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*Full Length Research Paper*

# Characterization of a newly isolated green microalga *Scenedesmus* sp. as a potential source of biodiesel

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**An oil-producing microalga SP-01 belonging to the genus *Scenedesmus* was shown to be able to grow on a media containing 5 to 30 g $L^{-1}$ NaCl. Under mixotrophic condition, the maximum cell dry weight of 3.1 g $L^{-1}$ and specific growth rate of 0.034 $h^{-1}$ were obtained at 0.5 g $L^{-1}$ $NaNO_3$ and 6 g $L^{-1}$ acetate. The effects of salinity on the biomass, lipid, and carotenoid productions of the alga SP-01 in mixotrophic mode were investigated. The biomass productivity increased with increasing $NaNO_3$ concentrations, and addition of NaCl resulted in a higher biomass while $NaNO_3$ was present. The maximum lipid content was obtained while no $NaNO_3$ and NaCl was added, and the lipid content decreased with increasing $NaNO_3$ concentrations or addition of NaCl. The maximum lipid productivities of 67.44 to 68.44 mg $L^{-1}day^{-1}$ were obtained while $NaNO_3$ and NaCl were not added or while 20 g $L^{-1}$ NaCl and 0.13 g $L^{-1}$ $NaNO_3$ were added. The algal lipid was mainly composed of C16 and C18 fatty acids accounting for more than 90% of total fatty acids. Furthermore, lutein and astaxanthin were the main carotenoids.**

**Key words:** Halotolerant, microalga, *Scenedesmus* sp., mixotrophic culture, lipid, carotenoid.

## INTRODUCTION

Microalgal biodiesel appears to be the renewable biofuel that has the potential to replace petroleum-derived transport fuel (Chisti, 2008). However, many key technologies at almost all stages of the microalgal biodiesel production should be further developed and optimized (Gong and Jiang, 2011). Wijffels and Barbosa (2010) advised that it might take 10 to 15 years for the development of a sustainable and economically viable process for the commercial biofuel production from microalgae. Mutanda et al. (2011) indicated that the microalgal biodiesel production mainly depends on the isolation and selection of ideal algal strains.The key factors determining the potential of microalgal strains as biodiesel material are their growth rate, lipid content, and lipid productivity (Mutanda et al. 2011; Gong and Jiang, 2011; Griffiths and Harrison, 2009). The ideal algal strain for biofuel production should have the highest biomass productivity and the highest lipid content. Unfortunately, this is not always achievable, because the microalgae with higher lipid content have mostly a slower growth rate, such as *Botryococcus braunii* (Rao et al., 2007). On the other hand, some microalgae with lower lipid content have a higher cell growth rate; for example, *Chlorella vulgaris* (Griffiths and Harrison, 2009). Much of early research on biodiesel production from microalgae focused on the search for species with high lipid content. However, those species grew slowly, and were conta- minated easily by other microorganisms in large-scale culture conditions.

Numerous studies have shown that different culture conditions play an important role in biomass and lipid production of microalgae, such as carbon source (Hu et

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al., 2008), nitrogen source (Li et al., 2008; Lin and Lin, 2011), C: N ratio (Chen and Johns, 1991), salinity (Rao et al., 2007), and light intensity (Lv et al., 2010). Moreover, microalgae can be cultivated under different modes such as autotrophic, heterotrophic, and mixotrophic cultivations. The previous investigation on searching for species was carried out mostly under the autotrophic growth mode; for example, the Aquatic Species Program (ASP) (Sheehan et al., 1998).

However, it is difficult to reach a high density of microalgal biomass under autotrophic mode, resulting in the increases of the biomass harvesting cost (Chen and Johns, 1991). The heterotrophic cultivation can eliminate the requirement for light, and thus offer the possibility of greatly increased cell density and productivity, but it cannot utilize solar energy and $CO_2$ directly (Li et al., 2011). High cell density and productivity can be obtained using mixotrophic cultivation in theory although bacterial infection may be a challenge (Li et al., 2011). The presence of an organic substrate means that cell growth is not strictly dependent on photosynthesis in mixotrophic cultivation (Andrade and Costa, 2007). Some microalgae were mixotrophic, such as *Haematococcus pluvialis* (Orosa et al., 2001), *C. zofingiensis* (Ip et al., 2004), *B. braunii* (Zhang et al., 2011), *Nannochloropsis* sp. (Hu and Gao, 2003), and *Spirulina platensis* (Andrade and Costa, 2007).

In the present study, a new strain of *Scenedesmus* sp. SP-01, which has a wide adaptability to salinity and can grow well mixotrophically using glucose and acetate as carbon sources, was isolated and identified. The optimization of the culture conditions, including carbon source and nitrogen source, were carried out in order to promote the growth of this alga. Moreover, the effects of salinity on the biomass, lipid, and carotenoid productions in the alga SP-01 at different $NaNO_3$ concentrations were investigated.

## MATERIALS AND METHODS

### The microalgal strain and culture conditions

The green microalga *Scenedesmus* sp. SP-01 was originally isolated from a fresh lake located in Guangzhou, China, and maintained in the China Center for Type Culture Collection (No. CCTCC AF 2011131). Axenic cultures of this alga were cultivated on 100 ml of the modified CZ-M1 medium (Ip et al., 2004), which consisted of 0.5 g $L^{-1}$ $NaNO_3$, 0.175 g $L^{-1}$ $KH_2PO_4$, 0.075 g $L^{-1}$ $K_2HPO_4$, 0.15 g $L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.025 g $L^{-1}$ $CaCl_2 \cdot 2H_2O$, 0.025 g $L^{-1}$ NaCl, 5 mg $L^{-1}$ $FeCl_3 \cdot 6H_2O$, 0.287 mg $L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.169 mg $L^{-1}$ $MnSO_4 \cdot H_2O$, 0.061 mg $L^{-1}$ $H_3BO_3$, 0.0025 mg $L^{-1}$ $CuSO_4 \cdot 5H_2O$, and 0.00124 mg $L^{-1}$ $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, in 250 ml Erlenmeyer flasks at 25°C. The medium was adjusted to pH 7.3 prior to autoclaving at 121°C for 20 min.

A 12/12 h light/dark photoperiod and 20 µmol photons $m^{-2}$ $s^{-1}$ light intensity measured at the surface of the flask were used. In order to investigate the salinity tolerance of this alga SP-01, 5 to 30 g $L^{-1}$ of sodium chloride was added to the basic medium. In order to optimize the growth conditions, sodium acetate and glucose (both 2 g $L^{-1}$), were adopted and added to the basic medium to investigate the effect of carbon on cell growth under mixotrophic (with 20 µmol photons $m^{-2}$ $s^{-1}$ light intensity) and heterotrophic conditions (in dark). Three nitrogen sources, sodium nitrate, urea, and ammonium nitrate, were adopted at five different initial concentrations (0.29, 0.58, 2.9, 5.8 and 11.6 mM) to investigate the effect of nitrogen on the growth of this alga.

### Effects of salt, nitrogen-deficient, and high-light stresses on the algal biomass, lipid and carotenoid accumulations

The combined nitrogen deficiency, salt (sodium chloride) stress, and high-light stress study were conducted with a mixotrophic culture grown on minimal medium supplemented with sodium acetate (6 g $L^{-1}$) as the carbon source under two different illumination condition. Sodium nitrate (0.13 to 0.5 g $L^{-1}$) was used as the nitrogen source and three nitrogen-deficient (0, 0.13, and 0.25 g $L^{-1}$ of $NaNO_3$) and one nitrogen-sufficient (0.5 g $L^{-1}$ of $NaNO_3$) concentrations were tested. A 10-day experiment was conducted in batch cultures and the nutrients were only added once at the beginning of the culture.

Cultures were not replenished with nutrients but sodium chloride (20 g $L^{-1}$), which was added to the algal cultures after 5 days of cultivation when the cells reached the late exponential phase in order to investigate the effect of salt stress on the biomass, lipid and carotenoid productions of the alga SP-01. The algal cultures with sodium chloride were divided into two groups: (1) maintaining the 12/12 h light/dark photoperiod at 20 µmol photons $m^{-2}$ $s^{-1}$ (NaCl/LL group), and (2) transferring the culture to a continuous 200 µmol photons $m^{-2}$ $s^{-1}$ high light intensity condition (NaCl/HL group). The culture without addition of NaCl was used as the control group. All cultures were further incubated for 5 days before being harvested for subsequent analysis. Culture flasks were manually shaken thrice a day. All the experiments were conducted in triplicate.

### Growth evaluation

For the determination of the alga cell dry weight (g $L^{-1}$), 5 ml aliquots of the algal culture were filtered through a pre-dried Whatman GF/C paper, and washed three times with water, and then the filters containing alga cells were dried until constant weight. Specific growth rate (µ, $day^{-1}$) was calculated from the alga cell dry weight, during the logarithmic phase of growth, using the equation $\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1)$, where $X_2$ and $X_1$ represent the dry weight values at times $t_2$ and $t_1$, respectively.

### PCR amplification and bioinformatic analysis of ITS gene

The ITS gene was amplified by polymerase chain reaction (PCR) using two sets of universal primers ITS1 and ITS4 (ITS1, 5′-TCCG TAGG TGAA CCTG CGG; ITS4, 5′-TCCT CCGC TTAT TGAT ATGC) (Jürgen et al., 2008) and the PCR products were sequenced by Sangon Biotech Co., Ltd (Shanghai, China). Sequence similarity were searched on the National Center for Biotechnology Information (NCBI) database with BLAST search (http://blast.ncbi.nih.gov/Blast.cgi) and calculated by pairwise alignment. The software package MEGA version 4.0 (Tamura et al., 2007) was used for analysis of the ITS gene sequence data. The phylogenetic tree was inferred using the neighbor-joining methods. Bootstrap analysis based on 1,000 replications was undertaken to test the robustness of the phylogenetic tree.

### Lipid and fatty acid analysis

Algal cells were harvested by centrifugation and dried by

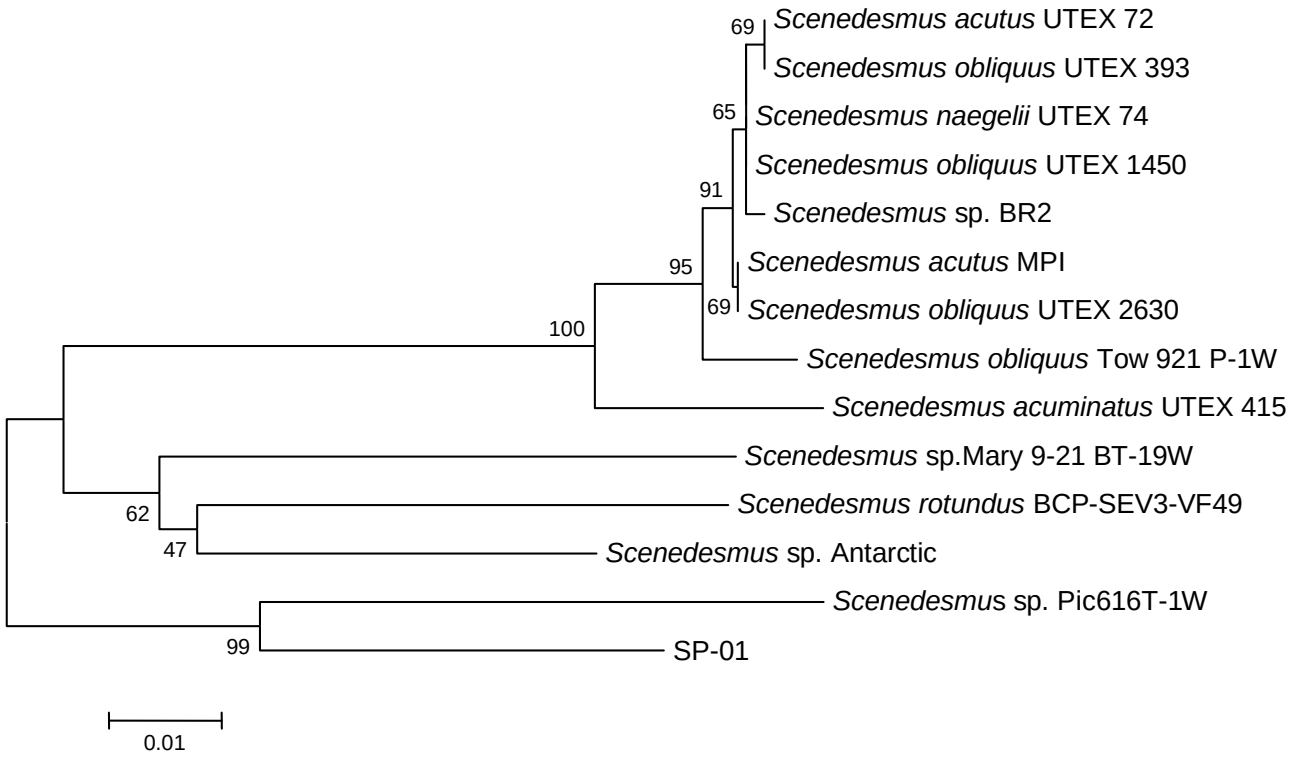


**Figure 1.** Phylogenetic tree based on ITS gene sequences of the alga SP-01 and species within *Scenedesmus.*

lyophilization. Total lipids were extracted from 200 mg of dried alga samples according to the modified Folch procedure (Guo et al., 2010). Fatty acids were transmethylated with sulphuric acid in methanol. Fatty acids were analyzed by gas chromatography-mass spectrometry (GC-MS) and gas chromatography (GC). The GC-MS analysis was carried out in a Shimadzu GCMS-QP2010 Plus gas chromatography-mass spectrometry (Shimadzu, Tokyo, Japan) equipped with a capillary column (50 m length × 0.25 mm i.d., 0.25 µm film thickness, CP-7419, Varian, Palo Alto, USA). The carrier gas was He at a constant flow rate of 1.0 ml/min.

The following temperature program was used: initial oven temperature of 130°C, held for 1 min; first program rate, 6°C/min to 190°C and held 4 min; second program rate, 20°C/min to 250°C, and held for 2 min. Electron ionization (EI) voltage was 70 eV and sample injection volume was 1 µl. Fatty acids were identified by comparing the mass spectrum with that of the library spectrum. Fatty acids were also analyzed by GC (GC-2010, Shimadzu, Tokyo, Japan). The carrier gas was $N_2$ at 3.0 ml/min. The temperature conditions were the same as above GC-MS. Heptadecanoic acid (C17:0, Sigma Chemical Co., St. Louis, MO, USA) was used as internal standard. Before sampling, blank runs were performed to ensure that there was no carryover of analytes from previous extractions. All of the samples were analyzed in triplicate.

#### Pigment analysis

The lyophilized alga cells were ground with the extraction solvent mixture (methanol: dichloromethane, 75:25, v/v) under nitrogen until the alga cell debris was almost colorless. The pigment extracts (20 µl aliquots) were separated and analyzed on an HPLC system (Waters, Milford, MA, USA) equipped with two 1525 pumps and a 2996 photodiode array detector, using a Waters Spherisorb® $ODS_2$ $C_{18}$ column (4.6 mm × 250 mm, 5 µm) according to the method described by Wang and Peng (2008). Briefly, a gradient mobile phase consisted of solvent A (acetonitrile/methanol/0.1 M Tris-HCl, pH 8.0, 84:2:14 by volume) and solvent B (methanol/ethyl acetate, 68:32 by volume) was used: 0 to 15 min, 0 to 100% B; 15 to 25 min, 100% B; 25 to 28 min, 100 to 0% B; 28 to 35 min, 0% B.

The flow rate was 1.2 ml $min^{-1}$ and column temperature was 25°C. The injection volume was 20 µl. The detecting wavelength was set between 300 and 650 nm, and the chromatographic peaks were measured at a wavelength of 450 nm to facilitate the detection of chlorophylls and carotenoids. Chromatographic peak was identified by comparing the retention times and spectra against the known standards or by comparing their spectra (obtained by photodiode array detection) with published data. Quantification of individual carotenoid was carried out using calibration graphs obtained from its authentic standard (Sigma Chemical Co., St. Louis, MO, USA).

## RESULTS AND DISCUSSION

### Identification and characterization of the newly isolated green microalga strain

The ITS gene sequence of the alga SP-01 was a continuous stretch of 662 bp and was recorded in the NCBI under the accession number JN832676. The BLAST result of the amplified sequence with other sequenced *Scenedesmus* strains in NCBI showed the highest degree of identity to the ITS of *Scenedesmus* sp. Pic 616T-1W (94.2%). The phylogenetic analysis based on ITS gene sequences (Figure 1) demonstrated that the newly isolated strain was identified as belonging to the

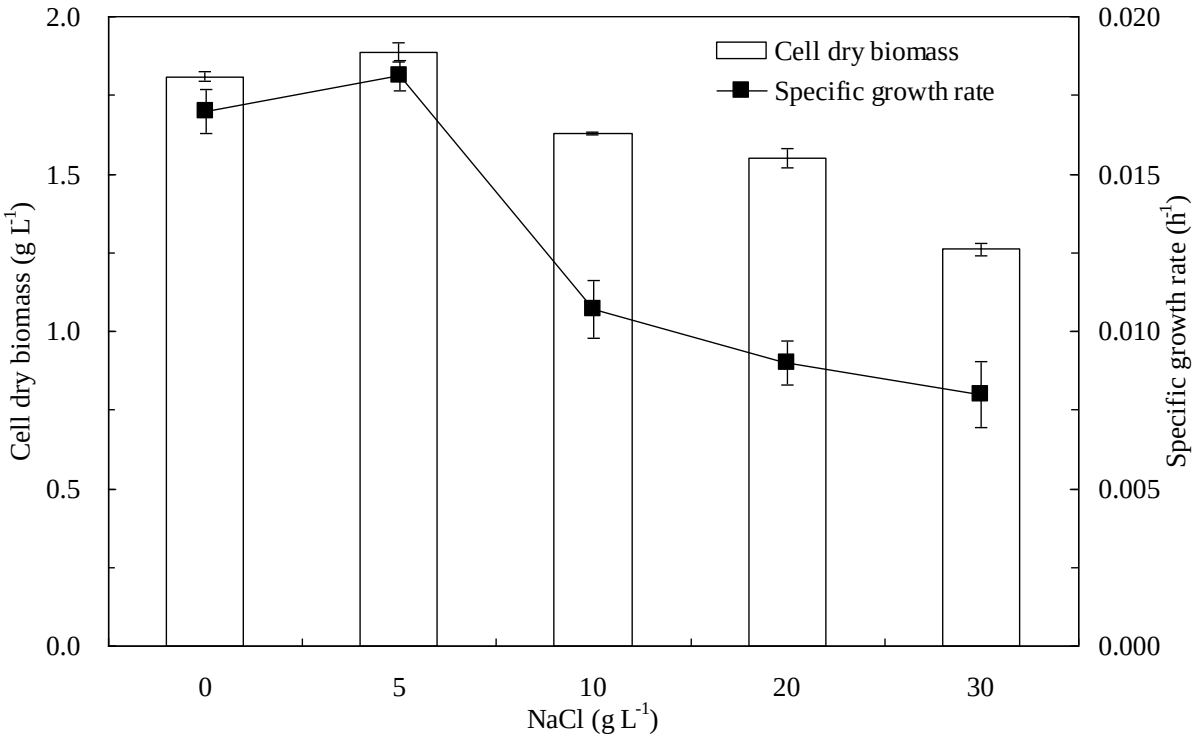


**Figure 2.** Average and standard deviation of the cell dry weight and specific growth rate of *Scenedesmus* sp. SP-01 grown on the modified CZ-M1 medium containing different concentrations of NaCl (*n*=3).

genus *Scenedesmus*.

### The salinity tolerance of alga SP-01

As shown in Figure 2, the results for the salinity tolerance showed that the alga SP-01 was able to grow on the culture medium containing 5 to 30 g $L^{-1}$ (85.5 to 512.8 mM) of sodium chloride, indicating that the alga SP-01 has a wide adaptability to salinity. The biomass yields increased with increasing concentrations (from 0 to 5 g $L^{-1}$) of sodium chloride and the maximum cell dry weight (1.89 g $L^{-1}$) and specific growth rate (0.02 $h^{-1}$) were obtained in 5 g $L^{-1}$ NaCl. However, further addition of NaCl (10−30 g $L^{-1}$) would cause a decrease in cell dry weight and specific growth rate (Figure 2). Higher salinity can cause reduced cell growth due to low turgor and decrease in photosynthetic rate (Hart et al., 1991).

The strains of the *Scenedesmus* genus have been considered to be tolerant to wide ranges of temperature, pH, light intensity, nutrient concentration, and heavy metals (Soeder et al., 1985; Nalewajko et al., 1997; Lin and Lin, 2011; Awasthi and Rai, 2005). Sanchez et al. (2008) showed that *S. almeriensis* tolerated up to 5 g $L^{-1}$ of NaCl, and the biomass productivity decreased remarkably above this salt concentration. Fedina and Benderliev (2000) reported that the cell growth of *S. incrassatulus* Bohl R-83 was seriously inhibited when NaCl concentration was up to 10 g $L^{-1}$. *S. rubescens* (formerly *Chlorella fuscavar. rubescens*) was found to be able to grow at 3% of NaCl (Huss et al., 1999).

### The growth of alga SP-01 with the presence of glucose or sodium acetate under mixotrophic and heterotrophic conditions

Time-course data for batch cultures of the alga SP-01 grown on sodium acetate or glucose under mixotrophic and heterotrophic conditions (in dark) are presented in Figure 3. As shown in the figure, with the presence of glucose or acetate under mixotrophic conditions, the maximum cell dry weight was 2.22 or 1.70 g $L^{-1}$, while it was only 1.50 or 1.27 g $L^{-1}$ in heterotrophic culture, respectively. The algal cells grew well in both growth modes, although the growth was much better under mixotrophic conditions. Moreover, under autotrophic conditions, the cell growth of alga SP-01 was lower in comparison with that under mixotrophic growth (data not shown).

It is likely that the combination of photosynthesis and assimilation of an organic substrate can promote the cell growth under mixotrophic condition. And obviously, between glucose and sodium acetate, sodium acetate addition produced more significant effect. Similar results were found in other microalgae, such as *H. pluvious*

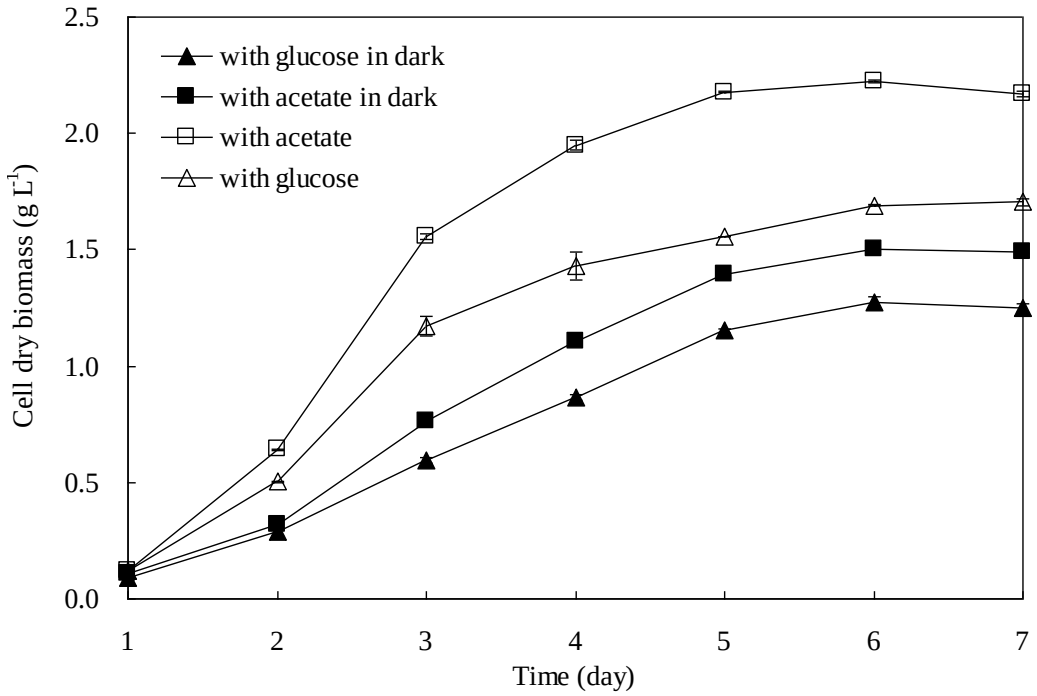


**Figure 3.** Time-course for batch cultures of *Scenedesmus* sp. SP-01 grown on the modified CZ-M1 medium with the presence of glucose or sodium acetate (both 2 g $L^{-1}$) under mixotrophic (with 20 µmol photons $m^{-2}$ $s^{-1}$ light intensity) and heterotrophic conditions (in dark); *n*=3.

(Orosa et al., 2001), *C. zofingiensis* (Ip et al., 2004), *B. braunii* (Zhang et al., 2011) and so on.

Acetate is an important carbon source enhancing both growth and carotenogenesis in *H. pluvious* (Orosa et al., 2001). Zhang et al. (2011) found that *B. braunii* can all grow well on six kinds of organic carbon source under mixotrophic condition and the growth rate and cell dry weight were significantly higher than those under autotrophic mode. However, among these carbon sources, glucose was the best. Ip et al. (2004) also reported that glucose plays a vital role in promoting cell growth of *C. zofingiensis* in mixotrophic culture.

In this study, our results suggest that, in alga SP-01, the oxidative metabolism of acetate was more effective than that of glucose due to their different assimilation mechanisms in this alga under the mixotrophic growth condition. In order to investigate the effects of sodium acetate concentration, five different sodium acetate concentrations (2, 4, 6, 8 and 10 g $L^{-1}$) were designed. The result indicates that different sodium acetate concentrations had different effects on cell growth under mixotrophic conditions (Figure 4). The specific growth rate increased with increasing concentrations (from 0 to 6 g $L^{-1}$) of sodium acetate and the maximum cell dry weight (3.09 g $L^{-1}$) and specific growth rate (0.034 $h^{-1}$) were obtained in 6 g $L^{-1}$ sodium acetate.

Higher sodium acetate concentration (6 to 10 g $L^{-1}$) would cause a decrease in cell dry weight and specific growth rate (Figure 4). In *H. pluvious*, growth rates were enhanced by the addition of 2.5 g $L^{-1}$ sodium acetate with respect to control cultures without this compound, but a concentration of sodium acetate higher than 5 g $L^{-1}$ caused growth inhibition (Orosa et al., 2001). Compared with *H. pluvious*, alga SP-01 can utilize sodium acetate at higher concentration. This may be due to its wide adaptability to acetate.

## The growth of alga SP-01 with different nitrogen sources and concentrations under mixotrophic condition

According to aforementioned results, three nitrogen sources, sodium nitrate, urea, and ammonium nitrate, were adopted at different initial concentrations with the presence of 6 g $L^{-1}$ sodium acetate to investigate the effect of nitrogen on cell growth of this alga in mixotrophic culture. The results are shown in Figure 5. As shown in Figure 5a, the cell growth of alga SP-01 improved significantly when the sodium nitrate concentration increased from 0.29 to 5.8 mM. However, cell growth was negatively impacted when sodium nitrate concentration further increased from 5.8 to 11.6 mM. Both the maximum cell dry weight and specific growth rate reached the highest values at 5.8 mM (0.5 g $L^{-1}$), which were 3.1 g $L^{-1}$ and 0.034 $h^{-1}$, respectively.

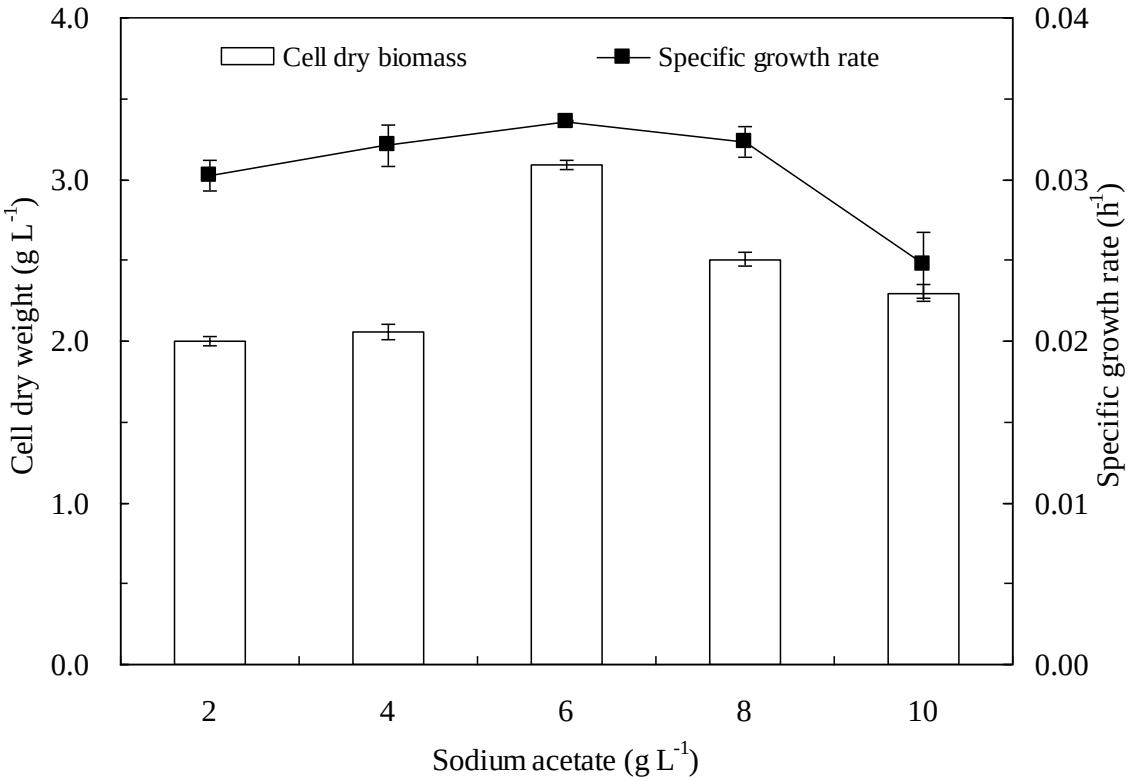


**Figure 4.** Average and standard deviation of the cell dry weight and specific growth rate of *Scenedesmus* sp. SP-01 grown on the modified CZ-M1 medium containing different concentrations of sodium acetate under mixotrophic condition; *n*=3.

Ammonium nitrate had the same trend of cell growth as sodium nitrate and obtained 2.42 g $L^{-1}$ of cell dry weight at 5.8 mM although it contains double nitrogen than sodium nitrate (Figure 5b). As shown in Figure 5c, urea supported the growth of alga SP-01 and obtained 2.36 of cell dry weight at 11.6 mM. Obviously, among the three tested compounds, that is, sodium nitrate, urea, and ammonium nitrate, sodium nitrate was the best nitrogen source.

Same results was found in *Neochloris oleoabundans*, one of the most promising oil-rich microalgal species, which obtained the highest cell dry weight (3.2 g $L^{-1}$) at 10 mM sodium nitrate (Li et al., 2008). These results also suggest that, under the investigated conditions, cell growth was limited when the initial nitrogen level was lower than a critical value, beyond which the nitrogen limitation was released. On the other hand, excessive nitrogen would cause growth inhibition, which may be due to other factors, such as N/P ratio, limiting the use of nitrogen (Bulgakov and Levich, 1999).

## The biomass and lipid production of the alga SP-01 in response to stress conditions

Figure 6 illustrates the effects of NaCl (20 g $L^{-1}$), $NaNO_3$ (0, 0.13, 0.25, and 0.5 g $L^{-1}$), and light intensity on the biomass productivity, lipid content, and lipid productivity of the alga SP-01. Biomass and lipid productivities were evaluated after 10-day culture period. As shown in Figure 6A, biomass productivity increased evidently with increasing initial concentrations of $NaNO_3$ (from 0 to 0.5 g $L^{-1}$) in the control, NaCl/LL, and NaCl/HL groups. When the $NaNO_3$ concentration increased from 0 to 0.5 g $L^{-1}$ (5.8 mM), the average biomass productivity increased from 125 to 289 mg $L^{-1}$ $d^{-1}$ for NaCl/LL group, from 118 to 261 mg $L^{-1}$ $d^{-1}$ for NaCl/HL group, and from 167 to 251 mg $L^{-1}$ $d^{-1}$ for control group.

The biomass productivities of NaCl/LL and NaCl/HL groups were higher than that of control group when $NaNO_3$ (0.13 to 0.5 g $L^{-1}$) was added to the initial culture media, and the biomass productivity of NaCl/LL group was higher than that of NaCl/HL group. Obviously, the addition of 20 g $L^{-1}$ NaCl after 5 days of cultivation when the algal cells reached the late exponential phase can result in a higher cell biomass, which was similar to the finding of Pal et al. (2011). The maximum biomass productivity (289 mg $L^{-1}$ $day^{-1}$) was obtained in NaCl/LL group with 0.5 g $L^{-1}$ of $NaNO_3$.

The increase of light intensity resulted not in an enhancement of biomass productivity; may be due to other factors limiting the use of light. However, these were not investigated in the present study. Baroli and Melis

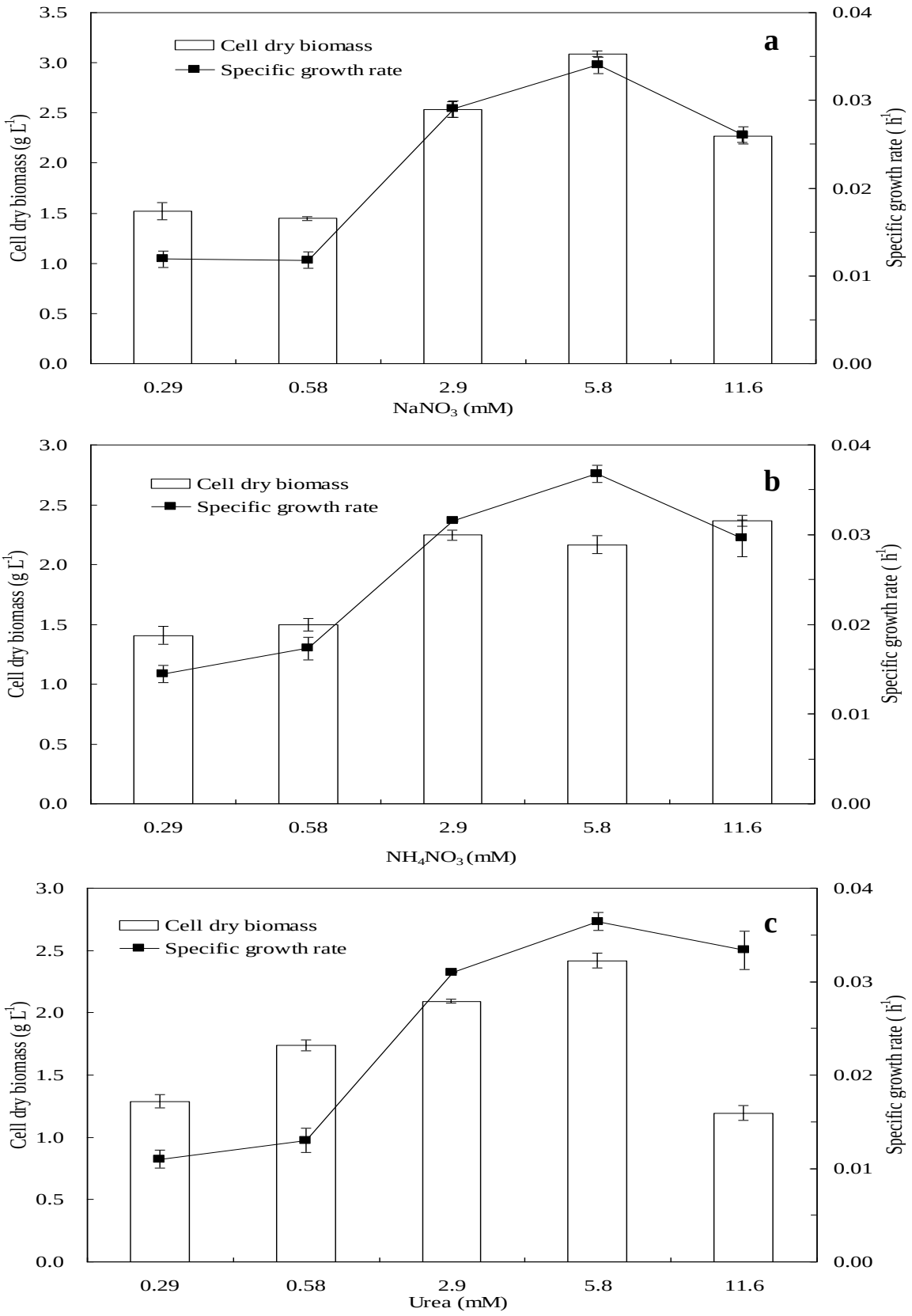


**Figure 5.** Average and standard deviation of the cell dry weight and specific growth rate of *Scenedesmus* sp. SP-01 grown on the modified CZ-M1 medium containing different concentrations of sodium nitrate ($NaNO_3$), urea, and ammonium nitrate($NH_4NO_3$) under mixotrophic condition ($n$=3).

(1996) reported that the diminished cell growth and productivity of alga *Dunaliella salina* under high light were attributed to photoinhibition of photosynthesis. The cell growth was limited and the biomass productivity decreased remarkably under nitrogen-deficient condition. Moreover, both NaCl/LL and NaCl/HL groups caused a significant decrease in biomass productivity when no $NaNO_3$ was added and the lowest biomass productivity (118 mg $L^{-1}$ $d^{-1}$) was obtained in NaCl/HL group.

There was a 2-fold increase in the lipid content of the alga SP-01 in all the three groups when the initial $NaNO_3$ concentration added to the culture media was reduced from 0.5 to 0 g $L^{-1}$ (Figure 6B), and the maximum lipid content of 41% was obtained in the control group, which was consistent with the results of Illman et al. (2000), who showed that the decrease in nitrogen concentration in the medium raised the lipid contents in algae *Chlorella* strains. This lipid content in the alga SP-01 was higher than a moderate lipid content of 10 to 39% obtained from the genus of *Scenedesmus* by Gouveia and Oliveira (2009), Mandal and Mallick (2009), and Ho et al. (2010), respectively. The addition of 20 g $L^{-1}$ NaCl in the late exponential phase resulted in a decrease in the lipid content of the alga SP-01, and this decrease was more obvious in NaCl/HL group. This result was not consistent with the finding by Rao et al. (2007), who found that the lipid content of alga *B. braunii* could be increased under given salinity level.

The effects of salt stress, nitrogen-deficiency, and light illumination on the lipid productivity of the alga SP-01 are shown in Figure 6C. As can be seen in Figure 3C, while $NaNO_3$ (0.13 to 0.5 g $L^{-1}$) was added as nitrogen source, no significant differences in the lipid productivity of the alga SP-01 were found between the control group and the NaCl/LL group and the lipid productivity in NaCl/HL group was lower than that in the control and NaCl/LL groups. Thus, the addition of 20 g $L^{-1}$ NaCl in the late exponential phase had no significant effects on the lipid productivity of the alga SP-01. The maximum lipid productivities of 67.44 to 68.44 mg $L^{-1}$ $day^{-1}$ were obtained in the control group without $NaNO_3$ and in the control and NaCl/LL groups with 0.13 g $L^{-1}$ $NaNO_3$.

Moreover, the lipid productivity in NaCl/HL group was about 50% lower than that in the control group when no $NaNO_3$ was added as nitrogen source, indicating that high salinity decreased lipid productivity of the alga SP-01 in the absence of $NaNO_3$, which was also consistent with the result of Pal et al. (2011). These results indicate that the nitrogen level ($NaNO_3$ concentration), which was the most commonly referenced nutrient-limiting factor in the growth and lipid accumulation of microalgae (Li et al., 2008), had more obvious effect on lipid productivity than salinity for the alga SP-01.

The lower sodium nitrate concentration leads to higher lipid content, indicating that lipids preferred to accumulate in N-limitation rather than N-saturation (Li et al., 2008; Lv et al., 2010). Lv et al. (2010) found that N-deprivation could stimulate the lipid accumulation, but the biomass productivity was reduced. The same result was obtained in the present study. When initial N concentration declined, the biomass productivity of the alga SP-01 was also reduced drastically. The fatty acid compositions of the alga SP-01 in control, NaCl/LL, and NaCl/HL groups with different concentrations of $NaNO_3$ in culture medium were determined and the results are shown in Table 1. As can be seen from Table 1, among the individual fatty acids, $C_{18:1}$ (oleic acid, 32.4−42.4%), $C_{16:0}$ (palmitic acid, 17.7 to20.8%), $C_{18:3}$ (linolenic acid, 10.6 to 14.8%), and $C_{18:2}$ (linoleic acid, 8.1 to 17.4%) were the main fatty acids and constituted 78.8 to 86.6% of the total fatty acids in the alga SP-01 under different culture conditions.

It is worth noting that the percentage of $C_{18:2}$ increased with increasing concentrations of sodium nitrate and the percentage of $C_{18:2}$ of the alga SP-01 in NaCl/HL group was markedly higher than that in the control group. The criteria for the selection of algal species suitable for biodiesel production should include not only high lipid productivity but also an appropriate composition of fatty acids in the lipids produced from those strains. It will have advantages on biodiesel production due to the suitable length of carbon chain and higher $C_{18:1}$ content increasing the oxidative stability for longer storage and decreasing the cold filter plugging point for use in cold regions (Knothe, 2005; Mutanda et al., 2011).

## The production of carotenoids in the alga SP-01 in response to stress conditions

The contents of carotenoids and chlorophylls in the alga SP-01 grown under different culture conditions are shown in Table 2. As can be seen from Table 2, lutein and astaxanthin were the main carotenoids, followed by canthaxanthin and zeaxanthin. Lutein, zeaxanthin and canthaxanthin in the alga SP-01 were present in free (unesterified) forms, whereas astaxanthin was esterified primarily as monoesters. Other carotenoids present in the alga SP-01 included neoxanthin, β-cryptoxanthin, and β-carotene.

The results demonstrate an opposite trends of changes in the contents of secondary carotenoids such as astaxanthin and primary carotenoids such as lutein. Thus, the maximal percentage content of lutein (68% of total carotenoids) and the highest lutein to astaxanthin ratio (17.4) were recorded at the highest $NaNO_3$ concentration tested (0.5 g $L^{-1}$), whereas the maximal astaxanthin percentage content (50%) and the lowest lutein to astaxanthin ratio (0.4) were found at a lower $NaNO_3$ concentration (0.13 g $L^{-1}$) in NaCl/HL group, indicating that stress conditions (salt stress, nitrogen-deficient, and high-light stress) could promote the production of astaxanthin. As shown in Table 2, an increase in the contents of secondary carotenoids was observed in both NaCl/LL and NaCl/HL groups at different $NaNO_3$

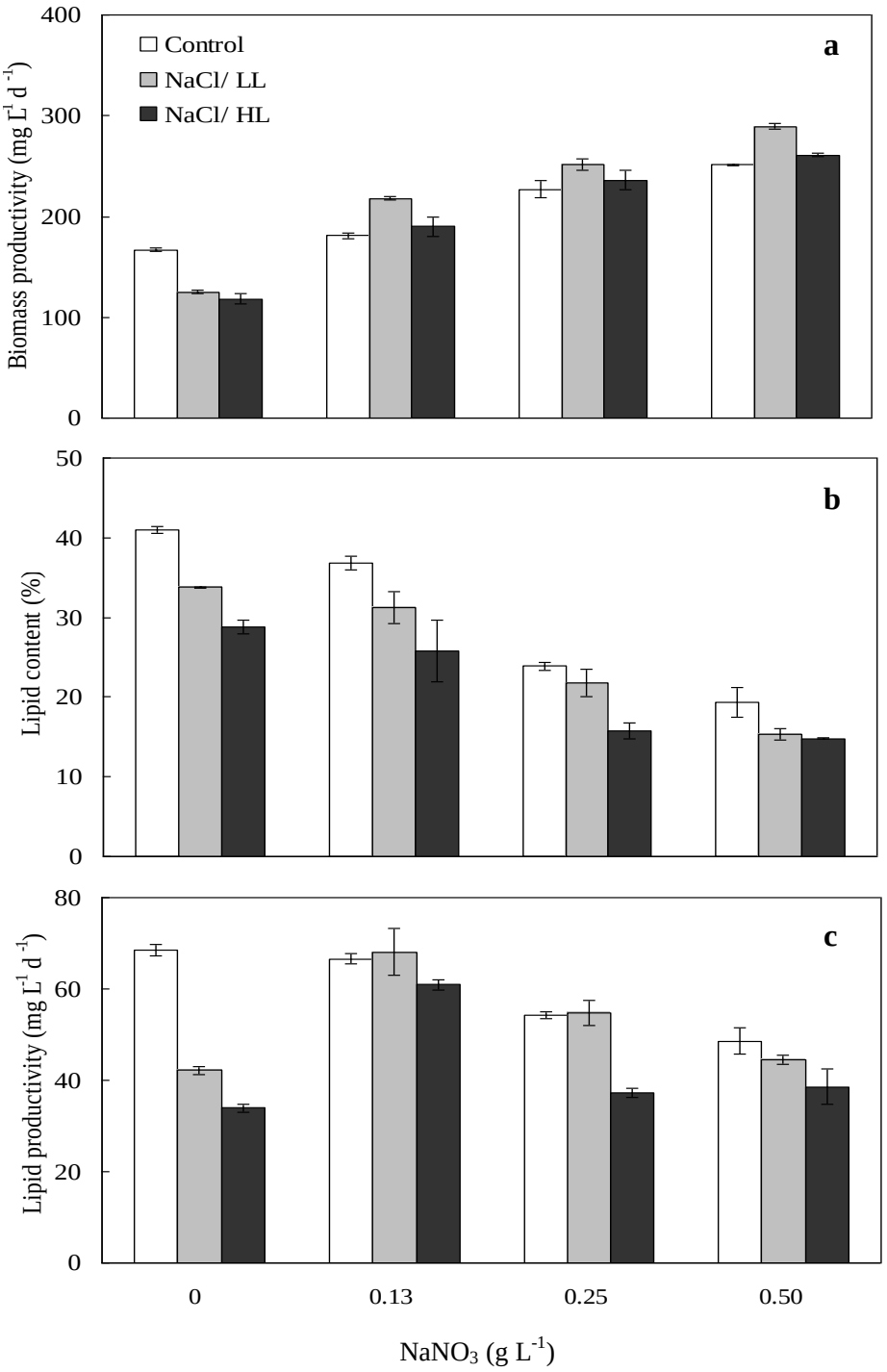


**Figure 6.** Average and standard deviation of biomass productivity (A), lipid content (B), and lipid productivity (C) of *Scenedesmus* sp. SP-01 grown on the modified CZ-M1 medium containing different concentrations of $NaNO_3$. Control, no additional NaCl was added; NaCl/LL, 20 g $L^{-1}$ of NaCl was added at late exponential phase; NaCl/HL, 20 g $L^{-1}$ of NaCl was added at late exponential phase and the culture was transferred to continuous illumination (200 µmol photons $m^{-2}$ $s^{-1}$); *n*=3.

concentrations.

The alga SP-01 accumulates lutein and astaxanthin as main carotenoids. Lutein is usually considered to be a primary carotenoid and plays an important role in light harvesting and protecting the photosynthetic apparatus and processes against damage that might be caused by

**Table 1.** Fatty acid composition (% of total fatty acid) of *Scenedesmus* sp. SP-01 in the control, NaCl/LL, and NaCl/HL groups with different concentrations of $NaNO_3$ in culture medium. The mean values (RSDs < 5%) of three determinations are presented.

| Fatty acid | 0 g $L^{-1}$ of $NaNO_3$ | | | 0.13 g $L^{-1}$ of $NaNO_3$ | | | 0.25 g $L^{-1}$ of $NaNO_3$ | | | 0.5 g $L^{-1}$ of $NaNO_3$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | NaCl/LL | NaCl/HL | Control | NaCl/LL | NaCl/HL | Control | NaCl/LL | NaCl/HL | Control | NaCl/LL | NaCl/HL |
| 16:0 | 19.9 | 19.8 | 20.8 | 19.0 | 19.0 | 19.7 | 18.0 | 19.7 | 19.7 | 17.7 | 19.1 | 18.6 |
| 16:1 | 3.7 | 2.7 | 3.1 | 2.9 | 2.7 | 3.2 | 4.5 | 3.6 | 3.1 | 5.2 | 3.2 | 3.8 |
| 16:3 | 7.3 | 5.0 | 4.5 | 4.8 | 4.5 | 4.2 | 5.0 | 4.6 | 3.9 | 5.6 | 4.3 | 4.0 |
| 18:0 | 6.8 | 7.9 | 4.5 | 9.6 | 9.3 | 5.0 | 7.7 | 8.4 | 4.2 | 7.0 | 7.5 | 6.4 |
| 18:1 | 39.7 | 42.4 | 37.1 | 41.1 | 41.1 | 37.1 | 36.6 | 35.5 | 36.6 | 35.3 | 32.4 | 34.0 |
| 18:2 | 8.5 | 8.1 | 14.6 | 9.6 | 10.8 | 15.3 | 12.3 | 13.8 | 15.5 | 13.2 | 17.1 | 17.4 |
| 18:3 | 10.7 | 11.3 | 13.1 | 10.6 | 10.6 | 13.4 | 12.1 | 11.8 | 14.8 | 12.8 | 13.1 | 13.9 |
| 17:3 | 3.5 | 2.8 | 2.4 | 2.3 | 2.0 | 2.1 | 3.8 | 2.7 | 2.3 | 3.3 | 3.3 | 1.9 |

**Table 2.** The content of pigments (mg/g) of *Scenedesmus* sp. SP-01 in the control, NaCl/LL, and NaCl/HL groups with different concentrations of $NaNO_3$ in culture medium.

| Pigment | 0 g $L^{-1}$ of $NaNO_3$ | | | 0.13 g $L^{-1}$ of $NaNO_3$ | | | 0.25 g $L^{-1}$ of $NaNO_3$ | | | 0.5 g $L^{-1}$ of $NaNO_3$ | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | NaCl/LL | NaCl/HL | Control | NaCl/LL | NaCl/HL | Control | NaCl/LL | NaCl/HL | Control | NaCl/LL | NaCl/HL |
| Chlorophyll a | 0.01 | ND* | 0.01 | 0.72 | 0.69 | 0.63 | 1.56 | 1.21 | 0.70 | 1.77 | 1.33 | 0.83 |
| Chlorophyll b | 0.06 | 0.07 | 0.06 | 0.44 | 0.46 | 0.32 | 0.77 | 0.80 | 0.46 | 1.18 | 0.87 | 0.46 |
| Neoxanthin | 0.01 | ND* | 0.01 | 0.07 | 0.05 | 0.04 | 0.12 | 0.09 | 0.07 | 0.16 | 0.10 | 0.09 |
| Lutein | 0.10 | 0.09 | 0.10 | 0.43 | 0.38 | 0.26 | 0.74 | 0.69 | 0.49 | 0.87 | 0.72 | 0.59 |
| Zeaxanthin | 0.03 | 0.03 | 0.02 | 0.07 | 0.07 | 0.03 | 0.07 | 0.05 | 0.07 | 0.10 | 0.05 | 0.09 |
| β-Cryptoxanthin | ND* | 0.01 | 0.02 | ND* | 0.02 | 0.02 | 0.01 | 0.02 | 0.02 | 0.01 | 0.03 | 0.03 |
| β-Carotene | 0.01 | 0.01 | 0.02 | 0.01 | 0.02 | 0.03 | 0.03 | 0.02 | 0.03 | 0.03 | 0.02 | 0.03 |
| Astaxanthin | 0.05 | 0.14 | 0.17 | 0.10 | 0.36 | 0.59 | 0.06 | 0.32 | 0.53 | 0.05 | 0.30 | 0.35 |
| Canthaxanthin | 0.03 | 0.06 | 0.11 | 0.07 | 0.16 | 0.21 | 0.06 | 0.13 | 0.16 | 0.06 | 0.07 | 0.12 |
| Primary carotenoids | 0.15 | 0.14 | 0.17 | 0.58 | 0.54 | 0.38 | 0.97 | 0.87 | 0.68 | 1.17 | 0.92 | 0.83 |
| Secondary carotenoids | 0.08 | 0.20 | 0.28 | 0.17 | 0.52 | 0.80 | 0.12 | 0.45 | 0.69 | 0.11 | 0.37 | 0.47 |
| Total carotenoids | 0.23 | 0.34 | 0.45 | 0.75 | 1.06 | 1.18 | 1.09 | 1.32 | 1.37 | 1.28 | 1.29 | 1.30 |

The mean values (RSDs < 5%) of three determinations were used; ND, not detected.

excess solar energy or other stresses (Sanchez et al., 2008). Astaxanthin, in contrast, is a typical secondary carotenoid which accumulates in lipid bodies located outside the chloroplast (Grünewald et al., 2001), and has considerable potential and promising applications in human health. Potential production of astaxanthin from microalgae has been a subject of intensive investigation (Orosa et al., 2001; Wang and Peng, 2008).

## Conclusion

A new oil-producing microalga SP-01 is salt-tolerant, and can grow on media containing 5 to 30 $gL^{-1}$ of NaCl. Lipid content (41%) in the alga SP-01 was higher than a moderate lipid content obtained from the genus of *Scenedesmus.* Although the lipid content of SP-01 decreased with increasing $NaNO_3$ concentrations or addition of 20 $gL^{-1}$ NaCl, the biomass productivity increased with adding $NaNO_3$ and NaCl. The maximum lipid productivities were obtained while no $NaNO_3$ and NaCl was added or while 20 $gL^{-1}$ NaCl and 0.13 $gL^{-1}$ $NaNO_3$ were added. The composition of fatty acids was mainly $C_{18:1}$, $C_{16:0}$, $C_{18:3}$, and $C_{18:2}$. SP-01 is a mixotrophic alga, and may achieve high biomass by optimization of culture conditions for biofuel or combined production of biofuel and high-value carotenoids such as lutein and astaxanthin.

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