Biodiesel Production from Castor oil in the Presence of Lipase/ Functionalized Mesoporous SBA-15

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Biodiesel production from castor oil in the presence of lipase/ functionalized mesoporous SBA-15

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Abstract

Background

Enzyme immobilization is efficient tool to reduce enzymatic processes cost and improve catalyst stability. The enzymatic catalytic transesterification reaction of castor oil was studied for the production of biodiesel. In this research, castor seeds oil was extracted and used along with methanol as substrates for enzymatic transesterification reaction. Porcine pancreatic lipase (PPL) immobilized on calcium alginate (Ca-Alg) and mesoporous SBA-15 supports identified, and its potential was investigated for used as the reaction catalyst.

Results

The mesoporous SBA-15 supports with a high specific surface area ($519.25 \text{ m}^2/\text{g}$) and the pore diameter ($83.2 \text{ \AA}$) was suitable for immobilization of the PPL with $4.6 \times 2.6 \times 1.1 \text{ nm}^3$ dimensions. The effect of temperature, biocatalyst concentration, methanol/oil molar ratio and water content on biodiesel conversion were investigated in presents of PPL immobilized on mesoporous SBA-15-OH-NH₂-GA. The biodiesel conversion in the presence of PPL immobilized on SBA-15-OH-NH₂-GA and Ca-Alg was calculated 88.6% and 77.65% in optimal conditions respectively.

Conclusions

The result indicate that castor plant has a very high potential for use in the future of the biodiesel industry as a substrate for transesterification reaction. The biodiesel production efficiency is higher when the PPL is immobilized on the SBA-15-OH-NH₂-GA toward than conditions that immobilized on the Ca-Alg.

Keywords

Biodiesel; Transesterification; Castor oil; Lipase; SBA-15

1. Introduction

The ever-increasing need of human to energy resources, more or less, been a great challenged for all countries of the world. The decline in fossil resources and environmental concerns were persuaded the researchers to find new sources of non-fossil energies, especially for the transportation. The biofuels are appropriate alternatives that can be used for transportation applications instead of fossil fuels. Biodiesel is a diesel fuel which produced from renewable sources such as vegetable oils (edible and non-edible oil) or animal fats [1-3]. Among the benefits of biodiesel into petroleum diesel can be noted to these cases: (I) renewable; (II) biodegradable; (III) almost contains no sulphur, thus SO_x will not be released; (IV) the emission of hydrocarbons, carbon monoxide and soot decreases; (V) it can be used without modifying existing engines [4, 5]. Transesterification is the most common process of biodiesel production. Transesterification reaction has been carried out using short chain alcohols with lipids in the presence of an acid, alkali, and enzymatic catalysts. The most of the world biodiesel is produced from edible oils such as olive oil, sunflower oil, soybean oil, and palm oil [6-9]. The edible oilseeds are easily available on large scale. However, the extensive use of these food-based feedstocks can cause serious food shortages. As a result, non-edible oilseeds (e.g., castor oil) have drawn attention for biodiesel production. Also, cultivation these oilseeds leads to consuming of carbon dioxide and reducing greenhouse effect. *Ricinus Communis* (Castor) seed oil is one of the options being considered for biodiesel production due to suitable annual production and high oil content (40-55% oil) [10, 11].

The special properties of lipases make them an effective biocatalyst for many reactions. Lipases have high catalytic activity and specificity in transesterification of triglycerides. One of the most significant limitation for using enzymes as catalyst is relatively high production cost. Enzyme immobilization is efficient tool to reduce enzymatic processes cost and improve catalyst stability. Enzyme immobilization on substrate surface has been developed through several techniques including Physical adsorption, covalent binding, cross-linking, and encapsulation [12, 13].

The various solid supports such as calcium alginate, alumina, ion-exchange resins, and nanoparticles have been applied as support for enzymes immobilization. The covalent bonding of the enzyme to the SBA-15 nanomaterials has been carried out, which have a large intrinsic surface area and thus lead to high enzyme loading and consequently high enzyme activity. It is common to modify nanomaterials with glutaraldehyde, which has a positive effect on the ability of SBA-15 to interaction with enzymes, thereby the catalytic activity of the immobilized enzyme has significantly improved [14, 15]. In one research, *Candida rugosa* lipase was

immobilized on SBA-15/chitosan-glutaraldehyde, and its hydrolytic activity measurement has been shown an increase in the stability of the biocatalyst in the reaction medium [16]. In another study, *Candida antarctica* lipase was immobilized on SBA-15 and ionic liquid modified SBA-15 ($\text{CH}_3\text{-IL-SBA-15-BF}_4$), and the results has indicated an improvement in catalytic activity in the modified SBA-15 [17].

In this research, castor seeds oil was extracted in two steps and used along with methanol as substrates for enzymatic transesterification reaction. The mesoporous SBA-15 was created in a typical synthesis and modified by APTES and GA. The PPL was immobilized on Ca-Alg and SBA-15-OH-NH₂-GA carriers, by entrapment and covalent bonding methods, respectively. The amount of immobilized lipase activity as well as the produced biodiesel from it during transesterification reaction were investigated.

2. Results and discussion

2.1. Enzyme supports characterization

The field-emission scanning electron microscope (FE-SEM) was carried out to explore the synthesized Ca-Alg polymeric beads morphology. The spherical shape of Ca-Alg beads were obvious on FE-SEM image (Figure 1).

Figure 1: The FE-SEM micrograph of Ca-Alg polymeric beads.

The FE-SEM micrographs of synthesized mesoporous SBA-15 and SBA-15-OH-NH₂-GA are shown in figure 2. According to FE-SEM images, the morphology of both bare and modified SBA-15 are uniform and rod-like particles which illustrates functionalization has no effect on the particle morphology.

Figure 2: The FE-SEM micrographs of a) SBA-15 and b) SBA-15-OH-NH₂-GA

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy was used to confirm the synthesis, activation, functionalization and modification of mesoporous SBA-15. As shown in **Error! Reference source not found.3**, the FTIR spectrum of mesoporous SBA-15 indicates two peaks at 1050 cm⁻¹ and 794 cm⁻¹, which are related to the Si-O-Si network vibrating (Figure 3a). After activation of mesoporous SBA-15 (Figure 3b), the broad peak appeared around 3000-3800 cm⁻¹ related to the OH stretching vibration.

The functionalization step of SBA-15-OH with APTES (Figure 3c) was identified by the peak appeared at 3279 cm^{-1} related to N-H stretching mood, as well as the peaks in the 1579 cm^{-1} and 689 cm^{-1} are characteristic of bending NH_2 . Also, the peak at 2924 cm^{-1} is attributed to the stretching C-H mood of CH_2 groups in the propyl chain, indicated that the amine group is grafted to mesoporous structure. In SBA-15-OH- NH_2 -GA FT-IR spectrum (Figure 3d), with grafting of GA to SBA-15-OH- NH_2 , two peaks were appeared at 1648 cm^{-1} and 1574 cm^{-1} can be related to the stretching mood of C=O and C=N, respectively. Also a peak was appeared at 2854 cm^{-1} related to the aldehyde C-H bonds.

Figure 3: The FTIR spectra of (a) SBA-15, (b) SBA-15-OH, (c) SBA-15-OH- NH_2 , (d) SBA-15-OH- NH_2 -GA.

As shown in the SAXS pattern of mesoporous SBA-15 (**Error! Reference source not found.4**), there is a strong peak at $2\theta=0.92$, which is related to the (100) plane, as well as two weak peaks at $2\theta=1.83$ and $2\theta=2.09$ which are related to the (110) and (200) planes respectively, the presence of such peaks are representing the P6mm space group (hexagonal), which well illustrates the two-dimensional and hexagonal regular structure of the mesoporous SBA-15. Peak intensities decrease significantly in the SBA-15-OH-NH₂-GA pattern compared to SBA-15 due to the proper functionalization and modification of mesoporous SBA-15. Peak intensity reduction in the SBA-15-OH-NH₂-GA pattern means that the order of the mesoporous structure decreases in the event of modification, meanwhile, the mesoporous structure is preserved; modification occurs mainly inside mesoporous channels.

Error! Reference source not found.4: The SAXS pattern SBA-15 and SBA-15-OH-NH₂-GA

The porosimetry measurements of nitrogen gas adsorption-desorption, Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) methods were used to characterize SBA-15 and determine the pores volume, surface area and diameter. The diagrams of N₂ adsorption-desorption isotherms for SBA-15 and SBA-15-OH-NH₂-GA were shown in **Error! Reference source not found.5a**. As shown, isotherms are type IV with hysteresis type 1 (H1), which is characteristic of mesoporous structures with cylindrical cavity geometry. The lack of deformation of the hysteresis loop after modification of the mesoporous SBA-15 indicated that a two-dimensional hexagonal structure was preserved. The BET technique was performed for determination of the total volume and surface area of SBA-15 and SBA-15-OH-NH₂-GA porous structures (table 1). According to the results, the total volume for SBA-15 and SBA-15-OH-NH₂-GA were 0.88 cm³.g⁻¹ and 0.66 cm³.g⁻¹ and the specific surface area were 519.25 m².g⁻¹ and 397.12 m².g⁻¹, respectively. The BJH method was used for determination of the size of

cavities (**Error! Reference source not found.5b**). As shown, the diameter of the cavities for SBA-15 and SBA-15-OH-NH₂-GA samples were 8.3 and 6.2 nm, respectively [33]. For SBA-15-OH-NH₂-GA, the decrease of total pore volume, special surface area and the average pore diameter which were observed compared with the SBA-15, confirms the functionalization and modification of the mesoporous SBA-15 [41].

Table 1: The BET and BJH results of SBA-15 and SBA-15-OH-NH₂

	Total pore volume (V_p)	Surface area (S_{BET})	Pore diameter size (d_p)
SBA-15	0.8798 cm ³ .g ⁻¹	519.25 m ² .g ⁻¹	8.32 nm
SBA-15-OH-NH₂-GA	0.6592 cm ³ .g ⁻¹	397.12 m ² .g ⁻¹	6.24 nm

Figure 5: a) N₂ adsorption-desorption isotherms b) distribution of cavities radius size for bare and modified SBA-15 mesoporous.

2.2. PPL immobilization and PPL activity assay

The amount of enzyme loading on supports was evaluated using Bradford's method (table 2). Since mesoporous SBA-15-OH-NH₂-GA was provided more surface area compared with Ca-Alg polymeric beads, enzyme loading on SBA-15-OH-NH₂-GA was 3 time higher than Ca-Alg polymeric beads. Hydrolysis and Esterification activity of immobilized PPL on supports were evaluated and results were reported in table 2. As shown in this table, hydrolytic activity of biocatalysts decreased after immobilization on support due to partial conformational change of enzyme during immobilization. But hydrolysis activity of PPL/SBA-15-OH-NH₂-GA biocatalyst showed the highest activity which was comparable with free PPL. Grafting of APTES and GA to mesoporous supports creates a wider spherical area for enzyme attachment which facilitate accessibility of substrate to enzyme active sites. The similar trend was observed in esterification reaction in present of both biocatalysts.

Table 2: loading and Hydrolysis and Esterification activity of immobilized PPL.

Carrier	Immobilized lipase (mg PPL/g carrier)	hydrolysis activity (U/g)	Esterification activity (%)
PPL	-	34.91	91.8
PPL/Ca-Alg	9.5	23.75	92
PPL/ SBA-15-OH-NH ₂ -GA	31.95	32.47	93.15

2.3.Design of experiments

To obtain the maximum of biodiesel production efficiency and the effect of different parameters on the transesterification reaction, experimental design was performed via Taguchi method. An orthogonal array with 9 runs (L9) including 4 parameters in 3 levels was selected (table 3). The reactions in the presence of SBA-15-OH-NH₂-GA-PPL were performed according to the L9 orthogonal array. Each run was repeated twice to increase the signal to noise ratio (SNR). The yields of produced fatty acid methyl esters (biodiesel) were determined via ¹H NMR technique. Table 3 is the experimental design table containing levels of parameters and the result of each reaction run. The SNR diagram for various parameters was obtained using Taguchi design analysis, which is visible in figure 6. As shown, the optimal is the reaction run with parameters at level 3. the statistical analysis (ANOVA) of the results was done and the effect of each parameter on the biodiesel production yield was determined. As shown in **Error! Reference source not found.**, catalyst amount, alcohol to oil molar ratio, the reaction water content, and temperature were the most effective parameters, respectively.

From Taguchi method prediction, the biodiesel production yield at level 3 (the optimum), should be 90.3% and the experimental result was 88.6% which was close to the prediction. For comparison, the optimum condition was used for biodiesel production in the presence of immobilized lipase on Ca-Alg seeds. The yield of biodiesel production was 77.65%, which indicates that mesoporous SBA-15-OH-NH₂-GA carriers are better than Ca-Alg seeds for PPL immobilization. All biodiesel production yield values were obtained after purification of the samples by removing the catalyst, glycerol, remained methanol, and hexane.

Table 3: The experimental design table.

	Temperature (°C)	Catalyst (g)	Methanol (g)	Water content (g)	FAMEs yield (%)
1	42	0.14	0.203	0	17.65
2	42	0.4	0.27	0.1	42.34
3	42	0.5	0.405	0.4	86.6
4	47	0.14	0.27	0.4	29.7
5	47	0.4	0.405	0	59.1
6	47	0.5	0.203	0.1	37.6
7	52	0.14	0.405	0.1	44.18
8	52	0.4	0.203	0.4	43.3
9	52	0.5	0.27	0	70.16

Figure 6: The SNR diagram for various parameters, affecting biodiesel production yield.

2.4. The ^1H NMR analysis to identify the chemical structure and determine the yield of produced biodiesel

The ^1H NMR spectrum of castor oil and biodiesel production in present of PPL/SBA-15-OH-NH₂-GA and PPL/Ca-Alg biocatalyst in optimal conditions (all factors at level 3) are shown in figures 7 and 8, respectively. Deuterated chloroform was used as a proton-free solvent. Tetramethylsilane (TMS) was used as internal standard. Regarding to figures 7 and 8, a single peak appeared at 3.7 ppm of the biodiesel spectra, which is related to the methoxy group ($\text{CH}_3\text{-O-CO-}$) of methyl ester and is a confirmation for the synthesis of biodiesel from castor oil. Triplet peaks present at 2.3 ppm are attributed to methylene protons of α -carbonyl, which is visible in all derivatives of glyceride and ester. Peaks in 4.1-4.3 ppm and 5.3 ppm are relevant to the methylene and methine protons ($\text{CH}_2\text{-CH-CH}_2$) existent in the castor oil triglyceride

structure, respectively, which are not observed in the biodiesel spectra when converted to methyl ester. Observed peak in 3.6 ppm is arising from hydrogen which connected to hydroxyl group-containing carbon. Triplet peak at 0.89 ppm is related to the protons of the end methyl (-CH₃). Peaks in 5.3-5.6 ppm are attributed to the olefin protons (-CH=CH-). Peak recorded in 1.6 ppm is related to methylene protons of β -carbonyl and peaks observed in 1.3 ppm are arising from methylene protons of the carbon chain. The biodiesel (methyl ester) production yield from castor oil is calculated by ¹H NMR spectrum using the Eq. (4):

$$Y(\%) = \frac{2A_{\text{methoxy}}}{3A_{\text{methylene}}} \quad (4)$$

which in this equation Y is the biodiesel production yield according to percent, A_{methoxy} is the integral amount of methoxy protons and A_{methylene} is the integral amount of methylene protons in the α -carbonyl position. The yield of biodiesel production by immobilized lipase on SBA-15-OH-NH₂-GA and Ca-Alg is shown in table 4.

Table 4: Optimal biodiesel production yield

	PPL/SBA-15-OH-NH ₂ -GA	PPL/Ca-Alg
Biodiesel production yield	88.6%	77.65%

Figure 7: The ¹H NMR spectrum of castor oil.

3. Conclusions

In this study, the efficiency of oil extraction from castor seeds was determined by the oil expeller machine, followed by the Soxhlet apparatus, at about 60% W/W. Also, the main triglyceride component of castor oil is ricin oleic acid, which is an unsaturated fatty acid with a hydroxyl group, which makes it polarized, so it is suitable for the transesterification reaction in the presence of methanol. The above points indicate that castor plant has a very high potential for use in the future of the biodiesel industry as a substrate for transesterification reaction. The results of the SAXS analysis and N₂ adsorption-desorption isotherms confirm that the two-dimensional hexagonal structure and highly ordered of mesoporous SBA-15, which this good feature provides functionalization, modification and infiltration of the reactants through the pore of the SBA-15. The results of the ¹H NMR spectra of the produced biodiesel samples in the optimum state indicate that the biodiesel production efficiency is higher when the PPL is immobilized on the SBA-15-OH-NH₂-GA toward than conditions that immobilized on the Ca-Alg. This is due to the fact that, due to the decrease in material size, the SBA-15 nanostructure can provide a higher surface for linkage of the PPL, consequently, it will increase the amount of enzyme loading per unit of the mass carrier. Also, the larger surface area for the mass transfer between particles decreases the resistance of the substrate transfer to the particle containing the immobilized enzyme, which in turn leads to an increase in the activity of the immobilized lipase and thereby increase in overall speed of the reaction. Therefore, nano-scale substances are ideal substrate materials for enzymes immobilizing used for biocatalysis reactions. The covalent bond of lipase on the mesoporous SBA-15 has the advantage of a strong interaction between the enzyme and the carrier in the immobilization process, which makes the PPL sturdier in its place during the catalytic process, thus it's less talented for denaturation and also the sustainability of PPL is much increasing in organic solvents. As well as, the immobilization of lipase on Ca-Alg involves the physical entrapment of the enzyme in a small space that it causes problems such as leakage and emit of the enzyme inside the solution, plus a significant amount of infiltrating limitations, which reduces the biodiesel production efficiency than use of the mesoporous SBA-15 as the carrier.

4. Materials and methods

4.1. Materials

Powder PPL, poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Pluronic P123, $M_n \approx 5800$), tetraethyl orthosilicate (TEOS), (3-aminopropyl) triethoxysilane

(APTES), glutaraldehyde (GA), sodium alginate (Na-Alg), bovine serum albumin (BSA), phosphoric acid 85%, ethanol, toluene, calcium chloride, hexane and acetone were purchased from Sigma-Aldrich. Triacetin, Coomassie Brilliant Blue G-250, absolute methanol, sodium hydroxide (99.5%), and hydrochloric acid (36%) were purchased from Merck. The castor seeds Omid industrial oilseed press workshop, Iran.

4.2. Extraction of oil from castor seeds

The castor oilseed used in this study was collected by Omid industrial oilseed press workshop in Iran. Two steps extraction was applied for Castor oil extraction. In the first step, oil extraction was done via oil expeller pressing machine. The seeds were squeezed under high pressure, either in one step or more steps. This expeller pressing machine was equipped with a temperature control device to avoid any damage to the oil that may affect its properties. Then the oil extracted was collected and filtered. To extract the residual oil from press cakes solvent-extraction method was used as second step treatment. The oil was separated by the Soxhlet extractor apparatus in the presence of a hexane solvent at 70 °C for 5 h. In the end, hexane solvent was separated using a rotary evaporator (ROTAVAPOR- RE, BUCHI, Switzerland) and the total content of the oil existent in castor seeds was determined by using the Eq. (1).

$$\text{Oil extraction yield (\%)} = \frac{\text{The weight of extracted oil}}{\text{The weight of castor seeds}} \times 100 \quad (1)$$

4.3 PPL/Ca-Alg biocatalyst preparation

A 100 mL of Na-Alg solution containing 0.2 g of PPL was added into the 200 mL CaCl₂ solution (2 M) dropwise with syringe at 4 °C. Ca-Alg gels were formed instantly through cross-

linking by dripping the solution of Na-Alg and PPL into the CaCl_2 solutions. As soon as the Na-Alg polysaccharide is placed in a solution of calcium ions, Ca^{2+} ions are exchanged with the Na^+ ions in the polymer. All formed gels were stored at 4 °C for 2 h to harden then separated from the solution by vacuum filtration and washed with deionized water. Figure 9 is a graphical showing of PPL/Ca-Alg biocatalyst preparation.

Figure 9: The schematic of PPL/Ca-Alg biocatalyst preparation.

Protein content loading on Ca-Alg beads was measured with the Bradford's method using BSA as a standard and calculated according to Eq. (2):

$$C_L \frac{C_0 V_0 - C_f V_f}{m_b} \quad (2)$$

where C_L is the amount of entrapped lipase per gram of Ca-Alg (mg L/g Ca-Alg), C_0 the initial concentration of lipase in the Na-Alg solution (mg L/ml), V_0 the Na-Alg solution volume (ml), C_f the concentration of lipase in the filtered solution (mg L/ml), V_f the volume of the filtered solution (ml) and m_b the amount of Ca-Alg beads (g) after drying.

4.4 PPL/SBA-15-OH-NH₂-GA biocatalyst preparation

4.4.1 Mesoporous SBA-15 synthesis

For mesoporous SBA-15 preparation, 5 g of Pluronic P-123 as a structure-directing agent was dissolved to 25 mL HCl 37% W and 150 mL distilled water. Then stirred at 35 °C for 20 h. P-123 forms the micelle in an aqueous medium. Then, 10.5 g of silica source (TEOS) was added to the medium containing micelles and the resulting solution was stirred at the same temperature for 20 h. The TEOS hydrolysis along with polymerization occurs in an acidic medium and forms a silica network around the template. Finally, the mixture is transferred to a Teflon-lined autoclave for particle growth and crystallization at 100 °C for 24 h. After filtration and washing with distilled water, the powder was dried in the hot air oven at 40 °C and calcined to remove the surfactant (P-123) at 550 °C for 5 h in a muffle furnace.

4.4.2. Activation of mesoporous SBA-15.

The problem of the calcination process is that many active silanol groups at the surface of the silica network are converted into siloxane bridges, in which case the mesoporous are not suitable for functionalization; thus, for activation of mesoporous SBA-15 and the formation of hydroxyl groups on its surface, mesoporous SBA-15 was dissolved in 42.5 mL HCl (2 M) and kept under stirring at 50 °C for 4 h. Therefore, mesoporous SBA-15-OH material washed with distilled water and dried at 40 °C for 10 h.

4.4.3. Functionalization of mesoporous SBA-15-OH.

Amine functionalizing of mesoporous was performed by adding 0.5 mL APTES to 1 g of

activated SBA-15 nanostructures in 40 mL of anhydrous toluene. The mixture was exposed to reflux under the argon atmosphere for 10 h. Finally, the mesoporous SBA-15-OH-NH₂ were collected by filtration, washed with acetone and dried overnight at 50 °C.

4.4.4. Modification of mesoporous SBA-15-OH-NH₂.

Modifying of mesoporous SBA-15-OH-NH₂ was done by soaking 1.25 g in a mixture of 4 mL of aqueous GA 25% and 36 mL phosphate buffer (0.1M) with pH=7.5 for 2 h under reflux and the inert atmosphere of argon. Then, the chemically modified mesoporous SBA-15-OH-NH₂-GA was washed with 50 mL of the same phosphate buffer and then washed with 50 mL of distilled water on a sintered glass filter and dried overnight at 50 °C.

4.4.5. Nanoparticle characterization

The ultraviolet-visible (UV-Vis) spectroscopy (Lambda 850, Perkin Elmer) was used to measurement the amount of enzyme loaded on carriers and lipase esterification activity assay. Surface morphology of Ca-Alg and SBA-15 carriers was investigated by field-emission scanning electron microscopy (FE-SEM) (MIRA3, TESCAN, Czech Republic). The spectra of SBA-15, SBA-15-OH, SBA-15-OH-NH₂, and SBA-15-OH-NH₂-GA were recorded by attenuated total reflectance Fourier-transform infrared (ATR-FTIR) spectroscopy (Tensor 27, Bruker) to determine the structure and functional groups. Small-angle X-ray scattering (SAXS) (X'Pert PRO MPD, PANalytical, Netherlands), was used to determine the crystal structure of mesoporous. N₂ adsorption-desorption isotherms (BELSORP-mini, Japan) technique, was used to determine the capacity of mesoporous and the diameter of its pores. The optimization of biodiesel production was done by Minitab (version 17.3.1) software, using the design of experiments (DOE) with the Taguchi method. To determine the chemical structure of obtained biodiesel and the amount of the production yield, proton nuclear magnetic resonance (¹H NMR) (INOVA, 500 MHz) was performed.

4.4.6. Immobilization of PPL on mesoporous SBA-15-OH-NH₂-GA

For immobilization of lipase through covalent bonding to mesoporous SBA-15-OH-NH₂-GA, 1-1.75 g of carrier was dissolved in 625 mL phosphate buffer with pH=7.5 then 1 mg/ml of PPL solution was added. The mixture was stirred at 25 °C for 24 h. After completion of the reaction, the solution was incubated at 4 °C for a short time.

4.5. Hydrolysis activity assay of immobilized PPL

Free and immobilized PPL activity were investigated in the 1,2,3-triacetoxyp propane hydrolysis reaction. 2 g triacetin, 50 mL distilled water and 25 mL phosphate buffer (1M, pH=7) was stirred for about 10 min to prepare a triacetin emulsion [32]. When the pH solution was reached to a constant value, a certain amount of free (0.25 g) and immobilized PPL was

added to the above mixture. Acetic acid is produced as a processed product that reduces the pH of the environment. The resulting mixture was then continuously titrated with NaOH solution (0.01 M) achieve to a pH of about 12. The consumption volume was recorded exactly and PPL activity was calculated (one unit of lipase activity is defined as the amount of enzyme required to release 1 μmol of fatty acid at 37 °C per minute). The reaction was performed once in the presence of immobilized lipase on Ca-Alg polymeric beads (3 g) and again in the presence of immobilized lipase on mesoporous SBA-15-OH-NH₂-GA (0.5 g).

4.6. Esterification activity assay of PPL

Esterification activity of biocatalysts were measured in an enzymatic alcoholysis reaction. The certain amount of free (0.0225 g) and immobilized lipase was added to 0.631 mL of oleic acid dissolved in 10 mL n-Hexane. Then 0.183 mL of n-Butanol (molar ratio of oleic acid to n-Butanol 1:1) was added reaction medium. The reaction was carried out at 37 °C for 4 h and the intensity of stirring was 250 rpm. To determine the amount of residual free fatty acid, the test specimens were transferred to a 15 mL falcon tube and then added 2 mL of copper(II) acetate-pyridine reagent (5% W/V in distilled water and adjust the pH to 6.1 with pyridine) to each of the falcon tubes and were intensively stirred with a vortex mixer for 90 s [36]. Then, was allowed the solutions to be converted into two phases. The absorbance of the upper layer at the wavelength of 715 nm was measured by UV-Vis spectrophotometer. Blank solutions were made in the absence of oleic acid. The amount of free fatty acid was determined using the standard oleic acid solutions in the range of 120-165 μmol . The reaction was performed once in the presence of free lipase and again in the presence of immobilized lipase on Ca-Alg (0.2 g) and mesoporous SBA-15-OH-NH₂-GA (0.045 g), which the amount of free and immobilized PPL activity according to Eq. (4) was 340, 38.3 and 172.5 U/g, respectively. Also, the amount of yield of the produced fatty acid methyl esters (FAME) by the free and immobilized PPL as a catalyst was determined according to Eq. (5):

$$\text{The FAMES production yeild (\%)} = \frac{\text{The consumed fattey acids}}{\text{The primery fatty acids}} \times 100 \quad (5)$$

4.7. Biodiesel production

The reactions were carried out in clear tubular screw cap vial 40 mL containing 2 g castor oil, a certain amount of catalyst (immobilized PPL on mesoporous SBA-15-OH-NH₂-GA), different temperatures, different molar ratios of alcohol to oil and variable water content (in the form of phosphate buffer with pH=7). To each one reaction vessels, 5 mL of hexane solution were added. The reactions were carried out in a bath of oil set up on a magnetic stirrer with a speed of 250 rpm. Optimization of the effective parameters on transesterification reaction in

order to biodiesel production was performed based on Taguchi experimental design using Minitab software. The above experiments were performed in two repetitions [34]. In order to compare, the optimal conditions of the above experiments were used to produce biodiesel using immobilized PPL on Ca-Alg.

4.8. Biodiesel purification

To remove biocatalysts and glycerol from the reaction medium, the product was centrifuged for 20 min at 3500 rpm. Then, biodiesel was collected. A mixture of 2:1 volume ratio of deionized water to biodiesel was stirred at 50 °C for 30 min. Again, the upper layer which contains fatty acid methyl esters and hexane was separated via separatory funnel. After separation, a rotary evaporator was used to remove the hexane solvent and pure biodiesel was obtained.

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Figures

Figure 1

The FE-SEM micrograph of Ca-Alg polymeric beads.

Figure 2

The FE-SEM micrographs of a) SBA-15 and b) SBA-15-OH-NH₂-GA

Figure 3

The FTIR spectra of (a) SBA-15, (b) SBA-15-OH, (c) SBA-15-OH-NH₂, (d) SBA-15-OH-NH₂-GA.

Figure 4

The SAXS pattern SBA-15 and SBA-15-OH-NH2-GA

Figure 5

a) N₂ adsorption-desorption isotherms b) distribution of cavities radius size for bare and modified SBA-15 mesoporous.

Figure 6

The SNR diagram for various parameters, affecting biodiesel production yield.

Figure 7

The ¹H NMR spectrum of castor oil.

Figure 8

The ¹H NMR spectrum of the biodiesel production in present of a) PPL/SBA-15- OH-NH₂-GA and b) PPL/Ca-Alg biocatalyst in optimal conditions.

Figure 9

The schematic of PPL/Ca-Alg biocatalyst preparation.

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