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In silico optimization of lipid yield utilizing mix-carbon sources for biodiesel production from *Chlorella minutissima*

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ABSTRACT

Single-stage cultivation strategy is more favorable than two-stage cultivation owing to the lesser technical obstacles and being more cost-effective in the microalgal biodiesel production. Hence, in this study, mixotrophically grown *Chlorella minutissima* MCC 27 biomass was optimized for synergistic maximization of biomass yield and intracellular lipid accumulation using Central Composite Rotatable Design (CCRD). The effect of mix-carbon sources (glucose + acetate), nitrate concentration and culture time on biomass accumulation and lipid content (% DCW) was evaluated separately using Response Surface Methodology (RSM). Out of the three strategies, viz., biomass optimization (BO), lipid content (% DCW), and simultaneous production of biomass and lipid content and their maximization, the third strategy was found to be most effective to obtain the maximum lipid productivity (108.81 mg L^{-1} d^{-1}), which was approximately 10-fold higher than the autotrophically grown *C. minutissima* culture. The optimized conditions for maximum lipid productivity were found to be 5.96 g L^{-1} glucose, 4.12 g L^{-1} acetate, 0.73 g L^{-1} nitrate and incubation period of 10 days. Palmitic acid was the principal FAME composite followed by oleic acid and the ratio of saturated fatty acids (SFA):monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) was found to be 1.9:1.8:1. Accordingly, simultaneous biomass and lipid content optimization was found to be a better strategy for the maximization of lipid yield as compared to the other strategies followed in the present study.

1. Introduction

Environmental benefits such as high biodegradability, non-toxicity and renewable nature of microalgae have always excited the scientific community to explore more about these photosynthetic microorganisms. Microalgae-to-diesel production and its usage are found to be implicitly carbon-neutral and proficient in CO_2 emission performance [1], operable in traditional internal combustion engines, and comparably safer for storage and distribution than the petroleum-based fuels [2–5]. Further, higher volumetric biomass productivity and lack of requirements of huge arable lands and fresh water for the cultivation of these non-food based energy crops surmount the previous two generations of biofuel feedstocks [6–8]. Some microalgae are known to accumulate very high concentrations (up to 85%) of lipid [7,9,10] and Chlorophyceae is one of the major classes of lipid hyper accumulating green microalgae. Some of the potential oleaginous Chlorophycean microalgae and the effective strategies for enhancing their intracellular lipid have been enlisted in Table 1.

The nature of synthesis/breakdown of Triacylglycerol (TAG) is a developmental-related phenomenon in oil plants; on the contrary, in microalgae, it is apt to be regulated by a stress linked metabolism [11]. Changing of nutritional regimes and culture conditions, and making them extreme often enforce microalgae to deposit neutral lipid as a protective measure to cellular photo oxidation [12]. Nutrient starvation stops cell division and on the contrary intracellular photosynthetic activities disharmony to cell growth augment carbon assimilation and carbon fluxes towards intracellular neutral lipid (mostly TAGs) accretion [11]. It is one of the conventional yet most effective strategies to drive TAG accumulation in microalgae. However, chiefly the strategic-increase of lipid content (% DCW) based on stress physiology does not reciprocate in enhanced total lipid yields [13]. The lipid content achieved beyond 40% DCW is often at the cost of biomass stock of the cells [14], which leads to reduced gross lipid yield imperative for biodiesel productivity [15]. Some researchers propose a 2-stage cultivation strategy could be effective in overcoming the enhanced lipid/reduced biomass dilemma. In this method, the first stage usually involves concentrating of algal biomass through a heterotrophy/mixotrophy optimization and the second stage involves substantial conversion of lipid via N-starvation/limitation. However, dense cultures become impenetrable to light due to self-shading, and thereby aggravate

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Table 1
Critical conditions and enhancement of lipid yield in various Chlorophycean microalgae.

Microalga	Stress/strategy	Lipid accretion	Reference
Dunaliella tertiolecta ATCC 30929	Salinity	67.00%	[64]
Chlorella vulgaris	Fe-supplementation	56.60%	[65]
Haematococcus pluvialis	Continuous irradiance of high light intensity	34.85%	[66]
Scenedesmus sp. 11-1	High light intensity	41.10%; 1600.00 mg L^{-1}	[67]
Scenedesmus sp.	Salinity, N-limitation	68.44 mg L^{-1} d^{-1}	[68]
Chlorella zofingiensis	N-starvation	65.10%; 87.10 mg L^{-1} d^{-1}	[69]
Chlorella zofingiensis	P-starvation	44.70%; 44.70 mg L^{-1} d^{-1}	[69]
Scenedesmus obliquus HM103382	Salinity	34.00%	[70]
Chlamydomonas mexicana GU732420	Salinity	37.00%	[70]
Pseudokirchneriella subcapitata	High light intensity	39.30 mg g^{-1} CDW	[71]
Chlamydomonas sp. JSC4	Gradient salinity stress	59.40%; 223.20 mg L^{-1} d^{-1}	[72]
Chlorella pyrenoidosa	Two-stage	68.00%	[73]
Scenedesmus sp.	$NaHCO_3$, $NaH_2PO_4{\cdot}2H_2O$, $NaNO_3$ optimized medium	304.02 mg L^{-1}	[74]
Scenedesmus abundans	Inoculum concentration, $NaHCO_3$ supplementation	26.28%	[75]
Chlorella vulgaris	Sparging of CO_2 (8% v/v)	29.50 mg L^{-1} d^{-1}	[76]
Chlamydomonas reinhardtii	S-starvation	36.98%	[77]
Chlorella vulgaris	Two-stage	53.97%; 9.21 mg L^{-1} d^{-1}	[78]
Dunaliella tertiolecta UTEX LB 999	N-starvation	28.00%	[79]
Acutodesmus dimorphus	N-starvation	29.92%	[80]
Chlamydomonas reinhardtii CCAP 11/32C	Inoculum concentration, N, acetate optimized medium	10.58 mg L^{-1} d^{-1}	[81]
Chlorella minutissima MCC 27	One-stage; Glucose, acetate, N optimized medium	36.21%; 1129.75 mg L^{-1}	This study

maximization of lipid productivity and again within a legitimate time frame questioning the efficacy of the process [16]. Besides, involvement of multiple harvesting steps usually makes the process technically elaborate and economically unfeasible. In this regard, single-stage mixotrophic cultivation could be more cost- and energy-efficient and the target should be optimum yield of the total lipid as it is the immediate raw material for biodiesel production.

Although the one-variable-at-a-time (OVAT) approach for enhancing the lipid pool of oleaginous microalgae is extensively studied, it lacks a clear and complete interpretation of the interactions among other variables influencing the response of interest. Relatively few reports are available on the interactive studies when a number of crucial attributes have been considered at a time. Response Surface Methodology (RSM) is one such multivariate statistical technique, which was utilized in this study for exploring and establishing the approximate functional relationship between the response and cluster of design variables. Among other designs, in this study, the Central Composite Rotatable Design-RSM (CCRD-RSM) was selected owing to its capability of optimizing experiments with several variables, and further with relatively less number of experiments, as is reported, without dropping the differentiation power and retaining its sensitivity to the incidence of outliers in the design [17]. In addition, it can also target the stability region of the experimental design round about a centre point ascertained by its properties of rotatability and orthogonality and fit the quadratic polynomials [18]. Hence, in the present study, multifactor optimization of crucial parameters for maximization of biomass yield and lipid content in terms of percent dry weight of mixotrophic *C. minutissima* MCC 27 was carried out using CCRD-RSM. Further, a simultaneous biomass and lipid content optimization was conducted and compared with the former two maximization strategies. The optimized lipids were then enzymatically-transesterified and the FAME-characterization of the produced *C. minutissima* MCC 27-diesel was carried out and compared among the three different optimized conditions.

2. Materials and methods

2.1. Strain and cultivation medium

The strain *C. minutissima* MCC 27, purchased from the Center for Conservation and Utilization of Blue Green Algae (CCUBGA), IARI, India, had been selected for this study. The cultures were maintained in N 11 medium, which is composed of KNO_3 1000 mg L^{-1}, $Na_2HPO_4{\cdot}H_2O$ 83 mg L^{-1}, KH_2PO_4 52 mg L^{-1}, $MgSO_4{\cdot}7H_2O$ 50 mg L^{-1}, $CaCl_2{\cdot}H_2O$ 10 mg L^{-1}, Fe-EDTA stock solution (10 g of chelate per liter) 1 mL L^{-1}, and SAZ stock solution (Trace metal mixture) 1 mL L^{-1}. The SAZ is composed of $MnCl_2{\cdot}4H_2O$ 99 mg L^{-1}, $NiSO_4{\cdot}6H_2O$ 23.6 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 63 mg L^{-1}, $CuSO_4{\cdot}5H_2O$ 5 mg L^{-1}, $CoSO_4{\cdot}7H_2O$ 2.8 mg L^{-1}, NH_4VO_3 2.9 mg L^{-1} and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 1.8 mg L^{-1}. The pH of the medium was adjusted to 6.8 (pH meter, M/s. ELICO, India) prior to autoclaving (15 psi, 121 °C, 15 min). The logarithmic phase culture was used as inoculum with an initial cell concentration of 115 mg L^{-1}. The batch cultures of *C. minutissima* were maintained in 250 mL Erlenmeyer flasks containing 100 mL of N 11 medium. Air conditioning system was used to maintain the temperature of the culture room at 25 ± 2 °C. The cultures were exposed to a light intensity of 75 µmol photon m^{-2} s^{-1} PAR and 14:10 h light-dark cycle. The cultures were hand-shaked three times a day for uniform growth and to avoid biofouling.

2.2. Assay of biomass and lipid

The growth of *C. minutissima* MCC 27 was monitored by measuring the optical density at 540 nm by using a UV-1800 (SHIMADZU, Japan) spectrophotometer. Harvesting of algal biomass was done by means of centrifugation (Eppendorf Centrifuge 5810R, 5000 rpm, 15 min) with a two-time washing with distilled water to remove the excess of salts. Moisture was removed from the freshly harvested biomass in an oven at 60 °C till it attained a constant weight. The volumetric biomass productivity (P_B) was measured as per the following equation:

$$P_B\ (\mathrm{g\ L^{-1}\ d^{-1}}) = \frac{X_f - X_i}{t_f - t_i} \quad (1)$$

where X_f and X_i are the final and initial biomass concentrations (g L^{-1}) at time t_f and t_i (days), respectively.

The extraction of lipid was carried out as per the protocol of Bligh and Dyer [19]. The solvent-extracted lipid was then filtered with Whatman No. 1 filter, dried and weighed as per the method described by Dash and Banerjee [20]. In addition, the volumetric lipid productivity (P_L) was measured as per the following equation:

$$P_L\ (\mathrm{mg\ L^{-1}\ d^{-1}}) = P_B \times LC_f \times 10 \quad (2)$$

where P_B is the volumetric biomass productivity (g L^{-1} d^{-1}) and LC_f is the final lipid content (% DCW).