Indian Journal of Geo Marine Sciences
Vol. 49 (02), February 2020, pp. 326-332

Short Communication

Reciprocal Response of Nitrogen for Enhancing Growth and Proximate Compositions of Marine Microalga *Tetraselmis* sp. under low Saline Conditions

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Received 23 July 2018; revised 08 October 2018

The present investigation was aimed to study reciprocal response of nitrogen to enhance the cell multiplication and proximate balance from marine microalgae *Tetraselmis* sp. under low saline conditions. The strain has been isolated from Yellow Sea, South Korea and the algae were cultured in the different nitrogen concentrations (0N, 1N & 2N) under low saline conditions (15, 20 and 25 psu).The results revealed that the *Tetraselmis*sp. have significantly higher (P < 0.001) optical density, cell density, biomass concentration and total lipid in moderate nitrogen concentration (1N) under 25psu of salinity condition. However, the total protein and carbohydrate were significantly higher (P < 0.001) in the high nitrogen concentration (2N) under 20 psu of salinity. The present study reveals that the combined effects made a huge impact on growth and biochemical variation in the microalgae.

[**Keywords:** Carbohydrate; Lipid; Nitrogen; Protein; Salinity; *Tetraselmis* sp.]

Introduction

Most of the microalgae has the ability to produce a value-added products like pigments, vitamins, poly unsaturated fatty acids (PUFA) and in addition, they are also highly potential candidates and act as raw materials for medicine, nutritional, beauty products and food industries[1-3]. Certain physicochemical, nutrient parameters play a vital role in microalgae culture. Among which nitrate and ammonium salts are considered as a nitrogen-rich source which plays a key role in growth and metabolism of microalgae; while salinity parameter has an ability to change the cell multiplication and proximate compositions. Adaptation to salinity differs between algae, based on their groups like halotolernt and halophilic[4]. The changing salinity (preparedly reducing) is preparable methods to enhance the proximate structures of saline algae. While the sodium chloride fluctuations made an impact on starch metabolism and revealed that the cultivation condition and particular species nature may depends[5]. So the current research work was aimed to investigate the reciprocal response (increase and decrease) of nitrogen in low saline conditions to get a maximum algal cell multiplication and biochemical structures such as proteins, lipids and carbohydrate in marine microalgae *Tetraselmis* sp. The main purpose of selecting *Tetraselmis* sp. for this experiment, is it a big green flagellate microalgae which has a capacity to store natural lipids in their cells. So that the successful outputs can be implemented in the large-scale industries and hatcheries under optimized culture conditions.

Material and methods

Stock culture maintenance

Marine microalgae *Tetraselmis* sp. was acquired from Center for Marine Bioenergy Consortium, Inha University, Seoul, South Korea. The inoculum were cultivated in LED chamber (HST-120LE-4, Hanbaek St. Co., South Korea) which were fed with Walne's medium[6] at optimized culture condition which were explained in our previous publication[7]. For cultivation experiments, 0.250 mL round bottom conical flask were used by filling 0.200 mL saline water. The experimental inoculum were obtained from the 5–8 days of cultivation.

Experimental conditions

The experimental design (Fig. 1) for the nitrogen treatments (0N (no nitrogen), 1N (100 g L^{-1}sodium nitrate) and 2N (200 g L^{-1}sodium nitrate)), Walne's composition was incorporated for this experiment and their variations is listed out in the Table 1. The working volume is 500 mL and water medium has three different salinities such as 15, 20 and 25 psu. The pre-measured inoculum was shifted to 0.500 mL of conical flasks (Byrex, South Korea) which were

occupied with 0.400 litre of experimental filtered saline water. Then the experimental flask was kept in working orbital shaker (Fine PCR, SH30). The whole experiment was carried outunder controlled conditions[7]. During the experiment, once in two days Optical Density (OD), cell numbers, dried algal biomass was estimated and on 14th day of experiment proximate compositions was estimated.

Analysis

The growth of algae were estimated by using UV visible Spectrophotometer (3200, X-MA, Human Corporation, South Korea) by measuring optical density at 680nm[7]. The dried algal biomass was analyzed every two days of except using standard method[8] and the concentration was estimated as per following formula.

$$Biomass\,(gL^{-1}) = \frac{W1 - W0}{10/1000}$$

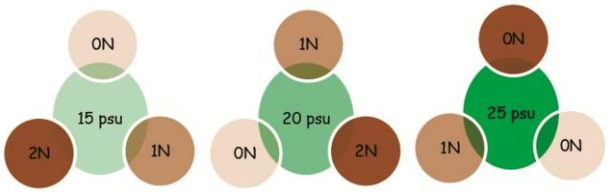

Fig. 1 — Schematic representation of experimental plan

Table 1 — Medium composition for experiment.

Components (g)	1N	2N	0N
Solution A			
Sodium nitrate	100	200	-
Ferric chloride hexahydrate	1.3	1.3	1.3
Manganese(II) chloride tetrahydrate	0.36	0.36	0.36
Boric acid	33.6	33.6	33.6
Ethylene diaminete traacetic acid	45.0	45.0	45.0
Sodium dihydrogen phosphate dihydrate	20.0	20.0	20.0
In to 1 liter of distilled water			
(B) Trace metal solution			
Zinc chloride	4.2	4.2	4.2
Cobaltous chloride hexahydrate	4.0	4.0	4.0
Ammonium heptamolybdate	1.8	1.8	1.8
Copper(II) sulfate pentahydrate	4.0	4.0	4.0
In to 1 liter of distilled water			
(C) Vitamin solution			
Cyanocobalamin	0.01	0.01	0.01
Thiamine HCl	0.01	0.01	0.01
Biotin	0.0002	0.0002	0.0002
In to 100 ml of distilled water			

The lipid content was analyzed as per Bligh and Dyer[9] method with minor changes. Lowry *et al*.[10] has been followed for estimation of total protein from the algal cells. Carbohydrate assessment wascarried out based on the Dubois *et al*.[11].

Statistical analysis

Collected data were analyzed by using the SPSS Ver. 17.0 (Statistical Program for Social Sciences 17.0). Two-way analyses of variance (ANOVA) were performed to find out the significant differences among the treatments.

Results and Discussion

Growth of *Tetraselmis* sp. cultivated in different salinity and nitrogen conditions

In recent times, numerous research[12] studies have concentrated on sustainable energy and they have demonstrated that microalgae plays a key role as the main source for biofuel production[12]. For boifuel production the biomass productivity, biochemical compositions, growth of microalgae, optimization of regulating factors such as physicochemical parameters and nutrients are considered very essential. Among the essential parameters the availability of nutrients, pH, salinity and light are the majorreasons which were modifying the algal cell multiplications and their regular process like photosynthesis.

Figure 2 shows the cell density (a-c) and optical density (d-f) of the *Tetraselmis* sp. cultured under the low saline conditions with reference to the different nitrogen conditions on the culture medium. At the 14th day of experiment, the highest cell density of *Tetraselmis* sp. was observed in culture having in 25 psu of salinity showed cell density of 98 ± 2.35×10^4 cells mL^{-1} under optimum nitrogen condition (1N). The concentration of *Tetraselmis* sp. cell observed to be in 25 psu is significantly higher than the other experimental salinities such as 20 psu (68 ± 1.62×10^4 cells mL^{-1}) and 15 psu (82 ± 1.96×10^4 cells mL^{-1}) under the same nitrogen concentration. During the 14th day of experiment, the *Tetraselmis* sp. at the salinity of 25 psu had the highest optical density (0.40 ± 0.007 Abs) under the optimum nitrogen concentration (1N). This current study dealt with the algal cell multiplication and proximate composition with various combination of salinity and nitrogen.The earlier studies suggested that the, microalgae need optimum growth factors to enhance their growth and biochemical compositions[13]. The present results show that the *Tetraselmis* sp. cells can extend their life with minimum or without nitrogen sources, however, the cell multiplication was

significantly len owing to nitrogen unavailability. Previous studies states that the algal cell growth rate was decreasing while nitrogen content was low in the culture medium[14]. The conversion of chlorophyll into the cell wall, protein and nucleic acid may help to increase their cell multiplication when the nitrogen storages were inactive[15]. This study clearly explains that neither the too low nor too high salinity level but moderate salinity and the availability of optimum nutrients in the medium will have the ability to have the higher growth. Fatma *et al*.[16] support our present findings and they explained that at higher saline water medium, the microalgae growth was put off when the osmo protectant is used tometabolism enzymes stabilization with the help of compatible solutes.

Biomass production of *Tetraselmis* sp. cultivated in different salinity and nitrogen conditions

During the 14th day of experimental period, the *Tetraselmis* sp. at salinity 25 psu under optimal nitrogen concentration (1N) represented the maximum dried algal biomass concentration(0.75±0.01mg L^{-1}, $p<0.001$), followed by 25 psu:2N and 15 psu:1N and concentration of biomass was 0.66±0.01 and 0.6±0.01mg L^{-1} respectively (Fig. 3). The daily biomass production was significantly varied ($p<0.001$) with reference to nitrogen depletion and replication under different saline conditions. During the present experiment in 25 psu:1N combination, biomass production was 34.66 % which was the maximum level noticed, it may due to their growth

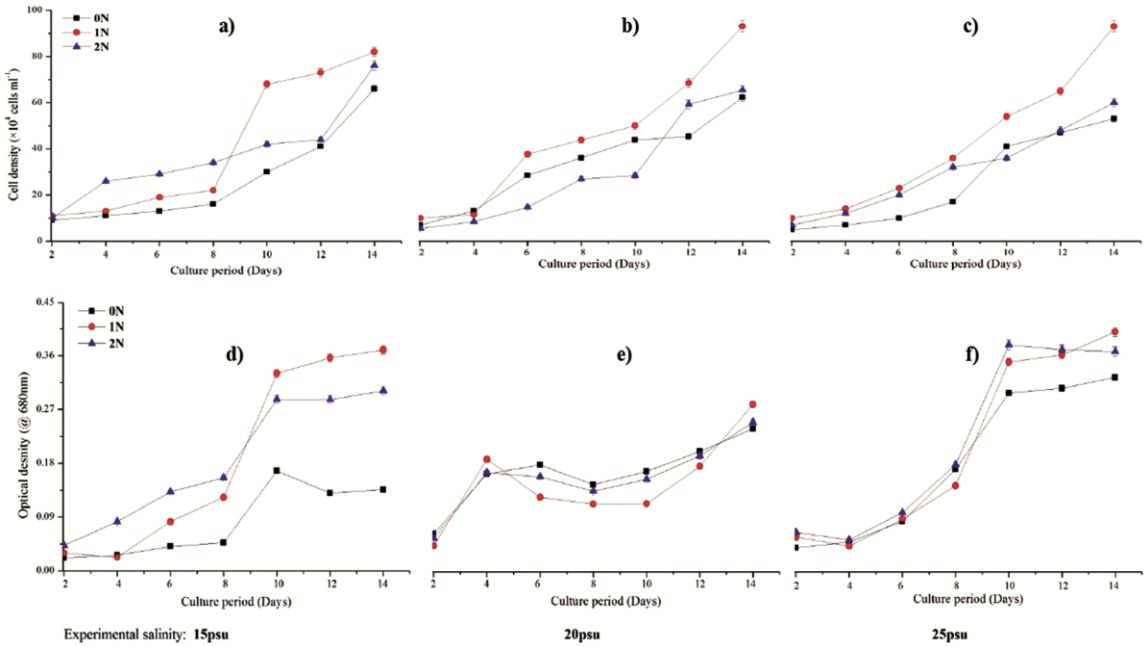

Fig. 2 — Cell density (a,b,c) and optical density (d,e,f) vs culture period (day) for culturing *Tetraselmis* sp. in three different salinities (15, 20, 25) under diiferent nitrogen (0N, 1N, 2N) concentreation (Mean ± SE).

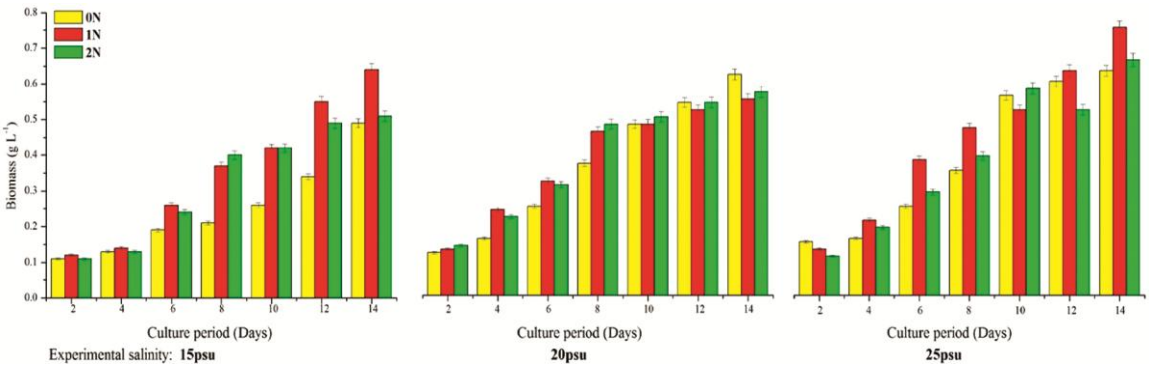

Fig. 3 — Biomass production vs culture period (day) for culturing *Tetraselmis* sp. in three different salinities (15, 20, 25) under diiferent nitrogen (0N, 1N, 2N) concentreation (Mean ± SE).

which was high during the entire experimental period (14 days) (Fig. 1a-b). The same trend has been observed by Gu *et al*.[13]. Vazquez-Duhalt and Arredondo-Vega[17] found that, the biomass production was relatively high when the *Tetraselmis* sp. and *Botryococcus braunii* adopted into the different salinity levels. Dammak *et al*.[12] proved that the, *Tetraselmis* sp. cultured in the low or moderate nitrogen containing medium their biomass production was high during entire culture period. The present result corroborates and satisfies the hypothesis of Yao *et al*.[18] which too states that, the higher biomass concentration (5.72 g L^{-1}) after 8 days of growth of *Tetraselmis subcordiformis* when the nitrogen concentration was low.

Total protein content of *Tetraselmis* sp. cultivated in different salinity and nitrogen conditions

Gu *et al*.[13] stated that the, fluctuation of salinity in the water would affect the biomass fabrication and also protein level of microalgae. The total protein contents of *Tetraselmis* sp. under different combination of nitrogen and salinity conditions were represented in Figure 4a. The replicated nitrogen condition under 25 psu of salinity showed a significantly higher protein content than the other combination of nitrogen and salinity. The different combination of salinity and nitrogen are15 psu:0N, 15 psu:1N, 15 psu:2N, 20 psu:0N, 20 psu:1N, 20 psu:2N, 25 psu:0N, 25 psu:1N, 25 psu:2N and their protein yield was 25.92 ± 2.1 %, 29.28 ± 1.3 %, 33.92 ± 3.8 %, 32.49 ± 1.2 %, 38.95 ± 2.9 %, 41.61 ± 1.3 %, 28.52 ± 1.5 %, 28 ± 3.2 %, 31.5 ± 2.6 %, respectively. The *Tetraselmis* sp. show the lowest protein concentration at the combination of 15 psu salinity and nil nitrogen condition (0N) than the other salinity and nitrogen combination tested. This study shows that the total protein content reducedonce the salinity increase beyond the limits. Renaud and Parry[19] experimental findings revealed that protein content will be heavily affected in *Isochrysis* sp., *Nannochlorospsisoculata* and *Nitzschia* sp. when salinity level changes in the water especially while increasing their limits and also agreed our results. The protein synthesis may active when the ATP secretions getting stop during sodium chloride concentration is unstable[20]. The same principle was advisable for cultivation of *Tetraselmis* sp. with reference to different salinity levels and finally higher amount of protein content was obtained in 20 psu of salinity. *Arthrospiraplatensis (Spirulina)* getting down their protein content from 50 to 38 % when culturing at salinity increased from 13 to 35 g/L[21].

Total carbohydrate content of *Tetraselmis* sp. cultivated in different salinity and nitrogen conditions

For photoautotrophic microalgae adequate supply of light intensity, nitrogen, salinity and pH stronglystimulates the growth and biochemical compositions such as carbohydrates, lipids and may also change the carbon dioxide[22]. The effect of various salinity and nitrogen combination (15, 20 and 25psu vs 0N, 1N and 2N) on carbohydrate content of *Tetraselmis* sp. were displayed in Figure 4b.

The carbohydrate results show significant difference ($p < 0.001$) between the salinities and nitrogen variations (Fig. 4b). The highest carbohydrate content (23 ± 0.5 %) was found in replicated (2N) nitrogen level under 20 psu of salinity and lowest carbohydrate (16.2±0.2%) content was found in 0N (nil nitrogen) concentration in low saline (15 psu) condition. However, the carbohydrate content was eventually low in the nitrogen depleted conditions and high in the nitrogen replicated conditions. On the other hand, the accumulation of carbohydrate significantly increased when the salinity concentration was pumped into beyond the limit. Siaut*et al*.[23] stated that there is a direct proportional

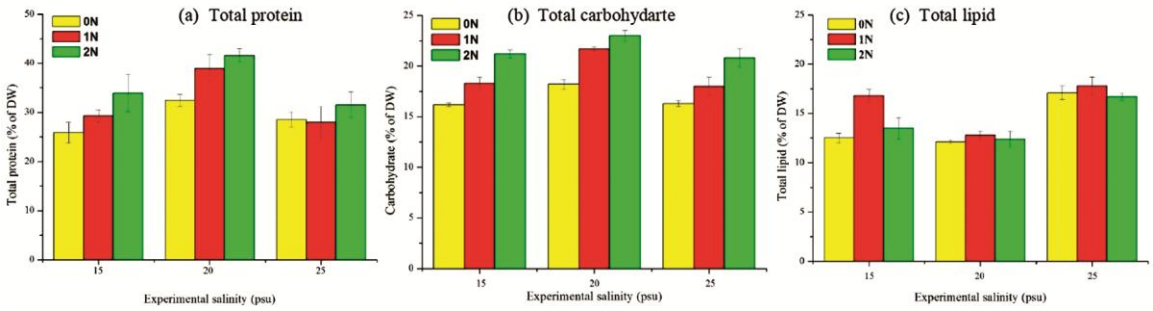

Fig. 4 — Total protein (a), carbohydrate (b) and lipid (c)production vs culture period (day) for culturing *Tetraselmis* sp. in three different salinities (15, 20, 25) under diiferent nitrogen (0N, 1N, 2N) concentrations(Mean ± SE).

relationship in carbohydrate content with saline culture medium in the fresh water microalgae *Chlamydomonas reinhardtii* culture. But at the same time on the other hand, according to Chen and Jiang[24] marine microalgae *Dunaliella* sp. revealed that there is an inversely proportional relationship in starch content with salinity variations.Yao *et al*.[18] too explained that the salinity plays a fickle role in starch metabolism and it varies from species to species and is specific to cultivation environmental conditions.

The present study revealed significantly higher carbohydrate (23 ± 0.5 %) content in moderate salinity (20 psu) than the lower (15 psu) and higher (25 psu) salinity which shows 18.2 ± 0.5 % and 21.7 ± 0.2 % of carbohydrate respectively. Li *et al*.[15] suggested that the "fattening" conditions like nitrogen desolation or inhibition was one of the successful methods to increase or modify the biochemical composition such as lipid and carbohydrates in microalgae. However, Ho *et al*.[25] found the slight surge of carbohydrate and lipid content when the nitrogen starvation extended in fresh water microalga *Scenedesmus obliquus*. Contrasting to these results the present study revealed that the replication of nitrogen could support the carbohydrate production than the nitrogen starvation which could trigger the iron or trace element particles against the saline conditions.

Total lipid content of *Tetraselmis* sp. cultivated in different salinity and nitrogen conditions

The construction and gathering of fatty acids methyl esters profile in microalgae it fully depends on the lipid pathway and it may change their direction when microalgae face the unfavourable conditions[26]. The total lipid content of *Tetraselmis* sp. were deciphered in Figure 4c. Among the three salinity concentrations tested (15, 20 and 25 psu), the 25 psusalinity revealed the higher amount of total lipid concentration than the other salinities (15 and 20 psu). In the nitrogen variations, moderate nitrogen (1N) combination has resulted higher amount of lipid concentrations than the depleted (0N) and replicated (2N) nitrogen combination. *Tetraselmis* sp. exhibited considerably higher (P<0.001) lipid concentration (17.8 ± 0.9 %) when cultured at 25 psu:1N combination compared to other combinations shown in Figure 4c. Roopnarain *et al*.[27] and Wan *et al*.[28] stated that the biomass production of *Isochrysis galbana* and *C. sorokiniana* was low due to least cell growth with reference to ammonium as a nitrogen source. In the time of experimental period, the total lipid productivity was high (17.8 ± 0.9%; Fig. 4) in moderate nitrogen concentration (1N) with higher saline concentration (25 psu) owing to the high biomass production in same combination (1N:25 psu; Fig. 3) of nitrogen and salinity which indicate that the high biomass production lead the algal lipid production and this observation was agreed by Kim et al.[14]. The present research part revealed that the depleted and moderate nitrogen cultures exhibited higher lipid production than the replicated nitrogen combination.

The earlierstudy Kim *et al*[14] alsostated that the nitrogen deficient cultures have higher amount of lipid and other biochemical properties than the nitrogen sufficient cultures. Among the various stress factors, salinity made a huge impact on the physiological and biochemical profile of microalgae which was incorporated with algal growth. Also they can trigger to enhance the algal lipid profile which were playing major role in the fatty acid metabolic changes[29]. Presence of high quantity saturated and mono unsaturated fatty acids in the microalgae leads to the good quality of biodiesel or animal feed extraction[30]. In the present study, lipid profile was increased when salinity was increased from 15 psu to 25 psu with response to osmotic pressure and this part of the result was supported by Hu *et al*.[26]. The microalgae growth were constant when their proteins and osmo protecting solutes are preparing iron exchange process into the cell wall with reference to the various salinity[31,32].

Conclusion

This current work validates that growth, biomass, carbohydrate, protein and lipid production of oceanic microalga *Tetraselmis* sp. were considerably boosted by different nitrogen and salinity combinations under experimental conditions. The proper combination of nitrogen and salinity is considered a key factor inducing the growth and biochemical profile. The highest cell density, optical density, biomass and lipid production obtained were 98 ± 2.35×10^4 cells mL^{-1}, 0.40 ± 0.007abs, 0.75 ± 0.01mg L^{-1}, 17.8 ± 0.9 % in 25 psu:1N combination; the maximum protein and carbohydrate was 41.61 ± 1.3 % and 23 ± 0.5 % in dry weight observed in 20 psu:2N combination which are higher than the earlier reported values. Further, it can be stated that the combination of physico-chemical parameter especially salinity and nitrogen exhibit the better result rather than relying upon individual parameters. The present study inferred the

useful data on for mass scale production of *Tetraselmis* sp. for biofuel and associated commercial products.

Acknowledgement

Authors are thankful to the National Marine Bioenergy R & D Consortium, Ministry of Ocean & Fisheries, South Korea for providing financial support (Project Number-200255) for this work. Further, the author S. Dinesh Kumar is respectful to UGC (Ref. No. F./31-1/2017/PDFSS-2017-18-TAM-13681 dated 19.06.2017) and the author S. Latha is grateful to DST-SERB-NPDF (File Number: PDF/2016/003456, dated 28.03.2017), Govt. of India, for providing post-doctoral Fellowships.

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