at docosahexaenoic acid (4,7,10,13,16,19-22:6) [15].

Various environmental conditions have an affect on cell growth, DHA productivity, and fatty acid composition. Bajpai *et al.* [6] previously reported that fungal biomass production can be substantially increased with a proportional increase in DHA yield by manipulating the culture medium constituents, while optimizing and controlling parameters, such as the aeration, pH, and light in the fermentors. Parameters including the me-

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dium composition [1,16], carbon source, nitrogen source [6], nutrient starvation [17], fatty acid precursor addition [18], pH, temperature [19-21], light intensity [22], aeration and culture age [15] have all been reported to play important roles in the biosynthesis and accumulation of polyunsaturated fatty acids in most microorganisms.

In the present study, the physiological characteristics of *T. aureum,* including the cell growth, DHA production, lipid content, and sugar uptake, were investigated as functions of the culture temperature, rotation speed, and culture time.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Thraustochytrium aureum ATCC 34304 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The strain was maintained on a 3% agar slant based on an artificial sea-water medium supplemented with yeast extract (1 g/L) and peptone (1 g/L). The medium for the inocula and main culture was the same artificial sea-water, which was composed of 24 g/L NaCl, 12 g/L $MgSO_4$ $7H_20$, 1 g/L $CaCl_2 \cdot 2H_2O$, 0.75 g/L KCl, 0.04 g/L $NaNO_3$, 0.001 g/L K_2HPO_4, 1 g/L $C_4H_{11}NO_3$, 12 mg/L Na_2EDTA, 2 mg/L $ZnSO_4 \cdot 7H_2O$, 1 mg/L $NaMoO_4$, 0.5 mg/L $FeCl_3 \cdot 6H_2O$, 0.2 mg/L $MnCl_2 \cdot 4H_2O$, 2 μg/L $CoCl_2 \cdot 6H_2O$, 2 μg/L $CuSO_4 \cdot 5H_20$, 300 μg/L $C_{12}H_{17}ClN_2OS \cdot HCl$, 20 μg/L $H_2NC_6H_4COOH$, 10 μg/L $C_{18}H_{32}CaN_2O_{10}$, and 4 μg/L cyanocobalamin. In addition, glucose was used as the carbon source and yeast extract and peptone as the nitrogen sources. The concentrations of the nitrogen sources were fixed at 1 g/L for the yeast extract and 1 g/L for the peptone for all cultures, whereas the carbon source concentration was varied between 5 g/L and 35 g/L to investigate the effect of the concentration on the physiological characteristics of *T. aureum.*

The inocula were prepared in 250-mL Erlenmyer flasks containing 50 mL of the medium, and were grown at 24°C for 48 h with orbital shaking at 100 rpm. Then the production cultures were performed using the 48 h inoculum at a rate of 5%(v/v). in 250-mL Erlenmeyer flasks containing 60 mL of the medium over 7 days. The cultivation temperature was changed from 4°C to 32°C and the rotation speed from 50 rpm to 200 rpm. Samplings were carried out every 24 h to analyze the dry cell weight, glucose consumption, lipid content, and DHA production.

Analytical Methods

The dry weight of the biomass was determined by centrifuging a known volume of the fugal cell suspension, washing the cells with distilled water two or three times, and drying them at 70°C for more than 20 h. The glucose concentration was then determined using a glucose analyzer (TOA, GLU-11, Japan).

The dried cells were weighed in a 10-mL teflon-lined screw cap vial and the lipids extracted using methanol at 60°C for 24 h. The debris was removed by centrifuging, then the extracted lipids were dried at 30°C under a vacuum and weighed. The dried lipids were estrified with an acetyl chloride-methanol solution using the method of Lepage and Roy [23]. The methyl esters were dissolved in hexane and analyzed using a Hewlett-Packard 6890 gas chromatography equipped with a flame ionized detector (FID) and HP 19091J-413 capillary column. The column temperature was raised from 150°C (2 min) to 265°C (2 min) at 7°C /min.

RESULTS AND DISCUSSION

Effect of Rotation Speed

The biomass production patterns for various rotation speeds (50, 100, 150, and 200 rpm) are illustrated according to the cultivation day in Fig. 1. At all rotation speeds, the biomass production increased with the cultivation day, yet the maximum biomass concentration was achieved with 100 rpm compared with the other rotation speeds.

The effect of the rotation speed on the lipid content in the biomass and DHA content in the lipids is presented in Fig. 1. The lipid content was higher in the order of 200, 100, 150, and 50 rpm.

However, the differences in the lipid production in the cells between the rpms were too small to conclude that rotation speed had a specific effect on the production. The rotation speed did have a slight affect on the DHA content in the lipids. Which was higher in the order of 150, 200, 100, and 50 rpm. In the case of 50 rpm, the DHA content was much lower compared to that with the other rpms. When the rotation speed was changed from 100 to 200 rpm, the difference in the DHA content according to the rpm was minimal.

Bajapai *et al.* used a rotation speed of up to 300 rpm to culture the same strain, *T. aureum* [6]. However, a rotation speed higher than 250 rpm disrupted the cells, so that the morphology was found to be severely changed and the DHA content also greatly reduced. As such, since the DHA content in lipids would appear to be significantly affected by shear force, the agitation speed is seemingly a key factor for optimal DHA production.

Table 1 represents the various yields defined by Eqs. (1) to (4). Every value was calculated on the basis of five days of cultivation.

$$Y_{X/S} = (X_{5day} - X_0)/(S_0 - S_{5day}) \quad (1)$$

$$Y_{L/X} = (L_{5day} - L_0)/(X_{5day} - X_0) \quad (2)$$

$$L_{D/X} = (D_{5day} - D_0)/(X_{5day} - X_0) \quad (3)$$

$$Y_{D/L} = (D_{5day} - D_0)/(L_{5day} - L_0) \quad (4)$$

Where, X, S, D, and L are the biomass, glucose, DHA and lipid concentration. The subscripts 5 day and 0,

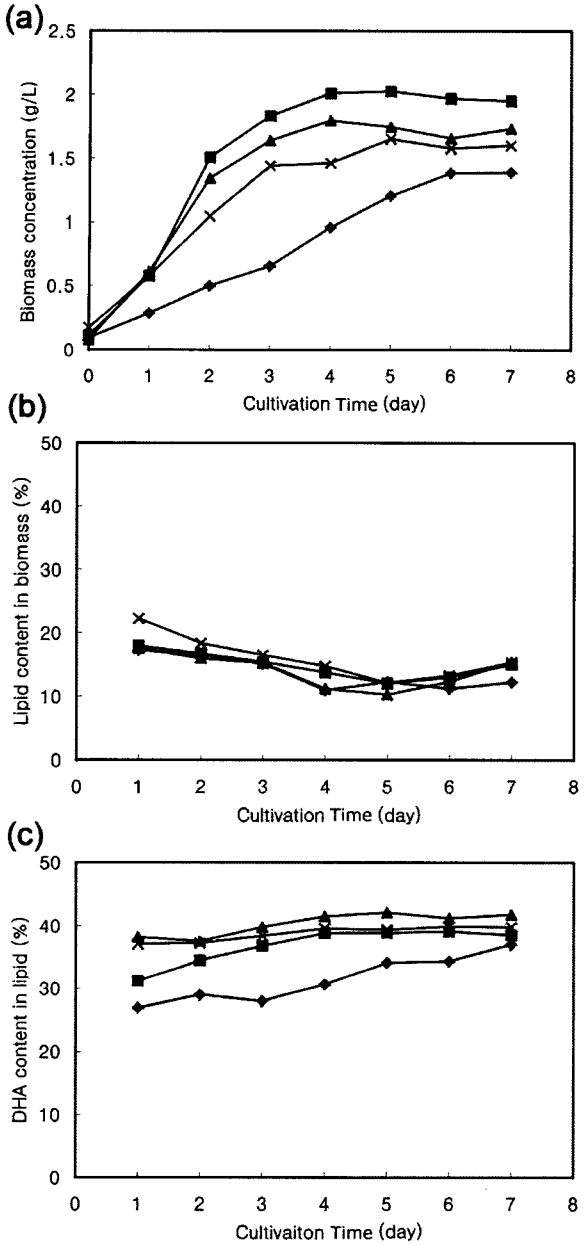

Fig. 1. Effect of rotation speed on biomass production (a), lipid content in biomass (b), and DHA content in lipid (c). ◆, 50 rpm ; ■, 100 rpm ; ▲, 150 rpm ; ×, 200 rpm.

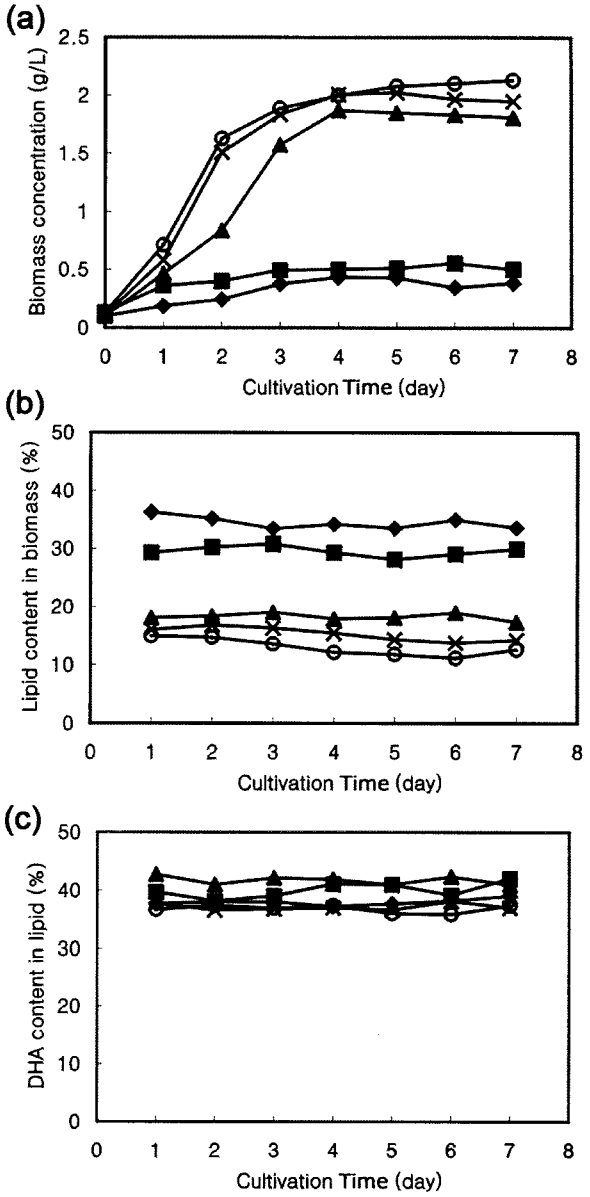

Fig. 2. Effect of cultivation temperature on biomass production patterns (a), lipid content in biomass (b), and DHA content in lipids (c). ◆, 4°C; ■, 11°C; ▲, 18°C; ×, 24°C; O, 32°C.

mean after 5 days of cultivation and before cultivation respectively.

The biomass yield, $Y_{X/S}$, ranged from 0.37 to 0.48, the lipid yield to biomass, $Y_{L/X}$, from 0.53 to 0.66, and the DHA yield to biomass, $Y_{D/X}$, from 0.046 to 0.054. In the current study the various yields exhibited no increasing or decreasing trend with an increase in the rotation speed. The effect of the temperature and glucose concentration on the physiological characteristics of *T. aureum* was also investigated to ensure maximum cell growth and stable DHA production when fixing the rotation speed at 100 rpm.

Effect of Cultivation Temperature

The cultivation temperatures considered in the current study were 4, 11, 18, 24, and 32°C. The cultivation was carried out for 7 days. However, in the case of 4°C, the cultivation was continued for 14 days because the cell growth was too slow. The growth profiles observed at different temperatures are illustrated in Fig. 3. The biomass concentration relative to the cultivation day increased up to the fourth day, yet thereafter, the cell growth reached the stationary phase. When the cultivation temperatures were 4 and 11°C, the growth was

Table 1. Yield of biomass, lipid, DHA according to various rotation speeds (five days of cultivation)

Rotation speed	$Y_{X/S}$	$Y_{L/X}$	$Y_{D/L}$	$Y_{D/X}$
50 rpm	0.3785	0.0689	0.6648	0.0458
100 rpm	0.3899	0.0940	0.5286	0.0497
150 rpm	0.3657	0.0692	0.6572	0.0455
200 rpm	0.4837	0.0914	0.5850	0.0535

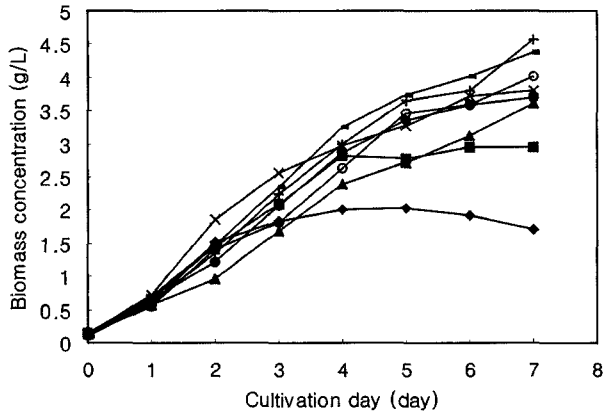

Fig. 3. Effect of initial glucose concentration on the cell growth of T. aureum. ◆, 5 g/L ; ■, 8 g/L ; ▲, 11 g/L ; X, 14 g/L ; O 17 g/L ; ● 23 g/L ; +, 29 g/L ; –, 35 g/L.

very slow and also stopped after the fourth day. The biomass production patterns at 4 and 11°C were very similar to each other, yet quite different from the patterns at 18, 24, and 32°C. However, the differences among 18, 24, and 32°C were too small to be distinguished.

According to the previous report by Bajapai *et al.* [6], the biomass concentration increased relative to the temperature and reached a maximum value at 28°C. Therefore, they investigated other culture characteristics at 28°C. Meanwhile Yokochi *et al.* [14] also found that the growth of *Schizochytrium limacium* SR21 was a function of the cultivation temperature, where the optimum temperature was 25°C. Other marine microorganisms are also affected by the cultivation temperature and show different optimum temperatures for the maximum biomass [15].

The effect of the cultivation temperature on the lipid content in the biomass and DHA content in the lipids is presented in Fig. 3, respectively the lipid content became higher with a decrease in the cultivation temperature (Fig. 3).

Bajapai *et al.* [6] also reported the same trend where the lower the temperature, the higher the lipid content.

The DHA content in the lipids was not significantly affected by the cultivation temperature compared with the lipid content in the biomass and remained almost constant throughout the culture period (Fig. 3).

Table 2 represents the values of the four different yields defined by Eq. (1) at various cultivation temperatures. The lipid yield, $Y_{L/X}$, increased with a decrease in the cultivation temperature and became at 0.34 at 4°C. The DHA yields, $Y_{D/L}$ and $Y_{D/X}$, showed the same tendency as the lipid yield.

Table 2. Yield of biomass, lipid, DHA according to various cultivation temperatures (five days of cultivation)

Cultivation temperature	$Y_{X/S}$	$Y_{L/X}$	$Y_{D/L}$	$Y_{D/X}$
4°C	0.7681	0.3374	0.6153	0.2076
11°C	0.7247	0.3656	0.4109	0.1502
18°C	0.3736	0.1771	0.4317	0.0764
24°C	0.3899	0.0940	0.5285	0.0497
32°C	0.4411	0.1084	0.4156	0.0450

Table 3. Effect of initial glucose concentration on biomass, lipid, DHA yields and the sugar conversion ratio

Initial glucose concentration (g/L)	5	8	11	14	17	23	29	35
Biomass yield	0.330	0.360	0.362	0.388	0.348	0.316	0.340	0.387
Lipid yield	0.371	0.394	0.439	0.472	0.550	0.705	0.810	0.733
DHA yield	0.224	0.246	0.233	0.225	0.186	0.199	0.182	0.280
Sugar conversion ratio	0.937	0.971	0.913	0.768	0.735	0.612	0.530	0.405

Effect of Initial Sugar Concentration

The effect of the initial glucose concentration on the lipid content in the biomass, DHA content in the lipids, biomass yield, lipid yield, and DHA yield was investigated within a range of 5 g/L to 35 g/L of glucose. The effect of the initial glucose concentration on the biomass production of *T. aureum* is shown in Fig. 3. The biomass increased relative to the cultivation day with all initial glucose concentrations. The initial glucose concentration exhibiting the highest biomass at the end of the culture was 29 g/L with a biomass value of 4.5 g/L. The biomass yields with the various initial glucose concentrations and sugar conversion ratios are illustrated in Table 3, as defined by Eqs. (5) and (6) respectively.

$$Y_{x/s} = \frac{\Delta X}{\Delta S} \tag{5}$$

$$Y_{S/So} = \frac{\Delta S}{So} \tag{6}$$

Here, ΔX is the difference between the biomass concentrations on the seventh day and the initial day of cultivation, and ΔS is the difference between the sugar concentrations. As shown in Table 3, the biomass yield remained nearly constant, regardless of the initial sugar concentration. However, the sugar conversion ratio decreased according to the increase in the initial sugar con-

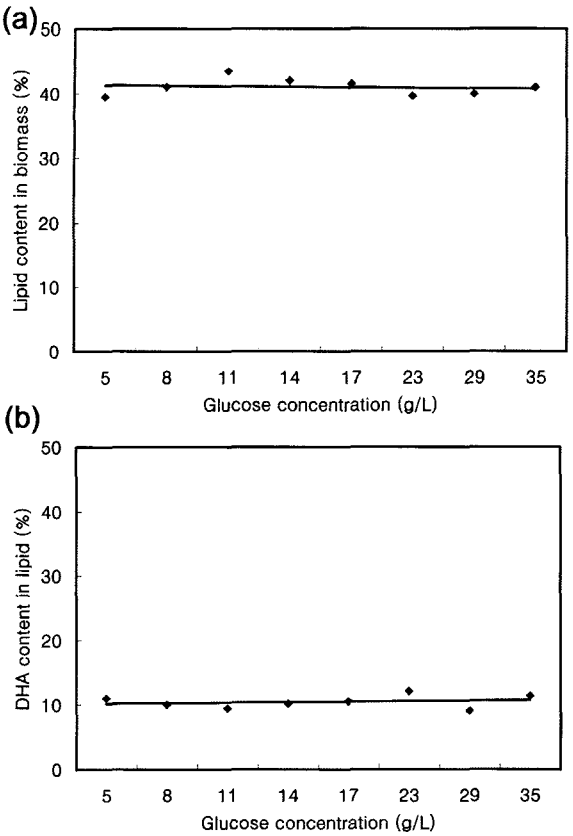

Fig. 4. (a) Effect of initial glucose concentration on lipid content in biomass after 7 days of cultivation. (b) Effect of initial glucose concentration on DHA content in lipids after 7 days of cultivation.

centration. Many researchers have attempted to optimize the medium composition and environmental factors with a fixed initial sugar concentration [6,14,24]. As such, there are quite a few reports on the effect of the initial sugar concentration on the culture characteristics.

The lipid and DHA contents at the end of the culture are presented in Fig. 4. The lipid content in the biomass remained around 13%, regardless of the initial sugar concentration, while the DHA content in the lipids also remained nearly constant at 40%.

The lipid and DHA yields, as defined by Eqs. (7) and (8), are also illustrated in Table 3.

$$Y_{L/S}=\frac{\Delta L}{\Delta S}\left(\frac{mg}{g}\times\frac{1}{100}\right) \quad (7)$$

$$Y_{D/S}=\frac{\Delta D}{\Delta S}\left(\frac{mg}{g}\times\frac{1}{100}\right) \quad (8)$$

Where ΔL, ΔS, and ΔD represent the difference between the seventh day concentration and the initial concentration for the lipids, glucose, and DHA, respectively. As shown in Table 3, the DHA and lipid yields remained nearly constant, regardless of the initial glucose concentration.

CONCLUSION

The effect of the initial concentration, cultivation temperature, and rotation speed on the growth, lipid production, and DHA production characteristics was investigated.

The growth of *T. aureum* exhibited a significant relationship with the rotation speed and was found to be very sensitive to the shear force. The cell growth was optimal around 100 rpm. The effect of the rotation speed on the lipid content in the biomass was minimal, yet the DHA content in the lipids was significantly reduced with a low rotation speed of 50 rpm. Changing the rotation speed from 100 to 200 rpm had no obvious affect on the DHA content. The conversion rates ($Y_{X/S}$, $Y_{L/X}$, $Y_{D/X}$, $Y_{D/L}$) also remained unchanged with rotation speeds within a range of 50 to 200 rpm.

As the cultivation temperature was lowered, the lipid and DHA contents in the biomass increased, yet the biomass production became lower. The cultivation temperature had a significant effect on growth, the lipid content in the biomass, and DHA content in the lipids. In the case of a cultivation temperature of 4 and 11°C the growth was very slow and stopped after the fourth day at which point the final biomass was less than 0.5 g/L. The growth with cultivation temperatures of 18, 24, and 32°C was very similar. However, the lipid content in the biomass and DHA content in the lipids increased according to a decrease in the temperature. It was found that a low temperature induced more lipid and DHA accumulation in the biomass, as such, the optimal temperature for cell growth was not the best condition for lipid and DHA production.

The biomass increased with the cultivation period, regardless of the initial glucose concentration. At a low glucose concentration below 8 g/L, a dependency of the cell growth on the initial glucose concentration appeared, and similar growth curves were also found with a higher glucose range. The biomass yields remained nearly constant, irrespective of the initial glucose concentration, yet the sugar conversion ratio decreased with an increase in the initial glucose concentration. This reflects the inappropriateness of the medium composition at a high glucose concentration, which will be investigated in a further study. The lipid content in the biomass and DHA content in the lipids remained almost constant, regardless of the initial glucose concentration.

Accordingly, the current study demonstrated that the growth of *T. aureum*, its lipid content, and DHA content are partially or completely affected by environmental and medium factors, therefore these factors must be synthetically considered in order to optimize DHA production when using *T. aureum*.

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