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Full Length Research Paper

The effects of nitrogen and phosphorus deficiencies and nitrite addition on the lipid content of *Chlorella vulgaris* (Chlorophyceae)

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The effect of 50% N, 100% N, 50% N plus 50% P and 50% P deficiencies and nitrite addition were treated on *Chlorella vulgaris* (Chlorophyceae) was studied in laboratory conditions with the aim to determine the effects of the deficient nutrient and different nitrogen sources on lipid and protein contents. Protein and lipid values of the biomass were found as 50.8 and 12.29% for the control group, 20.3 and 17.5% for 50% N(-), 13.01 and 35.6% for 100% N(-), 21.37 and 20.5% for 50% N(-) and 50% P(-), 38.16 and 16.7% for 50% P(-) and 41.03 and 13.04% for the nitrite group that was added. The highest lipid content was recorded with the culture to which 100% N(-) was treated with 0.18 g/L dry-weight.

Key words: *Chlorella vulgaris*, lipid, nitrogen and phosphorus deficiencies, nitrite.

INTRODUCTION

Microalgae (photosynthetic microorganisms) have the ability to use solar energy in combining water with carbon dioxide to create biomass. In recent years, a study on microalgal lipid for biodiesel sources has been done in many countries. Microalgae, which can be cultured throughout the year, have a simple reproducing system, use water most effectively and do not need rich soil for growth. Beginning in the late 1940's, *Chlorella* was investigated for its possible wide-scale production and could be used for nutritional purposes, such as a source of protein, lipids, carbohydrates, vitamins, and minerals to help fill the "protein gap" and feed an ever expanding world population (Becker, 2007).

Hundreds of microalgal strains capable of producing high content of lipid have been screened and their lipid production metabolisms have been characterized and reported (Sheehan et al., 1998). Several studies have shown that the quantity and quality of lipids within the cell can vary as a result of changes in growth conditions, such as temperature and light intensity, or nutrient media characteristics, concentration of nitrogen, phosphates and iron (Illman et al., 2000; Liu et al., 2008). However, the aim of this study is to compare the lipid production of *Chlorella vulgaris* at different nutrient compositions.

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MATERIALS AND METHODS

Algae material and culture conditions

Chlorella species are eukaryotic, unicellular and non-motile freshwater green algae that belong to the 'Chlorophyta division' (Kay, 1991). *Chlorella* cells have hemicellulotic cell walls and are spherical with a diameter ranging from 2 to 10 µm (Kay, 1991; Becker, 2007). Microalga *C. vulgaris* cultures were kept at a constant room temperature of 22 ± 2°C and illuminated with fluorescent lamps (PHILIPS TLM 40W/54RS) at an irradiance level of 80 µmol/m^2/s with photoperiod 16:8 (L:D). As such, the irradiance was measured by a radiation sensor LI-COR (LI-250). The microalgae were grown in 8 L glass jar in a batch culture system and the culture was continuously stirred by air.

The cultures were grown in Jaworsky medium and the content of the medium consists of the following composition (g/200 ml): 4 $Ca(NO_3)_2.4H_2O$, 2.48 KH_2PO_4, 10 $MgSO_4.7H_2O$, 3.18 $NaHCO_3$, 0.45 EDTA FeNa, 0.45 EDTA Na_2, 0.496 H_3BO_3, 0.278 $MnCl_2.4H_2O$, 0.2 (NH_4) $6Mo_7O_{24}.4H_2O$, 16 $NaNO_3$ and 7.2 $Na_2HPO_4.12H_2O$.

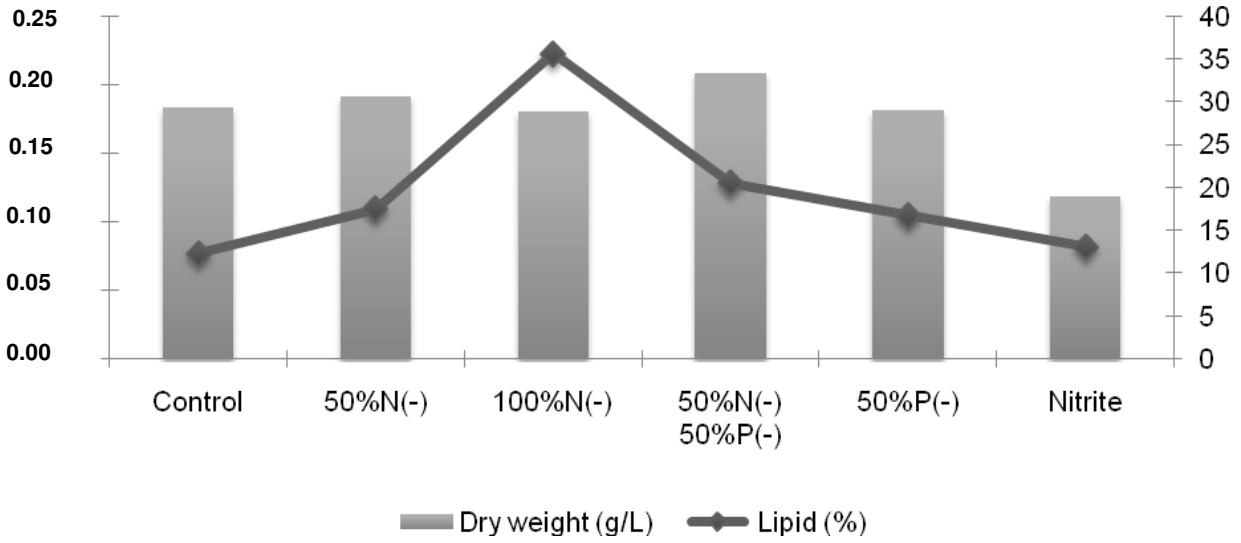

Figure 1. Dry weight and lipid values of the cultures applied in different nutrient combinations.

The experiment was performed in 50 and 100% nitrogen, 50% nitrogen plus 50% phosphorus and 50% phosphorus deficiency according to the amounts in Jaworsky medium, and as such, the nitrogen source of the nitrite was performed. The control culture groups, at which the original Jaworsky medium was used, were also formed. Consequently, all the applications were repeated in triplicate.

Analytical methods

C. vulgaris cell concentration was determined daily by optical density measurements at 500 nm with a UV–vis spectrophotometer (Liu et al., 2008). Dry weight was determined by the filtering of different volumes of algal culture through Whatman GF/C glass fibre. Algae biomass was dried at 105°C for 2 h and was then weighed (Boussiba et al., 1992). Chlorophyll a (chl *a*) content of the algae was determined spectrophotometrically after extraction with acetone (Parsons and Strickland, 1963). As such, all measurements were carried out in triplicate.

For lipid and protein analyses, samples of microalgae were collected in the stationary growth phase. *C. vulgaris* cells were separated from the medium by centrifugation at 7500 rpm for 10 min, using the centrifuge model of Hereaus. However, biomass was dried at 55°C for 2 h, pulverized in a mortar and stored at -20°C for later analysis.

Dry extraction procedure according to Zhu et al. (2002) as a modification of the wet extraction method by Bligh and Dyer (1959) was used to extract the lipid in microalgal cells. Typically, cells were harvested by centrifugation at 7500 rpm for 10 min. After drying, the samples were pulverized, overnight, in a mortar and extracted using a mixture of chloroform: methanol (2:1, v/v). About 120 ml of solvents were used for every gram of dried sample in each extraction step. The solid phase was separated carefully using filter paper (Advantec filter paper, no. 1, Japan) in which two pieces of filter papers were applied twice to provide complete separation. The solvent phase was evaporated in a rotary evaporator under vacuum at 60°C and the procedure was repeated three times until the entire lipid was extracted. The effects of solvents having different polarities for extracting the lipid, as well as the effect of drying temperature and ultrasonication time were investigated in this study. As a result, the total protein was determined by Kjeldahl method (AOAC, 1998).

Statistical analysis

Two-way analysis of variance (ANOVA) was used to test the effects of nutritional deficiencies and nutritional factors on lipid amount in biomass. When differences were found in the two-way ANOVA, Duncan multiple comparison test (HSD) of the one-way ANOVA was used to compare the mean differences (Zar, 1999) by the Statistical Package for the Social Sciences (SPSS) (Version 12.0, SPSS, Chicago, IL). As such, the differences were considered to be significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

The lipid contents were compared in the study planned to determine the stress factors, nutrient deficiencies (50% N, 100% N, 50% N 50% P, 50% P) and nitrite addition on *C. vulgaris* cell lipid content. It is known that the different nitrogen sources and levels were effective on the growth of microalgae and biochemical composition (Brown et al., 1989; Gökpinar, 1991; Fidalgo et al., 1995; Valenzuela-Espinoza et al., 1999; Xu et al., 2001). In this study, different nutritional combinations were tried on *C. vulgaris* (Chlorophyceae) and it was reported that the highest lipid accumulation was short of the 100% N.

Economically, the fuel from algae is important than the lipid content of algae beside biomass productivity. In microalgae-lipid studies, dry matter and lipid content are considered together. In this study, dry matter of 0.18 g/L and 35.60% lipid were determined from the culture upon which the nitrate source was decreased to 100%. It was also observed that these values were similar to the dry matter (0.18 g/L) of the control group to which the optimum culture conditions were supplied (Figure 1). The lowest chl *a* content of 9.25 µg/L was reported for the culture of which the lipid content was the highest and the nitrate content was absent. As such, the stress factor of

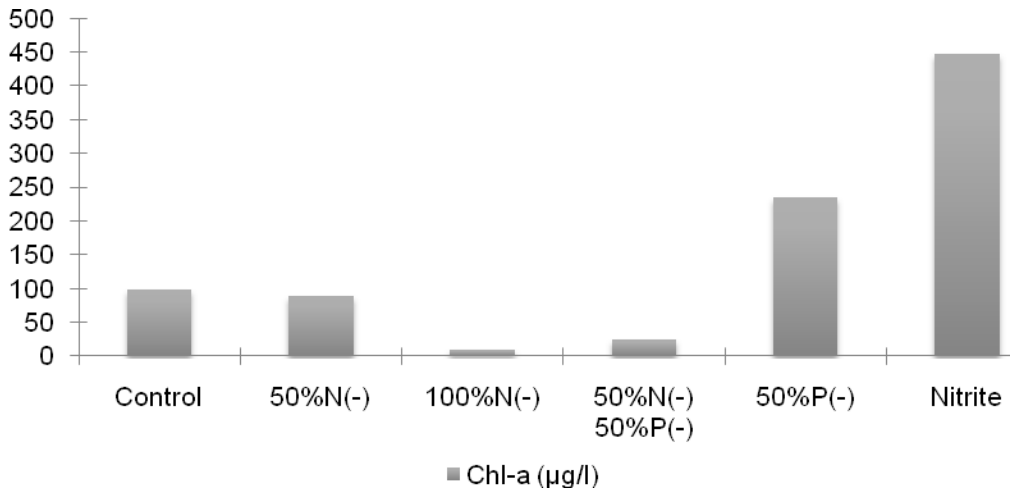

Figure 2. Chlorophyll *a* values of the cultures applied in different nutrient combinations.

Table 1. The lipid, dry weight and protein contents of *C. vulgaris*.

Parameter	Control	50%N(-)	100%N(-)	50%N(-) plus 50%P(-)	50%P(-)	Nitrite
Lipid (%)	12.29±3^{c}	17.5±4^{bc}	35.6±8^{a}	20.5±1^{b}	16.7±2^{bc}	13.04±1^{c}
DW (g/L)	0.18±0.02^{ab}	0.19±0.05^{a}	0.18±0.008^{ab}	0.21±0.05^{a}	0.18±0.03^{ab}	0.12±0.007^{b}
Protein (%)	50.8±0.01^{a}	20.3±0.02^{e}	13.01±0.01^{f}	21.37±0.02^{d}	38.16±0.05^{c}	41.03±0.03^{b}

*Different letters between the lines indicate significant difference at 5% by Duncan.

lacking nitrate led to a lack of chl *a*. The highest chl *a* value of 446.97 µg/L was observed from the culture to which nitrite was added as a nitrogen source at the last day of growth. However, it was thought that nitrite application stimulates the increase of chl content (Figure 2).

Green algae *Chlorella* contains 50 to 60% protein and is known as one of the richest chlorophyll sources (Singh and Chandra, 1988), in that different factors are effective on the production and storage of lipid for different microalgae species. While nitrogen is deficient, protein decreases in general (Shifrin and Chisholm, 1981). In this study, protein decreased in *C. vulgaris* due to a lack of the 50% N, 100% N, 50% N plus 50% P and 50% P. In the control group, 50.8% protein and 12.29% lipid was reported (Table 1). Reitan et al. (1994) cultured the microalgae *Phaeodactylum tricornutum, Chaetoceros* sp. (Bacillariophyceae), *Isochrysis galbana* (clone T-Iso), *Nannochloris atomus* (Chlorophyceae), *Tetraselmis* sp. (Prasinophyceae) and *Gymnodinum* sp. (Dinophyceae) at different lacking nitrogen media and observed the increase of lipid, whereas Widjaja (2009) cultured *C. vulgaris* in the nitrogen deficient medium for the periods of 7 and 17 days. At the end of the 17 days culture period, the total lipid was found to be higher. At the second part of the study, the different ratios of CO_2 (0, 20, 50 and 200 mL/min.) were added to the cultures. As such, the higher lipid amount was determined with the group that had an addition of 20 mL/min CO_2 in 17 days. However, Lv et al. (2010) studied the effect of KNO_3 concentration on the lipid level. Different KNO_3 concentrations of 0.2, 1.0, 3.0 and 5.0 mM were applied to the cultures of *C. vulgaris* and it demonstrated that the lipid content decreased with the increase of KNO_3 concentration. In another study, *Chlorella* (*C. vulgaris*, *Chlorella minutissima*, *Chlorella emersonii, Chlorella protothecoides and Chlorella sorokiniana*) species were cultured in deficient N media and it was determined that the growth of *C. vulgaris* was better than the other four species (Illman et al., 2000). Shifrin and Chisholm (1981) and Sukenik et al. (1989) reported that while the restricted N in microalgae cultures caused a decrease in cell number and chl *a*, organic carbon compounds which were seen as lipids increased. In addition, while chl *a* decreases in the cells, carotene increased and turned yellow in cultures.

In this study, it was observed that chl *a* contents of *C. vulgaris* culture to which N-starvation was treated decreesed and also, a yellowish colour was recorded in the culture. On the other hand, P-starvation (50%) and an addition of nitrite caused an increase in the chl *a* level of the cultures. While the dry matter was found to be low in the group that nitrite was added as a nitrogen source, the highest chl *a* value was reported in the same culture.

Recently, for biodiesel production, some *Chlorella* sp. has been cultured heterotrophically in fermenters.

However, it is thought that the heterotrophic microalgal culture of the industrial scale is quite expensive and its implementation is very difficult in practice. The major difficulties in using organic matter as a nutrient and in cultures could be considered to be an axenic one. Liang et al. (2009) investigated biomass and lipid production of *C. vulgaris* in different conditions. The results show that the higher lipid content (38%) was supplied with heterotrophic growth than autotrophic growth using acetate, glucose or glycerol. Dry matter of 2 g/L and 54 mg/L lipid production were reached at the 1% glucose level. Miao and Wu (2006) cultured *C. protothecoides* autotrophically and found 52.64% protein and 14.57% lipid. In the same study, it was reported that the protein level decreased to 10.28% and the lipid level increased to 55.20% during heterotrophic growth. In this study, it was observed that while the protein content decreased, the lipid level increased in the groups that had 50% N, 100% N, 50% N + 50% P and 50% P deficiencies. The lipid content of 35.60% with the 0.18 g/L dry matter was recorded for the group that lack the 100% N. These results obtained from laboratory conditions should be tried outdoors in photobioreactors and open ponds.

In recent years, microalgae and non-toxic biodiesel fuel sources are being investigated as renewable energy sources. In addition, to determine the microalgae species that contain high lipid, the studies of determining stress conditions which stimulate the increase of the current lipid content have been continued. In this context, biotechnological studies, which are a means of product optimization, will shed light on the utilization of algae as a source of fuel.

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