Optimization of parameters for the production of biodiesel from rubber seed oil using onsite lipase by response surface methodology

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Abstract

In the present study, we demonstrate the properties of rubber seed oil (RSO) and its potential application for producing biodiesel by lipase-catalyzed transesterification. Rubber seed contains 39.45% of oil on a dry weight basis. Lipase was secreted from novel bacterial isolate of *Pseudomonas aeruginosa* strain BUP2. Response Surface Methodology (RSM) *cum* Box–Behnken Design (BBD) was employed to optimize the combined effect of different independent parameters namely oil–methanol ratio, enzyme unit, reaction temperature and reaction time. Biodiesel yield of 99.52% was obtained in the validation experiments at the optimal level of lipase (750 U), methanol ratio (1:10), temperature (45 °C) and time (4 h). The fuel properties of biodiesel obtained under the validation condition met the specifications as mentioned in ASTM D6751 and EN 14214 standards. Biodiesel aliquots were characterized using thin-layer chromatography (TLC), gas chromatography (GC), fourier transform infra-red spectroscopy (FTIR) and elemental analysis. The present study demonstrates an important application of a potential substitute for fossil fuel from raw feedstocks with high economic value.

Keywords Rubber seed oil · *Pseudomonas aeruginosa* BUP2 · Transesterification · Lipase · Biodiesel · Response surface methodology

Introduction

The fast growth of global market, especially in industrialization and motorization, has led to sharp rise in the demand of petroleum fuels. The research and development group has great anxiety on the increase in the number of vehicles, depletion of petroleum fuels, dynamic fuel pricing and environmental concerns. Therefore, it is very essential to find out sustainable alternative substitutes to petroleum fuels such as biodiesel, biomethane, biogas, electric batteries, hydrogen cells, non-fossil natural gas and other biomass sources

(Sarkar et al. 2012). Among these, biodiesel seems to be more reliable and cost effective. Biodiesel refers to mono-alkyl esters or a mixture of fatty acid short-chain alcohol esters (methanol/ethanol). Usually, it comes from renewable sources such as plant or animal and as such it is biodegradable, non-toxic and sulphur free. Commonly, synthesis of biodiesel is done by transesterification processes. It has two basic routes: (i) chemical-catalyzed transesterification and (ii) enzyme-catalyzed transesterification. Compared to conventional chemical methods for biodiesel production, lipase-catalyzed transesterification is getting more attention these days due to its ability to carry out both esterification and transesterification simultaneously, for easiness in the separation of product from enzyme as well as from glycerol and decrease in the inhibition rate (Onoji et al. 2016b). Moreover, lipases are insensitive to the bulk of free fatty acid (FFA) in contrast to the chemical transesterifying agents. Recently, our group introduced a novel strain of *Pseudomonas aeruginosa* BUP2, a potent producer of lipase (Unni et al. 2016).

Lipases (EC 3.1.1.3) are a subclass of esterase that catalyze the breakdown of fats to glycerol and long-chain fatty acids and are produced by several organisms including

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bacteria, fungi and yeast, as well as animals and plants (Benjamin and Pandey 1996). In addition, lipase can involve in hydrolysis, esterification, inter-esterification, and transesterification reactions. Microbial lipases are widely used in industry because of its versatile catalytic activities, ease of genetic manipulation and high yield, coupled with exponential growth of the producing microorganisms in inexpensive media and absence of seasonal fluctuations (Priji et al. 2016). Many species of bacterial genera such as *Pseudomonas*, *Bacillus*, *Serratia*, and *Alcaligenes* and fungi such as *Aspergillus*, *Penicillium* and *Candida* are the best known producers of lipases (Jaeger and Reetz 1998).

In recent times, most of the biodiesel studies exploit the edible oil plants such as soybean, rapeseed, sunflower, safflower, oil palm and canola as substrates and these vie with food, pharmaceutical and cosmetic uses, resulting in high cost of living and high price of the biodiesel generated. The use of non-edible oils from the seeds of plants such as rubber, jatropha, castor, linseed, moringa, cotton, karanja, neem and tobacco is being proposed to avert any impending fuel-food crisis (Kumar and Purushothaman 2010). In India, *Jatropha* is the targeted feedstock in biodiesel production by chemical transesterification or using commercially available lipases, whose cultivation needs special attention due to conversion of agricultural land. Raw materials account for about 70–90% of the production cost of biodiesel and producing biodiesel from the non-edible seed oils will ultimately reduce its price. The aforesaid background highlights the importance of rubber tree, being the least investigated raw material source for biodiesel production.

Hevea brasiliensis (para rubber tree) is a perennial tree belonging to the family *Euphorbiaceae* and is native to Brazil. It is primarily cultivated for the production of the milky latex as a source of natural rubber, while the seeds are ancillary. India is the sixth largest producer and fourth largest consumer of natural rubber in the world. In India, rubber plantations are highly concentrated in the state of Kerala (approximately 80%). A rubber plantation is estimated as able to produce about 2 tonnes of rubber seeds per hectare per year (Tea et al. 2002). Commercially, several methods are employed for oil extraction such as mechanical pressing, solvent extraction and soxhlet extraction. Recent studies show that soxhlet extraction method could be practiced at industrial level, by dint of its higher oil yield, low operating cost and shorter time. Rubber seeds contain significant amount of oil (35–50 wt%). RSO has several industrial applications and is mainly used in paint and coating formulations as semidrying oil (Aravind et al. 2015), as biolubricant, in hydraulic oil formulations (Yang et al. 2014; Kamalakara et al. 2013) and in production of oleochemicals (Hosamani and Katagi 2008). Other uses include biodiesel synthesis (Reshad et al. 2015), cosmetic (Nian-Yiana et al. 2014)

pharmaceutical products (Takase et al. 2015) and alkyd resin and polyurethane biocomposite production (Onoji et al. 2016a; Bakare et al. 2010; Ikhuoria et al. 2007).

Conventional single-factor at-a-time strategy for optimizing multiple parameters to obtain a suitable condition with the maximum yield is a tedious task since large number of combinations would be involved in the process. Therefore, the present study is employing process designs software Minitab 14 (USA). Response surface methodology (RSM) plus Box–Behnken design (BBD) is a technique being employed for the optimization, modeling and analysis of production of biodiesel (Faisal et al. 2014). Box–Behnken design (BBD) is one of the most common designs in RSM studies, which in combination with RSM has been applied here for the optimization of different variables so as to attain the maximum catalytic efficiency in the process of production of biodiesel. To the best of our knowledge, there are no reports on the production of biodiesel with reference to RSO having high FFA using partially purified onsite lipase. Thus, this study will be very helpful to develop a self-sustained system for the production of biodiesel with an industrial perspective from non-edible and untapped rubber seed oil (RSO) by lipase-mediated transesterification using low-cost microbial lipase.

Materials and methods

Materials

Analytical- and bacteriological-grade chemicals were procured from Himedia (India) and Merck (India) Ltd. Fresh rubber seeds of RR II 105 cultivar were collected from the local rubber plantation at University of Calicut (11.1340°N, 75.8952°E).

Oil extraction and characteristics of rubber seed oil

Collected seeds were decorticated and cleaned manually to free the kernel. Subsequently dried at 80 °C for 3 h in an oven and stored in air-tight containers to avoid contamination. Forty grams of flour was bagged in a porous tissue paper and placed in a soxhlet extraction thimble, containing 150 ml of n-hexane as extraction solvent and boiled for 3 h (Mohd-Setapar et al. 2014). The yield of oil content by soxhlet process was calculated in percentage using Eq. (1). The oil was recovered using a rotary evaporator and kept in an oven at 70 °C for 1 h. Collected oil was filtered using 0.2-µm syringe filter to remove solid impurities. Subsequently, standard methods of ASTM D6751 were performed to determine the physicochemical properties of RSO with acid value, iodine value, saponification value, density and viscosity:

$$\text{RSO yield (wt\%)} = \frac{\text{Mass of RSO collected (g)}}{\text{Mass of rubber seed kernel used (g)}} \times 100 \quad (1)$$

Fatty acid profile of RSO

RSO sample (1 μl) was diluted with laboratory grade n-hexane (9 μl) and injected (220 $^{\circ}\text{C}$) in splitless mode into a gas chromatograph (Agilent, 7697 A, USA) equipped with a DB-WAX capillary column (Agilent, 30 m $\times$ 250 μm , 0.25 μm film thickness) and mass spectrophotometer (Agilent, 5973N). Helium was used as the carrier gas with the flow rate of 1 ml min^{-1} . The program was set from 50 $^{\circ}\text{C}$ at an interval of 5 min, rising to 65 $^{\circ}\text{C}$ at a rate of 2 $^{\circ}\text{C min}^{-1}$. Then it was raised at the rate of 5 $^{\circ}\text{C min}^{-1}$, to reach 200 $^{\circ}\text{C}$ and held for 5 min. Finally, the temperature was increased at the rate of 10 $^{\circ}\text{C min}^{-1}$ so as to reach 250 $^{\circ}\text{C}$ and maintained for 10 min. The spectrum analysis of compounds was done using Wiley 7n.1 database.

Microorganism and medium

Pseudomonas aeruginosa BUP2; a novel Gram-negative, rod-shaped aerobic rumen bacterium, was used for this study (Unni et al. 2015). Mother cultures were maintained in semi-synthetic BUP medium; which comprises of the following ingredients (g/l): 5 NH_4NO_3 ; 5 peptone; 4 $(\text{NH}_4)_2\text{SO}_4$; 3 beef extract; 2 K_2HPO_4 ; 2 NaCl ; 0.1 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 L-cysteine hydrochloride in double-distilled water. The medium was autoclaved at 15 psi, at 121 $^{\circ}\text{C}$ for 15 min, and supplemented with pre-sterilized groundnut oil (1%, v/v) as an inducer. Subsequently, the 24 h-old culture (2%) was inoculated into autoclaved BUP medium and incubated on an orbital shaker (Inlabco, India) at 28 $^{\circ}\text{C}$ and 150 rpm for 24 h.

Purification by ammonium sulphate precipitation

The turbid culture was centrifuged at 12,000 $\times g$ at 4 $^{\circ}\text{C}$ for 12 min and collected the supernatant as crude lipase. Actual quantities of ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$ were gradually added to the crude lipase, keeping on magnetic stirrer in a cold room (4 $^{\circ}\text{C}$) and stirred for 30 min so as to reach 20% of saturation and the precipitate was collected by centrifugation (9400 $\times g$ for 10 min at 4 $^{\circ}\text{C}$). Subsequently, the process was continued up to 20–40%, 40–60% and 60–80% of salt saturations, and the precipitates were collected separately in each step. The pellets were collected in minimum volume of 0.1 M Tris-HCl buffer (pH 8.0), subsequently dialyzed (dialysis membrane-60, Himedia) against 0.1 M Tris-HCl buffer

(pH 8.0) for 24 h at 4 $^{\circ}\text{C}$ under continuous stirring with two buffer changes at an interval of 12 h. Then the dialysate was centrifuged (9400 $\times g$ for 10 min at 4 $^{\circ}\text{C}$) and the clear supernatant was collected and store in the refrigerator.

Lipase assay

Lipase activity of each dialysate fraction was assayed using modified method of Gupta et al. (2002) as briefly described hereunder. The assay tube containing 1.98 ml of 0.1 M Tris-HCl buffer with 0.15 M NaCl and 0.5% Triton X-100 was pre-incubated with 20 μl of purified lipase at 37 $^{\circ}\text{C}$ for 10 min and subsequently, 20 μl of substrate (50 mM *p*NPP in acetonitrile) was added to the reaction mixture at 37 $^{\circ}\text{C}$ for 30 min. The intensity of light absorbed by the reaction mixture (chartreuse yellow color) was monitored using UV-visible spectrophotometer (Shimadzu, Germany) at λ_{405} . The lipase activity was calculated using Eq. (2). From these, the best active fractions were used for transesterification reactions.

$$\text{Lipase activity (U/ml)} = \frac{A \times V_f}{V_s \times \epsilon \times t \times d} \quad (2)$$

where A represents absorbance at λ_{405} ; V_f is the final volume of assay buffer, V_s is the volume of lipase added (ml), ϵ is the extinction coefficient of enzyme (0.017), t is the time of hydrolysis, and d is the diameter of cuvette (1 cm).

Transesterification reaction

Experimental setup

The reaction was carried out in a 100-ml three neck round bottom (RB) flask attached to a reflux condenser. The RB flask was placed on a hot plate magnetic stirrer (2 MLH-Remi, India). RB flask contains refined RSO with methanol in the presence of the purified lipase (dialyzed ammonium sulphate fraction). The magnetic stirrer was adjusted to the defined temperature intervals (30, 45 and 60 $^{\circ}\text{C}$) and constant stirring (200 rpm). Diagrammatic representation of the reaction setup is shown in Figure S₁. The lipase breaks the ester bond of triglycerides to form fatty acid methyl esters as presented in Fig. 1.

Experimental design

The statistical software Minitab (version 14, USA) was used for designing and analyzing the experimental trials of BBD in RSM (Sudha et al. 2017). The four-level three-point (high, medium and low) design of BBD was employed for this study, which includes L_{27} trails. The four independent variables and range have been shown in Table 1.

Fig. 1 Action of lipase; RSO with methanol in the presence of lipase to form fatty acid methyl esters and glycerol

Table 1 Independent variables and levels used in RSM

Independent variables	Symbols	Levels		
		-1	0	+1
RSO–methanol ratio (mol/mol)	X_1	1:6	1:9	1:12
Amount of lipase (U/ml)	X_2	500	750	1000
Temperature (°C)	X_3	30	45	60
Reaction time (h)	X_4	2	4	6

Box–Behnken design (BBD) and validation experiment

Significant reaction parameters such as molar ratio of methanol (1:6–1:12), temperature (30–60 °C), time (2–6 h), and lipase unit (500–1000 U/ml) were selected on the basis of preliminary studies. To reduce errors, 27 trials were performed in triplicate and the fatty acid methyl esters (FAMES) produced were taken as the response variable (Table 2). The

Table 2 Experimental trials according to BBD model for the optimization of biodiesel production

Trial no.	Variable levels				Biodiesel production yield	
	RSO-to-methanol ratio (mol/mol) X_1	Amount of lipase (U/ml) X_2	Temperature (°C) X_3	Reaction time (h) X_4	Observed	Expected
1	1:9	1000	45	6	74.56 ± 0.23	76.00
2	1:6	500	45	4	68.13 ± 0.11	68.04
3	1:9	750	60	6	85.43 ± 0.16	85.31
4	1:6	750	60	4	73.15 ± 0.54	70.17
5	1:9	750	30	2	81.12 ± 0.61	80.89
6	1:9	500	45	2	78.23 ± 0.24	76.79
7	1:12	1000	45	4	76.12 ± 0.36	75.94
8	1:12	750	45	2	77.28 ± 0.58	77.89
9	1:9	750	45	4	96.46 ± 0.08	97.29
10	1:9	500	30	4	68.24 ± 1.24	66.13
11	1:9	1000	30	4	68.32 ± 1.32	64.25
12	1:9	750	45	4	97.23 ± 0.84	97.29
13	1:9	500	45	6	59.89 ± 0.92	59.04
14	1:6	750	45	2	70.46 ± 1.45	72.72
15	1:9	1000	60	4	66.72 ± 1.23	69.07
16	1:9	750	60	2	72.15 ± 0.68	70.06
17	1:12	750	45	6	84.22 ± 0.54	82.19
18	1:6	1000	45	4	57.46 ± 0.24	57.03
19	1:9	500	60	4	65.24 ± 0.86	69.54
20	1:9	750	45	4	98.18 ± 0.21	97.29
21	1:6	750	45	6	69.56 ± 0.16	69.19
22	1:9	750	30	6	64.51 ± 1.54	66.33
23	1:12	750	30	4	72.16 ± 0.82	75.14
24	1:6	750	30	4	70.00 ± 0.38	71.57
25	1:12	750	60	4	86.34 ± 0.46	84.77
26	1:9	1000	45	2	56.63 ± 0.18	57.48
27	1:12	500	45	4	67.13 ± 0.12	67.29

results were analyzed by fitting to the second-order polynomial equation (Eq. 3). To check the validity of quadratic model, four trials were predicted by software. Biodiesel produced was calculated and compared with predicted values.

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} X_i X_j, \quad i < j \quad (3)$$

where Y represents the response variable, β_0 is the interception coefficient, β_i is the coefficient of the linear effect, β_{ii} is the coefficient of quadratic effect, β_{ij} is the coefficient of interaction effect when $i < j$, and k is the number of involved variables.

Production of biodiesel by onsite lipase

The progress of the reaction was monitored by removing aliquots of reactants at particular time intervals. Instantly, the mixture was poured into a separating funnel and allowed to settle for gravity separation. Two layers were formed, the top biodiesel layer was phase separated from the bottom glycerol layer to remove excess amount of methanol, and the biodiesel layer was collected. Then, it was washed thrice in deionized warm water to remove dissolved glycerol and impurities. Washed aliquots were then dried over anhydrous sodium sulphate and kept in oven at 110 °C for 20 min. The clean and dried biodiesel was stored under controlled room temperature (below 28 °C) for further use.

Analysis of biodiesel

Thin-layer chromatography (TLC)

Semi-quantitative analysis of biodiesel was carried out using TLC method. Silica gel-G coated on glass plate was activated for overnight at 60 °C and 0.5 µl of samples was applied. For biodiesel analysis, the solvent mixture of n-hexane:ethylacetate:acetic acid (94:5:1, v/v/v) was used as mobile phase (Samukawa et al. 2000). After chromatographic development (~30 min), plate was allowed to air dry at room temperature (i.e., 30 °C) for half an hour. Followed by saturated with iodine chamber (5–10 min) and spots were recorded.

Gas chromatography (GC)

The yield of biodiesel was analyzed by Shimadzu Gas Chromatography (2010 Plus), attached with flame ionization detector and connected with MXT biodiesel column (17 m, 32 mmID and 0.10 µm df). Initially, 1 g of FAMES was

weighed into 10 ml of glass vial and derivatized with 100 µl of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). Subsequently, the aliquot was vortexed and allowed to stand for 20 min. For analysis, aliquotes were diluted with 4 ml of GC/HPLC grade n-heptane (99%). Aliquot was withdrawn using Hamilton microliter syringe and was injected into GC system. High-purity (99.99%) nitrogen was used as the carrier gas and it had 30 ml/min flow rate. GC oven was conditioned at 55 °C for 1 min and heated to 380 °C at 15 °C/min. Finally, it was maintained at 380 °C for 15 minutes and total analytical time was 37 min.

Characterization of biodiesel

Elemental analysis

Carbon (C), hydrogen (H), nitrogen (N) and sulphur (S) contents were estimated using elemental analyzer (Flash 2000 Elemental Analyzer; Thermo Fisher Scientific). Aqueous samples were weighed (1.0–3.0 mg) in soft universal tin containers (O.D: 0.5 mm × H: 0.8 mm) and sealed tightly without air gap. Sample was loaded into the combustion reactor via the autosampler with oxygen. Elemental analysis was carried out in triplicate and average value was reported for each element. The oxygen (O₂) content was calculated by mean difference. After combustion, the resulting gases (N₂, O₂, H₂O, and SO₂ or SO₃) were carried by running helium gas, then swept through a GC column that provided separation of the combustion gases, and finally, detected by a Thermal Conductivity Detector (TCD). Total run time was around 12 min.

FTIR spectra of rubber seed oil and biodiesel

RSO and biodiesel aliquots (5 mg) were mixed with IR-grade potassium bromide (KBr), and fixed on a sample holder for analysis. FTIR analysis of the samples was carried out at mid-infra-red region of 400–4000 cm⁻¹ (Jasco FTIR 4100 Series, Japan).

Fuel properties of biodiesel

The fuel properties of produced biodiesel were determined using the standard protocol of ASTM D-6751 and EN 14214.

Results and discussion

Physicochemical properties of rubber seed and rubber seed oil

General characters of rubber seed as well as physicochemical properties of refined RSO are tabulated in Table 3. Mottled

Table 3 Physicochemical characterization of rubber seed and rubber seed oil

Properties	Composition
Rubber seed	
Seed weight (g)	3.68–5.039
Kernel weight (g)	2.43–3.41
Seed kernel ratio (%)	66.67 ± 3.2
Moisture content (%)	1.75 ± 0.45
Rubber seed oil	
Color	Golden yellow
Density (g/cm ³)	0.854
Viscosity (mm ² /s @ 40 °C)	38.46
pH	6.14
Oil content (%)	39.5
Acid value (mg KOH/g oil)	45.32
Free fatty acid	22.66
Iodine value (g I ₂ /100 g oil)	135.52
Saponification value (mg KOH/g oil)	192.36

brown-colored seeds weighing from 3.68 to 5.039 g enclosed kernels with 2.43–3.41 g of weight which was 66.03–67.67% of the total seed weight. However, average weight percentage of the kernel was 66.67% of seed weight (seed kernel ratio).

Fatty acid profile of RSO

The gas chromatograms in Figure S₂ depict the conversion of the triglycerides from RSO to its respective fatty acid methyl esters. It can be seen that refined RSO comprises of linoleic acid (33.43%), oleic acid (29.73%), linolenic acid (15.90%), palmitic acid (12.59%) and stearic acid (8.35%) which agrees with the results of the previous studies of Kitigowittana et al. (2013) and Ramadhas et al. (2005). The total unsaturated fatty acid content of RSO biodiesel is found to be 79.06%. The respective mass spectra are detected and presented in Fig. 2.

Fig. 2 Mass spectrum of biodiesel from rubber seed oil. **a** Linoleic acid methyl ester (33.43%), **b** oleic acid methyl ester (29.73%), **c** palmitic acid methyl ester (12.59%), **d** stearic acid methyl ester (8.35%) and **e** linolenic acid methyl ester (15.90%)

Statistical optimization

RSM was employed to develop a statistical cum mathematical correlation between different independent parameters such as molar ratio of the reactants, reaction temperature, reaction time and catalyst unit on the production of biodiesel. Three main statistical tools, i.e., analysis of variance (ANOVA), regression analysis (R^2) and 3D plotting of response variables were performed to optimize the optimum conditions for maximum production of biodiesel. The quadratic regression model of BBD is based on the uncoded levels of the independent variables. A second-order polynomial equation was fitted to the optimization of biodiesel production, which resulted in the following regression equation:

$$Y = -150.5 + 14.58 x_1 + 0.3224 x_2 + 2.859 x_3 - 6.12 x_4 - 1.225 x_1 * x_1 - 0.000307 x_2 * x_2 - 0.04822 x_3 * x_3 - 2.692 x_4 * x_4 + 0.00655 x_1 * x_2 + 0.0613 x_1 * x_3 + 0.327 x_1 * x_4 + 0.000093 x_2 * x_3 + 0.01813 x_2 * x_4 + 0.2491 x_3 * x_4 \quad (4)$$

where X_1 is RSO to methanol molar ratio, X_2 is lipase unit, X_3 is reaction temperature and X_4 is reaction time.

Analysis of variance (ANOVA) is a method to find out the significance and strength of the experimental result as well as the effects of significant variables and their interaction on the selected responses. The results of ANOVA are tabulated in Table 4. The quadratic regression model has an F value of 29.68 and p value is zero, which indicates that the model is significant. The p value represents the probability of error, and it is used to check the importance of each regression coefficient. The p value is also pinpointing the interactive effect of each response with independent variables. In general, if the quadratic regression model shows a lack of fit, this indicates that the model does not sufficiently describe the relationship between the independent variables (i.e., methanol-to-oil molar ratio, lipase unit, temperature and time) and the dependent variable. Generally, a high R^2 value justifies that there is good correlation between the model and experimental data. Thus, the R^2 value is close to unity (0.9719); which shows that there is good agreement

between the model and experimental data. In addition, the coefficient of variation (C.V.) is found to be 0.99, which gives a guarantee that the experimental data are accurate and confirmed (Ali et al. 2017).

Residual is explained as the difference or variation between the experimental and predicted values. Hence, the residuals were normalized and divided with an estimate of their standard deviations, resulting in standardized residuals. Normal distribution versus the standardized residuals obtained from experiments as a straight line. Moreover, validation experiments showed a good correlation coefficient of 0.994 and very low p value, i.e., the quadratic polynomial model was highly significant and indicated high correlation between the predicted and observed values of biodiesel pro-

duced. Ali et al. (2015) reported the quadratic response models to be successfully applied for the production of biodiesel using onsite lipase-mediated reaction with central composite design. Similar studies were conducted by Chen et al. (2002) and Shieh et al. (2003).

Effect of parameters

Four variable parameters hold significant impact on the production of biodiesel from rubber seed oil and that encompasses the second-order polynomials. The best way of expressing the effect of all parameters on biodiesel yield within the experimental parameters under investigation is to generate response surface and contour plots of the equation. The 3D response surfaces and 2D contour plots are plotted as shown in Figs. 3 and 4. From the shape of contour plots, the significance of the mutual interactions between the independent variables could be estimated. An elliptical profile of the contour plots indicates remarkable interaction between the independent variables. The responses obtained were of

Table 4 Analysis of variance (ANOVA) for Box–Behnken model

Source	Degree of freedom	Sum of square	Mean square	F -value	p -value
Regression	14	3203.04	228.79	29.68	0.000
Linear	4	302.77	75.69	9.82	0.001
Square	4	2205.13	551.28	71.51	0.000
Two-way interaction	6	695.13	115.86	15.03	0.000
Residual error	12	92.52	7.71		
Lack-of-fit	10	91.03	9.10	12.26	0.078
Pure error	2	1.48	0.74		
Total	26	3295.55

Fig. 3 Response surface plots (3D) and contour plots (2D) showing the effect of different parameters on production of biodiesel over onsite lipase from *P. aeruginosa* BUP2

Fig. 4 Response contour plots (3D) and contour plots (2D) showing the effect of different parameters on production of biodiesel over onsite lipase from *P. aeruginosa* BUP2

convex nature suggesting that there were well-defined optimum operating conditions. Biodiesel yield was sensitive to methanol molar ratio, enzyme concentration, temperature and time.

A reaction with mid-range of molar ratio 1:9 (RSO:methanol) and amount of enzyme of 750 U favored maximal yield and conversely it decreased at molar ratio of 12:1 (Fig. 3a). The biodiesel yield is minimal with optimum amount of enzyme and molar ratio; however, as the molar ratio is increased while keeping the amount of enzyme at optimum level (750 U) the biodiesel yield reaches the maximum value. This indicates the effectiveness of the lipase enzyme being utilized in the minimum amount. Previous studies reported that the yield was improved as the amount of enzyme was increased in the reaction system (Silitonga et al. 2016; Bello et al. 2016; Al-Zuhair 2005).

Figure 3b shows the interaction of temperature and molar ratio on biodiesel production at the hold values of 750 U of lipase and time of 4 h. The molar ratio of methanol to RSO was one of the most important parameters determining the production of biodiesel. Biodiesel yield initially increased with the increase of methanol–oil molar ratio and further increase in the molar ratio led to a decrease in the yield, in that unresolved methanol inhibited the lipase by destroying the active sites of enzyme (Li et al. 2013). This could be due to the fact that methanol is a denaturing agent of enzymes and insoluble in the oil at high concentration, which made proteins unstable and denatured (Qin et al. 2008). Figure 4a depicts the response between the amount of enzyme, temperature and transesterification reaction percentage yield at constant molar ratio of 9:1 and time of 4 h. Temperature is a key parameter that affects the conformation of lipase and it can initially boost up the yield and then cause a drastic reduction. In the present study, the temperature varies from 30 to 60 °C where the reaction is slow at low temperature. Figure 4b represents the effect of changing amount of enzyme and time on alcoholysis at 45 °C temperature and molar ratio of 9. The highest conversion (99.52%) was obtained at 750 U of enzyme and at 45 °C of temperature. The yield is then decreased by the increase in molar ratio of methanol and temperature.

FAME production was more sensitive to both the amount of enzyme and molar ratio compared to temperatures and time. Similar trend was also shown by Azocar et al. (2010). But, the time of 4 h was recorded to give optimum yield near to 80% and at similar temperature range of 45 °C as depicted in Fig. 4c. In this study, the maximum yield of 99.52% was obtained at 1:10 molar ratio of oil-to-methanol with the hold values of 45 °C of temperature and 4 h of reaction time (Table 5). The present findings agree with the results obtained by other researchers (Jeong and Park 2009; Köse et al. 2002; Chiang et al. 2003), who establish that at temperature above 50 °C, the yield of FAME was decreased due to the deactivation of enzyme at higher temperatures.

Analysis of biodiesel

Gas chromatography (GC) analysis was employed to determine the conversion of RSO to biodiesel. The standard methyl esters of palmitic, stearic, oleic and linoleic acids were used as standards. Area percentage of RSO to biodiesel was calculated and interpreted with the help of peak integration software GC solution (Shimadzu cooperation). Unutilized rubber seed oil seems to be a promising feedstock for producing biodiesel by dint of its low cost and its role in enhancing the economy of rubber farmers. Recently, Amini et al. (2017) demonstrated biodiesel production from *Ocimum basilicum* (sweet basil) seed oil by lipase-catalyzed transesterification. It resulted in 94.58% FAME yield after reaction at 47 °C for 68 h in the presence of 6% (w/w) catalyst and a methanol to oil ratio of 10:1. Charpe and Rathod (2011) compared the transesterification efficiency of lipases from *Aspergillus oryzae*, *P. fluorescens*, *P. cepacia*, and *C. rugosa* using waste frying oil and found that *P. fluorescens* lipase exhibited the highest production of fatty acid methyl esters, 63.8% after optimizing the reaction conditions along with the stepwise addition of methanol. Sebastian et al. (2016) reported comparative studies between chemical and enzymatic transesterifications of rubber seed oil for biodiesel production. From this study, they concluded that enzyme-catalyzed reaction is superior to chemical method and it has given more pure biodiesel as well as glycerol than that from chemical method. Recently, our group demonstrated

Table 5 Experimental trials for the validation of the predicted model

Trials	RSO to methanol ratio (mol/mol)	Lipase (U/ml)	Reaction temperature (°C)	Reaction time (h)	Observed biodiesel (%)	Predicted biodiesel (%)
1	9	800	40	4	96.23	97.36
2	10	750	45	4	99.52	98.12
3	7	900	50	6	78.65	76.32
4	8	600	35	2	83.95	82.31

Fig. 5 Characterization of RSO and biodiesel; **a** TLC plate showing RSO and biodiesel samples with reference standards; *MO* monolein, *DO* diolein, *TO* triolein, *FAMEs* fatty acid methyl esters, *OA* oleic acid, *BS* biodiesel, *RSO* rubber seed oil. **b** FTIR analysis of crude rubber seed oil and biodiesel produced from RSO

biodiesel from used cooking oil by using alkaline lipase (Priji et al. 2017). Till date, there are only a few reports on lipase-mediated transesterification of RSO to biodiesel (Vipin et al. 2016; Sebastian et al. 2017). Similarly, the present study shows that partially purified onsite lipase could transesterify the high-FFA-contained RSO to pure biodiesel by single-step method.

TLC plate saturated with iodine chamber is an effective tool to identify the appropriate product in transesterification reaction. Spots were corresponding to substrates and products were confirmed with the presence of external

standards (oleic acid, monolein, diolein, triolein and fatty acid methyl esters) run in parallel and are presented in Fig. 5a. Fourier transform infrared spectroscopic (FTIR) analysis can be used to determine the various functional groups present in the sample and it gives high-spectral data over a wide range of absorption. The comparative FTIR spectrum depicts the frequency ranges and their corresponding functional groups and indicated compounds are represented in Fig. 5b. The FTIR spectrum shows definite vibrations of alkanes and poly-aromatic groups and the lack of sulphur. In the biodiesel spectrum, a doublet

Table 6 Elemental analysis of Rubber seed oil versus Biodiesel

Sample	Elemental composition (%)				
	C	H	O*	S	N
Rubber seed oil	78.81	11.89	9.20	0.10	0
Biodiesel	72.67	11.14	16.19	0	0

*Calculated by mean difference

Table 7 Fuel properties of biodiesel

Properties	Methods	Unit	Limits	Standards	Biodiesel
Ester content	EN14103	wt%	96.5 min	EN14214	99.52
Free glycerol	EN14105	wt%	0.02 max	EN14214	0.00
Monoglyceride	EN 14214	wt%	0.8	EN14214	0.30
Triglyceride	EN 14214	wt%	0.20	EN14214	0.18
Total glycerol	EN14105	wt%	0.25 max	EN14214	0.20
Acid value	EN14104	6751	4.82		
Density	EN ISO 3675	g/m ³	860–900	EN14214	884

peak in the range of 945.17 and 1036.56 cm⁻¹ due to C–O stretching is absent for the RSO spectrum. Presence of a broad peak around 945 cm⁻¹ represents the presence of –OH bending for carboxylic dimers in the product, which describes the complete conversion of free fatty acid to its ester. Absorbance in the region 2856–3006 cm⁻¹ is due to the stretching of C–H vibration, and the absorption peak at 1650 cm⁻¹ RSO represents C=C stretching vibration. The 1464 and 1376.91 cm⁻¹ absorptions are characteristic of C–H bending vibration for the alkyl groups –CH₂– and CH₃– of the hydrocarbon skeleton. The single sharp peak at 720 cm⁻¹ due to C–H bending characterizes the presence of C–(CH₂)_n–C group. Absence of peaks in the range of 720–945 cm⁻¹ and 1750–2800 cm⁻¹ confirm the absence of –C–O–O–C stretching and alkynic residues, respectively, in the fatty ester composition. From the spectrum, the wide peak of C=O vibration band (in the range 1744–1712 cm⁻¹) of methoxy carbonyl group in biodiesel was observed. Hence, esters were characterized by the strong absorption due to C=O stretching frequency near 1740 cm⁻¹ and by the strong absorption involving the stretching of C–O near 1240 cm⁻¹ (Fig. 5b). Previous studies (Ndana et al. 2013; Coates 2000) show the same spectrum of RSO and biodiesel compound produced as methyl ester.

Elemental analysis is used qualitatively as well as quantitatively for the detection of organic matter based on combustion technique. In this method, sample is burned in an excess of oxygen, and trapped the product as CO₂, H₂O, N₂

and SO₂ or SO₃. From this analysis, the major components of the samples have been found to be carbon, hydrogen and oxygen, respectively. The percentages of those components are shown in Table 6. As compared to petroleum diesel, biodiesel produces lesser amounts of carbon and unburned hydrocarbons. Absence of sulphur implies that it can be directly used in diesel engines. Thus, this study provides good practical information on biodiesel produced from RSO.

The fuel characteristics of the biodiesel produced over onsite lipase under the predicted optimum reaction parameters were determined, and it meets the standard specifications of ASTM D 6751 and EN 14214 (Table 7). Thus, the novel thermotolerant alkaline lipase from *Pseudomonas aeruginosa* BUP2 is found to be a potent candidate for transesterification of RSO and suitable for the industrial scale up.

Conclusion

In this study, we have tested and employed the locally available rubber seed oil to produce biodiesel by lipase-mediated transesterification. Lipase was secreted from *Pseudomonas aeruginosa* BUP2, a novel bacterial isolate. Principles of Response Surface Methodology cum Box–Behnken design were used for the optimization of different reaction parameters. The optimum synthesis of biodiesel was found to be 99.52% under the suggested conditions of lipase (750 U), methanol ratio (1:10), temperature (45 °C) and time (4 h). The response and observed values showed better correlation coefficient, which implies that this quadratic model was highly significant. The biodiesel products obtained were tested and characterized by TLC, GC–MS, FTIR and elemental analysis and it was confirmed that the fuel properties met the specification of biodiesel as mentioned in international standards of ASTM D6751 and EN 14214. Thus, this preliminary study of biodiesel produced by *Pseudomonas aeruginosa* BUP2 may broaden its application to fuel and motor industries as potential for green technologies.

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Compliance with ethical standards

Conflict of interest The authors declare the nonexistence of competing or other interests.

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