Journal of Industrial Microbiology & Biotechnology (2000) 25, 218–224

www.nature.com/jim

Heterotrophic production of eicosapentaenoid acid by the diatom *Nitzschia laevis*: effects of silicate and glucose

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The effects of silicate and glucose on growth and eicosapentaenoic acid (EPA) production by the diatom *Nitzschia laevis* were studied. By alternately altering the concentrations of silicate (2.7–64 mg l^{-1}) and glucose (1–40 g l^{-1}) in the medium, the highest cell dry weight (ca. 5.5 g l^{-1}) was obtained at 20 g l^{-1} glucose and 32 mg l^{-1} silicate, while the highest specific growth rate (ca. 0.65 day^{-1}) was obtained at a relatively low glucose concentration (5 g l^{-1}) and high silicate concentrations (32–64 mg l^{-1}). At glucose levels of 5 and 20 g l^{-1}, EPA content was higher with lower silicate concentrations (2.7 and 16 mg l^{-1} silicate, respectively), while at a silicate level of 16 mg l^{-1}, higher glucose concentrations (20–40 g l^{-1}) facilitated EPA formation. The highest EPA yield (131 mg l^{-1}) was obtained at 20 g l^{-1} glucose and 32 mg l^{-1} silicate, while the highest EPA productivity (15.1 mg l^{-1} day^{-1}) was obtained at 20 g l^{-1} glucose and 64 mg l^{-1} silicate. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 218–224.

Keywords: diatom; *Nitzschia laevis*; EPA; glucose; silicate; heterotrophic

Introduction

$\omega-3$ Polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA), are of increasing interest owing to their therapeutic and pharmaceutical applications. Clinical studies have indicated that EPA plays an important role in the prevention of arrhythmia, atherosclerosis, cardiovascular disease and cancer [10].

At present, the commercial source of EPA is marine fish oil. However, there are several disadvantages of fish oil, such as peculiar taste and odour, stability problems and high purification cost. Because fish obtain $\omega-3$ polyunsaturated fatty acids from zooplankton which consume algae, many efforts have been devoted to developing a commercially feasible technology to produce EPA directly from microalgae [3,14,26,29,30,33].

As most EPA production processes are based on photoautotrophic growth, which is often hindered by light limitation, the EPA yield and productivity are very low because of the low growth rate and low cell density. To enhance EPA production by microalgal cultures, development of a commercial heterotrophic process is desired [1,4]. To date, several diatoms have been reported to be capable of producing EPA under heterotrophic conditions [2,28].

Silicate is an essential nutrient for diatom growth because the cells need silicate to form rigid external frustule shells. The effects of silicate on the growth of diatoms have been reported in some ecological investigations [16,17]. The influence of silicate on lipid synthesis has also been demonstrated in some diatom cultures such as *Cyclotella cryptica* [20], *Chaetoceros gracilis* [15], *Hantzschia* sp. [27], *Phaeodactylum tricornutum* [33] and *Nitzschia inconspicua* [6].

Glucose is another important nutrient for heterotrophic growth of microalgae; it is the most commonly used carbon source for microbial lipid production [18]. Production of polyunsaturated fatty acids by microalgae is affected by initial glucose concentration in a number of heterotrophic cultures of microalgae, e.g., in the production of DHA by *Crypthecodinium cohnii* [13], *Thraustochytrium* sp. [23] and *Schizochytrium limacinum* SR 21 [32]; arachidonic acid (AA) by *Mortierella alpina* [21] and *Rhytidiadelphus squarrosus* [11]; EPA by *Pythium irregulare* [24]; and docosapentaenoic acid (DPA) by *Pythium acanthicum* [22].

Recently, a diatom species, *Nitzschia laevis*, was investigated in our laboratory for its EPA production potential [31]. The initial results indicated that this diatom is a good EPA producer under heterotrophic conditions, as the cells showed not only better growth, but also a higher EPA content than under photoautotrophic conditions. However, there is a lack of detailed information on the effects of nutrients, especially silicate and glucose, on cell growth and EPA production by *N. laevis*. The aim of the present work was to obtain quantitative information on the effects of glucose and silicate on the growth and EPA production by *N. laevis*. The information would be useful for development of a cost-effective fermentation process for EPA production by the microalga.

Materials and methods

Cell cultures

The diatom *N. laevis* UTEX 2047 (University of Texas Culture Collection) was used. The preculture conditions were the same as in the previous study [31]. Exponentially growing cells in shake flasks were used as inoculum. Experiments were first performed by varying the concentrations of silicate (supplied in the form of $Na_2SiO_3 \cdot 9H_2O$) at a given glucose concentration (5 g l^{-1}) to study the effects of silicate on cell growth and EPA production. Then, the effects of glucose were investigated by varying glucose concentrations at a given silicate concentration (16 mg l^{-1}; 60 mg l^{-1} $Na_2SiO_3 \cdot 9H_2O$). After the optimal

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Received 8 May 2000; accepted 21 July 2000

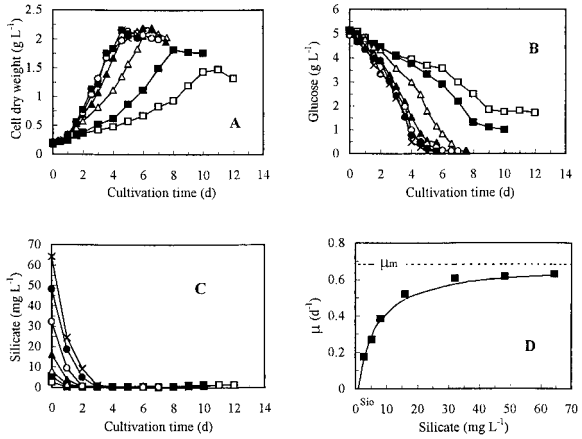

Figure 1 Time course of cell dry weight (A), glucose consumption (B), silicate consumption (C) and specific growth rate (μ) vs. silicate (D) of the diatom *N. laevis* at different initial silicate concentrations (with 5 g l^{-1} glucose). Symbols for initial silicate concentrations in (A), (B) and (C): (□) 2.7 mg l^{-1}; (■) 5.4 mg l^{-1}; (△) 8 mg l^{-1}; (▲) 16 mg l^{-1}; (○) 32 mg l^{-1}; (●) 48 mg l^{-1}; (×) 64 mg l^{-1}. (D) Scatter points, experimental values; solid line, calculated values.

glucose concentration (20 g l^{-1}) for EPA production was determined, the effects of silicate on EPA production was reinvestigated at this glucose level by varying the silicate concentration. The cells were cultured in triplicate in 500-ml Erlenmeyer flasks each containing 200 ml medium, at 25°C with orbital shaking at 160 rpm in darkness.

Analyses

Cell dry weight, glucose concentration (by HPLC) and silicate concentration (by spectrophotometry) in the medium were determined as reported previously [31]. The cells at early stationary phase were harvested and lyophilized for fatty acid analysis. Fatty acid methyl esters were prepared by *trans*-methylation with methanol–acetyl chloride [12] and analyzed by GC. Details of the GC analytical procedures were described previously [31].

Results and discussion

Effects of silicate at low glucose concentration

Cell growth and nutrient consumption: Figure 1 shows the kinetics of cell growth and nutrient consumption of *N. laevis* at different silicate concentrations. As shown in Figure 1A, both cell growth rate and maximum cell dry weight were lower at lower silicate concentrations. When silicate concentrations were greater than 16 mg l^{-1}, both the growth rate and maximum cell dry weight were at a high level.

Figure 1B and C shows the time course of glucose and silicate consumption, respectively. At lower initial silicate concentrations (2.7 and 5.4 mg l^{-1}), glucose was not completely consumed at the end of cultivation which is probably due to the limitation of silicate. At higher initial silicate concentrations (8–64 mg l^{-1}), all of the glucose was consumed by the cells by the end of cultivation. Increasing the glucose concentration from 5 to 20 g/l led to an increase in the maximum cell dry weight (Figure 3A). This suggested that glucose may be the limiting substrate at high silicate concentrations. The cell growth yield based on glucose, $Y_{X/Glu}$, was approximately 0.43 (g g^{-1}) over the range of silicate levels from 2.7 to 64 mg l^{-1}. As shown in Figure 1C, in all cases, silicate decreased sharply during the initial stage of cultivation. After 3 days, although the cells still grew, the net silicate uptake ceased and a residual amount of about 0.9 mg l^{-1} was present in the medium (Figure 1A and C). This pattern of silicate consumption was also observed in other

Table 1 Fatty acid composition (% TFA) of the diatom *N. laevis* at different initial silicate concentrations (with 5 g l^{-1} glucose)[a,b]

Fatty acids	Silicate (mg l^{-1})						
	2.7	5.4	8.0	16	32	48	64
14:0	10.09±0.72	11.20±0.47	10.82±0.46	11.05±0.53	9.81±0.06	10.61±0.48	10.40±0.20
14:1	0.52±0.07	0.54±0.02	0.59±0.02	0.57±0.04	0.54±0.01	0.59±0.00	0.57±0.06
16:0	20.78±1.43	22.78±0.86	25.63±0.78	25.54±0.27	27.40±0.26	25.91±0.32	27.60±1.02
16:1	37.16±1.23	37.12±1.00	35.88±1.50	33.97±0.41	33.16±1.17	33.43±0.73	30.08±1.10
18:0	1.46±0.08	1.05±0.07	0.81±0.01	1.65±0.12	0.97±0.10	1.32±0.05	1.64±0.02
18:1	5.63±0.53	4.83±0.43	3.31±0.11	3.95±0.20	3.98±0.38	3.72±0.32	4.10±0.27
18:2	4.05±0.45	3.52±0.28	3.18±0.24	2.54±0.07	2.97±0.25	3.34±0.49	3.54±0.41
18:3 ($\omega-6$)	0.94±0.07	0.79±0.33	0.69±0.14	1.04±0.07	0.85±0.06	1.06±0.12	1.24±0.03
18:3 ($\omega-3$)	1.20±0.10	0.98±0.39	0.73±0.39	1.03±0.09	0.95±−0.13	0.88±0.14	1.32±0.12
20:4	4.31±0.43	4.75±0.25	4.50±0.25	4.48±0.74	4.28±0.49	4.29±0.59	5.11±0.12
20:5	13.75±0.84	12.43±1.21	14.15±0.58	14.09±0.17	14.07±0.55	14.82±0.50	14.4±0.76
Unsatd (% TFA)	67.67±1.38	64.97±1.79	62.74±1.01	61.76±0.94	61.82±0.64	62.16±0.27	60.36±0.98
∇/mol	1.44±0.04	1.36±0.07	1.39±0.03	1.38±0.03	1.40±0.02	1.41±−0.01	1.42±0.05
TFA/DW (%)	13.89±1.05	13.43±1.75	11.02±0.86	10.07±1.37	8.17±0.46	7.55±0.51	7.5±1.23
$Y_{TFA/Glu}$	0.055±0.004	0.056±0.007	0.045±0.006	0.040±0.003	0.032±0.002	0.029±0.002	0.028±0.004

∇/mol: degree of unsaturation = [1.0(% monoenes) + 2.0(% dienes) + 3.0(% trienes) + 4.0(% tetraenes) + 5.0(% pentaenes)]/100.
TFA/DW: TFA content (%) = (TFA/cell dry weight) × 100%.
YTFA/Glu: TFA yield based on glucose, unit: (g TFA) (g glucose)$^{-1}$.
[a]Data are expressed as mean±SD of three replicates.
[b]Unsatd: percentage of unsaturated fatty acids (% of TFA).

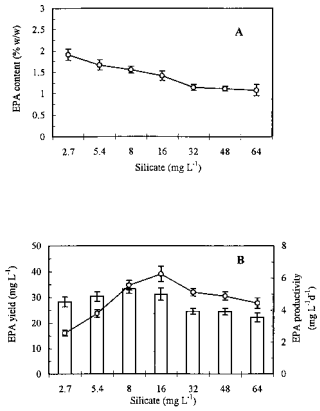

Figure 2 EPA content (A), and yield (circles) as well as productivity (bars) (B) of the diatom *N. laevis* at different initial silicate concentrations (with 5 g l^{-1} glucose). The EPA productivity was determined by calculating the EPA yield over the culture time. Data are means of three replicate measurements and the error bars show standard deviations.

diatom species [16,17,20]. Silicate uptake was mainly restricted to a brief period during cell division and a small amount of silicate remained in the medium when extracellular silicate uptake ceased. The reason for this phenomenon has been explained for the culture of *Thalassiosira pseudonana* cells [16]. To describe the growth kinetics of diatoms under silicate-limited conditions, Paasche [17] proposed a modified Monod model to correlate the specific growth rate with the silicate concentration, i.e.,

$$\mu = \frac{\mu_m\,(S_i - Si_o)}{K_s + (S_i - Si_o)} \qquad (1)$$

where μ_m is the maximum specific growth rate, K_s is the Monod half-saturation constant, S_i is the initial silicate concentration in the medium and Si_o is the silicate concentration remaining in the medium when the uptake of medium silicate ceased.

In the present work, the specific growth rates of *N. laevis* under different silicate concentrations could also be described by the modified Monod model (Equation 1). As shown in Figure 1D, the model gave a satisfactory fit (r^2=0.987) to the experimental data. The values of μ_m, K_s and Si_o were 0.683 day^{-1}, 5.92 mg l^{-1} and 0.89 mg l^{-1}, respectively.

Fatty acid profile and EPA production: The fatty acid profile of *N. laevis* at different silicate concentrations is shown in Table 1. In all cases, C14:0, C16:0, C16:1 and C20:5 were the major fatty acids in the cells. The cells also contained small amounts (less than 5% of total fatty acids, TFAs) of C14:1, C20:4 and C18 fatty acids (C18:0, C18:1, C18:2, C18:3 $\omega6+\omega3$). The percentage of C16:0 increased, and C16:1 decreased slightly with increasing silicate concentrations, while the proportions of other fatty acids were not very different at different silicate concentrations. Table 1 also shows that the percentage of unsaturated fatty acids (unsatd) decreased slightly with increasing silicate concentrations, but the degrees of fatty acid unsaturation (∇/mol) were unchanged. However, silicate greatly influenced the content of TFA; with an increase of silicate concentration from 2.7 to 64 mg l^{-1}, the TFA content decreased from 13.89% to 7.5% (w/w). This result is similar to those of previous investigations in which the lipid content of several marine diatoms increased under silicate-limited conditions [9,20,27]. The reason for this may be that in silicate-limited cultures, the cell alters its metabolism and diverts energy which was previously allocated for silicate uptake into storage lipid [7].

To test this explanation, the TFA yield based on glucose, $Y_{TFA/Glu}$, (g g^{-1}) was investigated. As shown in Table 1, the values of $Y_{TFA/Glu}$ were higher at the lower silicate levels (i.e., 2.7 and 5.4 mg l^{-1}), and then decreased with increasing silicate concentration from 5.4 to 64 mg l^{-1}. This suggests that more glucose was converted to lipids in the cultures with low, or limited silicate.

With respect to EPA production, as shown in Figure 2A, the content of EPA in the cells decreased with increasing silicate concentration. This was due to the decrease in TFA content with an increase in silicate concentrations as the EPA proportions in TFA were not apparently different (Table 1). Figure 2B shows that the highest EPA yield (33.3 mg l^{-1}) was obtained at an initial silicate concentration of 8 mg l^{-1}, whereas the highest productivity (6.25 mg l^{-1} day^{-1}) was obtained at a higher initial silicate concentration (16 mg l^{-1}) because of the higher growth rate at this silicate level.

Effects of glucose

Growth and EPA production by the diatom *N. laevis* was further investigated at differential initial glucose concentrations. The initial silicate level was set at 16 mg l^{-1} silicate as this was the optimal silicate concentration for EPA productivity (Figure 2B).

Cell growth and glucose consumption: As shown in Figure 3A and B, the cell growth kinetics at different glucose concentrations were quite different. This was reflected not only by maximum cell dry weight, but also by the specific growth rate. The maximum cell dry weight increased with glucose concentration ranging from 1 to 20 g l^{-1}, then leveled off at glucose concentrations of 20 to

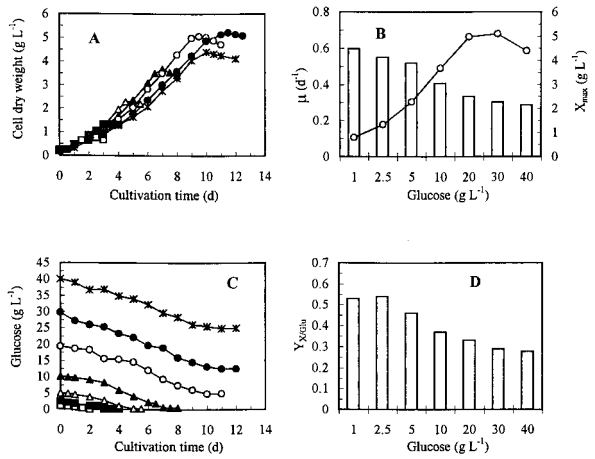

Figure 3 Time course of cell dry weight (A), specific growth rate (μ, bars) as well as maximum cell dry weight (X_{max}, circles) vs. glucose (B), glucose consumption (C), and growth yield based on glucose ($Y_{X/Glu}$, g g^{-1}) of the diatom *N. laevis* at different initial glucose concentrations (with 16 mg l^{-1} silicate). Symbols for initial glucose concentrations in (A) and (C): (□) 1 g l^{-1}; (■) 2.5 g l^{-1}; (△) 5 g l^{-1}; (▲) 10 g l^{-1}; (○) 20 g l^{-1}; (●) 30 g l^{-1}; (✶) 40 g l^{-1}.

Table 2 Fatty acid composition (% TFA) of the diatom *N. laevis* at different initial glucose concentrations (with 16 mg l^{-1} silicate)[a,b]

Fatty acids	Glucose (g l^{-1})						
	1	2.5	5	10	20	30	40
14:0	10.30±0.02	9.91±0.86	10.37±0.86	10.21±0.78	10.98±0.29	10.18±0.80	10.86±0.35
14:1	0.46±0.00	0.56±0.07	0.81±0.04	0.71±0.05	0.74±0.02	0.54±0.02	0.69±0.02
16:0	22.19±1.02	22.63±1.97	25.05±2.07	26.30±0.98	25.98±0.78	24.93±1.01	23.95±0.28
16:1	31.79±1.84	32.44±2.98	32.57±1.50	34.12±2.06	35.24±3.12	39.32±1.97	38.91±0.87
18:0	6.02±0.10	4.25±0.41	2.70±0.12	2.97±0.06	2.04±0.09	1.54±0.07	1.26±0.38
18:1	4.65±0.14	3.29±0.30	4.75±0.12	4.26±0.08	4.62±0.17	4.20±0.14	4.81±0.09
18:2	3.66±0.09	3.25±0.27	3.08±0.00	2.80±0.01	3.87±0.12	2.97±0.13	3.72±0.29
18:3 ($\omega-6$)	0.77±0.04	1.26±0.08	1.28±0.04	0.91±0.00	0.97±0.21	0.84±0.03	1.04±0.09
18:3 ($\omega-3$)	0.87±0.07	1.10±0.09	1.29±0.05	1.06±0.07	0.92±0.27	0.87±0.19	1.10±0.05
20:4	5.43±0.54	5.89±0.50	4.65±0.17	3.92±0.20	3.77±0.67	3.60±0.29	3.46±0.28
20:5	15.17±1.07	15.12±0.63	13.64±0.62	12.44±0.74	10.43±0.74	9.95±0.14	9.91±0.66
Unsatd (% TFA)	62.22±1.41	63.21±0.98	61.88±0.53	60.52±0.47	61.00±2.58	63.35±1.04	63.53±1.26
∇/mol	1.42±0.03	1.50±0.05	1.38±0.00	1.28±0.01	1.22±0.04	1.24±−0.02	1.21±0.01
TFA/DW (%)	6.70±0.14	6.98±0.33	10.04±0.04	14.31±0.24	21.90±1.61	22.54±0.78	21.45±0.32

[a]Data are expressed as mean±SD of three replicates.
[b]The denotation of unsatd (% TFA), ∇/mol, TFA/DW (%) are the same as in Table 1.

30 g l^{-1}, and dropped off at 40 g l^{-1}. The specific growth rate monotonically decreased with increasing glucose from 1 to 40 g l^{-1}. This pattern of growth kinetics on glucose was also reported in cultures of *C. cohnii* [8] and *Thraustochytrium* sp. [23].

Figure 3C shows the time course of glucose consumption by *N. laevis*. When the initial glucose concentration was over 20 g l^{-1}, glucose was not completely consumed at the end of cultivation. Further investigations showed that increasing the silicate concentration from 16 to 32 mg l^{-1} silicate at 20 g l^{-1} glucose level increased the maximum cell dry weight (see Figure 5B). This suggests that silicate may be the limiting substrate under these conditions.

The changes of $Y_{X/Glu}$ with glucose concentration are given in Figure 3D. The higher value of $Y_{X/Glu}$ was obtained at lower initial glucose concentrations, which suggests that at low glucose levels, the cells could utilize glucose more efficiently for growth. The low $Y_{X/Glu}$ values obtained at higher glucose concentrations may be due to the inhibitory effect which required more maintenance energy [5].

Fatty acid profile and EPA production: The effects of glucose concentration on the fatty acid composition of *N. laevis* are shown in Table 2. With an increase in glucose concentration, the percentage of C16:1 increased; this increment was considered to be offset by the decrease in the proportions of C18:0, C20:4 and C20:5 because other fatty acids were not different in terms of proportions at different glucose concentrations (Table 2). Also, the TFA content increased significantly (from 6.7% to 21.45%) with increasing glucose concentrations, perhaps because more storage lipids accumulated in the cells with carbon abundance [19]. Although more storage lipids might form in cells at high glucose concentrations, the lipids are usually triglycerides, which consist

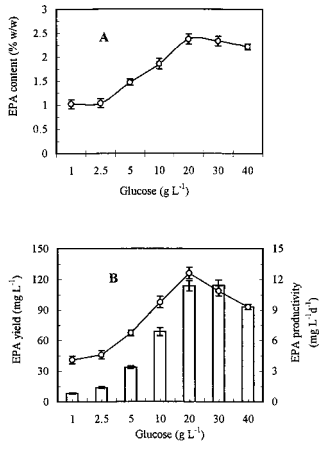

Figure 4 EPA content (A), and yield (circles) as well as productivity (bars) (B) of the diatom *N. laevis* at different glucose concentrations (with 16 mg l^{-1} silicate). The EPA productivity was determined by calculating the EPA yield over the culture time. Data are means of three replicate measurements and the error bars show standard deviations.

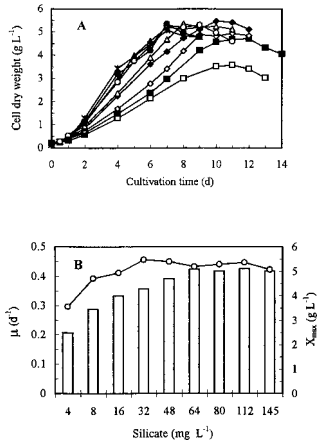

Figure 5 Time course of cell dry weight (A) and specific growth rate (μ, bars) as well as maximum cell dry weight (X_{max}, circles) vs. silicate (B) of the diatom *N. laevis* at different initial silicate concentrations (with 20 g l^{-1} glucose). Symbols for initial silicate concentrations in (A): (□) 4 mg l^{-1}; (■) 8 mg l^{-1}; (◇) 16 mg l^{-1}; (◆) 32 mg l^{-1}; (△) 48 mg l^{-1}; (▲) 64 mg l^{-1}; (○) 80 mg l^{-1}; (●) 112 mg l^{-1}; (✶) 145 mg l^{-1}.

Table 3 Fatty acid composition (% TFA) of the diatom *N. laevis* at different initial silicate concentrations (with 20 g l^{-1} glucose)[a,b]

Fatty acids	Silicate (mg l^{-1})								
	4	8	16	32	48	64	80	112	145
14:0	12.19±0.29	12.86±0.07	11.26±0.04	10.91±0.07	10.08±0.24	10.28±0.10	10.19±0.21	10.08±0.04	10.07±0.20
14:1	0.71±0.03	0.62±0.00	0.61±0.01	0.56±0.00	0.55±0.04	0.58±0.02	0.56±0.09	0.52±0.01	0.54±0.02
16:0	20.77±0.29	23.12±0.13	26.6±0.12	25.82±0.28	26.75±0.31	27.97±0.13	27.46±0.54	27.88±0.34	27.71±1.39
16:1	39.25±0.49	37.69±0.73	35.51±0.18	36.85±0.29	36.52±0.73	33.82±0.27	35.58±1.39	30.36±1.00	30.04±0.48
18:0	1.42±0.86	1.15±0.57	1.24±0.53	1.24±0.08	1.48±0.26	1.75±0.16	1.65±1.69	2.05±2.12	2.80±1.35
18:1	5.45±0.15	5.27±0.08	5.06±0.27	5.12±0.04	4.18±0.17	4.37±0.02	4.91±0.12	5.29±0.25	5.32±0.25
18:2	4.02±0.04	3.84±0.02	4.41±0.51	3.82±0.00	3.75±0.07	3.77±0.05	3.68±0.12	4.53±0.21	4.44±0.08
18:3 ($\omega-6$)	0.91±0.01	0.87±0.04	1.05±0.09	1.09±0.01	1.19±0.07	1.32±0.01	1.17±0.02	1.68±0.08	1.97±0.31
18:3 ($\omega-3$)	1.16±0.08	1.07±0.05	1.12±0.01	1.23±0.00	0.99±0.14	1.41±0.01	1.43±0.09	1.73±0.15	1.75±0.09
20:4	3.17±0.04	3.04±0.14	3.26±0.06	3.16±0.02	4.07±0.12	3.96±0.05	2.86±0.14	4.26±0.02	3.74±0.28
20:5	10.89±0.02	10.42±0.18	10.10±0.22	10.15±0.04	10.38±0.07	10.71±0.05	10.46±0.11	11.07±0.16	11.08±0.81
Unsatd (% TFA)	65.62±0.80	62.87±0.37	60.90±0.61	62.03±0.30	61.69±0.18	60.00±0.23	60.7±2.14	59.49±1.78	58.92±0.16
∇/mol	1.25±0.01	1.21±0.01	1.20±0.00	1.20±0.00	1.23±0.01	1.24±0.00	1.20±0.02	1.28±0.03	1.26±0.03
TFA/DW (%)	19.62±0.41	19.99±0.71	24.73±0.91	23.1±2.06	20.82±0.43	18.88± − 1.65	17.94±0.82	14.93±1.87	13.51±1.94

[a]Data are expressed as mean±SD of three replicates.
[b]The denotation of unsatd (% TFA), ∇/mol, TFA/DW (%) are the same as in Table 1.

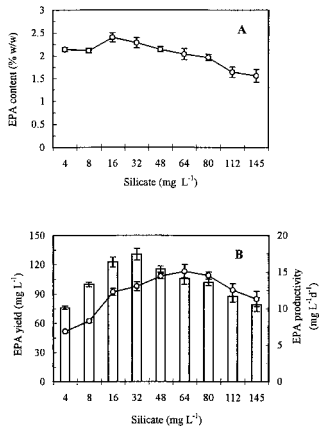

Figure 6 EPA content (A), and yield (circles) as well as productivity (bars) (B) of the diatom *N. laevis* at different silicate concentrations (with 20 g l^{-1} glucose). The EPA productivity was determined by calculating the EPA yield over the culture time. Data are means of three replicate measurements and the error bars show standard deviations.

largely of saturated and monosaturated fatty acids [25]. This may explain why the degree of fatty acid unsaturation (∇/mol) at a higher glucose concentration was lower than that at a lower glucose concentration (Table 2).

The changes of EPA content at different glucose concentrations are shown in Figure 4A. Although the EPA proportion of TFAs was lower at a higher glucose concentration (Table 2), high glucose is favorable to cellular EPA content because the content of TFAs increased greatly with increasing glucose concentrations (Table 2 and Figure 4A). The highest EPA content (ca. 2.4%, w/w) was obtained at 20 g l^{-1} glucose or above (Figure 4A).

Figure 4B shows EPA production at different glucose concentrations. The highest EPA yield (ca. 110 mg l^{-1}) was obtained at 20 and 30 g l^{-1} glucose. However, because growth rate at 20 g l^{-1} glucose was higher than that at 30 g l^{-1} glucose, the highest EPA productivity (12.6 mg l^{-1} day^{-1}) was obtained at 20 g l^{-1} glucose.

Effects of silicate at high glucose concentration

As the optimal glucose concentration for EPA production is 20 g l^{-1} (Figure 4B), the effects of silicate on *N. laevis* cultures were investigated at that concentration.

Cell growth characteristics: Figure 5A shows the time course of cell growth at different silicate concentrations. The specific growth rate and maximum cell dry weight are presented in Figure 5B. As shown in Figure 5B, with silicate concentrations increasing from 4 to 32 mg l^{-1}, both the maximum cell dry weight and specific growth rate increased with the silicate concentration. When silicate was increased to 32 mg l^{-1}, the maximum cell dry weight reached the highest level (ca. 5.5 g l^{-1}) and started to level off. However, the specific growth rate kept increasing up to 64 mg silicate l^{-1}.

Fatty acid profile and EPA production: Table 3 shows fatty acid profiles of the cells at different silicate concentrations. Compared with the data in Table 1, the pattern of fatty acid composition was similar to that at low glucose concentration. The percentage of C16:0 increased, and C16:1 decreased slightly with increasing silicate concentration, while the proportions of other fatty acids were not different for different silicate concentrations. As shown in Table 3, the changes of percentage of unsaturated fatty acid, degree of fatty acid unsaturation (∇/mol), and content of TFA (TFA/DW) with the silicate concentrations were also similar to those in Table 1. However, the absolute values of ∇/mol were lower, but the values of TFA/DW were higher at 20 g l^{-1} glucose than those at 5 g l^{-1} glucose (Table 1). As for the EPA production, Figure 6A shows that the highest EPA content (ca. 2.4%, w/w) was obtained at 16 mg l^{-1} silicate, while the highest EPA yield (131 mg l^{-1}) and productivity (15.1 mg l^{-1} day^{-1}) were obtained at 32 and 64 mg l^{-1} silicate, respectively (Figure 6B).

In conclusion, silicate and glucose are two important factors influencing the growth and EPA production of heterotrophic cultures of the diatom *N. laevis*. By optimizing the silicate and glucose concentrations, a high EPA yield (131 mg l^{-1}) and productivity (15.1 mg l^{-1} day^{-1}) were obtained. Compared with previous reports [3,14,26,29,30,33], the results of the present work indicated that the EPA yield and productivity obtained were relatively high. To further improve EPA production, it will be necessary to investigate the effects of other nutrients (e.g., nitrogen source, phosphorus) and environmental factors (temperature, salinity, medium pH, dissolved oxygen level).

Acknowledgements

The authors gratefully acknowledge the support of Hong Kong Research Grants Council, the Industry Department of the Hong Kong Government, and the University of Hong Kong Committee on Research and Conference Grants.

References

1 Apt KE and PW Behrens. 1999. Commercial developments in microalgal biotechnology. *J Phycol* 35: 215–226.
2 Barclay WR, KM Meager and JR Abril. 1994. Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. *J Appl Phycol* 6: 123–129.
3 Blanchemain A and D Grizeau. 1999. Increased production of eicosapentaenoic acid by *Skeletonema costatum* cells after decantation at low temperature. *Biotechnol Tech* 13: 497–501.
4 Chen F. 1996. High cell density culture of microalgae in heterotrophic growth. *Trends Biotechnol* 14: 421–426.
5 Chen F and MR Johns. 1996. Relationship between substrate inhibition and maintenance energy of *Chlamydomonas reinhardtii* in heterotrophic culture. *J Appl Phycol* 8: 15–19.
6 Chu WL, SM Phang and SH Goh. 1996. Environmental effects on the growth and biochemical composition of *Nitzschia inconspicua* Grunow. *J Appl Phycol* 8: 389–396.
7 Coombs J, PJ Halicki, O Holm-Hansen and BE Volcani. 1967. Studies on the biochemistry and fine structure of silicate shell formation in diatoms: II. Changes in concentration of nucleoside triphosphates in silicon-starvation synchrony of *Navicula pelliculosa* (Breb.) Hilse. *Exp Cell Res* 47: 315–328.
8 de Swaaf ME, TC de Rijk, G Eggink and L Sijtsma. 1999. Optimisation of docosahexaenoic acid production in batch cultivation by *Crypthecodinium cohnii*. *J Biotechnol* 70: 185–192.
9 Enright CT, GF Newkirk, JS Craigie and JD Castell. 1986. Growth of juvenile *Ostrea edulis* L. fed *Chaetoceros gracilis* Schutt of varied chemical composition. *J Exp Mar Ecol* 96: 15–26.
10 Gill I and R Valivety. 1997. Polyunsaturated fatty acids, part 1: occurrence, biological activities and application. *Trends Biotechnol* 15: 401–409.

11 Hansen CE and P Rossi. 1991. Effects of culture conditions on accumulation of arachidonic and eicosapentaenoic acids in cultured cells of *Rhytidiadelphus squarrosus* and *Eurhynchium striatum*. *Phytochemistry* 30: 1837–1841.
12 Jiang Y and F Chen. 1999. Production potential of docosahexaenoic acid by the heterotrophic marine dinoflagellate *Crypthecodinium cohnii*. *Process Biochem* 34: 633–637.
13 Jiang Y and F Chen. 2000. Effect of medium glucose concentration and pH on docosahexaenoic acid content of heterotrophic *Crypthecodinium cohnii*. *Process Biochem* 35: 1205–1209.
14 Molina Grima E, JA Sanchez Perez, JL Garcia Sanchez, F Garcia Camacho and D Lopez Alonso. 1992. EPA from *Isochrysis galbana*. Growth conditions and productivity. *Process Biochem* 27: 299–305.
15 Mortensen SH, KY Borsheim, JR Rainuzzo and G Knutsen. 1988. Fatty acid and elemental composition of the marine diatom *Chaetoceros gracilis* Schutt. Effcets of silicate deprivation, temperature and light intensity. *J Exp Mar Biol Ecol* 122: 173–185.
16 Paasche E. 1973. Silicon and the ecology of marine plankton diatoms: I. *Thalassiosira pseudonana* (*Cyclotella nana*) grown in a chemostat with silicate as limiting nutrient. *Mar Biol* 19: 117–126.
17 Paasche E. 1973. Silicon and the ecology of marine plankton diatoms: II. Silicate-uptake kinetics in five diatom species. *Mar Biol* 19: 262–269.
18 Ratledge C. 1989. Microbial Lipids, Vol. 2. Academic Press, London.
19 Roessler PG. 1990. Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions. *J Phycol* 26: 393–399.
20 Shifrin NS and SW Chisholm. 1981. Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light–dark cycles. *J Phycol* 17: 374–384.
21 Singh A and OP Ward. 1997. Production of high yield of arachidonic acid in a fed-batch system by *Mortierella alpina* ATCC 32222. *Appl Microbiol Biotechnol* 48: 1–5.
22 Singh A and OP Ward. 1998. Docosapentaenoic acid (C22:5, $\omega-3$) production by *Phythium acanthicum*. *J Ind Microbiol Biotechnol* 20: 187–191.
23 Singh A, S Wilson and OP Ward. 1996. Docosahexaenoic acid (DHA) production by *Thraustochytrium* sp. ATCC 20892. *World J Microbiol Biotechnol* 12: 76–81.
24 Stinson EE, R Kwoczak and M Kurantz. 1991. Effect of culture conditions on production of eicosapentaenoic acid by *Phythium irregulare*. *J Ind Microbiol* 8: 171–178.
25 Suen Y, JS Hubbard, G Holzer and TG Tornabene. 1987. Total lipid production of the green alga *Nannichloroposis* sp. QII under different nitrogen regimes. *J Phycol* 23: 289–296.
26 Sukenik A. 1991. Ecophysiological consideration in the optimization of eicosapentaenoic acid production by *Nannochloropsis* sp. (Eustigmatophyceae). *Biores Technol* 35: 263–269.
27 Taguchi S, JA Hirata and EA Laws. 1987. Silicate deficiency and lipid synthesis of marine diatoms. *J Phycol* 23: 260–267.
28 Tan CK and MR Johns. 1996. Screening of diatoms for heterotrophic eicosapentaenoic acid production. *J Appl Phycol* 8: 59–64.
29 Vazhappilly R and F Chen. 1998. Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth. *J Am Oil Chem Soc* 75: 393–397.
30 Vazhappilly R and F Chen. 1998. Heterotrophic production potential of omega-3 polyunsaturated fatty acids by microalgae and algae-like microorganisms. *Bot Mar* 41: 553–558.
31 Wen ZY and F Chen. 2000. Production potential of eicosapentaenoic acid by the diatom *Nitzschia laevis*. *Biotechnol Lett* (in press).
32 Yokochi T, D Honda, T Higashihara and T Nakahara. 1998. Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR21. *Appl Microbiol Biotechnol* 49: 72–76.
33 Yongmanitchai W and OP Ward. 1991. Growth of and omega-3 fatty acid production by *Phaeodactylum tricornutum* under different culture conditions. *Appl Environ Microbiol* 57: 419–425.