African Journal of Biotechnology Vol. 9(23), pp. 3431-3436, 7 June, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

*Full Length Research Paper*

# Enhancement of the performance of covalently immobilized lipase using alcohol quenching technology

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Accepted 12 May, 2010

**In order to modulate the microenvironment for enzyme covalently attached on support and improve the covalent immobilization of lipase, alcohol molecules were used to quench the excessive activated functional group on support surface. Effects of kind and content of alcohol molecules on the relative activity of the immobilized enzyme and the characteristics of the immobilized enzyme were examined carefully. The maximum relative activities of the immobilized lipase quenched with methanol and n-propanol, were 224.3 and 224.5%, respectively, both 1.96 fold of the just immobilized lipase which was not quenched with alcohol. Residual activity of the immobilized lipase using methanol to quench the excessive activated groups on support surface was 65.9% after heating at 50°C for 60 h, 1.29 folds higher than that of the ordinarily immobilized lipase (with no blockage). Alcohol molecules could alter the physical and chemical properties to modulate the microenvironment on support surface by changing the hydrophobicity. Suitable microenvironment, resulted from the methanol quenching the excessive active groups, would further favor the activity and the stability of lipase at higher temperature.**

**Key words:** Alcohol quenching, mesocellular siliceous foams, covalent immobilization, microenvironment, lipase.

## INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are widely used as versatile biocatalysts in industrial biotechnology and modern organic chemistry especially for synthesis of enantio-enriched compounds and polymerization reactions, esterification and interesterification (Chaubey et al., 2009; Chisti, 2007, 2008). However, native enzyme application is often hampered by its lack of long-term stability under process conditions, and also by difficulties in its recovery and reuse. To solve these problems, much attention has been paid to enzyme immobilization (Xie et al., 2009). The immobilization of the enzyme on solid supports increases the robustness of the enzyme towards organic media (Xie et al., 2009), non-physiological pH and temperature (Sio and Quax, 2004). There are some other advantages, for example repetitive uses, the possibility to stop the reaction easily and the availability which cannot be contaminated by the enzymes (Gomez et al., 2006).

Moreover, enzyme immobilization has been revealed as a very powerful tool to enhance almost all enzyme properties, if properly designed: example, stability, activity, specificity and selectivity, reduction of inhibition, etc (Blanco et al., 2007). Availability of the immobilized enzyme catalyst with improved activity and stability is also expected to reduce the expense of products (Parmar et al., 2000). To date, biologists and chemists have also been searching for the high efficient and stable biocatalyst through computational approaches and recombinant DNA technology (Burton et al., 2002; Korkegian et al., 2005). Unfortunately, computational approaches must rely on simplified models that only approximate real proteins and their interactions (Chen et al., 2009) and there is still

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**Abbreviations: MCFs,** Mesocellular siliceous foams; **GPTS,** 3-glicydoxypropyltriethoxysilane; **p-NPB,** p-nitrophenyl acetate; **FT-IR,** fourier transform infrared.

considerable effort required to extend its use towards obtaining novel enzymes with more complex catalytic mechanisms (Hibbert and Dalby, 2005).

Now, many attempts were focused on multipoint covalent immobilization which was reported to increase clearly the thermal stability of immobilized enzymes. However, stabilizing effect increases with the number of covalent bonds between enzymes and the support until some critical value (a limit) is achieved and further increase in the number of bonds does not lead to further stabilization (Martinek and Mozhaev, 1991). Excessive active groups on support surface would increase the possibility of enzyme deactivation by subsequent slow reaction between the enzyme and them (Cao and Schmid, 2005). In the present study, lipase was covalently attached on functional MCFs and alcohol molecules were used to quench the unwanted activated groups and modulate the micro-environment in mesopores in expectation of perfecting the immobilized lipase. The properties of the lipase immobilized depending on alcohol quenching technology were also investigated.

## MATERIALS AND METHODS

### Materials

Lipase, gift from Dr. Zhenming Chen, was from the recombinant strain *Escherichia coli* BL21 (pET28a-lip2). This strain contains the gene from *Geobacillus* sp. Tetraethylorthosilicate (Shanghai, Sinopharm) and 3-glicydoxypropyltriethoxysilane (GPTS) (Jingzhou Jianghan Fine Chemical Co., Ltd.) obtained from the company was used as such. *p*-Nitrophenyl acetate (p-NPB) was obtained from TCI (Shanghai) Development Co., Ltd. Poly (ethylene glycol)-block-poly (propyleneglcol)-block-poly (ethyleneglycol) (P123) of the highest grade commercially available was obtained from Sigma-Aldrich. Methanol, ethanol, iso-propanol, butanol, n-propanol and other chemicals were all provided by Sinopharm Chemical Reagent (Shanghai, Sinopharm) and were analytical grade. Deionized water with a resistance greater than 18 MQ was obtained from a Millipore-Q Plus water purifier.

### Preparation, functionalization and characterization of MCFs support

Mesocellular Siliceous Foams (MCFs) was prepared and characterized using the methods as reported by Schmidt-Winkel et al. (1999) and Wang et al. (2008). The epoxy-functionalized MCF was prepared by post-condensation method. 1.0 g MCF was suspended in 30 mL of toluene. Then 5 mL of GPTS was added into the mixture and the post-condensation reaction was carried out at 110℃ with reflux for 20 h. The white solid was filtered off, washed with toluene and hexane, respectively, dried under vacuum at 80℃. Then they were characterized by FT-IR spectroscopy (Zeiss, Specord M 80) using KBr method.

### Covalent immobilization of lipase in the epoxy-functionalized MCFs

20 mg of Epoxy-functionalized MCFs incubated in 3 ml of a phosphate buffer solution (pH 8.0, 0.1 M) containing a suitable amount of lipase and the mixture was shaked mildly for 20 h. Then the immobilized enzyme was separated and washed using phosphate buffer (pH 7.0, 0.1 M) until no protein could be detected in the supernatant. The amount of enzyme remaining in the supernatant was measured using a Bradford assay.

### Enzyme activity assay

The activity of free and immobilized lipase was determined by a previously reported assay method using *p*-nitrophenyl acetate as a substrate (Ihara et al., 1991). The reaction mixture (3.0 mL) was composed of 2.85 ml phosphate buffer (pH 6.5, 0.01M), enzyme solution of appropriate dilution (0.15 mL) and appropriate immobilized CAL-B (3 - 5 mg) in which the buffer was used for the blank. The mixture was incubated for 2 min at 37℃. Reactions were initiated by addition of 0.15 mL of a *p*-nitrophenyl acetate solution and stopped by the addition of acetone (3 mL) after 5 min. The mixtures were clarified by filtration and the absorbance of solution due to the release of *p*-nitrophenol was measured at 405 nm. One unit of lipase activity was defined as the amount of enzyme which liberates 1 µmol of *p*-nitrophenol per min. Experiments were carried out in triplicate and standard error was never over 5%. Coupled yield and relative activity of immobilized enzyme were calculated as in the previous work by (Wang et al., 2008).

### Stabilization and thermo-stability of the covalently immobilized lipase

In order to improve the thermo-stability of lipase preparations, 20 uL of alcohol reagent was added into the immobilization system at 5 h before the immobilization of enzyme was finished, and then the immobilized enzyme was separated by centrifugation. For thermo-stability, free enzyme and immobilized enzyme were transferred into reaction medium and incubated at 50℃ for different time period. Periodically, samples of the suspension were withdrawn and their remaining activities were assayed as described above. Thermo-stability is given as residual activity of the immobilized derivatives or soluble enzyme.

## RESULTS AND DISCUSSION

### Effect of alcohol on the activity of the covalently immobilized lipase

In the present work, lipase was covalently immobilized in the mesocellular siliceous foams (MCFs) which was functionalized with 3-glicydoxypropyltriethoxysilane (GPTS). Alcohol molecules were used to quench the excessively functional group and proposed to stabilize the enzyme protein structure and modulate the microenvironment on support surface (Figure 1).

To investigate the effect of alcohol reagents on the activity of the immobilized lipase, different amount of alcohol reagents were offered to the immobilization mixture after the immobilization was carried out for 20 h. Alcohol reagents exert a lesser influence on the coupled yield of the immobilized enzyme (above 90%). Maximum coupled yield (99.1%) was obtained when the content of methanol was 2%. Figure 2 show that alcohol reagents exert a positive influence on the relative activity of the covalently immobilized enzyme.

As can be seen in Figure 2, 2.0% alcohol in the immo-

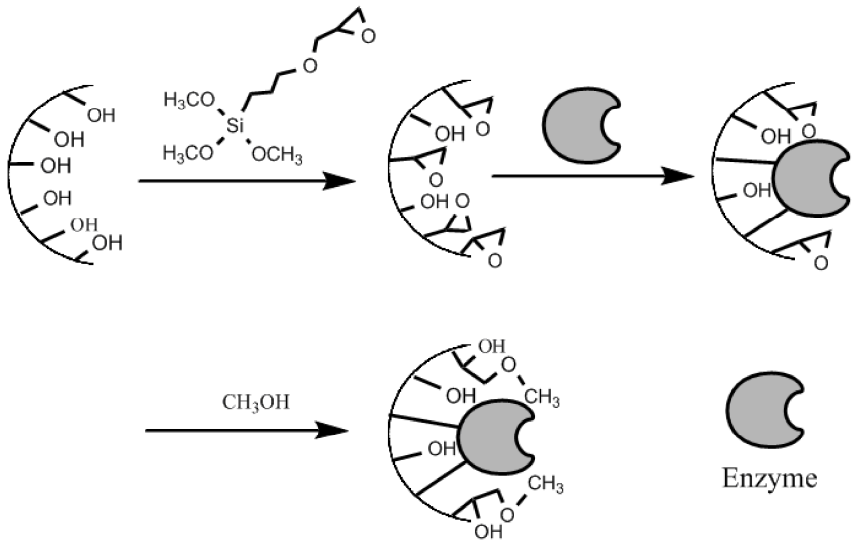


**Figure 1.** Preparation of routine of the immobilized lipase using methanol to quench the excessive functional groups.

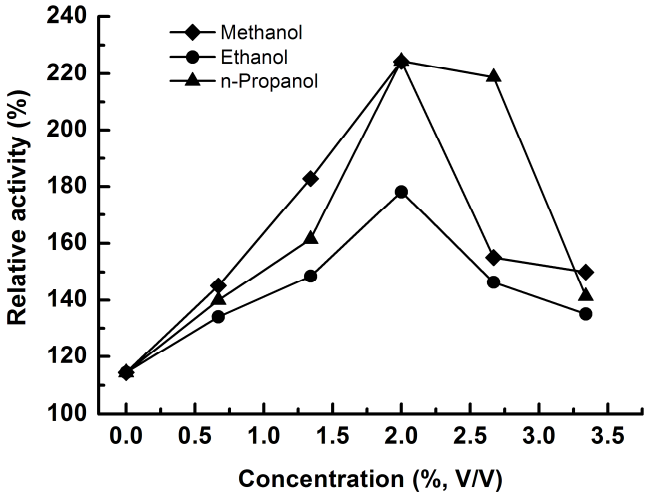


**Figure 2.** Effect of the content of alcohol on the relative activity of the immobilized lipase. The immobilized enzymes were all prepared with 100 mg enzyme protein offered to 1 g support.

bilization mixture was the most suitable to quench the excessive function group and modulate the micro-environment on support surface to improve the activity of immobilized enzyme. At this alcohol content for lipase immobilization, the maximum relative activities of the immobilized lipase quenched with methanol and n-propanol, respectively, were the nearly same, 224.3 and 224.5%. They are both 1.96 fold of the just immobilized lipase which was not quenched with alcohol, also much higher than that indicated in the previous report (86%) (Yu et al., 2006). This increment in the catalytic activity of lipase possibly resulted from the change in the physical and chemical properties of support surface (Blanco et al., 2007; Klotzbach et al., 2006; Wang et al., 2009). The presence of alcohol molecule on support surface decreases the hydrophobicity in the pore of MCFs support and improves the activities of the corresponding derivatives (Blanco et al., 2007).

## Kinetic parameter of lipase preparations

The biocatalytic activity of the immobilized lipase preparations was examined using p-nitrophenyl acetate (p-NPB) as the substrate. Michaelis-Menten parameters, *K*m and *k*cat, interpreted from the Lineweaver-Burk plots, were shown in Table 1. The data indicate that the methanol quenching clearly affects the reaction kinetics of the immobilized lipase preparation. Methanol on the support surface of MCFs enhanced the catalytic efficiency of the corresponding lipase preparation by 1.2 times. The corresponding catalytic efficiency (Kcat/Km) was up to 1.74$min^{-1}$ $mM^{-1}$, however, it is no more than 1.45$min^{-1}$ $mM^{-1}$ for the immobilized lipase preparation without quenching excessive functional group.

## Thermal stability of the covalently immobilized lipase

Methanol on the support surface was also found to affect the thermal stability of lipase preparations. As shown in Figure 3, methanol used in the blockage presented significant improvement of thermo-stability of the lipase in MCFs. Residual activity of the immobilized lipase using methanol to quench the excessive activated groups on support surface was 65.9% after heating at 50℃ for 60 h, 1.29 folds higher than that of the just immobilized lipase

**Table 1.** Kinetic parameter of lipase preparations.

| Kinetic Parameters | Immobilized lipase preparations | | |
|---|---|---|---|
| | JIME | IMEM[a] | IMEP[a] |
| Km (mM) | 0.66 | 1.21 | 1.02 |
| Kcat/Km ($min^{-1}$ $mM^{-1}$) | 1.45 | 1.74 | 1.47 |

[a]When the covalent immobilization of lipase was carried out for 20 h, 60 uL of methanol and n-propanol were added into the mixture, respectively, then the mixture were stirred again for 5 h. The immobilized enzymes were all prepared with 100 mg enzyme protein offered to 1 g support. JIME, just immobilized enzyme; IMEM, immobilized enzyme with methanol quenching; IMEP, immobilized enzyme with n-propanol quenching.

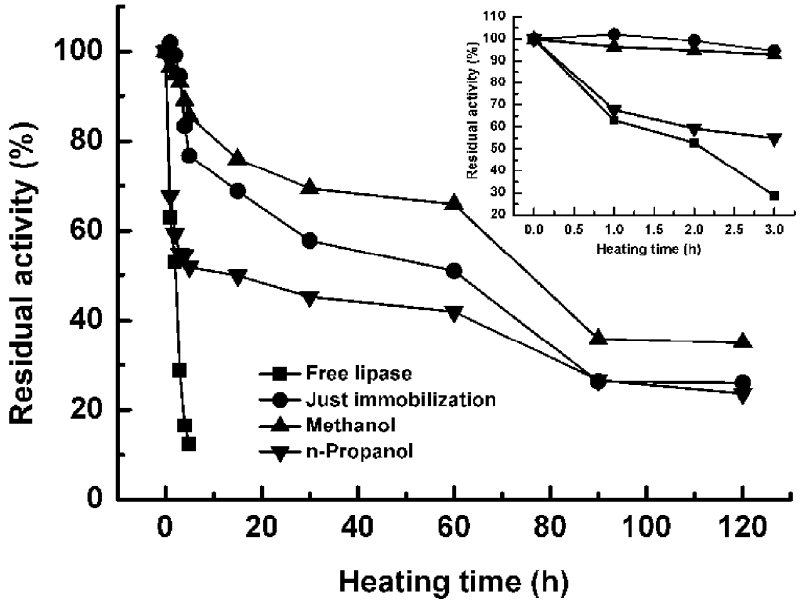


**Figure 3.** Thermal stability of free lipase and immobilized lipase preparations. The immobilized enzymes were all prepared with 100 mg enzyme protein offered to 1 g support. When the covalent immobilization of lipase was carried out for 20 h, 60 uL of methanol and n-propanol were added into the mixture, respectively, then the mixture were stirred again for 5 h.

with no blockage. In contrast to the immobilized lipase preparations, free lipase lost 88.7% of the initial activity after heating at 50℃ for only 5 h. The thermal stability of the immobilized lipase preparation was also better than that in the previous report (Dalal et al., 2007).

However, n-propanol on the support surface did not increase the thermal stability of the immobilized lipase preparation as methanol. It has been reported that multi-point covalent attachment of proteins on supports clearly increased the thermal stability of immobilized enzymes. However, stabilizing effect increases with the number of covalent bonds between enzymes and the support until some critical value (a limit) is achieved and further increase in the number of bonds does not lead to further stabilization (Martinek and Mozhaev, 1991). Modification of support using low molecular weight reagent could accommodate enzyme protein and improve the immobilization (Itoh et al., 2007). Therefore, alcohol molecules attached on the wall of mesopores could quench the excessive activated groups which might deactivate enzyme by subsequent slow reaction between the enzyme and them (Cao and Schmid, 2005). Moreover, the alcohol used modified the physical and chemical properties of support surface which might be one of key factors affecting thermo-stability of immobilized enzyme. Appropriate modification would make the surface suitable for lipase to modulate its structure and enhance the thermal stability of the enzyme preparation.

## Storage stability of the covalently immobilized lipase

Figure 4 illustrates the storage stability of free and immobilized lipase preparations at 4℃ for long terms of

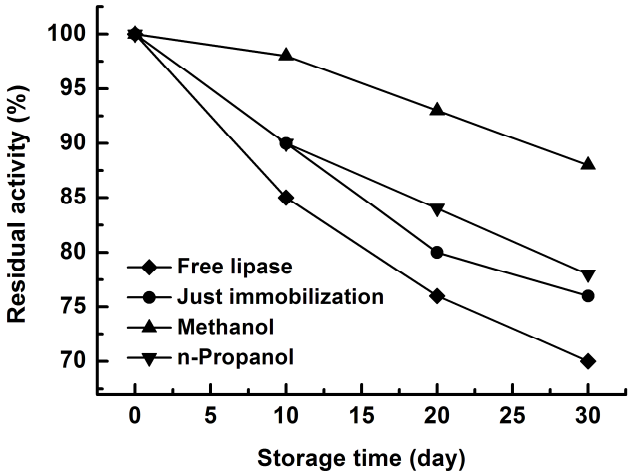


**Figure 4.** Storage stability of free lipase and immobilized lipase preparations. The immobilized enzymes were all prepared with 100 mg enzyme protein offered to 1 g support. When the covalent immobilization of lipase was carried out for 20 h, 60 uL of methanol and n-propanol were added into the mixture, respectively, then the mixture were stirred again for 5 h.

storage. The enzyme activity was measured every ten days. The activity of immobilized enzyme using methanol quenching the excessive functional groups remained more than 88.5% at 4℃ after 30 days. However, the activity of free enzyme only remained 70.1%. These results indicate that the immobilized enzyme using alcohol quenching the excessive functional groups had good storage stability. Free lipase showed the lowest stability, while in any case of immobilized lipase based on alcohol quenching technology, less loss in activity was observed. The severe decrease in activity of free lipase and just immobilized lipase might be due to protein conformational changes. The decrease in the activity of immobilized lipase using methanol quenching the excessive functional groups was slight and slow. It can be concluded that the physical and chemical properties of support surface have a significantly beneficial effect on the storage stability of the immobilized lipase preparations, as they can stabilize the protein structure and protect it from unfolding even more.

## Conclusions

In this work, alcohol molecules were used to quench the excessive activated functional group on support surface to modulate the microenvironment for enzyme covalently attached on support. Effects of kind and content of alcohol on the relative activity of the immobilized enzyme and the characteristics of the immobilized enzyme were investigated carefully. Under the optimal condition, the immobilized lipase using methanol quenching technology exhibit excellent activity and good thermal and storage stability. Methanol quenches and blocks the excessive epoxy functional group on support surface, which alters the physical and chemical properties to create the suitable microenvironment for stabilize lipase. Suitable micro-environment, resulted from the methanol quenching the excessive active groups, would further favor the activity and the stability of lipase at higher temperature. This technology makes the immobilization a suitable one to produce an industrial biocatalyst and enhance the enzymatic preparation of some chemicals and products in biocatalysis.

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