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Effect of salt concentration on production of polyunsaturated fatty acids in *Thraustochytrium aureum* ATCC 34304

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Abstract–This study attempted to characterize the effects of NaCl and $MgSO_4$ as the primary components of sea salt affecting the yields of production of polyunsaturated fatty acids (PUFAs) by *Thraustochytrium aureum* ATCC 34304. Reductions in the NaCl concentration of the culture medium suppressed the formation of palmitic acid (PA, C16 : 0) but enhanced the production of PUFAs, which induced an increase of the concentration of docosahexaenoic acid (DHA, C22 : 6) up to 46.65% from 44.26%. $MgSO_4$ revealed a similar, yet more highly significant, effect on the fatty acids profile than NaCl. The yields of PUFAs and DHA showed maximum values such as 67.10% and 49.47%, respectively, at the concentration of 10 g L^{-1} for NaCl and 0 g L^{-1} for $MgSO_4$. However, the quantities of mono- and diunsaturated fatty acids, oleic acid (OA, C18 : 1) and linoleic acid (LA, C18 : 2) at the same concentrations, were compared with those of normal culture medium. Additionally, it was investigated that the increase of culture temperature reduced PUFAs contents but the reductions were recovered by the removal of $MgSO_4$ form the culture medium, which showed that concentration of salts and culture temperature affected independently the production of PUFAs in *T. aureum*.

Key words: *Thraustochytrium aureum*, Polyunsaturated Fatty Acids (PUFAs), Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA), Salt Effect

INTRODUCTION

Polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA, C20 : 5 ω-3) and docosahexaenoic acid (DHA, C22 : 6 ω-3) are essential components of the glycolipids and phospholipids which form the plasma membranes [1]. They also function as both precursors of certain hormones and signaling molecules [2]. Furthermore, these fatty acids have been known to yield beneficial effects in the prevention and treatment of heart disease, high blood pressure, inflammation, and certain types of cancer [3-5].

Mammals, including humans, cannot synthesize PUFAs *de novo*, but instead transform from linoleic acid (LA, C18 : 2 ω-6) or α-linolenic acid (ALA, C18 : 3 ω-3) to PUFAs [6]. However, since the transformation efficiency is quite low, the direct intake of PUFAs has been recommended, in particular for infants, pregnant women, elderly men, and some patients [7,8].

The most abundant source of PUFAs is ocean fish, including herring, mackerel, salmon, and sardines [8,9]. Fish oils, processed through extraction, concentration and refining, have been widely used as a principal source for commercial applications of PUFAs [10,11]. However, the current production of PUFAs from fish oils may be restricted, due primarily to the variable quality of oil components depending on fish species and the season and location of catching and the accumulation of environmental pollutants, especially heavy metals including mercury, in fishes which occupy high positions in the marine food chains [12].

A great deal of attention has recently centered on the microbial production of PUFAs, largely due to the potential for overcoming the problems inherent to the use of fish oils [13-17]. A variety of microorganisms capable of generating PUFAs have thus far been identified, and these species are largely marine algae or protists [8]. Improvements in biomass and production yields of desired PUFAs in the cultivation of microorganisms will, however, be necessary in order to compete with the relatively low cost of fish oils for the production of PUFAs. A great deal of effort has been devoted to the elucidation of the environmental factors affecting PUFAs production yields for the purposes of strain development and the optimization of culture conditions [8,16]. Associated with the critical roles of fatty acids in membrane fluidity, temperature has been identified as a primary factor influencing fatty acid profiles, e.g., increases in the proportions of PUFAs under lower temperature conditions [18-22]. Salt is likely to be another environmental factor that determines fatty acid profiles [23]. However, few reports have been made about the effects of salt on PUFAs production on the molecular basis. In this study, some attempts were made to evaluate the effects of the salts of sea water, NaCl and $MgSO_4$, on fatty acid profiles, particularly PUFAs profiles, in the marine protist *Thraustochytrium aureum* ATCC 34304 that is capable of production of DHA up to 45% of the fatty acids [8,20,21,24]. And another investigation was made to elucidate the relationship between salt concentration and cultivation temperature with regard to PUFAs production.

EXPERIMENTAL

1. Microorganism and Medium

Thraustochytrium aureum ATCC 34304 was employed in our examination of the composition of PUFAs. The medium used for

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the cultivation of *T. aureum* ATCC 34304 was modified slightly from the medium developed by Solomon Goldstein [25] and its components were glucose 10 g L^{-1}, yeast extract 1 g L^{-1}, peptone 1 g L^{-1}, NaCl 24 g L^{-1}, $MgSO_4$ 12 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 1 g L^{-1}, KCl 0.7 g L^{-1}, $NaNO_3$ 0.04 g L^{-1}, K_2HPO_4 0.001 g L^{-1}, Na_2EDTA 12 mg L^{-1}, $ZnSO_4 \cdot 7H_2O$ 2 mg L^{-1}, $NaMoO_4 \cdot 2H_2O$ 1 mg L^{-1}, $FeCl_3 \cdot 6H_2O$ 0.5 mg L^{-1}, $MnCl_2 \cdot 4H_2O$ 0.2 mg L^{-1}, $CoCl_2 \cdot 6H_2O$ 2 μg L^{-1}, $CuSO_4 \cdot 5H_2O$ 2 μg L^{-1}, thiamine hydrochloride 300 μg L^{-1}, ρ-amino benzoate 20 μg L^{-1}, calcium pentothenate 10 μg L^{-1}, and cyanocobalamin 4 μg L^{-1}. The effects of salts were investigated at various concentrations of NaCl and $MgSO_4$ such as 5, 10, 20, 30 g L^{-1} and 3, 6, 9, 15 and 18 g L^{-1}, respectively.

2. Cultivation

A colony of *T. aureum* was inoculated into 50 ml of liquid medium in a 250-mL Erlenmeyer flask, and cultivated for 24 hours at 24 °C with a shaking speed of 100 rpm. The culture was transferred to fresh media containing a variety of NaCl or $MgSO_4$ concentrations and cultivated for 10 days under the same conditions. The cells were harvested by centrifugation at 3,000 rpm for 15 minutes every day, and used to determine dry cell weight and fatty acid profile.

3. Lipid Extraction and Fatty Acid Analysis

The harvested cells were washed three times with distilled water and dried completely in a drying oven for 20 hours at 70 °C. The dried cells were weighed, and the lipids were extracted through treatment with 3 ml methanol, followed by 1 hour of incubation at 100 °C. Fatty acid profiles were determined by the method of Lepage [26] using a gas chromatograph (Hewlett Packard 6890, USA) equipped with a flame-ionized detector (FID) and a DB23 (30 m×0.25 mm×0.26 μm, Agilent Technologies, USA) capillary column. The column temperature was increased from 150 to 270 °C (2 min) at 7 °C/min. Heptadecanoic acid (C17 : 0) was used as a standard. All the results presented below were obtained from at least three independent experiments.

RESULTS AND DISCUSSION

1. Effect of NaCl Concentration on the Yield of PUFAs Production

NaCl and $MgSO_4$ are the principal constituents of sea salt medium for the cultivation of the marine protist *T. aureum*. In order to evaluate the effects of these salts on fatty acid profiles, in particular the yield of PUFAs production including DHA, *T. aureum* was grown in culture media at a variety of concentrations of these two salts (Fig. 1). *T. aureum* failed to grow when NaCl was omitted completely from the culture medium (data not shown), indicating the indispensability of the salt. Fatty acid profiles of *T. aureum* grown at various NaCl concentrations were tabulated as shown in Table 1. Palmitic acid (PA, C16 : 0) was found as primary saturated fatty acid of *T. aureum* and featured about 15% of the total fatty acids. The cellular quantity of PA increased very slowly with increasing concentration of NaCl in the cultivation medium and peaked at a concentration of 20 g L^{-1} but decreased at higher concentrations. The proportion of eicosapentaenoic acid (EPA, C20 :5 ω-3), docosapentaenoic acid (DPA, C22 : 5 ω-6) and docosahexaenoic acid (DHA, C22 : 6 ω-3) was not affected much by the concentration of NaCl in the range from 5 to 30 g L^{-1}. This finding is consistent with the results of previous studies [20,27]. The content of PUFAs in the cell decreased from 62.10% at 5 g L^{-1} NaCl to 60.06% at 30 g L^{-1} NaCl. Interestingly, arachidonic acid (AA, C20 : 4) contents were shown to be quite low, less than 1%, under our experimental conditions. This result did not match previous reports in which two C20 PUFAs, AA and EPA, were presented at similar levels under typical conditions of 25 °C [20].

Table 1. Effect of NaCl concentration on production yield of PUFAs in *T. aureum*

NaCl (g L^{-1})	5	10	15	20	30
DCW[a] (g L^{-1})	1.75	3.30	3.56	3.73	3.14
C16 : 0	14.19	15.53	15.98	16.04	15.65
C18 : 1	8.55	7.85	8.18	7.66	7.89
C18 : 2	2.57	1.86	1.91	1.81	1.94
C20 : 4	0.99	0.67	0.64	0.66	0.72
C20 : 5	4.07	4.80	4.55	4.73	4.48
C22 : 5	10.39	11.57	11.14	11.44	10.27
C22 : 6	46.65	44.26	44.76	44.49	44.59
PUFAs[b]	62.10	61.30	61.09	61.32	60.06

[a]Dry cell weight.

[b]PUFAs are composed of AA, EPA, DPA, and DHA. Production yields (relative ratio) of fatty acids were presented by the average value of the contents of each stage of growth.

2. Effect of $MgSO_4$ Concentration on the Yield of PUFAs Production

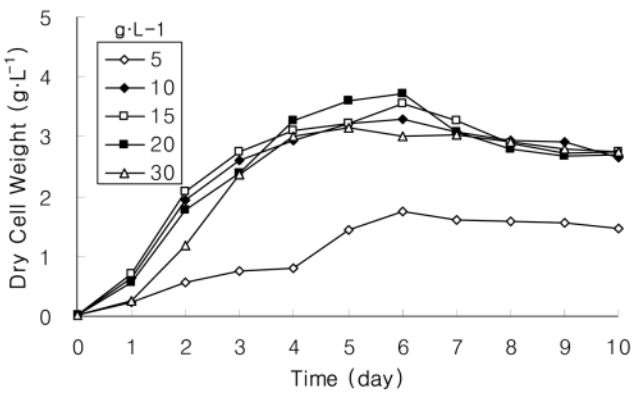

Fig. 1. Growth of *T. aureum* at various concentrations of NaCl.

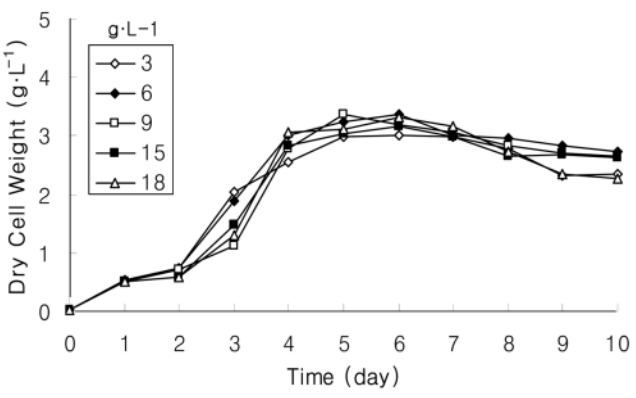

Fig. 2. Growth of *T. aureum* at various concentrations of $MgSO_4$.

Table 2. Effect of $MgSO_4$ concentration on production yield of PUFAs in *T. aureum*

$MgSO_4$ (g L^{-1})	0	3	6	9	15	18
DCW[a] (g L^{-1})	1.80	3.01	3.37	3.38	3.16	3.32
C16 : 0	13.80	14.55	14.90	15.30	15.26	15.01
C18 : 1	7.38	6.87	6.28	5.82	6.73	7.74
C18 : 2	1.95	2.27	2.04	1.94	2.14	2.35
C20 : 4	0.60	0.53	0.56	0.56	0.65	0.77
C20 : 5	5.19	5.16	5.40	5.28	5.00	4.74
C22 : 5	11.22	12.22	13.10	13.24	12.27	10.99
C22 : 6	46.55	43.75	42.85	41.93	41.92	42.13
PUFAs[b]	63.56	61.66	61.91	61.01	59.84	58.63

[a]Dry cell weight.
[b]PUFAs are composed of AA, EPA, DPA, and DHA. Production yields (relative ratio) of fatty acids were presented by the average value of the contents of each stages of growth.

The effect of $MgSO_4$ concentration was small on the growth of cells as shown in Fig. 2. The cell weight increased slowly for the first two days and rapidly for the next two days, and marked the peak on the 5-day. The same effect on fatty acid profiles was seen in conjunction with $MgSO_4$ (Table 2). The cellular PA content decreased with increasing $MgSO_4$ concentration. However, $MgSO_4$ was found to have a more significant influence on the yield of PUFAs production, particularly DHA. The proportion of PUFAs in the total cellular fatty acids increased from 58.63% at 18 g L^{-1} Mg SO_4 to 63.56% at 0 g L^{-1} Mg SO_4 and that of DHA from 41.92% at 15 g L^{-1} $MgSO_4$ to 46.55% at 0 g L^{-1} $MgSO_4$ for DHA.

3. Accumulative Effects of Both NaCl and $MgSO_4$ on the Yield of PUFAs Production

Based on the results of the salts effects, the integrated effects of both salts on fatty acid profiles were examined at various concentrations of the essential salt, NaCl, in absence of $MgSO_4$. Maximum yields of PUFAs and DHA were 67.10% and 49.47%, respectively, at 10 g L^{-1} NaCl and 0 g L^{-1} $MgSO_4$ (Table 3). In addition, the cellular contents of mono- and diunsaturated fatty acids, oleic acid (OA, C18 : 1) and linoleic acid (LA, C18 : 2), were gradually reduced in proportion to the reducing concentrations of both salts. According to these results it was concluded that low salt concentrations stimulate the desaturation of fatty acids, increasing the cellular amount of PUFAs, in particular, the most highly unsaturated DHA in *T. aureum*.

Table 3. Integrated effect of both NaCl and $MgSO_4$ on production yield of PUFAs in *T. aureum*

NaCl (g L^{-1})	5	10	15	20	30
DCW[a] (g L^{-1})	0.26	2.07	2.29	2.12	1.96
C16 : 0	17.28	14.67	15.02	14.14	13.94
C18 : 1	5.08	6.06	6.94	7.71	7.25
C18 : 2	1.24	1.57	1.62	1.87	2.17
C20 : 4	0.15	0.48	0.54	0.59	0.66
C20 : 5	6.25	5.20	5.47	5.83	5.14
C22 : 5	13.23	11.95	11.25	11.09	11.28
C22 : 6	44.50	49.47	48.08	46.84	46.46
PUFAs[b]	64.13	67.10	65.34	64.35	63.54

[a]Dry cell weight.
[b]PUFAs are composed of AA, EPA, DPA, and DHA. Production yields (relative ratio) of fatty acids were presented by the average value of the contents of each stage of growth.

Table 4. Effects of temperature and salt on production of PUFAs in *T. aureum*

$MgSO_4$ (12 g L^{-1})	+		-	
Temperature (°C)	25	30	25	30
C16 : 0	15.44	17.12	13.81	15.87
C18 : 1	6.49	7.77	7.38	7.54
C18 : 2	2.10	2.79	1.95	2.39
C20 : 4	0.59	0.51	0.60	0.49
C20 : 5	5.07	3.88	5.19	3.94
C22 : 5	11.95	12.80	11.22	14.08
C22 : 6	43.02	41.86	46.55	42.28
PUFAs[a]	60.63	59.05	63.56	60.79

[a]PUFAs are composed of AA, EPA, DPA, and DHA. Production yields (relative ratio) of fatty acids were presented by the average value of the contents of each stage of growth.

4. Salt Effect Compensated for Temperature Effects on the Yields of PUFA Production

PUFAs production per dry cell decreased with increasing temperature of cultivation, e.g., lower PUFAs yields per total fatty acids in the cell are observed at higher temperatures. Consistent with this fact, the yields of PUFAs and DHA production per total fatty acids in the cell were reduced from 60.63% to 59.05% and from 43.02% to 41.86%, respectively, when the temperature was increased from 25 °C to 30 °C (Table 4). Cellular PA contents also increased from 15.44% to 17.12%, thereby corroborating the effect of temperature. We also examined whether the effect of salt could overcome the effect of temperature on PUFAs yields. The reduced cellular PUFAs content at 30 °C was recovered up to a similar level at 25 °C by excluding $MgSO_4$ from the culture medium. This result suggested that the environmental factors such as temperature and salt concentration act independently on the control of cellular amount of PUFAs in *T. aureum*.

CONCLUSION

In this study, attempts were made to determine the effects of the concentration of the salts NaCl and $MgSO_4$, the primary components of sea salt, on the fatty acid profile and the yields of PUFAs production of *T. aureum* ATCC 34304. Reduction of NaCl concentrations in the culture medium caused a gradual decline in the content of principal saturated fatty acid, palmitic acid (PA, C16:0) from 15.65% at 30 g L^{-1} NaCl to 14.19% at 5 g L^{-1} NaCl but induced an increase in the concentration of PUFAs from 60.06% to 62.10%. $MgSO_4$ evidenced a more significant effect on the fatty acid profile. The content of PA increased from 58.63% at 18 g L^{-1} $MgSO_4$ to 63.56% at 0 g L^{-1} $MgSO_4$ and that of PUFAs. The integrated effect of the salts showed the highest yields of PUFAs and DHA 67.10% and 49.47%, respectively, at concentration of NaCl and $MgSO_4$ of 10 g L^{-1} and 0 g L^{-1}. A gradual reduction in the cel-

lular contents of mono- and diunsaturated fatty acids, OA and LA, was also caused by the integrated effects of the salts. These results support the conclusion that a reduction in salt concentrations stimulates a desaturation of fatty acids, and an increase in the cellular quantities of PUFAs, in particular, the most highly unsaturated DHA, in *T. aureum*. Furthermore, the reduced PUFAs contents observed at higher temperatures were recovered by removal of $MgSO_4$ from the culture medium, which suggests that two factors, temperature and salt concentration, act independently on the control of the cellular quantity of PUFAs in *T. aureum*. Further investigations into the molecular mechanisms of the effects of salts are desirable to increase the understanding on the biological functions of PUFAs, and also to facilitate the development of industrial strains with improved the yields of desired PUFAs production.

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