Comparative Study of Production of Biodiesel Utilizing Calcinated Eggshell and Immobilized Lipase-Eggshell Catalysts by Waste Cooking Oil, Catalysts Characterization and Yield Optimization

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Due to the high demand, cost and environmental toxicity of fossil fuels, mainly petroleum and diesel, there is a massive demand for alternative fuels based on biological origin. The production of biodiesel from cheap resources has gained more attention. Present study conducted biodiesel production from waste cooking oil utilizing calcinated eggshells and immobilized lipase. The eggshell was calcinated at temperature ranges from 500-800 °C and characterized by TGA, SEM, XRD and FTIR analysis; the study of characteristics of calcinated eggshell was helpful in the production of biodiesel, it is around ~42% yield production achieved at 60 °C, 1000 rpm revolution of stirrer for O/A (oil/alcohol) molar ratio 1:6. Now the lipase of activities 40 µmol/g min immobilized with eggshell, the eggshell-lipase catalyst (ELC) gives the yield of ~92% at 40 °C, 500 rpm revolution of stirrer and O/A molar ratio 1:6 after transesterification process on waste cooking oil (WCO) with calcined eggshell and reusability application of ELC. The production of biodiesel by immobilized catalyst at a higher temperature will reduce the thermal stability of lipase. To get the maximum biodiesel yield, the transesterification process must undergo at an optimum higher temperature. This is overcome by immobilizing the commercial lipase with an eggshell that can withstand even higher temperatures (40-45 °C) without losing its activities. The processes and optimization are globally integrated to improve the feasible evaluation of economic improvement for biodiesel production.

Keywords: Lipase, Calcinated eggshell, Biodiesel, Thermal stability, Waste cooking oil, Eggshell lipase catalyst.

INTRODUCTION

The exploitation of biodiesel for various energy consumption strategies has been utilized in diverse parts of the world as its high efficacy and eco-friendly. The production of biodiesel from cooking oil is not a new phenomenon. However, the evolution of developing a technique to produce a high yield of biodiesel at minimum cost is still a challenging task. These studies for developing an innovative approach to making history for the evolution of high yield biodiesel production at a low price. The comparization made of existing technology for producing biodiesel with our advanced technique for biodiesel production in all aspects.

Production of biodiesel from various sources like vegetable oils, including waste cooking oils and animal origin fats, has gained more attention [1]. Waste cooking oil can be considered a significant route for biodiesel production because of its easier cracking and waste resource utilization. Various reports reveal that the biodiesel production *via* different routes suggests the possible utilization of cheap substrates as the sources of biodiesel production. Various oil sources, such as edible and non-edible oils, have been used as foodstuffs for biodiesel production [2,3]. The uninterruptedly rising amount of edible vegetable oils makes them uneconomical as a long-term biodiesel source. Though waste vegetable oils are considered environmental pollution, they can be used as a potential raw material for biodiesel production and are cheap and readily available. The transesterification process of the vegetable oil is conducted to produce biodiesel in the presence of a catalyst.

The catalyst can be homogeneous, heterogeneous, enzymatic or nanoparticles. Homogeneous catalysts are considered more effective than their heterogeneous counterparts because of reduced mass transfer limitations and high conversion [4]. In this study, eggshell immobilized lipase was used as the catalyst

for transesterification of waste cooking oil, which brought about high strength biodiesel. Several authors [5-8] demonstrated the conversion of edible waste oil into useful biodiesel. The utilization of palm oil [9], oil palm wastes [10], fishing wastes [11], shark liver oil [12], *Posidonia oceanica* (L.) and frying oil wastes [13], waste clay oil [14], cooking and non-edible oil [15] reveals marked impact of bioenergy production. In this study, waste cooking oil was used as the substrate for biodiesel production utilizing eggshell immobilized lipase (ELC) as a catalyst. A study on optimization brought about highly stable ELC, which enhanced biodiesel production. Boey *et al.* [16] reported that the cost of production of per liter biodiesel is still high. Furthermore, Shwetha *et al*. [17] reported that eggshell consist of 94-98.5% $CaCO_3$, which can be calcined to CaO at different temperatures and acted as a catalyst for the production of biodiesel.

This study carried biodiesel production from waste cooking oil (WCO) with calcinated eggshell and eggshell immobilized lipase catalysts. A comparative study was done on the efficacy of transesterification of WCO using calcinated eggshells and eggshell immobilized lipase catalyst. After the transesterification, the produced biodiesel was purified by successive purification steps, which will remove undesirable impurities and thus enhance the yield of the final product.

EXPERIMENTAL

The chemicals *viz*. anhydrous Na_2HPO_4, HCl, HNO_3, CH_3OH (90% pure), triglyceride, olive oil and gum Arabic were purchased from Uma Scientific Pvt. Ltd., India. All the chemicals were of analytical grade and used without further purification.

Calcination of eggshell: Hen's eggshells were collected from the cafeteria, washed with demineralized water and dried in an oven at 80-85 ºC. It was ensured that the eggshell was free from moisture, crushed in a grinder and sieved from 100 µ size [18]. The crushed eggshells were weighed for calcination of each 50 g and heated for a temperature of 500, 600, 700, 800 and 900 ºC in an electrode buffer furnace (N_2 inert gas atmosphere) [19] for a different time intervals of 1, 2, 3, 4 and 5 h for each temperature range [20], samples were collected from calcined eggshell and sent for TG analysis to know the optimum temperature for calcination of eggshell. The calcined samples were also examined for SEM, XRD and FTIR analysis to find the change in the molecules szie, availability of the different types of components and availability of functional groups characterizing covalent bonds, respectively.

Preparation of eggshell powder for lipase immobilization

Preparation of SDS solution: The collected eggshells were washed with demineralized water to remove dust particles from the surface and 0.1% (w/v) of SDS solution was prepared to boil the eggshell [21,22]. The washed eggshells were boiled in 2 L of SDS solution. The required SDS solution is based on the amount of eggshell to be boiled. The eggshells must be fully immersed in SDS solution. It was heated at 60 ºC [21,23] for 20-25 min. During boiling, the interatomic bond of the eggshell matrix elongated, which was easily adsorbed the lipase.

Drying and crushing of boiled eggshells: The boiled eggshells were filtered from SDS solution, which may contain some SDS solution to their surface. It was washed with demineralized water thrice and then washed again with acetone thrice to remove moisture content on the eggshell surface. The boiled and washed eggshells were dried at 60 ºC in an oven for 4-5 h. The eggshells were crushed in a grinder and sieved to a uniform size of 100 µ [21].

Lipase immobilization: The lipase solution was prepared to immobilize it by stabilized material to extract the protein from the lipase, prepared with 50 mM of Na_2HPO_4 solution [21,23] and neutralized with HCl to get 50 mM (pH = 7) sodium buffer solution [24], to extract the protein from lipase, 1 g of crude commercial lipase was dissolved in sufficient amount of sodium buffer solution (50 mM, 7 pH) and stirred the solution for 15-20 min at room temperature [25]. It was centrifuged at 4000 rpm (2560 ×g) for 20 min at 4 ºC [26]. The supernatant was stored and residue lipase dissolved in a sufficient amount of sodium buffer solution and again centrifuged. This process was repeated until the milky colour of the lipase solution disappeared. The supernatant of the solution was used as lipase solution for further experimental work.

Immobilization of lipase solution with boiled powdered eggshell: There are different methods to immobilize the lipase on eggshells. According to Vasudevan & Fu [27], the crosslinking method of immobilization gives a high yield of biodiesel. This study also have a similar result by using the deeping method to immobilize lipase on eggshells. The boiled and crushed eggshell (5 g) was dipped in 250 mL of lipase solution. According to the requirement of immobilized catalyst, the immobilization did for a batch of 1 g of eggshell dissolved in 50 mL of lipase solution in a 250 mL capacity conical flask. The five numbers of 250 mL capacity of the conical flasks were taken to make five batches of each 50 mL supernatant solution for incubation. After the completion of incubation for 4 h, it was stored at 4 ºC for 36 h. It was washed with sodium buffer solution (50 mM) five times to remove unbounded lipase from the eggshell surface. Due to immobilization of lipase on eggshell, lipase gets adsorbed on the size of the surface of eggshell increased, which get confirmed by SEM and XRD characterization, thermal stability of the immobilized lipase-eggshell catalyst increased 13 times; a similar result was obtained by Hu *et al.* [28], which increase the rate of production of biodiesel.

Activity determination of ELC: The activity of commercial lipase initially was 40 µmol/g min. Among all methods for determining of activity of ELC, the titration method was easier and more accurate to calculate the ELC activity [23,27]. The ELC activity was expressed as fatty acid produced per unit mg of ELC [25].

Preparation of ELC solution: Prepared sodium buffer solution (50 mM, 7 pH) and prepared olive emulsion solution, which consists of 2.5 mL H_2O and 3 mL of olive oil; these emulsion solutions were added in 1.0 mL of Na_2HPO_4 (50 mM, 7 pH) [29-31], the gum arabic emulsion solution (5% w/v) was prepared. The ELC solution, olive oil emulsion solution, gum arabic emulsion solution and 3.0 mL of 95% ethanol were mixed and incubated at 37 ºC with a speed of 125 rpm for 30 min;

the resulted solution was obtained as burette solution for titration with 0.5 M NaOH solution [31,32]. The ELC prepared solution was titrated with 0.5 M NaOH in phenolphthalein as an indicator; the amount of lipase required to liberate 1 µM of FFA per min is 1 IU (international unit) activity of the lipase [23]. The specific activity of ELC was calculated as [21]:

$$\text{Specific activity of ELC} = \frac{\Delta V \times M}{w \times t} \quad (1)$$

where ΔV = change of NaOH volume; M = molarity of NaOH; W= amount of protein; and t = reaction time.

The ELC activity was calculated as 30 µmol/g min. It was observed that the activity of ELC was reduced from crude lipase activity; the same observation was reported by Chattopadhy & Sen [21]. However, this activity of immobilized lipase-eggshell catalyst is stable even at 40-50 ºC for maximum biodiesel yield and stable at 50-70 ºC for optimum yield of biodiesel [33], the pH of immobilized lipase-eggshell was also stable [34].

Method for production of biodiesel with calcinated eggshell

Method for measuring FFA from WCO: The collected WCO from the cafeteria of Sathyabama Institute of Science and Technology was heated at 60 ºC to remove undissolved impurities and moisture content. The amount of FFA present in WCO will decide the type of reaction for the production of biodiesel; the FFA was calculated by titration method, taking 10 g of WCO dissolved in 50 mL of isopropyl alcohol and drops of 0.1 N NaOH [35], it was heated at 60 ºC then cooled at 27 ºC, it was titrated with 0.1 N NaOH with phenolphthalein indicator, the FFA was calculated at the neutral point of reaction by titration formulae, the FFA was obtained as 2.4221 wt.%, which was less than 4, a similar result was obtained as the presence of FFA as 2.41 wt.% by Hsiao *et al.* [36] in WCO. If the amount of FFA < 4, the transesterification reaction is economical; otherwise, esterification reaction would be economical [35,37]. The physical properties of WCO are given in Table-1.

TABLE-1
PHYSICAL PROPERTIES OF WASTE COOKING OIL

Description of properties	Value
Molecular weight	874 u
Kinematic viscosity (25 °C)	32.12 c St
FFA	2.4221 wt%
pH	7.38
Density (25 °C)	916.9 Kg/m^3

Transesterification reaction for the production of biodiesel

Transesterification reaction in presence of calcinated eggshell: The WCO consist of triglyceride, which was reacted with methanol in the presence of calcinated eggshell (CaO); it was first converted to diglycerides anion and rearranged catalyst formed the methyl ester and glyceride. This process is called transesterification [16] and methyl ester is the biodiesel and glyceride is the byproduct. The methanol was measured as per O/A molar ratio as 1:3, 1:4, 1:5,1:6 and 1:7 [38]. The amount of methanol was stirred with calcinated eggshell for 1 h, with a fixed amount of catalyst (3% w/v) and with a speed range of 500-1000 rpm, then the WCO was heated separately at 60 ºC

to remove undissolved particles and moisture content, these moisture-free WCO was permitted to react with methanol in the presence of calcined eggshell for the different time interval from 1 h to 5 h and at a different temperature ranging from 50-65 ºC [16,39] and operating parameters were optimized for a maximum yield of methyl ester (FAME). The biodiesel yield was calculated using eqn. 2.

Transesterification reaction in presence of immobilized lipase-eggshell catalyst: The immobilized lipase-eggshell catalyst would be stable at 50 ºC for maximum yield beyond this temperature [33,34,40], it would be stable for a long duration for optimum yield, thus the transesterification process was conducted from 38-45 ºC of O/A various molar ratio 1:3, 1:4, 1:5, 1:6 and 1:7 for a time duration 1 h to 5 h [38]. In presence of a continual amount of catalyst 1% w/w for maximum yield of biodiesel, Mendes *et al.* [41] tested the production of biodiesel at 0.1-0.5% w/w immobilized lipase catalyst of waste cooking oil and yield of biodiesel will increase with the increase in the amount of catalyst. The biodiesel yield was calculated using eqn. 2. The operating parameters were optimized for a maximum yield of biodiesel by RSM [42].

$$\text{Yield of biodiesel (\%)} = \frac{\text{Mass of biodiesel (g)}}{\text{Mass of WCO (g)}} \times 100 \quad (2)$$

The percentage yield of biodiesel production was also verified using the following eqn. 3 [43]:

$$\text{Yield of biodiesel (\%)} = \frac{\sum \text{Mass of FAME (g)}}{\text{Mass of WCO (g)}} \times 100 \quad (3)$$

where percentage FAME was referred from GC-MS analysis of biodiesel fuel.

Purification of biodiesel: By using gravity method, biodiesel was separated from glycerol and other impurities like water, FFA and alcohol. Due to the differences in the density of biodiesel and others like glycerol, water, acid and other heavy particles, the biodiesel was in the top phase and others were in the bottom phase [44]. The immobilized catalyst was separated by filtration. The biodiesel purification was performed by centrifuging the biodiesel with hot water (60 ºC) for 5-10 min to extract biodiesel from heavy elements. It was permitted to settle down in a separating conical flask for 20-30 min to visualize the phases. The top phase was again repeated 3-4 times so that the top phase should have a yellow colour and the bottom phase must have a clear colourless solution; the bottom phase may have content of glycerol, acid and water was discarded and the top phase had a content of biodiesel, these biodiesels were dried in an oven at 100 ºC for 3-4 h in order to vapourize a significant amount of moisture content in it.

RESULTS AND DISCUSSION

SEM analysis of calcinated eggshell: The sample was collected from calcined eggshell for SEM analysis, shown in Fig. 1a, while the change in the size of calcined eggshell particles was observed in the SEM result, which is depicted in Fig. 1b. The average diameter of calcinated eggshell particles was 5 µm. It is visible that the size of the eggshell particles
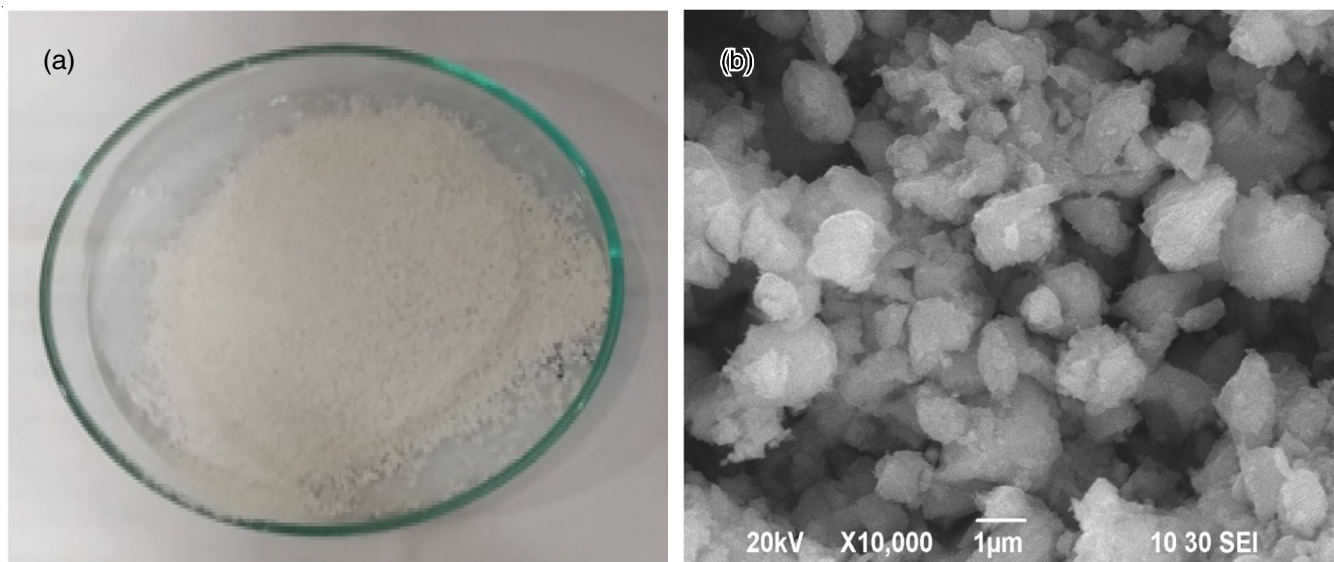
Fig. 1. (a) Image of calcined eggshell, (b) SEM image of calcined eggshell

has increased. The smooth surface of the particles of eggshell becomes cuboidal, which indicates the surface of an atom of calcined eggshell must increase; the image says some cavity and some hills shape formation indicates the size and shape of the eggshell particles was increased and the surface area of calcined particles also increases due to the calcination of eggshell [21]. The above SEM result was obtained from a calcined eggshell at 800 °C for 4 h.

The cavity and hill form structure in the boiled eggshell disappeared in the immobilized lipase-eggshell catalyst, which is attributed due to the adsorption of lipase on the surface of particles of eggshell. The particles size of eggshell was obtained as 1 μm from the SEM image of the boiled eggshell. In contrast, the particle size of immobilized lipase-eggshell was increased to 10 μm, so there is adsorption on the surface of the eggshell by lipase [45]. These lipases are bonded with eggshell however, some of the bonds will be break and electrons will be free to form a new bond with suitable subtract and when lipase come in contact with the boiled eggshell powder, it forms a strong bond between lipase and eggshell [45]. Two types of adsorption may occur during lipase immobilization in eggshells,

one is due to the physical contact and another is due to chemical contact [21].

XRD studies: The X-ray diffraction (XRD) analysis was performed for calcined eggshell and immobilized lipase-eggshell to identify the crystallinity and phases of the calcined grown structures. The XRD analysis of the calcined eggshell at 700 °C for 3 h is depicted in Fig. 2a and immobilized eggshell and $2\theta = 25.30°$, the joint committee on powder diffraction standards (JCPDS) [46,47]. Peaks for the catalyst appeared at $2\theta = 44°$ for calcined for immobilized eggshell-lipase, which is characteristic of calcium oxide. The peak characteristics of $Ca(OH)_2$ were observed at 49.2° and 60.5° for calcined eggshells and 35.7° and 45.5° for ELC were high intensity. The peaks obtained at 73.4° and 82.3° were characteristics of activated carbon in calcined eggshells depicted in Fig. 2a. The eggshell was immobilized by lipase; its intensity increased 99% to 200%, 160%, 140%, 120%, 100% and 120% for $2\theta = 25.3°$, 39.8°, 44.5°, 45.0°, 65.0° and 79.0°, respectively, the maximum intensity of 99% generated at $2\theta = 25.30°$, 44.50°, 48.50° and 50.00° for CaO only, the similar result was also obtained for chicken eggshell catalyst by Fayyazi *et al.* [48]. Other comp-
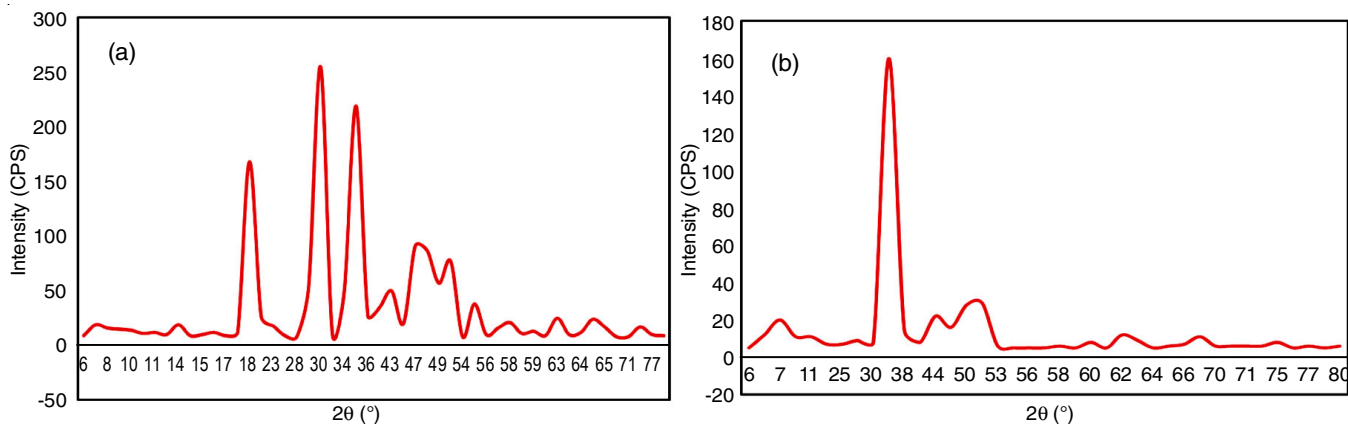

Fig. 2. (a) Image for XRD of ELC, (b) image for XRD of calcinated eggshell

onent peaks are less intensity for a few instead of time, it indicates that the catalyst consists of mostly CaO (Fig. 2a). When eggshell was immobilized by lipase, its intensity increased 99% to 200%, 160%, 140%, 120%, 100% and 120% for 2θ = 25.3º, 39.8º, 44.5º, 45.0º, 65.0º and 79.0º, respectively (Fig. 2b). The peaks were compared with the joint committee on powder diffraction standards (JCPDS) [46,47]. Peaks for the catalyst appeared at 2θ = 44º for calcined for immobilized lipase-eggshell is the characteristic of calcium oxide. The ELC intensity increased from 99% to 200%, 160%, 140%, 120%, 100% and 120% for 2θ = 25.3º, 39.8º, 44.5º, 45.0º, 65.0º and 79.0º, respectively.

It means that some additional elements must be added to the surface of the eggshell so that its intensity has increased from 99% to 200%, 160%, 140%,120%, 100% and 120% for 2θ = 25.0º, 39.8º, 44.5º, 45.0º, 65.0º, 79.0º, respectively [26], as result lipase must be adsorbed on the surface of the eggshell. Hence, the intensity was increased, which may help increase biodiesel yield, similar results were also obtained by Babajide *et al.* [46].

FTIR studies: Fig. 3a-b shows the IR spectra of both raw and calcined eggshells. The FTIR spectra before the calcination process embodies the raw eggshells and showed that the broad transmission bonds at around 2863 cm^{-1} can be attributed to OH stretching vibration from the residual waste [49]. The weaker bond at 2360 cm^{-1} is attributed to C=O bonds from carbonate [50]. Infrared bands at 1398 and 872 cm^{-1} show the C–O stret-ching and bending of $CaCO_3$. In the meantime, the strong bond at 710 cm^{-1} represents a Ca–O bond [29]. The IR spectrum after calcination shows the existence of -OH in $Ca(OH)_2$ for the peak of around 3600 and 2360 cm^{-1}. Another band exists at 670 cm^{-1} attributed to the Ca-O band [26]. The functional peaks of 1981, 1414, 1398, 1063 and 710 cm^{-1} represent the stretching vibration of CO_3^{2-} group present in the eggshell [51,52]. The FTIR results showed that $CaCO_3$ had completely converted to CaO as Ca-O bond existed in the calcined eggshell.

FTIR studies of immobilized eggshell-lipase catalyst: The major absorption bands occurred at 1411.88 and 871.90 cm^{-1} are attributed to asymmetric stretch and out-plane bending CO assigned to calcium oxide [19]. The band at 3430.24 cm^{-1}

was assigned to OH^-, which is indicative the presence of calcium hydroxide, formed by absorption of atmospheric moisture onto the surface of calcium oxide. The band at 2514.77 cm^{-1} is attri-buted to alkanes CH stretching from immobilized lipase-egg-shell; the Ca-O bond exists in the calcined eggshell. Kumar *et al.* [53] also reported a similar result for immobilized catalyst from pseudomonas cepacian lipase.

Production of biodiesel

Transesterification reaction with calcinated eggshell: The composition of waste cooking oil is mentioned in Table-1. It was undergone a chemical transesterification reaction of waste oil with methanol to produce biodiesel by varying one parameter and keeping other parameters constant.

Yield of biodiesel with O/A with varying different para-meter: Biodiesel was produced at different O/A molar ratios and other constant parameters like temperature, time and amount of catalyst. The O/A molar ratios were taken as 1:3, 1:4, 1:5, 1:6 and 1:7 for 1.0 h, with 3% (m/W) of catalyst at 30 ℃ by trial-and-error method and data obtained was compared with RSM predicted value for optimization of the parameters. The ANOVA model was designed and obtained the actual value of the different parameters for the maximum biodiesel yield. The GC-FID analysis of produced biodiesel was carried out and the intensity of FAME was observed.

The different peaks of voltage, some of the similar peaks of high intensity and some of the average peak were obtained (Fig. 4). It is based on voltage intensity developed due to the different carbon percentages of hydrocarbon; these hydro-carbons represent the FAME present in biodiesel. The voltage peak represents the specific FAME (Table-2).

Optimization of process parameters for renewable catalyst

ANOVA for quadratic model: The Model F-value of 60.34, 76.77, 245.49 denotes that the model is significant. Due to noise, there is only a 0.01% chance of an F-value. The model is significant and the probability of noise effect did not exceed 0.02% [54]. The *p*-value implies that less than 0.0500 indicates that model terms are significant. Significant model terms are A, B, C, D, AB, AC, AD, BC, BD, CD, A^2, B^2, C^2 and D^2. The values greater and above than 0.1000 indicate that the model
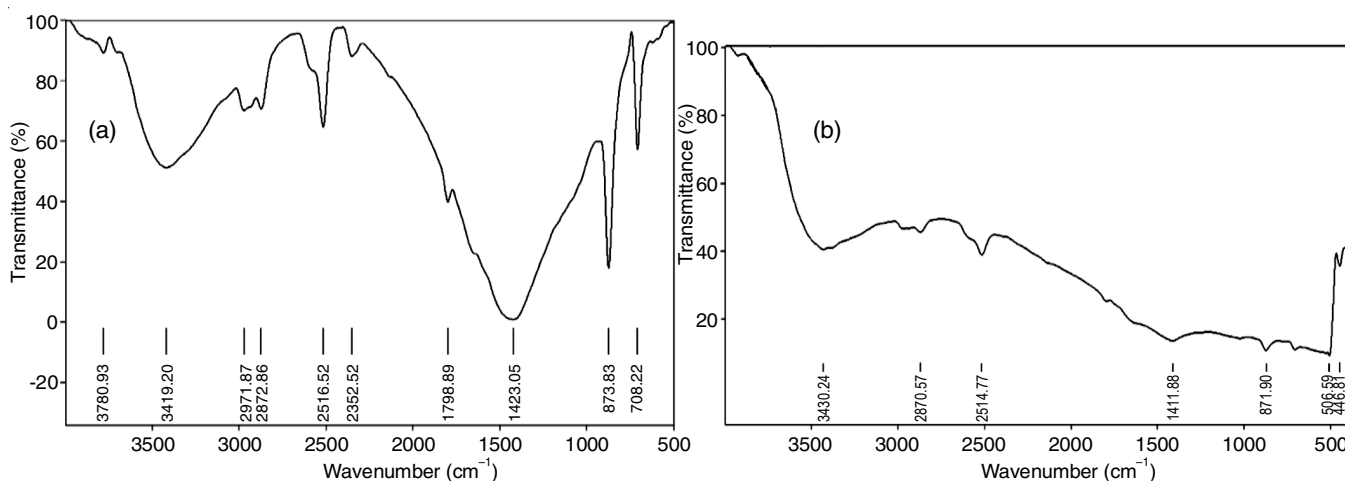

Fig. 3. (a) FTIR spectra of calcinated eggshell catalyst and (b) immobilized ELC
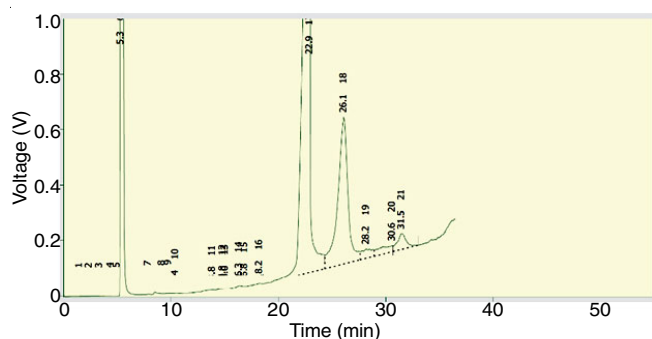
Fig. 4. GC-FID for produced biodiesel (voltage peak response with retention time)

TABLE-2 FAME COMPOUND NAME CORRESPONDING TO VOLTAGE PEAK IN GC FID OF PRODUCED BIODIESEL		
Name of compound (FAME)	Time (min)	Number of carbon contained
Methyl hexanoate	5.3	C18
Methyl myristate	10	C4
Methyl stearate	11	C8
Methyl myristoleate	13	C9
Methyl palmitate	14	C6.3
Methyl oleate	15	C6.8
Methyl linoleates	16	C 8.3
Methyl lignocerate	22.9	C 16
Methyl docohexanenoate	26.1	C 18
Octadecanoic acid methyl ester	28.2	C19
Methyl arachidate	30.6	C 20
Eicosanoid acid methyl ester	31.5	C 21

stipulations are not significant. In case there are many insignificant model terms, model reduction may convalesce your model. The lack of fit F-value of 0.1967, 1.20, *p*-value 0.1967, 0.3978 suggests the lack of fit model is not significant in relation to the actual true error. There is a 90.77% chance that a lack of fit F-value of considerable value could occur due to noise.

ANOVA quadratic model for calcined eggshell: The quadratic model for optimizing parameters for maximum yield

of biodiesel by calcinated eggshell as catalyst is shown in Table-3.

Design actual for calcinated eggshell: The design of optimization parameters for biodiesel production by calcinated eggshell is shown in Table-4.

Plotting of graph with different parameters using RSM data for calcined eggshell catalyst

Graph between oil: Alcohol ratio & time *vs.* biodiesel yield: The maximum yield obtained at oil: alcohol ratio (1:6) for 120 min is 43%, as shown in Fig. 5a.

Graph between the amount of catalyst & time *vs.* yield of biodiesel: The maximum yield of biodiesel obtained at 5 g of catalyst amount for 120 min is 43%, as shown in Fig. 5b.

Graph between oil: alcohol ratio & temperature *vs.* yield of biodiesel: The maximum yield obtained at 50 ºC and 1:6 oil: alcohol ratio is 42%, as shown in Fig. 5c.

Comparison of yield of biodiesel of experimental value and RSM design value: The comparative graph between actual created data for biodiesel yield and RSM data is shown in Fig. 5d. It is observed that both data are co-related, with almost 99.9% of correlated points on the straight line; a few points are just away from the regression line. It means that the present experimental values are optimum and accurate.

ANOVA quadratic model for immobilized eggshell-lipase catalyst: The ANOVA quadratic model for immobilized eggshell-lipase is depicted in Table-5.

Design actual for immobilized eggshell-lipase catalyst: The design value of optimization of parameters for biodiesel production by immobilized lipase eggshell is shown in Table-6.

Graph between RSM parameters for production of biodiesel by immobilized eggshell-lipase catalyst

Graph between time *vs.* oil: alcohol for maximum yield: The biodiesel was produced at different O/A molar ratios and other constant parameters like temperature and catalyst amount. The O/A molar ratios were taken as 1:3, 1:4, 1:5, 1:6 and 1:7

TABLE-3 ANOVA QUADRATIC MODEL FOR CALCINED EGGSHELL						
Source	Sum of square	Df	Mean square	F-Value	P-Value	Response
Model	344.66	14	24.62	76.77	< 0.0001	Significant
A-Time	22.87	1	22.87	71.32	< 0.0001	Significant
B- Temperature	0.0556	1	0.0556	0.1733	0.6831	Not significant
C-Oil: Alcohol ratio	1.39	1	1.39	4.33	0.0550	Not significant
D- Catalyst amount	4.50	1	4.50	14.03	0.0019	Significant
AB	0.0625		0.0625	0.1949	0.6652	Not significant
AC	0.0625	1	0.0625	0.1949	0.6652	Not significant
AD	10.56	1	10.56	32.94	< 0.0001	Significant
BC	14.06	1	14.06	43.85	< 0.0001	Significant
BD	0.0625	1	0.0625	0.1949	0.6652	Not significant
CD	0.5625	1	0.5625	1.75	0.2052	Not significant
A^2	3.37	1	3.37	10.50	0.0055	Significant
B^2	7.88	1	7.88	24.58	0.0002	Significant
C^2	79.23	1	79.23	247.09	< 0.0001	Significant
D^2	30.87	1	30.87	96.26	< 0.0001	Significant
Residual	4.81	15	0.3207			
Luck of fit	1.10	9	0.1217	0.1967	0.9848	Not significant
Pure Error	3.71	6	0.6190			
Cor. Total	349.47	29				

TABLE-4
ACTUAL DESIGN VALUE FOR CALCINATED EGGSHELL CATALYST

| Std. | Run | Factor 1 | Factor 2 | Factor 3 | Factor 4 | Response 1 |
| | | A | B | C | D | Biodiesel yield (%) |
		Time (min)	Temp. (°C)	Oil:alcohol ratio (v/v)	Catalyst amount (g)	
26	1	120	50	6	5	40
15	2	60	70	8	9	35
19	3	120	30	6	5	42
10	4	180	30	4	9	30
20	6	120	70	6	5	42
7	7	60	70	8	1	34
1	8	60	30	4	1	34
2	9	180	30	4	1	33
6	10	180	30	8	1	35
16	11	180	70	8	9	31
5	12	60	30	8	1	36
18	13	240	50	6	5	40
22	14	120	50	8	5	35
24	15	120	50	6	9	36
25	16	120	50	6	5	40
3	17	60	70	4	1	35
11	18	60	70	4	9	36
27	19	120	50	6	5	40
14	20	180	30	8	9	33
30	21	120	50	6	5	42
17	22	120	50	6	5	40
28	23	120	50	6	5	40
8	24	180	70	8	1	33
9	25	60	30	4	9	34
4	26	180	70	4	1	35
23	27	120	50	6	1	38
29	28	120	50	6	5	41
12	29	180	70	4	9	32
21	30	120	50	4	5	35

TABLE-5
ANOVA QUADRATIC MODEL FOR IMMOBILIZED EGGSHELL-LIPASE CATALYST

Source	Sum of square	Df	Mean square	F-Value	P-Value	Response
Model	3237.24		231.23	245.49	< 0.0001	Significant
A: Time	45.78	1	45.78	48.60	< 0.0001	Significant
B: Temperature	0.4659	1	0.4659	0.4946	0.4927	Not significant
C: Oil:alcohol ratio	0.0311	1	0.0311	0.0330	0.8582	Not significant
D: Catalyst amount	7.82	1	7.83	8.30	0.0114	Significant
AB	10.56	1	10.56	11.21	0.0044	Significant
AC	0.5625	1	0.5625	0.5972	0.4517	Not significant
AD	85.56	1	85.56	90.84	< 0.0001	Significant
BC	0.0625	1	0.0625	0.0664	0.8002	Not significant
BD	33.06	1	33.06	35.10	< 0.0001	Significant
CD	39.06	1	39.06	41.47	< 0.0001	Significant
A^2	15.24	1	15.24	16.18	0.0011	Significant
B^2	44.44	1	44.44	47.18	< 0.0001	Significant
C^2	1045.97	1	1045.97	1110.4	< 0.0001	Significant
Residual	14.13	15	0.9419			
Lack of fit	7.24	7	1.03	1.2	0.3978	
Pure error	6.89	8	0.8611			
Cor Total	3251.37	29				

for the different time intervals from 1.0 h to 3 h with 3% (m/W) of catalyst at 30 °C by trial-and-error method and data obtained was plotted (Fig. 6a). The maximum yield of biodiesel for a specific O/A ratio was considered the optimum O/A molar ratio.

Graph between oil: alcohol *vs.* amount of catalyst for maximum yield: The biodiesel was produced at different O/A

molar ratios and different amounts of catalyst, keeping other parameters like temperature and time constant. The O/A molar ratios were taken as 1:3, 1:4, 1:5, 1:6 and 1:7 for 1.0 h with catalyst amount varying from 1 g to 4 g at 30 °C by trial-and-error method and data obtained (Fig. 6b). The maximum yield of biodiesel for a specific O/A ratio was considered the optimum O/A molar ratio.
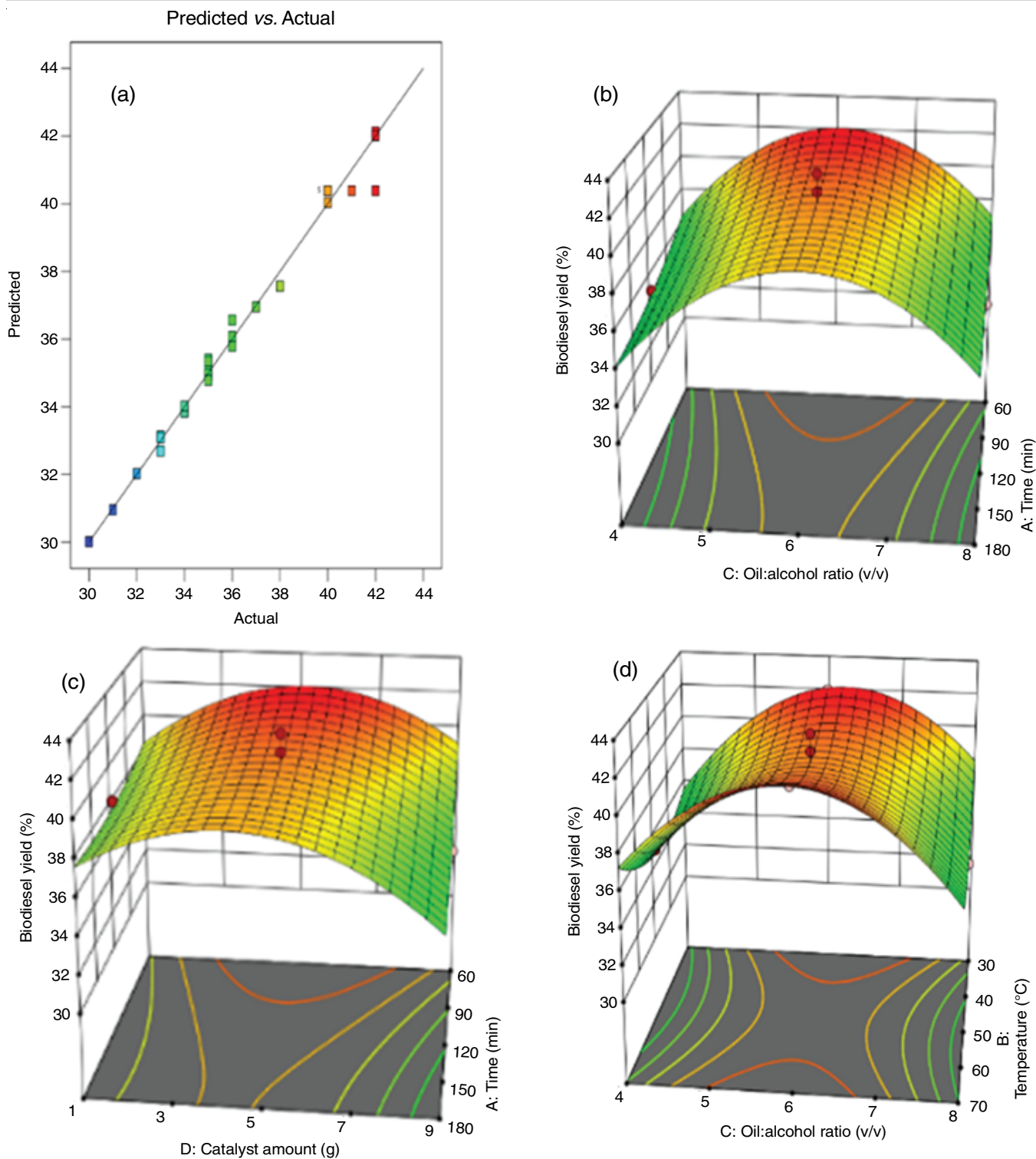
Fig. 5. (a) Graph for oil: alcohol ratio & time *vs.* yield of biodiesel (calcined eggshell), (b) amount of catalyst and time *vs.* yield (calcined eggshell), (c) Oil:alcohol ratio & temperature *vs.* yield of biodiesel (calcined eggshell), (d) the graph between actual biodiesel yield and predicted yield (calcined eggshell)

Amount of catalysts *vs.* time & maximum yield: The biodiesel was produced at different amounts of catalyst and at different times intervals, keeping other parameters like oil: alcohol ratio (O/A) and temperature constant by the trial-and-error method. The maximum yield of biodiesel for a specific O/A ratio was considered the optimum O/A molar ratio (Fig. 7c).

Graph between the predicted amount of biodiesel yield with actual experimental yield: The biodiesel yield was obtained experimentally and RSM obtained the yield of predicted biodiesel RSM. The graph was plotted with both data and found the relation between them. The correlation was obtained; almost all the points are on the straight line and very
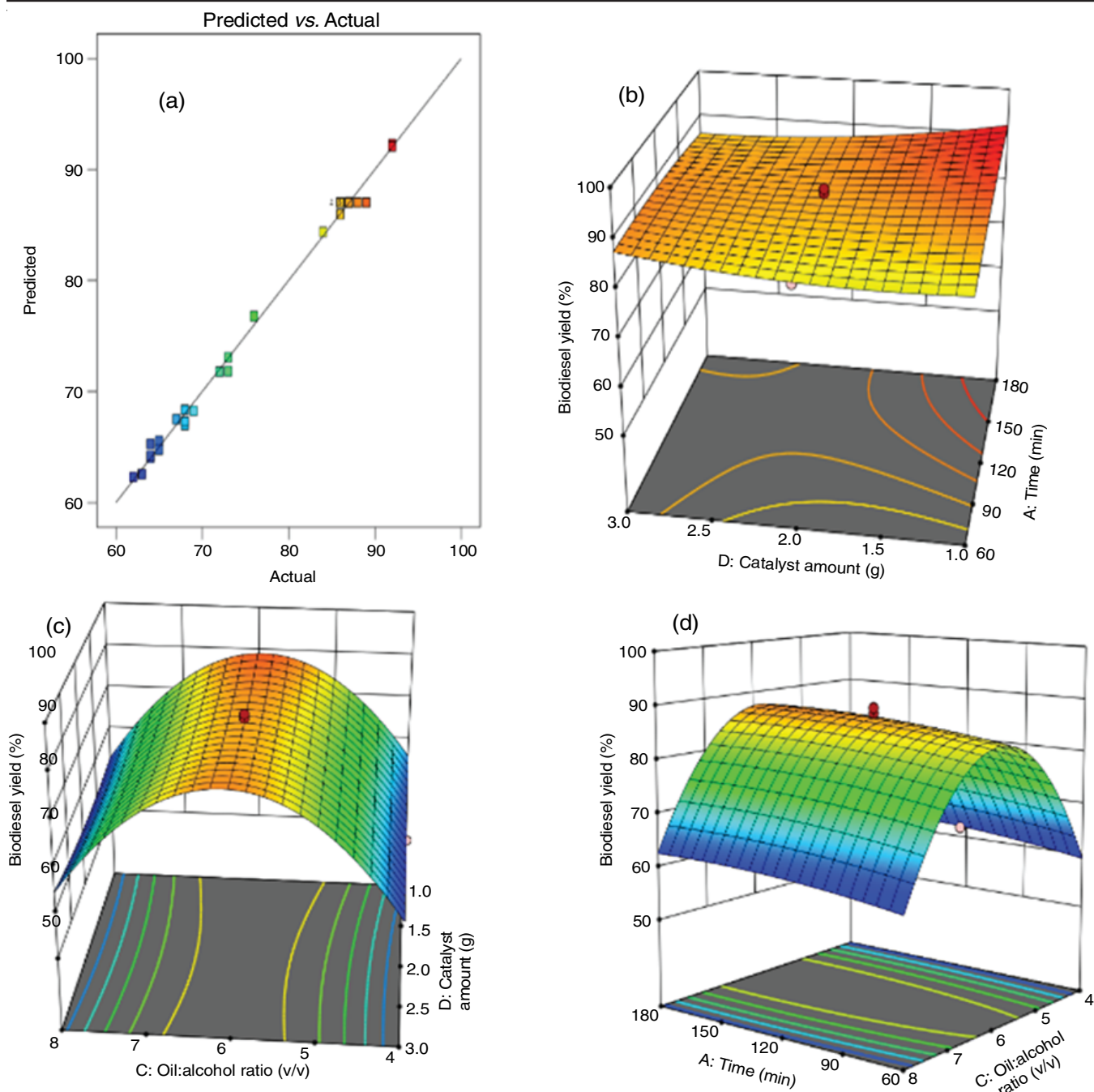
Fig. 6. (a) Graph between time *vs.* oil:alcohol and yield (ELC), (b) graph between oil:alcohol *vs.* catalyst and yield (ELC), (c) the graph between the amount of catalyst *vs.* time and yield (ELC), (d) the graph between predicted yield *vs.* actual yield (ELC)

few points are a little far from the straight line (Fig. 7d). It is too regrated to each other, as a result, the experimental value hold valid.

Comparative optimized parameters for the production of biodiesel by calcined eggshell and immobilized eggshell-lipase catalyst: The obtained value of all parameters for biodiesel production is studied by using calcinated eggshell and immobilized lipase-eggshell catalyst. The optimum value of all the parameters for both catalysts is given in Table-7. It was found that all the biodiesel properties by each method were same. This property was compared with the ASTM D 06751: 2012 standard [55,56], which is shown in Table-8.

Optimization of transesterification process: The effect of process parameters like temperature, time, O/A molar ratio, amount of catalyst and yield of biodiesel was studied by RSM. These factors are employed in the optimization study with 30 experimental runs using CCD (central composite design) for calcined eggshell and immobilized eggshell-lipase as a heterogeneous catalyst for biodiesel production. The optimized parameters for the production of biodiesel by calcined eggshell are O/A molar ratio (1:6), reaction time (120 min), temperature (30-50 ºC and amount of calcined eggshell (5 g wt.%) and the optimized parameters for an immobilized eggshell-lipase catalyst for the production of biodiesel are O/A molar ratio
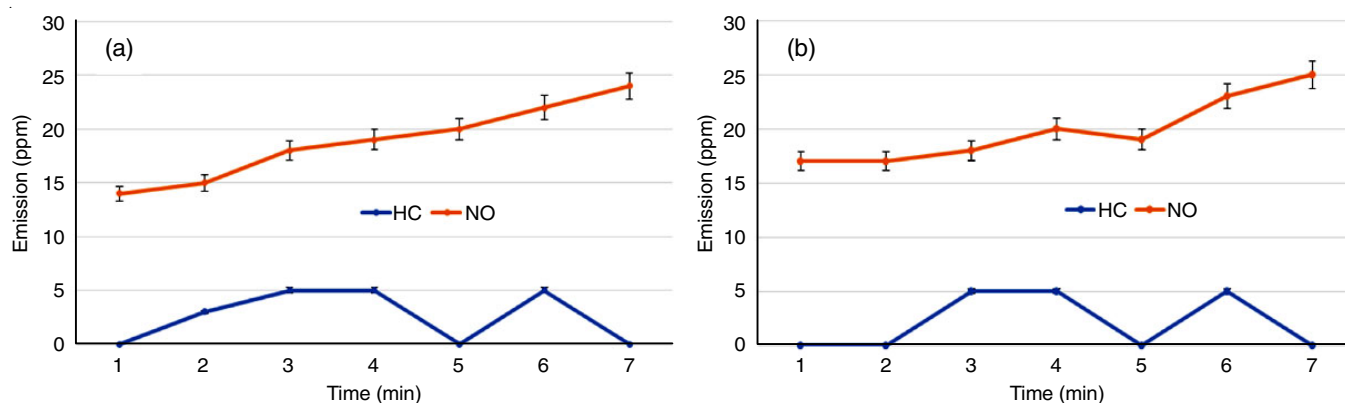
Fig. 7. (a) Emission hydrocarbon and NO *vs*. time (ELC), (b) emission of HC and NO *vs*. time (calcined eggshell)

TABLE-6
ACTUAL DESIGN VALUE FOR IMMOBILIZED LIPASE EGGSHELL CATALYST

Std.	Run	Factor 1	Factor 2	Factor 3	Factor 4	Response 1
		A	B	C	D	Biodiesel yield (%)
		Time (min)	Temp. (°C)	Oil:alcohol ratio (v/v)	Catalyst amount (g)	
5	1	60	30	8	1	63
12	2	180	50	4	3	65
13	3	60	30	8	3	72
24	4	120	40	6	4	92
20	5	120	40	6	2	87
2	6	180	30	4	4	73
26	7	120	40	6	2	87
4	9	180	50	4	1	76
27	10	120	40	6	2	89
18	11	240	40	6	2	86
29	12	120	40	6	2	86
1	13	60	30	4	1	64
7	14	60	50	8	1	64
6	15	180	30	8	1	68
15	16	60	50	8	3	67
14	17	180	30	8	3	68
3	18	60	50	4	1	68
9	19	60	30	4	3	69
19	20	120	50	6	2	92
30	21	120	40	6	2	87
16	22	180	50	8	3	68
11	23	60	50	4	3	64
10	24	180	30	4	3	65
17	25	60	40	6	2	84
8	26	180	50	8	1	73
28	27	120	40	6	2	86
21	28	120	40	6	2	87
25	29	120	40	6	2	88
22	30	120	40	4	2	62

(1:6), reaction time (120 min), temperature (40 °C) and amount of catalyst (2 g wt.%). As per the optimum data, the ELC is more economical compared to the eggshell catalyst, since the less amount of catalyst required for maximum yield of biodiesel. The above four factors for the production of biodiesel were correlated and every factor is significantly optimized. The model for correlation of these factors was analyzed and formed a quadratic relation among them [57]. The four factors designed quadratic equation for process optimization for calcined eggshell catalyst and immobilized lipase-eggshell catalyst is represented by eqn. 4:

$$Y\ (\%) = \beta_1 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 AB + \beta_6 AC + \beta_7 AD + \beta_8 BC + \beta_9 BD + \beta_{10} CD + \beta_{11} A^2 + \beta_{12} B^2 \beta_{13} C^2 \beta_{14} D^2 \quad (4)$$

where Y is the percentage yield of biodiesel, A, B, C, D are the independent variable(input) and AB, AC, AD, BC, BD and CD are the interaction variables(input) [19,57].

Transesterification for calcined eggshell and immobilized eggshell-lipase catalyst: The evaluated parameters for maximum yield of biodiesel by using calcined eggshell as a catalyst are temperature (30 to 70 °C) with O/A molar ratio (1:4 to 1:8), time (60 min to 180 min) and amount of catalyst

TABLE-7
A COMPARATIVE OPTIMUM VALUE OF ALL
PARAMETERS FOR CALCINATED EGGSHELL AND
IMMOBILIZED EGGSHELL-LIPASE CATALYST

Parameters	Calcinated eggshell	Immobilized eggshell-lipase
A: Time	120 min	120 min
B: Temperature	50 °C	40 °C
C: Oil:alcohol molar ratio	1:6	1:6
D: Catalyst amount	5 g	2 g
R^2	0.9862	0.9978
Adjusted R^2	0.9734	0.9959
Predicted R^2	0.9659	0.9737
Lack of fit	Not significant	Not significant
Biodiesel yield (%)	40.38	92

(1-9) g wt.% and evaluated parameters for immobilized eggshell-lipase catalyst with temperature (30-50 °C) with an O/A molar ratio (1:4 to 1:8), time (60-180 min) and amount of catalyst (1-4) g wt.%. The significant model descending order: D, D^2, C, B, B^2, A^2, AB, C^2 and A for the biodiesel production using both calcined eggshell and immobilized lipase-eggshell catalyst. The value of $p < 0.05$ for calcined eggshell implies the significance and the p-value < 0.0001 indicates the most significance. Most of the terms are significant. The A, B, C, D, AB, AC, AD, BC, BD, A^2, B^2, C^2 and D^2 are the most significant for calcined eggshell and immobilized lipase-eggshell catalyst. The Adjusted value of R^2 (0.9734) and p-value (< 0.0001) for calcined eggshells indicate that model terms are significant. The lack of fit value (0.1967) is not significant concerning pure error [31,57]. The suggested model for the calcined eggshell catalyst is represented by eqn. 5:

$$Y (\%) = 42.1295 + 1.208\ A + 28.0\ B - 11.375\ C + 21.855\ D + 0.0979\ AB - 0.2486\ AC + 17.916\ AD + 19.08\ BC - 0.1845\ BD + 18.339\text{-}CD + 18.054\ A^2 - 111.782\ B^2 + 0.875\ C^2 + 1.5833\ D^2 \quad (5)$$

For immobilized lipase-eggshell catalyst, the p-value (< 0.001) implies significance, the adjusted R^2 value (0.9959), p-value (< 0.0001) indicates model terms are significant, lack of fit value (0.8611) and value of P (0.3978) are not significant. The suggested model for the immobilized lipase-eggshell catalyst is represented by eqn. 6:

$$Y (\%) = 99.61 - 1.02\ A - 0.468\ B + 3.19\ C + 6.86\ D + 2.33 \times 10^{-2}\ AB - 0.55\ AC + 0.077\ AD + 0.20\ BC + 0.092\ BD + 0.86 \times 10^{-2}\ CD + 0.87 \times 10^{-2}\ A^2 + 5.43 \times 10^{-2}\ B^2 + 0.34\ C^2 + 0.12\ D^2 \quad (6)$$

Engine and emission test for produced biodiesel

Engine details: IC Engine set up under test is research diesel having power 3.50 kW @1500 rpm which is 1 cylinder, four-stroke, constant speed, water cooled, diesel engine, with cylinder bore 87.50 (mm), stroke length 110.00 (mm), connecting rod length 234.00 (mm), compression ratio 18.00, swept volume 661.45 (cc).

Combustion parameters: Specific gas const. (kJ/kg K): 1.0, air density (kg/m³): 1.17, adiabatic index: 1.41, polytrophic index: 1.34, number of cycles: 10, cylinder pressure reference: 6, smoothing 2, TDC references-16.

Performance parameters: Orifice diameter (mm): 20.00, orifice coeff. of discharge: 0.60, dynamometer arm length: 185, fuel pipe dia (mm): 12.40, ambient temp. (ºC): 27, pulses per revolution: 360, fuel type: diesel, fuel density (Kg/m³): 772, calorific value of fuel (kJ/kg): 38000.

Change of thermal efficiency and brake thermal efficiency of engine with load applied: The embedded system-engine plotted graph for change in the engine's efficiency with an applied load on the engine (Fig. 8a). When the load increases from 0.1 kg to 12 kg, the engine's efficiency decreases from 92% to 68% but not proportionally. The load varies from 0.1 kg to 3.5 kg. The efficiency decreases proportionally in a line segment with a slope of 72.82º, the load from 3.5 kg to 6.5 kg, the slope has decreased to 1.90º, the load change from 6.5 kg to 9.5 kg then the slope again decreases to 63.43. Finally, further efficiency decreases with a slope of 57.99º when the load increases from 9.5 kg to 12 kg, as indicated in the blue colour graph in Fig. 8a. Now break down applied to the engine, so load decreases gradually from 12 kg to 9.5 kg, 9.5 kg to 6.5 kg, 6.5 kg to 3.5 kg and 3.5 kg to 0.1 kg, the efficiency also decreases to 1.0% as shown by the red graph in Fig. 8a. The break thermal efficiency with load for a fuel produced by calcined eggshell is similar to that of ELC as shown in Fig. 8b.

Change of SFC (specific fuel consumption) and fuel consumption with load applied: The fuel consumption per unit time is called specific fuel consumption. The SFC and fuel consumption changed with a change in load on the engine (Fig. 8c) for fuels produced with ELC catalyst. The SFC drastically decreases proportionally from 2.8 kg/h to 0.5 kg/h when load increases from 0.4 kg to 3.5 kg and further decreases normally from 0.5 kg/h to 0.25 kg/h when load increases from 3.5 kg to 12.5 kg, *i.e.* at the initial stage when the engine started the rate of change of fuel consumption was more and after-
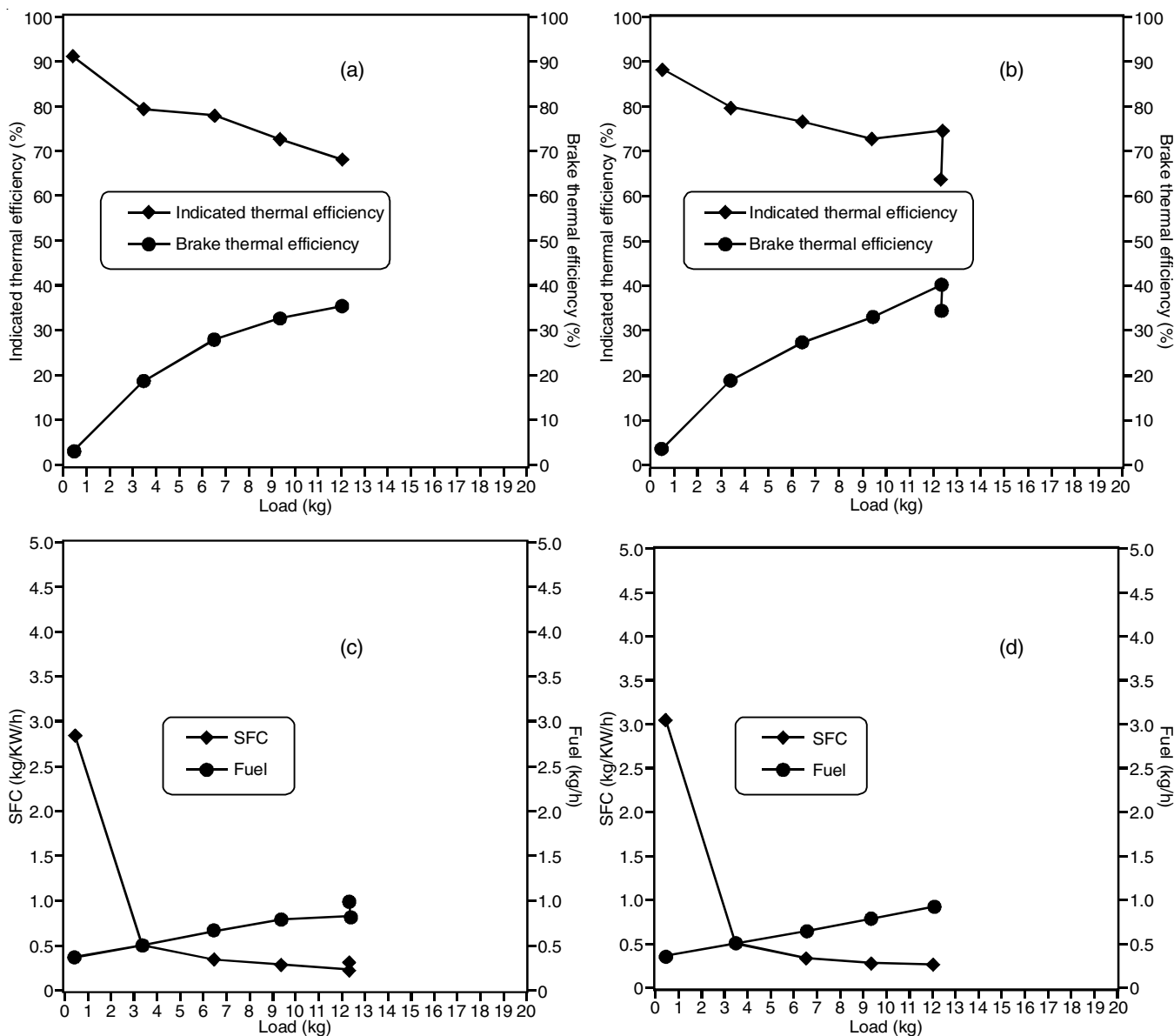
TABLE-8
PHYSICAL PROPERTIES OF BIODIESEL PRODUCED BY CALCINATED
EGGSHELL AND IMMOBILIZED EGGSHELL-LIPASE CATALYST

Physical parameters	Calcinated eggshell	Immobilized eggshell lipase	ASTM D 6751
Acidic value	0.60 mg NaOH/g	0.48 mg NaOH/g	0.50 mg KOH/g
Density	883 kg/m³	862 kg/m³	860-900 kg/m³
Kinematic viscosity @ 40 °C	5.65 cst	4.40 cst	3.5-5.0 mm²/s
Flash point by PMCC method	186 °C	152 °C	Min 130 °C
Fire point by PMCC method	198 °C	168 °C	–
Cloud point	5 °C	-1 °C	Report D 2500
Pour point	-4 °C	-4 °C	–
Moisture content	0.029%	0.024%	500 mg/kg
Calorific value	7463.612 cal/g	6899. 87893 cal/g	–

Fig. 8. (a) Graph of image brake thermal efficiency *vs*. load, (b) graph of image brake thermal efficiency *vs*. load (calcined eggshell) (ELC), (c) graph for SFC and fuel consumption *vs*. load (ILE), (d) graph for SFC & fuel consumption *vs*. load (calcined eggshell)

wards rate of change of fuel consumption was less with the load as indicated by blue graph in Fig. 8c for the fuel produced with ELC; the fuel consumption increases proportionally from 0.4 kg to 0.8 kg when load increases from 0.4 kg to 12.5 kg as indicated by red graph in Fig. 8c, *i.e.* total fuel consumption per hour was 0.4 kg at load which is too marginal. The fuel consumption for the fuels produced with calcined eggshells is similar to ELC, as shown in Fig. 8d. Hence, the fuel behaviours with the engine is almost same as whether it is produced with ELC or calcined eggshell catalyst.

Emission test: The emission of different gases was moni-tored during the engine test of produced biodiesel using immo-bilized lipase-an eggshell catalyst in the transesterification reaction. The emitted gases were monitored as hydrocarbon, nitric oxide, carbon monoxide, carbon dioxide and carbon mono oxide with time.

Emission of hydrocarbon (HC) and nitric oxide (NO): The monitoring of hydrocarbon gases and NO were observed during the operation of the engine from 1st min to 7th min, the maximum amount of emission of hydrocarbon was 5 ppm and the maximum amount of emission of NO was 25 ppm, as shown in Fig. 7a for a fuel produced with ELC and in Fig. 7b for fuel produced with the calcined eggshell, which is too low compared to the emission of hydrocarbon by the conventional source of energy [58]. Shirneshan [58] reported the emission of hydrocarbon was 24-36 ppm and the emission of CO was a minimum of 25 ppm from diesel and petrol fuels, which is too high compared to biodiesel [58]. Similarly, few researchers [59,60] reported the emission of 40-70% for hydrocarbon and 250-650 ppm for NOx from diesel and petrol fuels, which is too high as compared to produced biodiesel fuel. There is no emission of CO from the engine using produced biodiesel
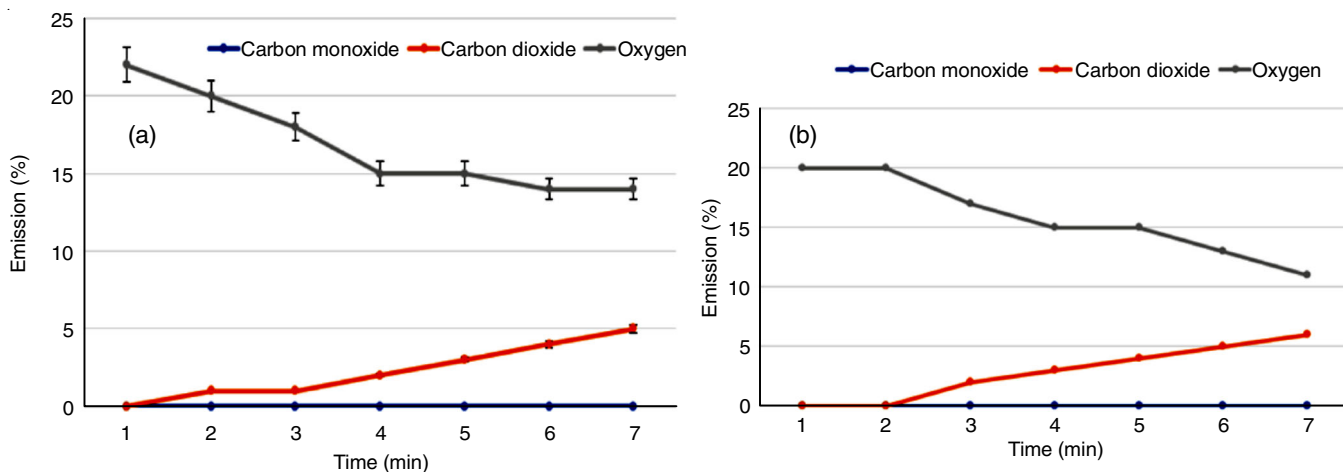
Fig. 9. (a) Emission of carbon monoxide, oxygen and carbon oxide *vs.* time (ELC), (b) emission of CO, oxygen and carbon dioxide *vs.* time (calcined eggshell)

fuel, whereas CO_2 and O_2 emitted a maximum of 5 and 22 ppm, respectively from the engine, as shown in Fig. 9 for the fuels produced with ELC and in Fig. 9b for the fuel produced with calcined eggshell catalyst. Moreover, the emission of CO and CO_2 was a minimum of 25 and 7 ppm, respectively, from the diesel engines [58], so the emission of HC, NOx, CO and CO_2 from biodiesel engine is too negligible compared to conventional fuel engine for fuels produced with ELC as well as with calcined eggshell. Hence, biodiesel produced with ELC and calcined eggshell catalyst is good environmentally friendly.

Conclusion

The catalyst derived from immobilized lipase-eggshell had excellent activity in heterogeneous transesterification of waste cooking oil for biodiesel production. The calcination of the catalyst upsurged the surface area, which led to better catalytic activity. Reusing eggshells to prepare the catalyst could recycle the waste, minimise contaminants, reduce the cost of catalyst and make the catalyst environmentally friendly. Hence, eggshell immobilized with lipase showed high activity and it also has a better yield of biodiesel production than the calcined eggshell catalyst. The engine and emission test and fuel properties for immobilized-lipase eggshell catalyst biodiesel gave better performance than calcined eggshell catalyst biodiesel. However, the cost of biodiesel production by lipase will be somewhat costlier than calcined eggshell, which has reduced the overall production cost of biodiesel by producing the biodiesel with immobilized eggshell-lipase catalyst.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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