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Evaluation and optimization of two stage sequential *in situ* transesterification process for fatty acid methyl ester quantification from microalgae

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ABSTRACT

This study demonstrates a direct transesterification (DT) method for reliable quantification of microalgal lipid. Primary screening of various transesterification methods and the types of biomass (wet, oven dried and lyophilized) were performed with heterotrophically grown *Chlorella* sp. FC2 IITG which revealed two stage DT with lyophilized biomass using NaOH in first stage and H_2SO_4 in second stage as the best combination with fatty acid methyl ester (FAME) yield of 39.17% (w/w, dry cell weight). Further optimization of transesterification parameters for selected method using response surface methodology, predicted the optimum values for catalyst to biomass ratio 0.67 (w/w) and 2.07 (v/w), methanol to biomass ratio 49.51 (v/w) and 61.07 (v/w) and reaction time 19.33 (min) and 10 (min) for first and second stages respectively. The optimum conditions showed 462.6% and 445.4% increment in FAME yield when compared with Bligh and Dyer method for *Chlorella* sp. FC2 IITG and *Chlorella sorokiniana* FC6 IITG respectively with highest transesterification efficiency of 98.96%. Improved transesterification efficiency of two stage DT was attributed to efficient destabilization of cell wall as confirmed by scanning electron microscopic imaging. FAME produced via DT of *Chlorella* sp. FC2 IITG satisfied most of the biodiesel properties as per ASTM D6751 and hence, could be an alternative to petro-diesel.

1. Introduction

Microalgae based biodiesel production has gained significant interest as a potential alternative source that can effectively fulfill the energy requirements due to its competitive combustion efficiency with less sulfur emission and carbon negative properties [1]. Innate abilities of the microalgae such as higher specific growth rate, greater photosynthetic efficiency than plants and the ability to grow in marginal lands, marine and waste waters has made them a promising feedstock for biodiesel generation [2].

Under optimal cultivation conditions, many microalgal species are able to accumulate up to 50–70% (w/w) of lipid in dry biomass [2]. Lipid content of microalgae reflects energy yield from the biomass and hence, has largest impact on overall process economy for biodiesel production [3]. Therefore, accurate and reliable quantification of microalgal lipid content is the key towards selection of suitable algal strains, optimizing growth condition and process monitoring. The traditional method of lipid estimation proposed by Bligh and Dyer [4] relies on extraction of intracellular lipid from algal biomass using chloroform and methanol. However, this method of lipid estimation may suffer from various limitations: (1) incomplete extraction of lipid from algal biomass, (2) extraction is dependent on the polarity of the solvent as well as composition of the algal lipids [5,6] and (3) possibility of simultaneous extraction of non-saponifiable compounds such as pigments [6]. It has been demonstrated that fine tuning of process parameters of Bligh and Dyer method resulted in increased lipid yield, pointing towards incomplete extraction of the original method [7,8]. Further extraction of the oil from algal biomass remains the major challenge in the overall process due to the rigid cell wall structure and smaller size of the algal cells [9]. Due to lower efficiency of

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conventional extraction method, various physical and chemical pretreatment methods were incorporated in the extraction procedure, which augmented further expenses without considerable increase in extraction efficiency. These methods involve large amount of solvents such as hexane, chloroform, and methanol causing adverse effects on health and environment [10]. Therefore, there is a need to develop an efficient and reliable method for laboratory scale quantification of algal lipid eliminating separate extraction step.

Use of *in situ* transesterification or direct transesterification (DT) of the algal biomass for converting lipids into biodiesel may be a viable option which can bypass expenses of extraction and the harmful effects of the solvents used in conventional methods over the environment [11]. Johnson and Wen [9] demonstrated that one step DT of *Schizochytrium limacinum* yielded higher amounts of FAME than conventional extraction based methods. A sequential two stage DT method with higher FAME yield was reported for three different algal strains [12]. However, development of efficient and rapid DT method is evolving in the literatures. Moreover, the DT methods reported in the literatures need to be further optimized to increase transesterification efficiency. To that end, a reliable DT method with increased hydrolysis and FAME conversion efficiencies need to be designed, which is a prerequisite for selection of strains with high lipid content. Patil et al. [13] reported the application of response surface methodology (RSM) to optimize the effect of catalyst concentration, dry biomass to methanol ratio and reaction time involved in microwave assisted DT to increase the FAME yield. Heterogeneous acid (hetero polyacid) catalyzed transesterification conditions were statistically optimized using RSM and 96% of transesterification efficiency was achieved in the optimized method [14]. Similarly, the effects of reaction temperature, catalyst amount and oil to methanol molar ratio on FAME yield were optimized using RSM for the production of animal fatty acid methyl esters [15].

The present research reports the quantification of algal lipid via DT of whole biomass (a) to eliminate separate step for lipid extraction; (b) to be applicable to various microalgal strains and; (c) to improve transesterification efficiency. Initially, array of 24 experiments were conducted to select the best combination of transesterification and the type of biomass (wet, oven dried and lyophilized biomass). Each biomass type was then tested against eight different transesterification methods to screen the best combination in terms of FAME yield. The combination with maximum FAME yield was further optimized using statistically designed RSM experiments. The process parameters: catalyst to biomass ratio, methanol to biomass ratio and reaction time were optimized for maximizing the transesterification efficiency. Finally, suitability of microalgal FAME as biodiesel was evaluated by comparing its properties with ASTM Specifications for Biodiesel Fuel (B100).

2. Materials and methods

2.1. Algal culture and biomass preparation

Seed culture of *Chlorella* sp. FC2 IITG [16] and *Chlorella sorokiniana* FC6 IITG was grown in a 250 mL flask containing 100 mL BG11 medium [17]. The flasks were incubated in an orbital shaker with continuous shaking at 150 rpm, temperature 28 °C and illumination of 20 μmol photons m^{-2} s^{-1} with a light: dark cycle of 16:8 h. Mid log phase grown seed culture was used as inoculum (1%, v/v) to obtain lipid rich biomass via heterotrophic cultivation of *Chlorella* sp. FC2 IITG and photoautotrophic cultivation of *C. sorokiniana* FC6 IITG in a 5.0 L automated bioreactor (Biostat B Plus, Sartorius Stedim Biotech, Germany) containing 3.0 L BG11 medium and grown at temperature 28 °C, agitation 400 rpm and aeration 1 vvm. Under heterotrophic cultivation BG11 medium was supplemented with 15 g L^{-1} of glucose as the sole source of energy and carbon [18]. The pH was maintained at 7.4 through addition of 0.25 M NaOH/HCl. Under photoautotrophic condition 1% (v/v) CO_2 mixed with air was used as carbon source and illumination of 20 μmol photons m^{-2} s^{-1} with a light: dark cycle of 16:8 h was used as energy source. Once the culture reached the stationary phase, cells were harvested by centrifugation at 8000 × *g* at 4 °C for 10 min. The pellets were washed twice in saline (0.85%, w/v NaCl) and frozen immediately at −80 °C. The frozen cells were directly used as wet algal biomass (water content 80%, w/w) whereas the cells dried in a hot air oven at 80 °C for 24 h were used as oven dried algal biomass (water content 0%, w/w). The cells were lyophilized for 12 h at 0.08 mtorr vacuum to obtain the freeze dried algal biomass (water content ~33%, w/w). Depending upon the experimental designs, different biomass type in the form of lyophilized or oven dried or wet biomass was used for transesterification. All the screening experiments and DT optimization were performed in the heterotrophic biomass of *Chlorella* sp. FC2 IITG and the evaluation of the optimized method was carried out in photoautotrophic biomass of *C. sorokiniana* FC6 IITG.

2.2. Selection of best combination of transesterification method and biomass type

Eight different transesterification methods were carried out to screen the best combination of transesterification method and type of biomass for FAME production (Fig. 1). While M1 represents conventional method of lipid extraction proposed by Bligh and Dyer [4] followed by transesterification, the remaining seven methods (M2–M8) represent *in situ* transesterification and their combinations in one or two stage process. All methods were carried out with three different types of algal biomass which includes wet, oven dried and lyophilized biomass to evaluate the effect of biomass on transesterification. Wet biomass of 100 mg and its equivalent lyophilized biomass of 30 mg and oven dried biomass of 20 mg were used in each method. In case of Bligh and Dyer method (M1), 3.75 mL of 1:2 chloroform: methanol was added to 100 mg wet biomass, and sonicated at 30% amplitude for 30 s. Additional chloroform of 1.25 mL was added and sonicated once again followed by addition of 1.25 mL distilled water. The two phase systems were separated through centrifugation. However, extraction of lipid from lyophilized and oven dried biomass was carried out with extra addition of 0.8 mL and 0.7 mL of distilled water respectively, to make the final volume equal in all three types of biomass for the method M1. All the transesterification experiments were performed with methanol as the solvent and acid (H_2SO_4 or HCl) and/or base (NaOH) as catalysts, at a temperature of 90 °C and a constant shaking of 150 rpm in a water bath. Glyceryltriheptadecanoate (C17 triacyl glycerol from Sigma Aldrich, USA) was used as the internal standard to assess the transesterification efficiency of each method. The transesterification efficiency was calculated as follows:

$$\text{transesterification efficiency}(\%) = \frac{\text{amount of C17 methyl ester obtained}}{\text{amount of C17 triacyl glycerol added}} \times 100$$

In the conventional method (M1) the extracted algal oil was treated with 1 mL of 0.5 N NaOH in methanol for 40 min, whereas in one step DT method, algal biomass was directly treated with 1 mL of 0.5 N NaOH in methanol (M2), 1 mL of 5% HCL (v/v) in methanol (M3) and 1 mL 5% (v/v) H_2SO_4 in methanol (M4) for 40 min (Fig. 1). In the two step DT methods, the sequential treatment of algal