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Activity Performance and Stability of Functionalized SBA -15 Mesoporous Silica for Lipase Immobilization

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Activity Performance and Stability of Functionalized SBA -15 Mesoporous Silica for Lipase Immobilization

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Abstract

SBA -15 Mesoporous Silica was synthesized and functionalized with aminopropyl-, phenyl- and chloropropyl- using post-synthesis method. The esterification of citronellol with lauric acid in batch mode at 37 °C was used to measure the performance of the immobilized enzymes. Characterization of the supports revealed the satisfactory development of mesoporosity with uniform internal channels distribution. Pure SBA-15 showed the typical hexagonal pore and straight channels while the functionalized SBA-15 with triethoxysilane caused pore size to decrease and the distortion of internal channels occurred to subsequently improved the degree of immobilization. The best support among the four was the 3-aminotriethoxysilane functionalized SBA-15 that showed about 77 % conversion after 24 h. The stability of lipase immobilized on 3-aminotriethoxysilane functionalized mesoporous SBA-15 was also investigated. It was found that the immobilized enzyme was stable with minimal leaching. Repetition of reaction using the same immobilized enzyme also showed promising result with nearly 60 % acid conversion after 3 cycles.

Keywords: Activity, stability, lipase, functionalized mesoporous silica.

Introduction:

All processes in living organisms require some form of enzyme to cause the reactions to occur at a rate sufficient to support life. One example of enzyme is Candida rugosa lipase, which is the enzyme that catalyzes esterification reaction. Lipase (triacylglycerol acylhydrolases) is an important enzyme with a broad variety of industrial applications due to the multiplicity of reactions [2, 3, 8, 9, 10, 12, 13]. Due to their unique structural characteristics, lipases can catalyze reactions including organic substrates at the interface of organic and aqueous phases and can preserve their catalytic activity in organic solvents, biphasic systems and in micellar solutions. Even though enzymes proved to be very useful catalysts for industrial applications, their usage is currently limited by their sensitivity to temperature, stability, high cost and the difficulty in recovering active enzyme for reuse [6]. From all the factors, the difficulty to recover the active enzyme appears to be the main factor that influences the cost and efficiency of a catalyst in any reaction. As the cost of catalyst is quite high, the inability to reuse the enzyme will contribute to the production cost. This proves to be a very big obstacle for the industries and the current focus is on the approaches to solve this problem.

In order to overcome the inability to recover reusable active enzymes, many researchers developed many approaches to immobilize enzyme, such as sol-gel encapsulation, crossing-linking crystals and protein polymer conjugates [2, 8, 9, 10, 12, 13]. Mesoporous materials (MPSs) have shown to be more exciting candidates in bioimmobilization compared with conventional materials because of their uniform and adjustable pore size, large surface area, pore volume and opened pore structures. Mesoporous materials have important applications in a wide variety of fields such as separation, catalysis and adsorption. One of them is the immobilization of enzyme in mesoporous silica to catalyze biochemical reaction. The immobilization of enzyme enables continuous activity of enzyme which can be recovered and reused.

Santa Barbara Amorphous (SBA-15) possess highly ordered hexagonal array of channels, which involved the use of a coblock polymeric surfactant as template [2]. They have pore size raging between 20 and 300 Å and possess large BET (Brunauer–Emmett–Teller) surface area (>700 m^2/g) with large pore wall thickness. The large wall thickness results in higher hydrothermal stability than M41S (Mesoporous materials). SBA-15 has proved to be very promising for the size selective separation of large biomolecules, because pore diameters are in the range required for these separations, and the silica framework is well suited for the development of bonded and selective sorption phases. Functional groups like amines, chlorides and phenyl may be added and attached to the surface of mesoporous molecular sieves via tethering alkyl chains. These groups subsequently provide different interactions between the surface of the support and the enzyme molecule. By examining the functional groups on the surface of an enzyme molecule, a

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suitable 'counter-functional group' on the surface of the support could provide strong interaction for immobilization [2, 8, 9, 10, 12, 13].

The aim of this work is to investigate the activity and stability of immobilized lipase in different functionalized mesoporous SBA-15.

Experimental:

Synthesis of SBA-15:

Pluronic P123 triblock copolymer EO_{20}-PO_{70}-EO_{20}, BASF (code used by chemical company) was used as surfactant template. The molar composition of the gel was 1 SiO_2: 0.017 P123: 2.9 HCl: 202.6 H_20. The surfactant solution first was dissolved in distilled water and followed by the addition of HCl (35% in water) at 40°C. Tetraethylorthosilicate (TEOS) was added as the silica source and stirred for 24 hours. The mixture was then transferred to glass bottle and heated at 100°C for 24 h. The white precipitate was then filtered, washed with distillated water and air dried. Then, the dry precipitate was calcined at 550°C for 4 h in a using furnace.

Functionalization of SBA-15:

Three functional agents used to functionalize the SBA-15 were phenyltriethoxysilane $(EtO)_3Si$-Ph, 3-aminopropyltriethoxysilane $(EtO)_3Si$-$PrNH_2$ and 3-chloro-propyl-tri-etho-xysilane $(EtO)_3Si$-PrCl. The functionalization was done post-synthesis. Calcined SBA-15 (2 g) was suspended in 40 ml of toluene. Then, 10 ml of triethoxysilane with desired functional group was added and the reaction was heated to reflux for 8 h. The white solid was filtered off, washed with toluene and dried under vacuum.

Immobilization of Enzymes:

Direct immobilization was used to immobilize the *Candida rugosa* lipase. Lipase (20 mg) and pH 7.5 phosphate buffer (10 ml) were added to 2 g of functionalized or unfunctionalized SBA-15 support. The mixture was then stirred at 37°C for 24 h. The supernatant was then separated from the solid material by filtration and the BCA (bicinchoninic acid assay) kit testing was performed to determine the amount of enzyme immobilized. The SBA-15 immobilized lipase was then washed with phosphate buffer (pH=7.5) solution. The wet SBA-15 was then kept in vacuum oven overnight for complete drying.

Assay of Enzyme Activity:

30 mM (millimole) of citronellol and 20 mM of lauric acid were dissolved into 10 ml solution and added to 0.6 g of immobilized SBA-15 and stirred for 24 h at 37°C. After that, the sample (1 ml) was quenched with 1 ml of quenching reagent (50% acetone and 50% ethanol) to stop the reaction. Then the solution was titrated with 0.2 mole/liter of NaOH and the volume of NaOH consumed was recorded. The activity of the enzyme was calculated in the standard way.

Results and discussion:

Activity of esterification reaction:

The activity of esterification reaction was investigated by using different types of support with different enzyme loadings. The four different supports, i.e. pure SBA-15, SBA-15 functionalized with phenyltriethoxysilane, 3-aminopropyltriethoxysilane and 3-chloro-propyl-triethoxysilane were immobilized with *Candida rugosa* lipase and was used as catalyst for the esterification reaction. After that, different loadings (5 g/L, 20 g/L and 60 g/L) were used to catalyze the reaction to test the activity of the immobilized enzyme. The amount of enzymes immobilized for each support is shown in Table 1. It is noted that the amounts of enzymes immobilized onto each support were different. Phenyltriethoxysilane and 3-chloropropyltriethoxysilane functionalized SBA-15 showed higher percentage of immobilization with 74.2% and 74.9%, respectively compared to pure SBA-15 or 3-aminopropyltriethoxysilane functionalized SBA-15. Pure SBA-15 had the lowest percentage of absorption with only 62.1% while 3-aminopropyltriethoxysilane functionalized SBA-15 had 68.4%. Pure SBA-15 had weaker interaction with enzyme compared to the functionalized SBA-15 because of larger pore size and only Si-OH bonds in pure SBA-15 were involved in the immobilization. On the other hand, chemical bonds were involved in functionalized supports and hence stronger interaction with the enzymes resulted. Chloro and amine groups are thought to be able to react with the amine groups available on the surface of the enzyme structure. For phenyl group, the large surface area was thought to result in better enzyme immobilization compared to other materials [5].

Table 1: Percentage of enzyme immobilized

Support	Enzyme Immobilized (%)
SBA-15	62.14
Amino	68.39
Phenyl	74.22
Chloro	74.93

Enzyme support is an important factor in enzyme immobilization for the esterification reaction. The ideal support should allow the effective utilization of the enzyme by having the enzyme molecules accessible to the substrates [1]. The result obtained from the activity of esterification is shown in Figure 1. It is obvious that SBA-15 functionalized with 3-aminopropyltriethoxysilane had the highest acid conversion compared to the other three supports. All four types of support showed an increasing trend of conversion with increasing amount of immobilized enzyme. Pure SBA-15 also proved to be a promising enzyme support with nearly 78% acid conversion at 60 g/L loading. For phenyltriethoxysilane functionalized SBA-15, it showed a very high conversion even at 5 g/L loading but the conversion remained constant at 20 g/L with no significant difference between 20 g/L and 60 g/L loading. For 3-chloropropyltriethoxylsilane, it had a very low conversion at low loading of enzyme. At 60 g/L, the acid conversion was higher than phenyltriethoxysilane functionalized SBA-15 but lower than pure SBA-15. It is clearly noted that 3-aminopropyltriethoxysilane was the best support among the four different types of support.

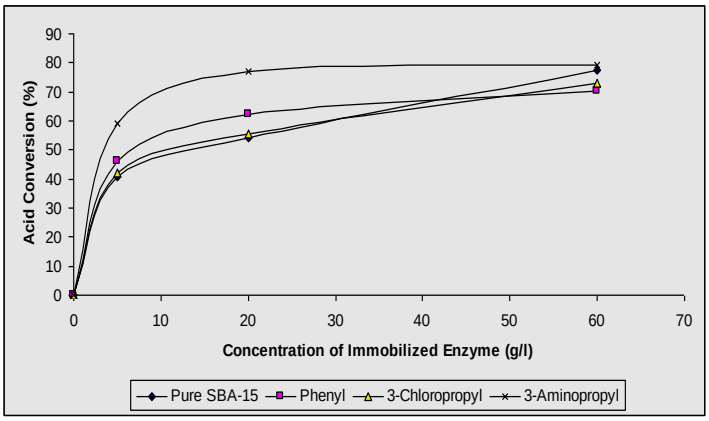

Figure 1: Effect of different types of support for lipase immobilization for the enzymatic synthesis of citronellyl laurate in iso-octane.

From Table 1, 3-aminopropyltriethoxysilane has the lowest percentage of enzyme immobilized compared to other supports. However, the activity is relatively the highest among them all. This was due to small pore diameter support of the SBA-15 (4-5 nm) which is measured using Autosorb1, Quantachrome Autosorb Automated Gas Sorption System supplied by Quantachrome. The small pore size but with high loading of enzymes could inhibit substrate and product diffusion and subsequently reduced specific activities [5].

Solid support used in physical adsorption method is mainly classified as three types: hydrophilic, hydrophobic, and amphiphilic supports. Generally, a hydrophilic support is preferred for lipase immobilization since lipases are spontaneously soluble in aqueous solutions. Such a support not only maximizes available area for lipase attachment and improves the efficient immobilization of lipase, but also keeps the essential water layer that surrounds the biocatalysts and prevents the impairment of catalytic activity. However, in the case of catalytic reaction, the substrate catalyzed by lipase is commonly insoluble. Hence, the support with strong hydrophilicity unavoidably decreases the accessibility of substrate to the immobilized

lipase, thus decreasing the lipase activity. As for conclusion, an amphiphilic support with both hydrophobic and hydrophilic characteristics is needed [7]. SBA-15 functionalized with triethoxysilane groups created an amphiphilic condition suitable for the immobilization and subsequently esterification reaction between citronellol and lauric acid. The post synthesis grafting with organic moieties like triethoxysilane groups on SBA-15 created a hydrophobic condition suitable for the substrates [7]. This result is in accordance with other findings that reported that functionalization increased the interaction between support and immobilized enzymes. It is thought that the amine groups available on the surface of Candida rugosa lipase interacted complementally with the amine groups available in the triethoxysilane groups which enhanced the interaction level compare to other functional groups like phenyl or chloro groups which could inhibit the reaction [4]. Hence, 3-aminopropyltriethoxysilane functionalized SBA-15 was used for further investigation purposes as it was the best among the four types of support.

Stability of Immobilized Enzyme:

The stability of immobilized enzymes was investigated in two aspects i.e. the leaching test and repetition of reaction. In leaching test, the immobilized enzyme was subjected to centrifugation at 2000 rpm and was tested for any leaching phenomenon. In the repetition of reaction, immobilized enzymes were used as catalyst for repeated reactions to test the activity of the immobilized enzymes.

Leaching Test:

The amount of enzyme leached out from the support was tested by using Pierce BCA (bicinchoninic acid) protein assay kit. From known amount of free enzymes, one can get the amount of lipase immobilized by subtracting the amount leached out from the free amount of lipase. In this test, the stability of the immobilized enzymes on the support was investigated by subjecting the immobilized enzymes to a very high degree of centrifugation at 2000 rpm. Supernatant collected was then tested by using Pierce BCA kit to determine the amount of protein leached out. Figure 2 shows the result of leaching test conducted by using 3-aminopropyltriethoxysilane functionalized SBA-15, the best support among the four types of materials. It was observed that protein leached out from the support in the first 10 minutes was the highest and the amount decreased with time until 40 minutes after centrifugation. This showed that leaching process only took place in the first 10 minutes and although there was some leaching from the support, 3-aminotriethoxysilane functionalized SBA-15 still able to retain a significant amount of enzymes for reaction purposes. Figure 3 shows the percentage of leaching in the conducted test. It is noted that the percentage of leaching is gradually decreasing until the amount became nearly constant. This trend showed that leaching only occurred significantly in the first 10 minutes of centrifugation. After that, no significant leaching of immobilized enzyme occurred. The support could retain the immobilized enzymes even though it was centrifuged at 2000 rpm through strong chemical interactions between the enzyme molecule and support. It could be concluded that leaching was not a problem for 3-aminopropyltriethoxysilane functionalized SBA-15 and it had the potential to be used as a support for enzyme immobilization.

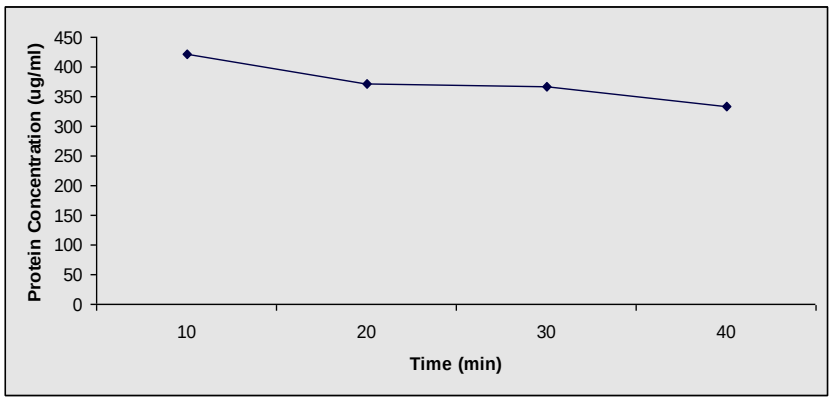

Figure 2: Stability of immobilized enzyme in leaching test upon centrifugation at 2000rpm.

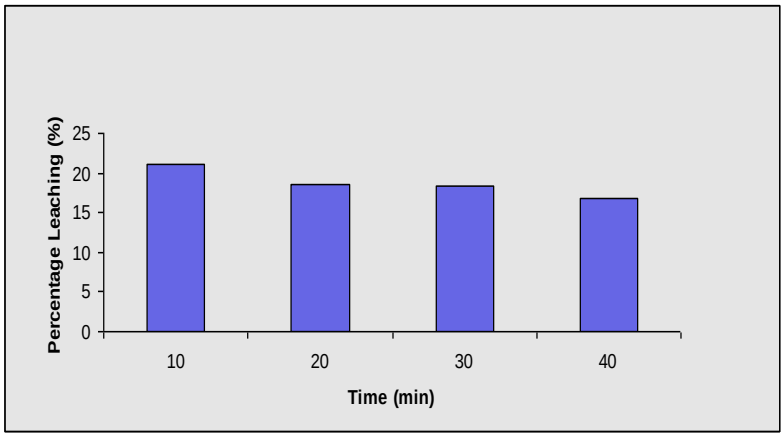

Figure 3: Percentage of leaching in leaching test.

Recovery and Reusability Test:
In this test, the immobilized enzyme was used as catalyst for the esterification reaction and recovered repeatedly. This test shows the feasibility of the support as an ideal catalyst where repeated usage is a must for economic purposes. Figure 4 shows the result obtained from the test by using 3-aminotriethoxysilane functionalized SBA-15. It is obvious that the activity of the immobilized enzyme decreased as the number of reuse increased. This was due to the leaching phenomenon and also the decrease in the amount of active immobilized enzymes. Leaching normally occur due to the weak bonding of support and enzyme after a few repetition reactions. On the other hand, the amount of active enzymes also decreased with time as the enzymes undergo conformational change. Consequently, the enzymes could not catalyze the esterification reaction anymore [7]. The acid conversion dropped from 80% for new immobilized enzyme to 65% acid conversion after three times of reused. The conversion can be considered high after few times of reuse compared to the other supports like polymer where leaching occurs and causes the activity to drop drastically [11].

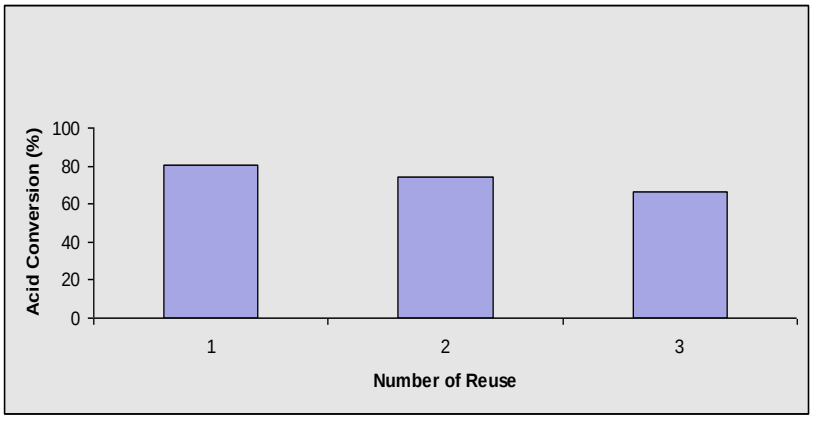

Figure 4: Stability of immobilized enzyme on aminotriethoxysilane functionalized SBA-15 in repetition reaction

Conclusion:
Immobilization of *Candida rugosa* lipase on various SBA-15 supports was performed using physical and chemical adsorptions. The immobilized enzymes were then used for the synthesis of citronellyl laurate ester in batch system using lauric acid and citronellol as the reactants. The best support among the four was the 3-aminotriethoxysilane functionalized SBA-15 which showed a very high activity with minimal leaching. The acid conversion using this support was the highest with approximately 77 %

conversion after 24 h of reaction.

The stability of the immobilized lipase in 3-aminotriethoxysilane functionalized SBA-15 was also investigated. It was found that the immobilized enzyme was stable with minimal leaching phenomenon. Repetition reaction using the same immobilized enzyme also showed promising result with nearly 60 % acid conversion after three times of usage.

Characterization of the supports also revealed the internal channel distribution and pore size characteristics. Pure SBA-15 showed the typical hexagonal pore and straight channels while the functionalized SBA-15 with triethoxysilane caused pore size to decrease and the distortion of internal channels was visible to subsequently improve the degree of immobilization.

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فعالية و استقرار السليكات الوظيفية متوسطة المسامية لتثبيط الليباز

أحمد مبارك السباعي

الملخص

تم تحضير السليكات متوسطة المسامية كحامل للمجموعات الوظيفية من أمينو بروبيل فينيل و كلوروبروبيل باستخدام طريقة التحضير المتقدمة. وقد تم قياس أداء الأنزيمات المثبطة باستخدام عملية أسترة السترونيلول مع حمض اللوريك في مفاعل متقطع عند رجة حرارة 370 م. وبينت دراسة الخواص التركيبية تطوير المسامات بشكل جيد مع توزيع منتظم للقنوات الداخلية. حيث أظهرت السليكا النقية مسامات سداسية وقنوات مستقيمة بينما بعد إضافة المجموعات الوظيفية انخفض حجم المسامات وتغيرت القنوات الداخلية مما أدى إلى تحسين المواصفات. وقد أظهرت النتائج أن أفضل حامل هو ثلاثي أمينوترثوكسيسلان وذلك من خلال الحصول على أعلى نسبة تحويل تقدر ب 77 % بعد مرور 24 ساعة. كما تمت دراسة مدى استقرار تثبيط الليباز على ثلاثي أمينوتريثوكسيسلان وقد أظهرت النتائج استقرارا عاليا مع الحد الادنى من الرشح. وبتكرار التفاعل باستخدام الانزيم نفسه فقد أظهرت نتائج جيدة مع نسبة تحويل للحمض تقارب 60 % بعد ثلاث دورات.

الكلمات المفتاحة: النشاط، استقرار ، الليباز ، السليكات الوظيفية المتوسطة المسامية.