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Biodiesel production via lipase catalysed transesterification of microalgae lipids from *Tetraselmis* sp.

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ABSTRACT

Tetraselmis sp. is a green marine microalgae and known to produce lipids that can be transformed into biodiesel. The influence of nitrate concentration (0.00 g/L, 0.10 g/L, 0.14 g/L and 0.18 g/L) on the growth rate of *Tetraselmis* sp. was investigated. The marine microalgae were harvested during the exponential phase and lipid was extracted by chloroform-methanol solvent and quantified using Nile Red method. The conversion of lipid to biodiesel was performed via i) alkali-based transesterification reaction which utilized sodium hydroxide (NaOH) and ii) enzyme catalysed transesterification process which utilized immobilized lipase. The fatty acid methyl esters (FAME) components were identified using gas chromatography (GC) and then compared with the FAME standard. The results revealed that 0.18 g/L nitrate concentration was the optimal for cultivation of microalgae. However, the highest lipid content was achieved in the absence of nitrate (0.0 g/L). The biodiesel yield from the lipase catalysed transesterification process was 7 folds higher compared to the alkaline based transesterification.

1. Introduction

Recently, biodiesel from oil crops such as soy and palm, animal fat, waste cooking oil [1,2], bioethanol and other alcohols from sugarcane and corn starch, hydrogen long-chain hydrocarbons, and biogas [3] have attracted much attention as an important fuel option and coined as biofuel. However biofuel from food crops will require large areas of arable land and has to compete with the cultivation of food crops and thus is currently a huge controversial issue [4]. The biofuel utilization in the European Union is growing rapidly and great efforts were made in developing the technology [5]. For the last few decades, microalgae have been recognized as a potential source of sustainable substrate to produce biofuel due to the numerous advantages such as rapid growth potential and biomass generation, higher photosynthesis efficiency and no requirement for large arable land area [6–8]. Microalgae can be cultivated near to sewage or next to power plant smokestacks so as to digest the pollutants and generate lipid. In the late 1990's, some microalgae were discovered to produce neutral lipids from carbon dioxide during photosynthesis process with efficiency of 30 times more than that of plants in terms of the amount of neutral oil produced per unit area of the land allocated [9]. In the cultivation of microalgae the amount of nitrogen is an important factor to be addressed because it is an important component of amino acids, as well as being vital for chlorophyll hence is likely to promote microalgae growth. Previous studies have shown that under nitrogen (nitrate) limitation conditions, nitrogen metabolism in the algal cells decreases, contributing to the enhancement of the synthetic activities of secondary carotenoids and other non-nitrogen compounds. The nitrogen-deficient condition increases lipid content due to the lack of $NaNO_3$ which limits protein biosynthesis, reduce biomass of microalgae but promotes lipid/protein ratio [10].

Biodiesel is a mixture of fatty acid methyl esters (FAMEs) which is produced from the transesterification of lipids and is rapidly gaining acceptance by the majority as an alternative source of energy for the future [11]. In production of biodiesel from marine microalgae, the low yield from the alkali-catalysed transesterification is one of the major challenges faced.

It is also believed that the lipase- catalysed transesterification can improve the yield of biodiesel. Lipases can be categorised into three classes based on their specificity or selectivity such as regio- or positional specific lipases; fatty acid type specific lipases and specific lipases for a certain class of acylglycerols (mono-, di- or triglycerides). Several examples are lipases from *Pseudomonas fluorescens, Pseudomonas cepacia, Candida rugosa, Candida antarctica, Candida cylindracea, Rhizopus oryzae* and *Mhizomucor miehei*.

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Enzymes can be linked to insoluble matrices by a variety of methods such as adsorption, covalent coupling and entrapment; a process called immobilization which can provide stability to enzymes. Stability has always been an issue during transesterification, especially when the media is predominantly non-aqueous. In aqueous media, where enzymes are freely soluble, immobilization provides a method to recover the biocatalyst by centrifugation or filtration. In anhydrous media, enzyme molecules though mostly insoluble, tend to clump together. Thus, immobilization is a suitable method to increase the surface area of the biocatalyst.

For the last few decades, there were many reports on biodiesel production using lipase as biocatalyst [12–17] but most of these studies investigated the use of lipase catalysed transesterification for biodiesel production from vegetable oils such as sunflower, jatropha, soybean and palm oil. In some of these applications the lipases were immobilized in various supports such as acrylic resin, textile membrane, polypropylene, celite, diatomaceous earth, polyglycidylmethacrylate beads [15,16]. Enzymatic catalysed transesterification requires mild environment and can tolerate the presence of water and free acids in crude oil. In addition the recovery of glycerol and fuel purification is minimized and low amounts of waste is produced which overcomes the negative effect of alkaline catalysed transesterification [14]. Nearly all of the research focused on the use of lipase catalysed transesterification for vegetable oils for biodiesel production and to the best of our knowledge no work has been reported on the use of lipase catalysed transesterification for microalgal oils to biodiesel.

Thus in this study biodiesel from microalgae (*Tetraselmis* sp.) lipids was synthesized using lipase- catalysed transesterification. In order to reduce cost and increase life span of enzymes the lipases were immobilised in alginate beads thus allowing recycling of the enzymes. Alginate is a common immobilization matrix and has been used successfully for the immobilization of lipases [18–21] and in biodiesel production [22]. In addition, biodiesel was also synthesized by conventional alkali transesterification method under the same temperature and pH so as to compare their production yields.

2. Materials and methods

2.1. Microalgae cultures and medium

The marine microalgae strains were bought originally from Algae Tech (Ltd). *Tetraselmis* sp. was maintained in F/2 medium which contains 0.075 g $NaNO_3$; 0.005 g $NaH_2PO_4.H_2O$; 0.023 g $ZnSO_4.7H_2O$; 0.152 g $MnSO_4.H_20$; 0.0073 g $Na_2M0O_4.2H_2O$; 0.014 g $COSO_4.7H_2O$; 0.0068 g $CuCl_2.2H_2O$; 4.6 g $Fe(NH_4)_2(SO_4)_2.6H_2O$ and 4.4 g $Na_2EDTA.2H_2O$ per litre; 0.0005 g Biotin; 0.022 g Thiamine and 0.0027 g Vitamin B_{12} per 20 ml. *Tetraselmis* sp. was stored in a 2 L conical flask as a batch culture at 25 °C ± 0.5 °C, pH 7.8 ± 0.2 and was exposed to continuous illumination.

2.2. Cultivation of marine microalgae under different nitrate concentration

The marine microalgae, *Tetraselmis* sp. was cultivated in F/2 medium with 10% starting inoculum taken from stock culture. The marine microalgae, *Tetraselmis* sp. was cultivated at ambient temperature (27 °C) and pH 7 with aeration condition as the control growth environment. The marine microalgae were also cultivated under different nitrate concentrations (0.00 g/L, 0.10 g/L, 0.14 g/L and 0.18 g/L). The marine microalgae growth was monitored in terms of turbidity using the optical density (OD) method. Samples were taken every two days for twenty-two days where OD readings were taken using a UV–vis spectrophotometer (Shimadzu UVmini-1240) at a wavelength of 620 nm. The lipid content was measured using the Nile Red method [23]. The biomass measured by the spectrophotometer only indicated an absorbance value, thus in order to obtain a more accurate *Tetraselmis* sp. growth data, cell dry weight measurement was performed simultaneously. 2 ml of the sample was collected from each different nitrate concentration cultures and was placed onto aluminium foil. The sample was then placed in the incubator at 60 °C and then left overnight. After one day, the sample weight was measured. Each experiment was performed in triplicates for the different nitrate concentrations so as to ensure reproducibility of data.

2.3. Measurement of lipid – Nile red staining method

Lipid content was determined rapidly using the Nile red staining method by Perkin Elmer LS-55 fluorescence spectrophotometer. Nile red (NR, 9-diethylamino-5H-benzo[α] phenoxa-phenoxazine-5-one) was diluted to 0.5 mg/mL in acetone [23]. An aliquot 1 mL of the culture broth was taken from the conical flasks and centrifuged at 2000 rpm for 5 min. The supernatant was discarded and the pellet was washed with 1 mL of phosphate buffer saline (PBS) followed by centrifugation at 2000 rpm for 5 min. This process was repeated for 3 times. The sample was diluted for 1000 fold (10^3 dilution) and an aliquot of 2.8 mL was withdrawn from the test tube and added with 1.8 μL of Nile Red solution and then transferred into a cuvette. The solution was kept in the dark for 20 min to achieve full-staining. The excitation and emission wavelength was set at 566 and 600 nm respectively; whereas excitation and emission slit width were set at 10.0 nm. The fluorescent intensity was then recorded.

2.4. Lipid extraction

100 mL of sample was withdrawn from the culture bottle when the growth of cultivated microalgae reached the exponential phase and the lipid extraction was performed according to Bligh and Dyer method [24]. The sample was centrifuged repetitively at 4000 rpm for 15 min. The biomass was washed with 20 mL of distilled water was freeze-dried overnight at −40 °C under vacuum. 1 g of the dried sample was grinded by a mortar and pestled into powder form and dissolved in 100 mL distilled water. Cell disruption was performed by microwave oven at 100 °C, 2450 MHz for 5 min and subsequently dissolved in 30 mL solvent mixture of chloroform and methanol (ratio of 2: 1). 10 mL of lysozyme (1 %w/v) was then added into the solution so as to disrupt the extracellular cell wall and then sonicated for 10 min at (4 °C, pulse 0.7, time 2 min and amplitude 5%) so as to ensure complete lysis of the cell walls. The sonicated mixture was left for 24 h to allow the formation of bilayers which consists of a methanol upper layer and chloroform lower layer. The chloroform layer containing the extracted lipid was then passed through a filter paper for 2 or 3 times so as to ensure that any residual algal biomass from the extract was totally removed. The lipid was then placed in a rotary evaporator at 50 °C under vacuum for 15 min so as to remove any remaining solvent. The crude microalgae oil was stored at 4 °C for the following transesterification process.

2.5. Alkali-based transesterification

Methanol was mixed with 0.5 g of NaOH and stirred for 20 min at 400 rpm at approximately 65 °C. The ratio of methanol to oil in the mixture was kept to 6:1. The mixture of catalyst and methanol was then poured into the conical flask containing the algae oil to initiate the transesterification process. The conical flask was stirred continuously for 3 h at 300 rpm and then allowed to settle for 16 h