Compromising Between Growth and Oil Production of *Nannochloropsis oculata* Cultivated Under Halo-Stress

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Abstract: The marine micro-alga *Nannochloropsis oculata* was batch-wise grown under different salinities ranged from 0 to 40 g^{-1} up to the stationary phase. The aim was to identify the best salinity that could enhance the maximum cell mass and oil for biodiesel production. Growth data showed an increase in cell number and dry weight of micro-alga as a function of salinities up to 30 g^{-1}, while the amounts of algal oil continually increased over all experimental salinity range. In order to get a maximum oil yield whilst maintaining a high cells concentration of the alga, the cells at stationary-phase were transferred from the preferred growth medium with salinity of 30 to salinity of 32 g^{-1}. The cells have been re-inoculated in the up-next concentrations of 34, 36, 38, and 40 g^{-1} in the same step-wise fashion. By this trend of cultivation, algal cell division showed a good adaptation to the highest salinities and the cells would not go into osmotic shock and die. On the other hand, the composition of fatty acid methyl esters (biodiesel) has been identified as: C14:0, C16:0, C16:1, C16:2, C18:0, C18:1, C18:2, C18:3, C20:4 and C20:5. Data showed a clear significant effect, positively or negatively, of halo-stress on the proportional amounts of fatty esters. However, based on fatty acid type, halo-stress showed no effect on fatty acid profiles comprising biodiesel.

Keywords: Biodiesel; fatty acids; growth; *Nannochloropsis oculata*; salinity

1. Introduction

Research for the development of a sustainable alternative fuel is being carried out to overcoming increasing demands for energy and environmental problems caused by burning of fossil fuel. Biodiesel is a fuel that composed of triglyceride fatty acid methyl esters and produced by many biological systems like plant oil. Many crops like soybean, canola and palm are major feedstock for biodiesel production, but encounter limitations regarding their consuming as valuable nutritive and edible crops (Koh, 2007; Amaretti et al., 2010). Alternatively, algae could be substituted as a sustainable feedstock for biodiesel production (Chisti, 2007; Pienkos and Darzins, 2009). Algae have been characterized by their high lipid content and fast growth rate. In addition, algae can be cultured on marginal lands using saline water and hence avoiding competing with arable land and agricultural crops. The production of micro algal biomass for biodiesel must be economically feasible and competitive with liquid fuels. Therefore, the success of biodiesel production from microalgae depends on high biomass productivity and considerable lipid yields (Chisti, 2007).

Manipulating culture conditions of the growth medium, this is known as biochemical engineering of algal cultivation (Courchesne et al., 2009), influences downloading of algae biomass and intracellular accumulation of oil (Liu et al., 2008).

Factors influence algae cultivation conditions including temperature (Behrens, 2005), light (Carvalho et al., 2006), chemical composition of the growth medium, pH (Harrison and Berges, 2005) and salinity (Takagi et al., 2006; Rao et al., 2007; Trobajo et al., 2011).

Without a doubt, raised water temperatures arisen from the global warming can cause increasing in salinity due to extensive evaporation in the open algal production system. High salinity creates a physiological stress to channel metabolic fluxes to lipid accumulation in microalgae (Courchesne et al., 2009). Hence, halo-stress could be applied to boost lipid accumulation in microalgae for biodiesel production purposes. However, while halo-stress increases lipid amount in algae, a decrease in cell division has markedly been observed (Takagi et al., 2006). Therefore, compromising between high cell density and oil production by means of biochemical engineering of algal cultivation is an urgent matter.

Nannochloropsis are eustigmatophytes marine algae (Doan and Obbard, 2010), which are known as good lipid producers and fast grown microalgae (Chiu et al., 2009). The genus is recommended as a bio-fuel feedstock by many authors (Rosenberg et al., 2008; Rodolfi et al., 2009). In addition, *Nannochloropsis* tolerates many environmental stresses including salinity (Pal et al., 2011).

In this respect, the goal of the present study was to compromise between the maximum oil yields of *N. oculata* grown under halo-stress conditions whilst maintaining a high cell concentration. Chronologically, our first efforts were to identify the best salinity concentration (s) that stimulates the maximum biomass and oil production, with the emphasis of fatty acid composition. Later, we designed a trend of cultivation to achieve our goal.

2. Material and Methods
Experimental Organism and Culture Conditions
The eustigmatophyte *Nannochloropsis oculata* (Droop) Hibberd, was obtained from the Culture Collection of Algae (UTEX), Austin, Texas, USA. Two sets of triplicate of 1.5 L batch cultures of the micro-alga were grown in medium f/2 (Guillard and Ryther,1962; Guillard, 1975). The first set was cultivated under salinities: 0, 10, 20, 30, and 40 g^{-1} (Starr, 1964). For the second set, an inoculum of about 22×10^6 ml^{-1} cell number stationary-phase *N. oculata* grown under 30 g^{-1} salinity was transferred into the up-next concentration of 32 g^{-1} salinity and keep to grow till the beginning of the stationary growth phase. The process was repeated for other salinities (32, 34, 36, 38 and 40 g^{-1}) in the same fashion. All culture flasks were continuously agitated by bubbling with sterile air, enriched with 0.5% CO2 at $25 \pm 1°C$ in a temperature-controlled room. Illumination was provided with an irradiance of 300 µmol m^{-2} s^{-1}, under a 16 h/8 h light/dark regime.

Growth Evaluation
Cell growth was measured by daily counting cell number under a microscope using a haemacytometer. Culture samples washed and dried at 70°C. Dry weight was measured gravimetrically on triplicates of 10mL culture. At the beginning of the stationary growth phase of algal cultures (12 days for the first set and 6-8 days for the second one), the cells were harvested by a centrifuge for oil extraction and analysis.

Oil Extraction and Fatty Acid Methyl Esters Analysis
Algal oil was extracted using n-hexane (Miao and Wu, 2006). The crude oil extract was esterified according to Radwan (1991), and analyzed by a Shimadzu gas liquid chromatography (GLC), equipped with a flame ionization detector with packing column material Hp-5. The carrier gas was nitrogen and the short speed was 5 mm/min. Identification of fatty acid methyl esters (FAME) was carried out by comparing their retention times with those of standards.

Statistical Analysis
Data were analyzed using two-way analysis of variance (ANOVA), using COSTAT 2.0 statistical analysis software. Means were tested with least square difference (LSD), where the difference of $P \leq 0.05$ was significant. The mean value of triplicate data and the standard deviations (SD) were also calculated.

3. Results and Discussion
Algal growth
Growth data of *N. oculata* (Table 1) showed tolerance to changes in salinity within the experimental range of 0-40 g^{-1}. The cell number and dry weight showed an increase from 17.1 ± 0.4 to $26.6. \pm 0.1$ ml^{-1}, and 6.4 ± 0.1 to 9.7 ± 0.3 mg^{-1} respectively. However, the growth declined under stress of 40 g^{-1} salinity. Accordingly, these results indicate that 30 g^{-1} is the best salinity for creating a cell division in *N. oculata*. A similar result was reported for many other halo-tolerant species (Ben-Amotz et al., 1985; Takagi et al., 2006). A lot of halo-tolerant microalgae have a response mechanism that permits their existence in a saline medium. These strains produce metabolites that help protect them from salt injury and maintain an osmotic balance between the cell and its surrounds (Rao et al., 2007).

Algal oil
Oil amounts (Table 1) were gradually increased (from 31% to 46% of the dry weight, $P \leq 0.05$) over the applied salinities. Accumulation of oil in algal cells grown under halo-stress has been reported for many microalgae, such as *Nannochloropsis sp.* (Pal et al., 2011), *Dunaliella tertiolecta* (Takagi et al., 2006), some diatoms (Trobajo et al., 2011), and many of phytoplankton (Abid et al., 2008).

In this study, although 30 g^{-1} is identified as the best salinity that boosts oil formation in *N. oculata*, the increase is negated overall by the drop in cell division. Large biomass production and successful culture growth are essential to producing large volumes of lipids overall. Unfortunately, high proportions of lipids and high biomass productivity appear mutually exclusive.

Table 1: Growth and oil % of *Nannochloropsis oculata* grown under stress of 0-40 g^{-1} salinities

Salinity conc. (g^{-1})	0	10	20	30	40
Cell number (x 10^6) ml^{-1}	17.1 ± 0.4	19.3 ± 0.1	24.4 ± 0.3	26.6 ± 0.1	22.1 ± 0.2
Dry wt* (mg^{-1})	6.4 ± 0.1	7.3 ± 0.1	9.1 ± 0.5	9.7 ± 0.3	8.4 ± 0.1
Oil (%) **	31	34	39	43	46

Dry wt*: Data standardized as mg^{-1}; Oil**: Data standardized as % of the dry weight

Table 2: Growth of *Nannochloropsis oculata* grown under stress of 32-40 g^{-1} salinities

Salinity conc. (g^{-1})	32	34	36	38	40
Cell number (x 10^6) ml^{-1}	24.3 ± 0.4	24.5 ± 0.1	25.4 ± 0.3	26.06 ± 0.1	26.1 ± 0.2
Dry wt* (mg^{-1})	9.6 ± 0.3	9.3 ± 0.1	9.7 ± 0.1	9.9 ± 0.2	10.01 ± 0.2

Dry wt*: Data standardized as mg^{-1}

For increasing oil content whilst maintaining high cell concentration, stationary phase *N. oculata* - starting with the optimum growth salinity of 30 g^{-1} - have been transferred in a concentration of 32 g^{-1}. After achieving stationary growth, the cells have been re-inoculated in the up-next concentration of 34, 36, 38 and finally into the optimum salinity for oil production 40 g^{-1}. By this cultivation manner, data showed a gradual increase in cell number (from 24.3± 0.4 to 26.1± 0.2), which is corresponding to downloading biomass (from 9.6± 0.3 to 10.01± 0.2), as a function of the experimental salinities (Table 2). Accordingly, algal cell division showed good adaptation to the highest salinities and the cells do not go into osmotic shock and die. While this will not be cost effective as production cost will increase due to the extra processing steps, the idea to use salt marsh habitats for applied purposes should be further researched. Many previous studies (Mullar and Gottschalk, 1973; Chrisman et al., 1985; Hieks et al., 1989; Mohammady et al., 2005), demonstrated the production of certain types of functional proteins working as a cell protective against surroundings. These proteins enhance the cell division-machinery and operate the mechanism of gene regulation efficiently. However, none of these studies was concerned with salinity stress. Thus, further efforts should be done to correlate between the observations here and the protein profiles produced by *N. oculata* grown under the above conditions to understand the mechanism that controls the process.

Fatty acid methyl esters composition

The dominant FAME fractions detected (Table 3) are C16:0 (palmitic acid), C16:1 (palmitoleic acid), and C20:5 (pentaenoic acid, EPA), together with C18:1 (oleic acid) and C18:2 (linoleic acid). However, C14:0 (myristic acid), C16:2 (hexadecadienoic), C18:0 (stearic acid), C18:3 (linolenic acid), and C20:4 (arachidonic) were detected in relatively small amount under the practical limit of the salinities. Data showed an increase in the amounts of both C16:0 and C16: 1 over the experimental salinities (P≤0.05). Moreover, a decrease in the fatty esters amounts of C18:2 and C18:3 were detected (P≤0.05).

A negative correlation between the experimental salinities and fatty acids C16:2, C18:0 and C18:1 is observed under salinities of 30 and 40 g^{-1}. While no significant effect on fatty acids C14:0 and C20:4 as a function of salinity. In view of that, *N. oculata* shows tolerance against halo-stress by modifying their cellular fatty acids content.

Biodiesel is composed mainly of palmitic (C16:0), stearic (C18:0), oleic (C18:1), and linolenic (C18:3) acids. Definitely, the proportions of fatty acid methyl esters contribute strongly to the quality of biodiesel fuel (Knothe, 2008). Moreover, increasing palmitic acid is desirable for good quality biodiesel (Mandal and Mallick, 2011). Data in this research demonstrated a significant effect of salinity on the proportions of FAME of *N. salina*. In addition; the applied salinity range boosts the C16:0 formations. Therefore, in a correlation with the above studies, the authors postulate cultivation of the examined strain under halo-stress to improving biodiesel quality. However, in this work, both C18:0 and C18:3 are found in relatively small amounts. The ideal mixture of fatty acid comprising biodiesel has been suggested to be C16:1, C18:1and C14:0 in the ratio 5:4:1 (Schenk et al., 2008). The closest ratio to that idea (4.9: 2.04: 1) has been shown here in the cells grown under salinity of 40 g^{-1}. This means that oil of *N. oculata* needs to be enriched with more C18:1, since this fraction has been reported to have a reasonable balance of biodiesel fuel properties (Rashid et al., 2008). In this study, the highest amount of C18:1 (12.4%) was obtained in the cells grown under 20 g^{-1} salinity stress. In addition, a decline in the total amount of unsaturated fatty acids associated with an increase in saturated fractions has been shown as a function of experimental salinities. Accordingly, the ratio of unsaturated to saturated fatty acids is gradually decreased (Table 3). The proper percentage of saturated and unsaturated fatty acid is very important to microalgae as a biodiesel feedstock (Deng et al., 2009). However, high concentration of saturated fatty acid pool is desirable for good-quality biodiesel (Mandal and Mallick, 2011).

4. Conclusions

Halo-stress shows a great influence on biodiesel from *N. oculata*. The examined alga demonstrated an ability to produce large percentage of oil under salinity stress up to 40 g-$^{-1}$.

Table 3: Fatty acids composition of *Nannochloropsis oculata* grown under stress of 0-40 g^{-1} salinities

FA***	Salinity concentrations (g^{-1})				
	0	10	20	30	40
C14:0	5.0 ± 0.2	5.4 ± 0.1	5.4 ± 0.2	5.0 ± 0.1	4.9 ± 0.1
C16:0	13.0 ± 0.2	20.8 ± 0.2	22.0 ± 0.3	25.0 ± 0.5	25.8 ± 0.1
C16:1	18.1 ± 0.5	20.0 ± 0.3	21.0 ± 0.3	23.0 ± 0.9	24.0 ± 1.2
C16:2	3.4 ± 1.2	3.0 ± 1.2	3.1 ± 1.3	1.8 ± 0.3	0.2 ± 1.1
C18:0	5.0 ± 0.2	5.1 ± 0.1	5.0 ± 0.1	3.1 ± 0.1	3.0 ± 0.5
C18:1	10.0 ± 1.1	11.0 ± 0.1	12.4 ± 0.2	10.4 ± 0.4	10.0 ± 0.1
C18:2	9.0 ± 0.7	8.0 ± 0.1	8.0 ± 0.2	6.0 ± 0.1	4.2 ± 0.8
C18:3	4.9 ± 0.3	4.6 ± 0.5	3.0 ± 0.1	1.9 ± 0.6	1.3 ± 0.6
C20:4	5.5 ± 0.5	5.7 ± 0.1	5.4 ± 0.4	5.4 ± 0.1	4.6 ± 0.1
C20:5	26.1 ± 0.2	18.2 ± 0.4	14.7 ± 0.7	18.4 ± 0.4	23.0 ± 0.2
Total saturated	23.0	30.5	32.4	33.1	33.7
Total unsaturated	77.0	69.5	67.6	66.9	66.3
Unsat / Sat ratios	3.35	2.28	2.09	2.02	1.97

FA***: fatty acid methyl esters (%)

The decrease in the cell density at this salinity grade shows a trade off between cell division and oil production. For increasing oil content whilst maintaining a high cell concentration, we designed a trend of cultivation depending on re-inoculation of the stationary grown algal cells starting with its preferred salinity of 30 g^{-1} into the concentration of 32 g^{-1}. After the alga attain the same previous age, the process is repeated to the up next concentrations of 34, 36, 38 and 40 g^{-1}, in a step-wise fashion. By this trend of cultivation algal cell division showed good adaptation to the highest salinities and the cells do not go into osmotic shock and die. Accordingly, the authors suggest that 40 g^{-1} is the best salinity concentration to compromise between high cell division and maximum oil production, if *N. oculata* was cultivated under the above conditions. However, as this will not be cost effective due to the extra processing steps, the idea to use salt marsh habitats for applied purposes should be further researched.

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