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Immobilization of lipase onto mesoporous silica KIT-6 and montmorillonite K10 for enzymatic hydrolysis of tributyrin

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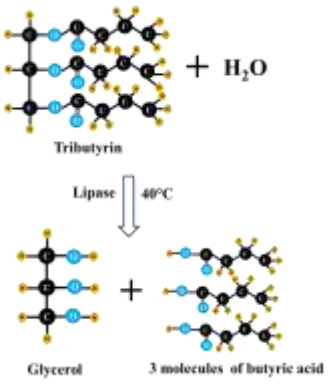
GRAPHICAL ABSTRACT



ABSTRACT

Mesoporous silica KIT-6 and montmorillonite (MMT) K-10 clay were prepared and used for immobilization of the enzyme, *Candida rugosa* lipases (CRL), aiming at their use as biocatalysts for the hydrolysis of tributyrin. Immobilization of the enzymes onto the supports was performed by physical adsorption using 0.1 M phosphate buffer solutions (pH 7) as the dispersion medium. The activity of the immobilized CRL for tributyrin hydrolysis was investigated at incubation temperature of 40 °C during 120 min and different concentration of the lipase solution for both the supports. Characterization by XRD showed that the long-range ordering in the KIT-6 and crystallinity of the MMT K-10 materials were affected slightly by the lipase immobilization. These results give an indication to the presence of lipase-support interaction in the immobilized lipase systems. Additionally, the results of FTIR spectroscopy verified the presence of silanols on the surfaces of MMT K-10 and KIT-6 materials. Nitrogen adsorption data showed the resulting immobilized enzyme catalysts were rendered porous, with the KIT-6 giving significantly higher specific surface areas than the MMT K-10. The immobilization of CRL on KIT-6 and MMT K-10 through hydrogen bonding with the silanol groups, led to an increase in the hydrolysis activity compared to that of free lipase. However, the activity of KIT-6 immobilized CRL was higher than was observed on MMT K-10 immobilized CRL. Furthermore, lipase immobilized on mesoporous silica KIT-6 was shown to be recyclable up to 5 times in aqueous medium. The high surface area and large pore diameter of the mesoporous silica KIT-6 may be the crucial factors that play an important role in retaining the enzyme in the support, and consequently, improving the lipase activity and stability.

Keywords: mesoporous silica KIT-6, montmorillonite K-10, immobilization, *Candida rugosa* lipase, hydrolysis.

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1. INTRODUCTION

Candida rugosa lipase (CRL), a mesophilic lipase having high activity and broad specificity in the reaction medium, is one of the enzymes most frequently employed in biotechnology, with application in food and flavouring, fine chemicals, pharmaceutical and cosmeceutical industries [1-3]. Lipases have been widely found in biological systems, where they catalyze the hydrolysis of triacylglycerols to glycerol and fatty acids in aqueous media [4]. In the presence of enzymes as biocatalysts, the chemical reactions such as hydrolysis, esterification, transesterification, and aminolysis were possible, where the lipases showed high specificity and catalytic activity [5-7]. However, the difficulties in their recovery from the reaction systems, poor recyclability and low long-term operational stability, impose limitations on the commercial uses of lipases. Nevertheless, these inherent properties of enzymes may be overcome through immobilization of the enzymes, structure modification and the use of additives [8, 9].

Enzyme immobilization is the most commonly used strategy to impart the desirable features of conventional heterogeneous catalysts onto biological catalysts. Immobilized enzymes are often more stable and easier to recover than free enzyme in solution, enabling the reuse of the immobilized enzyme without a significant loss in biological activity [10]. Furthermore, they showed superior activity compared to the free enzyme, including greater thermal and pH stability [11-13].

The synthesis of stable, enzyme supported materials represents a significant practical fundamental challenge. There are several methods available for immobilization of enzymes on various supports, including adsorption, ionic bonding, covalent binding, cross-linking, entrapment, and encapsulation [14, 15]. Binding of the enzyme to a support through physical adsorption is the most simple

immobilization method and therefore also frequently used in large-scale production [16].

Promising materials as supports for enzymes are inorganic materials (silica, clay, alumina) [17-19], inorganic salt (calcium carbonate; $CaCO_3$, calcium sulfate; $CaSO_4$) [20,21], ion-exchange resin (Amberlite, Dowex), natural resin (colophony) [22], natural biopolymer (sodium alginate) [23], synthetic polymer (polypropylene, polyethylene) and zeolites [24]. Aluminosilicate clay such as montmorillonite K-10 and silicate mesoporous materials such as MCM-41, SBA-15, KIT-6, have potentially interesting properties such as hydrophobic and hydrophilic behavior, electrostatic interactions as well as mechanical, chemical and bacterial resistance [25-29]. Such materials are in high demand in traditional areas, e.g. catalysis, adsorption/separation, and ion-exchange, and the more specialized fields, e.g. biotechnology. Furthermore, mesoporous silicate materials, including KIT-6, have the advantages over traditional support materials by providing a high surface area, minimum diffusion limitation and high mass transfer [30]. In addition, the well-ordered and tunable pores make them suitable as hosts for enzymes [31]. These materials also have abundant surface silanol groups which are important for the surface functionalization of enzymes; hence, a key feature that allows for the adsorption of molecules through hydrogen bonding and/or electrostatic interaction.

Montmorillonite (MMT), a naturally occurring 2:1 phyllosilicate, is the most common member of the smectite clay family. It consists of negatively charged, crystalline aluminosilicate platelets with an average platelet thickness of 1 nm, and length and width of up to 1 μm. The MMT crystal lattice is composed of an octahedral alumina layer fused between two tetrahedral silica layers. Swelling phyllosilicates, especially the MMT K-10, constitute a naturally occurring class of inorganic catalysts and have been widely used as a catalyst support [32]. Immobilization of lipases on robust, solid supports is one way to improve their stability and activity, and can be reused for large scale applications with cost effective and high loading capacity.

In the present work, the lipase from Candida regusa (CRL) was immobilized in montmorillonite (MMT) K-10 and mesoporous materials (KIT-6) through physical adsorption, and these two materials were compared with regard to their catalytic properties in the hydrolysis of tributyrin. Tributyrin is a triglyceride naturally present in butter. It is an ester composed of butyric acid and glycerol. Triglyceride is the main component of natural oil or fat, and stepwise converted into diacylglycerol, monoacylglycerol and glycerol by hydrolysis accompanied with the liberation of a fatty acid at each step [33]. Glycerol and fatty acid are widely used as raw materials in the food, cosmetic and pharmaceutical industries [34].

At present, the Colgate-Emery method has been industrially used for the hydrolysis of triglycerides [35]. This process utilizes steam of high-temperature (523 K) and high-pressure (5.00×10^6 Pa), resulting in high energy consumption and thermal damage of the products. A hydrolysis method using lipase as a biocatalyst instead of the Colgate-Emery method has been reported before [36,37].

The enzymatic hydrolysis using lipase may be carried out in mild conditions; at room temperature or typically at 35°C and atmospheric pressure [38-41]. The enzymes have substrate and positional specificities [42-47], so that the side reactions such as saponification, polymerization and oxidation are prevented to enhance the yield of the desired product. For optimization of the reaction conditions, the influence of important parameters, namely, enzyme concentration, type of support and the reusability of immobilized enzymes, with regard to the immobilization efficiency and hydrolytic activity were investigated.

2. EXPERIMENTS

2.1 Chemicals and Materials

All chemicals used in this study were of analytical grades, unless otherwise stated. All the chemicals were used directly as received without further purification Candida rugosa lipase Type IV containing ~1176 U/mg lipase, poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol); PEG-PPG-PEG (Pluronic® P123), tetraethyl orthosilicate; TEOS, 1-butanol, 99.8%, montmorillonite K-10 (MMT K-10), bovine serum albumin; BSA, were obtained from Sigma–Aldrich (St. Louis, USA). Tributyrin; 98%, was obtained from Acros Organic (USA), while sodium hydroxide pellets and Bradford reagent were purchased from Merck (Darmstadt, Germany). Hydrochloric acid, 37 % and ethanol, 96 %, were purchased and used without additional purification process. Deionized water from MiliQ was obtain from a water purification system and use throughout this experiments.

2.2. Method

Synthesis of KIT-6

In a typical synthesis, approximately 6.0 g of Pluronic® P123 was dissolved in 217 mL of distilled water and 11.8 g of concentrated HCl (37%), and then added to 6.0 g of 1-butanol under constant stirring at 35 °C. After one hour stirring, 12.9 g of TEOS was added dropwise, and the mixture was continuously stirred at 35 °C for 24 h for KIT-6 silica phase formation. The final solution was transferred to a Teflon bottle and heated at static conditions at 130 °C for 24 h. The product obtained after hydrothermal treatment was filtered and air-dried at 100 °C without washing. Afterward, the product was washed with an HCl–ethanol mixture for three times to remove the template and was subsequently subjected to calcination at 550 °C.

Treatment of Montmorillonite K-10

Prior to use, MMT K-10 was calcined for 2 h at 550 °C to remove the impurities and water.

Immobilization of Lipase

Each of the Candida rugosa lipase (10 mg, 25 mg and 50 mg) was dissolved in 5 mL 0.1 M phosphate buffer solutions (pH 7), respectively, and centrifuged for 15 min to remove insoluble impurities. Prior to use, MMT K-10 was calcined during 2 h at 550 °C to remove impurities and water. Then, approximately 50 mg of KIT-6 and MMT-K10 was dispersed in 5 mL of (2, 5 and 10 mg/mL) of lipase solution. The immobilization of lipase was carried out by continuous stirring of the mixtures for 2 h at room temperature. The immobilized lipase samples were separated by centrifugation and washed with deionized water to remove the unabsorbed soluble enzyme. Both precipitate and supernatant were collected for further experiments. The samples were dried under vacuum at room temperature and the supernatant underwent UV illumination to determine protein assay.

In this study, lipase immobilized on KIT-6 and MMT K-10 were denoted as KIT-6/crl and MMT K-10/crl respectively. Then, for the immobilized lipase on KIT-6 and MMT K-10 with different enzyme concentrations, the series of prepared samples were denoted as KIT-6/crl and MMT K-10/crl respectively; the lipase concentration was given in the parenthesis, namely, KIT-6/crl(2); KIT-6/crl(5) KIT-6/crl(10); and MMT K-10/crl(2); MMT K-10/crl(5) and MMT K-10/crl(10).

Protein Assay

The amount of protein loading and leakage of the samples were determined according to the Bradford assay method [48] using BSA as the standard. The amount of bound protein was determined by means of UV-Visible spectroscopy using the wavelength programmed at 592 nm. Thus, the percentage of immobilization (%) is given as follows:

$$\% \text{ immobilization} = (C_i - C_f)/C_i \text{ (mg/mL)} \times 100$$

where C_i is the total amount of protein in supernatant before immobilization (mg/mL); C_f is the total amount of protein in supernatant after immobilization (mg/mL).

The immobilization efficiency of the immobilized enzyme on support, η, was estimated using the following relation, where (E_0) and (E_f) is the hydrolytic activity of the lipase solution before and after immobilization respectively, the hydrolytic activity is given in (U/mL) and the corresponding volumes (V_0 and V_f), given in mL.

$$\eta = [(E_0V_0 - E_fV_f)/V_0E_0] \times 100$$

Hydrolysis Assay

The hydrolysis activity of tributyrin was measured by the titration method at 40 °C and the pH 7.0 was maintained using 0.1 M sodium hydroxide solution (NaOH). The 18 mL phosphate buffer (0.10 M, pH 7.0) was incubated in a thermostated vessel at 25 °C and stirred constantly. Then, 2.0 mL of tributyrin was added and the pH was stabilized at pH 7.0. When the pH stabilizes, 5 mg of catalyst was added and the consumption of NaOH was determined. One unit (U) of hydrolytic activity is defined as 1 µmol butyric acid released per min per mg lipase [49], according to the following relation,

$$\text{Hydrolytic activity} = (V \times M \times 100)/ (E \times t)$$

where, V is the difference in volume in mL of NaOH between the blank and samples after time t (period of incubation in min), which is a measure of the butyric acid release due to hydrolysis, M is the molarity of NaOH and E is the amount of the enzyme employed in mg.

Leaching analysis

The leaching test was performed by dispersing 50 mg of silica and clay supports to 5 mL lipase (5 mg/mL) in 0.1 M phosphate buffer solution at pH 7 in a covered vessel to prevent evaporation. The mixture was stirred at 40 °C for 120 min. Then, the leaching of the enzymes from the supports, mesoporous silica KIT-6 and MMT K-10 clay was measured by the lipase content of the supernatant at different time intervals using the Bradford assay method.

Reusability Procedure

Approximately 50 mg of each sample was undergoing hydrolysis reaction. After the first reaction, the remaining precipitates in the solution were collected by centrifugation and washed with distilled water, then dried at room temperature in the desiccator. After that, the solid catalyst was used for other reactions consecutively. In this study the data were collected up to 5 cycles.

Characterization

Specific surface areas were calculated by the single point Brunauer–Emmett–Teller (BET) method. Fourier transform infrared spectroscopy (FTIR) was performed on a Perkin Elmer Spectrum One spectrometer with KBr pellet technique, in the wavelength range from 4000-400 cm^{-1}. XRD was performed using a Bruker D8 Advance X-ray diffractometer operated at 40 kV voltage and 40 mA current, using Cu Kα radiation (λ = 1.5406 Å). UV–visible spectroscopy was performed on a Lambda 25 Perkin-Elmer spectrometer, with wavelength program detection at 592 nm.

3. RESULTS AND DISCUSSION

3.1 Immobilization of Lipase on Support

Information on lipase molecules bound to the support surface with different lipase loading was obtained by FTIR spectroscopy and the spectra are depicted in Fig. 1 and Fig. 2. From the spectra obtained, the lipase displays a typical spectrum of the proteins, with the absorption bands associated with their characteristic amide I group (1500–1700 cm^{-1}), with COO^- (1650 cm^{-1}) and NH_2^+ (1550 cm-1) in both samples of MMT K-10 and KIT-6. The presence of these two peaks was used as markers for the presence of the enzyme on the support surface. Besides, the peak at 2900 cm^{-1} was due to C–H stretches that originated from the lipase and became more intense as the concentration of lipase increased. Table 1 presents the area ratios of amide I as compared to Si–O–Si peaks in both supports.

Table 1 Area ratio of an amide group (CONH) to (Si–O–Si) band

Lipase concentration (mg/mL)	$A_{CONH}/A_{Si\text{-}O\text{-}Si}$ (MMT K-10)	$A_{CONH}/A_{Si\text{-}O\text{-}Si}$ (KIT-6)
2	0.6977	0.3916
5	0.7582	1.0726
10	0.7634	1.2562

Furthermore, the FTIR spectra of all the MMT K-10 samples show general similarity in the 4000–1500cm^{-1} region, as seen in Fig. 1 (b - e). The main absorption bands in MMT K-10 are in the regions 4000–3000 cm-1 and 1200–400 cm^{-1}. The band at 3638 cm^{-1} is attributed to the stretching vibration of OH groups bonded to A1 or Mg.

The bands in the region 1200–400 cm^{-1} give more information about the structural characteristics of clay minerals and are attributed to the lattice vibration [50, 51]. Bands at 1037, 578, and 620 cm^{-1} were originated from the clay lattice Si–O stretching, Al–O–Si vibrations and coupled A1–O and Si-O out of plane vibrations. Besides, the asymmetric and symmetric vibration peaks of the tetrahedral Si-O-Si linkages in the KIT-6 silicate framework were exhibited at around 1200 cm^{-1}, 1080 cm^{-1}, 804 cm^{-1} and 471 cm^{-1}.

As the support materials are predominantly silica based materials, so all the samples show the typical Si–O stretching band in the FTIR spectra. Then, generally, the band at around 972 cm^{-1} is assigned to Si–OH stretching vibration in all the samples. Additionally, the vibrational band at around 3400 cm^{-1} is typical of the stretching frequency of free O–H bond as shown in all the spectra.

Based on the results of the BET analysis (Table 2), the MMT K-10 showed the highest percentage decrease of surface area after adsorption of lipase, i.e., up to 99%, as compared to KIT-6; 67%. The marked decrease in surface area suggests that the lipase molecules could be intercalated in the clay minerals [52, 53], or could be due to the layered structure of MMT K-10 which has been collapsed during the immobilization process. However, MMT K-10 seemed to be more applicable for enzyme immobilization compared to other clay minerals because the inter-lamellar region of montmorillonite is constituted by pillars, which can reduce the capacity of cationic exchange. This factor can explain the lowest yields of immobilization obtained with this support, in spite of the higher accessibility of the enzyme to the support [54]. Otherwise, the mesoporous silica KIT-6 could be considered as a promising candidate for the lipase immobilization within its surface. The KIT-6 has a large surface area with porous and tortuosity structure.

Table 2 BET Surface area data of all the samples

Support Material	BET Surface Area (m^2/g)	Surface area decrease (%)
KIT-6	827.2	-
KIT-6/crl (2)	452.9	45.2
KIT-6/crl (5)	304.3	63.2
KIT-6/crl (10)	277.5	66.5
MMT K-10	253.4	-
MMT K-10/crl (2)	3.65	98.5
MMT K-10/crl (5)	3.43	98.6
MMT K-10/crl (10)	3.28	98.7

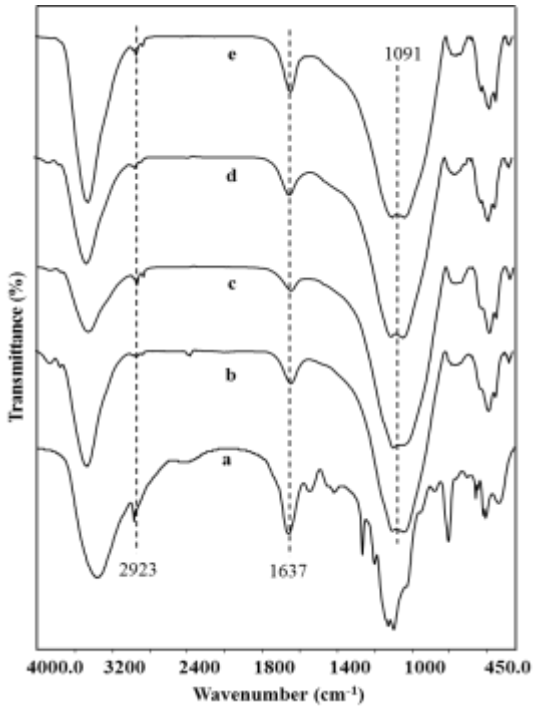

Fig. 1 FTIR Spectra of (a) free lipase (b) MMT K-10, (c) MMT K-10/crl(2), (d) MMT K-10/crl(5), (e) MMT K-10/crl(10)

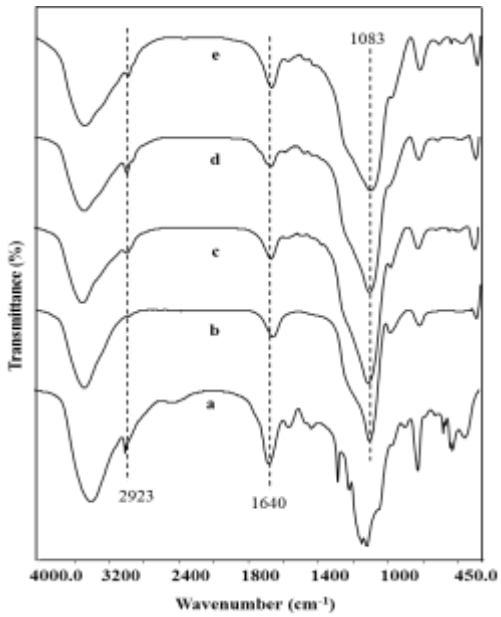

Fig. 2 FTIR Spectra of (a) free lipase, (b) KIT-6, (c) KIT-6/crl(2), (d) KIT-6/crl(5) and (e) KIT-6/crl(10)

Besides, the relative crystallinities of the various MMT K-10 and KIT-6 materials were compared using the XRD patterns of MMT K-10 shown in Fig. 3 while those of KIT-6, shown in Fig. 4. Also, the d-001 spacing values for these samples are given in Table 3.

Table 3 XRD data of MMT K-10 and KIT-6 materials with different lipase loading

Catalyst	2θ(degree)	d- 001(nm)
MMT K-10	8.826	1.011 nm
MMT K-10/crl(2)	9.024	0.998 nm
MMT K-10/crl(5)	9.052	0.983 nm
MMT K-10/crl(10)	9.032	0.985 nm
		d-211 (nm)
KIT-6	0.950	9.431
KIT-6/crl(2)	0.987	8.835
KIT-6/crl(5)	0.976	8.819
KIT-6/crl(10)	0.985	8.828

From the XRD patterns (Fig. 3), the high intensity as well as the sharpness of the XRD peaks for the MMT K-10 samples clearly indicate that they are highly the materials. Apart from that, all the samples exhibit similar diffraction patterns, signifying that the MMT lamellar structure has been kept even after the introduction of the enzyme. For the parent MMT K-10, the peak at 2θ = 8.826° corresponds to a d-spacing of 1.011 nm. This peak represents the (001) plane analogous to the inter-layer spacing. After the immobilization of lipase, the intensity of the (001) peak does not change significantly. There is a slight shifting of the peak to a higher 2θ angle, suggesting that very few of the layers are intercalated by the enzymes. As enzyme loading was increased, the intensity of the new peak also increased, confirming the intercalation of the enzyme into the clay layers [55-57].

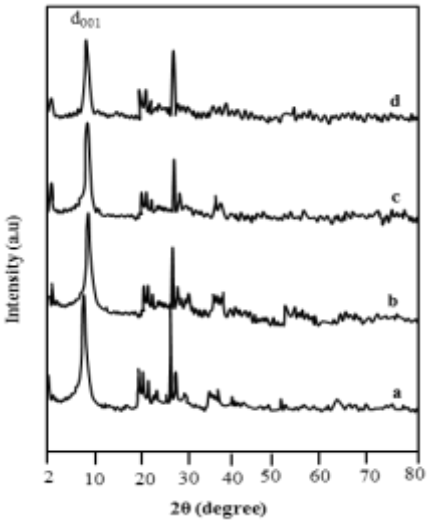

Fig. 3 XRD patterns of (a) MMT K-10, (b) MMT K-10/crl(2), (c) MMT K-10/crl(5), (d) MMT K-10/crl(10)

Then, the XRD patterns of KIT-6 before and after immobilization of lipase are presented in Fig. 4, which clearly show an intense peak due to the (211) reflection, that is consistent with the 3-D cubic symmetry of the space group *Ia3d*. This peak showed evidence of the long range ordering in the KIT-6. The XRD patterns of all the KIT-6 based samples are similar, suggesting that the mesoporous cubic structure is retained even after immobilization of lipase.

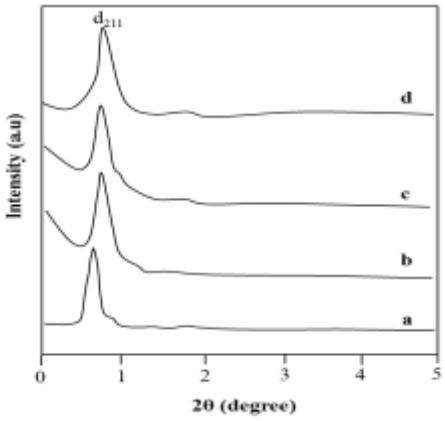

Fig. 4 XRD patterns of (a) KIT-6, (b) KIT-6/crl(2), (c) KIT-6/crl(5), (d) KIT-6/crl(10)

However, this peak was broadened with the increase in the lipase loading, owing to the lipase-support interaction. In addition, the position of the characteristic peak of KIT-6/crl

with different concentration of lipase was slightly shifted to higher 2θ angles. It could happen, only if the immobilization occurred inside the KIT-6 mesopores, as well as on the external surfaces. Filling of the pores of the support materials have already been confirmed by the decrease in surface areas with increasing protein loading, as shown in Table 2.

3.2 Effect of lipase concentration on hydrolytic activity

The hydrolytic activity of the immobilized lipase has been determined by hydrolysis of tributyrin at pH 7.0 and 40 °C using the titrimetric method and the results are depicted in Table 3.

Table 3 Activity and efficiency of lipase immobilization on different support using different lipase concentration

Support Material	Protein Loadings (mg/g)	Hydrolytic activity (U/mg)	Immobilization efficiency (%)
Free lipase	NA	120	NA
KIT-6	NA	8	NA
KIT-6/crl (2)	11.6	35	62
KIT-6/crl (5)	90.2	74	76
KIT-6/crl(10)	119.0	104	89
MMT K-10	NA	5	NA
MMT K-10/crl (2)	5.9	32	43
MMT K-10/crl (5)	11.8	36	54
MMT K-10/crl (10)	14.9	50	67

*NA = Not available

As can be deduced from the results, the lipase activity increased as the lipase loading is increased in both of the MMT K-10 and KIT-6 supports. This suggests that less area was available for the lipase to spread itself with the increase of lipase loading. Accordingly, more of its active conformation is retained and the loss in hydrolytic activity was reduced, in agreement with the previous study [58]. However, at 5 and 10 mg/mL protein loading, respectively, both the supports did not show any significant increase in protein loading as well as hydrolytic activity. This is due to the addition of larger lipase quantities in the small amount of the solution was not practical since the mixing of matrix and lipase together made the solution extremely viscous, so not helping to further increase the hydrolysis yield. Also, it should be noted that at low loadings, there is a large excess of surface area that the lipase can occupy and the lipase attempts to maximize its contact with the surface, which results in a loss of conformation, and consequently in a reduction of activity. Therefore, working in the most suitable concentration range would have a positive effect on the enzyme activity.

Furthermore, the results also signify that surface properties of the support surface play an important role in the lipase activity. It has been established that the area available for protein adsorption and the equilibrium behavior of the lipase/support in the immobilization systems depends on the physical and structural properties of both the support and the lipase [59,60].

In this study, the KIT-6 displayed higher protein loading and the corresponding higher activity towards the hydrolysis of tributyrin compared to MMT K-10. The KIT-6 based samples achieved up to 89% immobilization efficiency with hydrolytic activity of 104 U/mg. The higher loading of lipase on KIT-6 support is due to their characteristics such as having a large surface area, interwoven structure, abundance of silanol group and adjustable pore size in a range suitable for biomolecules such as enzymes. Moreover, the porous structure enables a large quantity of the enzyme to be immobilized compared to a flat surface in MMT K-10. Entrapment in the pores may also protect the enzymes from the surrounding media.

Hence, the amount of lipase immobilized in MMT K-10 is low compared to that found in the mesoporous KIT-6 materials due to the different pore structures, which are layered and porous with gyroid structure, respectively. Free hydroxyl groups present on the surfaces of these supports can form hydrogen bonds with the functional groups of amino acid chains of the lipase, promoting higher efficiency to the immobilization process. The van der Waals forces can also facilitate the adsorption of the enzyme to the surface of the support. Additionally, the hydrophobic interactions of lipase-support molecules can contribute to the adsorption process [61-63]. At this point, it should be noted that hydrophobicity of the support would have an effect on the extent of enzyme binding. Mesoporous silica KIT-6 has higher hydrophobicity compared to MMT K-10; as the result, the immobilization of lipase resulted in higher yields.

During enzyme immobilization, concentration of the enzyme and not the amount, was the critical factor since excess protein was available in all cases. At a too high enzyme concentration, the support material becomes saturated and little additional protein can be adsorbed even at higher concentrations. It was apparent that simply adsorbing a large amount of enzyme on a support material was not sufficient to produce a high activity [64].

3.3 Leaching test

Leakage of the enzyme might occur because the protein is non-covalently linked to the matrix, especially in aqueous medium which would also mean desorption of the lipase from the matrix. Therefore, the possibility that the activity was lost because of the enzyme desorption during the washings was considered and studied. The activity of the supernatant indicated the end point of the immobilization process.

The percentage of the enzyme leaching out from different supports is shown in Fig 5. According to the graph plotted, the MMT K-10 suffers a continuous leaching, without reaching an equilibrium point in the time range studied which is within 2 hours. It is obvious that the leaching out of the immobilized lipase increases with the prolonging of incubation time for MMT K-10. Besides, the mesoporous silica KIT-6 also shows traces of the lipase leached out from the KIT-6 matrix, but this materials exhibit a marked saturation profile, reaching a maximum at short times around 60 to 120 minutes that is not exceeded when they are left longer under suspension.

The leaching of lipase is possibly due to the weak interactions between lipase molecules and the silanol groups of both samples and also because of desorption or detachment from the surface, and the subsequent diffusion out of the support material [65]. Generally, detachment processes are controlled by the strength of the interactions with the surface, while the diffusion process is dependent mainly upon the geometry of the pores.

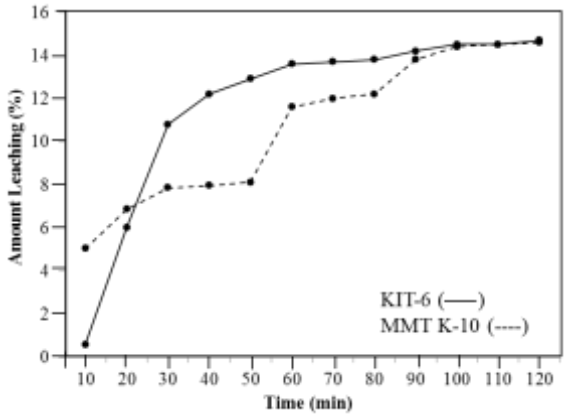

Fig. 5 Graph of percentage of lipase (5 mg/mL) leaching out from KIT-6 and MMT K-10 in aqueous solution (pH 7) at 40 °C

The result shows that although the better connectivity did not result in any improvement of the enzyme content or contact time, the tortuosity inherent to three-dimensional network of silica KIT-6 sample effectively retains the lipase, thus preventing its leaching. Above all, the abundance of interconnecting elbows facilitates the blockage of the pore networks. This effect is contrary to that observed in drug delivery systems, in which the use of three-dimensional networks is known to ease the leaching of the guest molecules [66].

3.4 Reusability of immobilized lipase

The results of repeated usage of immobilized lipases are shown in Fig. 6. The reusability results confirmed the superior catalytic performance of the immobilized lipase materials obtained using the KIT-6 as compared to MMT K-10. The reusability studies were carried out by using the same reaction and repeatedly using the same catalyst. The data were collected up to the 5 cycles.

In the first run, the hydrolytic yields were high for both samples and declined in the subsequent reuses. The MMT K-10 samples demonstrated a rapid drop in hydrolytic activity compared to KIT-6 materials. This could probably be due to the discharge of enzyme from the support during the course of the reaction. For the lipase immobilized on MMT K-10, the hydrolytic activity was significantly decrease after each cycle compared to the KIT-6 samples; the latter system could retain its activities up to 75 % after 5 cycle of reactions.

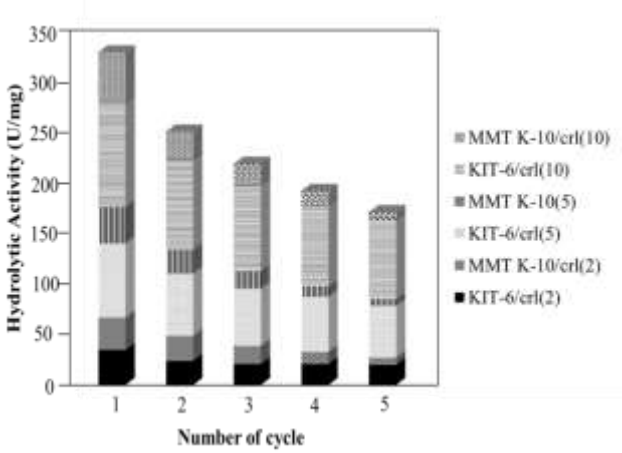

Fig. 6 Bar chart of hydrolytic activity (U/mg immobilized lipase) through 5 cycles of reactions

4. CONCLUSION

Candida rugosa lipase was successfully immobilized on both supports by the physical adsorption method, and mesoporous silica KIT-6 showed the satisfactory data on the effect of enzyme concentration solutions, hydrolytic activity and leaching. From this study, immobilization of lipase on KIT-6 and MMT K-10 was achieved via hydrogen bonding between the protein and the silanol groups of the supports. However, steric factors may also be related to the textural attributes of the materials; they should be taken into account when explaining the differences in the hydrolytic behaviour of the supports. From the results, lipase immobilized in KIT-6 showed higher loading and less protein leaching out from the support than the MMT K-10. The mesoporous silica KIT-6 also exhibited satisfactory recoveries, giving high yield of the product during 5 recovery cycles. In conclusion, the utilization of mesoporous silica KIT-6 resulted in an enhanced catalytic performance as compared to the silicate clay, montmorillonite K-10. The mesoporous silica materials such as KIT-6, offer vast prospects for numerous future applications in immobilization of biocatalyst, due to their high surface areas and pore volumes, combined with

highly accessible and highly connected open pore networks.

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