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The Effect of Nitrogen Deficiency on the Growth and Lipid Content of *Isochrysis affinis galbana* in Two Photobioreactor Systems (PBR): Tubular and Flat Panel

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ABSTRACT
Energy is becoming one of the most expensive production inputs nowadays. Energy reserves are starting to run out and their polluting nature has become undeniable. Therefore, there is an urgent necessity for renewable energies. One of these energy sources is algae, which are seen as promising for biofuel production. Algae can be cultured in non-agricultural land, high photosynthetic activity, harvested throughout the year high biomass production. High lipid from algae is possible by reducing some elements of growth conditions from the nutrient medium. In this study, *Isochrysis affinis galbana* species were cultured in two reactors; flat panel photobioreactors with different light paths (1, 3, 5, 7 and 10 cm) and tubular photobioreactors, with 50% nitrogen reduction and 20% inoculation densities. Biomass, lipid and protein ratios were determined. The highest lipid content of 33.13% was obtained from *I. aff. galbana* with 12.11% protein in flat panel photobioreactors with 50% nitrogen reduction and 10 cm light path, and a 0.991 g L^{-1} biomass rate was obtained. The highest optical density was found in the 10 cm light path flat panel photobioreactor with a 50% nitrogen reduction.

Keywords: *Isochrysis affinis galbana;* Photobioreactor; Lipid; N deficiency

1. Introduction

This study was conducted in order to determine the effects of N restriction on growth, lipid, protein, and chlorophyll contents in *Isochrysis affinis galbana* species of Prymnesiophyceae class and to produce renewable, non-toxic biofuels from microalgae.

Increasing interest in microalgae biotechnology in recent years is due to their high amounts of valuable bioactive metabolites (Becker 1994). In recent years, fat and fatty acid products obtained from single-celled algae have attracted considerable attention. Initially, the 'Solar Energy Research Institute' focused on the use of algal lipids as biofuels (Neenan et al 1986). Microalgae are potential sources of biodiesel with a lipid content of 20% to 50, even 80% (Chisti 2007). Efforts are underway to exploit the use of renewable, non-toxic, biodiesel fuel from microalgae as an energy source. For this purpose, in addition to the identification of microalgae species with high lipid content and growth rate, studies on determination of serious stress conditions such as N limitation, P deficiency, Si deprivation, high salinity etc. have begun (Lynn et al 2000; Zhila et al 2005; Mandal & Mallick 2009; Zhila et al 2011). Those stress conditions stimulate the increase of lipid content in the cell are being evaluated in many countries.

The purpose of the present study was to investigate the effect of N on the growth and lipid content of *I. aff. galbana* in two photobioreactor systems (PBR): tubular and flat panel. In addition, we investigated the maximum lipid and

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biomass in order to determine which PBRs are more suitable for culture of *I. aff. galbana* for biofuels production purpose.

2. Material and Methods

2.1. Algae and culture conditions

The microalgae *I. aff. galbana* UTEX LB 2307, was supplied by the University of Texas at Austin Collection, which is a single-celled marine species with two whips, with no haptonema and a single yellow-brown chloroplast. The cell size is 4-8 μm (Hoff 1987). The inoculum for the tubular and flat panel PBRs were grown under laboratory conditions on F/2 medium (Guillard et al 1973). Cells were cultured under a constant light intensity of 80 μmol photonm^{-2} s^{-1} at 20 °C were used. The irradiation was measured using the Radiation Sensor LI-COR (LI-250, Inc. USA). The microalgae stock culture was cultured in an 8 L glass jar and air was continuously supplied. The volume of the tubular photobioreactor system is 110 L. The tubular photobioreactor system was a horizontally installed reactor made of transparent acrylic tubes with an inner diameter of 2.6 cm. In order to keep the craw flow rate constant, a flow rate of the circulation pump was set to 0.3 m s^{-1}. A collection tank of about 150 L was built for the culture collection chamber. CO_2 gas inlet flowmeter was provided. pH and temperature were measured continuously by probes. Outdoor flat panel PBRs were 10 mm thick transparent glass material, 50.0 cm wide and 50.0 cm high with 1, 3, 5, 7 and 10 cm light paths (Hu et al 1996a) in batch systems. The volumes of the PBR (without bubbling gas) was 2 L in 1 cm, 6 L in 3 cm, 10 L in 5 cm, 15 L in 7 cm and 21 L in 10 cm. The culture mixture was provided with 2% CO_2 enriched air as described by Hu et al (1996b). pH was arranged with a pH controller as 7 and light intensity was measured 3 times a day.

In the experiment, *I. aff. galbana* was cultured in different flat panel and tubular PBRs. Experimental work is carried out on 50% N deficiency according to F/2 medium and with inoculation densities of 20%. All the applications were made in three replicates. The experiments were completed on different days and indicated in the tables.

2.2. Analytical methods

Chlorophyll *a*, total carotenoids, dry weight (biomass) and optical density (OD) analysis were performed daily. The dry weight was determined according to Hu & Richmond (1994). The *I. aff. galbana* cell concentration was determined daily by measuring the optical density at 680 nm (Lin et al 2007), by a UV-visible spectrophotometer (Shimadzu, UV mini, 1240 model, Japan). Chlorophyll *a* and total carotenoids contents were determined on a spectrophotometer at 665, 645, 630 and 480 nm as described by Parsons & Strickland (1963). All measurements were made in three replicates.

Microalgae were harvested for lipid and protein analysis in the stationary growth phase. *I. aff. galbana* cells were separated by centrifugation at 7500 rpm for 10 min, using the centrifuge model of Hereaus Supragufe 22. However, the biomass was dried at 55 °C for 2 hours, triturated with a grinder and then stored at -20 °C for analysis. Lipid extraction from microalgae cells was performed by the method described by Bligh & Dyer (1959). The total protein was calculated by the determination of N content (Nx6.25) according to Kjeldahl method (AOAC 1995).

2.3. Statistical analysis

The data were subjected to a one-way analysis of variance and Duncan's multiple range test was used as a post-hoc test. Statistical Package for the Social Sciences (SPSS) (Version 12.0, SPSS, Chicago, IL) (Zar 1999) was adapted to a personal computer. The differences were considered at a significance level of $\alpha = 0.05$.

3. Results and Discussion

The measured temperatures were between 21.7 ºC and 24.7 ºC and light intensities were recorded between 224 μmol photonm^{-2} s^{-1} and 284 μmol photonm^{-2} s^{-1} in culture at different light path lengths in flat PBR systems. In tubular photobioreactor systems, the temperature was determined between 21.6 ºC and 22.3 ºC and the light intensity between 281 μmol photonm^{-2} s^{-1} and 286 μmol photonm^{-2} s^{-1}.

The biomass, protein, and lipid contents of *I. aff. galbana* were presented in Table 1 and Table 2. In the control group and N deficiency group, the highest biomass was determined as 1.068 and 0.991 g L^{-1}, respectively, in flat panel PBR with 10 cm light path. The highest amount of lipid was detected in the flat panel PBR with 10 cm light path where the highest biomass was obtained. The highest lipid in the control group was 15.19%, while the N deficiency group was

33.13%. The lowest amount of protein was obtained with 12.11% in the flat panel PBR with 10 cm light path where the highest lipid was obtained. The protein and total lipid ratios were similar in all control groups (P>0.05). In N deficient cultures, the lowest biomass and lowest lipid amount was obtained in the flat panel PBR with 1 cm and 3 cm light path.

Table 1- Main parameters of biomass, lipid and protein content of *I. aff. galbana* at control groups in flat panel PBRs and tubular PBR

Reactors	*Biomass (g L^{-1})*	*Protein (%)*	*Total lipid (%)*
Tubular PBR (16 days)	0.902±0.02^{c}	28.65±10^{a}	15.09±0.70^{a}
FP-PBRs 1 cm light path (15 days)	0.801±0.02^{d}	28.67±0.60^{a}	15.31±0.30^{a}
FP-PBRs 3 cm light path (16 days)	0.813±0.03^{d}	28.80±0.50^{a}	14.98±0.50^{a}
FP-PBRs 5 cm light path (16 days)	0.893±0.02^{c}	28.83±0.20^{a}	15.07±0.60^{a}
FP-PBRs 7 cm light path (18 days)	0.958±0.01^{b}	28.65±0.60^{a}	15.12±0.60^{a}
FP-PBRs 10 cm light path (19 days)	1.068±0.02^{a}	28.80±0.50^{a}	15.19±0.70^{a}

Means values, n= 3, different letters between the lines indicate significant difference at 5% by Duncan multiple range test

Table 2- Main parameters of biomass, lipid and protein content of *I. aff. galbana* at N deficiency groups in FP-PBRs and tubular PBR

Reactors	*Biomass (g L^{-1})*	*Protein (%)*	*Total lipid (%)*
Tubular PBR (12 days)	0.875±0.03^{bc}	13.50±0.70^{b}	32.10±0.40^{b}
FP-PBRs 1 cm light path (11 days)	0.772±0.01^{d}	15.05±0.50^{a}	30.01±0.10^{c}
FP-PBRs 3 cm light path (13 days)	0.785±0.01^{d}	15.01±0.40^{a}	30.16±0.30^{c}
FP-PBRs 5 cm light path (13 days)	0.832±0.02^{c}	13.07±0.20^{b}	32.09±0.80^{b}
FP-PBRs 7 cm light path (14 days)	0.913±0.01^{b}	13.02±0.40^{b}	32.18±0.50^{ab}
FP-PBRs 10 cm light path (15 days)	0.991±0.02^{a}	12.11±0.30^{c}	33.13±0.80^{a}

Means values, n= 3; different letters between the lines indicate significant difference at 5% by Duncan multiple range test.

Optical density, chl *a* and total carotenoid amounts in the control and N deficiency groups were summarized in Table 3 and Table 4.

Table 3- Main parameters of optical density, chl *a* and total carotenoid amounts of *I. aff. galbana* at control groups in flat panel PBRs and tubular PBR

Reactors	*OD*	*Chl a (μg L^{-1})*	*Total carotenoid (μg L^{-1})*
Tubular PBR (16 days)	0.487±0.001^{c}	489±2^{a}	0.626±0.001^{a}
FP-PBRs 1 cm light path (15 days)	0.457±0.010^{d}	358±5^{c}	0.408±0.002^{d}
FP-PBRs 3 cm light path (16 days)	0.497±0.020^{c}	361±2^{c}	0.419±0.001^{d}
FP-PBRs 5 cm light path (16 days)	0.568±0.001^{b}	422±6^{b}	0.459±0.002^{b}
FP-PBRs 7 cm light path (18 days)	0.625±0.002^{a}	484±9^{a}	0.438±0.001^{c}
FP-PBRs 10 cm light path (19 days)	0.588±0.010^{b}	501±10^{a}	0.467±0.001^{b}

Means values, n= 3; different letters between the lines indicate significant difference at 5% by Duncan multiple range test.

Table 4- Main parameters of optical density, chl *a* and total carotenoid amounts of *I. aff. galbana* at N deficiency groups in flat panel PBRs and tubular PBR

Reactors	*OD*	*Chl a (μg L^{-1})*	*Total carotenoid (μg L^{-1})*
Tubular PBR (12 days)	0.398±0.002^{c}	180±3^{a}	0.810±0.001^{a}
FP-PBRs 1 cm light path (11 days)	0.301±0.001^{d}	130±1^{b}	0.479±0.002^{c}
FP-PBRs 3 cm light path (13 days)	0.437±0.003^{b}	130±1^{b}	0.476±0.001^{c}
FP-PBRs 5 cm light path (13 days)	0.441±0.001^{b}	172±1^{a}	0.543±0.002^{b}
FP-PBRs 7 cm light path (14 days)	0.453±0.001^{a}	130±2^{b}	0.534±0.001^{b}
FP-PBRs 10 cm light path (15 days)	0.458±0.001^{a}	125±1^{b}	0.546±0.001^{b}

Means values, n= 3; different letters between the lines indicate significant difference at 5% by Duncan multiple range test

Initial optical density values were 0.263 for all groups. At the end of the experiment, the highest optical density value was be 0.625 in the flat panel PBR with 7 cm light path in the control group. The highest optical density was 0.458±0.001 in the flat panel PBR with 10 cm light path in the N deficiency group. The minimum optical density was determined in the flat panel PBR with a 1 cm light path as 0.457±0.01 and 0.301±0.001 the control group and N deficiency, respectively.

The initial chl *a* and total carotenoid were determined as 245±2 µg L^{-1} and 0.242±0.001 µg L^{-1} in the control group and in the N deficiency group, respectively. At the end of the experiment, the lowest chl *a* was detected in the N deficient groups in flat panel PBRs with 1, 3, 7 and 10 cm light path. But the highest total carotene was measured in the tubular PBR with 0.810 µg L^{-1} in the N deficient group. The lowest carotene in the group with N deficiency was determined in the flat panel PBR with 1 cm light path.

The primary objective of producing phototrophic organisms is to provide a continuous culture with optimal cell density. Algae requires that cultured cells constantly react to these conditions, as the various environmental factors during the cultivation of an algae show great changes both daily and seasonally. The biochemical composition of algal biomass is affected by environmental factors. The most important of these are growth conditions such as light, temperature, nutrient medium, salinity and pH (Brown et al 1989; Roessler 1990; Sukenik 1991; Cohen et al 1988; Lourenço et al 2002). Nutrient elements and their concentrations used in culture media with physical conditions may cause changes in microalgae growth and their biochemical structure. Growth affects concentrates as well as the nutrient content used in media (Brown et al 1989). Nitrogen (N) limitation has been shown to cause changes in the biochemical structure of many algal groups. Especially the increase in the amount of lipids (Illman et al 2000). For this purpose, in this study, N concentration in F/2 medium was reduced to fifty percent. It is known that different N levels can affect the biochemical composition and growth of microalgae (Fidalgo et al 1995; Xu et al 2001).

Microalgal biomass is very important for the study of lipid, and lipid is the main objective of increasing both simultaneously biomass. In this study, the reduction in the amount of N in the medium caused an increase in the amount of lipids in the cell and a decrease in the amount of protein. The highest biomass in the study was determined to be 0.991 g^{-1} and the highest lipid was 33.13% with a 50% N deficiency in the flat panel PBR with 10 cm light path. However, the lowest amount of protein was found in this group. In another study, it was determined that low N concentration decreased the growth rate in *N. oculata* and did not affect growth rate in *C. vulgaris* (Converti et al 2009). In a research conducted by Xu et al (2001), *Ellipsoidion* sp. was cultured in different N sources and in a N free medium, the growth in the N free medium was low. Adenan et al (2016) reported that lipid ratio is increased in *Chlorella* and *Chaetoceros* species cultured with N deficiency applied to culture, but growth with protein and carbohydrate amounts decreases. The amount of biomass obtained due to the lipid content of the algae is also important. Biomass is generally reduced in cultures where N deficiency is applied. Thomas et al (1984) reported that *P. tricornutum* cultured in medium containing N and in the medium where N deficiencies were applied, and that the biomass amount was low in the nutrient medium where N deficiency was applied. Similar studies have suggested that the N restriction is responsible for the decrease in cell density and the decrease in biomass quantities in species (Kilham et al 1997; Pruvost et al 2009). In this study, the lowest biomass amount in *I. aff. galbana* was determined as 0.772 g L^{-1} in the flat panel PBR with 1 cm light pathway, in the 50% N (-) group with 30.01% lipid. N and P are the most essential elements for cell growth. These two elements are involved in the synthesis of intracellular structure. Therefore, cell growth instead of lipid synthesis will be dominant when these nutrients are present. Nitrogen deficiency limits algal growth and protein synthesis, resulting in increased lipid content (Converti et al 2009). In similar studies it was reported that the N limitation caused increases lipid; *D. tertiolecta* contained maximum total lipid in low N containing medium conditions (Fábregas et al 1989); *P. tricornutum* accumulates a high amount of total lipid in N deficiency (Thomas et al 1984); when the amount of N in *I. galbana* was increased from 0.04 mmol L^{-1} to 0.7 mmol L^{-1}, the total lipid content decreased from 22% to 16.9% (Utting 1985). Tornabene et al (1983) reported that freshwater algae *N. oloeabundas* was cultured in growth medium with N deficiency at different rates, and the total fat percentage in N deficient groups varied between 35-54% by dry weight. Ben-Amotz et al (1984) reported that different algal species were cultured in a nutrient-deprived medium and showed significant increases in total lipid content in all species. Sukenik & Wahnon (1991) reported that when *I. aff. galbana* was cultured in a N deprived medium, both carbohydrate and total lipid ratios increase. Zhila et al (2005) reported that *B. braunii* was cultured in 75% N reduced medium and reported a 21% increase in total lipid. Weldy & Huesemann (2007) reported that *D. salina* was cultured in a photobioreactor system with N deficiency and observed the change in total lipid ratio, which increased from16% at the beginning of the stagnation phase to 44%. In the study conducted by Rodolfi et al (2009), studied the dry matter and total lipid ratio of 4 species (2 marine and 2 freshwater species) by culturing them in a 20 L flat alveolar panel reactor with N deficiency. In the marine species, *Nannochloropsis* sp. a total lipid content of 60% was determined in N deprived medium. Gouveia et al (2009) found

that the maximum total lipid ratio in *N. oleabundans* species was 56% when N restriction was applied for 6 days. Damiani Cecilia et al (2010) reported that when they cultured *H. pluvialis* under different stress conditions (high light and high light-nitrogen deficiency), the total lipid ratio increased from 15% to 32.99% in N deficiency and high light intensity. Bulut Mutlu et al (2011), in their study, cultured *C. vulgaris* for five in different nutrient media under laboratory conditions, they found that the highest total lipid content was 35.6% in the group with 100% N excision. In a study conducted by Uslu et al (2011), *S. platensis* increased total lipid (17.05%) when cultured in a 100% N starvation medium. Uslu et al (2013), also investigated *C. vulgaris* in tubular photobioreactors in a 50% reduced N medium; lipid ratios were 12.34% and 38.16% for the control and 50% N limitation groups, respectively. In another research Uslu et al (2014) cultivated *P. tricornutum* with different light path lengths of 1, 3, 5, 7 and 10 cm and with a deficiency of 50% N in order to determine lipid, protein and biomass contents. The highest lipid, protein and biomass of *P. tricornutum* was 34.6%, 8.50% and 1.064 g L^{-1}, respectively, in the flat panel PBR system with 7 cm light path. Ak et al (2015) studied *P. tricornutum* tubular reactor systems and in medium containing 50% N, and found 35.04% lipid, 0.980±0.02 g L^{-1} biomass and 8.87% protein ratios. whereas 16.93% lipid, 1.036±0.025 g L^{-1} biomass and 31.05% protein were detected in the control group. Kamalanathan et al (2016) observed physiological changes on *Chlamydomonas* cultured in N and phosphorus (P) deficiency. They have reported that the physiology of *Chlamydomonas reinhardtii*, with N starvation and also with N plus P starvation combined, shows a deeper influence on the P starvation. At the same time, the photosynthetic performance of *C. reinhardtii* showed major changes under N starvation but was comparatively unchanged by P starvation. The lipid concentration per cell was at least 2.4 times higher in the N deficient groups than in the control group, however, the amount of protein is lower in groups with N deficiency. In general, N deficiency has a more dramatic effect on *C. reinhardtii*'s physiology and lipids and protein levels than P deficiency.

In this study, similarly, the N limitation reduces the growth rate, which increases the cellular lipid rate. However, the amounts of biomass were not very low in groups with N deficiency. What is important is that the biomass ratio can be obtained at reasonable levels so that the increased lipid content can be economically assessed.

The algae biomass is affected by many parameters including the nutrient medium used for the culture of algae, the surface area and material of the system used, and the path taken by the light in the water column. Light is an important parameter especially in algal cultures. The angle at which the photobioreactor receives the light is important, in fact the surface area of the material plays a significant role in the efficiency to get enough light for the algal culture. According to Zijffers et al (2008), it is important for the reactor to take sunlight in the most efficient way. They generally recommend the use of flat and rectangular systems or linear and cylindrical shaped systems for good light energy distribution. Linear and cylindrical systems can be effective in focalizing light (Richmond 1986; Zou & Richmond 1999; Durmaz & Erbil 2017). In the study of Durmaz (2000), he investigated the growth of *Chlorella* sp. in natural light/dark periods outside the laboratory in reactors with different light path lengths (10 cm, 15 cm and 20 cm). *Chlorella* sp. reached the highest concentration of cells density of $49.5x10^6$ mL^{-1} in the photobioreactors with 20 cm light path. While, the highest cells density was $49x10^6$ cells mL^{-1} in the panels with 15 cm and $36.5x10^6$ cells mL^{-1} in the panels of 10 cm. In his study, it was stated that the length of light path should be adjusted according to the type of culture. It was determined that panels with 15 cm and 20 cm light paths have a high specific growth rate. It is emphasized that the sunlight intensity and duration is important in microalgae culture which is made in glass panels with different light path lengths and it is stated that the appropriate light path length for the species is important for efficiency. In this study, it was also determined that the growth increased as light path length increased.

In a study investigating the effects of N restriction on metabolites in microalgae cultures, an increase in the proportion of organic carbon compounds such as lipid was observed, while a decrease in cell number and chl *a* was detected. However, yellowing of colors was observed in cultures due to increased carotene content (Shifrin & Chisholm 1981; Sukenik et al 1989). Marín et al (1998) investigated the effects of *D. salina* on different N rates on carotene and chl *a* amount. As the N deficiency increased, total carotene and chl *a* decreased. In this study, chlorophyll content decreased in 50% N (-) cultures and increases in total carotene content occurred in *I. aff. galbana*. The highest OD values in *I. aff. galbana* was obtained in the control group and in the panel system with 7 cm light path. The lowest OD value was obtained in the group of 50% N (-) in the tubular photobioreactor system in *I. aff. galbana*. This provides us with the result that the optical density is lower in the N deficiency groups, as in other parameters.

4. Conclusions

As a result, microalgae biomass can be used as an alternative source for the production of renewable energy. The studies on microalgae lipid production are mostly carried out under laboratory conditions. It is important to ensure the commercial production of microalgae. However, it is also significant to reduce the cost of culture to produce lipids.

Finally, the results obtained in this study have shown that the lipid content of *I. aff. galbana* may be a good source of biodiesel. It would be more appropriate to use algae which can be cultured in nonagricultural lands for year-round instead of production of oil crops in agricultural lands as energy source.

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