Accumulation potential of lipids and analysis of fatty acid profile of few microalgal species for biodiesel feedstock

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ABSTRACT

High lipid content with adequate fatty acid profile is required for focusing any microalgal species as a renewable feedstock for biodiesel production. The present study examines the lipid accumulation potential of two green and two blue-green microalgal species, and analyzes the fatty acid profiles under various conditions for possible recommendation. Maximum lipid accumulation of ~40% of dry cell weight (dcw) was recorded under N and P starvation in the green microalgae, Scenedesmus obliquus and Chlorella vulgaris. For cyanobacterial species (Nostoc muscorum and Synechocystis PCC 6803), the response was poor under all the conditions studied. The relative percentage of saturated fatty acids (SFA) was maximum in green as well as blue-green microalgal species under control and all the treated conditions. Oleic acid (monounsaturated fatty acid, MUFA) content was increased, and the relative percentage of polyunsaturated fatty acids (PUFA), i.e. linoleate and linolenate decreased against control in all the four microalgae under the treated conditions. The green microalgae, Chlorella vulgaris and Scenedesmus obliquus, are found to be the suitable feedstock for biodiesel production with lipid accumulation ~40% (dcw) under specific conditions. A significant rise in the relative percentage of SFA and MUFA, and fall in PUFA contents are desirable for production of good quality biodiesel.

Keywords: Green and blue-green microalgae; lipids; monounsaturated fatty acids; polyunsaturated fatty acids; saturated fatty acids

INTRODUCTION

Carbon neutral renewable liquid fuels are needed to eventually replace the petroleum-based transport fuels that contribute to global warming. In this context, microalgal biodiesel could be a potential and suitable alternative, without adversely affecting the agricultural production [1]. Although microalgae are not yet produced at large scale for bulk applications, recent advances particularly in the fields of system biology, genetic engineering and bio-refining present opportunities to develop this process in a sustainable and economical way within the next decades [2]. The main bottleneck of commercial application of biodiesel is its high cost of production [3]. To reduce the overall cost of biodiesel production, increase in lipid content per unit microalgal biomass is one of the efficient strategies [4]. The most efficient trigger to enhance lipid accumulation in microalgal cell is nutrient limitation [5]. But high lipid content and high lipid productivity are in contradiction with each other, due to the low microalgal biomass productivity in nutrient-limited conditions [6]. The fatty acid composition of algae can vary both quantitatively and qualitatively with their physiological status and culture conditions. The properties of biodiesel are mainly determined by its fatty acid methyl esters [7]. Structural features of a fatty acid methyl ester molecule that influence the physical and fuel properties are chain length and degree of unsaturation [7]. On heating unsaturated
fatty acids, polymerization of glycerides will occur; this may lead to gum formation and engine failure [8]. To ensure the quality of biodiesel as alternative fuel, fatty acids analysis of the feedstock is required. Currently, oleaginous microalgae are in focus in the microbial biofuel field because of their ability to produce substantial amounts of triacylglycerols (TAGs) as storage lipids. Although cyanobacteria usually do not accumulate neutral lipids, their photosynthetic membranes are made of diacylglycerols, and they also have a robust lipid metabolism [9]. Additionally, cyanobacteria are much more genetically manipulatable compared to eukaryotic microalgae [10]. Overall, researchers predict that a hectare of algae could produce 90000 L of biodiesel, and has the potential to go even higher [11].

Biodiesel is a clean burning alternative fuel to fossil diesel, which can be produced from domestically grown renewable resources. Biodiesel consist of alkyl (methyl, propyl, or ethyl) esters which can be derived by chemical reaction of the lipids (vegetable oil, animal fat) with an alcohol [12].

In this study, our effort was to raise the lipid pool of two cyanobacterial and two green microalgal species by taking into account of some important variables such as nitrogen, phosphorous and iron starvation, and sodium thiosulphate supplementation to the culture medium, and also to analyze the variations in fatty acid profile under the above conditions.

MATERIALS AND METHODS

2.1. Organisms and growth conditions
Established cultures of *Chlorella vulgaris*, *Scenedesmus obliquus*, *Nostoc muscorum* and *Synechocystis PCC* 6803 were used in this study, and maintained in a culture room at 25 ± 2°C under a photoperiod of 14:10 h at light intensity of 75 µmol photon m⁻² s⁻¹ PAR. Axenic cultures of *C. vulgaris* and *S. obliquus* were grown in 150 ml Erlenmeyer flasks containing 50 ml of N11 medium [13], at pH 6.8. *N. muscorum* and *Synechocystis PCC* 6803 were grown respectively in nitrate-free and nitrate-supplemented BG 11 medium [14], at pH 7.5. The culture media and pH were selected based on the optimum growth of the respective organism.

2.2. Dry weight measurement
Dry cell weight (dcw) was determined gravimetrically according to Rai et al. [15]. A known volume of algal culture was centrifuged at 5000 rpm for 10 min and the harvested biomass was dried at 60°C till to reach a constant weight.

2.3. Extraction of lipid from algal biomass
Extraction of lipid was done following the protocol of Bligh and Dyer [16], which is a liquid-liquid extraction method.

2.4. Effects of nitrogen, phosphorus, iron starvation and thiosulphate supplementation on biomass and lipid yield
To study the effects of nitrogen, phosphorus and iron starvation on biomass and lipid yield, *C. vulgaris* and *S. obliquus* cultures were grown in N-deficient, P-deficient and Fe-deficient conditions. N-deficiency was achieved by substituting KNO₃ of N11 medium with equimolar concentrations of KCl. For P-deficiency, the microalgal cultures were transferred to the mineral salt medium, where Na₂HPO₄·H₂O and KH₂PO₄ were replaced with equimolar concentrations of Na₂SO₄ and KCl, respectively. Fe-deficiency was achieved by substituting FeSO₄·7H₂O of the medium with equimolar concentration of Na₂SO₄.

In case of *N. muscorum* and *Synechocystis PCC* 6803, cultures were grown in P-deficient BG 11 medium, where K₂HPO₄ of the medium was replaced by equimolar concentration of KCl. N-deficiency was achieved by substituting NaNO₃ and Na₂MoO₄·2H₂O of the medium with equimolar concentrations of NaCl, and Co(NO₃)₂·6H₂O with CoCl₂·6H₂O, respectively. For Fe-deficient condition, ferric citrate of the medium was substituted by sodium citrate. Effect of sodium thiosulphate on growth and lipid accumulation was studied by supplementing the nutrient medium with 0.5 g l⁻¹ of sodium thiosulphate.

2.5. Analytical procedure
The acid catalyzed transesterification of algal oil was carried out using 60:1 molar ratio of methanol to oil and the top organic layer was taken for analysis by using a GC system (Clarus 500, Perkin-Elmer, Shelton, CT, USA) equipped with Elite-1® dimethylpolysiloxane capillary column (30 m x 0.25 mm x 0.25 µm) and flame ionization
RESULTS AND DISCUSSION

3.1. Effects of N, P and Fe starvation, and sodium thiosulphate supplementation on biomass yield

Severe reduction in biomass yield was recorded under nitrogen starvation followed by phosphorus- and iron-starved conditions for an incubation period of 7 days (Fig. 1). Our observation that biomass can be harvested on day 7 after cultivation suggests that early harvest of the cells for lipid production is suitable.

In *S. obliquus*, biomass yield was ~6 fold lower than the control under nitrogen starvation, whereas ~4 fold lower for phosphorus-starved and ~2 fold lower for iron-starved cultures. A similar trend for *C. vulgaris* was also recorded; i.e. ~5 fold lower than the control for nitrogen-starved cultures, whereas ~3.5 fold lower for phosphorus-starved, and ~3 fold lower for iron-starved conditions.

Nitrogen starvation in the medium reduced the biomass yield to 0.13 g l\(^{-1}\) against 0.21 g l\(^{-1}\) control in *N. muscorum*, and 0.21 g l\(^{-1}\) against 0.39 g l\(^{-1}\) in *Synechocystis* PCC 6803 on day 7 of incubation. Phosphorous starvation however, had comparatively less adverse effects than nitrogen starvation for an incubation period of 7 days. Under Fe starvation, biomass yield was reduced to 0.19 g l\(^{-1}\) against 0.21 g l\(^{-1}\) in *N. muscorum*, and 0.36 g l\(^{-1}\) against 0.39 g l\(^{-1}\) in *Synechocystis* PCC6803 on day 7 of incubation. It is quite apparent from these experiments that cyanobacterial biomass yield was less affected than green microalgal cultures under nutrient-deprived conditions. This could be due to the unique ability of cyanobacteria to store phosphate as polyphosphate bodies and nitrogen as cyanophycin reserves [17]. Cyanobacteria also sequester Fe involving small organic ligands, called siderophores, and Clarke et al. [18] reported that the siderophore system in *Anabaena* sp. had developed primarily to overcome the iron starvation. The impact of 0.5 g l\(^{-1}\) sodium thiosulphate supplementation on biomass yield of the test microalgae is also presented in Figure 1. The biomass yield was reduced to 0.13 g l\(^{-1}\) against 0.85 g l\(^{-1}\) in *C. vulgaris*, 0.14 g l\(^{-1}\) against 0.67 g l\(^{-1}\) in *S. obliquus*, 0.11 g l\(^{-1}\) against 0.21 g l\(^{-1}\) in *N. muscorum* and 0.25 g l\(^{-1}\) against 0.39 g l\(^{-1}\) in *Synechocystis* PCC6803 on day 7 of sodium thiosulphate supplementation. This is well in agreement with Feng et al. [19], where growth of *Chlorella* sp. was severely affected under sodium thiosulphate supplementation.

3.2. Effects of N, P and Fe starvation, and sodium thiosulphate supplementation on lipid accumulation

Starvation of nitrate, phosphate and iron, and thiosulphate supplementation appeared to be suitable stimulants for lipid accumulation, particularly for the green microalgae (Fig. 2). Lipid content in *C. vulgaris* and *S. obliquus* was increased up to 41.2% (dcw) against 7.8, and 42% (dcw) against 6.5, respectively under N-starvation. This is well agreement with the report of Shiflin and Chisholm (1981), where accumulation of fatty acid derivatives in 30 microalgal species belonging to chlorophyceae and diatoms was observed under nitrogen starvation) [20]. In *N. muscorum* and *Synechocystis* PCC6803 the rise was marginal, i.e. only up to 9.8% against 5.2, and 4.0% against 2.9 controls. This could be ascribed to the fact that under nitrogen starvation, the carbon dioxide fixed is converted into carbohydrate or lipid rather than protein due to unavailability of nitrogen source [21]. The another possible reason could be that under nitrogen starvation, NADPH consumption was decreased due to unavailability of nitrogen pool, which blocks the amino acid synthesis pathways, especially the reaction from α-ketoglutarate to glutamate, thus resulting into accumulation of excess NADPH in the cells [22]. It is well known that for synthesis of fatty acid, NADPH acts as a reducing power and plays a very important role in the two step reduction process of fatty acid biosynthesis [23].

Under P-starvation, lipid content in *C. vulgaris* and *S. obliquus* was raised up to 40.1% (dcw) and 31%, respectively on 7th day of incubation (Fig. 2). This confirms the earlier findings of Rhee et al. [24], where lipid accumulation was enhanced when growth was restricted due to unavailability of phosphate. Phosphate limitation also increases the accumulation of reducing power Konopka and Schmura [25], thus might be resulted into higher lipid biosynthesis.
Fig. 1. Impact of nutrient starvation and STS-supplementation on biomass yield of the selected microalgae on day 7 of incubation

Fig. 2. Impact of nutrient starvation and STS-supplementation on lipid accumulation of the selected microalgae on day 7 of incubation
Fig. 3. Fatty acid profiles of the four microalgae (a) under controlled condition, (b) nitrogen starvation, (c) phosphorous starvation, (d) iron starvation, and (e) sodium thiosulphate supplementation.
Phosphorus is an important component of DNA and RNA; the lack of this element will cause defective cell division, leading to arrest of cell growth. The lack of P also could impair phospholipids synthesis, which will promote the synthesis of TAGs [26]. In contrast, N. muscorum and Synechocystis PCC 6803 again registered an insignificant rise.

Effect of iron starvation on lipid accumulation of the selected microalgae was also investigated. When the culture flasks were incubated under Fe-deficient condition, in C. vulgaris and S. obliquus, lipid content was increased up to 19.6 and 24.4% (dcw), respectively (Fig. 2). On deficiency of iron, the biomass yield was also decreased (Fig.1). This affirms the earlier findings of Weng et al. [27] that iron starvation can misalign chloroplast lamellas, and metamorphose and twist thylakoid of microalgae Prorocentrum micans and Cryptomonas sp., leading to a retarded growth rate. Both the cyanobacterial species again depicted a marginal rise against control. Under 0.5 g l\(^{-1}\) sodium thiosulphate supplementation, lipid pool of C. vulgaris and S. obliquus was raised up to 26.9 and 29.7% (dcw), respectively. Rao and Mutharasan (1988) observed that the possible reason could be that sodium thiosulphate supplementation cause to be a reducing environment leads to increased pool size of NADH [28]. Under such condition, acetyl-CoA could not enter the tricarboxylic acid cycle (TCA) cycle as the high concentrations of NADH inhibit the enzyme citrate synthase, one of the key enzymes of TCA cycle, leading to an increase in the pool of acetyl-CoA. The later might be converted to malonyl CoA, catalyzed by acetyl-CoA carboxylase (ACCase), the central carbon donor for fatty acid synthesis [29]. In N. muscorum and Synechocystis PCC6803 the rise was however, up to 8.6 against 5.2%, and 4.9 against 2.9% (dcw) control. Under all the selected conditions, the response of cyanobacteria to lipid accumulation was very poor. It is in continuation with the earlier findings that cyanobacteria do not accumulate high amounts of lipids [30, 31].

3.3. Variations in SFA, MUFA and PUFA contents under N, P and Fe starvation, and sodium thiosulphate supplementation

The gas chromatographic study demonstrates the relative percentage of various fatty acid methyl esters (FAME) present in the transesterified oil or biodiesel of the four test microalgae under the above conditions. Under control condition (Fig. 3a), the relative percentage of saturated fatty acids (SFA) in all the four microalgae was higher than unsaturated fatty acids, i.e. ~70-75% (SFA) and 25-30% (MUFA and PUFA), respectively. Palmitic acid is the most abundant fatty acid in all the test microalgae. S. obliquus showed maximum percentage (~34%) of MUFA (oleic acid). Only S. obliquus and Synechocystis PCC6803 contained linolenic acid that was ~17.0 and 8.4%, respectively. Oleic acid content was increased in all the test microalgae under the treated conditions (Figs. 3b-e); maximum rise was recorded for S. obliquus (52%) followed by C. vulgaris (44%) in Fe-starved condition.

The ratio SFA/PUFA and MUFA/PUFA (Tables 1 & 2) reveals high relative percentage of saturated fatty acids and monounsaturated fatty acids than polyunsaturated fatty acids. It can be clearly observed that proportions of SFA/PUFA and MUFA/PUFA were increased in both the conditions (starved and supplemented) than control which is desirable for good quality biodiesel as these offers oxidative stability to the fuel. Oxidative stability is one of the major issues affecting the use of biodiesel because of the presence of polyunsaturated methyl esters in algal oil [32]. Polyunsaturated fatty acids with four or more double bonds are quite common in microalgal lipids. These bonds are susceptible to oxidation during storage, thus reduce the acceptability of microalgal oil for production of biodiesel [33]. Bucy et al. [34] reported that several species of microalgae that are suitable for large scale cultivation as genus Nanochloropsis accumulate lipids that consist of long chain polyunsaturated fatty acids. These although are of high value products but problematic in terms of biodiesel properties such as ignition quality and oxidation stability.

Table 1. Fatty acid ratios of the test green microalgae under nutrient starvation and thiosulphate supplementation

<table>
<thead>
<tr>
<th>Fatty acid ratio</th>
<th>Control</th>
<th>Starved nitrogen</th>
<th>Starved phosphorous</th>
<th>Starved iron</th>
<th>Supplemented sodium thiosulphate</th>
<th>S. obliquus</th>
<th>C. vulgaris</th>
<th>PCC 6803</th>
<th>S. obliquus</th>
<th>C. vulgaris</th>
<th>PCC 6803</th>
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<tbody>
<tr>
<td>Chlorella vulgaris</td>
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<tr>
<td>SFA/PUFA</td>
<td>5.28</td>
<td>3.61</td>
<td>3.40</td>
<td>4.36</td>
<td>4.90</td>
<td>1.33</td>
<td>2.53</td>
<td>4.42</td>
<td>2.34</td>
<td>2.58</td>
<td>3.58</td>
</tr>
<tr>
<td>MUFA/PUFA</td>
<td>0.76</td>
<td>2.90</td>
<td>2.90</td>
<td>4.23</td>
<td>3.67</td>
<td>2.71</td>
<td>3.58</td>
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To be a suitable biodiesel feedstock a multitude of factors have to be considered, one of which is lipid accumulation and for this it was found that green microalgae, *Chlorella vulgaris* and *Synechococcus obliquus*, were suitable feedstocks with lipid accumulation ~ 42% of dry cell weight (dcw). In case of cyanobacteria lipid accumulation was insignificant. *C. vulgaris* and *S. obliquus* oils mainly consisted of methyl esters of palmitate and stearate, (50-65%) and (35-50%) respectively, followed by oleate (25-45%) with a small fraction of linoleate and linolenate. This advocates their high oxidative stability. Increase in the relative percentage of monounsaturated fatty acid (MUFA), i.e. oleic acid, *vis-a-vis* decrease in the relative percentage of polyunsaturated fatty acid (PUFA), i.e. linoleic and linolenic acids against the control was observed in all the four microalgae under the stress conditions studied. Rise in oleic acid is desirable for a good quality biodiesel. Amongst the four treatments nitrogen and phosphorous deprivation are found to be the most suitable stimulants for accumulation of high amount lipids with desired fatty acids for microalgal biodiesel production.

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