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Lipase Immobilized into Novel GPTMS: TMOS Derived Sol-Gels and its Application for Biodiesel Production from Waste Oil

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Abstract

In this essay, lipase from Burkholderia cepacia was immobilized into 3-glycidoxypropyltrimethoxysilane (GPTMS) and tetramethoxysilane (TMOS) derived sol-gels. GPTMS:TMOS molar ratio of 1:3 was found to yield the best result. The morphological characteristics were investigated based on SEM and BET analysis. Sample mean pore diameter was 39.1 nm, it had a specific surface area of 60 m^2/g prior to enzyme addition which decreased to 7.49 m^2/g after immobilization. The enzyme activity was assessed through transesterification of waste cooking oil in the presence of ethanol with optimal conditions of: 40℃, 15% immobilized lipase, 9:1 alcohol to oil molar ratio in 24 h of reaction which resulted in 91.70% biodiesel production. In 6-hour reaction time, 86.87% biodiesel was obtained which is much shorter than conventional enzymatic transesterification which is 72 h. Ethyl esters were characterized by determining their viscosity, density, and flash point based on ASTM D 6751-07b standards.

Keywords: *Immobilization, Lipase, Sol-gel, Biodiesel, Enzymatic Transesterification*

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1. Introduction

Global warming and limited fossil fuels forced countries to find alternatives like renewable sources. Biofuels are environmentally friendly, renewable and a sustainable substitute produced from biomass which is the largest renewable energy supply (77.4% of all available energy resources) [1]. Among different biofuels, biodiesel (the mixture of monoalkyl esters of long chain fatty acids) attracts greater attention because it has similar energy content, physical and chemical structure of petrodiesel. It also emits lower amounts of toxic exhaust gases like CO and unburned hydrocarbons [2]. 86 K higher flash point (>423K) of biodiesel makes it categorized as a non-flammable, non-explosive, and transportable fuel. Another advantage of biodiesel is its biodegradability according to Makareviciene and Janulis, within 21 days, 98% of pure biodiesel decomposed, which is 38% higher than petroleum diesel. The main drawback of biodiesel production is the high cost of fresh feedstock (oil), which is about 80-90% of total production cost, this causes the price of biodiesel to be double that of petrodiesel [3].

The simplest and most efficient route for biodiesel production is transesterification which involves vegetable oils with short chain alcohols as reactants and a catalyst. Chemical catalyst like a strong acid or base has high yield in short reaction time. However, the process requires high temperature (80°C), low content of free fatty acids in (oil) to avoid saponification as this side reaction would make the downstream process even harder and eventually reduce biodiesel yield. To prevent this problem (saponification), the reactants must be dry and have less than 0.1 wt% of free fatty acid which is another limit for a suitable feedstock in chemically catalyzed reactions [4]. A biological catalyst like lipase has the advantage of performing its catalytic activity in a more moderate condition with different triglyceride substrate including oils with high levels of free fatty acids. However, there are some drawbacks for enzymatic transesterification too, like slower reaction rate, possibility of enzyme deactivation and high cost of enzymes [5].

One way to compensate for high enzyme cost and increase its stability is immobilization, which is defined as the attachment of the molecule into a solid support to limit its movement in space. Based on the enzyme type and its application, the immobilization technique used will be different. There are five major techniques used for immobilization namely, adsorption, covalent binding, crosslinking, encapsulation, and entrapment. Adsorption involves contamination of the enzyme and support which is easy, but weak connections would increase enzyme loss. Covalent binding provides the strongest bond between the enzyme and support; it is rather expensive and may affect the active site of the enzyme. In crosslinking method, reagents are used to develop a high active structure; the binding can be intra/intermolecular. High enzyme content is an advantage, but small particle size would make them hard to separate. Encapsulation is somehow similar to entrapment, but the environment is much more restricted, and the mass transfer rate is relatively low. Entrapment of lipase refers to capture of enzyme within a polymer matrix. In this method, the enzyme loading is high

(80-100%) but substrate diffusion is restricted. Entrapped enzyme can be recovered easily during continuous operation. [2,6]. The main advantage of entrapment is the lipase physical interaction with the polymer, and consequently low enzyme denaturation [7]. The sol-gel matrix has the advantage of balancing stability and activity loss, the enzyme is entrapped by strong bonds with simple chemical reaction [8]. This method involves the enzyme solution and an acid or base catalyst and alkoxysilanes as the matrix precursor. The immobilization platform is synthesized by hydrolysis and condensation of the precursors, and eventually an amorphous silica matrix is obtained [9]. Noureddini *et al*. (2005) studied iso-butyltrimethoxysilane (iso-BTMS) and tetramethoxysilane (TMOS) derived sol-gels. Results indicated that 65 mol% of ethyl esters were formed in one-hour reaction [10]. Meunier and Legge (2012) studied double immobilization of lipase (NS44035), first into PTMS/TMOS sol-gels and then on Celite® support. Lipase activity was investigated through transesterification of triolein, 60% conversion was reported in six-hour reaction [11]. This research is dedicated to GPTMS:TMOS sol-gels for lipase immobilization. The difference between 3-glycidoxypropyltrimethoxysilane (GPTMS) and other precursors used in sol gel process is its organic functional group that will associate crosslinks in sol-gel polymerization process [12].

In our previous study, temperature and pH effects on the hydrolytic activity of free and immobilized enzymes, reusability and its reaction kinetics were studied [13]. In this research, the immobilization platform was prepared and the enzyme was immobilized in the synthesized gel. The characteristics of the enzyme were evaluated using BET and SEM. Then the immobilized lipase activity was tested during transesterification of ethanol and waste oil to produce biodiesel.

2. Materials and methods

3-(2,3-epoxypropoxy) propyltrimethoxysilane (GPTMS) and tetramethylorthosilicate (TMOS) were purchased from Merck Millipore (Darmstadt, Germany), Lipase *Burkholderia cepacia*, *Mucor Miehei*, *Procine Pancrease* and *Candida Rugosa* were purchased from Sigma-Aldrich Company. All phosphate buffers were prepared using the sodium salt. Other chemicals used for the synthesis were of analytical grade.

2-1. Sol-Gel immobilization procedure

Sol-gels were prepared according to Reetz and Hara with some modifications [13], GPTMS and TMOS (different molar ratios) as precursors were mixed with acid or base catalyst (HCl or NaF). The mixture was sonicated for one hour to have a clear, homogeneous solution by Ultrasonic bath (Wiseclean® model WUC-D10H), until a clear single phase solution was formed and no alcohol smell was detected. The partially hydrolyzed sol was put in an ice bath to cool and hydrolysis stage was completed. Afterward the lipase solution was added and the mixture was vigorously shaken until gelation occurred, then it was moved to a Petri dish and dried at room temperature for 24 hours. Next, the immobilized lipase was broken up using a spatula. The immobilized

enzyme was dried at room temperature, then crushed in a mortar and pestle and stored at 4°C [14,15].

2-2. Morphological characteristics

General morphological characterization was investigated by scanning electron microscopy (SEM) (S360 Cambridge 1990) at an accelerating voltage of 20 kV. The samples were coated with a thin layer of gold before the imaging process. Brunauer–Emmett–Teller (BET) method was used to estimate morphology, specific surface area, mean pore diameter and total pore volume of the sol-gels. The nitrogen adsorption experiments were carried out at 77 K in Microtrac BELSORP-MINI II, USA. The adsorption/desorption data were processed through the BELSORP analysis program. Manipulation of the desorption isotherms with this software provides a fit of the data to other models such as Barrett, Joyner and Halenda (BJH) model, which ultimately results in the information about the pore size and the pore size distribution of the tested material. To evacuate the residual water, prior to BET measurements the sample was heated at 200°C under high vacuum for 90 min.

2-3. Immobilized enzyme activity

Hydrolytic activity is the amount of free fatty acids liberated during the hydrolysis process which is determined through titration and UV spectrophotometry approaches [8,16]. The test results of the immobilized enzyme have been compared with those obtained using the same amount of free enzyme under identical conditions (reported as Relative activity). Lipase transesterification activity was determined by assessing the amount of Fatty Acid Ethyl Esters (FAEE) liberated which can be determined through gas chromatography/mass spectrometer GC-MS equipped with a HP-5 column 30 m long, internal diameter 0.25 mm. The column temperature was held at 160°C for two minutes, heated to 300°C with 8°C/min rate and then maintained for 5 min. The temperatures of the injector and detector were set at 280 and 230°C, respectively. For GC-MS analysis, 5 µL of the aforementioned mixture and 300 µL of 1.4 mmol/L heptadecanoic acid methyl ester (hexane as the solvent) which has served as the internal standard were precisely measured and mixed thoroughly. 0.5 µL of the treated sample was injected into a gas chromatograph column. Analysis of waste oil, ethanol and immobilized lipase was carried out in various conditions to find the best possible result. The experiments were conducted twice and data presented are mean values. The best produced ethyl ester was characterized by determining its viscosity, density, and flash point based on ASTM D 6751-07b standards.

3. Results and discussion

3-1. Enzyme immobilization

3-1-1. Acid or base catalyst for sol-gel hydrolysis stage

As hydrolysis stage is quite slow, especially regarding alkoxides, therefore acids or bases are applied to speed up the reaction. According to the results, the activity of immobilized lipase in HCl catalyzed sol-gels are 3.5 times higher than those with base catalyst (NaF). This catalyst usually affects the final network structure: Acids lead the

formation of polymer-like expanded structure which is more favorable for immobilization, rather than bases which form highly branched dense structures (Fig. 1) [17].

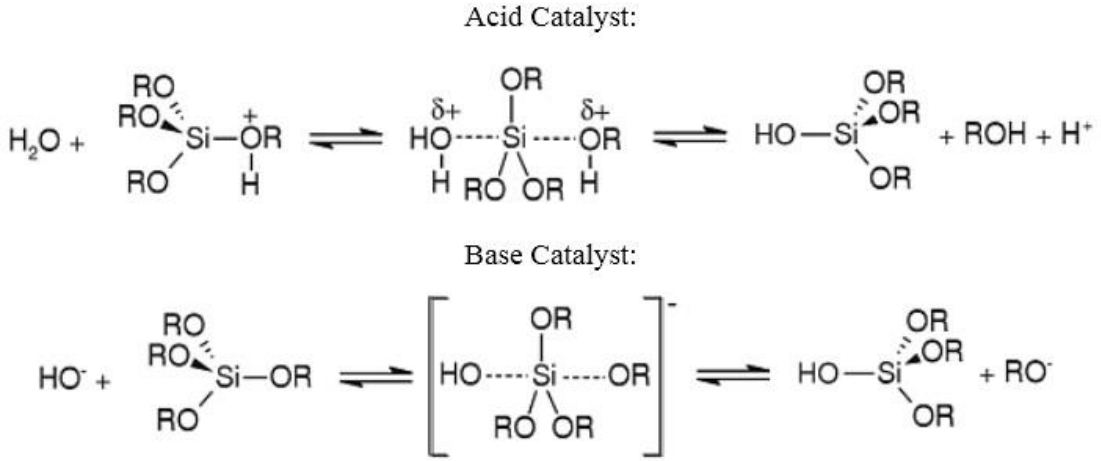

Figure 1. Sol-gel hydrolysis reaction by acid or base catalyst.

3-1-2. Precursors-water molar ratio

In previous studies other precursors were used to immobilize lipase [10,11], therefore, to find the best immobilization platform, catalytic activity of different sol-gels were investigated under same condition. The best relative activity was obtained by GPTMS:TMOS molar ratio of 1:3 (Fig. 2).

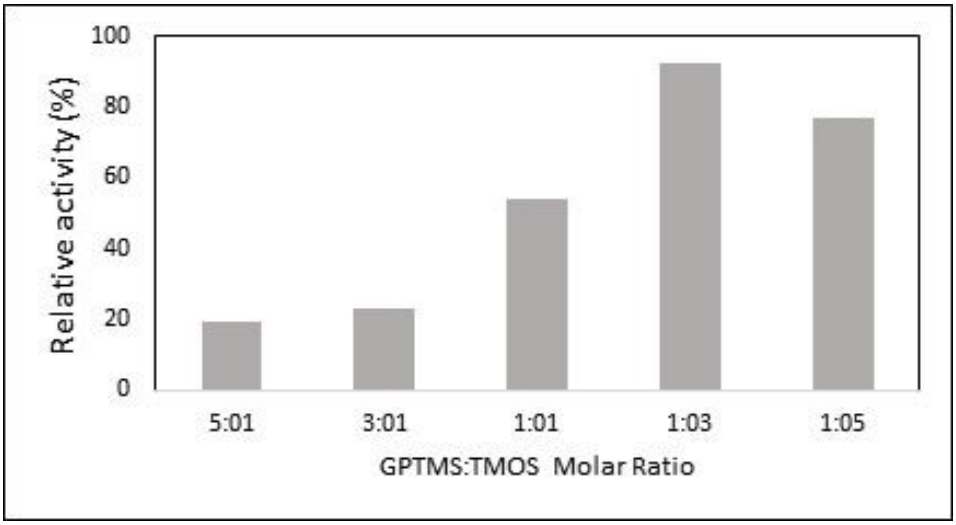

Figure 2. Precursors molar ratio effect on relative activity (Olive oil emulsion, Temperature: 40°C, pH 7.5).

The reason may lie in sol-gel stages (Fig. 3); as the condensation reactions proceed, a network polymer results from polysiloxane formation. The polycondensation reactions occur repeatedly until the active silanol sites form siloxane. TMOS constructs three to six Si–O unit rings, in the next step, the GPTMS precursor molecules were added to the cyclic TMOS-only structures. In this procedure, one to three glycidoxypropyl groups from the previously GPTMS precursor molecule replaced the corresponding number of hydroxyls of the siloxane (TMOS) ring elements [18].

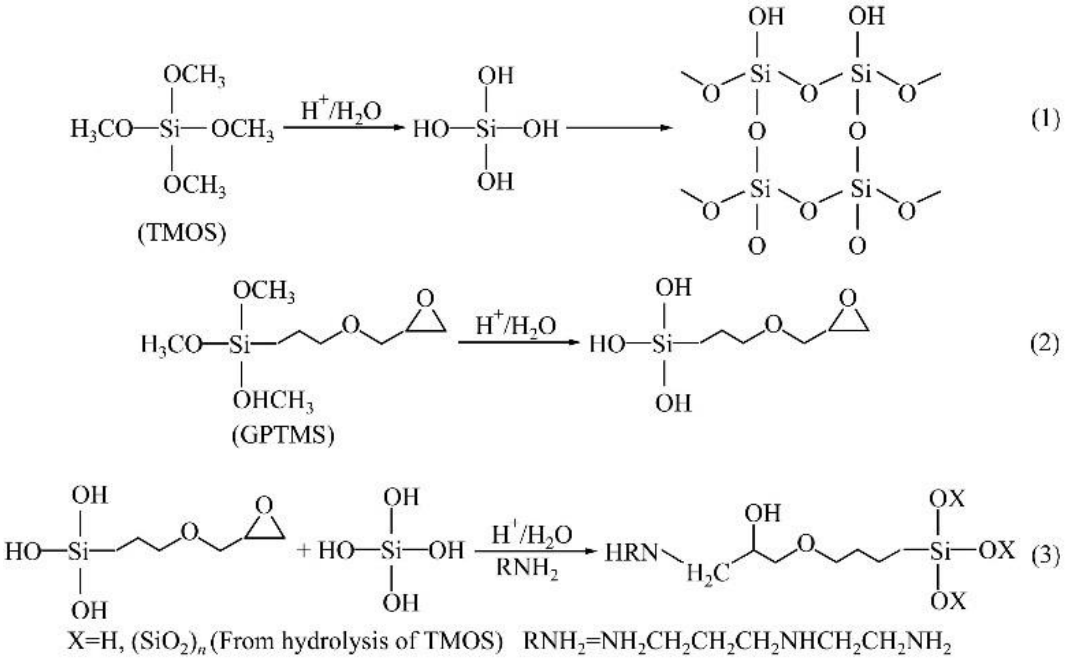

Figure 3. TMOS and GPTMS Acidic hydrolysis (See Ref. [19]).

In this case, as the molar ratio of GPTMS increases, a smooth surface is formed due to its coating properties rather than a porous media to immobilize the enzyme ("ball and stick form") (Fig. 4). An increase in the GPTMS content would chemically stabilize the network and therefore the network makes it harder for substrates to access the enzyme.

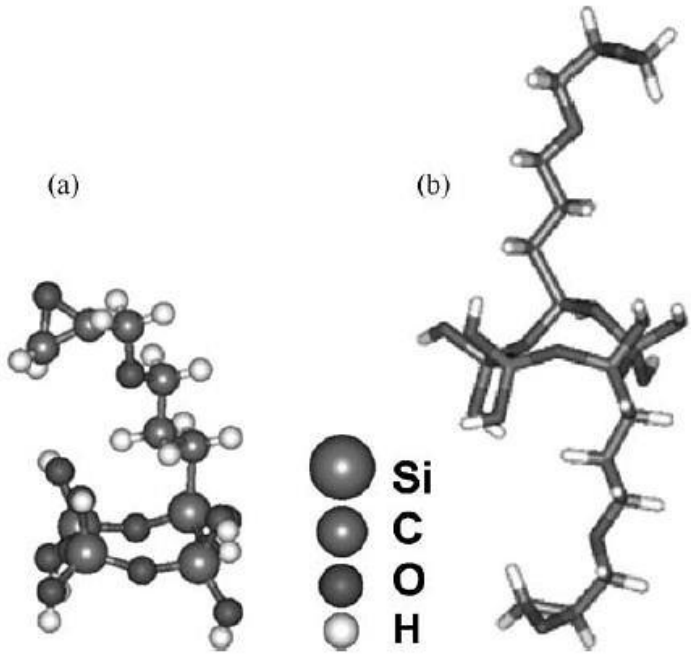

Figure 4. GPTMS molar ratio effect on gel molecular structure (a) 3 TMOS/1 GPTMS ("ball and stick") and (b) 3 TMOS/2 ("stick format") [20].

3-1-3. Additives

Several additives have been used to boost enzyme activity [21]. Salt additions enables better enzyme dispersion in the matrix [22]. In this study, KCl and PVA were tested and according to the obtained results they decrease the enzyme activity by 83.9 and 66.8% respectively. Therefore, none of the additives used seems to have any positive effect on lipase activity. Persson *et al.* (2002) also reported that KCl decreases the enzyme loading, which would lead to lower activity [23]. PVA (Polyvinyl alcohol) was also reported to possibly prevent pores collapse and stabilize the enzyme, but in this case it may not interact favorably with GPTMS:TMOS materials [14].

3-1-4. Lipase type

Before biodiesel production, the best enzyme was chosen based on its relative activity. Four lipases: *Burkholderia cepacia*, *Mucor Miehei, Procine Pancrease* and *Candida Rugosa* were screened for their hydrolytic activity [13]. As Fig. 5 illustrates, *Burkholderia cepacia* showed the best activity compared to the other three enzymes. Free enzyme initial activity and its interaction with sol-gels affects the catalytic activity.

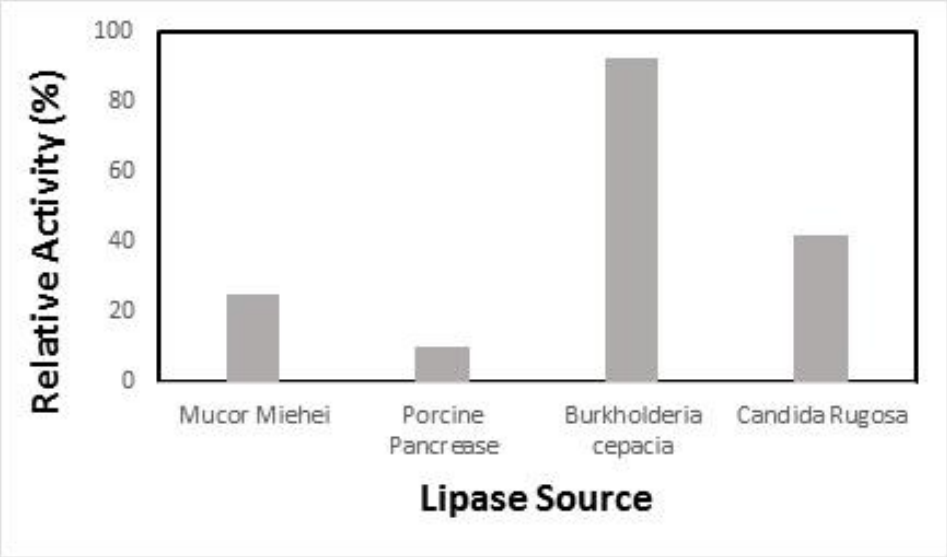

Figure 5. Relative activity of different enzymes (Olive oil emulsion, Temperature: 40°C, pH 7.5).

3-2. Biocatalyst characterizations

SEM micrographs of the sol-gels alone and after immobilization are shown in Fig. 6, which show the disordered pore structure of the synthesized platform for lipase immobilization. In image (a) the surface of the sol-gel alone has different structure compared to image (b) which was taken in the presence of lipase molecules. It appears that lipase molecules change the properties of the immobilization platform. BET analysis also show different trends before and after immobilization (Fig. 7).

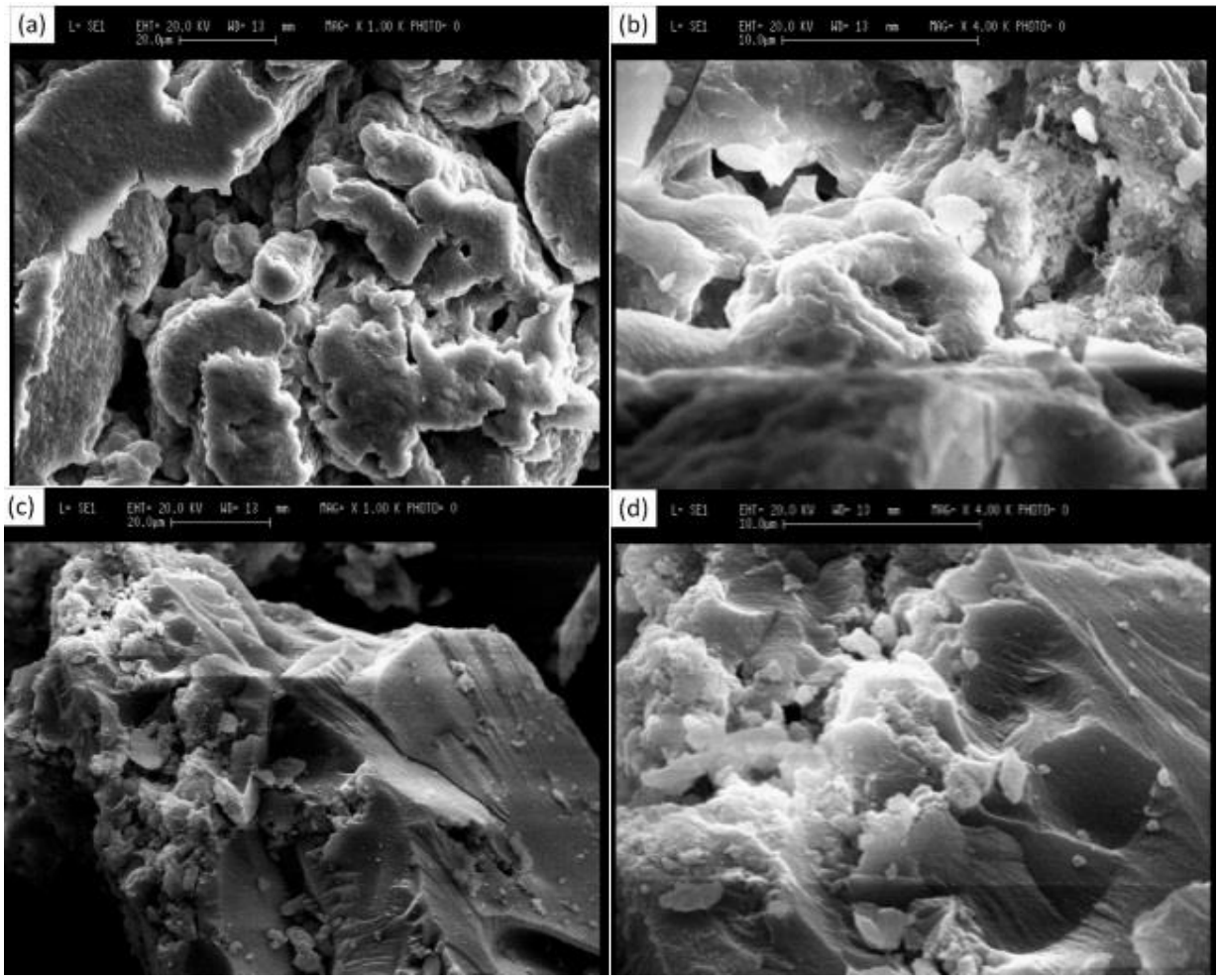

Figure 6. SEM images of GPTMS/TMOS Sol-Gel at 1000x and 4000x magnification. (a,b) before; and (c,d) after immobilization.

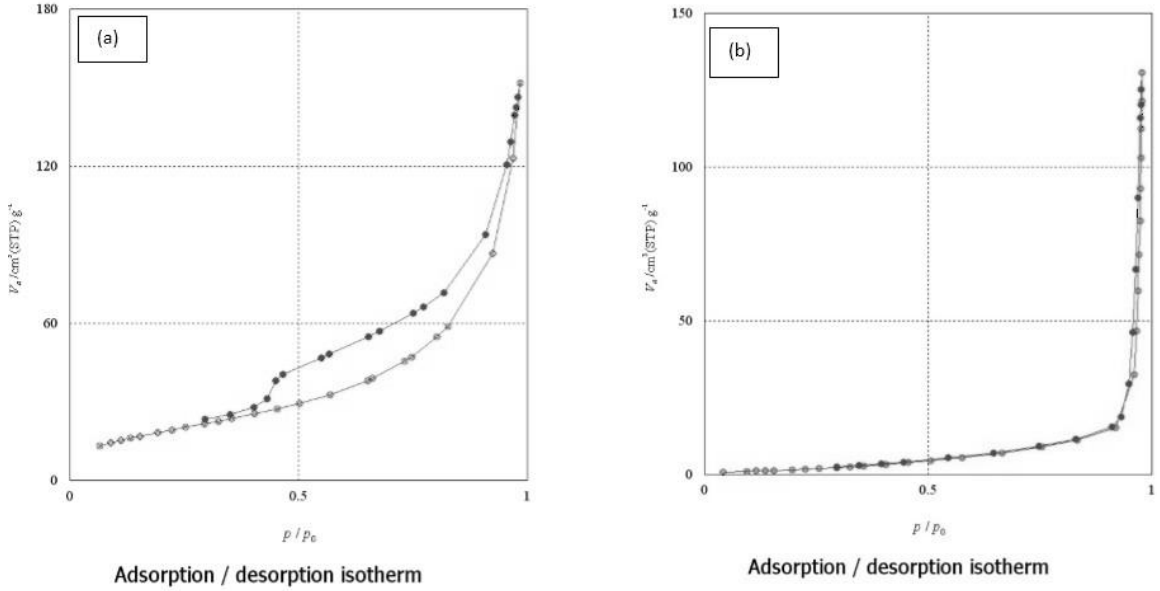

Figure 7. Adsorption/desorption isotherm curve (a) before (b) after immobilization.

Prior to enzyme addition, sol-gel exhibits an adsorption-desorption isotherm type IV (representative for a mesoporous material) but after immobilization, it showed Isotherm type III characterization categorized as the non-porous structure because of lipase molecules in sol-gels. The hysteresis loop of GPTMS:TMOS sol-gel was of type H3 which shows plate-like particles that would develop slit-like pores. Best pore diameter is reported to be more than 35 nm to allow the lipase to penetrate the sol-gel network (39.1 nm in this study). Without enzymes some of the pores would collapse during the drying stage. The reported 50-70% shrinkage is evidence of this phenomenon; therefore, to fully understand the correct morphological characteristic, an exact similar component should be used instead of lipase which can be extracted during drying step without damaging the gel structure [17]. In this research glucose-D was tested for this reason but, because of molecule size difference (Glucose 1nm-Lipase 5 nm) and interaction between precursors and glucose, the gel turns into a paste, so the analysis was conducted on sol-gels before and after immobilization (Table 1). Compared to other reports, the prepared sample had a lower specific surface area which is considered to be in direct correlation with higher enzyme activity as Noureddini *et al*. (2007) which confirmed that, with increase of specific surface area lower catalytic activity is expected [24]. In case of large amount of enzymes, large surface area would lead to a better dispersion of enzyme but less enzyme content, large surface area will inactivate the catalyst [25].

Table 1
Characteristics of the samples based on BJH analysis.

Sample	Specific surface area (m^2/g)	Mean Pore Diameter (nm)	Total Pore Volume (cm^3/g)
Sol-Gel without Enzyme	60.95	13.488	15.983
Immobilized lipase	7.49	39.1	0.2

3-3. Biodiesel production

3-3-1. Alcohol type and addition method

The system criteria had to be developed to obtain the desired results. Ethanol was chosen as a better reactant based on our prior experiments showing higher product (46.94% more biodiesel). In most studies, in order to prevent enzyme denaturation caused by alcohol, the three-step addition was suggested due to toxic effect of alcohol on enzyme activity [26]. In our study, this method resulted in less biodiesel content of the final product. In single step addition of alcohol, the FAEE reached 86.5% (compared to 35% for multiple step addition). One explanation with this controversy might be the possibility of ethanol evaporation in the steps and according to the results for this immobilized enzyme, the sol-gel materials increased lipase resistance toward large amount of alcohol in single step addition.

According to transesterification stoichiometry, the minimum alcohol to oil molar ratio should be 3:1. More alcohol would increase the amount of biodiesel, but at the same time, it will inhibit lipase activity

and denature the enzyme. Most enzymes are denatured by an excessive amount of methanol. In this study, the maximum activity appeared at 9:1 ethanol to oil molar ratio but further increase in alcohol caused 56% decrease in FAEE production. This could demonstrate that immobilized lipase can tolerate high ethanol concentration in transesterification, and thus the stepwise feeding strategy is no longer needed [10].

3-3-2. Water

Water is an essential element for lipase activity since the reaction occurs in the aqueous interface. For this general rule, water was added to the reaction mixture (optimum 10%based on enzyme content) [27]. Results indicated that water would not only accelerate the reaction, it also decreased the FAEE content by 20.4%. This might be due to the fact that the amount of water presented in the reaction medium (in waste oil or entrapped inside the platform) was sufficient for lipase activity and there was no need to add more water as another component, otherwise it would lead to FFA formation rather than FAEE which is not favorable for our prime purpose. It was also reported by Ban (2001) that because of excess water in waste oil, lipase is considered the most efficient catalyst for this substrate [28].

3-3-3. Hexane

According to previous studies, hexane improves the solubility of reactants. To investigate this concept, the reaction was carried out with and without hexane, results showed that hexane lowered the biodiesel content to 13.63%; as it strongly interacts with the water layer coating enzyme molecules and it is not compatible with the sol-gels causing the powder turn into a slurry, similar results were also reported by Hsu *et al*. (2003) [29].

3-3-4. Temperature

In most cases, rise in temperature would increase the reaction rate. In enzymatic reaction, at high temperatures, the enzyme starts to denature and its tertiary structure is deformed. Enzymes perform their catalytic activity in a certain thermal range. First, the catalytic activity rises sharply in the range of 30°C, at 40°C it reached a peak of 86.5% and then decreased drastically to 49.38% at 50°C. As the increase in temperature may weaken and destabilize the bonds that link enzymes with necessary cofactors, the rate of deactivation of enzymes increases.

3-3-5. Enzyme content

Increasing the enzyme content would affect the reaction rate, as more catalyst is available for the substrate. Fig. 8 illustrates the effect of the enzyme content on the transesterification with ethanol and waste oil using entrapped enzyme. There is little difference between different enzymes content, even 5% of the enzyme is quite enough to catalyze the process. Higher enzyme content increases the chance of sedimentation rather than a dispersed reaction mixture.

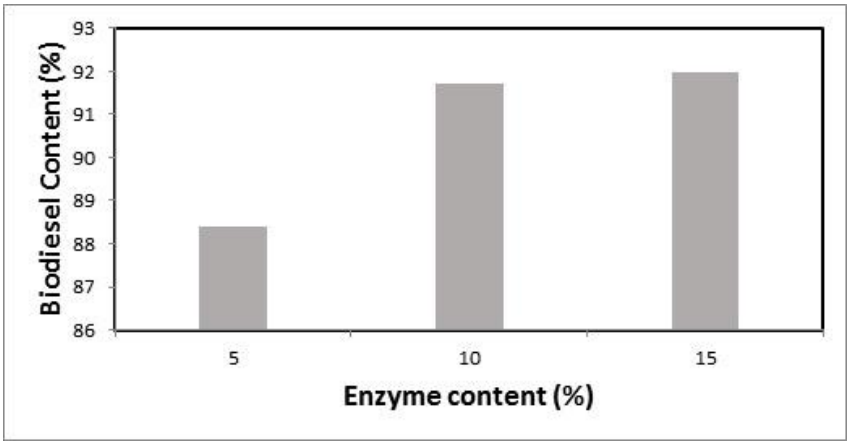

Figure 8. Biodiesel content as a function of enzyme content (10 g of waste oil, alcohol to oil molar ratio of 9:1 agitation rate of 200 at 40°C in 24 h).

3-3-6. Agitation speed

One of the main factors of every process is the way reactants would be mixed and dispersed to increase the amount of products. On the other hand, higher agitation speed means higher input power is required, so there should be a balance between agitation speed, and desired product concentration. According to the results demonstrated in Fig. 9, changing (adjusting) agitation speed from 200 to 500 increased biodiesel content from 63.85% to 86.50% (26.18% increase), but afterwards speed slightly decreased, it can be due to the effect of high shear stress at high speeds on both enzyme structure and dispersion status as most of the immobilized lipase stuck to the wall of the container at high speeds [30].

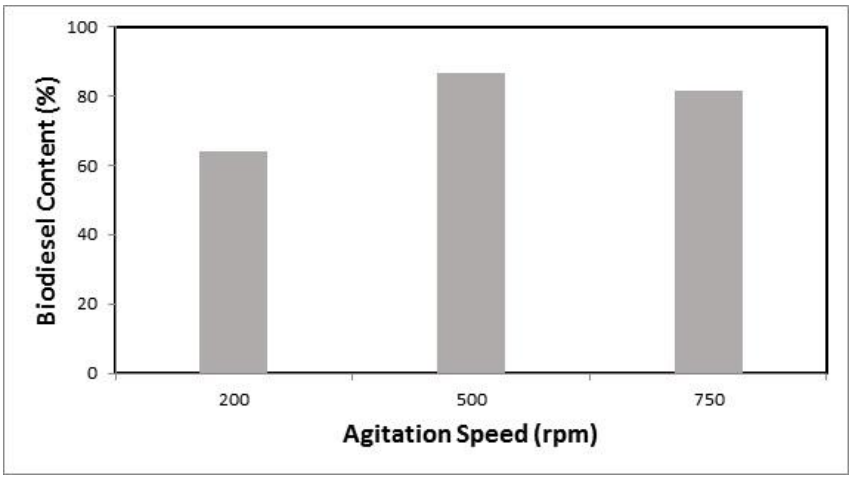

Figure 9. Effect of agitation speed on the transesterification of 10 g of waste oil, 1 g of immobilized enzyme, alcohol to oil molar ratio of 9:1 at 40°C in 24 h.

3-3-7. Time

Time is a key factor in every process due to its effect on other adjustments and the final cost of the product and also the fact that reactions are completed in a specific time due to equilibrium or finished reactants or accumulated products. To find how long it takes for the reactants to produce high amount of biodiesel, the same reaction was carried out in different time courses. As

shown in Fig. 10, in six-hour reaction, 86.87% biodiesel was obtained and after that only minor changes occurred (five percent more after 24 h). Previous reports suggested longer time periods, but this system achieved high performance in a short time period [31–33].

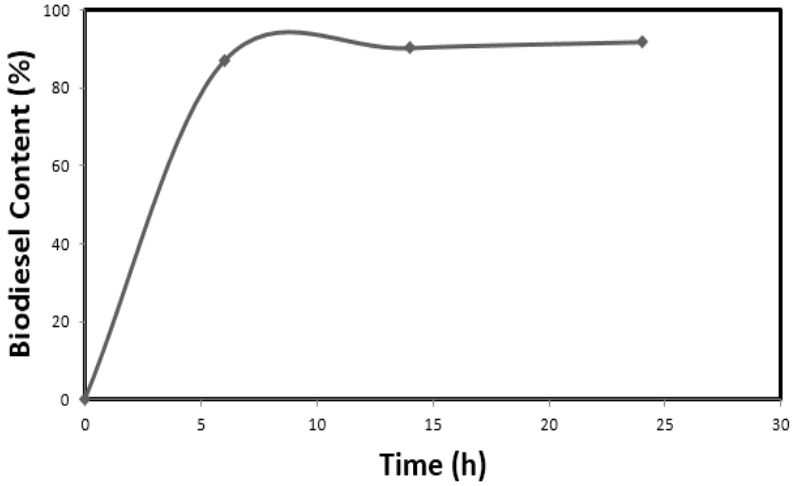

Figure 10. Time course of the transesterification of 10 g of waste oil, 1 g of immobilized enzyme, alcohol to oil molar ratio of 9:1, agitation rate of 200 rpm at 40°C.

3-3-8. Final product characteristics

The best obtained biodiesel content and the specifications of the American ASTM D 6751-07b Standard which is applied to ethyl esters is summarized in Table 2. Flash point is the lowest temperature at which a fuel will ignite and is a very important factor for fuel safety, shipping and storage criteria. The biodiesel produced in this research work had a flash point of 110°C higher than the diesel fuel flashpoint.

Table 2
Biodiesel characteristics base on ASTM D 6751-07b Standard.

Parameter	Specification	Measured	Unit
Ethyl ester content		91.7	Wt%
Viscosity 40°C	1.9-6.0	5.17	mm^2/s
Density 15°C		0.85	g/cm^3
Flash point	>130	174.2	°C

4. Conclusions

The sol-gel immobilized enzyme obtained is applicable for transesterification of waste oil to produce biodiesel fuel as a promising alternative for fossil fuel within even six hours which is shorter than the conventional 72 hours required time. Using waste oil instead of fresh feedstock would decrease the ultimate cost of the process significantly and using ethanol, which can be also produced from renewable sources is another advantage of this system. Higher flash point would make this product safe to store. Mesoporous media protected the enzyme toward alcohol and temperature.

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