Research Articles

Effect of salinity on growth, biochemical composition, and lipid productivity of *Nannochloropsis oculata* CS 179

Effect of salinity (15, 25, 35, 45, and 55\(^\circ\)e) on growth, biochemical composition, and lipid productivity of *Nannochloropsis oculata* CS 179 was investigated under controlled cultivation in a 19-day study. The results demonstrate that the dry biomass of *N. oculata* was the highest at a salinity of 25\(^\circ\)e among the treatments in the first 10-day cultivation (*P*<0.05). During days 14–19 (stage III), the dry biomass productivity was the highest at a salinity of 35\(^\circ\)e (*P*<0.05). The algae had the highest chlorophyll content (26.47 mg g\(^{-1}\)) at 25\(^\circ\)e in stage I, and it decreased continuously at stage III. Protein content (as% of dry biomass) of algae reached the highest value of 42.25 ± 2.10% at 15\(^\circ\)e, and the lipid content was the highest of 32.11 ± 1.30% of dry biomass at 25\(^\circ\)e. However, the lipid productivity of these algae was the highest at 35\(^\circ\)e (64.71 mg L\(^{-1}\) d\(^{-1}\); *P*<0.001). C16 series content was the highest among the total fatty acid methyl esters (FAME), and eicosapentaenoic acid C20:5n-3 (EPA) content was high at the low salinity. Fatty acid profiles of *N. oculata* varied significantly under different salinities.

**Keywords:** Biomass / Chlorophyll a / Fatty acid / *Nannochloropsis oculata* / Salinity

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1 Introduction

Because of the shortage of fossil fuel, environmental problems, and emergency of global warming, alternative energy resources have recently received worldwide attention [1]. One of the promising alternatives is biodiesel. However, the conventional materials, such as soybean oil, rapeseed oil, corn oil, animal fat, and waste cooking oil, are far from meeting demands [2]. The potential value of microbial, and particularly microalgal, photosynthesis to produce biofuels, has been widely recognized [3–6]. The interest of microalgae for alternative fuel production is due to its alterable lipid content in some species by varying cultivation conditions [7]. Many studies have focused on the appropriate conditions to increase the lipid content and growth rate during microalgal cultivation [4–6, 8–11]. Previous studies showed that the lipid content in some microalgae could be increased by changing the temperature, CO\(_2\) aeration fixation, salinity, iron concentration, phosphate concentration, light intensity, and nutrient concentration [8–12].

For the salt-water species, salinity is one of the most important factors affecting the growth, lipid content, and biochemical composition of microalgae [13–15]. The adaptability of microalgae to salinity is different because of the physiological difference in some species [16]. Borowitzka and Brown [17] reported that *Dunaliella tertioleeta* could effectively exclude salt from cells and accumulate glycerol during growth. As direct responses to extra-cellular salt concentration, the protein, lipid, and carbohydrate contents can be affected by a wide range of salinity for most microalgae species [16, 18]. Rao et al. [19] reported that *Botryococcus braunii* LB 572 cultured at low salinity contained enhanced amounts of lipids. The lipid content and the carotene to chlorophyll ratio of *Navicula* sp. increase with increasing salinity [20] and the lipid content in *Dunaliella* cells increased at higher salinities [21].

*Nannochloropsis oculata* is an important microalga in the field of marine biotechnology, and it has been widely used as feedstock in aquaculture [22, 23]. It has been identified as one of the most promising photoautotrophic producers of eicosapentaenoic acid (EPA) [24]. Converti et al. [8] studied the effects of temperature and nitrogen concentration on growth and lipid content of *N. oculata* for biodiesel production. Chiu et al. [25] evaluated the lipid accumulation of *N. oculata* in response to CO\(_2\) aeration. The present study aimed to evaluate the effect of salinity
on growth, biochemical composition, and lipid productivity of microalgae *N. oculata* in the indoor cultivation.

## 2 Materials and methods

### 2.1 Algae strain and culture conditions

*Nannochloropsis oculata* CS 179 was purchased from the Key Laboratory of Aquaculture of Ocean University of China (obtained from Australian CSIRO Collection of Living Microalgae, http://www.cmar.csiro.au/microalgae). Seawater used in the experiment was pumped directly from the Daya Bay (near Hong Kong) of South China Sea and treated with sand filtration.

Roux bottle (RB) (Pyrex, Sigma-Alorich, USA) (25-cm height, 11.5-cm width, and 5.5-cm depth) with 800 mL of media was used for cultivating *N. oculata*. Magnetic stir plates (Scholar 171, Corning, Co. Ltd., Germany) were used to continuously mix the cultivation medium in RB. The light source was composed of fluorescent tubes (Speethalux™ T5HO54W 6500K, China), which were placed horizontally and parallel to the front side of RB. Pure CO₂ and air (2:3 by volume) were injected into the RB. The cultivation was conducted under continuous illumination and the temperature, pH, and light intensity were maintained at 26 ± 1°C, 7.7 ± 0.2, and 160 ± 5 μmol photons m⁻² s⁻¹, respectively.

### 2.2 Experiment design

Five salinity treatments (15, 25, 35, 45, and 55‰), each with three replicates, were conducted to evaluate the effect of salinity on growth and lipid accumulation of *N. oculata*. Before the experiment, different salinity levels were achieved by adding the sodium chloride or distilled water, and the algae was acclimated into the corresponding salinity levels from 35‰ within 6 days. Then, five treatments were set up and each bottle was inoculated with the algae at the level of 0.35 ± 0.05 g L⁻¹ dry biomass (DB).

Once starting the experiment, the process was conducted as following: During the first 10 days (stage I), the algae were cultivated with the nutrient media (1.5 mL Guillard’s F/2 trace metal solution and 4 mL modified Guillard’s F/2 formula (24 g urea, 6 g NaH_{2}PO_{4} · H_{2}O, 0.2 g MnCl_{2} · 4H_{2}O, 4 g Na_{2} · EDTA, 0.05 g vitamin B1, and 0.0001 g vitamin B12) per liter of the sterilized fresh seawater). Then, 100 mL of the culture media from each RB were daily sampled (8:00 a.m.) to measure the growth (absorbency and biomass) and biochemical composition and replaced by 100-mL media with corresponding salinity. On the 10th day, 200 mL of culture media in each RB was replaced with regular seawater with the corresponding salinity to dilute the algae concentration and on the same day, the algae in each RB was conducted by ceasing the nutrient media. During the days 10–13 (stage II), the algae was regarded as the acclimation of nutrient deficient. From days 14–19 (stage III), 50 mL of culture media was daily sampled for the measurement and replaced with 50 mL regular seawater with corresponding salinity in each RB.

### 2.3 Absorbency and DB

Absorbency and DB were used to represent the growth of *N. oculata*. Absorbency was measured at 680 nm by UV–vis spectrophotometer (model Lambda 25, PerkinElmer, Milan, Italy) [25]. A certain volume (depends on the concentration of sample, normally less than 10 mL) of sample was filtered through a predried (105°C, 2 h) and weighted glass microfiber filter disks (d = 47 mm, 0.7 μm nominal pore size) (Whatman CF/F). Then the filter with algae was dried in oven at 105°C for 3 h. The dry weight of algal was the DB [10].

Chlorophyll a concentration was measured using the spectrophotometric method [12]. Methanol (99.9%) was used to extract the chlorophyll a from each sample at room temperature for 3 h. The extracted matter was centrifuged (8000 rpm, 8 min) and then the 400–750 nm absorption spectrum was used to measure the pigments by spectrophotometer (VIS-7220/7220G, Rayleigh, Beijing, China).

Concentrations of chlorophyll a were determined according to the formulas of Ritchie [26]:

\[
\text{[Chlorophyll a] μg mL}^{-1} = 8.0962 \times A_{652} + 16.5169 \times A_{665}
\]

Absorbencies at 652 nm and 665 nm were corrected from turbidity by subtracting absorbency at 750 nm.
Table 1. The effect of salinity on the dry biomass (DB) productivity and absorbency increment of *N. oculata* CS 179 during 19-day cultivation. Stage I, (0–10th); stage III, (14th–19th). Values are mean ± SD. Within the same row, significant differences (a, b, and c) are indicated by different superscripts ($p<0.001$).

<table>
<thead>
<tr>
<th>Salinity</th>
<th>DB productivity (mg L$^{-1}$ d$^{-1}$)</th>
<th>Absorbency increment (d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage I</td>
<td>Stage III</td>
</tr>
<tr>
<td>35‰</td>
<td>105.88 ± 9.43$^c$</td>
<td>224.71 ± 13.08$^c$</td>
</tr>
<tr>
<td>45‰</td>
<td>148.94 ± 12.71$^b$</td>
<td>257.52 ± 12.56$^b$</td>
</tr>
<tr>
<td>35‰</td>
<td>152.26 ± 9.50$^b$</td>
<td>287.61 ± 16.91$^a$</td>
</tr>
<tr>
<td>25‰</td>
<td>176.77 ± 11.29$^a$</td>
<td>232.75 ± 10.04$^c$</td>
</tr>
<tr>
<td>15‰</td>
<td>149.85 ± 8.93$^b$</td>
<td>209.92 ± 22.46$^c$</td>
</tr>
<tr>
<td></td>
<td>0.254 ± 0.001$^c$</td>
<td>0.242 ± 0.012$^b$</td>
</tr>
<tr>
<td></td>
<td>0.438 ± 0.030$^b$</td>
<td>0.286 ± 0.003$^a$</td>
</tr>
<tr>
<td></td>
<td>0.585 ± 0.055$^a$</td>
<td>0.283 ± 0.005$^{ab}$</td>
</tr>
<tr>
<td></td>
<td>0.624 ± 0.072$^a$</td>
<td>0.181 ± 0.001$^c$</td>
</tr>
<tr>
<td></td>
<td>0.510 ± 0.045$^{ab}$</td>
<td>0.265 ± 0.029$^{ab}$</td>
</tr>
</tbody>
</table>

2.5 Lipid and protein content

After 19 days of cultivation, the lipid content of the algae at each treatment was measured through the ultrasonic (Sonicator 4000, Misonix, USA) modified method [27]. A total of 30-mg sample was used to extract the lipid. The sample was firstly conducted with 3-mL chloroform/methanol solution (2:1, v/v) in the epoxy resin (EP) tube at 4 °C for 3 h and then, the sample in EP tube was broken ultrasonically for 30 min. The sample was bathed at 70 °C for 15 min and collected by centrifugation (2000 rpm, 9°C and 30 min). A solution of 0.5-mL sodium chloride (0.9%) was used to wash the sample again, and another centrifugation (2000 rpm, 10 min) was run. The upper aqueous phase was eliminated and the lower chloroformic phase was filtered through anhydrous sodium sulphate and collected. The remaining algae were collected and the above process was repeated twice. The combined chloroform phases were evaporated to dryness under nitrogen, and then lipid content was determined gravimetrically.

Total protein of *N. oculata* was measured after 19-day cultivation by Folen–Lowry method [28].

2.6 Fatty acids analysis

Direct transesterification for determination of free and bound fatty acid content of the algae was carried out. The surrogate standard and transesterification reagent were prepared, and approximately vacuum-filtered 3 mg of each algae sample stored at −80°C was used. Five milliliters of transesterification reagent was added to each sample, which was heated at 100°C for 20 min with vortexing at 0 min and 20 min. Samples were allowed to cool for 30 min and then extracted three times with 5 mL of GC-grade hexane. Fatty acid methyl esters (FAME) were combined and evaporated with N$_2$ gas to dryness on the Reacti-Vap III. Samples were redissolved in 300 μL of gas chromatography (GC) grade n-decane, and later transferred to a GC vial with poly spring insert and run using the FASTFAME method on the GC. For rapid GC analysis, a HP 6890 series GC system with HP 6890 series auto injector and FID was used in conjunction with a Restek FAMEWAX (30 m × 0.25 mm ID × 0.25 μm) film thickness column. Duplicates of each FAME analysis were done and FAMES identification was run by comparison with standard certificate, Supelco FAME 10 mix 37 (Bellefonte, PA, USA). Instrument conditions and operation methods were the same as used before by Lin and Lin [10].

2.7 Statistical analysis

The statistical analyses were performed using the software SPSS 17.0 (Statistical Program for Social Sciences 17.0) and Sigma PLOT 10.0. A one-way analysis of variance (ANOVA) was used to evaluate the differences among the treatments. If ANOVA effects were significant, comparisons between the different means were made using post-hoc least significant differences (LSD) test with a confidence level of 0.05.

3 Results

3.1 Dry biomass (DB)

During the first 10 days of cultivation (stage I), the algae at salinity of 25‰ had the highest biomass concentration (1920 ± 113.6 mg L$^{-1}$, $p<0.05$), DB productivity (176.77 ± 11.29 mg L$^{-1}$ d$^{-1}$, $p<0.05$), and absorbency increment (0.624 ± 0.072 d$^{-1}$, $p<0.05$) (Fig. 1A and B, Table 1). At stage III (from the 14th to 19th), absorbency of algae increased slowly, and algae at 45‰ had the highest absorbency increment (0.286 ± 0.003 d$^{-1}$). DB productivity (over 200 mg L$^{-1}$ d$^{-1}$) was higher than that at stage I, and algae at 35‰ had the highest DB productivity (287.61 ± 16.91 mg L$^{-1}$ d$^{-1}$), which was significantly higher than that at 15‰ ($p<0.05$) (Table 1). Totally, the algae at 35‰ had the highest absorbency, DB concentration, and DB productivity after the 19-day cultivation (Fig. 1A and B, Table 1).

3.2 Chlorophyll a

The chlorophyll $a$ content and concentration increased significantly during the first 6 days, and then changed slightly within the cultivation of stage I (Fig. 2A and B). The algae at 25‰ had the highest chlorophyll $a$ value (51.78 ± 2.82 mg L$^{-1}$, 26.47 ± 0.48 mg g$^{-1}$) ($p<0.001$). After the dilution at day 10, the chlorophyll $a$ concentration increased at stage II, and it still increased at the beginning of stage III at 15, 45, and 55‰ and then decreased (Fig. 2A). After the transition culture (stage II), the chlorophyll $a$ content of DB (mg g$^{-1}$) of algae decreased con-
Figure 2. Effect of salinity on (A) chlorophyll a concentration and (B) chlorophyll a content during the 19-day cultivation of N. oculata CS 179. The arrows indicate when the new cultivation media was added. Stage I, the nutrient supply stage; stage II, the media replacement acclimation stage; stage III, the without nutrient supply stage.

3.3 Protein and lipid contents

Protein content (% of DB) of N. oculata at the salinity of 15‰ reached the highest level of 42.25 ± 2.10%, and then it decreased to the lowest level (25.70 ± 2.48%) at 55‰ after 19 days of cultivation (p<0.001) (Fig. 3). The highest lipid content (32.11 ± 1.30% of DB) occurred at 25‰, while the lowest lipid content of the algae was 25.70 ± 2.64% at 15‰ (p<0.05) (Fig. 3).

3.4 Lipid profiles and productivity

Fatty acids of FAME of the algae at 25, 35, and 45‰ were measured after 19 days of cultivation. C16 series content (% of total FAME) was the highest among all the fatty acid profiles, and C16:0 was the highest at 25‰ but C16:1 content was the highest at 45‰. C18:1, C18:2, and C18:3 contents increased with the increase of salinity. C20:5n-3 (EPA) was the highest at 25‰ (Table 2).

The mean DB productivity of N. oculata after 19 days of cultivation was the highest at 35‰ (p<0.001), and the algae had the highest lipid productivity (64.71 mg L⁻¹ d⁻¹) at 35‰, which was significantly higher than that in other treatments (p<0.001). The total C16 series and C18 series (∑C16–C18) productivity of the algae did not differ significantly among 25, 35, and 45‰ (Table 3).

4 Discussion

This study addressed the effect of salinity on growth and biochemical composition of N. oculata. In preliminary studies, microalgae commonly prefer the appropriate factors for their growth or accumulation of some special biochemical composition [8–10, 29]. The appropriate salinity range of N. oculata has been documented between 10‰ and 35‰ [30,31]. In the present...
The optimum salinity for the algal growth was 25%e, but the species could grow better at 35%e during the without nutrient supply stage. Kirst [32] explained that the microalgae would expend energy while attempting to maintain the turgor pressure, and this led to a decrease in DB productivity or reduction in growth. Hart et al. [33] also showed that the reduced growth at high salinities was due to decrease in photosynthetic rate in some marine microalgae.

Within the cultivation of stage I, the algae grew fast at low salinity (15, 25, and 35%e), but the algae had the highest absorbance value and DB productivity at 35%e and 45%e at stage III (Fig. 1 and Table 1). At the beginning of the experiment, the nutrients were equally supplied to all the treatments and all the culture conditions were uniform except for the salinity. Thus, the differences of growth or DB productivity among the treatments should be contributed by the salinity in the present study. Previous study related to the effect of salinity on the growth also shows that the Dunaliella salina prefers the lower salt concentration [21, 34], and most microalgae grow relatively slow at high salinity [35, 36]. In contrast, the absorbency increments in stage III were lower than those at stage I (Table 1), and this is due to the high algae concentration in media. Moreover, when making the culture media of high salinity, the sea salt was used, which commonly resulted in the higher nutrient content than that in the low salinity treatments [37]. In the present study, the DB productivity at 35%e was the highest because of the relatively high growth throughout the 19 days of cultivation. The similar report by Vazquez-Duhalt and Arredondo-Vega [38] and Ben-Amotz et al. [39] found that the decreased yields of biomass were due to nonadaptability of the organism to different salinities in B. braunii.

Chlorophyll a concentration of the algae peaked at 25%e, and this partly reflected a relatively fast growth of algae (Figs. 1 and 2), which agrees with the results from other studies that low salinity favors chlorophyll a production in Dunaliella tertiolecta [40] and Tetraselmis chuii [13]. During the stage III, the chlorophyll a contents (mg g−1) decreased extremely especially in treatments of 25, 35, and 15%e, and this might be because of the light limitation over the increase of the biomass in the media. It is reported that the deficiency of K± and Na+ concentration in the media might also contribute to the decrease of the chlorophyll a of Trichodesmium sp. [41, 42]. At stage III, the chlorophyll a concentrations were still high in all treatments because of the high biomass concentrations in the media (Fig. 2). Further research should focus on the elucidation of the relationship between the chlorophyll a content and nutrient availability for the algae in the media.

The final protein content of the algae decreased with the increase of the salinity among the treatments. This result showed that N. oculata had the similar response that Isochrysis sp., N. oculata and Nitzschia (frustulkw) while affected by the salinity change [30]. Moreover, Ravelonandro et al. [41] showed that the protein content of Arthrospira (Spirulina) platensis was also reduced from 50 to 38% with the increase of the salinity from 13 to 35 g L−1. However, Mishra et al. [14] found that a significant increase in total protein was observed at high salinity (5.5 M NaCl) in N. oculata, and it was double to that in 0.5 M NaCl.

Under unfavorable conditions, many algae species commonly change their lipid biosynthetic pathways toward the formation and accumulation of fatty acids that serve primarily as the storage form of lipids rather than the formation of structural compounds [4]. In this study, lipid contents of the algae (≤ 32.11%) in all treatments were relatively lower than the reported value (approximately 40%) in the same species [25, 30]. The algae had the highest lipid content at 25%e but reached the highest lipid productivity (64.71 mg L−1 d−1) at 35%e after 19 days of cultivation, due to the high DB productivity of the algae at 35%e. This result was similar to that reported by Renaud and Parry [30], who found a highly significant increase of the total lipid of N. oculata when salinity increased from 10 to 35%e. In the present study, the lipid contents of algae decreased while the salinity was over 25%e. However, the process of the lipid accumulation in many algae species is different, the lipid content of Dunaliella increase with the increase of NaCl from 0.5 (equal to sea water) to 1.0 M [21]. Furthermore, Li and Qin [40] studied the effect of salinity on the total lipid content of three freshwater strains of B. braunii (CHN, UK and JAP) and found that the lipid content of the CHN strain did not change but that of UK and JAP strains decrease with the increase of salinity. The enhanced respiration indicated that the responses to salinity stress was an energy consuming process [43], and the mechanisms of the effect of salinity on the lipid accumulation have not yet been elucidated.

In this study, the predominant fatty acids of N. oculata were the palmitic acid (C16:0), palmitoleic acid (C16:1), and eicosapentaenoic acid (C20:5) as previously reported by Renaud and Parry [30] and Xu et al. [44] for this species. The fatty acid profiles at 25, 35, and 45%e were not significantly different, and unsaturated fatty acids (UFA) and saturated fatty acids (SFA) also did not differ among the three treatments in the present study, and this result was different from the report that the degree of fatty acid saturation of Dunaliella sp. increased with the increase of salinity [45].

Generally, the C16 and C18 series contents of microalgae were often used to evaluate the oil productivity from algae.

Table 3. Effect of salinity on the mean dry biomass (DB) productivity, lipid content, lipid, and ∑C16–C18 productivity of N. oculata CS 179 after 19 days of cultivation. Values are mean ± SD. Within the same row, significant differences (a, b, and c) are indicated by different superscripts (p<0.001).

<table>
<thead>
<tr>
<th>Salinity</th>
<th>Mean DB productivity (mg L−1 d−1)</th>
<th>Lipid content (%)</th>
<th>Lipid productivity (mg L−1 d−1)</th>
<th>∑C16–C18 productivity (mg L−1 d−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>55%e</td>
<td>157.58 ± 15.48b</td>
<td>26.50 ± 1.24b</td>
<td>41.76 ± 1.43b</td>
<td>—</td>
</tr>
<tr>
<td>45%e</td>
<td>195.64 ± 17.41a</td>
<td>28.17 ± 1.40b</td>
<td>55.12 ± 3.12ab</td>
<td>39.77 ± 3.11a</td>
</tr>
<tr>
<td>35%e</td>
<td>210.02 ± 20.33a</td>
<td>30.61 ± 3.14a</td>
<td>64.71 ± 5.10a</td>
<td>42.30 ± 2.37*</td>
</tr>
<tr>
<td>25%e</td>
<td>185.13 ± 14.58ab</td>
<td>32.11 ± 1.30a</td>
<td>59.45 ± 2.51a</td>
<td>38.48 ± 3.25*</td>
</tr>
<tr>
<td>15%e</td>
<td>163.67 ± 11.45b</td>
<td>25.70 ± 2.64b</td>
<td>42.07 ± 3.12b</td>
<td>—</td>
</tr>
</tbody>
</table>
In the present study, the $\sum C_{16}$–$C_{18}$ series content (of total FAME) of the algae (at 45%) was 70.8% and $C_{18}$ series content was low (only 14.3%) of total FAME. This composition was similar to *Monodus subterraneus*, which had the $\sum C_{16}$–$C_{18}$ series content of 90% and the $C_{18}$ series content of 5.3% [47], but it was different from *Haematococcus pluvialis*, which had the $\sum C_{16}$–$C_{18}$ series content of 76% and $C_{18}$ series content of 55% [46]. Although a comparison of fatty acid profiles was made among different algal species, it was difficult to evaluate their potential value for biodiesel because of the controlled culture conditions.

### Practical application

The interest of microalgae for alternative fuel production is due to its alterable lipid content in some species by varying cultivation conditions. For the salt-water microalgae species, salinity is one of the most important factors affecting the growth, lipid content, and biochemical composition of microalgae in the field of marine biotechnology.

In recent years, outdoor open pond raceways have been used for mass cultivation of microalgae because of biofuel production for its relatively low cost of construction and operation, large production capacity, and durability. However, microalgae in outdoor system are easily affected by the sudden or gradual salinity fluctuation from rainfall and evaporation. This study estimated the difference of growth, biochemical composition, and lipid productivity of microalgae *N. oculata*, which has been a potential candidate for biodiesel production, under different salinities through the controlled cultivation.

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### 5 References


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