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Tropho-metabolic transition during *Chlorella* sp. cultivation on synthesis of biodiesel

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ABSTRACT

Mixotrophic and heterotrophic cultivation modes were studied for enhancing the biomass and lipid productivities using light and carbon as critical factors. The adaptability of the newly isolated *Chlorella* sp towards transition from mixotrophic to heterotrophic cultivation mode was evaluated. Organic carbon illustrated higher affinity towards both biomass and lipid productivities. Specific changes in fatty acid profile were observed with respect to trophic condition. Maximum biomass productivity (4.21 g/l) and relatively higher lipid productivity (107.3 g/kg of DCW) was observed with mixotrophic (MXG) condition while heterotrophic mode showed higher lipid content (28.9%). Higher carbohydrate content (94.3 mg/g DCW) was observed in mixotrophic mode and maximum protein content (450 mg/g DCW) was obtained with heterotrophic condition. The synergism between total lipid content, fatty acid composition and biomass productivities during trophic transition was critically evaluated. The transition between the trophic modes have given deeper insights into the metabolic partitioning of carbon in photosynthetic and respiratory pathways during synthesis of biodiesel precursors. Mining of bio-based products from microalgae can create more sustainable economies and integrated approach will add paybacks to process signifying algal based biorefinery model.

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1. Introduction

Photosynthetic organisms have evolved direct and indirect mechanisms for sensing and responding to environmental changes [1]. Green microalgae are usually grown under various modes of nutrition for large scale biomass production in open pond and photobioreactors. In this regard, microalgae cultivation is gaining grounds due to ample advantages like biosequestration of CO_2 for biomass production, usage of solar energy for its metabolic process, cultivation on marginal lands and synthesis of high value products, etc [2]. The most important large-scale applications of these compounds are in food, fuel, pharmaceutical, nutraceutical, healthcare and cosmetic industries with very high commercial values [3]. Microalgae can be cultivated for these products under photoautotrophic, mixotrophic and heterotrophic modes based on the availability of light and CO_2 as energy and carbon sources respectively.

However, photoautotrophic process suffers with limitations like low biomass, growth rate and lipid productivity, insufficient light and fluctuating environmental parameters [4]. As an alternative solution, heterotrophic mode results in high cell densities when cultivated in closed bioreactors under controlled conditions with organic carbon substrates such as glucose, acetate, glycerol, etc [36] as energy sources thereby avoiding the light requirements. The major limitation of heterotrophic mode of nutrition is the cost of organic substrates used for the cultivation. In contrast, mixotrophic cultivation utilizes both organic and inorganic carbon sources for cellular growth in the presence of light as the energy source providing high cell densities and higher lipid contents as compared to other cultivation conditions [5,6]. Thus under mixotrophic cultivation, growth is not strictly limited by the availability of light or availability of carbon source as in the case for photoautotrophic and heterotrophic condition respectively [7]. Irrespective of the cultivation conditions, carbon supplementation is the strategy extensively employed to enhance the neutral lipid content of the cells but at the cost of reduced growth rate and biomass titer [8]. Thus, success of microalgae based biodiesel production largely depends on two important parameters - i.e., biomass productivity and lipid content which governs the techno economic feasibility of the whole process [9]. Two stage cultivation strategies are being

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developed in which first stage targets at high density biomass cultivation followed by enhancement of total/neutral lipid content by exposing the cells to higher carbon condition or by addition of lipid elicitors molecules [6]. Strain development, process optimization and engineering are imperative to make algal biofuels practical and economically viable.

In the present study, the isolated strain was evaluated for carbon partitioning pathways with effect of changes in nutritional trophic levels. Assimilation of carbon towards higher lipid or carbohydrate synthesis in different metabolic routes was elucidated. The transition in nutritional mode was reflected in the individual fatty acid production, saturation and unsaturation profiles. The activation of various metabolic pathways under presence and absence of light in mixotrophic mode was elucidated.

2. Materials and methods

2.1. Strain selection and cultivation conditions

Different microalgae cultures growing in wastewater streams were collected from Nacharam Lake located in the vicinities of Hyderabad. Prior to isolation, the cultures were washed several times in running tap water and filtered through sieve to remove sand particles, phytoplankton and debris. These lakes receive high load of inlet nutrients in form of domestic sewage and industrial effluents from surrounding residential areas and industries. The cultures were grown in rectangular plastic containers (36 cm × 24 cm × 12 cm) with open sunlight and domestic sewage as medium for growth [10]. During initial growth period, the biomass was mixed periodically to avoid settling and uniform distribution of sunlight to the cells. After visibly good amount of biomass and cell density was achieved, the algae samples were subjected to purification by serial dilution followed by spread plate and streak plating on specific algae agar medium (Himedia). The isolated strains were cultured in BG-11 and algae agar media under illumination of 56 $\mu E/m^2/s$ with 12:12 photoperiod at pH 7.1 and 25 °C. The morphology of pure cultures of algae was examined by bright field microscopy (Nikon Eclipse 80i) and 18S sequencing data identified the strain as *Chlorella* sp. After sub-culturing for 3 times by streaking, individual colonies were isolated and inoculated into 10 ml test tubes followed by 250 ml flasks containing BG-11 medium using the same culture conditions described above.

2.2. Experimental methodology

Experiments were carried out under two phases where first phase was operated in mixotrophic mode for 8 days and second phase was subsequently carried out in three sets of which one set was continued as mixotrophically grown culture (MXT condition). The second set was supplemented with 7.5 g/l of glucose at the end of 8th day (MXG). Third set was subjected to dark heterotrophic conditions (MHT) condition. The total cultivation period of all the three experimental sets were 16 days. Sterilized BG-11 media was used for cultivation with total/working volume of 0.5/0.4 l. Exponentially growing *Chlorella* sp. cells (10%) from stock culture were inoculated to flasks under aseptic conditions. Mixotrophic experiments were provided with light intensity measured as photosynthetic photon flux density (PPFD) of 56 $\mu E/m^2/s$ using white fluorescent tube lights (Phillips 40 W). The light intensity was measured using (Extech LT-300) Light meter. All the experiments were operated at 25 °C and 7.1 pH in light assisted shaker incubator at 120 rpm. Mixotrophic cultures were grown under a 12:12 (Light:Dark) cycle [11]. Heterotrophic cultivation (MHT) in total darkness was wrapped with aluminum foil. Glucose (7.5 g/l COD) was used as organic carbon source for biomass growth in mixotrophic and heterotrophic conditions. Ampicillin (0.2 mg/ml) was added to avoid the bacterial contamination. All the experiments were operated in triplicates and standard deviation is represented in the form of error bars.

2.3. Biochemical analyses

Cell growth was measured in terms of absorbance at 750 nm using spectrophotometer (Thermo Electron). For cell dry weight (CDW) determination, algae samples were centrifuged at 10,000 rpm for 5 min at 4 °C, washed twice with phosphate buffer and dried at 60 °C for overnight until constant weight was attained. The final weight was expressed in g/l. The chlorophyll 'a' and 'b' measurements were carried out by taking 10 ml of cell suspension and centrifuging at 8000 rpm for 5 min to obtain culture pellet. Then acetone and ethanol (1:1) was added to the pellet and the suspension was subjected to sonication at 40 khz frequency for 2 min using probe sonicator (Qsonica, Q55). The centrifugation step was repeated twice and the chlorophyll concentration was determined by measuring OD of the supernatant at 647 and 664 nm for chlorophyll 'a' and 'b' respectively [12]. Chlorophyll estimation was performed using the following equation.

$$\text{Chlorophyll a } (\mu g/ml) = (-1.93 \times A_{647}) + (11.93 \times A_{664})$$

$$\text{Chlorophyll b } (\mu g/ml) = (20.36 \times A_{647}) - (5.5 \times A_{664})$$

Substrate removal in terms of COD, nitrate and phosphate was measured using standard APHA methods [13]. Carbohydrate content was determined using the phenol-sulfuric acid method after acid hydrolysis of the algae biomass [14], and the protein content was determined using Lowry method [15]. The total lipids were extracted by modified Bligh and Dyer method using chloroform and methanol (2:1) as solvents [16] and hexane was used for neutral lipid extraction. Briefly, at the end of the experiment the entire cell suspension was centrifuged at 8000 rpm for 10 min and dried in hot air oven at 60 °C for 24 h to obtain a constant weight of biomass. Then dried biomass was ground into fine powder using mortar and pestle and subjected to alternating sonication (40 khz, 2 min) and centrifugation (8000 rpm, 10 min) thrice to extract maximum lipids from the biomass. The resulting solvent-lipid layer was transferred to pre-weighed round bottom flask and the solvent was evaporated. Total and neutral lipids were determined gravimetrically.

2.4. Fatty acid methyl esters (FAME) analysis

Lipid analysis was performed by concentrating the biomass (5000 rpm; 5 min; 30 °C). Dried microalgae biomass (100 mg) was subjected to methanol-sulfuric acid (2%) mixture and refluxed for 4 h. The reaction was monitored by thin layer chromatography (TLC) using n-Hexane and ethyl acetate (EA) mixture (90:10) as mobile phase (Venkata Mohan et al., 2011). Reaction was continued till the oil spot disappeared on the TLC plate. After the reaction time (4 h), the contents were washed with 25 ml of water, the aqueous layer was extracted with EA (2 × 25 ml) and pooled. The extract was dried over anhydrous Na_2SO_4 and concentrated under vacuum. The dried FAME was analyzed using GC-FID [32]. After conversion of fatty acids to methyl esters, the concentrated sample was used for the detection of FAME composition by GC with FID (Nucon-5765) through capillary column [Valcobond (VB) 30 mm (0.25 mm × 0.25 lm)] using nitrogen as carrier gas (1 ml/min). The temperature of the oven was initially maintained at 140 °C (for 5 min), later increased to 240 °C at a ramp of 4 °C/min for 10 min. The injector and detector temperatures were maintained at 280 and 300 °C respectively with a split ratio of 1:10. FAME composition

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