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Research paper

Augmentation and starvation of calcium, magnesium, phosphate on lipid production of *Scenedesmus obliquus*

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ABSTRACT

Theoretical evaluation of algal biodiesel shows extravagance and economical impedance. Lipid productivity has been a prominent strategy to attain economic viability. The present study investigates the effect of starvation and supplementation of Ca, Mg and P on growth and lipid accumulation in microalga *Scenedesmus obliquus*. Calcium and phosphate starvation induced lipid accumulation by 52.9% and 47.6% of DCW (dry cell weight) respectively; supplementation with 300 mgL-1 of magnesium induced lipid accumulation by 54.6% DCW. Interestingly growth was not affected at 300 mg/L of magnesium and conditions like calcium and phosphate starvation affected the growth but not completely retarded. The statistical studies with Box-Behnken model revealed that the macronutrients exerted their significant impact on growth and lipid accumulation individually than interactively.

1. Introduction

The strategic transformation of pilot scale biodiesel production from microalgae to sustainable commercial biodiesel industries heavily relies on its cost of production and return on investment. Rapidly growing microalgae possess low lipid content and in cases it's vice versa. Few well known examples are *Botryococcus braunii* for lipid rich and poor growth rate [1] and chlorella strains possess faster growth rate and lower lipid content [2,3] When fast growing microalgal species are subjected to starvation or nutritional stress to accumulate the lipid, it suffers with poor growth rate [4]. Amalgamation of biomass production with lipid production is of paramount importance in achieving economic feasibility. Such economic feasibility could be achieved only by attaining overall productivity which has been stated by myriad of reports worldwide [5]. Economic insight into the algal biodiesel production indicates that doubling the overall productivity can decrease the overall costs to 50% [6]. Although genetic manipulations may enhance the lipid content, biochemical engineering will further increase the productivity and it bears lot of attention in lipid content enhancement in microalgae.

Stressing the photosynthetic microalgae through substantial adjustments in their nutritional requirement with an intention of accumulating lipids has been familiarly followed as biochemical Engineering [7]. Various minerals like nitrogen, phosphate, iron, copper, silicon and alteration of culture conditions such as temperature, irradiance and CO_2 supply are explored to augment the lipid content of fast growing microalgae. Starvation of nitrogen and phosphorous in the photoautotrophic culture of microalgae is widely investigated [8,9].

Microalgal cell deviates the metabolic flux generated from photosynthesis towards lipid biosynthesis under nitrogen starvation [7]. In a low nitrogen medium, *Chlorella emersonii* and *Chlorella vulgaris* accumulate a fold increase in lipid content [2]. Nitrogen deficient condition coupled with high irradiance triggers 100% increase of total lipids in *Scenedesmus obliquus* CNW–N [10]. Similarly phosphate starvation augments the TAG (Triacylglycerol) up to six folds in *Monodus subterraneus* [11].

Various researchers proposed two stage cultivation processes, one for accelerating the growth and second stage for rapid accumulation of lipid [12,10]. For instance, nutrient replete conditions as growth inducting phase followed by nitrogen deficient with controlled phosphate and light conditions as lipid inducting phase yielded lipid content to 53% of dry cell weight [13]. Similarly another report established heterotrophic condition followed by photoautotrophic cultivation for augmenting the overall lipid

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productivity in *Chlorella sorokiniana* [14]. This process has its own inherent disadvantage of tedious cultivation and culture transfer facility which is economically not feasible. Although lipid accumulation was enhanced at starving particular nutrient, growth rate was severely affected consequently and reduces the lipid productivity [15]. However an ideal desideratum would be attaining the lipid productivity which couples lipid production and biomass production. Hence, finding a strategy that could stimulate the lipid accumulation without affecting the growth could be the challenge in front of researchers.

Though many studies revealed the fate of lipid accumulation at starved level, very few investigated it in surplus conditions. Extreme concentrations of nutrients like tenfold higher than required optimum concentrations are less studied in lipid perspective. Hence our study mainly aims in elucidating and comparing the stress phenomenon at both starved and surplus conditions. Another light of our present study concentrates on nutrient less condition for lipid accumulation. Due to physiological vitality, nitrate and phosphate have been the well-known for their influence in lipid accumulation. However, vital nutrients like calcium and magnesium which are essential macronutrients for microalgae are less studied.

Ca^{2+} plays vital role in cell wall and membrane structure and its low resting concentration and high diffusion coefficient provides useful intracellular messenger which coordinates response to environmental changes and signals appropriate development. Gerloff and Fishbeck [16] showed that minimum of 0.04% dry weight is Ca^{2+} in algal cells and the absence of intracellular calcium makes plasma membrane permeable, through which intracellular solutes will be lost. Apart from this, calcium modulates several enzyme activities in photosynthesis and promotes photosystem II [17]. Similarly magnesium being central metal ion in the chlorophyll molecule activates more enzymes and it is essential for aggregation of ribosomes [18]. Magnesium deficiency results in considerable decrease in photosynthetic rate and it affects the photosynthesis in both at high and low concentrations and eventually impairs the growth [19].

However different stresses resulted in different fashions over lipid accumulations; stress response without affecting the growth would be desideratum. Hence, in our present study, to investigate the effect of nitrate, phosphate, calcium and magnesium on growth and lipid accumulation individually and interactively with one factor at a time approach and Box-Behnken design respectively. Design expert software version 9.0 has been used to investigate the combined effect of all these four nutrients. Eukaryotic green microalga *Scenedsmus* sp, had been selected for its fast growing and robust quality for withstanding extreme environments [20,21].

2. Materials and method

2.1. Culture conditions

For the cultivation of microalga *Scenedesmus obliquus* Bold Basal medium with the composition of 250 mgL^{-1} of $NaNO_3$, 25 mgL^{-1} of $CaCl_2.2H_2O$, 75 mgL^{-1} of $MgSO_4.7H_2O$, 75 mgL^{-1} of K_2HPO_4, 175 mgL^{-1} of KH_2PO_4, 25 mgL^{-1} of NaCl, 50 mgL^{-1} of EDTA, 31 mgL^{-1} of KOH, 4 mgL^{-1} of $FeSO_4.7H_2O$, 11 mgL^{-1} of H_3BO_3 and micronutrients constituted 8.8 mgL^{-1} of $ZnSO_4.7H_2O$,1.4 mgL^{-1} of $MnCl_2.4H_2O$, 0.71 mgL^{-1} of MoO_3, 1.57 mgL^{-1} of $CuSO_4.5H_2O$, 0.49 mgL^{-1} of $Co(NO_3)_2.6H_2O$ was used. For experimental purposes, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, K_2HPO_4 and KH_2PO_4 were amended in the concentration of 0, 25 mgL^{-1}, 75 mgL^{-1}, 150 mgL^{-1}, 300 mgL^{-1}, 600 mgL^{-1}; 25 mgL^{-1}, 50 mgL^{-1}, 100 mgL^{-1}, 500 mgL^{-1}, 1000 mgL^{-1}; 50 mgL^{-1}, 250 mgL^{-1}, 500 mgL^{-1}, 750 mgL^{-1}, 1000 mgL^{-1} respectively. The cell suspension at 5% concentration was used uniformly as inoculum and the cultures were maintained at 25 °C by constant shaking at 14.5 rad s^{-1} under 100 μmol m^2s^{-1} illumination.

2.2. Experiment design for optimization

Box–Behnken design in Design Expert 9.0 was used to evaluate the interactive effect of calcium, magnesium, phosphate and nitrate. The response values were growth and lipid content of *Scenedesmus obliquus*. Interactive effect of all the four factors was analyzed using Design expert 9.0 software.

2.3. Growth measurements

The algal growth rate was monitored by measuring the turbidity of the growth medium (Optical density at 750 nm) with a UV–Visible spectrometer (Shimadzu UV-1800). On 15th day, algal biomass was centrifuged and dried at 105 °C to estimate dry weight.

2.4. Lipid extraction and estimation

The lipids were extracted by modified Folch procedure [22]. 1 g of lyophilized algal biomass was ground with 3 cm^3 of Chloroform/methanol (2/1 v/v), then vortexed and centrifuged at 956 g. The supernatants were collected while residues were re-extracted with 2 cm^3 of chloroform/methanol (1/1) v/v and the supernatants were pooled together. The solvents were evaporated in a rotary evaporator and then briefly dried under nitrogen to completely remove the solvents from lipid. The dried lipid was weighed and stored at −20 °C.

2.5. Lipid quantification by nile red fluorescence

The cellular neutral lipid content was quantified according to modified Huang et al. [23], procedure. Lyophilized algal biomass was diluted to 1.5×10^6 cells per cm^3 concentration with a haemocytometer. 5 cm^3 of algal suspension was mixed with Nile red (Sigma Aldrich) (9-diethylamino-5H-benzo[a]phenoxazine-5-one) solution in Dimethylsulfoxide and vortexed vigorously for 1 min to uniformly stain the algal cells. This suspension was analyzed in a Spectroflurometer with an excitation and emission wavelength of 480 nm and 570–590 nm respectively. The spectroflurometer conditions were excitation slit − 10 nm, emission slit − 10 nm and the photomultiplier was set at 400 V. Relative fluorescence intensity was precisely measured after subtracting the auto fluorescence of Nile red and algal cells.

2.6. Lipid analysis

The extracted lipids were fractionated using 500 mg Sep pak 3 cc silica catridge (Waters, Milford, USA) into glycolipids, phospholipids and neutral lipids using the solvents methanol, chloroform and acetone respectively. Initially silica gel was conditioned with the elution of 5 cm^3 chloroform and then 50 mg of lipids was subjected for separation using chloroform acetone and methanol for NL, GL and PL [24]. Following the drying of fractions with nitrogen gas, the fractions were weighed and quantified. Algal lipids were transesterified by the combination of acid and alkali catalysis. 10 mg of algal oil was mixed with 1 cm^3 of Saponification reagent (15% NaOH in methanol/water mixture, 1:1) and boiled for 30 min and then 2 cm^3 of 6N HCl and methanol (1:1 v/v) was mixed thoroughly and again boiled in a water bath at 80 °C for 30 min. The tubes were cooled and the upper layer of fatty acid methyl esters were extracted with 1.5 cm^3 of hexane and anhydrous petroleum