Enzymatic production of biodiesel using lipases immobilized on silica nanoparticles as highly reusable biocatalysts: effect of water, *t*-butanol and blue silica gel contents

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Abstract:

The enzymatic production of biodiesel by methanolysis of canola oil was studied using self-made biocatalysts. Mesoporous SBA-15 nanoparticles were prepared, characterized and functionalized by 3-glycidyloxypropyl trimethoxysilane. Lipases from *Candida antarctica* (CALB), *Thermomyces lanuginosus* (TLL) and *Rhizomucor miehei* (RML) were covalently immobilized onto SBA-epoxy. Thermal stability and the influence of methanol concentration on the catalytic activity were also evaluated. higher thermal stability and methanol tolerance for immobilized derivatives were achieved compared to the free enzyme. In an optimization study, the effect of water, *t*-butanol and blue silica gel as water adsorbent on the yield of FAME was considered. For the SBA-RML catalysed reaction, water had little effect in increasing FAME yield, but when 20 wt% water by substrate weight was added to the SBA-TLL catalysed reaction, the methyl ester content reached nearly complete conversion (98% FAME). *t*-Butanol had a great effect on yield, with almost complete conversion for SBA-RML and SBA-TLL. The effect of blue silica gel also was investigated. The immobilized TLL was quite stable and can be reused for 20 cycles without significant loss in activity (6%). RML and CALB also presented a good reusability, keeping 95% of their initial activities after 7 and 15 cycles of the reaction.

1. Introduction

In chemical reactions, the use of nontoxic solvents/reagents is desirable due to health as well as environmental concerns [1]. Due to limited energy reserves and the increasingly urgent problem with green-house gases coming from the fossil fuels, the utilization of biofuels has become a hot topic of recent years. Biodiesel, which is derived from triglycerides by transesterification with methanol, is a renewable, biodegradable, and nontoxic fuel [2]. This fuel is currently the most important alternative diesel fuel. Vegetable oils for biodiesel production vary noticeably with region, climate and specially feedstock availability. Thus, the search for additional regional biodiesel feedstocks is an important objective. For example, rapeseed and sunflower oils are predominantly used in Europe; palm oil predominates in tropical countries, and soybean oil and animal fats in the USA [3].

Acids, bases, both liquid and heterogeneous, as well as free or immobilized enzymes have been investigated as transesterification catalysts [4]. Today, alkaline

transesterification is the most commonly used method for biodiesel production in industry, [5, 6] but this method has some drawbacks, such as soap formation during the methanolysis of oils with high free fatty acid content and also difficulties in the recovery of glycerol and the need for removal of catalyst [7].

Contrary to alkaline catalysts, enzymes do not form soaps and produce biodiesel with a very high purity even from low quality feedstocks such as used cooking oil and greases [8, 9]. This has been long established in the scientific literature, although the economic aspect of large-scale enzymatic biodiesel production has not been addressed in nearly the same detail. However, developments over the last few years have taken enzymatic biodiesel from an academic curiosity to a real attractive alternative for biodiesel producers. This is based on a desire in the industry to allow for multiple feedstocks in the plants, combined with efforts from an enzyme producer like Novozymes to develop robust, efficient and economically attractive enzymatic processes. Hence, Novozymes is now promoting the BioFAME process based on liquid formulated enzymes [10]. Immobilization of enzymes on solid carriers is perhaps one of the most commonly used strategies to improve operational stability of biocatalysts, including better operation control, easier product recovery without catalyst contamination, and flexibility of reactor design [9, 11-16]. Unfortunately, immobilization also significantly adds to the cost of the already expensive catalysts. Therefore, for an immobilized enzyme solution to be competitive in production of a bulk product like biodiesel, cost is critical, and development of low-cost high-productivity immobilized lipases is of significant interest. One of the key questions related to the immobilization process is which kind of immobilization (chemical bonding or physical adsorption) is more effective? The major disadvantage associated with the immobilization of lipase by the adsorption method is a relatively weak interaction between the enzymes and surface which results in leaching of the enzyme during the reaction. Covalent bonding of the enzyme to the support can prevent the problem of leaching, increase the stability and enable multiple reuse of the enzyme derivatives. The enzyme is generally bonded to the surface through any one or more of a number of sites. The ideal immobilization of an enzyme should occur in such a way that it does not destroy the structure of the enzyme nor hinder diffusion of the substrate and product to and from the active site [17, 18].

While various methods are available for the enzyme immobilization, it is essential to note that the actual immobilization process used for each enzyme is unique and

depends on the specific enzyme and support used [19]. Numerous supports have been investigated with respect to the immobilization of enzymes including polymeric supports, metallic and glass surfaces, carbon nanotubes, porous and non-porous inorganic materials, colloidal supports and powdered materials.

Mesoporous silicates are promising candidates for enzyme immobilization with respect to the requirements for enzyme carriers such as large surface area, narrow pore size distribution, well defined pore geometry, thermal and mechanical stability, sufficient functional groups for enzyme attachment, hydrophilic character, water insolubility, regenerability, and toxicological safety [20, 21].

Up to now, several mesoporous silicas, such as MCM-41 [22], SBA-15 [23], and mesocellular foam (MCF) [24] have been synthesized and utilized for bioimmobilization [25]. Mesoporous materials can accommodate proteins and biomolecules in their inside pores by entrapping and on the outer surface by covalent bonding [26]. The highly ordered large pore mesoporous silica SBA-15 was introduced in 1998 [23, 27]. SBA-15 with pore sizes ranging from 5-30 nm depending on the synthesis conditions, can be synthesized in a various shapes such as rods, fibers, platelets and spheres [21]. SBA-15 has higher thermal and hydrothermal stability than previously synthesized mesoporous materials. Ordered mesoporous silicas due to their unique textural structure, represent valuable model systems to monitor aspects of the immobilization process [28].

In the physical adsorption of lipase, the ideal pore size of mesoporous materials should be ideally similar to or larger than that of lipase for successful enzyme adsorption. High pore size may lead to the leaching of lipases from the channels, which also accounted for a decrease in enzyme loading and enzyme instability [29-31]. On the contrary, covalent bonding can induce effective and almost irreversible immobilization.

The validation of the feasibility of immobilized lipases on mesoporous materials for biodiesel production represents the beginning of a new field of research [28, 32-34].

The major reported functionalization of the SBA-15 surface was modifying using amination (reaction of the silanols with aminopropyltrimethoxysilane), and subsequently reaction with glutaraldehyde which resulted introducing the -CHO functional group [35, 36].

In our previous work, a process for biodiesel production using immobilized lipases on epoxy-functionalized silica was developed successfully and optimal conditions were

obtained [37]. In the work reported here, a SBA-15 surface is modified using 3-glycidyloxypropyl trimethoxysilane (3-GPTMS) and three lipases *Candida antarctica* lipase B (CALB), *Thermomyces lanuginosus* lipase (TLL) and *Rhizomucor miehei* lipase (RML) are immobilized onto the epoxy functionalized SBA. The enzyme stability in methanol, its thermal stability and reusability in FAME production are evaluated. The enzyme derivatives are utilized for lipase-catalysed transesterification and esterification of canola oil to fatty acid methyl esters (FAME). Several reaction parameters influence reaction rate and FAME yield. Some of these parameters are studied here, aiming to obtain optimal conditions for the methanolysis of canola oil. Canola oil was selected for this study since it has the highest oil productivity per hectare in Iran.

2. Materials and methods

2.1. Materials

Lipases from *Thermomyces lanuginosus* and *Rhizomucor miehei*, methyl ester standards (methyl laurate, methyl stearate, methyl linoleate, methyl oleate, methyl palmitate and methyl myristate) were purchased from Sigma-Aldrich. Lipase B from *Candida antarctica* was kindly donated by Novozymes (Bagsvaerd, Denmark). Crude canola oil was purchased from a local market with 8.4% free fatty acid and the following composition in fatty acids (w/v) were determined by ISO 5508: 1990 method [38]: 58.8% oleic acid, 21.7% linoleic acid, 4.8% palmitic acid, 2.39% stearic acid. The molecular weight of the oil which was calculated from its saponification value was determined to be 880 g/mol. Water content in the oil measured by Karl Fischer titration method was determined to be 0.08% (w/w). Tetraethyl orthosilicate (TEOS) Methanol, *t*-butanol and triethylamine (Et₃N) were purchased from Merck chemicals. 3-Glycidyloxypropyl trimethoxysilane (3-GPTMS) was purchased from Acros. All the other chemicals were obtained commercially and were of analytical reagent grade.

2.2. Synthesis and functionalization of SBA

Mesoporous silica SBA-15 was synthesized and characterized according to the reported procedure [39]. Pluronic P123 triblock copolymer (EO20–PO70–EO20, BASF) was used as template. Briefly, pluronic P123 (4 g) were added to 2 M aqueous HCl (144 ml) at 40°C. Successively, tetraethyl orthosilicate (TEOS) was added

dropwise (mass ratio TEOS/P123 = 2:1) and then stirred for 2 h. Afterwards, the mixture was transferred to Teflon-lined sealed container and kept at 100°C for 48 h. The white solid was filtered, washed with distilled water and calcined at 550°C to remove the template.

The mixture of dry SBA (1 g) in dried toluene solution (30 ml), containing 3-GPTMS (1 ml) and Et₃N (0.15 ml) was refluxed under argon atmosphere and constant stirring for 4 h (Scheme 1). The SBA was then washed thoroughly with CHCl₃ and dried at 60°C for 2h.

2.3. Immobilization of lipase

Epoxy functionalized SBA (1 g) was mixed with lipase (40 mg) in 25 mM pH 7.0 phosphate buffer (10 ml) and incubated at 25°C for 24 h (Scheme 1). Periodically, an aliquot of the supernatant was withdrawn and analyzed for determination of the protein concentration by the Bradford's method [40]. Immobilized lipase was separated by filtration, washed thoroughly with distilled water, and then dried overnight at room temperature. The amount of lipase bound to the carrier was determined as the difference between the initial and residual protein concentration. The yield of bound enzyme was calculated as the ratio of the amount bound on silica to the initial amount.

2.4. Lipase activity assay

The activities of the immobilized preparations were analyzed spectrophotometrically by measuring the increase in absorbance at 348 nm, produced by the release of *p*-nitrophenol ($\epsilon = 5150 \text{ M}^{-1}\text{cm}^{-1}$) in the hydrolysis of *p*-NPB in 25 mM sodium phosphate buffer at pH 7 and 25°C. To start the reaction, 10 μl of the lipase suspension (4 mg immobilized enzyme in 1 mL phosphate buffer 25 mM pH 7) was added to 1 ml of the reaction mixture (0.8 mM *p*NPB in phosphate buffer 25 mM pH 7). Hydrolysis was followed by measuring the change in absorbance over 2 min.

2.5. Thermal stability studies

Thermal stability studies were carried out by incubating the immobilized enzyme (100 mg/mL of 25 mM sodium phosphate buffer, pH 7) and soluble enzyme (1 mg protein/mL of 25 mM sodium phosphate buffer, pH 7) at 50, 60, 70 and 80 °C. The

residual activity was measured using the *p*NPB assay after 24 h as described above. The residual activity was calculated as the ratio between the activity at a given time and the activity at time zero of incubation.

2.6. Stability in methanol as co-solvent

The stability of lipases and their immobilized derivatives were evaluated by incubation at 25 °C in aqueous conditions using methanol as co-solvents. The incubation in co-solvents was performed for 24 h in 10, 30, 50, 70 and 90% methanol solution by adding the appropriate amount of methanol to a 25 mM sodium phosphate solution, pH 7. Samples were taken after 24 h and their activity was measured by the *p*NPB assay as described above. The residual activity was calculated as the ratio between the activity measured at a given time and activity at time zero of incubation.

2.7. Biodiesel synthesis reaction

In a typical experiment, the reaction was performed in 10 mL screw-capped vials containing 0.65 g canola oil, anhydrous methanol at oil:methanol molar ratio of 1:3 and 3.8 wt% of biocatalyst (25 mg), correspond to 1 mg of lipases on SBA-15-epoxy. Methanol was added by a three-step procedure and each one molar equivalent of methanol was added at the reaction time of 0, 24, and 48 h, respectively (Scheme 2). The mixtures were incubated with the immobilized lipases on SBA-epoxy at 50 °C. Methanolysis reactions were carried out with varying amount of *t*-butanol, water and water adsorbent. To study the effect of water concentration on methanolysis, the amounts of *t*-butanol and water were varied from 10-60% and 10-30 wt% respectively by weight of oil. To study the effect of water adsorbent, blue silica gel (30–50 wt% oil) was added to the reaction mixture to remove the by-product water. Reactions were performed for a maximum period of 72 h under constant magnetic agitation of 250 rpm. For the time course studies, an aliquot of reaction medium was taken at various time intervals (24, 48 and 72 h), centrifuged (12000 rpm, 10 min), mixed with methyl laurate (as an internal standard) and analyzed by the GC method as described below.

2.8. GC analysis

The FAME contents in the reaction mixture were quantified using a Thermo-Quest-Finnigan (Plymouth, Minnesota, USA) GC instrument equipped with an RTX-1

column and a flame ionization detector (FID). Nitrogen was used as the carrier gas at a constant flow of 1.2 mL/min. A specified amount of methyl laurate as the internal standard and 0.5 ml hexane were added to the accurately weighted sample from the upper layer of the reaction mixture. Then, 2.0 μ l of the diluted sample was injected into the GC gas chromatograph. The column temperature was kept at 150 °C for 0.5 min, raised to 270 °C at 10 °C/min, and then maintained at this temperature for 3 min. The injector and detector temperatures were set at 220°C and 250°C, respectively. By comparing the retention times and peak areas of standard fatty acid methyl ester peaks, the total quantities of biodiesel in the reaction mixtures were calculated.

2.9. Enzyme reuse

The enzyme reuse was evaluated in the transesterification reaction of canola oil with methanol. After methanolysis reactions immobilized lipases were separated by centrifugation (4000 rpm, 5 min) and washed with *n*-hexane three times, and then the lipase derivatives were dried in a desiccator for 24 h. After this treatment, the immobilized enzymes were resuspended in a fresh aliquot of substrates for the next catalytic cycle. The ester yield of the first reaction was set as 100% and the ester yield in the subsequent reactions was calculated accordingly.

3. Result and discussion

3.1. Synthesis and characterization of support and immobilized lipases

The majority of reported methods for immobilization of lipases on mesoporous materials are based on amination and subsequently reaction with glutaraldehyde. This process consists of two steps, but in present work a single step functionalization has been introduced and resulting biocatalysts successfully were utilized for biodiesel production for the first time. Fig. 1 shows SEM images of SBA-15 before and after functionalization. Upon functionalization, the silica support maintains their structure and displayed pore diameter of about 8 nm, which are fitting with the dimensions of the enzyme. Both images exhibit a rather wavy or short, rod-shaped morphology. Enzyme immobilization on porous materials seems to be very effective, because they are able to spread enzyme molecules on their high surface areas [35].

Epoxy functionalized SBA was prepared by 3-GPTMS in presence of Et₃N according to the methodology previously described. Then, three lipases TLL, RML and CALB were immobilized on epoxy functionalized SBA. Specific activity and immobilization yields obtained for each lipase derivative are presented in Table 1. All derivatives prepared on epoxy functionalized SBA showed a very high yield of immobilization >90%. The specific enzyme loading of 36-38 mg per gram support was obtained for all three enzymes. The total amount of immobilized lipase on the support was four times higher than that observed with those formed by covalent binding between enzyme and epoxy functionalized silica (ca. 40 mg/g SBA vs. 10 mg /g silica). In general one of the advantages of nanometer-scale materials is high enzyme loading due to their large relative surface area. High enzyme loading leads to better biocatalytic activity and stability. This makes the nanopores materials an ideal support for enzyme immobilisation as compared to conventional materials [29]. Table 2 compare the improvement of the biocatalyst in this work to our previous report. As can be seen, specific activity of RML-SBA (1520 U/g) has improved versus RML-silica (1230 U/g), while the specific activity of other derivatives have decreased comparing silica derivatives, the thermal stability and methanol tolerance of RML-SBA have improved in comparison with RML-silica, but for the other derivatives these amounts are unchanged. In this method of immobilization, due to strong covalent bond between lipase and support, there was no leaching of enzymes during the washing process, indicating the stable covalent immobilization.

There are some reports about the influence of the support surface on the loading and the enzymatic activity of the immobilized lipases on SBA-15 and surface modified SBA-15 [28, 32, 35, 41-43]. Table 3 presents comparison between the epoxy-functionalized SBA in this investigation and previously reported immobilization on SBA support. As mentioned above, the key finding in this work is that all previous chemically modification of SBA-15 carried out by functionalization of SBA in two steps and using lower enzyme loading in the reaction.

In our previous investigation the oil methanolysis was performed at 50°C for 96 h with various amounts of methanol. When more than 3 molar equivalents of methanol were present initially in the oil mixture, methanolysis decreased dramatically [37]. To fully convert the oil to its corresponding methyl esters, at least 3 molar equivalents of

methanol are needed. Thus, the reaction was conducted by adding 3 molar equivalents methanol in 3-steps to avoid lipase inactivation.

3.2. Thermal Stability of the free and immobilized lipases

One of the main goals of this investigation was to improve the enzyme's thermal stability to be suitable for industrial use. As can be seen in Fig. 2, immobilization significantly improves thermal stability of all three enzymes. For *R. miehei* lipase, the increased thermal stability of the immobilized derivative is outstanding. For example at 50 °C, the immobilized enzyme retained nearly 100% of its relative activity for an incubation period of 24 h, whereas that of the free enzyme only reached 18%. However, at 60 °C, the free enzyme lost all its initial activity, whereas the immobilized enzyme still remained 89% of its relative activity. The immobilized RML retained about 69% of its initial activity even at 80 °C.

T. lanuginosus lipase which was immobilized on SBA-epoxy by covalent attachment showed a lower thermal stability than that of the free lipase at 50 °C. At 60 °C, the temperature at which the enzymes are preferably used in industries to avoid microbial contamination, TLL immobilized onto mesoporous silica nanoparticles retained 75% of their relative activity after 24 h. However, at 70 °C, the free enzyme lost 75% of its relative activity, whereas the immobilized enzyme retained 63% of its relative activity. At higher temperatures (80 °C) the free enzyme retained only 20% of its relative activity compared to 47% for the immobilized enzyme.

For free and immobilized CALB, increasing the temperature to 60 °C clearly unraveled the difference in the stability of the biocatalysts, as SBA-CALB retained 78% of its relative activity for an incubation period of 24 h, while liquid CALB decreased to 10% activity.

In general, thermal inactivation of enzymes is caused by disturbance of their 3D structure by the increasing temperature [44]. The (multipoint) covalent immobilization on porous SBA seems to offer significant stabilization of the enzyme structure. Clearly, this translates to a longer lifetime at elevated temperatures which are favorable for industrial use.

3.3. Methanol stability of free and immobilized lipases

Methanol is widely used as the acyl acceptor for the production of biodiesel and is often favored over other alcohols because of its low cost and also, the rate of lipase-

catalyzed transesterification reaction usually increases with increasing the length of hydrocarbon chain of alcohol [45]. However, several lipases are reported to be inhibited by methanol, which limits the possibility of increasing the alcohol/oil ratio and/or requires modified processes [46]. In present report the stabilities of different immobilized derivatives were evaluated in presence of 10-90 wt% methanol as described in section 2.6.

Fig. 3 shows the stability of the covalently immobilized preparations and corresponding free enzymes in the presence of methanol. In the presence of methanol, the difference between SBA-RML and free RML is very clear. SBA-RML became highly stabilized with 46% residual activity after 24h incubation in 90% methanol vs. no residual activity for free RML after 24 h incubation.

CALB seems to be the most methanol stable enzyme out of the three. Consequently, there were no significant differences between covalently immobilized CALB and the free enzyme. Both residual activities were gradually decreased till 75% activity at 90% methanol content.

In the case of TLL, the residual activity reached to 60% after the addition of 90% methanol. Therefore, TLL similar to CALB is highly resistant to methanol. , Comparing with our previous work, enzyme immobilization on SBA-epoxy compared to silica-epoxy, increased activity of TLL and RML in high concentrations of methanol, but had little effect on CALB activity.

3.4. Biodiesel reactions catalyzed by immobilized lipases

We have previously shown that immobilized lipases on epoxy functionalized silica can be used as biocatalysts for solvent free methanolysis of canola oil. Initially, only modest conversions (45–70%) to FAME were obtained, but in an optimization study, the effect of water, *t*-butanol and blue silica gel on the fatty acid methyl ester (FAME) yield was considered. Complete conversion to FAME was achieved under optimum conditions [47]. As a continuation of these studies, in the present work, the solvent system and water adsorbents were evaluated separately, to study the performance of the new developed biocatalyst.

As shown in Fig. 4-6, initial studies showed that the FAME content reached the modest conversion rate: 24, 33 and 34% after 72h when SBA-CALB, SBA-TLL and SBA-RML biocatalysts were used respectively. Large molecules such as

triacylglycerols and fatty acid methyl esters have to diffuse through small pores of SBA to reach the enzymes and the external environment respectively, so using modifiers might improve the FAME conversion rate. In order to get an in-depth understanding of the reaction mechanism and an improved result, the effects of some important variables on the transformation were examined.

3.4.1. Effect of water content on methanolysis catalyzed by lipases

The amount of water in the enzymatic reaction mixture is a critical parameter and has strong influence on the catalytic activity and stability of the lipases. It is known that the enzyme needs a certain amount of water to maintain its proper conformation. Water content has a thermodynamic effect on the equilibrium of the reaction. In the case of the lipases, high water content significantly decreases the ester production through increase in hydrolysis [48]. Furthermore, the enzyme conformation in reaction media with high water content may be more flexible, which may also decrease the stability of enzyme but increase the transesterification activity of enzyme [49].

To get a better view of the effect of water on canola oil methanolysis, reaction profiles were compared, using 0-30% added water (Fig. 4). Since we observed earlier that water suppressed the methanolysis reaction catalysed by Silica-CALB we did not evaluated the water effect for SBA-CALB.

For the SBA-RML catalysed reaction at 50 °C and 1:3 substrate molar ratio, poor conversion was obtained at all tested water concentrations (Fig. 4a)., with 10% (w/w) of added water, about 32% of alkyl esters were reached in 72 h, interestingly, the reaction proceeded nearly identically with 0% added water (Fig. 4a). With 20% (w/w) of added water, about 42% of methyl ester was reached in 72 h but additional increases of water did not result in further improvements in canola oil conversion, only about 27% FAME in 30% methanol. This decrease can be attributed to the progressive flooding of the pores in the enzyme support on which the enzyme was immobilized [50].

In contrast, with SBA-TLL (Fig. 4b), when 20-30 wt% water by substrate oil weight was used, the ME content reached nearly complete conversion, 98% FAME yield. It is likely that the TLL immobilized on epoxy functionalized SBA can be inactivated by methanol due to insufficient water in the reaction mixture, so added water improve the

FAME yield. However, with 10 wt% methanol the reaction rate was lower, and the ME content reached approximately 80%. The water (30% w/w of oil) also was essentially sufficient to give a nearly complete conversion of canola oil in 72 h.

*3.4.2. Effect of *t*-butanol on methanolysis catalyzed by lipases*

Transesterification can be carried out either using organic solvents or in solvent-free medium. Organic solvent reduces the viscosity of the reaction mixture and can improve solubility of methanol and/or glycerol. The most widely used solvent is *t*-butanol. Due to its moderate polarity is able to solve both glycerol and methanol and reduce the inhibitory effects of methanol [51-53].

Fig. 5a shows the effect of *t*-butanol on SBA-RML catalyzed reaction. It can be seen that in the presence of enough *t*-butanol, an increase in the biodiesel yield is observed. The best yields were obtained at 60 wt% of added *t*-butanol, 95% FAME in 72 h, which is in good agreement with those reported in the literature [54-56].

For SBA-TLL catalysed system (Fig. 5b), adding only 10% *t*-butanol to the medium, resulted in a significant increase in the conversion of oil to methyl ester. For the SBA-CALB reaction however, only high *t*-butanol concentrations had a positive effect.

As can be seen in Fig. 5c the FAME content obtained of canola oil methanolysis in a solvent-free medium catalyzed by CALB approximately reached 24% after 72 h reaction. Addition of *t*-butanol to reaction medium increased FAME yields gradually. FAME content reached to 61% by adding 40% *t*-butanol to the reaction medium.

3.4.3. Effect of water adsorbent on the methanolysis catalyzed by lipases

The esterification of free fatty acids present in the oil with methanol produce water in the reaction medium. Since 8.5% of the canola oil used in this investigation is free fatty acids, the effect of water adsorbent quantity on the reaction was investigated. The addition of water adsorbent to the biodiesel reaction prevents the aggregation of the enzyme and shifts the reaction equilibrium to ester production. Therefore, higher ME yield can theoretically be achieved with water adsorbent [57].

In this paper, the effect of blue silica gel quantity was investigated (30–50 wt% oil) on the reaction. In the reactions catalyzed by SBA-CALB and SBA-TLL, the blue silica gel had a positive effect on the ME yield, up to 30 wt%. ME yield increased (ca. 45% FAME) with increasing blue silica gel quantity at 30 wt% (Fig. 6), due to this fact that removal of water in the mixture by blue silica gel, shifting the reaction. Further

increase in blue silica gel quantity, however, resulted in a lower ME yield. The decrease in MEs yield at high blue silica gel quantity could be attributed to the adsorption of methanol onto blue silica gel, mass transfer limitation or too low water activity [57, 58]. When the reaction was carried out by RML, the addition of water adsorbent decreased ME yield.

Table 5 summarizes the optimal condition and maximal FAME yield for three immobilized lipases in presence of three modifiers (*t*-butanol, water and water adsorbent). SBA-CALB afforded maximum yield in 40 wt% of *t*-butanol as co-solvent (58.8 %) and RML-epoxy yielded 95.4% FAME in 60 wt% of *t*-butanol. The best biodiesel yield belongs to SBA-TLL derivatives which showed high conversion 94.2 and 98.6% FAME in 10 wt% of *t*-butanol and water as co-solvents respectively.

3.5. Operational stability of biocatalysts

To further examine the potential of self-made biocatalysts for biodiesel production, their operational stability was investigated. After each batch reaction, the immobilized lipases were recovered by filtration, dried in desiccator and the next batch was carried out with fresh substrates.

Experiments investigating reusability of biocatalyst indicate that immobilized lipases on epoxy-functionalized SBA show good capability to be used repeatedly (Table 4) thereby no leaching of lipase would occur under mechanical stirring during the methanolysis reaction. The initial activity prior to the first recovery was taken as 100%. The relative efficiency of the biocatalyst for the transesterification with repeated use is shown in Table 4. As can be seen, 93% of the initial activity of the derivatives was maintained after 20, 15 and 7 cycles for SBA-TLL, SBA-CALB and SBA-RML, respectively.

Thanks to their high tensile strengths, nanoscale materials are often robust and resistant to breakage through mechanical shear in the running reactor making them suitable for multiple reuses [29]. Arumugam and co-workers has reported *Candida antarctica* immobilization on SBA-15 by physical adsorption. The percentage yield of FAME decreased by 8.9 % after eight recycles, the gradual decrease in FAME yield was attributed to both loss of activity of immobilized lipase and loss of enzyme due to leaching [32]. The biocatalyst showed higher operational stability than the corresponding physisorbed enzyme since it can be reused 6 times against 6 consecutive runs for the physisorbed enzyme.

Mucor miehei lipase which was covalently bound on isocyanate functionalized SBA-15, was examined as biocatalyst for transesterification of colza oil with methanol. These reusability results suggested that the leaching of the enzyme is strongly diminished by covalently linking the enzyme and the biocatalyst can be reused for at least 6 runs [33].

4. Conclusion

The epoxy functionalized SBA-15 was designed as novel mesoporous support for covalent immobilization of lipases. The epoxy functionalization of SBA was successfully carried out in one-step, while all previously reported chemically modification of SBA-15 carried out by functionalization of SBA in two steps. All three enzymes were covalently immobilized in high yield onto modified support. Immobilization onto SBA strongly increased the enzyme's thermal stability and methanol tolerance compared to that of soluble enzymes. Comparing with other immobilization method on the SBA-15 support (chemically or physically), epoxy-functionalized silica resulted to higher catalytic activity and reusability. SBA-TLL, SBA-CALB and SBA-RML showed operational stability up to 20, 14 and 7 runs, respectively. Despite lipase thermal stability and its methanol tolerance, because of low FAME yield, SBA isn't suitable support for biodiesel reaction in this condition. In an optimization study, the effect of water, *t*-butanol and water adsorbent addition on the FAME yield were evaluated and the methyl ester content reached nearly complete conversion. Further investigation is underway to improve conversions for other sources of oils such as waste cooking oil in solvent-free methanolysis and scale-up reactions. The potential of this technology is also being explored to introduce other functional groups to prepare highly reusable biocatalysts and lower the cost of production of biodiesel.

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Table 1. Enzymatic catalytic activity of the immobilized biocatalysts ^a

Enzyme derivative	Immobilization yield (%) ^b	mg lipase/g support	Specific activity (U/mg enzyme) ^c	SD ^d
Crude CALB	-	-	1770	5.1
Crude RML	-	-	2050	7.5
Crude TLL	-	-	1280	3.8
CALB-SBA	90	36	1340	6.9
RML-SBA	91	36	1520	8.1
TLL-SBA	95	38	1150	9.2

^a Immobilizations were performed as described in Section 2.2.

^b Yield is defined as the percentage of soluble enzyme that attached to the support.

^c Specific activity (U/g lipase) is expressed as micromole of *p*NPB that is hydrolyzed per minute per gram of immobilized protein.

^d Standard deviation (SD) of the mean specific activity

Table 2. Comparing the main characteristics of epoxy functionalized SBA and silica.

	SBA-epoxy			Silica-epoxy [37]		
	CALB-SBA	RML-SBA	TLL-SBA	CALB-silica	RML-silica	TLL-silica
mg lipase/ g support	36	36	38	9.6	9.6	10
Specific activity U/g	1340	1520	1150	1510	1230	1700
Thermal stability (°C) (100 % residual activity)	25	50	50	55	45	50
Methanol concentration (wt %) (> 80% residual activity)	90	10	30	90	0	30

Table 3. Comparing the reaction components/conditions and biodiesel yields using the immobilized lipases in present work and previous works.

Support-functional group	Reaction condition	Biodiesel yield (%)	mg Protein/ g support	Reuse (reaction cycle)	Ref
SBA-15-CHO ^a	Ethanolysis of sunflower oil (8h) (alcohol:oil molar ratio) 8:1; 30 °C; 125mg of immobilized lipase (4 wt% of biocatalyst)	84	6.54	-	[35]
SBA-15-CHO ^a	Ethanolysis of sunflower oil (10h) (alcohol:oil molar ratio) 8:1; 30 °C; 4 wt% of biocatalyst, correspond to 35 mg of lipase on SBA-15-CHO)	100	300	20 (80% conversion)	[28]
SBA-15-CHO ^a	Ethanolysis of lipid extract from <i>Nannochloropsis gaditana</i> microalga, 40°C, 24 h, 500:1 oil:lipase mass ratio and 8:1 ethanol:oil mass ratio	74.1 (<i>CALB</i>) 28.5 (<i>TLL</i>) 59.2 (<i>Mucor miehei</i>)	36.1 (<i>CALB</i>) 40.2 (<i>TLL</i>) 15.7 (<i>Mucor miehei</i>)	10 (61.1%, <i>CALB</i>) 2 (25 % <i>TLL</i>) 2 (<10% <i>Mucor miehei</i>)	[41]
SBA-16-CHO ^a	Ethanolysis of lipid extract from <i>Nannochloropsis gaditana</i> microalga, 40°C, 24 h, 500:1 oil:lipase mass ratio and 8:1 ethanol:oil mass ratio	65.8 (<i>CALB</i>)	26.6 (<i>CALB</i>)	-	[41]
MCM-41-CHO ^a	Ethanolysis of lipid extract from <i>Nannochloropsis gaditana</i> microalga, 40°C, 24 h, 500:1 oil:lipase mass ratio and 8:1 ethanol:oil mass ratio	71.8 (<i>CALB</i>)	28.8 (<i>CALB</i>)	-	[41]
SBA-15-epoxy ^b	Methanolysis of canola oil, 50 °C, 72 h, 3:1 methanol:oil molar, 3.8 wt% of biocatalyst, correspond to 1 mg of lipases on SBA-15-epoxy)	24 (<i>CALB</i>) ^c 33 (<i>TLL</i>) 34 (<i>RML</i>)	36 (<i>CALB</i>) 38 (<i>TLL</i>) 36 (<i>RML</i>)	20 (82%, <i>CALB</i>) ^d 20 (94% <i>TLL</i>) 7(94% <i>RML</i>)	Present work

^a Functionalization of SBA in two steps: 1) Introducing NH₂ functional group (reaction with aminopropyltrimethoxysilane); 2) introduction aldehyde function (reaction with glutaraldehyde)

^b Functionalization of SBA in one step: Introducing epoxide group (reaction with 3-GPTMS)

^c Biodiesel yields are reported for non-optimized condition

^d The initial activity prior to the first recovery was taken as 100%

Table 4. The effect of repeated use of immobilized preparations on their activities in biodiesel production.

cycle	Activity (%)					
	SBA-CALB	SD ^a	SBA-TLL	SD	SBA-RML	SD
1	100	0	100	0	100	0
3	100	0	100	0	100	0
5	100	0	100	0	97	1.5
7	98	1.1	100	0	94	1.2
9	96	1.7	100	0	62	2.5
11	95	1.0	98	1.2	40	1.4
13	95	1.5	98	1.4	-	-
15	95	2.3	95	2.3	-	-
20	82	1.8	94	1.9	-	-

^a Standard deviation (SD) of the mean activity

Table 5. The optimal condition and maximal FAME yield for three immobilized lipases

Modifier	CALB		TLL		RML	
	Modifier (%)	Yield (%)	Modifier (%)	Yield (%)	Modifier (%)	Yield (%)
no modifier	-	24	-	33	-	34
water	-	-	10	98.6	20	42.9
<i>t</i> -butanol	40	58.8	10	94.2	60	95.4
water adsorbant	30	41.6	30	47.3	0	34

Fig 1. Scanning electron micrographs of SBA before (a,b): pore sizes shown in (b) are 6.32, 7.89 and 11.1 nm and after enzyme immobilization (c,d): pore sizes shown in (d) are 6.79, 8.93, 10.3 nm.

Fig. 2. Thermal stability of CALB, TLL and RML. The incubation was carried out in 25 mM sodium phosphate buffer, pH 7, 24 h at different temperatures (25, 50, 60, 70 and 80 °C). Hydrolytic activity of enzyme derivatives was then determined by measuring the increase in absorbance at 348 nm produced by the release of *p*-nitrophenol during the hydrolysis of *p*-nitrophenyl butyrate. Results are the mean of duplicates. (-) Crude CALB, (*) Crude TLL, (●) Crude RML, (◆) SBA-CALB, (■) SBA-TLL, (▲) SBA-RML.

Fig. 3. Methanol stability of CALB, TLL and RML. The incubation was carried out in 25 mM sodium phosphate buffer, pH 7, 24 h at different methanol concentration (10, 30, 50, 70 and 90%). Hydrolytic activity of enzyme derivatives was determined by measuring the increase in absorbance at 348 nm produced by the release of *p*-nitrophenol during the hydrolysis of *p*-nitrophenyl butyrate. Results are the mean of duplicates. (×) Crude CALB, (*) Crude TLL, (●) Crude RML, (♦)SBA-CALB, (■) SBA- TLL, (▲) SBA- RML.

Fig. 4. Effect of water content on methanolysis of canola oil. a) SBA-RML, b) SBA-TLL. Reactions were performed at 50 °C with 0.65 g of oil and 25 mg immobilized enzyme. Methanol to oil molar ratio: 3, methanol was added three times at 0, 24, and 48 h. The conversion to biodiesel was measured quantitatively by GC. The amount of added water was 0% (♦), 10% (■), 20% (▲), 30% (×).

Figure

(a)

(b)

(c)

Fig. 5. Effect of *t*-butanol on methanolysis of canola oil. a) SBA-RML, b) SBA-TLL, c) SBA-CALB. Reactions were performed at 50 °C with 0.65 g of oil and 25 mg immobilized enzyme. Methanol to oil molar ratio: 3, methanol was added three times at 0, 24, and 48 h. The conversion to biodiesel was measured quantitatively by GC. wt% of *t*-butanol in the reaction mixture: 0% (◆), 10% (■), 20% (▲), 30% (×), 40% (*), 50% (●), 60% (+).

Figure

(a)

(b)

(c)

Fig. 6. Effect of water adsorbent quantity on methanolysis of canola oil. a) SBA-RML, b) SBA-TLL, c) SBA-CALB. Reactions were performed at 50 °C with 0.65 g of oil and 25 mg immobilized enzyme. Methanol to oil molar ratio: 3, methanol was added three times at 0, 24, and 48 h. The conversion to biodiesel was measured quantitatively by GC. The amount of blue silica gel was 0% (×), 30% (◆), 40% (■), 50% (▲).