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High-density fed-batch culture of a thermotolerant microalga *Chlorella sorokiniana* for biofuel production

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HIGHLIGHTS

- Reveal the relationship between nitrogen consumption and pH change.
- Obtain a high growth rate of 0.133 h^{-1} through optimization.
- Achieve high biomass and lipid concentrations of 103.8 g L^{-1} and 40.2 g L^{-1}.
- Perform lipid class analysis for the microalga *Chlorella sorokiniana.*
- Evaluate biodiesel characteristics based on the fatty acids compositions.

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ABSTRACT

Culturing microalgae heterotrophically for producing lipid-based biofuels such as biodiesel and renewable hydrocarbons has attracted increasing attention due to the advantages of fast growth and high lipid yield under this growth mode without being subjected to light limitation. High cell density in the culture broth is desirable for reducing downstream processing costs. Oleaginous microalga *Chlorella sorokiniana* was investigated for high cell density culture with glucose as the carbon source. Best growth performance was obtained first with batch culture at pH 7.0 when ammonium was the nitrogen source. Then, two-stage fed-batch fermentation was conducted under the optimal conditions. The algal biomass grew linearly in the first stage with a productivity of 24.2 g L^{-1} d^{-1}, and the lipid content increased from 14.5% to 38.7% in the second stage. This fermentation strategy resulted in algal biomass and lipid concentrations of 103.8 g L^{-1} and 40.2 g L^{-1} respectively. Analysis of lipid and fatty acid profiles showed *C. sorokiniana* accumulated a large amount of neutral lipids (92.9% of total lipids), triacylglycerols (82.8% of neutral lipids), and high contents of palmitic, oleic and linoleic acids, which are ideal form of lipid for making biodiesel. These results suggest that heterotrophic culture of *C. sorokiniana* holds great potential for lipid-based biofuel production.

1. Introduction

Microalgal biomass as a feedstock for producing renewable fuels has attracted great attention in the recent years due to primarily its high potential productivity [1]. Phototrophy and heterotrophy are two growth modes of microalgae that both have commercial potential. Phototrophic culture of algae in open ponds is a more favorable option than closed photobioreactor systems due to the low production costs [2,3]. However, prior to large scale application of phototrophic microalgal biomass cultivation for biofuel production, a series of key challenges have to be resolved, i.e., susceptible to contamination, light limitation, low productivity and difficulties in harvesting [4,5]. Heterotrophic cultivation eliminates the requirement for light and takes advantage of fast growth, high production rate, high degree of process control and low cost harvesting due to the high cell density [6]. Heterotrophic cultivation can be accomplished with mature fermentation technologies and facilities, such as those used for industrial beverages, medicines and food additives production, which results in a significant reduction in costs compared with closed photobioreactor systems [2,7]. A major limitation of heterotrophic algal cultivation is the sustainability and cost of organic carbon source, but using of lignocellulose-derived sugars offers a solution [8].

Among algal species with industrial potential, the green microalga *Chlorella* is attractive because it can grow both phototrophically and heterotrophically with a high biomass concentration [9]. *Chlorella* is one of the commercially important microalgae with

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world annual sales of more than US$ 38 billion [10]. Many high-value chemicals can be produced from *Chlorella*, such as β-1,3-glucan (an active immunostimulator, a free radical scavenger and a reducer of blood lipids), and carotenoids (protection against UV-caused skin damage, macular degeneration, cancers and age-related degenerative diseases) [10,11]. Recently, heterotrophic culture of *Chlorella* has shown promise for biodiesel production, which is attributed to its high growth rate and oil content [9]. Biodiesel made from heterotrophic *Chlorella* meets the ASTM standard in terms of density, viscosity, flash point, cold filter plugging point, solidifying point, and heating value [12]. Liu et al. [13] also demonstrated that the oil extracted from heterotrophically grown cells was more feasible for biodiesel production than the photoautotrophic cells due to the higher yields of total lipids, neutral lipids, triacylglycerol (TAG) and oleic acid. Cell density is one of the most important factors affecting the economics of algae based biofuels. Studies on high density culture were performed in order to reduce the process cost. Liu et al. [9] cultured *Chlorella zofingiensis* with fed-batch fermentation, and the lipid concentration reached 20.7 g L^{-1}. Higher cell density of 51.2 g L^{-1} and lipid concentration of 25.8 g L^{-1} were achieved by *Chlorella protothecoides* [14]. However, the biomass concentration is still low compared to some commercial oleaginous microalgae like *Crypthecodinium cohnii* and *Schizochytrium* sp. that were reported to reach biomass concentration higher than 100 g L^{-1} [6,15].

It was demonstrated that the green alga *Chlorella sorokiniana* could grow at a higher rate on glucose in the dark compared with the phototrophic cultivation [5]. In our previous work, we found that the thermotolerant characteristics of *C. sorokiniana* (tolerate up to 42 °C with optimal growth at 37 °C) greatly enhanced its growth performance. Cell density of 37.6 g L^{-1} with the lipid content of 31.5% was obtained in only 72 h under optimal batch fermentation conditions [16]. The high growth rate makes *C. sorokiniana* a potential species for high density cultivation. However, further optimization is necessary since our data showed that the consumption of different nitrogen sources by *C. sorokiniana* might result in pH change of the culture broth, which subsequently affected the algal growth. Moreover, screening a less expensive nitrogen source (i.e. ammonium) can benefit the cost reduction. Actually, not all lipids produced from microalgae are equally suitable for biofuel production. Only neutral lipids especially triacylglycerols (TAGs) are ideal starting material to make high energy density transportation fuels, i.e., biodiesel and renewable hydrocarbons [1]. Therefore, detailed lipid and fatty acid profiles of *C. sorokiniana* will be useful for assessing their appropriateness as biofuel feedstocks.

The objective of this study was to further improve the biomass productivity and lipid concentrations of *C. sorokiniana*, and investigate the potential of this algal species as a feedstock for lipid based biofuel production. Firstly, the relationship between pH change and nitrogen consumption was studied in order to improve the growth rate and select a low cost nitrogen source for *C. sorokiniana*. Then, fed-batch cultivation was tested for achieving high cell density and oil accumulation. Finally, evaluation of the lipid and fatty acid profiles extracted from *C. sorokiniana* were performed for the purpose of making biodiesel.

2. Materials and methods

2.1. Organism and medium

The green microalga *C. sorokiniana* (UTEX 1602) was obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). This strain was maintained at 4 °C on an agar slant of Kuhl medium [17] supplemented with 10 g L^{-1} glucose. The minimal medium was used in all batch and fed-batch cultivations and consisted of (per L) 621 mg $NaH_2PO_4{\cdot}H_2O$, 89 mg $Na_2HPO_4{\cdot}2H_2O$, 246.5 mg $MgSO_4{\cdot}7H_2O$, 9.3 mg EDTA, 0.061 mg H_3BO_3, 14.7 mg $CaCl_2{\cdot}2H_2O$, 6.95 mg $FeSO_4{\cdot}7H_2O$, 0.287 mg $ZnSO_4{\cdot}7H_2O$, 0.01235 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.169 mg $MnSO_4{\cdot}H_2O$, and 0.00249 mg $CuSO_4{\cdot}5H_2O$.

2.2. Batch culture

To investigate the effects of pH on the growth of *C. sorokiniana*, batch cultures were carried out in a 1-L fermentor (New Brunswick Scientific, CT, USA) containing 0.5 L minimal medium supplemented with 20 g L^{-1} glucose and 2.0 g L^{-1} KNO_3. The pH values were maintained at 5.0, 6.0, 7.0, 8.0 and 9.0 by feeding with 2 mol L^{-1} NaOH or H_2SO_4 solutions. To study the nitrogen sources on cell growth, NH_4Cl, KNO_3, NH_4NO_3 and yeast extract at the same nitrogen concentration of 20 mmol L^{-1} were added into the culture medium respectively with or without pH control.

2.3. Fed-batch culture

In order to achieve high biomass and lipid concentration, *C. sorokiniana* was cultured in fed-batch mode. Primary fed-batch culture was conducted in a 5-L fermentor (New Brunswick Scientific, CT, USA) containing 2.0 L minimal medium supplemented with 20 g L^{-1} glucose and 2.0 g L^{-1} KNO_3 (C/N ratio 29/1) [16]. The glucose concentration was maintained between 10 g L^{-1} and 60 g L^{-1} by feeding with concentrated culture medium containing 500 g L^{-1} glucose, 50 g L^{-1} KNO_3 and 25 × minimal medium. The pH value was maintained at 6.0 by feeding with 2 mol L^{-1} H_2SO_4 solution.

To improve the biomass and lipid concentration, a two-stage fed-batch fermentation strategy was conducted. The initial culture contained 2.0 L minimal medium supplemented with 20 g L^{-1} glucose and 1.1 g L^{-1} NH_4Cl. Nitrogen stock solution containing 185 g L^{-1} NH_4Cl and 50 × minimal medium was fed with glucose stock solution (700 g L^{-1} pure glucose) at C/N ratio of 29/1 in the first 48 h of cultivation, and later on only glucose stock solution was added. The pH value was maintained at 7.0 by feeding with 10 mol L^{-1} NaOH solution.

The fermentors for batch and fed-batch cultures were covered with aluminum foil to keep the cultures in darkness. The dissolved oxygen concentration was maintained at 50% by changing the agitation speed and the aeration rate. The temperature was controlled at 37 °C. All the media including the stock solution were autoclaved at 121 °C for 20 min before cultivation.

2.4. Analytical procedure

For algal biomass determination, 5 mL cell suspension samples were transferred to a centrifuge tube and centrifuged at the speed of 1000*g* for 5 min. The cell pellet was washed three times with distilled water to remove the residual sugars and chemicals, and then dried in a pre-weighed aluminum dish at 105 °C for 3 h. Glucose and nitrate concentrations were analyzed according to Li et al. [16]. The high range (2–47 mg L^{-1}) ammonia TNTplus testing kit (Hach Company, CO, USA) was used in ammonium nitrogen analysis, and Hach Method 10205 was followed.

The lipids were extracted with a solvent mixture of chloroform–methanol–water (2:1:0.75, v/v) based on the modified Folch procedure [18]. The analysis of lipid and fatty acid profiles was performed according to the description by Bates and Browse [19]. Individual lipid class was separated with one-dimensional thin-layer chromatography (TLC), silica gel 60 20 × 20 cm glass plates (EMD Millipore, MA, USA), using a solvent mixture of hexane-diethyl ether-acetic acid (70:30:2, v/v). Visualization was carried