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Enhancing performance of lipase immobilized on methyl-modified silica aerogels at the adsorption and catalysis processes: Effect of cosolvents

Siliang Gao, Yujun Wang*, Wenwen Wang, Guangsheng Luo, Youyuan Dai

State Key Laboratory of Chemical Engineering, Department of Chemical Engineering, Tsinghua University, Beijing 100084, China

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ABSTRACT

Hydrophobic silica aerogels modified with methyl group were applied as support to immobilize *Candida rugosa* lipase (CRL). At the adsorption process, different alcohols were used to intensify the immobilization of CRL. The results showed that *n*-butanol wetting the hydrophobic support prior to contacting with enzyme solution could promote lipase activity, but the adsorption quantity onto the support decreased. Based on this, a novel immobilization method was proposed: the support contacted with enzyme solution without any alcohols, and then the immobilized enzymes were activated by 90% (V) *n*-butanol solution. The experimental results showed that this method could keep high adsorption quantity (413.0 mg protein/g support) and increase the lipase specific activity by more than 50%. To improve the stability of immobilized lipase, the support after adsorption was contacted with *n*-octane to form an oil layer covering the immobilized lipases, thus the leakage can be decreased from over 30–4% within 24 h. By utilizing proper cosolvents, a high enzyme activity and loading capacity as well as little loss of lipase was achieved without covalent linkage between the lipase and the support. This is known to be an excellent result for immobilization achieved by physical adsorption only.

1. Introduction

Lipases are macromoleculars of proteic nature, with molecular weights about 40,000–60,000 Da, and molecular sizes about 4–5 nm diameter roughly [1–3]. They have a broad variety of applications in industry due to the multiplicity of reactions they catalyzes, such as ester hydrolysis, ester synthesis, ester exchange, alcoholysis, and their natural substrates are long-chain acyl glycerols, which have low aqueous solubility [4–6]. It is common practice to use lipases in immobilized form to enhance their activity, selectivity, and operational stability. The attachment to the support can be through simple adsorption, ion exchange or covalent linkage. Each approach has its own positive and negative attributes that impact enzyme performance, and undoubtedly adsorption is the most simple and cost-effective way preferred in industry [7].

The nature of the support is known to be an important factor that can influence lipase catalytic performance [8,9]. It has been reported that among all the structural parameters, pore size and hydrophobicity of the supporting materials are the most important in the immobilization process and determine the yields of enzyme, catalytic efficiency and possible leakage. The ideal size of pores was found to be at least 3–5 times of the protein diameter in order to prevent restrictions to the access of immobilized enzyme [10–12]. Modification of hydrophilic surfaces with hydrophobic groups resulted in higher loading capacity and better stability [4,13], perhaps because of the multi-interactions between the support and lipase. In the meanwhile, hydrophobic support may provide feasible contact between the hydrophobic substrates and the immobilized lipase and enhance the non-aqueous catalytic reactions. However, the inner surface may not be fully utilized for lipase adsorption even if the pore size is big enough during the immobilization process, because of an increased capillary resistance for the aqueous phase to diffuse into the hydrophobic support materials [14,15]. Thus, the polarity of the enzyme solution is supposed to be reduced in order to improve the access of protein to the inner surface of the pores. Attentions have also been paid to optimize the immobilization conditions to enhance enzyme activity. Solution pH, temperature, surfactants are the commonly used ways [16]. However, less is known about the effect of cosolvents, which may affect the protein–protein association or the 3D structure of the enzyme and thus may also impact enzyme activity. Another unfavorable effect is the high risk of enzyme leaching from the support materials. Fernandez-Lorente et al. [17] studied the durability of adsorbed lipase on alkyl-modified agarose and found that nearly 100% of the immobilized enzyme was desorbed, which indicated weak protein-support multi-interactions. In consideration of the hydrophobicity of the support, an oil film, which has more affinity with hydrophobic materials, may be used to cover and protect the immobilized lipases, providing a new method to solve the leaching problem since it is difficult to strengthen the interac-

* Corresponding author. Tel.: +86 010 62773017; fax: +86 010 62770304.
E-mail address: wangyujun@mail.tsinghua.edu.cn (Y. Wang).

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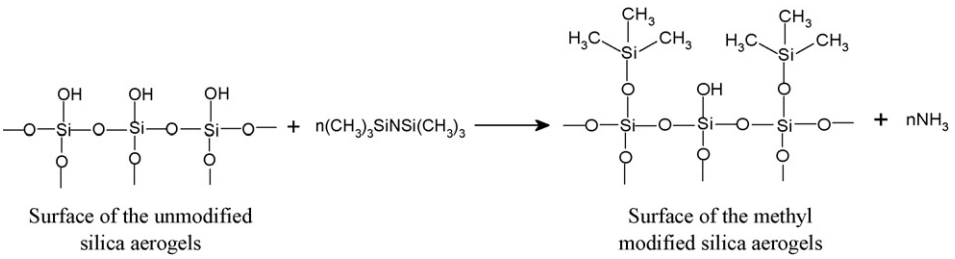


Scheme 1. The principle of the methyl-modification reaction at the surface of silica aerogels.

tions between the support and lipase without introducing chemical bonds. Therefore, we believe that the proper use of organic cosolvents may improve the inherent problems existed in the normal lipase immobilization process, and its effect on loading capacity, activity, enzyme leakage is worth discussing.

Aerogels based on silica, which are extremely porous materials with high specific surface area, are chosen as immobilization support in our work. The diameter of the pore is larger than 25 nm and can provide sufficient space for enzyme molecules. Coating of silica surface with methyl groups transforms the hydrophilic material into a highly hydrophobic one, providing excellent properties to interact with both the hydrophobic lid of lipase and the substrates. Principle of the modification reaction was shown in Scheme 1, and hexamethyldisiloxane was used as a siliane reagent. Fig. 1 shows the ^{29}Si NMR spectra of the modified support, in which the three peaks from left to right present Si connected with methyl groups, hydroxyl groups and oxygen atoms, respectively. The characteristics of the methyl-modified silica aerogels used in the experiment are as below: surface area: 234.0 $m^2\ g^{-1}$; pore volume: 2.232 ml g^{-1}; average pore diameter: 38.15 nm; contact angle with water: 99.2°, mass fraction of methyl groups: 5.46%. The size of particles was about 10 μm, thus the restrictions of external diffusion due to the particle size could be neglected according to the research of Serra's group [11]. Our previous work has shown that lipase could be successfully immobilized into this kind of material by physical adsorption [18]. Nevertheless, such high hydrophobicity might not be suitable environment for enzyme dissolved in an aqueous medium to penetrate into the pore channels. Blanco et al. [19] have found that the capacity for lipase immobilization in the highly hydrophobic octyl-modified silica has been increased in a twofold factor by the use of 10% ethanol to pre-wet the support,

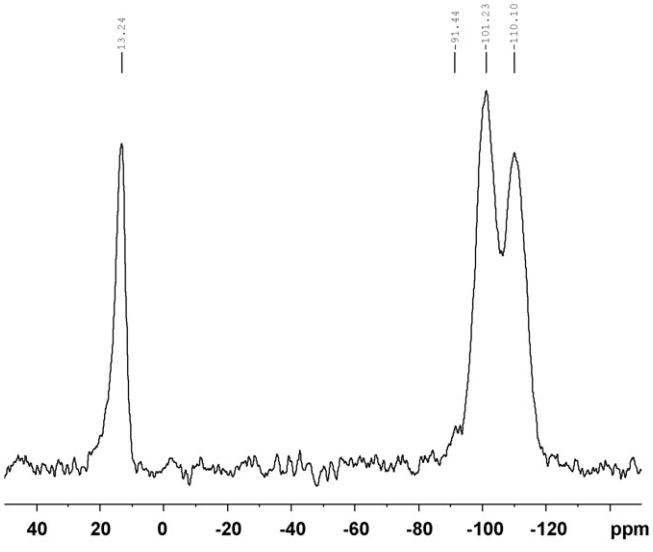


Fig. 1. ^{29}Si NMR spectra of methyl-modified silica aerogels.

possibly due to the decrease of the hydrophobicity of the microenvironment and improvement of the lipase distribution. It is also reported recently that the treatment with some polar solvents on immobilized lipases may enhanced their activity and stability, perhaps because of the changes in secondary structure of enzymes in presence of alcohols [20,21]. However, more general effects of even low concentration of alcohols on the enzymes loading and activity and detail reasons for that enhancement are still under investigation. In this paper, *Candida rugosa* lipase (CRL) with diameter below 5 nm was used as model enzyme to be immobilized on silica aerogels. Ethanol, *n*-propanol, isopropanol, *n*-butanol, *t*-butanol were applied as cosolvents to investigate the enhancing effect on lipase adsorption and activity. Also, we found that dispersing the hydrophobic support after lipase immobilization into *n*-octane before two-phase catalytic reaction can prevent lipase leaching efficiently. Reasons for why organic cosolvents can improve lipase immobilization on the hydrophobic support are mainly discussed in this paper.

2. Materials and methods

2.1. Materials

C. rugosa lipase type VII was purchased from Sigma–Aldrich (Dorset, UK). Highly refined olive oil was obtained from Shanghai Agent Company with a saponification value of 192. Methyl-modified silica aerogels were obtained from Shao Xing Na No Company Limited, China. It was produced through supercritical dry method [22] and methyl-functioned by hexamethyldisiloxane which was evaporated into a gas phase for a gas–solid reaction. Ethanol, *n*-propanol, isopropanol, *n*-butanol, *t*-butanol, and other chemicals were of analytical grade and were used without further purification.

2.2. Methods

2.2.1. Characterization

A certain amount of methyl-modified silica aerogels particles were tabletting for contact angle measurement by Dataphysics OCA-H200. The mass fraction of methyl groups was obtained by thermogravimetric analysis, which was performed by the STA 409 PC. The ^{29}Si NMR spectra was measured by solid-state NMR (CPMAS). Nitrogen adsorption–desorption isotherms were measured at 77 K using a Quantachrome Autosorb-1-C Chemisorption–Physisorption Analyzer after the samples were outgassed for 30 min at 200 °C. The BET surface area was calculated from the adsorption branches in the relative pressure range of 0.05–0.25, and the total pore volume was evaluated at a relative pressure of about 0.99.

2.2.2. Immobilization procedure by physical adsorption (equilibrium experiment)

The immobilization of *C. rugosa* lipase on methyl-modified silica aerogels via hydrophobic interactions is given as below:

1. Pre-wet the methyl-modified silica aerogels by adding alcohols to the aerogels;
   2.5 ml of four kinds of alcohols were added to 0.1 g of methyl-modified silica aerogels, respectively, and each was left in a closed conical flask to pre-wet the support.
2. Suspend the pre-wetted aerogels in the aqueous solution of lipase for 12 h at 25 °C;
   Lipase solutions were prepared by adding appropriate amounts of lipase powder to 25 ml of phosphate buffer solution (pH 7.0) and then added to the closed conical flask containing the pre-wetted support and the remained alcohols. The temperature during adsorption was controlled at 25 °C.
3. The solution was filtrated after adsorption, the support with immobilized lipase was washed with deionized water for three times and dried in the air before activity measurement;
   In the study of the activation effect of alcohols on immobilized lipase, 0.6 g of modified silica aerogels without any alcohol pre-wetting was used for adsorption. 10 ml of *n*-butanol solution at a certain concentration was slowly dropped onto 0.1 g of the support taken from the above aerogels after adsorption during the filtration process for 10 min and then open-air-dried for 30 min before activity measurement.

The original enzyme protein concentration and the concentration after filtration and washing were measured by the Lowry method [23] with the Agilent 8453 UV–vis spectrophotometer.

2.2.3. Kinetics of adsorption and desorption

0.02 g of methyl-modified silica aerogels with or without ethanol pre-wetting was added to 25 ml of lipase solution, respectively. After magnetic stirring for a certain time, 0.25 ml of the enzyme solution was taken out and centrifugated for 5 min before the protein concentration was measured in the supernatant. The total adsorption time was 5 h.

0.1 g of plain or pre-wetted aerogels with immobilized lipase was suspended in 25 ml of phosphate buffer solution, respectively. 0.25 ml of the solution was taken out and protein concentration was checked in the supernatant after 5 min centrifugation. The whole desorption process last for 24 h.

0.1 g of plain or pre-wetted support with immobilized lipase was suspended in 10 ml of *n*-octane for 20 min before 10 ml of phosphate buffer solution was added. After stirring for 24 h, the whole aqueous phase was separated for protein measurement and 10 ml of fresh phosphate buffer solution was added for another desorption cycle. Five cycles were repeated, and desorption time was 2 h for each cycle. The whole processes were controlled at 25 °C.

2.2.4. Measurement of the immobilized lipase activity

The activity of immobilized lipase was measured by following the method of Yamada and Machida [24] with some modifications. The measurement was based on hydrolysis of olive oil. The substrate was prepared by thoroughly mixing 50 ml of olive oil with 150 ml of *n*-octane. Then 0.1 g of modified silica aerogels with immobilized lipase was first dispersed into 2.5 ml of *n*-octane and then 7.5 ml of the oil mixture was added. After warming-up to 40 °C for 20 min, 10 ml of phosphate buffer solution (0.05 mol/l) was added. The reaction emulsion was stirred for 30 min at the same temperature and was later centrifuged at 5000 rpm for 15 min to separate the three phases. Then 1 ml of both aqueous phase and oil phase was taken out and diluted to 10 ml by deionized water and ethanol–aether (volume ratio = 1:1) solution, respectively. Finally, 0.01 mol/l of NaOH standard solution was titrated for fatty acid (FFA) measurement. Specific enzyme activity is defined as the amount of FFA produced per minute per mg protein under the assay conditions.

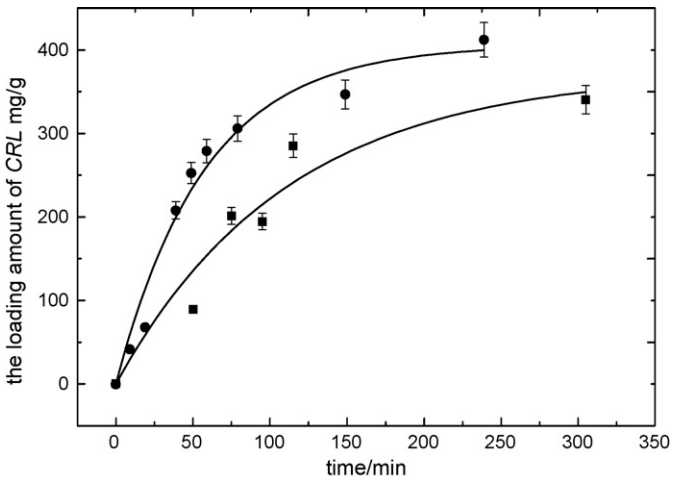


Fig. 2. Adsorption kinetics of lipase in non-wetted (■) and pre-wetted support with 10% (v/v) of ethanol (●) (the initial concentration of lipase solution is 771.0 mg protein/l; 0.02 g of support was used).

3. Result and discussion

3.1. Effect of alcohols on the lipase adsorption process

It has been reported that the structure of protein may change in different concentration of alcohols [25,26]. However, the change of the support surface caused by the alcohol film pre-wetting has not drawn much attention. In order to study the effect on adsorption kinetics of lipase caused by alcohols, ethanol was taken as an example for detailed research. In the comparative experiments of adsorption, one set of aerogels was prepared without ethanol pre-wetting; the other was pre-wetted with 10% (v/v) of ethanol. When mixed with the lipase, the non-pre-wetted aerogels floated over the solution while the pre-wetted aerogels were totally immersed in the solution. A lot of bubbles were also observed in the pre-wetted aerogels/lipase mixture, which indicated that the solution has penetrated to the pore channel of the support. As shown in Fig. 2, the adsorption rate of lipase in the pre-wetted aerogels is higher than the blank sample. The smoother adsorption curve implies a continuous diffusion of lipase into the pore of the pre-wetted support. Table 1 shows the significant difference in contact angle of the aerogels with water and ethanol. With a very hydrophobic surface, the contact angle between the non-pre-wetted aerogels and the buffer solution is more than 90°, and consequently the lipase can only reach the very outer part of the channel. Associated with random desorption, the adsorption curve of the blank sample became rough. The contact angle between the non-pre-wetted aerogels and ethanol, ethanol solution was 16.65° and 89.2°, respectively, suggesting that the presence of miscible alcohol at low concentration is enough to create a less hydrophobic environment with significant decrease in interfacial tension thus enable the penetration of lipase into inner surface of the pores.

Table 1
Contact angle data of methyl-modified silica aerogels with alcohols and alcohol solutions.

Liquid	Contact angle	Log *P*[a]	Solution (10% V)	Contact angle
Ethanol	16.65°	−0.24	Ethanol–water	89.2°
n-Propanol	17.7°	0.25	*n*-Propanol–water	89.4°
Isopropanol	15.2°	0.05	Isopropanol–water	91.8°
n-Butanol	17.8°	0.88	*n*-Butanol–water	59.4°
t-Butanol	24.35°	0.8	*t*-Butanol–water	100.2°

[a] Data were from Laane et al. [31].

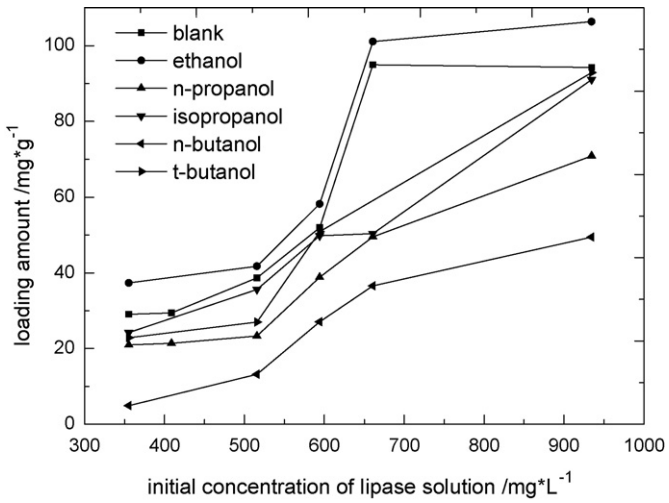


Fig. 3. Effect of different alcohols on the lipase loading (0.1 g of support was used in each sample).

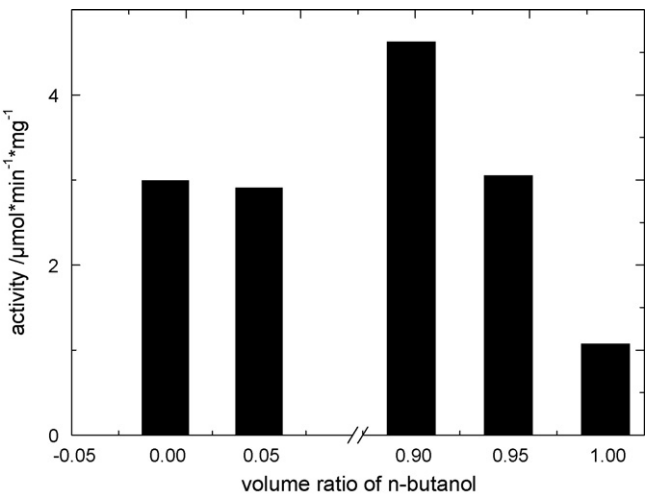


Fig. 4. Effect of volume ratio of *n*-butanol on lipase activity in the activation process (0.1 g support with 4.5 mg immobilized lipase used in each sample).

When it comes to the effect of alcohols on lipase loading capacity, a blank sample with non-pre-wetted support was also included for comparison with pre-wetted support. As shown in Fig. 3, the loading capacity of lipase was increased by about 10% by pre-wetting the silica aerogels with 10% of ethanol at different lipase initial concentrations. However, with other alcohols pre-wetting, enzyme loading was lower than the blank sample, which is an unexpected result and is very different from the results obtained by Blanco's group [19]. This is probably due to the difference between the hydrophobicities provided by C_1 and C_8 chains. The methyl groups did not seem to possibly provide more than a moderate hydrophobicity to the silica aerogels and would not make necessary the presence of alcohols in order to decrease hydrophobicity and favor enzyme diffusion through the porous network. Therefore, the results of this work and Blanco's were not easily comparable. From the contact angle data shown in Table 1, we may see that all the used solutions containing 10% (V) alcohols can improve the wettability of the hydrophobic support except for *t*-butanol, and the *n*-butanol solution can facilitate the penetration of the enzyme solution most, however, it resulted in the least lipase loading. Thus there seems to be no obvious relation between the decrease of capillary resistance and adsorption efficiency, which indicated that the effect of alcohols on lipase loading is more complicated than the change of microenvironment of the hydrophobic support. Though the enzyme solution can infiltrate the pore and make more use of its inner surface, the interactions between lipase molecule, the outer of which is more hydrophobic, and the support may be weakened. It might be easier for water molecules to drag lipases back into the aqueous phase. Besides, it has also been reported that some alcohols may cause rearrangement of the secondary structure of lipase [27] and may stretch or compress the molecule, so as to promote or prevent its accessibility into the pores of the immobilization carrier. Further explanations may be obtained by means of spectroscopic analysis of lipase structure in the buffer solution with different alcohols.

3.2. Effect of alcohols on the activity of immobilized lipase

On the other hand, catalytic activity of lipase in the presence of low concentration of alcohols (10%) was not negatively affected by the change of microenvironment and structure of lipase molecule. Contrarily, the FFA production even more than doubled when 10% (V) of *n*-butanol solution was used in the adsorption process, though less lipase was immobilized into the support (Table 2). The result indicated that *n*-butanol may have some activation effects on lipase. Taking the obvious decrease of lipase loading caused by the addition of *n*-butanol into consideration, an improved way to achieve both high enzyme loading and high activity is developed, which is using methyl-modified silica aerogels not pre-wetted by any alcohols for adsorption and then activating the immobilized lipase by *n*-butanol solution during the filtration process to enhance its activity. Ethanol was not chosen to pre-wet the support because its addition made the operation complex while the amount of lipase loading did not increase obviously.

Further experiments were focused on the optimization of the *n*-butanol concentration used for lipase activation. Due to the two-phase region existed in the water–butanol phase diagram, only five concentrations were investigated. Fig. 4 shows that 90% (V) *n*-butanol solution can increase the lipase activity by more than 50%, and the activity retention could reach 52.2% compared with the free enzyme. It is worth noticing that the specific activity is always in inverse proportion to the amount of enzyme loading, however, the adsorption quantity was 45 mg/g in this experiment, higher than the general data obtained from industry [28].

Reasons for the effects of alcohols on lipase activity have drawn lots of attention. Blanco et al. found that the presence of alcohol may have an effect on the heterogeneity factor of the support surface, resulting in a more even monolayer distribution of lipase, which may help to increase the catalytic efficiency [19]. However, in our previous work [18], it was found that the specific activity of the immobilized lipase dropped as the loading amount increased,

Table 2
Effect of alcohols on FFA production and specific activity of immobilized lipase (initial enzyme concentration: 594.3 mg/l).

	Blank	Pre-wetted by 10% (v/v) alcohols				
		Ethanol	*n*-Propanol	Isopropanol	*n*-Butanol	*t*-Butanol
Lipase loading amount ($mg\,g^{-1}$)	52.0	58.2	38.8	49.8	27.1	46.0
Total amount of FFAs (μmol)	363	435	256	333	578	554
Special activity ($\mu mol\,min^{-1}\,mg^{-1}$) protein	2.33	2.49	2.20	2.22	7.13	3.63

which indicated a multilayer of the adsorbed lipases was formed in the immobilization process. The addition of alcohols might reduce the degree of lipase aggregation but cannot prevent enzyme aggregates especially at high lipase concentration. Therefore, it was not the main reason for the enhancement of lipase activity. Noticing that only *n*-butanol can improve the lipase activity obviously, we believed that the difference of the alcohols might help to find out another reason. According to the log *P* data shown in Table 1, lipase activity increased approximately upon the hydrophobicity parameters of the cosolvents used for pre-wetting. Some researchers were inclined to believe that when an enzyme came in contact with more hydrophobic environment such as a solution of polar solvent and water, its hydrophobic side chains were exposed more on the surface and the enzyme conformation changes to a more open form, which increased its ability to bind substrates, thus increasing rate limiting kinetic step [29,30]. This may explain the promoted effect of *t*-butanol solution on lipase activity. However, the results do not support that all alcohols would promote the specific activity of lipase, and also there seemed to be no corresponding relation between the alcohols' hydrophobicity parameters and their effects on lipase activity, which indicated that the polarity of the cosolvents can indeed affect the enzyme activity but was not the only factor of influence, whereas other factors were still under investigation. Similar conclusions were obtained by Wu's group [20].

3.3. Effect of n-octane on the desorption behavior of immobilized lipase

Table 2 shows that ethanol is better than other alcohols in improving the loading efficiency of lipase, thus it was used for pre-wetting the support in the study of desorption process.

Desorption of the enzyme from the support is very likely to occur in aqueous reaction media, given the non-covalent nature of the enzyme-support linkage. To determine whether the addition of ethanol onto the support has advantages on strengthening the interaction of lipase and the support, experiments of desorption were carried out. Two samples with immobilized lipase were dispersed into the buffer solution, one of which support was pre-wetted by ethanol while the other was blank. Shown in Fig. 5, almost 30% lipase leached during the first 30 min in both samples, and then the amount of desorption did not change over time basically. The final leached ratios after more than 12 h were 31.8 and 31.2% for the ethanol and blank sample, respectively. The result was consistent with Serra's [11], who also reported that nearly 30% of the enzyme was leached from the support within 2 h in their work. It seems that water molecules could easily break the interactions between lipase and the hydrophobic support and drag the enzyme into the aqueous phase no matter whether the support was pre-wetted by ethanol or not.

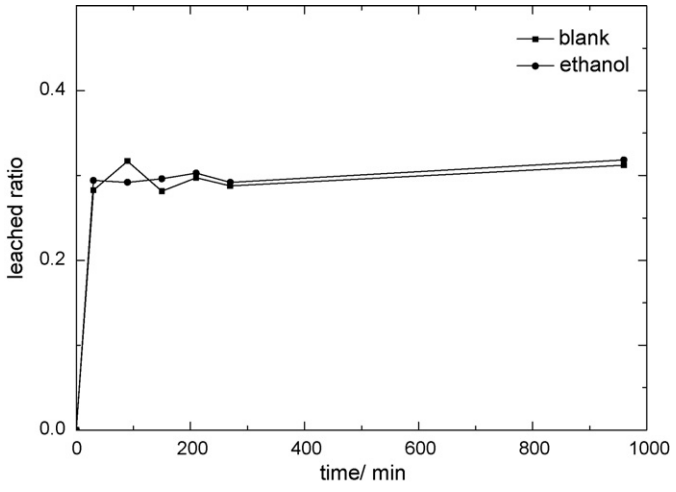


Fig. 5. Desorption kinetics of lipase from non-pre-wetted support (■, 8.80 mg of lipase on 0.1 g of support) and pre-wetted support with 10% (v/v) of ethanol (●, 9.64 mg of lipase on 0.1 g of support).

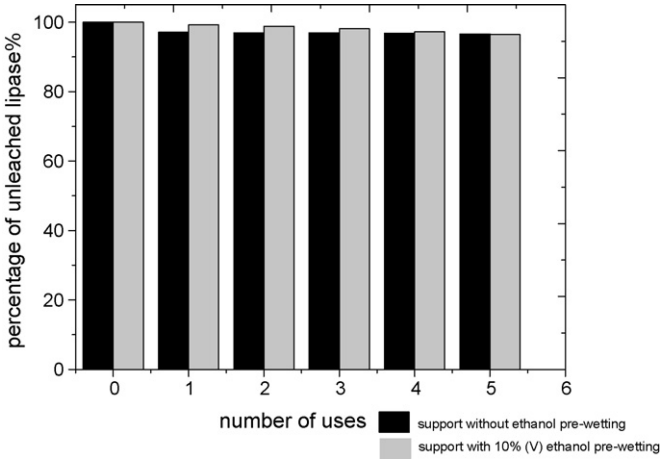
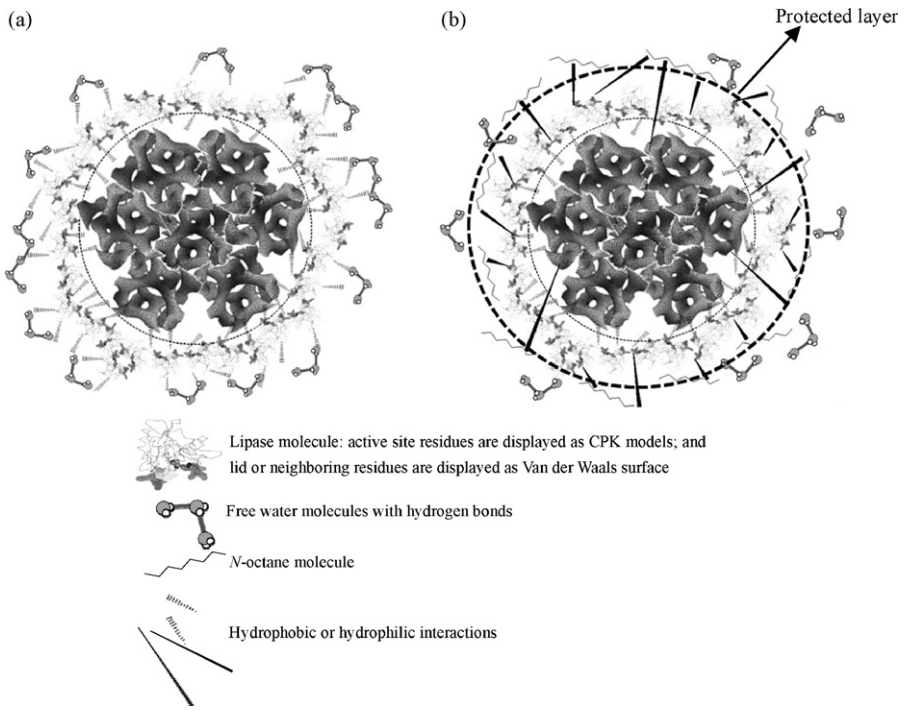


Fig. 6. Effect of *n*-octane film on the desorption behavior of immobilized lipase (the amount of immobilized lipase was 14.3 mg/0.1 g support (■) and 18 mg/0.1 g support ( ), respectively).

Since the contact angle of the modified silica aerogels with the buffer solution is 99.2°, it can be well dispersed into the oil phase while floating above the buffer solution, which indicates that the hydrophobic interaction between the support and immiscible solvent is much stronger than that between the support and aqueous phase. Thus introducing an immiscible organic film after lipase immobilization above the hydrophobic support may help to protect lipase molecules from desorption. *N*-Octane was chosen as the "protector". Considering that *n*-octane was used as solvent to dissolve olive oil for standard lipase activity measurement, it is harmless to lipase molecule.

The results shown in Fig. 6 illustrated that the loss of lipase in the aqueous phase was greatly reduced after immersed the support with enzyme into 10 ml of *n*-octane. Only 4% of immobilized lipase leached at the end of desorption process (24 h) for both blank sample and pre-wetted sample, though the loading amount of lipase was different after the adsorption process. More than 96% of lipase was still adsorbed in the support after five uses. It should be said that all the activity measurement mentioned above were carried out in the "*n*-octane protected" way to ensure the data obtained were the activity of immobilized lipase, not the free one. By utilizing the organic cosolvents, a high enzyme activity and little loss of lipase was achieved without covalent linkage between the lipase and the support. This is known to be a good result for immobilization achieved by physical adsorption only.

A supposed mechanism of the protection effect of *n*-octane film was speculated in Scheme 2. *N*-Octane was dispersed and covered the surface of the pores where the lipase was anchored. Since there was no aqueous medium inside, the disruption of hydrophobic interactions between the support and lipase may not happen. The strong hydrophobic interactions between the support and *n*-octane film trapped the lipase in the middle and help to complete the immobilization process. Consequently leaching is avoided irrespective that the immobilization had been carried out in the presence or in the absence of any alcohol solution. Water molecules may also contact with lipase because of the hydroxyl



Scheme 2. Desorption of immobilized lipase without organic solvents (a) and with the protection of *n*-octane layer (b).

group on the silica aerogels, thus the hydrolysis reaction can still happen.

4. Conclusion

Methyl-modified silica aerogel was subjected to several treatments by organic cosolvents in order to enhance its immobilization of *C. rugosa* lipase. Though the addition of alcohols can decrease the hydrophobicity and interface tension of the inner pore surface, only ethanol enabled the total access of the hydrophobic material to the aqueous enzyme solution, and the adsorption rate increased as well as the yields of immobilization of lipase were improved by about 10%. Other alcohols did not have positive effect on lipase loading, perhaps because of more complicated effects on the enzyme structure. Among the five alcohols used in the experiment, *n*-butanol treatment had the most obvious effect on enhancing the specific activity of immobilized lipase. With 90% (V) *n*-butanol solution activating the immobilized lipase, the specific activity increased by more than 50% compared with the not activated sample. Since alcohols used cannot strengthen the interactions between the lipase and support, *n*-octane, a more hydrophobic organic cosolvent was introduced to form a protective layer after the adsorption process to avoid lipase desorption in the aqueous phase. Almost no enzyme leached after the treatment of *n*-octane, due to the strong hydrophobic interaction between the support and the oil film. By using methyl-modified silica aerogels as support for adsorption, *n*-butanol solution for activation and *n*-octane for protection, high loading quantity, high activity and little loss of lipase can be achieved at the same time with the simplest operation.

Acknowledgements

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