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Effects of Nutritional Factors on the Growth and Heterotrophic Eicosapentaenoic Acid Production of Diatom *Nitzschia laevis*

CAO Xiaohong*, LI Songyao, WANG Chunling, and LU Meifang

Tianjin Key Laboratory of Food Nutrition and Safety, College of Food Science and Biotechnology, Tianjin University of Science and Technology, Tianjin 300457, *P. R. China*

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Abstract The effects of several nutritional factors on the growth and eicosapentaenoic acid (EPA) production of diatom *Nitzschia laevis* were studied. 4 LDM (quadrupled concentration of the nutrient salt) was the optimal concentration of nutrient salt for the growth and EPA production of *N. laevis*. The growth of *N. laevis* was inhibited when the glucose concentration was either lower than 10 gL^{-1} or higher than 15 gL^{-1}. Both sodium nitrate and urea were good nitrogen sources for the growth and EPA production, while ammonium chloride seriously decreased the dry cell weight (DW) and the EPA content. Silicate seriously influenced the growth of *N. laevis*. The maximum DW of 2.34 gL^{-1} was obtained in the presence of 150 mgL^{-1} $Na_2SiO_3 \cdot 9H_2O$. The EPA content remained almost the same when the silicate concentration was lower than 150 mgL^{-1}; however, higher silicate concentrations resulted in a steady decrease of EPA content. Low medium salinity (≤29) did not seem to influence the DW of *N. laevis*, and high salinity resulted in a decrease of DW. The highest EPA content (4.08%) and yield (110 mgL^{-1}) were observed at the salinity of 36 and 29, respectively.

Key words polyunsaturated fatty acid; eicosapentaenoic acid; microalga; *Nitzschia laevis*; heterotrophy

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1 Introduction

Recognition of the effects of ω-3 fatty acids has created a momentum for extensive nutritional and pharmacological studies on these substances in human physiology. Eicosapentaenoic acid (EPA) is one of the ω-3 fatty acids that has gained intensive attention of many researchers around the world. It has been considered as an important nutrition element for enhancing human health and treating a variety of diseases such as atherosclerosis, rheumatoid, arrhythmia, psoriasis, diabetes and cancers (Nettleton, 1993). Currently, marine fish oil is the major commercial source of EPA; however, EPA and other polyunsaturated fatty acids (PUFAs) contained in fish oil are often unsuitable for food application because of the contamination of diverse pollutants and problems associated with the typical fishy smell and unpleasant taste. In order to meet the expected rise in demand and to circumvent the drawbacks of fish oil, alternative production processes for PUFAs are currently being developed. These include the development of refining techniques of fish oil and the exploitation of microbial PUFA sources (Yamamura and Shimomura, 1997; Ratledge, 2001; Barclay *et al.*, 1994).

*Corresponding author. Tel: 0086-22-60601428;
E-mail: caoxh@tust.edu.cn

In recent years, marine microalgae have been proved to be the primary producer of PUFAs, and much effort has been devoted to developing a commercially feasible technology to produce EPA directly from microalgae (Barclay *et al.*, 1994; Lebeau and Robert, 2003a; Molina *et al.*, 2003). The diatom *Nitzschia laevis* had been selected and proved to be a good source of EPA due to its high EPA content (Lebeau and Robert, 2003a; Tan and Johns, 1996). This species has an additional advantage in EPA production; it produces little DHA, thus the potential problem of separating DHA and EPA can be avoided. Moreover, *N. laevis* has been proved to be able to grow heterotrophically by utilizing glucose as the only carbon source, and thus could accumulate relatively large amount of EPA (Wen and Chen, 2000a; Wen and Chen, 2001a).

In this work, the effects of several nutritional factors on the growth and EPA production of diatom *N. laevis* were studied.

2 Materials and Methods

2.1 Microalga and Culture Conditions

The microalga *N. laevis* (UTEX2047) was purchased from the Culture Collection of Algae at the University of Texas at Austin. The cells were maintained in LDM medium supplemented with 5 gL^{-1} glucose and 30 mgL^{-1}

$Na_2SiO_3 \cdot 9H_2O$. The components of basal LDM medium have been described by Starr and Zeikus (1993). Then the cells were innoculated into 500 mL shaking flasks each containing 200 mL medium and incubated at 25℃ in an orbital shaker (150 $r min^{-1}$). The initial acidity of the medium was adjusted to pH 8.2 prior to autoclaving at 121℃ for 20 min. Cultures were maintained at stationary phase and then analyzed for cell mass, lipid content and EPA production.

2.2 Biomass Measurement

A spectrometer (SP-756, Shanghai Spectrum Instruments CO., LTD) was used to measure the absorbance at 540 nm during growth to determine the cell density. The optical density (OD_{540}) was then converted to dry cell weight (DW), which was determined as described by Wen and Chen (2000a). To ensure that the optical density represented well the dry cell density, a relationship between OD_{540} and DW was established as described by Feng *et al.* (2005). *N. laevis* cells at the stationary phase were concentrated and then gradually diluted before the OD_{540} was measured. As shown in Fig.1, the value of OD_{540} within the range from 0.2 to 1.1 was linearly correlated with DW.

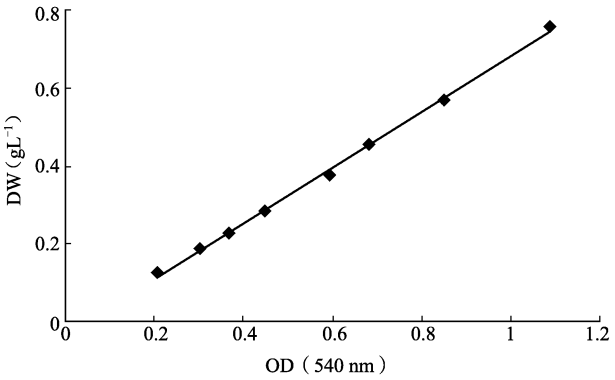

Fig.1 Relationship between optical density (OD_{540}) and dry cell weight (DW). The calibration equation is DW (gL^{-1}) $=0.7117OD_{540}-0.0319$ (correlation coefficient $R^2=0.9982$).

2.3 Lipid Extraction and Fatty Acids Analysis

N. laevis cells were harvested at the end of the exponential phase by centrifugation at 6000 $r min^{-1}$ for 10 min. The cells were washed twice by distilled water and then lyophilized and kept at −20℃ in test tubes filled with nitrogen before analysis. Lipid was extracted by the method of Bligh and Dyer (1959).

Lipid was methylated by a direct acid-catalyzed transesterification in 2 mL of 4% sulfuric acid in methanol (75℃ for 1 h). An appropriate amount of internal standard heptadecanoic acid (17:0) was added into the lipid extract before methylation. After the contents cooled, 2 mL water and 2 mL hexane were added. The fatty acid methyl esters (FAMEs) in the hexane layer were vortexed, centrifuged and collected. Then the hexane layer was analyzed by gas chromatography (GC 7890II, Techcomp. Shanghai) equipped with a flame ionization detector and a SE-54 capillary column (15 m×0.25 mm×0.25 μm). The column temperature was 200℃, the injector and detector temperature was 280℃. Nitrogen was used as the carrier. One microliter of samples were injected, and the quantification of EPA was identified by comparing its peak area with the internal standard heptadecanoic acid (17:0).

2.4 Statistical Analysis

Treatments were done in triplicate, and data were analyzed using the Statistical Analysis System (SAS 9.0). Differences were reported as significant when $P<0.05$.

3 Results

3.1 Concentration of Nutrient Salts

The LDM medium consists of 1 g tryptone, 892 mL seawater, 100mL Bristol solution, 6 mL PVI solution, 1 mL stock solutions of biotin and 1 mL vitamin B_{12} (per liter). Bristol solution and PVI solution were considered to supply essential metal elements for the growth of *N. laevis*. To study the effect of concentration of nutrient salts on the growth of *N. laevis*, the concentrations of the two solutions were increased by one, two and three times, and the media were defined as 2LDM, 3LDM and 4LDM, respectively. The control was the basal LDM medium, and defined as LDM. All the media were supplemented with 5 gL^{-1} glucose, 1 gL^{-1} tryptone and 120 mgL^{-1} $Na_2SiO_3 \cdot 9H_2O$. Results are presented in Fig.2. The growth of *N. laevis* was stimulated by increasing concentrations of Bristol and PIV solutions. This was probably because that higher nutrient salts concentration (4LDM) could supply sufficient metal elements which were vital to the growth of *N. laevis*, especially at the end of the exponential phase. No significant difference was observed when the concentration of nutrient salts was higher than 4LDM (data not shown). The DW was 1.55 gL^{-1} under 4LDM at the 9th day.

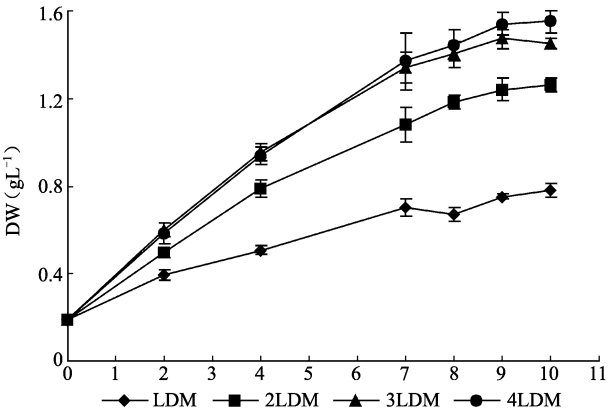

Fig.2 Effect of different concentrations of nutrient salts on the growth of *N. laevis*.

The EPA content was 2.10% for the basal LDM medium, but it increased significantly to 2.90% for 4LDM ($P<0.05$). Both the biomass and EPA content increased if

we quadruple the concentrations of Bristol and PVI solutions as shown in Figs.2 and 3. Therefore 4LDM was used in the following experiments.

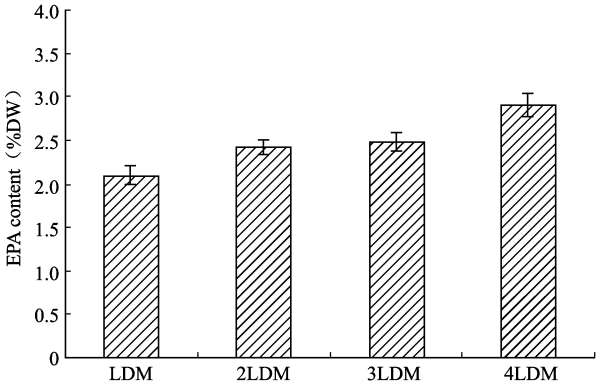

Fig.3 Effect of different nutrient salts concentrations on EPA content of *N. laevis*. $P<0.05$, compared with the control (LDM).

3.2 Glucose

Carbon source is necessary for providing the energy and carbon skeletons for cell growth. The heterotrophic microalga *Nitzschia laevis* can use glucose as the only carbon source (Wen and Chen, 2000a). The basal LDM medium was supplemented with 1 gL^{-1} tryptone and 120 mgL^{-1} $Na_2SiO_3 \cdot 9H_2O$. As shown in Fig.4, at low glucose concentrations (2 gL^{-1} and 5 gL^{-1}), the growth of *N. laevis* was seriously inhibited because of substrate limitation. The inhibitory effect of high initial glucose concentration (20 gL^{-1}, 25 gL^{-1}, and 30 gL^{-1}) was observed. The maximum cell biomass was obtained in the presence of 10 gL^{-1} (DW 2.06 gL^{-1}) and 15 gL^{-1} (DW 2.08 gL^{-1}) glucose. The glucose utilization efficiency was relatively lower at the glucose concentration of 15 gL^{-1}. So the optimal glucose concentration was 10 gL^{-1}. This result may be important for the development of batch and fed-batch cultivation processes in bioreactors because the initial glucose concentration should be appropriate to obtain the optimal growth rate, and at the same time avoid inhibition by high substrate concentrations.

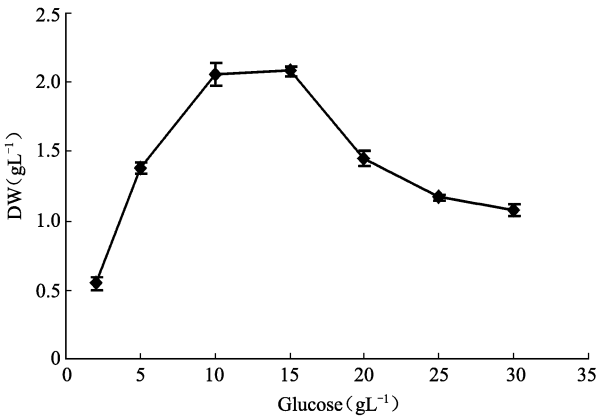

Fig.4 Effect of different glucose concentrations on the growth of *N. laevis*.

3.3 Nitrogen Source

Nitrogen is a major nutritional factor for microalgae cultivation. *Nitzschia laevis* was grown in 4LDM medium with $NaNO_3$, urea, NH_4Cl, tryptone and yeast extract as nitrogen sources, respectively. All the nitrogen sources were supplemented into the medium at the concentration of 1 gL^{-1} with 10 gL^{-1} glucose and 120 mgL^{-1} $Na_2SiO_3 \cdot 9H_2O$. Results are presented in Table 1. Sodium nitrate seems to be the best nitrogen source for the growth of *N. laevis*. When using ammonium as the sole nitrogen source, the DW was much lower than other nitrogen sources, probably because the pH of the medium containing NH_4Cl decreased sharply due to the assimilation of ammonium ions (Wen and Chen, 2001a). When using other nitrogen sources, the pHs of the media generally rise steadily from about 7.1 to about 7.8.

Table 1 Effect of different nitrogen sources on the growth and EPA production of *N. laevis*

Nitrogen (1 gL^{-1})	DW (gL^{-1})	Fatty acids composition (%TFA)					TFA content (% DW)	EPA	
		14:0	16:0	16:1	20:5	Others		Content (%DW)	Yield (mgL^{-1})
$NaNO_3$	2.22	14.85±1.03	11.73±0.19	41.47±2.08	20.20±0.18	7.61±0.69	13.22	3.07	68.1
Urea	1.94	13.31±0.30	8.31±0.82	34.92±0.60	27.62±0.96	15.04±0.46	13.18	3.64	70.6
NH_4Cl	0.94	21.24±0.25	14.48±0.28	46.55±1.58	17.70±0.54	0.03±0.01	2.88	0.51	4.8
Tryptone	2.01	12.69±0.27	18.21±0.29	49.64±0.84	12.68±1.07	2.72±0.80	10.88	1.38	27.7
Yeast extract	1.18	17.05±0.16	13.55±0.88	31.20±1.68	17.67±2.03	16.28±0.14	6.85	1.21	14.3

Notes: DW: dry cell weight; TFA: total fatty acids. Data are expressed as average ± SD of triplicate.

The major fatty acids produced by *N. laevis* were C14:0, C16:0, C16:1 and C20:5. Other fatty acids were either unknown or too little. No docosahexaenoic acid (DHA) was detected in the microalga *N. laevis*. When using sodium nitrate and urea as the sole nitrogen sources, the total fatty acid (TFA) content (%DW) was 13.22% and 13.18%, and the EPA content was 3.07% (%DW) and 3.64%, respectively. Both the TFA and EPA contents were higher than those of the other nitrogen sources. The EPA yields for $NaNO_3$ and urea were 68.1 mgL^{-1} and 70.6 mgL^{-1}, respectively. Table 1 indicates that both $NaNO_3$ and urea were good nitrogen sources for *N. laevis*, and thus the effects of different concentrations of the two nitrogen sources were studied in the following experiments.

3.3.1 $NaNO_3$

Fig.5 shows the effect of different $NaNO_3$ concentrations on the growth and EPA production of *N. laevis*. DW

increased with the increase of concentration of $NaNO_3$, and the highest DW (2.38 gL^{-1}) was obtained when the concentration of $NaNO_3$ was 2 gL^{-1}. The EPA content was also stimulated with increasing $NaNO_3$ concentration, and maximum EPA content was 3.38% when the concentration of $NaNO_3$ was 1.5 gL^{-1}. Higher concentrations were found to decrease the EPA content. The highest EPA yield (79.1 mgL^{-1}) was obtained when the concentration of $NaNO_3$ was 1.5 gL^{-1}.

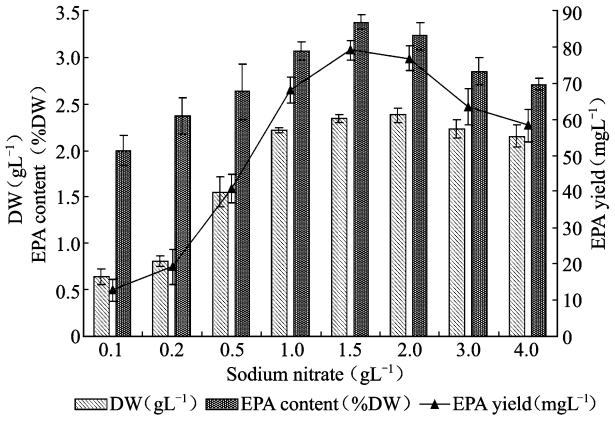

Fig.5 Effect of different $NaNO_3$ concentrations on the growth and EPA production of *N. laevis*.

The microalga *N. laevis* could be defined as an oleaginous microorganism as it was able to accumulate over 20%–25% lipid of the dry biomass (Ratledge and Evans, 1989). Most oleaginous microorganisms start to accumulate oil whenever carbon source is present, while at the same time, growth is limited by another nutrient, in practice often the nitrogen source (Kessell, 1968; Werner, 1977). Fig.6 shows that the lipid content decreased from 36.6% to 21.5% with the increasing concentrations of $NaNO_3$ from 0.1 gL^{-1} to 4.0 gL^{-1}, indicating that the lipid tended to accumulate at nitrogen-limited conditions. However, the EPA content (%DW and %TFA) increased from 2.00% and 13.52% to the highest value of 3.38% and 23.62%, respectively, indicating that EPA accumulated at relatively high nitrogen conditions which did not actually fit for lipid accumulation.

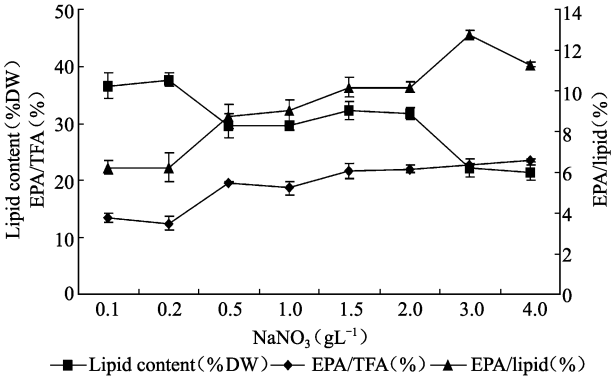

Fig.6 Effect of different nitrate concentrations on the lipid and EPA content production of *N. laevis*.

3.3.2 Urea

The effect of different urea concentrations on the growth and EPA production of *N. laevis* was investigated and the results are presented in Fig.7. DW, EPA content (% DW) and EPA yield increased with the concentration increase of urea, and reached the maximums 2.06 gL^{-1}, 3.89%, and 80.1 mgL^{-1} respectively when it was 1.5 gL^{-1}. A urea level above 1.5 gL^{-1} seemed to inhibit the EPA accumulation as shown in Fig.7. At the urea concentration of 4 gL^{-1}, the EPA content reduced to 2.65%.

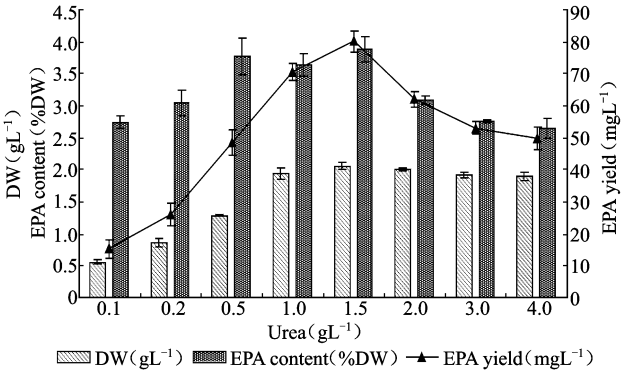

Fig.7 Effect of different urea concentrations on the growth and EPA production of *N. laevis*.

3.4 Silicate

Diatoms need silicon to form their frustules (cell walls composed of amorphous silica), thus investigations of the biomass and EPA production by *N. laevis* unavoidably involve this essential nutrient. *N. laevis* was cultivated in media containing 10 gL^{-1} glucose as the carbon source and 1.5 gL^{-1} $NaNO_3$ as the nitrogen source. As shown in Fig.8, under silicate-free (0 gL^{-1}) and silicate-deficient conditions, *N. laevis* can grow although such growth was seriously inhibited. This was probably because the *N. laevis* cell used its intracellular silicate pool to support its physiological activities (Werner, 1977). The maximum DW 2.34 gL^{-1} was obtained in the presence of 150 mgL^{-1} $Na_2SiO_3 \cdot 9H_2O$, and higher concentrations of $Na_2SiO_3 \cdot 9H_2O$ resulted in a steady decrease of biomass. At 500 mgL^{-1} $Na_2SiO_3 \cdot 9H_2O$, the DW decreased to 1.52 gL^{-1}.

The EPA content did not vary too much when the concentration of $Na_2SiO_3 \cdot 9H_2O$ was lower than 200 mgL^{-1}. Without silicate in the medium (to ensure that no silicate was contained in the medium, the inoculum was centrifuged at 479×g for 5 min and washed twice with fresh silicate-free medium before inoculating), the growth of *N. laevis* was inhibited. However, within a certain range the EPA content was 3.00% (% DW) under silicate-free condition, with no significant decrease compared to those at high silicate concentrations, which indicated that silicate was vital for the growth of *N. laevis* but not vital for EPA production. However, higher silicate concentrations (>200 mgL^{-1}) resulted in a significant decrease of the EPA content (Fig.8) (P<0.05 for 300, 400 and 500 mgL^{-1}

$Na_2SiO_3 \cdot 9H_2O$). When the concentration of $Na_2SiO_3 \cdot 9H_2O$ reached 500 mgL^{-1}, the EPA content was 1.94%, while it was 3.28% when the concentration was 150 mgL^{-1}. The maximum EPA yield 76.8 mgL^{-1} was also obtained at this concentration.

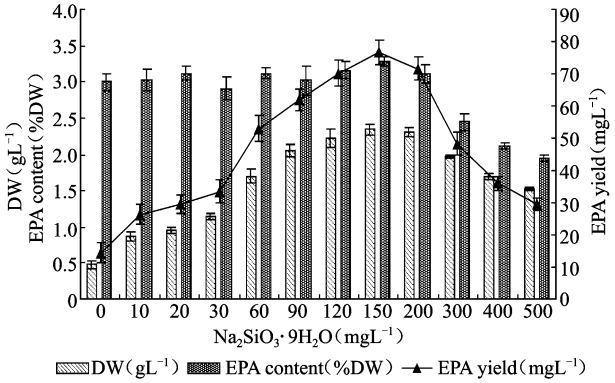

Fig.8 Effect of different concentrations of $Na_2SiO_3 \cdot 9H_2O$ on the growth and EPA production of *N. laevis*.

3.5 Salinity

Medium salinity may influence the physiological properties of marine microalgae. The effect of different medium salinities (ranging from 8 to 56) on biomass and EPA production was investigated in medium containing 10 gL^{-1} glucose, 1.5 gL^{-1} $NaNO_3$ and 150 mgL^{-1} $Na_2SiO_3 \cdot 9H_2O$. As shown in Fig.9, no significant decrease of DW was observed when the salinity was below 29. The growth of *N. laevis* was inhibited at the salinity of 36, 46, and 56 (P<0.05). The EPA content was stimulated when the salinity was below 36, and higher salinity resulted in an obvious decrease (P<0.05 for the salinity of 46 and 56). The lipid content was relatively stable (about 23%−25% of the dry biomass, details not shown). The maximum EPA yield 110 mgL^{-1} was obtained at salinity 29.

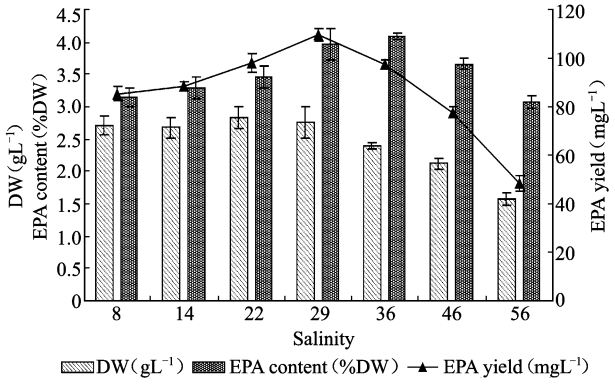

Fig.9 Effect of different medium salinities on growth and EPA production of *N. laevis*.

4 Discussion

The lipid content of the oleaginous microalga *N. laevis* was high at low nitrogen concentrations. The reason might be that in N-limited cultures, the availability of nitrogen caused an increase of cell chlorophyll to support cell metabolism, which was accompanied by an increase in the content of membrane lipids (Otero *et al*., 1997). However, the EPA content was enhanced, indicating that high nitrogen condition fit EPA accumulation but not lipid production. So EPA synthesis may not be totally associated with lipid accumulation.

It has been reported that the EPA content tended to increase when silicate became the limiting factor, and reasoned that in silicate-limited cultures, the cell tended to alter its metabolism and divert energy which was previously allocated for silicate uptake into lipid storage (Wen and Chen, 2000b; Wen and Chen, 2003). However, in this study, we found that the EPA content was stable under silicate-limited conditions ($\leqslant$150 mgL^{-1}), indicating that silicate was not vital for EPA production of *N. laevis*.

Culture medium of different salinity levels was obtained by adding various amounts of seawater condensate (SWC) to distilled water, and the salinity was determined by a baume detector. In our study, we found that the biomass was not influenced obviously by low medium salinity. This may be useful for large-scale cultivation, for the medium salinity should preferably be as low as possible in order to prevent potential bioreactor corrosion. Xu and Beardall (1997) reported that the content of ω-3 PUFAs in *Dunaliella* sp. decreased as the medium salinity increased. However, Seto *et al*. (1984) reported that *Chlorella minutissima* cells grown in SWC or NaCl-enriched medium contained more EPA than those in low NaCl medium.

In this study, the effects of several nutritional factors on the growth and EPA production of the microalga *N. laevis* were investigated. Further optimization is expected and high-yield EPA production process is also to be developed in flasks and bioreactors.

Acknowledgements

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