

Research Article

Lipase Functionalized Magnetic Nanobiocatalyst for Transesterification of Waste cooking oil into Biodiesel

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Abstract

The enzyme functionalized magnetic nanoparticle has great potential owing to its surface functionalities. In this work, nanobiocomposite (lipase bound magnetic nanoparticles) was used as catalyst for the transesterification of waste cooking oil into biodiesel. The average roughness and non-uniformity of the synthesized composite was analyzed using AFM, with certain aggregation over the surface of the particles. The chemical bonding of lipase with magnetic nanoparticles was confirmed by FT-IR and the XRD analysis confirmed the phase transition of lipase with magnetic particles. The magnetic property of composite after immobilization was confirmed by VSM. The biodiesel yield of 93.58% was obtained at 90 min of reaction time, 40°C of temperature using 36% (w/w) of catalyst loading and 1:5 (v:v) ratio of oil to methanol. The reusability characteristics were studied, indicating the stability over three runs. The presence of fatty acid methyl esters (biodiesel) was confirmed by GC-MS analysis.

Keywords: Biodiesel; Lipase; Immobilization; Transesterification; Magnetic nanoparticles.

Introduction

In recent times, alternative fuel has gained wide attention due to continuous depletion of fossil fuels around the world. Biodiesel has replaced the conventional energy sources due to various advantages associated with it. Biodiesel is produced by the transesterification reaction in the presence of a catalyst and methanol [1,2]. The two important criteria for the production process lies in the selection of suitable feedstock and catalyst as they directly depend on the cost of the production [3]. The most commonly used feedstock for the productions are edible, non-edible, animal fats, waste cooking oil and algae. The advantage of using waste cooking oil solves the issues of disposal after frying and helps in effective management of waste. The use of less expensive materials reduces the cost by 60-70 % of the total cost of production [4]. The enzyme catalyzed transesterification reaction ensures easy recovery of glycerol with high purity products in a single purification step. The two main drawbacks of using enzyme as catalysts are the cost of enzyme and the deactivation process on its interaction with methanol. The above mentioned problems were rectified mainly by

immobilization and stepwise addition of methanol [5,6].

Immobilization technologies offer an enhanced level of reusability and operational stability with high conversion rates and shorter reaction times. The rigid structure of the enzyme prevents from conformational variation and inactivation during the immobilization technique [7]. It was reported that the active sites of free lipase enzyme are formed with aggregates, whereas the active site of immobilized lipase was available for interaction and free from aggregation. In order to improve the performance, stability and separation process easier, immobilization of lipase was coated over magnetic particles, which are recovered by an external magnetic field. Nanomaterials as support for immobilization of enzyme are used widely because of their increased surface to volume ratio. Magnetic particles have a high specific area so that the binding of the enzyme could be easier with lower mass transfer resistance. The functionalized coating of nanoparticles are extensively used because of their selective recognition and capture of specific

molecules to maintain its stability conditions [8,9].

The present work focus on synthesis of functionalized nanobiocomposite and the use of nanocomposite for the production of biodiesel from waste cooking oil. The synthesized catalyst is characterized using Atomic Force Microscopy (AFM), X-Ray Diffraction (XRD), Fourier Transform Infrared spectroscopy (FT-IR) and Vibrating Sample Magnetometer (VSM). Optimization of various parameters relating to the production process was studied. The produced biodiesel was characterized for fatty acid methyl esters and compared with standards.

Materials and methods

Materials used

The chemicals used for the synthesis of magnetic nanoparticles such as Ferrous sulfate, Ferric chloride, Sodium hydroxide of laboratory grade were purchased from Rechem Laboratory. The materials used for surface modification like 3-Aminopropyl triethoxysilane (APTS), Tetra ethyl orthosilicate (TEOS), Sodium fluoride of analytical grade were purchased from Sigma Aldrich and Fisher Scientific respectively. Chemicals such as potassium Dihydrogen phosphate and Di-Potassium phosphate of analytical grade were purchased from Hi-Media. The glutaraldehyde and methanol were obtained from the Merck, India. The waste cooking oil used for the production was collected from commercial cooking area in bulk quantity. The enzyme lipase from (*Candida rugosa*) was commercially purchased from Sigma Aldrich (Bangalore) with the activity of 700 U/mg and these chemicals were used without any purification technique.

Synthesis of magnetic nanoparticles

Magnetic nanoparticles were synthesized by chemical co-precipitation method with certain modifications. 0.2 M of ferric chloride and 0.1 M of ferrous sulfate of 50 ml was prepared by keeping the solution under constant stirring condition in a magnetic stirrer at maximum speed. The precipitating agent, NaOH of 10% was added in drops to the above stirring solution for obtaining a black colored precipitate. The solution was left undisturbed until the particle settles at the bottom. The obtained precipitate was separated by magnetic decantation and washed twice with distilled water. The final

product was dried at 80°C for 24 hrs in hot air oven and grounded to fine particle [10-13].

Surface modification of magnetic nanoparticles

The prepared magnetite was suspended in 20 ml of distilled water. To this 1 ml of APTS, 3 ml of methanol, 1 ml of 1% Sodium fluoride was added and stirred continuously for 10 min. To the above solution, 20 ml of TEOS was added in drops and stirred for 24 hrs. The obtained precipitate was washed with ethanol, water and dried at 80°C for 24 hrs.

Immobilization of lipase with magnetic nanoparticles

The lipase bound magnetic nanoparticles was prepared by dispersing modified surface nanoparticles and lipase with respect to catalyst concentration in 100 mM phosphate buffer solution of pH 7. The binding agent, glutaraldehyde of 25% was added and kept at 30°C for 2 hours. The functionalized magnetic nanoparticles was then separated by magnetic decantation and then washed twice with phosphate buffer solution [12]. The supernatant obtained after immobilization was subjected to protein estimation by Bradford assay and the binding efficiency was calculated. The immobilized enzyme on the nanoparticles support was thoroughly washed with deionized water and buffer to remove any loosely bound protein.

Characterization of nanobiocomposite and biodiesel

The synthesized nanobiocomposite was characterized to study about the surface topology. Atomic Force Microscopy (AFM) was carried at different contact mode to examine the nature of particle at various positions using AFM (NTMDT, Ireland). The structural analysis was carried out by XRD (RIKAGU, Japan) confirming the structure of iron oxide. The study of functional group was performed by FT-IR-6300, (Jasco, International Co., Japan) which shows the chemical binding and its stability after the immobilization of enzyme. The magnetic property of magnetic nanoparticles after enzyme binding was confirmed by VSM. The biodiesel was characterized to confirm the presence of the biodiesel produced by a transesterification process using by Agilent 6890 gas chromatography equipped with 2 mm direct injector liner and 15m Alltech EC-5 column. The

obtained biodiesel was washed with distilled water twice to remove the impurities prior to GC-MS analysis. The oven temperature was programmed at 35°C with hold time of 2 min. It was then ramped at 20°C per minute to 300°C and hold for 5 min. The helium carrier gas was set to 2 ml/min flow rate at constant flow mode. Mass spectrometry was carried by JEOL GC mate II bench top double-focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-2000 [14].

Enzymatic transesterification reaction

Transesterification reaction was carried out in batch processes, under constant mixing conditions. The reaction was preceded by mixing a known amount of functionalized magnetic nanoparticles with methanol. Addition of methanol was done by stepwise to reduce the deactivation of the enzyme on interaction with methanol. The transesterification process was repeated for varied parameters like catalyst loading (4-40%, w/w), oil:methanol (1:1-1:6, v:v), temperature (25-45°C), agitation speed (100-300 rpm) and reaction time (30-180 min). Once the reaction is complete under fixed operating conditions, the mixture was transferred to separating funnel for the observation of phase separation. The apparatus was left undisturbed and the phase separation was noted with lighter and denser phase at the top and bottom of the separating funnel respectively. Biodiesel yield (% (w/w)) of was calculated using Eq. (1)

$$Yield = \left(\frac{V_B \times \rho_B \times M_B}{V_O \times \rho_O \times M_O} \right) \times 100 \quad \dots(1)$$

Where, V_B is the volume of the product, V_O is volume of oil, ρ_B is the density of biodiesel, ρ_O is density of oil, M_B is molecular mass of biodiesel and M_O is molecular mass of oil [14]. All the experiments were performed in duplicate and mean values is used for analysis.

Results and discussion

Surface morphology of nanobiocomposite using AFM analysis

The surface characteristics of nanobiocomposite were confirmed by AFM. The average roughness and non-uniformity of the synthesized particles was observed in AFM analysis and the particle was found to have certain aggregation over the surface. The analysis was performed in contact mode of 5*5

with the average roughness of 20.92. The size of the nanobiocomposite was found to be around 80-100 nm with certain agglomeration as shown in Fig. 1. The high roughness of the nanobiocomposite surface confirmed its influence on the spatial resolution and high surface area. The nanobiocomposite aggregate together without any space in between is resulted due to magnetic property, confirming the coating of lipase over magnetic particles. The pores of the synthesized particle were noted to be regular for the easy attachment of enzyme as compared to literature [15].

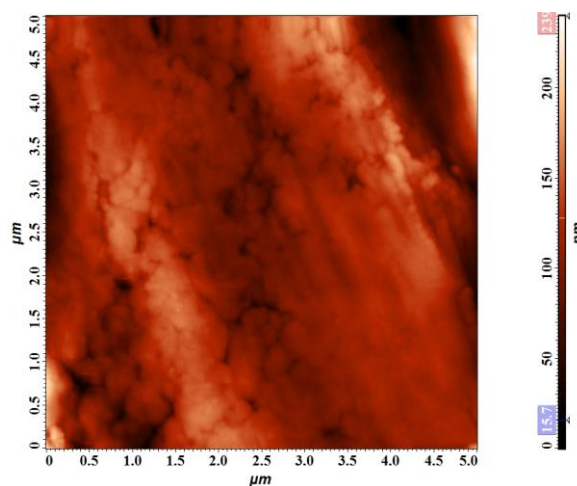


Fig. 1. 2D image of surface analysis of magnetic nanobiocomposite of lipase by Atomic Force Microscopy

Structural analysis of nanobiocomposite using XRD

The structural nature of lipase immobilized MNP was characterized by XRD analysis. The XRD pattern of lipase bound magnetic particles is shown in Fig. 2. The analysis was carried out at 2θ ranging from 15°C to 70°C. The characteristic peak confirms the presence of crystalline magnetic nanoparticles with cubical structure. The peak at 35.5, 53.4, 57 and 62.6° exhibits their corresponding indices such as (311), (422), (511) and (440). The phase characteristics of synthesized magnetic particle exhibited (3 1 1), (4 0 0), (5 1 1) and (4 4 0) planes confirming its cubical structure [16-18,20,23]. Thus, it was observed that the phase remains unchanged even after immobilization implying that magnetic property was retained during the production and separation process.

Functional group analysis using FT-IR

The chemical bonding between the enzyme and magnetic nanoparticle was analyzed by FT-IR as shown in Fig. 3. The peak at 1068 and 790 cm^{-1} of Si-O and NH_2 vibration mode confirms the presence of coating materials with the identification of their functional group. The characterization of free magnetic nanoparticle is reported in our previous work, which confirmed

the presence of primary amine group at 3462 cm^{-1} and weak vinyl bond (C-H) at 3076 cm^{-1} [19]. The existence of lipase was confirmed by peak at 1628 cm^{-1} and 1437 cm^{-1} . The vibration peak at 622 cm^{-1} confirms the presence of Fe-O bond and peak at 2920 cm^{-1} refers to C-H bond. The weak characteristic peak of lipase was noted due to the low lipase binding nature during the reaction [21,23,25].

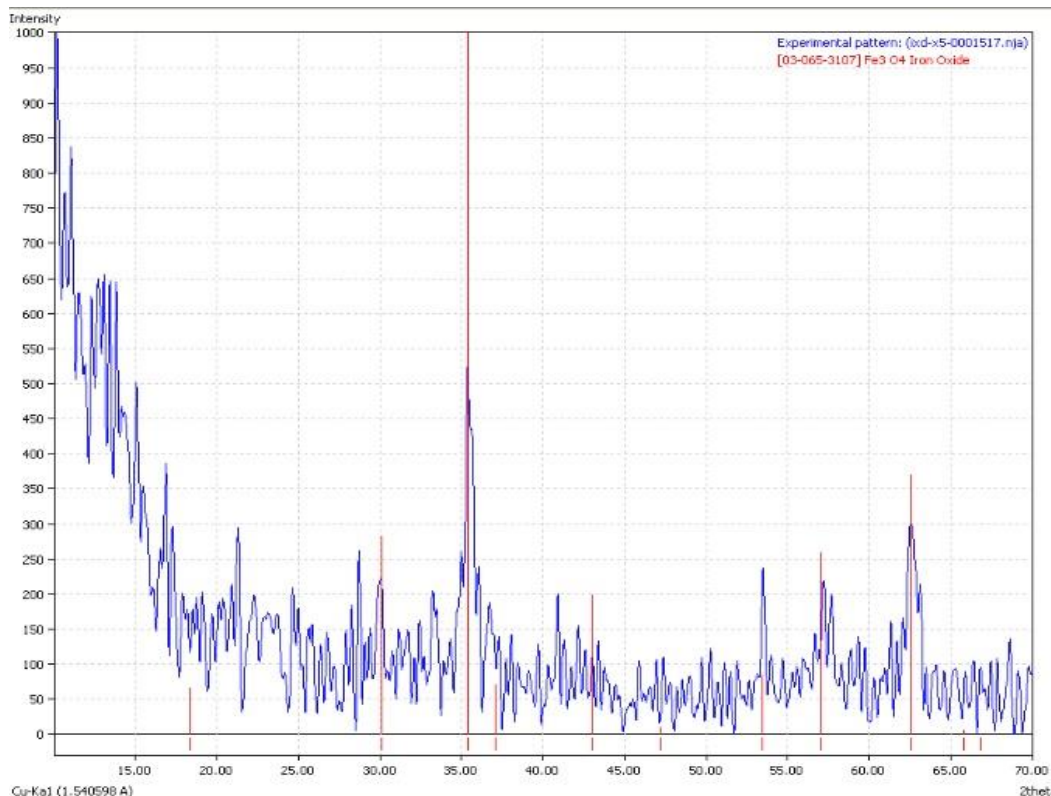


Fig. 2. XRD analysis of magnetic nanobiocomposite of lipase

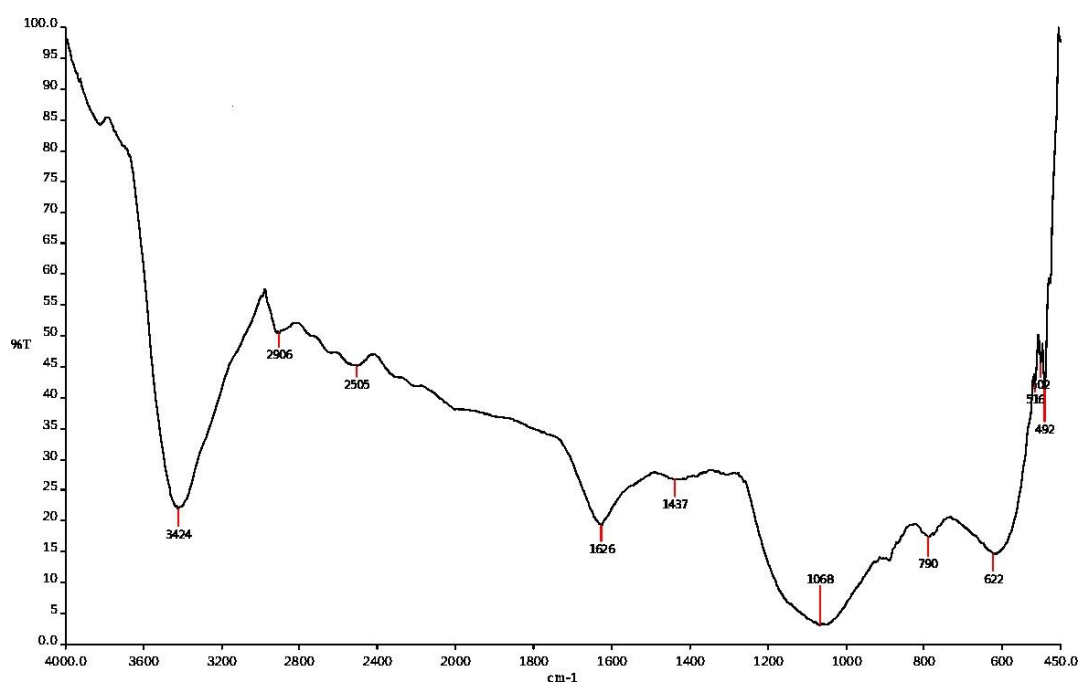


Fig. 3. FT-IR of magnetic nanobiocomposite of lipase

Analysis of magnetic particle of nanobiocomposite using VSM

The properties of nanocomposites were studied by Vibrating Sample Magnetometer as shown in Fig. 4. The sample was analyzed at room temperature with increase in coercivity noted due to the nature of the samples. The coercivity and retentivity was found to be 25.086G, $49.760E^{-3}$ emu respectively. The saturation magnetization was found to be 1.9838 emu/g and weak hysteresis confirmed the superparamagnetic property of the particle. The magnetic response showed a decline owing to its thickness obtained during the functionalization of magnetic particle. The higher saturation value showed positive effects on separation by applying an external magnet which could control the movement of the particle [24].

Effect of catalyst loading on transesterification reaction

The amount of catalyst used is an important criterion for the effective completion of reaction. The effect of catalyst loading was studied by varying the catalyst concentration from 4%-40% (w/w) under constant temperature and reaction time at 35°C and 90 min respectively. The reaction was also carried under constant stirring rate at 150 rpm and 1:4 of oil:methanol ratio. It was noted from Fig. 5, that the yield of biodiesel showed gradual increase from 12%-28% (w/w) with the maximum yield at 32% (w/w). The yield was maximum due to the formation of enzyme-substrate complex. However, the yield showed a decrease after 36% due to overloading of catalysts with high protein-protein interaction. The decrease in the yield of biodiesel owing to the limitation of mass transfer due to catalyst overloading [21,22].

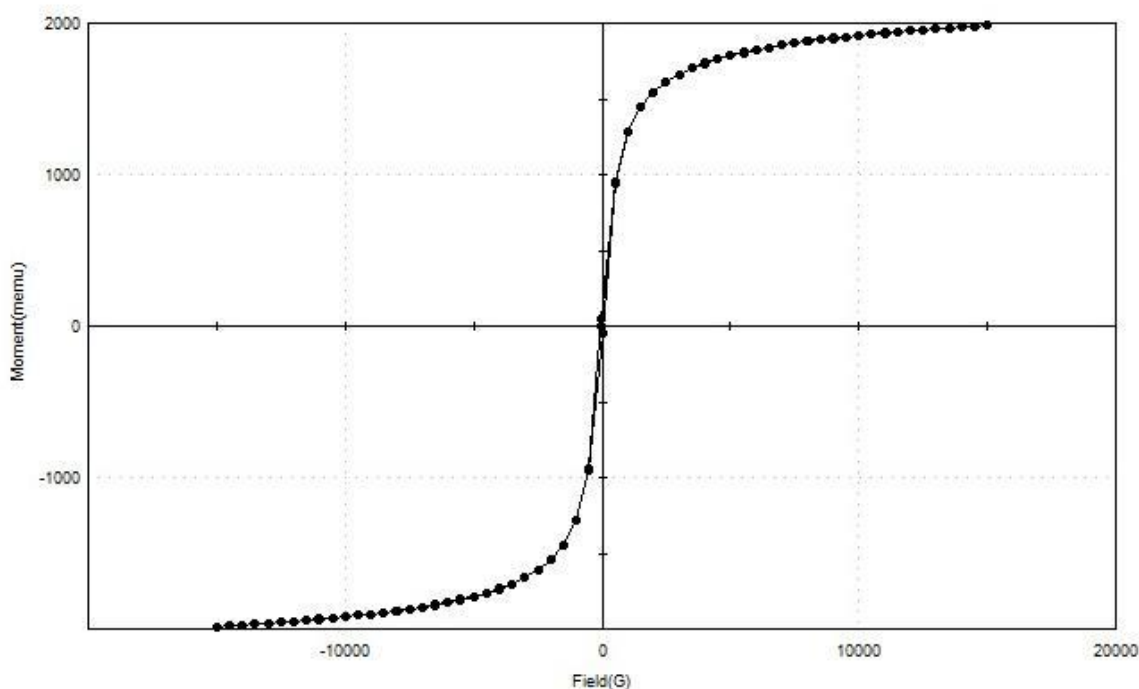


Fig. 4. Magnetisation effect on magnetic nanobiocomposite of lipase

Effect of temperature on transesterification reaction

Enzyme catalyzed transesterification reaction is highly influenced by temperature. The effect of temperature was studied effectively by varying temperature from 25-40°C. From Fig. 6, it is clear that the yield increase in the range of 30-40°C as the mass transfer limitation is very minimum between methanol and lipase bound magnetic nanoparticles. The minimal external

mass transfer limitation was might be due to the high dispersibility of nanocomposite in aqueous phases. This high dispersibility enhances the contact between solvent:oil mixture with kioase immobilized on surface of magnetic nanoparticles providing less mass transfer limitations. Also enzymes are immobilized in the surface of the magnetic nanoparticles. Thus there are no internal diffusional mass transfer resistance is possible. This is a major advantage of immobilizing enzyme on nanoparticles than

other solid support matrix which might show internal mass transfer limitations due to the immobilization of enzyme on internal structure of solid matrix. The yield showed a decrease after 40°C as the activity of catalyst was reduced due to exposure to a higher temperature range. Hence, the maximum yield was found to be 92.66% at 40°C. The reaction kinetics was assumed to follow first order and the rate constant k was calculated using yield as function of time at different temperature. The temperature dependency of transesterification reaction of waste cooking oil was studied using rate constants obtained at different temperature using the Arrhenius plot. The Arrhenius plot correlates the natural logarithm of the rate constant of the reaction $[\ln(k)]$ with the inverse of temperature ($1/T$), where T in Kelvin. The reason for the drop in second point of linear regression might be due to forward rate constants that cause abrupt change enzyme conformation during temperature change. The activation energy of enzyme catalyzed transesterification was found to be 15.56 KJ/mol which implies that the enzyme catalyzed reaction occurs at mild condition as shown in Fig. 7. The obtained Arrhenius plot was compared and consistent with the literature [26].

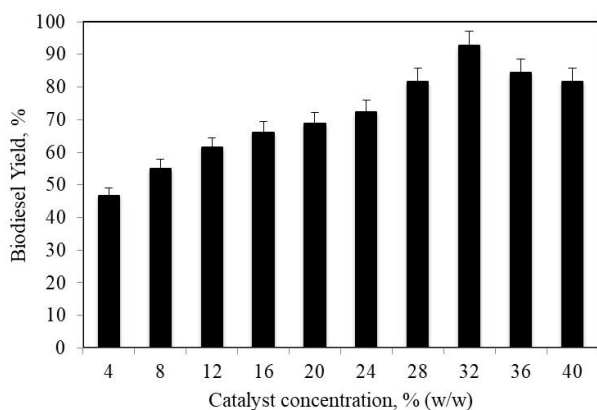


Fig. 5. Effect of catalyst loading on transesterification (Constant temperature 35°C; Reaction time 90 min; stirring rate 150 rpm, oil:methanol ratio 1:4 (v:v))

Effect on reaction time and stirring rate

Reaction time and stirring rate play a significant role in production of biodiesel. The reaction time was varied from 30-180 min by keeping other parameters at optimal conditions. The maximum biodiesel yield of 92.66% was obtained at 90 min. From Fig. 8, it was observed that the yield was maximum at initial stage and showed a

slight decrease latter which was attributed to long time exposure of catalyst, leading to over accumulation of reactants to the surface. The effect of stirring rate was studied by varying from 100-300 rpm with at optimal conditions. From Fig. 9 the maximum yield was observed at 200 rpm and the yield decreases at certain range due to change in structure on applying excessive mechanical agitation.

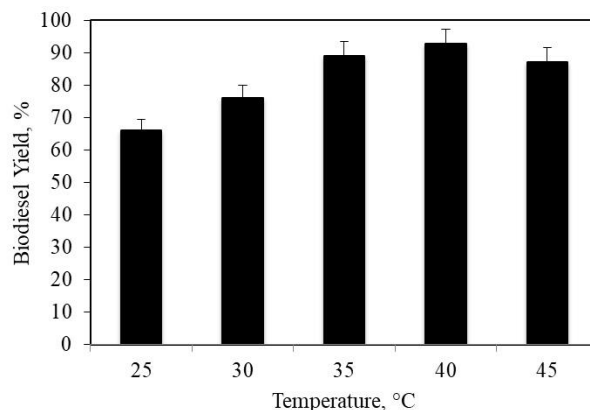


Fig. 6. Effect of temperature on transesterification (Catalyst loading 32% (w/w); Reaction time 90 min; stirring rate 150 rpm, oil:methanol ratio 1:4 (v:v))

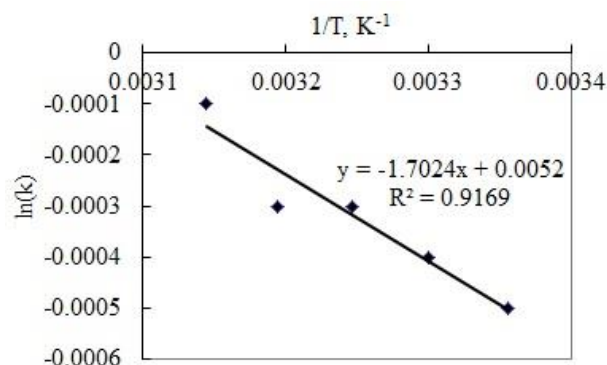


Fig. 7. Dependence of transesterification reaction on temperature as Arrhenius plot

Effect of oil to methanol ratio

The most important parameters for enzyme catalyzed reaction is the stoichiometric ratio of oil and methanol. Experiments were conducted with different ratio ranging from 1:1-1:6 (v:v) at optimal process parameters. The yield showed an increase on increasing the ratio of oil and methanol and reaches a maximum value of 1:5 (v:v) with a yield of 90.8% as shown in Fig.10. The decrease in the yield was noted, when the ratio was increased to 1:6 (v:v) and the obtained ratio was compared to the literature available [24]. The decrease in the yield was noted due to

deactivation of lipase over the functionalized magnetic particle. The possible mechanism for deactivation of the magnetic nanobiocatalyst is due to decrease in the hydrophilic interfaces between the solvent and enzyme with increase in number of cycles. The enzyme in nanocomposite might also get deactivated due to the formation of aggregates at high temperature. The solubility of methanol might be the other reason for decrease in yield as methanol has low solubility. Therefore, to reduce the negative impact of methanol over the lipase bound magnetic particle stepwise addition of methanol was followed to eliminate the deactivation of lipase on the addition of methanol.

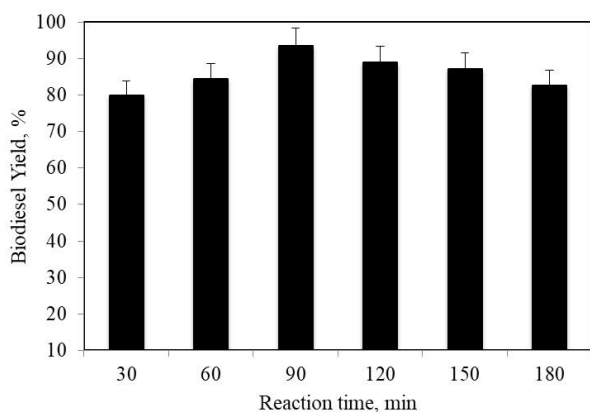


Fig. 8. Effect of reaction time on transesterification (Catalyst loading 32% (w/w); Constant temperature 40°C; stirring rate 150 rpm, oil:methanol ratio 1:4 (v:v))

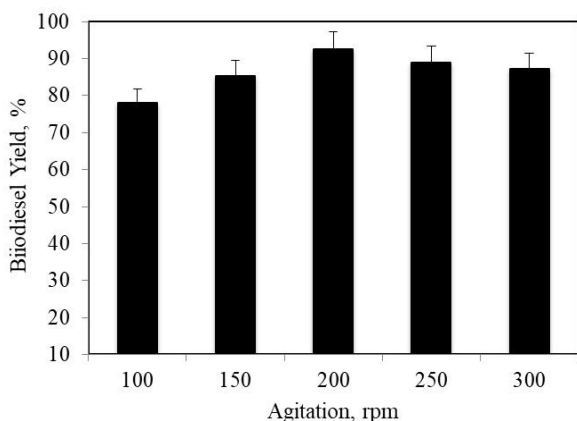


Fig. 9. Effect of stirring rate on transesterification (Catalyst loading 32% (w/w); Constant temperature 40°C; Reaction time 90 min; oil:methanol ratio 1:4 (v:v))

Reusability of catalyst

Reusability studies play a significant role during the production of biodiesel. As the nanocomposite is exposed to repeated usage,

stability should be studied extensively to monitor the activity of the nanocomposite. The Fig. 11 indicates, the activity of lipase bound magnetic particles was stable for 3 cycles, after which the yield was decreased. The decrease in the yield was noted due to loss of activity in the active sites with blocked pores on exposure to methanol.

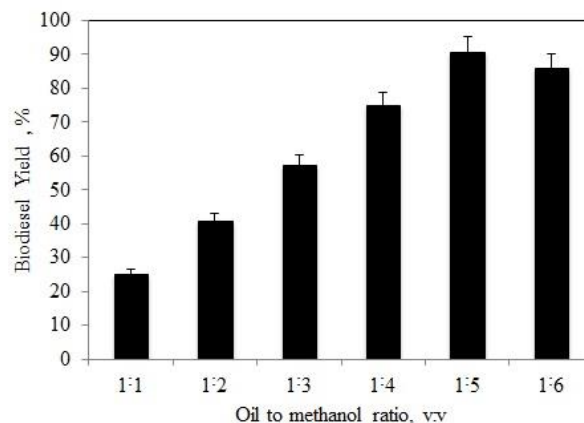


Fig. 10. Effect of different ratio of oil to methanol on transesterification (Catalyst loading 32% (w/w); Constant temperature 40°C; Reaction time 90 min; stirring rate 200 rpm)

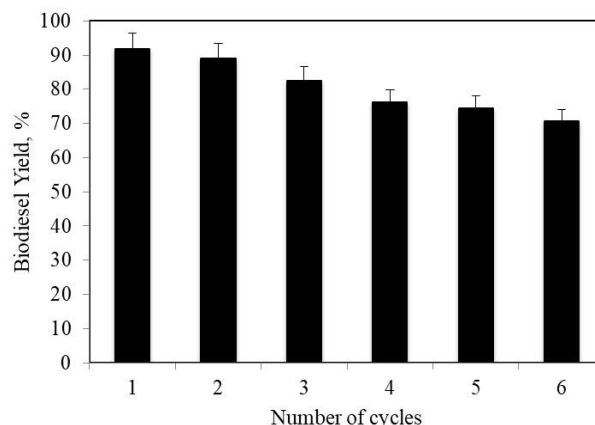


Fig. 11. Reusability magnetic nanobiocomposite of lipase

Analysis of fatty acid composition of biodiesel by GC-MS

The presence of fatty acid methyl esters were analyzed by Gas Chromatography Mass Spectrometry (GC-MS). The obtained biodiesel was pretreated in order to remove the impurities. The biodiesel was repeatedly washed with distilled water twice prior to GC-MS analysis. The obtained chromatogram confirms the presence of methyl esters against the retention time as shown in Fig. 12. The major components present in the biodiesel are 8-octadecenoic acid

methyl ester, Pentacosanoic acid methyl ester and 1-hexadecanol, 2-methyl with their retention time of 17.74, 16.13 and 14.19 respectively. The other components present in the biodiesel are 8, 11, 14-Eicosatrienoic acid, 9, 12, 15-Octavectrienoic acid, 2,3 dihydroxypropyl ester with the retention of 19.52 and 17.88

respectively. The GC-MS spectra of biodiesel showed the proportion of saturated fatty acid was higher than the unsaturated fatty acid. The MS components of biodiesel were found to match the data bank of mass spectra of NIST library VII provided by the instruments [14].

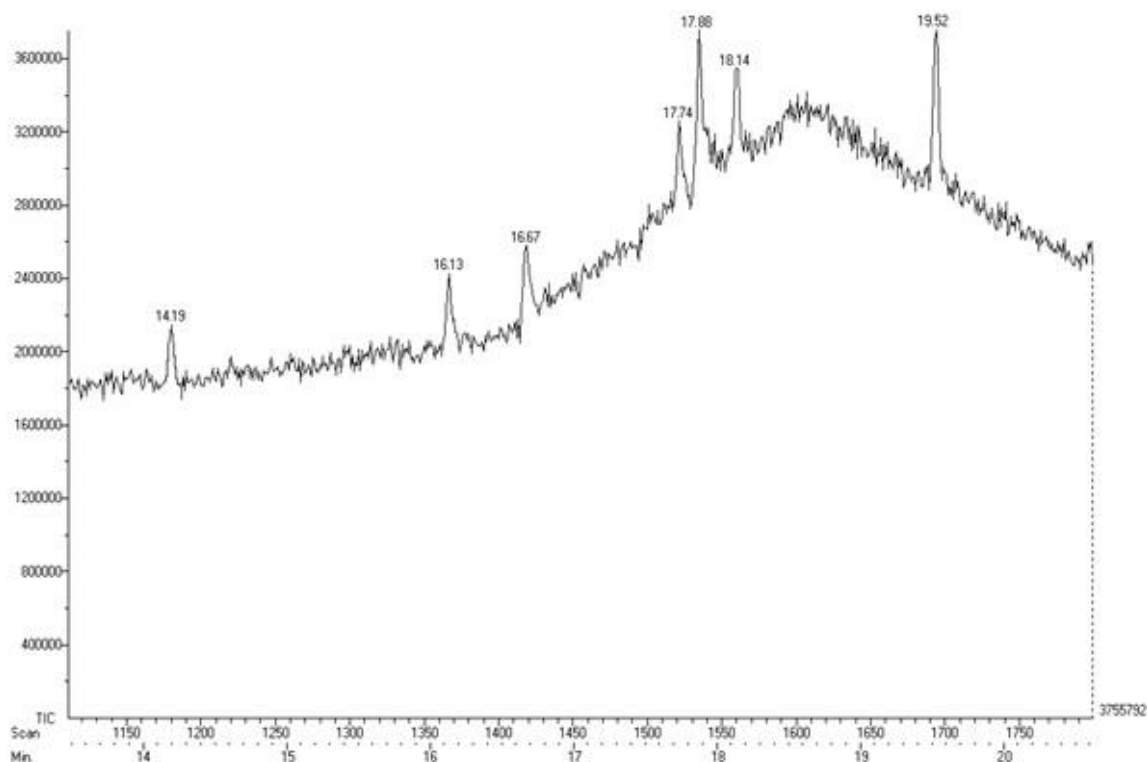


Fig. 12. GC-MS confirmation of presence of methyl esters in biodiesel

Conclusions

The production of biodiesel by lipase bound magnetic nanoparticles was found to be effective because of their surface characteristics. The magnetic properties were retained even after the immobilization of lipase, was confirmed by VSM. The optimized parameters and reusability studies showed significant production of biodiesel. The immobilization of lipase to magnetic particles proved that the production process was carried effectively at mild conditions. The yield of 93.58% was obtained under the optimized conditions and the presence of various fatty acid methyl esters was confirmed by GC-MS.

Conflict of interest

Authors declare no conflict of interest.

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