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Biocatalytic conversion of lipids from microalgae *Scenedesmus obliquus* to biodiesel using *Pseudomonas fluorescens* lipase

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HIGHLIGHTS

- Biocatalytic biodiesel conversion of microalgal lipids is greener and sustainable.
- Microalgal feedstock *Scenedesmus obliquus* was cultivated in an open circular pond.
- Immobilized *P. fluorescens* lipase showed better conversion among selected lipases.
- Immobilized *P. fluorescens* lipase can be reused for 4 repeated batches.
- Fuel properties of biodiesel showed compliance with the international standards.

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ABSTRACT

Conversion of microalgal lipids using biocatalyst is a novel and greener approach to produce biodiesel. Free and immobilized lipases from *Candida* sp. and *Pseudomonas fluorescens* along with free lipases from porcine pancreas and wheat germ were screened for biodiesel conversion of *Scenedesmus obliquus* lipids. Among selected lipases from various sources immobilized lipase from *P. fluorescens* showed superior biodiesel conversion. Optimization of reaction parameters viz. lipase amount, temperature, methanol to oil molar ratio and water content was carried out using response surface methodology. Best conversion of 66.55% was achieved at 35 °C, methanol to oil ratio of 3:1 with 10% enzyme amount and 2.5% water content based on oil weight. To tackle methanol tolerance step-wise methanol addition was applied, which improved biodiesel conversion upto 90.81%. Immobilized *P. fluorescens* lipase can be used for 4 batches without much loss in conversion efficiency (>95%). Biodiesel produced has the cetane number of 51.77, Calorific value of 37.67 MJ kg^{-1}. Most of the fuel properties of biodiesel met the specifications set by ASTM and EN standards.

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1. Introduction

Microalgal biodiesel has shown promising potential of alternate fuel in the light of dwindling petroleum fuel sources and food security concerns. Biodiesel is a mixture of monoalkyl esters of fatty acids derived from natural resources like vegetable oils, animal fats and microalgae. The most widely accepted method for synthesis of biodiesel is transesterification of oils using an alkali catalyst and methanol as the acyl acceptor [1,2]. Microalgae, the third generation feedstock have shown immense potential for biodiesel production compared to the previous feedstocks (food and non-food crops). Microalgae can accumulate substantial amounts of lipids and can be grown in marginal lands without competing with food crops. Use of wastewater as the nutrient medium, CO_2 sequestration and other value-added products adds to the benefits provided by microalgae [3–5].

In general, biodiesel can be synthesized from wide range of feedstocks like edible and non edible oils, animal fats, waste-cooking oil, microalgae, etc. Biodiesel can be used directly or blended with the diesel fuel for its application in compression ignition (CI) engines. Biodiesel has shown comparable calorific value to that of the diesel fuel and thus it can be directly used in CI engines with few modifications if required [1]. Biodiesel is synthesized by the process called transesterification where feedstock oils are converted into fatty acid acyl esters (FAAE) in presence of acyl acceptor (Mostly short chain alcohol) and catalyst. Catalysts used for transesterification includes homogeneous and heterogeneous chemical catalyst and enzyme (Lipase). Chemical catalyst and methanol as acyl acceptor are commonly used for transesterification process for biodiesel synthesis [1,2].

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Although the chemical conversion method has been widely used for biodiesel synthesis, it has some challenges: additional neutralization and product purification steps; high energy requirements and environmental concerns due to wastewater generation during downstream processing. On the other hand, the biocatalytic method offers the advantages of a high quality product which reduces downstream processing steps, less wastewater generation and energy consumption [6–8]. Lipase-catalyzed conversion of feedstock oils to biodiesel is greener and a sustainable approach because of its environmentally-benign nature and low energy requirements. High specificity and activity result in high quality pure products which need fewer purification steps for further processing. Lipases have been employed by many researchers as a catalyst for transesterification of various feedstocks for biodiesel synthesis [9–12]. Unlike alkali catalyst, lipases can be used for conversion of feedstock oils with high free fatty acids (FFA) [13]. Microalgal lipids have high FFA content, thus using lipase can provide significant advantages over the conventional conversion process using acid or alkali catalysts. Challenges faced when using lipases are its high cost, inhibition by short chain alcohols and slow reaction rate compared to chemical catalysts [7]. Enzyme-catalyzed biodiesel production is still under assessment stage for development of efficient process to overcome cost and inhibition constraints. There is comparatively sparse literature available about lipases being employed for conversion of microalgal lipids.

Conversion yields can be improved by screening suitable lipases from various sources and optimization of process parameters for selected lipases. Immobilization of lipase is a possible solution to reduce the cost, as immobilized lipase can be easily separated and reused [14]. Adding methanol to stoichiometric molar ratio or step-wise addition of methanol can overcome the methanol inhibition of lipases [8,15]. Another approach is to carry out reactions in suitable solvents which reduce the inhibitory effect by methanol [16]. Inhibition of lipase activity caused by methanol can be alleviated by step-wise addition of methanol to reaction mixture. For the optimization studies of lipase-catalyzed biodiesel synthesis, response surface methodology (RSM) has been successfully applied [17,18]. RSM studies not only predict optimized levels of selected parameters but also provide information about the extent of parameter's influence on the process.

Fuel properties of biodiesel have to meet specifications set by international standards for its successful implementation [1]. There are very few studies that report the fuel properties of biodiesel produced from microalgal feedstock. Fatty acid composition of lipids influences the fuel properties like oxidation stability, cold flow properties, cetane number, iodine value etc. ASTM 6751 and EN 14214 standards has set specifications for fuel properties of biodiesel.

This study explores the application of extracellular lipase for the conversion of microalgal lipids to biodiesel. *Scenedesmus obliquus* grown in open circular ponds was selected as the microalgal feedstock. Lipids extracted from *S. obliquus* were characterized. Lipases from selected sources were screened for their catalytic performance. The selected lipase was chosen for optimization of reaction parameters using response surface methodology. A three level four factor Box-Behnken experiment design was employed for response surface methodology optimization. Effect of various reaction parameters on the lipase-catalyzed biodiesel conversion was studied. Step-wise addition of methanol was investigated to observe further improvement in the biodiesel conversion. Reusability of the selected lipases was also evaluated. The resulting biodiesel product was characterized for its fuel properties and compared with standard specifications. To best of our knowledge there are not many studies for enzymatic conversion of microalgal lipids, and none of the previous study investigates this greener approach thoroughly as carried out in this study.

2. Material and methods

2.1. Chemicals and reagents

The free and immobilized lipases were obtained from Sigma–Aldrich. *Pseudomonas fluorescens* and *Candida rugosa* lipases immobilized on immobead 150 (Sigma–Aldrich, Netherlands) were used as immobilized lipases. Free lipases used were Amano lipase from *P. fluorescens* (Amano, Sigma–Aldrich, Japan), lipase from *Candida antarctica* (Sigma–Aldrich, Germany), lipases from porcine pancreas and wheat germ (Sigma–Aldrich, USA). A mixed fatty acid methyl ester (FAME) standard (37 components) and methyl heptadeconoate were obtained from Sigma–Aldrich, USA. All organic solvents and other chemicals purchased from Sigma–Aldrich were of analytical grade.

2.2. Biomass production and lipid extraction

Microalgal cultivation for biomass production and lipid extraction was done as described in our previous research [4]. *S. obliquus* FR751179.1 was grown in open circular pond (8000L) on BG11 nutrient medium. *S. obliquus* showed biomass yield of 1.16 g L^{-1}. Lipids were extracted from freeze dried biomass using microwave assisted solvent extraction. Solvents used were chloroform and ethanol in 1:1 (v/v) ratio. Acid value of extracted lipids was determined by ASTM method (D664-07). Lipids extracted from *S. obliquus* were used for lipase conversion study. Lipid content observed was 29% lipid g^{-1} of dry biomass, while acid value of lipid was 21 mg KOH g^{-1}.

2.3. Screening of lipases

Selected free and immobilized lipases (free lipases from *P. fluorescens*, *C. antarctica*, porcine pancreas and wheat germ and immobilized lipases from *P. fluorescens* and *C. rugosa*) were used for transesterification of *S. obliquus* lipids. Reaction conditions were: temperature, 40 °C; methanol to oil molar ratio, 4:1; water quantity, 5% of oil weight; enzyme concentration, 10% by oil weight with immobilized lipase and 1% by oil weight with free lipase based on lipase activity. For all the conversion experiments 0.1 g of *S. obliquus* lipids was used as feedstock. Solvent n-hexane was added to the reaction mixture to provide proper mass transfer. Samples were taken out at regular interval from the reaction mixture and analyzed using gas chromatography to determine percentage fatty acid methyl ester (FAME) conversion. Gas chromatography conditions were described in our previous research [4]. Methyl heptadeconoate was used as an internal standard and the 37 component FAME mix was used for peak identification. FAME conversion was calculated using formula by Lee et al. [19].

$$\text{FAME Conversion } (\%) = \frac{\sum \text{TA} - \text{IA}}{\text{IA}} \times \frac{C_I \times V_I}{m} \times 100 \quad (1)$$

where $\sum$TA: total area of peaks from C14 to C24, IA: peak area of internal standard, C_I: concentration of internal standard (mg L^{-1}), V_I: volume of internal standard (ml) and m: mass of biodiesel sample (mg). Based on FAME conversion and time required, a lipase was selected for further optimization studies.

2.4. Optimization of process parameters

A three level four factorial Box-Behnken response surface methodology experimental design using Minitab Statistical Software was employed in this study, comprising of 27 experiments with 3 replicates at centre point. Optimization of reaction conditions for lipase-catalyzed transesterification of *S. obliquus* lipids was

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