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Response surface methodology assisted biodiesel production from waste cooking oil using encapsulated mixed enzyme

Sirajunnisa Abdul Razack, Surendhiran Duraiarasan

Bioprocess Laboratory, Department of Chemical Engineering, Annamalai University, Annamalainagar, Tamil Nadu 608002, India

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ABSTRACT

In the recent scenario, consumption of petroleum fuels has increased to greater height which has led to deforestation and decline in fossil fuels. In order to tackle the perilous situation, alternative fuel has to be generated. Biofuels play a vital role in substituting the diesel fuels as they are renewable and ecofriendly. Biodiesel, often referred to as green fuel, could be a potential replacement as it could be synthesized from varied substrates, advantageous being the microalgae in several ways. The present investigation was dealt with the interesterification of waste cooking oil using immobilised lipase from mixed cultures for biodiesel production. In order to standardize the production for a scale up process, the parameters necessary for interesterification had been optimized using the statistical tool, Central Composite Design – Response Surface Methodology. The optimal conditions required to generate biodiesel were 2 g enzyme load, 1:12 oil to methyl acetate ratio, 60 h reaction time and 35 °C temperature, yielding a maximum of 93.61% biodiesel. The immobilised lipase beads remain stable without any changes in their function and structure even after 20 cycles which made this study, less cost intensive. In conclusion, the study revealed that the cooking oil, a residue of many dining centers, left as waste product, can be used as a potential raw material for the production of ecofriendly and cost effective biofuel, the biodiesel.

1. Introduction

Burgeoning population, surging globalization, soaring energy needs, rapid rise in oil prices and diminishing fossil fuels have urged on the biotechnologists to explore alternate fuels. To fulfill the incorrigible demand of petrofuels, biodiesel has been ascertained the most probable substitute. Biodiesel, generally referred to as fatty acid methyl esters (FAME), is a renewable, non toxic, sulfur-less, free from carbon dioxide and a clean fuel (Arumugam and Ponnusami, 2014; Leung et al., 2010). This could typically, be produced using vegetable oils, animal fats as feedstocks, that determine the generation of biofuels, through esterifying reactions aided by catalysts. Vegetable oils are potential substrates for yielding biodiesel since they are natural, renewable and ecofriendly but requirements of land for larger crop cultivation, deforestation and their high market values make them insatiable for larger biodiesel generation (Leung et al., 2010); fresh vegetable oils are also high-priced when compared to fossil fuels (Chhetri et al., 2008). Feedstock and biodiesel majorly account for 80% of the overall economy of the process (Chhetri et al., 2008; Kuan et al., 2013). Hence, waste cooking oil (WCO) could be a promising and cost effective candidate for synthesizing the biofuel, due to its low cost and availability elsewhere (Omar and Amin, 2011; Gnanaprakasam et al., 2013; Yan et al., 2014).

Commercially, biodiesel production is carried out by acid or alkali mediated transesterification of substrates to FAME. Though these conventional methods yield satisfactorily and require low reaction time, they possess their own disadvantages like difficult recovery of glycerol, low conversion rate, high-energy requirement, saponification and necessity of wastewater treatment (Rahimi et al., 2014; Jiang et al., 2014; Cervero et al., 2014; Lee et al., 2013; Rodrigues and Ayub, 2011; Kawakami et al., 2011). To overcome these difficulties, enzymatic mode has become a green and substantial means of biodiesel synthesis. Through this technique, easy recovery of by-product, elimination of salts and catalyst, higher yield under normal conditions and low temperature could be highly achieved (Lee et al., 2013; Gumbyte et al., 2011; Gharat and Rathod, 2013). Enzymatic biodiesel production has the ability to utilize low quality feedstocks like WCO with high FFA, which is another important criterion to perform using this method than that of the conventional (Kuan et al., 2013).

Lipase is one of the foremost and efficient enzymes implemented in enzymatic conversion of oil to FAME. Lipases could be utilized for the production since there is no production of soap, can be performed under milder conditions, ecofriendly and FAME

E-mail addresses: siraj.razack@gmail.com (S.A. Razack), suren_micro@yahoo.co.in (S. Duraiarasan)

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purification is easier than in conventional techniques (Zarei et al., 2014; Adachi et al., 2013). Lipases could be easily activated in the presence of oil water interface and has the capacity to maintain the catalytic activity in non-aqueous, biphasic systems and micellar solutions (Antczak et al., 2009).

However, biocatalytic esterification method has its own certain obstrucle like high cost of enzyme and slow reaction (Arumugam and Ponnusami, 2014). Recyclability of lipases is another major phenomenon that has to be considered essentially, which could be accomplished by immobilization. Various techniques to immobilise lipases had extensively been studied earlier (Modi et al., 2007; Kuan et al., 2013). In accordance with the report by Kuan et al. (2013), entrapment and encapsulation of the enzymes are more advantageous than any other immobilization method due to the prevalence of high mass transfer resistance.

Transesterification of oils to biodiesel is carried out in the presence of short chain alcohols such as methanol and ethanol. During the process, the prime by-product formed is glycerol, hindering the catalytic reactions and its downstream processing (Modi et al., 2007). Thus, interesterification using methyl acetate could be a better alternative due to fast reaction rates, high biodiesel yield and by-product being triacetin (triacetylglycerol), which does not deactivate lipase unlike glycerol. Moreover, triacetin is used commercially in polymers and explosives as gelatinizing agents and in tobacco, pharmaceutical and cosmetic industries as additives (Campanelli et al., 2010; Casas et al., 2011; Maddikeri et al., 2013). Moreover many researches are focused on finding optimal lipases to catalyze the biodiesel production. But natural oils are not homogenous substrates, as they are comprised of triglycerides with different fatty acids. Additionally, reaction mixture consists of triglycerides and free fatty acids, hence enzyme should own the ability to catalyze varied substrates; these make the finding of an optimal lipase tedious. Hence a mixture of different lipases with specific characters that act upon several substrates could act as an optimal biocatalyst (Rodrigues and Ayub, 2011; Garcia et al., 2011).

The two strains namely *Bacillus subtilis* and *Burkholderia cepacia* produce alkaline thermotolerant lipase which work at higher temperature in the ranges between 50 °C and 70 °C, more tolerant to organic solvents (Li and Yan, 2010; Sivaramakrishnan and Muthukumar, 2012) and can perform at high pH level (Dalal et al., 2008). Hence these two strains were selected to produce lipase for biodiesel synthesis. The main objective of the study of the present investigation is the enzymatic interesterification of WCO to biodiesel using mixed lipase from *B. subtilis* and *B. cepacia* (MTCC 1617), with methyl acetate as an acyl acceptor and optimizing statistically by Response Surface Methodology (RSM), based on 2 level 4 factor Central Composite Design (2^4 factorial design). These experiments let us learn the effect of each independent factor studied – biocatalyst loading, molar ratio, temperature and reaction time, and the interactive effects between the parameters on the dependent response variable. Central Composite Design (CCD) has the vantage of predicting the responses within the limits by varying the parameters simultaneously.

2. Materials and methods

2.1. Sample collection and pretreatment of WCO

Waste cooking oil (WCO) was purchased from local restaurant near Annamalai University campus, Annamalai Nagar. The WCO sample was filtered using a filter cloth to separate fried wastes in the oil. Then the filtered oil was washed with water to remove water soluble salts present in the oil sample. After that the WCO was heated at 110 °C in a beaker to evaporate the excess water. The saponification (SV) and acid value (AV) were analyzed for determining molecular weight of WCO (Sathasivam and Manickam, 1996; Xu et al., 2006). The molecular weight of waste cooking oil was found to be 855.68 g from the saponification value of 196.3 mg KOH g^{-1} and acid value of 1.68 mg KOH g^{-1}. The fatty acid compositions of WCO are palmitic acid (C16:0) – 6.73%, stearic acid (C18:0) – 4.11%, oleic acid (C18:1) 36.54% and linoleic acid (C18:2) – 52.62%.

2.2. Bacterial strains and culture medium

Two bacterial strains were used for lipase mediated biodiesel production (Fig. 1). *B. cepacia* (MTCC 1617) and *B. subtilis* were obtained from Department of Microbiology, Annamalai University, Tamilnadu, India. The two bacterial cultures were cultivated using 100 ml of two different nutrient broth consist of peptone (1.0), yeast extract (0.3), beef extract (0.3) and NaCl (0.3) (g/100 ml) and incubated at 37 °C for 24 h. These cultures were used as seed culture for lipase production.

2.3. Fermentation for lipase production

The lipase production was carried out in two different 250 ml Erlenmeyer flask using 100 ml basal medium containing 2% olive oil, 0.2% $CaCl_2{\cdot}2H_2O$, 0.01% $MgSO_4{\cdot}7H_20$, 0.04% $FeCl_3{\cdot}6H_2O$ and 0.3% NaCl. The contents were incubated for 48 h at 37 °C at 200 rpm. The pH was maintained at 7. After incubation, the cultures were centrifuge at 10,000 rpm for 10 min at 4 °C. The crude lipase (culture supernatant) from two bacterial cultures were mixed together and partially purified by 70% ammonium sulfate and kept at 4 °C for overnight. Then the precipitate was collected by centrifugation at 10,000 rpm for 30 min at 4 °C and it was used for immobilization.

2.4. Lipase assay and immobilization of partially purified lipase by encapsulation

Lipase activity was determined according to Burkert et al. (2004) and Padilha et al. (2012). The immobilization was carried by the encapsulation method using 2% sodium alginate. The partially purified lipase mixer was added with sodium alginate solution. The mixer was dripped into cold sterile 0.2 M $CaCl_2$ using sterile syringe from a constant distance and was cured at 4 °C for 1 h. The beads were hardened by suspending it again in a fresh $CaCl_2$ solution for 24 h at 4 °C with gentle agitation. After immobilization, the beads were separated by filtration and washed with 25 mM phosphate buffer (pH 6.0), to remove excess calcium chloride and enzyme.

2.5. Optimization of enzymatic biodiesel production using RSM

The production of biodiesel from WCO was developed and optimized using response surface methodology (RSM) provided by Design-Expert software version 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA). A standard RSM design tool known as Central Composite Design (CCD) was applied to study the catalyzed interesterification reaction variables. These experiments help in learning the effect of each independent factor and the interactive effects between the parameters on the dependent response variable. Central Composite Design (CCD) has the vantage of predicting the responses within the limits by varying the parameters simultaneously. Four independent variables were X_1: enzyme loading, X_2: oil to methyl acetate molar ratio, X_3: temperature and X_4: reaction time. The response chosen was fatty acid methyl ester (FAME) yields which were obtained from the reaction. Table 1 shows the ranges and levels of the four independent variables with actual