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EFFECT OF ENVIRONMENTAL STRESSES ON LIPID PRODUCTION BY *TETRASELMIS* SP. AND MODIFIED EXTRACTION METHODS FOR LIPID RECOVERY IN ASPECT OF PROSPECTIVE BIOFUEL PRODUCTION

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Abstract

Microalgae are known to be the most potential biomass for production of biofuel. Different environmental parameters like salinity, nitrogen deprivation and phosphorus deprivation were imposed on *Tetraselmis* sp. for examining their effects on algal growth and lipid accumulation. Additionally, various pretreatments namely thermal, ultrasound and ultraviolet were checked to enhance the lipid recovery from algal cells. Highest biomass was achieved from 35‰ salinity condition. Highest lipid content was found in thermal pretreated nitrogen deprived algal stock yielding 69.73% of dry weight. Lipid accumulation was highest in nitrogen deprivation yielding 42.83% of dry weight. Highest amount of lipid was recovered from thermal pretreatment yielding45.67%of dry weight. Thus this work has aided an optimized way to enhance biofuel production in terms of optimum biomass, lipid induction as well as lipid recovery from microalgal cell.

Keywords: *Microalgae, Tetraselmis, biofuel, lipid, parameter, pretreatment*

Introduction

Growing energy demand with global warming are two immediate threats affecting modern society due to increased population and uncontrolled urbanization. Therefore, the dependency on fossil fuel is rising day by day. It has been estimated that oil reserves would be exhausted by 2050. The subsequent emission of greenhouse gas leads to global warming as well as climate change. This concern is driving the researchers to produce alternative eco-friendly and renewable energy sources. Biofuel is considered a significant substitute for fossil fuels, because it is a biodegradable, sustainable and non-toxic source of fuel (1). In terms of biofuel, microalgae, prokaryotic or eukaryotic photosynthetic microorganisms, is a promising source having large amounts of lipid and no competition with other foodstuffs like corn, cereal etc. In addition, they have higher growth and photosynthetic rates utilizing very little land (2, 3). Moreover, algae 's ability to fix CO2 has been suggested as a way of reducing CO2 emitted by fossil fuel burning and can therefore be used to minimize greenhouse gas emissions (4). There are a number of species having the potentiality to produce higher lipid yield in their biomass. Among them the species strains of *Chlorella*, *Tetraselmis* and *Nanochloropsis* were extensively studied for their biodiesel-polyunsaturated fatty acid (PUFA) and co-product potential (5, 6). Dynamic experiments were carried out for enhancing the lipid content and improving lipid extraction efficiency to meet the biodiesel production at an optimum level.

Microalgal growth and its lipid synthesis can be modified by various environmental and nutritional stresses like salinity, nitrogen, phosphorus with an optimum range (7). Under nutritional or environmental stress algal cells produce large amounts of fatty acid mainly as triacylglycerides to resolve their cellular integrity. An increased accretion of triacylglycerides and a decrease of polar lipids have been noticed in diatom *Stephanodiscus minutulus* under limited supply of silicon, nitrogen or phosphorus in culture system (8). Nutrient deficiency in the green alga *Chlamydomonas moewusii* resulted in decreased contents of PUFA C16:3, C16:4 and C18:3 while total rates of C16:1 and C18:1 FA were improved. (9). Another challenge in the biofuel production is its extraction from algal cells. Species selection, cultivation, harvest, and cell disruption are some major steps of biofuel production. As algal cells sometimes contain thick multilayered cell walls, cellular disruption is a crucial step for efficient oil recovery (3, 10). There are variety of ways through which algal cells can be disrupted (11). However, the concerning issue is to select optimum stress condition along with extraction pretreatment in potential algal species for successful biofuel production. In this study higher saline condition, nitrogen and phosphorus starvations were used separately to know their effects on algal growth and optimum lipid accumulation from a potential algal species *Tetraselmis* sp. In addition, some potential pretreatments prior to extraction of lipids including thermal, ultrasonic and ultraviolet were used to check out their efficiency for algal oil recovery.

Methods

Culture Condition

The green microalga (*Tetraselmis* sp.) was obtained from Bangladesh Fisheries Research Station, Cox's Bazar and was reared at 30‰ saline water. A stock culture of microalgae (250mL) was prepared in 0.5 L conical flasks containing Guillard medium (12) and reared under continuous illumination with white 40 watt fluorescent bulb (Photosynthetic Photon Flux Density-PPFD ≈30 $\mu molm^{-2}s^{-1}$) for 13 days at 25 ± 1ºC.The initially inoculated algal cell concentration was (20-25)x10^4 cells/mL having log10 value of 5.3-5.4, reared with continuous aeration. The culture was scaled up to 1L and 3L to enhance the microalgal biomass. The growth rate was recordedby regular cell counting by Sedgewick-Rafter (S-R) cell counter.

Culture under different environmental parameters

Different environmental parameters (nitrogen deprivation, phosphorus deprivation and high salinity) were given at 1L stock biomass. For nitrogen and phosphorus deprivation, NaNO3 and NaH2PO4were omitted from the controlled Guillard medium, respectively. Salinity of 35‰ was considered as a higher saline condition. Every treatment was given as triplicate.

Harvesting of cells

On the fifth day of culture period when the algal stocks were in early stage of stationary phase, the culture biomass was flocculated with 200mg/L NaOH followed by filtration with 6 grade whatman filter paper. To remove the residual salt, the algal paste was rinsed using distilled water. Then the harvested stock was dried at 50°C with constant agitations for 48 hours using shaking hot water bath.

Algal cell pretreatment for oil extraction

The controlled culture of algal stock having controlled environmental parameters (e.g. 30‰ saline condition, nutrient rich guillard medium) was used for pretreatment after harvesting and drying. One gram of dried algal biomass was pretreated with an ultrasonicator at ≤50 °C for 40 minutes keeping in cool water bath to remove excess heat. Prior to that, 15 mL of distilled water was mixed with algal biomass. For UV radiation pretreatment 1gm dry weight of *Tetraselmis* sp. was mixed with 1 mL chloroform and 0.5 mL methanol in a glass centrifuge tube and treated at 4.5 W with a frequency of $9.6x10^{14}$Hz and 312 nm wave length for 30 minutes. In addition, a mixture of 1gm algal dried biomass and 15 mL distilled water was subjected to heat treatment using autoclave at 121°C and 103421.36 Pascal pressure for 15 minutes.

Lipid extraction

Total lipids of nutrient stressed and pretreated algal stocks were determined gravimetrically by extracting total lipids with modified Bligh and Dyer (13) method. 1 gm of algal stocks were mixed with 100 mL chloroform: methanol (2:1) ratio, homogenized for 15 minutes and kept into separating funnel. Then 50 mL distilled water was added and kept the mixture for 12 hours. Finally, the bottom layer containing lipid was transferred into fresh previously weighed beaker and placed it in the shaking hot water bath at 55 °C with 50 RPM for 10 hours in order to remove the solvent completely. After evaporation, the quantity of lipid was measured by an electric balance. The total lipid fraction was measured using the difference among final and initial flask weights. The lipid concentration was calculated with the following formula as dry weight ratio of extracted lipids to biomass (%w/w):

$$\text{Extracted lipid yield} = \frac{\text{Weight of extracted lipid}}{\text{Weight of algal biomass}} \text{ x } 100$$

The extracted lipid from unstressed and untreated algal biomass was considered as control for comparing lipid yield variation at different nutrient stress and pretreatment method.

Statistical Analysis

All types of statistical analysis were carried out with statistical software MINITAB, Version 17 (14). One-way ANOVA test for growth and lipid contents were done after confirming the normality and homogeneity of the data. All the growth cell count data were transformed into log data to perform the normality test. All the data sets were normally distributed. Tests for normality of the data were done according to Shapiro and Wilk (15). Test for homogeneity of variances was done based on Levene (16). Post-hoc analysis of the means were accomplished with Tukey's HSD (Honest Significant Difference) test (17). To compare each treatment with only the control, Dunnett's multiple comparison test was used (18).

Results and Discussion

Growth aspect of *Tetraselmis* sp. under different environmental conditions

The growth performance of microalga *Tetraselmis* sp. over the culture period is shown in Figure 1. The growth rate of *Tetraselmis* sp. has been expressed in log value of cell counts. While the first day counts resembled a 'lag phase' growth, from the second day onwards, there was a gradual increase of cells up to the fourth day and started to reduce on the fifth day. For nitrogen deprivation, the algal growth was increased up to the third day and reduced significantly ($F_{3,11}$= -9.78, P<0.05) from the fourth day onwards. Nitrogen salts are important inorganic sources for cell growth and metabolism. But again, the most favorable conditions for a concentration of nitrogen differ from species to species (19). However, deficiency in nitrogen inhibits algal growth significantly. This significant growth resulted from absorption of the inorganic nitrogen by algae which was immediately assimilated into biochemically active compounds

and subsequently recycled within cells in order to fulfill physiological needs (20). A Decreased yield of biomass under nitrogen deprivation in *Tetraselmis* sp. and *Nanochloropsis* sp. has also been reported by Alsull and Omar (21). Again in phosphorus deprivation the algal growth was also increased up to the third day and reduced significantly ($F_{3,11}$= -7.39, P<0.05) from the fourth day onwards. As essential nutrient phosphorus constitutes 1% of the dry weight of algae and its deprivation causes lower algal growth by reduction in the Calvin-Benson cycle substrates which consequently lowers the light utilization rate required for carbon fixation (22-24). Phosphorus limitation influencing lower steady-state growth rates have also been found in *Selenastrum minutum* (25). On the other hand, higher saline condition with 35‰ saline water caused a gradual increase ($F_{3,11}$= 9.99 P<0.05) in the growth of the algal stock having the peak at the fifth day. Fakhri et al. (26) showed higher specific growth rate with the increased salinity having an optimum level of 15‰ for *Tetraselmis* sp. Adenan et al. (27) reported that increasing salinity from 20‰ to 25‰ caused a significant increase in growth rate of *Chlorella* sp.

Lipid content under different environmental parameters

Lipid contents varied significantly ($F_{3,14}$= 88.49, P<0.05) when *Tetraselmis* sp. were subjected to nitrogen and phosphorous deprivation as well as increased salinity in Gulliard nutrient media as compared to standard control. The dry weight percentage of lipid content from *Tetraselmis* sp. under various experimental conditions is presented in Figure 2. *Tetraselmis* enriched with 30‰ saline water Guillard media in the presence of phosphorous and nitrogen was found yielded 11.74% lipid, but lipid contents significantly increased in nitrogen and phosphorus deprivation and in 35‰ saline condition as compared to the control. In this study, nitrogen deprivation yielded 42.83% ($F_{3,14}$= 15.44, P<0.05) lipid content. A rise in biosynthesis and accumulation of lipids has been found in algal culture due to the effect of nitrogen deficiency (20, 28). Furthermore, the cellular enzymatic balance responsible for lipid synthesis is largely altered in the nitrogen deprivation (29). Similarly, phosphorus, an important component of algal cells, leads to accumulation of lipids when it is being deprived from the culture medium by limiting light utilization required for carbon fixation. In this study, phosphorus deprivation yielded 24% ($F_{3,14}$= 6.09, P<0.05) lipid content. Working with *Monoraphidium* species, Dhup et al. (30) reported that the variation of different phosphorous concentrations in media improved the lipid content. Rodolfi et al. (31) also observed higher level of lipid induced by deprivation of nitrogen and phosphorus on several green algae, diatoms, red algae, eustimatophytes and prymnesiophytes. Working with *Scenedesmus* sp. Xin et al. (32) reported that limitation of phosphorus and nitrogen caused increase in lipids as high as 53% and 30%, respectively.

However, higher saline condition contributed 29.33% ($F_{3,14}$= 8.74, P<0.05) lipid yield in dry weight basis. The outcome of this present study having higher lipid content at high salinity corroborates some other studies (33-36). Enhancing NaCl concentration (0.4 to 4 M) in marine microalga *Dunaliella* resulted an increase in monounsaturated and saturated fatty acids (37). In another study with *Dunaliella tertiolecta*, intracellular lipid enrichment (60% to 67%) and rise in triglyceride concentration (40% to 56%) were achieved with NaCl concentration enhancement from 0.5 (freshwater concentration) to 1.0 M (38).

Lipid recovery under different pretreatment

Various pretreatment in the extraction method revealed a key role in the effective oil recovery from the algal stock. The total amount of lipid extracted from *Tetraselmis* sp. was considered an indication of the effectiveness of various methods of cell disruption used in the study. A significant difference was found ($F_{3,14}$= 159.72, P<0.05) among the studied techniques proving the ability of disrupting algal cell layer in varied way for efficient oil recovery as shown in the Figure 3. In the normal extraction method having no pretreatment contributed about 11.74% lipid yield. Thermal pretreatment carried out with autoclave in 121°C and 103421.36 pascal pressure contributed the highest lipid yield as 45.67% ($F_{3,14}$= 19.61, P<0.05).Temperature plays a major role in extracting the oil from the cell. In high temperature and moderate pressure the algal cells are denatured effectively due to the increase in the dissolution

capacity of the solvent system (39). Lee et al. (40) reported a maximum (7.9%) oil recovery from *Chlorella vulgaris* using autoclaving. Again autoclaving was found as the most efficient method of cell disruption yielding 7.88% of oil from *Ulva lactuca* (39).

Whereas, the ultrasound pretreatment showed less cell disruption effect contributing 27.33% ($F_{3,14}$= 9.01, P<0.05)lipid yield in dry weight. Ultrasonication is a physical method for disrupting algal cells. The intense sonication of liquid generates sound waves resulting alternate high-pressure and low-pressure cycles. During capitation the high pressure and high speed liquid jets generate shearing forces around the algae cells mechanically break the cell structure (11). Surendhiran and Vijay (41) found maximum oil recovery of 30.12% from *Nanochloropsis oculata* cells grown in normal nutrition condition. Ultrasonic extraction has resulted in a higher extraction output of approximately 2.25 times that of direct oil extraction from *Ulva lactuca* (39). In contrast, Lee et al. (40) found a least efficiency in *Botryococcus* sp. by ultrasonication. Similarly, ultraviolet pretreatment yielded 36% ($F_{3,14}$= 14.02, P<0.05) lipid yield in dry weight basis. As UV-C light carries more energy per photon (42), it has been used as a stress to break the cell layer. In addition, study on nitrogen deprivation in *Dunaliella tertiolecta* noted increased cell size following lipid accumulation by the UV ray exposure indicating a cell bursting phenomenon for lipid recovery (1). Similar phenomenon has been found in *Chlorella* sp. BR2 and *Tetraselmis* sp. M8 reported by Sharma (43).

However, a significant difference ($F_{2,14}$= 198.461, P<0.05) was found in the lipid content of normal and thermal pretreatment extraction method from control and nitrogen deprived algal stock as shown in figure 4. Similarly, lipid content was varied significantly ($F_{1,4}$= 68.083, P<0.05) in nitrogen deprived algal stock of normal and thermal pretreatment extraction method yielding 69.73% lipid from thermal pretreated nitrogen deprived algal stock. From the above discussion it is evident that the nitrogen deprivation causes highest lipid accumulation in *Tetraselmis* sp. In addition, thermal or autoclave pretreatment showed highest oil recovery from the algal cells. However, in case of growth determination, the salinity stress gave highest growth rate in response to 35 ‰ salinity stress. So, it is necessary to make a positive coordination among the growth of microalgae, stress-induced lipid production and its extraction through potential and effective pretreatment for the successful biofuel production.

Conclusion

This paper demonstrated the most effective way to induce lipid in microalgal cell. Furthermore, it depicted the most efficient way of oil recovery from the algal cell through disruption. Higher Saline condition induced highest algal biomass whereas nitrogen starvation caused highest lipid accumulation. In case of thermal pretreatment, maximum percentage of lipid was recovered. Furthermore, nitrogen deprivation in combination with thermal pretreatment gave highest lipid yield. These findings will be useful for maximizing lipid production in industrial level through cellular level biomass and lipid induction along with successful oil recovery. New insights could be focused on the combined use of nutrient stress supplemented by various pretreatment simultaneously for the successful biofuel production in the industrial level considering the balance of the cost.

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Figure 1. Growth aspect of *Tetraselmis* sp. under different environmental parameters in five day culture period. (Means that do not share a letter are significantly different). Nitrogen: culture grown under nitrogen starvation; Phosphorus:culture grown under phosphorus starvation; Salinity: culture grown under higher saline water (e.g: 35‰); Control: culture grown under 30‰ saline medium along with nitrogen and phosphorus enriched water.

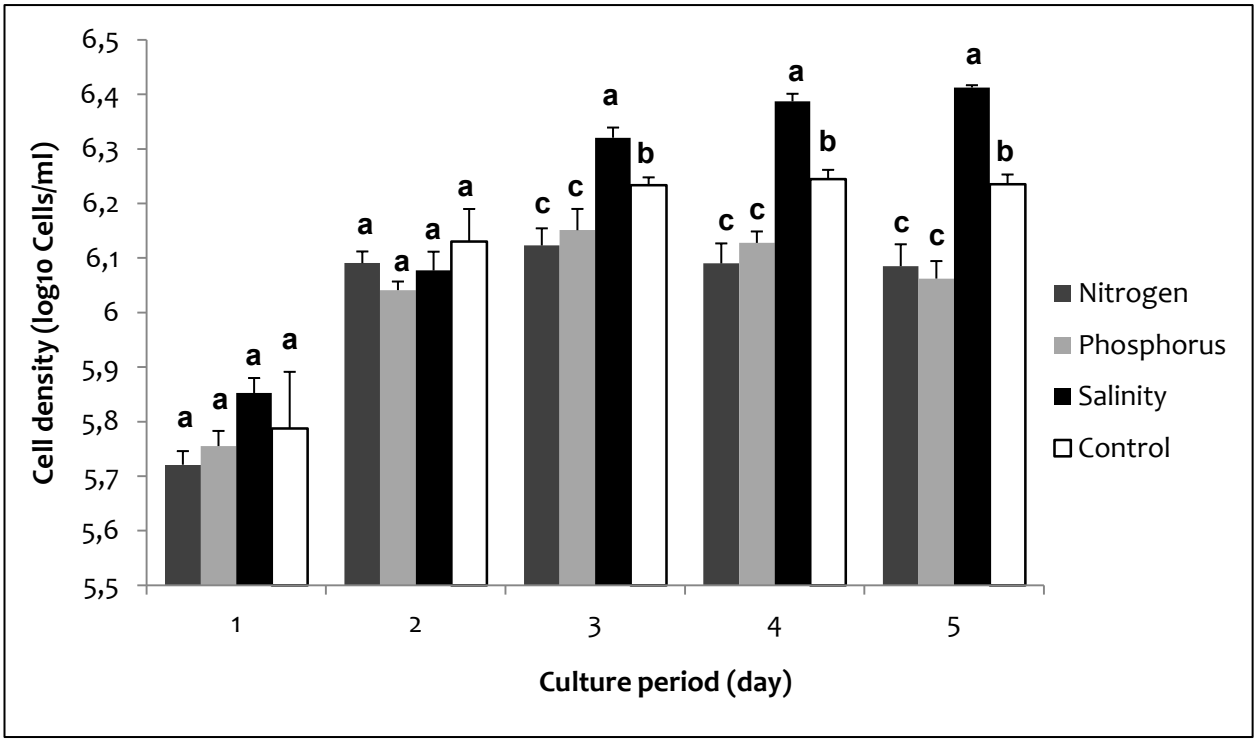

Figure 2. Lipid content under different environmental parameters. Means that do not share a letter are significantly different.

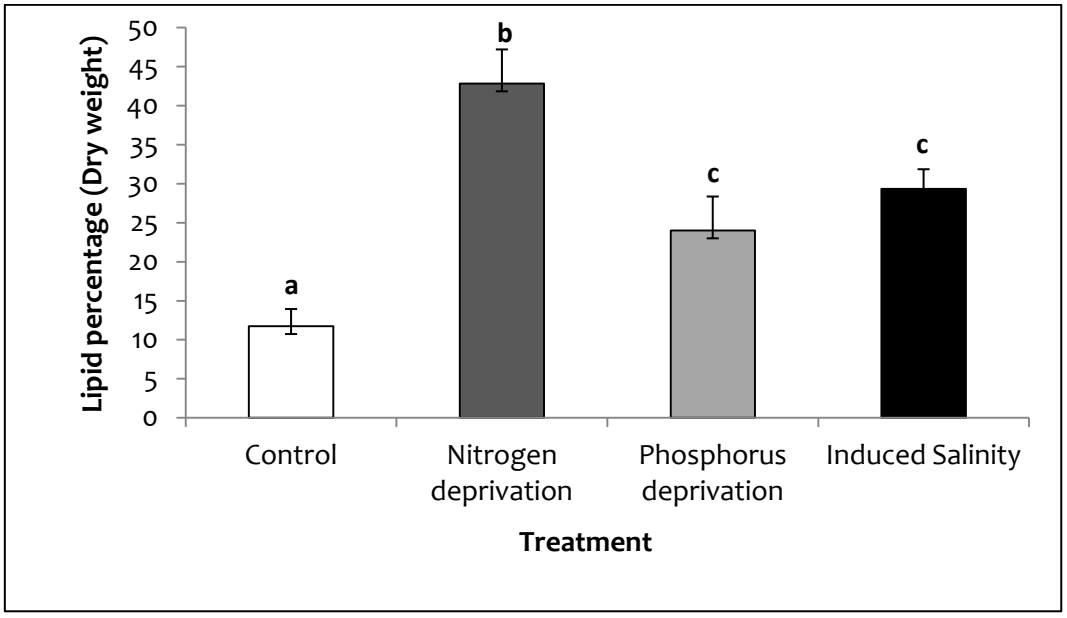

Figure 3. Lipid content recovered from different pretreatment. Means that do not share a letter are significantly different.

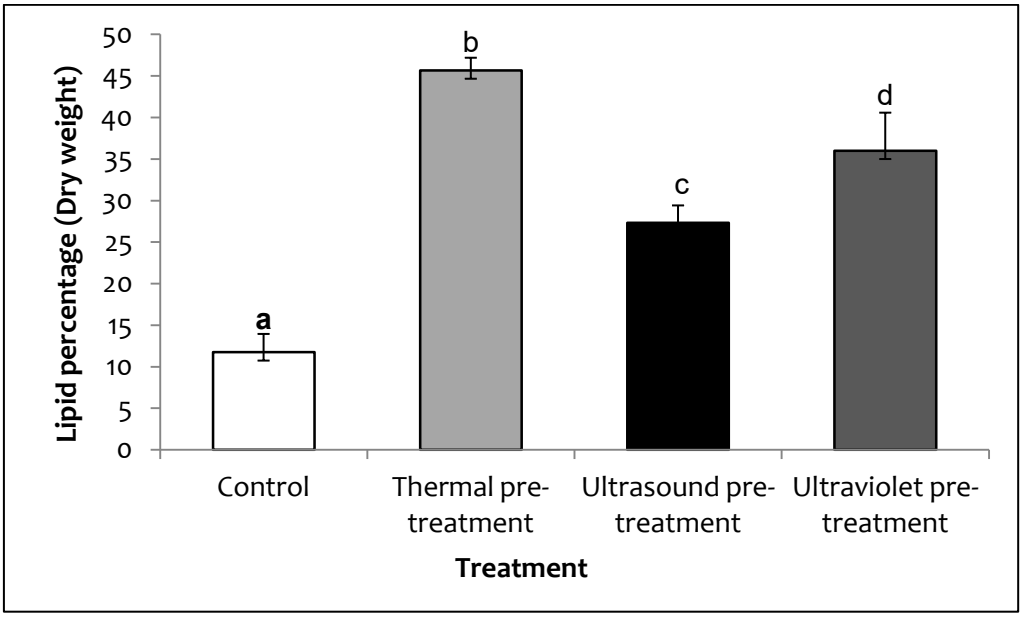

Figure 4. Lipid content of normal and thermal pretreatment extraction method from control and nitrogen deprived algal stock. Means that do not share a letter are significantly different.