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Suitable Culture Medium for *Synechocystis* sp. SY-4 and Biochemical Composition of the Cells at Various Growth Phases

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The optimum medium components and appropriate inoculum cell density for the mass culture of *Synechocystis* sp. SY-4 were examined. The highest growth rate of this alga was obtained in Oita medium. The optimum medium components for the growth of this alga were 80 mg of ammonium sulfate, 10 mg of calcium perphosphate, 6 mg of Clewat-32 and 7.5 mg of urea per liter of seawater (10‰). The appropriate inoculum density of this alga to give the maximum cell density was 10×10^4 cells ml^{-1}.

Furthermore, the boichemical composition (protein, carbohydrate, lipid and fatty acid) of *Synechocystis* sp. SY-4 at the various growth phases was investigated. The maximum protein and carbohydrate contents were 50 % and 13 % of cell dry-weight, respectively, at the early-stationary phase. On the other hand, the maximum lipid content (12 % of cell dry-weight) was observed at the late-logarithmic phase. Thus, the algal cells containing high amounts of protein, carbohydrate and lipid were found to be produced effectively at the late-logarithmic or early-stationary phase.

The percentage of 16:1n-7 and those of some other fatty acids comprising 16:0, 18:0, 18:2n-6 and 18:3n-3 tended to increase and decrease, respectively, in the later growth phases.

Introduction

A cyanophyte, *Synechocystis* sp. SY-4, was isolated from the Mekari Strait, Seto Inland Sea,[1] and found to be a potentially useful culture feed for rotifer[1] which is used widely as a food for fish larvae in aquaculture fisheries. This alga can grow at temperatures between 25 and 35℃[1] in a simple economical medium consisting of ammonium sulfate, calcium perphosphate and Clewat-32 which is commonly used for culturing *N. oculata* and *T. tetrathele.*[2] Although *Synechocystis* sp. SY-4 grows well in this medium, futher work is needed to examine optimum conditions for the maximum growth.

Planktonic diatoms as a mollusc feed have been mass-cultured in 100-1000-l circular polycarbonate tanks or less than 10-m^3 canvas tanks.[3] On the other hand, *N. oculata* as a rotifer feed has been mass-cultured in 150-600-m^3 concrete pools at outdoor sunny places.[4] However, the outdoor cultures have some difficulties to control the culture conditions because the culture environment varies complicatedly. From necessity for the outdoor culture of *Synechocystis* sp. SY-4, we investigated the optimum medium components and appropriate inoculum cell density for the mass culture of *Synechocystis* sp. SY-4 in this study.

An important thing to be considered with rotifer culture is that the constituents of rotifer reflect the biochemical composition of the feed.[2,5-8] Therefore, the production of microalgae with a high nutritional value is very important to obtain the high quality of rotifer. From this point of view, we also investigated here the biochemical compositions such as protein, carbohydrate, lipid and fatty acid of *Synechocystis* sp. SY-4 at the various growth phases to produce effectively the algal cells with a high nutritional value in this study.

Materials and Methods

Growth conditions

Synechocystis sp. SY-4 was cultured in 500-ml Sakaguchi flasks containing 200 ml medium (10‰), each with a silicon foam plug, under 3,000-lx light from fluorescent lamps (14 h: 10 h light-dark cycle) with shaking at 100 rpm in a temperature-controlled chamber (25 ℃). For the culture at an outdoor sunny place, this alga was cultured in a 1-m^3 polycarbonate tank with aeration.

Cell density (cells ml^{-1}) was determined by counting the cells in culture aliquots in

a Thoma chamber. *Synechocystis* sp. SY-4 grows through binary fission, the growth rate was calculated as doublings per day (K, day^{-1})[9] :

$$N_2 = N_1 \cdot 2^{K(t_2-t_1)}$$
$$K = [1/(t_2-t_1)][\log_2(N_2/N_1)]$$
$$= [3.322/(t_2-t_1)] \cdot \log(N_2/N_1)$$

where t_1 and t_2 are the times of the logarithmic growth phase in days, and N_1 and N_2 are the cell densities on days t_1 and t_2, respectively.

For the biochemical analysis of this algal cells, samples from cultures at the various growth phase were centrifuged at 3,000 rpm, washed with sterile seawater, freeze-dried, and then stored at -30℃ for one to two weeks until use.

Analytical methods

Frozen samples of *Synechocystis* sp. SY-4 were suspended in distilled water, and homogenized with an ultrasonicator UD-201 (Tomy), and then assayed for the biochemical composition. Proteins were assayed as described by Bradford[10] with bovine serum albumin as a standard. Carbohydrates were quantified by the phenol-sulfuric acid method[11] with glucose as a standard. Lipids were measured by the method of Bligh and Dyer[12] after extraction with chloroform-methanol (1:2, V/V). The extracted lipids were combined, concentrated and weighed. Lipids were then saponified with NaOH [0.3 M in 90 % (V/V) methanol]. The fatty acids thus obtained were esterified by methanolysis with HCl-methanol [5 % HCl in methanol, V/V], and then analyzed as their methyl esters with a Hitachi G-3000 gas chromatograph equipped with an FID and a fused-silica capillary column (30×0.25 mm DB-5, J & W Scientific).

Results

Growth

Four media, Yamaguchi, Oita, Nagasaki and Hiroshima (Table 1), which have been used for the culture of *N. oculata*,[13] and seawater (10‰) were tested for the growth of *Synechocystis* sp. SY-4 (Fig. 1-A). These media allowed growth rates (K, day^{-1}, days 0 to 3) of 1.05, 1.95, 1.53, 1.81 and 0.47, respectively. Oita medium, which gave the maximum growth rate in 5 days culture (Oita = Hiroshima > Nagasaki > Yamaguchi

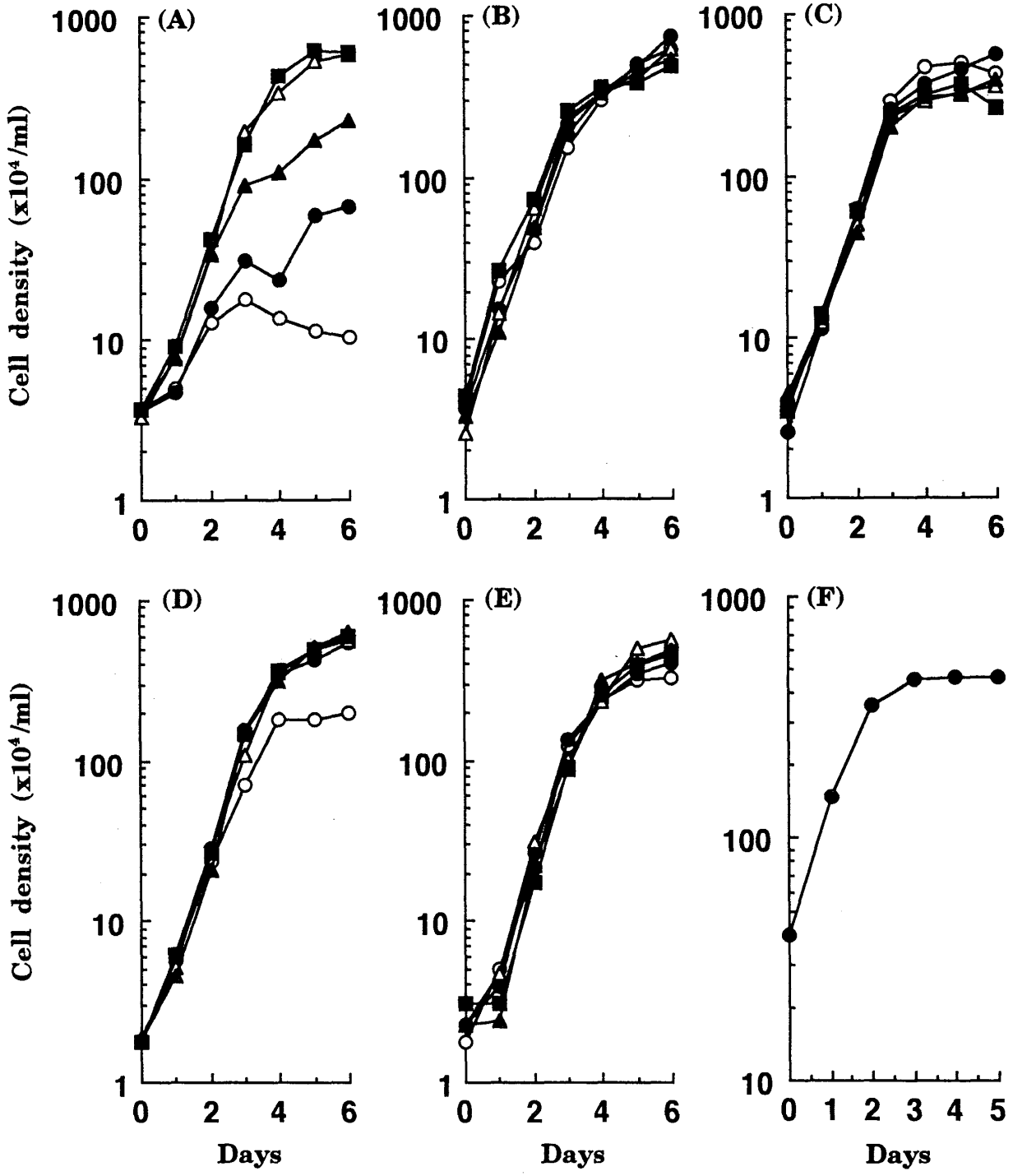

Fig. 1 (A) Growth of *Synechocystis* sp. SY-4 in the various fertilized media used for the mass culture of *N. oculata*. Media: ○, Seawater; ●, Yamaguchi; △, Oita; ▲, Nagasaki; ■, Hiroshima. See Table 1 for the medium compositions.
Growth of *Synechocystis* sp. SY-4 in Oita medium with different concentrations of (B) ammonium sulfate (mg/l): ○, 40; ●, 60; △, 80; ▲, 100; ■, 120, (C) calcium perphosphate (mg/l): ○, 5; ●, 10; △, 15; ▲, 20; ■, 25, (D) Clewat-32 (mg/l):○, 0; ●, 2; △, 4; ▲, 6; ■, 8, and (E) urea (mg/l): ○, 0; ●, 2.5; △, 5,0; ▲, 7.5; ■, 10.
(F) Growth of *Synechocystis* sp. SY-4 at an outdoor sunny place at water temperatures between 29.8 and 32.0℃.

seawater, $p < 0.05$), was chosen for further experiments. Next, the growth in this medium was examined by changing the concentration of the components one by one. This strain grew well at the concentrations of ammonium sulfate between 40 and 120 mg l^{-1} (Fig. 1-B), with the highest growth rate (K = 2.16, day^{-1}, days 0 to 3) with 80 mg l^{-1} ammonium sulfate (80 =100 = 120 > 60 > 40, $p < 0.05$), and the suitable concentration of calcium perphosphate for growth was 10 mg l^{-1} (K = 2.25, day^{-1}, day 0 to 3) (10 = 5 = 25 > 15> 20, $p < 0.05$)(Fig. 1-C). The highest growth was obtained with 6 mg l^{-1} Clewat-32 (K = 1.51, day^{-1}, days 0 to 3) (6 = 2 > 8 > 4 > 0, $p < 0.05$) (Fig. 1-D) and 7.5 mg l^{-1} urea (K = 1.84, day^{-1}, days 0 to 3) (7.5 = 10 > 5 > 2.5 > 0, $p < 0.05$) (Fig. 1-E). Thus, the optimum concentrations of medium components for the growth of *Synechocystis* sp. SY-4 were 80 mg of ammonium sulfate, 10 mg of calcium perphosphate, 6 mg of Clewat-32 and 7.5 mg of ureaper liter of seawater (10 ‰).

The growth of *Synechocystis* sp. SY-4 at an outdoor sunny place was shown in Figure 1-F. The maximum cell density (465×10^4 cells ml^{-1}) was obtained in 4-day culture. The water temperature during the culture changed between 29.8 ℃ and 32.0 ℃.

The growth rate and final density of *Synechocystis* sp. SY-4 in 5-day culture with various inoculum densities were shown in Fig. 2. The growth rate decreased with an increase in the inoculum density of this alga. On the other hand, the final density increased with an increase in the inoculum density up to 10×10^4 cells ml^{-1}, with a constant value at the inoculum density of more than 10×10^4 cells ml^{-1}.

Biochemical composition

The biochemical compositions (protein, carbohydrate, lipid and fatty acid) of *Synechocystis* sp. SY-4 harvested at the various growth phases (logarithmic, late-logarithmic, early-stationary and stationary) were determined, the results being shown in Tables 2 and 3. The maximum protein content was 50 % of the cell dry-weight at the early-stationary phase ($p < 0.05$). The maximum carbohydrate (13 %) and lipid (12 %) contents of the cell dry-weight were obtained at the early-stationary and late-logarithmic phases, respectively, but these values were not significant ($p > 0.05$) compared to those in the other growth phases except stationary phase.

The fatty acid composition of total lipids is given in Table 3. The major fatty acids in *Synechocystis* sp. SY-4 through all phases were 14:0, 16:0 and 16:1n-7. The percentage of 16:1n-7 increased in the later growth phases, but those of some other fatty acids such as 16:0, 18:0, 18:2n-6 and 18:3n-3 tended to decrease in these growth phases.

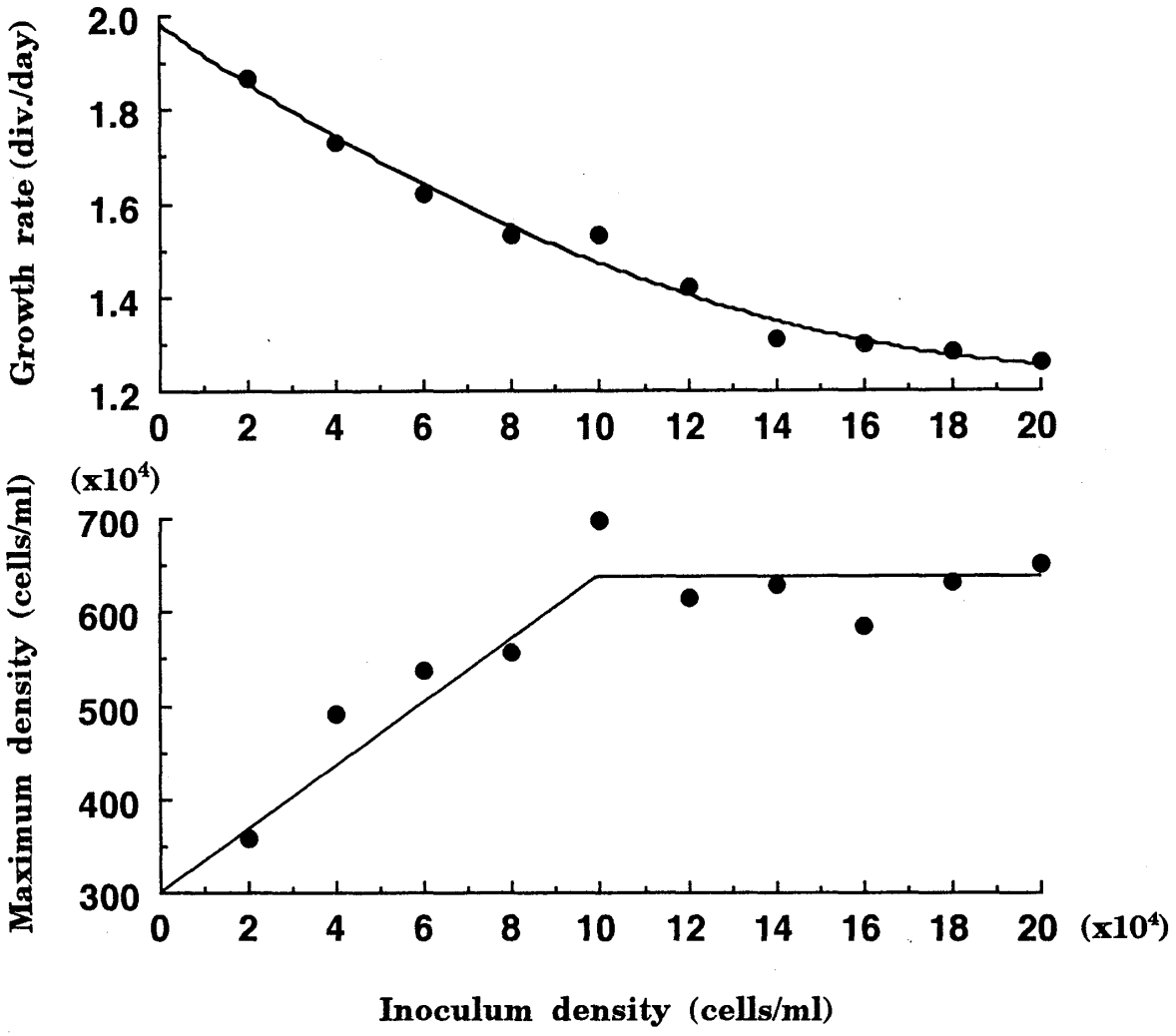

Fig. 2. Growth rate and maximum cell density of *Synechocystis* sp. SY-4 in 5 days culture with various inoculum densities at 25℃ and 3,000 lx.

Table 1. Compositions of fertilizers in the media adopted in four Prefectural Fisheries Institutes for the mass culture of *Nannochloropsis oculata.* [15]

Composition (mg l^{-1} seawater)	Medium			
	Yamaguchi	Oita	Nagasaki	Hiroshima
Ammonium sulfate	100	100	100	100
Calcium perphosphate	15	15	10	20
Clewat-32	0	5	0	4
Urea	5	5	10	0

Table 2. Biochemical composition of *Synechocystis* sp. SY-4 in the various growth phases.

Component (%) *	Growth phase			
	logarithmic	late-logarithmic	early-stationary	stationary
Protein	42	44	50	41
Carbohydrate	11	10	13	9
Lipid	10	12	10	7

* Expressed as a percentage of the cell dry-weight.

Table 3. Fatty acid composition of *Synechocystis* sp. SY-4 in the various growth phases.

Fatty acid (%)*1	Growth phase			
	logarithmic	late-logarithmic	early-stationary	stationary
12:0	6.1	6.2	6.4	6.4
14:0	36.1	35.6	37.5	37.1
15:0	1.2	1.0	1.0	0.8
16:0	13.2	13.3	10.8	10.5
16:1n-7	35.4	35.3	37.4	38.9
16:1n-5	Tr*2	0.2	0.2	0.2
16:2	0.2	0.2	0.3	0.1
16:3n-6	0.6	Tr	Tr	0.3
16:3n-3	0.6	0.1	0.4	0.4
18:0	2.6	3.4	1.1	1.1
18:1	0.6	0.7	0.7	0.7
18:2n-6	0.7	0.8	0.6	0.1
18:3n-6	0.4	0.2	0.3	Tr
18:3n-3	0.5	0.1	0.1	0.1

*1 Expressed as a percentage of the total fatty acid.
*2 Tr: trace amount (< 0.1).

Discussion

Synechocystis sp. SY-4 was cultured in various media[13] which have been used for the mass culture of *N. oculata*. Especially, the growth was greater in Oita or Hiroshima medium composed of ammonium sulfate, calcium perphosphate, urea and Clewat-32 than Yamaguchi or Nagasaki medium which does not contain Clewat-32. Thus, *Synechocystis* sp. SY-4 seems to require more metal ions for growth.

The mass culture media for *N. oculata* described above were obtained by improvements of Yashima medium proposed by Hirata[13] by changing the proportion of constituents. *N. oculata* is mass-cultured in medium containing of 100-200 mg l^{-1} of ammonium sulfate and 7.5-50 mg l^{-1} of calcium perphosphate in aquacultural institutes in Japan.[14] The production of *Synechocystis* sp. SY-4 is more economical because the good growth was obtained by the addition of less amounts of ammonium sulfate and calcium perphosphate compared with *N. oculata* culture.

Some of microalgae are not suitable for mass culture in outdoor because their growth rates decrease in the summer when the medium temperature rises to about 30 ℃, thus causing a shortage of live food in this season. In this study, *Synechocystis* sp. SY-4 was found to grow well in outdoor culture in spite of high temperatures between 29.8 ℃ and 32.0℃. *Synechocystis* sp. SY-4 was thus especially suitable for outdoor culture in the summer by virture of its high growth rate at high temperature.

In microalgal culture, the growth rate and the span of logarithmic growth phase are known to be affected by various inoculum cell densities. An inoculation with a low density of cells of *Chaetoceros decipiens* increased the maximum cell density at the stationary phase, and requires a long time to reach the maximum cell density.[15] Watanabe & Ota[16] also reported a similar tendency on the culture of *C. ceratosporum*. On the other hand, the value of the maximum cell density of *Tetraselmis tetrathele* was not affected with the inoculum cell density, but the time to reach the maximum density shortened by raising the inoculum density.[2] Tsunoda[17] found similar results on the culture of *C. ceratosporum* or *C. gracilis*. The growth rate of *Synechocystis* sp. SY-4 decreased with an increase in the inoculum density. The value of the maximum density of this alga in 5 days culture was enhanced with an increase in the inoculum density up to 10×10^4 cells ml^{-1}, and remained constant with the inoculum density of more than 10×10^4 cells ml^{-1}. Therefore, the appropriate inoculum density of *Synechocystis* sp. SY-4 is considered to be 10×10^4 cells ml^{-1}.

The biochemical composition of microalgae changed in the various growth phases.

Okauchi reported the change in chemical composition of *T. tetrathele*[2] and *N. oculata*[18] at the various growth phases. In the present study, we also found this phenomenon in *Synechocystis* sp. SY-4: the maximum protein (50 % of the cell dry-weight) and lipid (12 % of the cell dry-weight) contents were observed at the early-stationary and late-logarithmic phases, respectively. Shirota[19] calculated the average protein and lipid contents of microalgae to be 38.1 % and 8.2 %, respectively, based on the datum of sixteen microalgal species reported by Parsons et al.[20] and Hagino.[21] The protein [8] and lipid[22, 23] contents seem to be important factors in determining its nutritional value. Thus, the nutritional value of *Synecocystis* sp. SY-4 was evaluated high from this point of view. On the other hand, the maximum carbohydrate content (12 % the of cell dry-weight) was observed at the early-stationary phase. Carbohydrate also seems to be an important factor in determining a nutritional value.[6, 24, 25] Therefore, the most effective production of *Synechocystis* sp. SY-4 containing highest protein, carbohydrate and lipid was estimated to be attained at the late-logarithmic or early-stationary phase.

n-3 highly unsaturated fatty acid (n-3 HUFA) including eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) have been observed to play an essential role in fish larval growth.[22, 26-31] *Synechocystis* sp. SY-4 does not contain n-3 HUFA. Therefore, the nutritional value was evaluated lower than that of *N. oculata*. *N. oculata* is widely used in many aquaculture hatcheries as the initial step of an artificial food chain. The advantage of this alga over other microalgae lies primarily in its highly nutritious component i.e. EPA. Rotifers which consume the algal cells carry this nutrient to the fish larvae. Therefore, the nutritional value of the rotifer fed on *Synechocystis* sp. SY-4 could be effectively imploved on combination with *N. oculata*.

References

1) K. Sakamoto, E. Okimasu and A. Amemura : *J. Ferment. Bioeng.*, 82, 157-160 (1996).
2) M. Okauchi : *Bull. Natl. Res. Inst. Aquaculture*, 14, 1-123 (1988).
3) T. Ito, T. Ariyoshi and Y. Ito : *Bull. Saga Pref. Mariculture Center*, 97-103 (1985).
4) K. Kuronuma and K. Fukusho : *Bull. Int. Develop. Res. Inst. Canada*, 13-37 (1984).
5) I. Maruyama, T. Nakamura, T. Matsubayashi, Y. Ando and T. Maeda : *Suisanzoshoku*, 36, 259-263 (1988).

6) J.N.C. Whyte and W.D. Nagata : *Aquaculture*, 89, 263-272 (1990).
7) M. Caric, J. Sanko-Njire and B. Skaramuca : *Aquaculture*, 110, 141-150 (1993).
8) M.J. Fernandez-Reiriz, U. Labarta and M.J. Ferreiro : *Aquaculture*, 112, 195-206 (1993).
9) R.R.L. Guillard : *Cambridge Univ. Press, Cambridge*, 290-311 (1973).
10) M. Bradford : *Anal. Biochem.*, 72, 248-254 (1976).
11) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Reber and F. Smith : *Anal. Chem.*, 28, 350-356 (1956).
12) E.G. Bligh and W.J. Dyer : *Can. J. Biochem. Physio.*, 37, 911-917 (1959).
13) H. Hirata : *Youshoku*, 17, 79-82 (1980).
14) T. Nakano : *Bull. Yamaguchi Pref. Mariculture Center*, 4, 1-8 (1980).
15) Y. Matsuoka : *Bull. Kyoto Pref. Fish. Exp. Stat.*, 1-13 (1966).
16) K. Watanabe and Y. Ota : *Bull. Miyagi Pref. Fish. Exp. Stat.*, 24 (1967).
17) N. Tsunoda : *Bull. Yamaguchi Pref. Fish. Exp. Stat.*, 15, 31-42 (1977).
18) M. Okauchi, Wen-Jian Zhou, Wan-Hong Zou, K. Fukusho and A. Kanazawa: *Nippon Suisan Gakkaishi*, 56, 1293-1298 (1990).
19) A. Shirota : Suisan Jiryo Seibutsugaku, p. 303-333, *Koseisya Koseikaku, Tokyo* (1975).
20) T.R. Parsons, K. Stephens and J.D.H. Strickland : *J. Fish. Res. Bd. Canada*, 18, 1001-1016 (1961).
21) C. Ogino : *Nippon Suisan Gakkaishi*, 29, 459-462 (1963).
22) T. Watanabe, C. Kitajima, T. Arakawa, K. Fukusho and S. Fujita : *Nippon Suisan Gakkaishi*, 44, 1109-1114 (1978).
23) A.V. Frolov, S.L. Pankov, K.N. Geradze, S.A. Pankova and L.V. Spektorova : *Aquaculture*, 97, 181-202 (1991).
24) C.T. Enright, G.F. Newkirk, J.S. Craigie and J.D. Castell : *J. Exp. Mar. Biol. Ecol.*, 96, 1-13 (1986).
25) J.N.C. Whyte, N. Bourne and C.A. Hodgson : *Aquaculture*, 86, 25-40 (1990).
26) J.M. Owen, J.W. Adron, C. Middleton and C.B. Cowey : *Lipids*, 10, 528-531 (1975).
27) C.B. Cowey : *J. Fish. Res. Board. Can.*, 33, 1040-1045 (1976).
28) B.R. Howell : *Aquaculture*, 18, 215-227 (1979).
29) A.P. Scott and C. Middleton : *Aquaculture*, 18, 228-240 (1979).
30) T. Watanabe : In *Proc. 7 th Japan-Soviet Joint Symp. Aquaculture, Sept. 1978, Tokyo*, p. 29-66 (1979).
31) T. Watanabe, C. Kitajima and S. Fujita : *Aquaculture*, 34, 115-143 (1983).

要　約

藍藻 *Synechocystis* sp. SY-4 の好適培養液組成および好適接種密度を検討した。その結果，海水（10‰）1 ℓ当たり，硫安 80 mg，過燐酸石灰 10 mg，Clewat-32　6 mg および尿素 7.5 mg を施肥した培養液で最も高い増殖率が得られた。また，効率的に培養を行うための好適接種密度は，10×10^4 cells ml^{-1}と推察された。

Synechocystis sp. SY-4の各増殖相における細胞成分を分析した結果，静止期初期に蛋白質および炭水化物の各含量が最も高くなり，各々 50 %，13 %/cell dry-weight であった。一方，脂質含量は，対数期後期で最も高くなり，12 %/cell dry-weightであった。また，各増殖相における脂肪酸組成を分析した結果，対数期から静止期への移行に連れて 16:1n-7の割合は増加する傾向にあったが，16:0，18:0，18:2n-6 および 18:3n-3の割合は減少する傾向にあった。