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Loofah sponge activated by periodate oxidation as a carrier for covalent immobilization of lipase

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Abstract−Cellulose in loofah sponge was first oxidized by sodium periodate at positions 2 and 3 of anhydroglucose unit to introduce aldehyde groups, which were able to react with amino groups of lipase to form Schiff's base, and result in loofah sponge immobilized lipase. The result of periodate oxidation was confirmed by determination of aldehyde group content and analysis of Fourier transform infrared spectra. The optimal pH for both free and immobilized lipases was approximately 7.5. The maximum activity of immobilized lipase was observed at 40 °C, while the free lipase exhibited maximum activity at 37 °C. The immobilized lipase presented improved thermal stability, storage stability, and reusability.

Key words: Loofah Sponge, Periodate Oxidation, Lipase, Immobilization

INTRODUCTION

Compared with conventional chemical catalysts, enzymes are versatile biocatalysts which exhibit high catalysis efficiency and substrate specificity to biochemical reactions under mild reaction conditions. However, the main problems of using the soluble enzymes in industries are the instability of enzymes and difficulty of their recovery and reutilization. To overcome these problems, enzymes are often immobilized onto insoluble solid supports. Immobilization of enzymes not only can increase the stability of enzymes and facilitate the reuse of enzymes but also can reduce the cost of biocatalytic reaction.

Generally, the immobilization of enzymes includes the following four methods: adsorption onto insoluble solid material [1,2], entrapment or encapsulation in a polymeric matrix [3-6], crosslinking with bifunctional reagent [7,8], and covalent binding to insoluble solid carrier [9,10]. The immobilized enzyme by covalent binding has the following advantages: (1) the enzyme does not leak or detach from the support during utilization because of tight binding; (2) the immobilized enzyme can easily contact with the substrate because it is localized on the support surface; and (3) the stability of enzyme increases because of the strong interaction between the enzyme and support material [11].

In search of suitable materials for enzyme immobilization, cellulosic materials have been found to be among the top candidates due to their reproducibility, biocompatibility, biodegradability and processability [12-15]. Among the methods of enzymatic covalent immobilization, the aldehyde groups in cellulosic materials and the amino groups in the enzyme are a good choice to obtain highly stable immobilized enzymes by covalent bond [11,16,17]. Amino groups (in N-terminal and lysine residues) are very reactive, abundant on the enzyme molecules and form Schiff's bases with the aldehyde groups in cellulosic materials. The number of covalent bonds between support and enzyme depends on the degree of support activation and on the concentration of amino groups in the enzyme molecules [18,19].

Loofah sponge is the hard fibrovascular network found within the ripe fruit of *Luffa cylindrical.* As an inexpensive, biocompatible, hydrophilic cellulosic material, loofah sponge has been used as carrier for the immobilization of various biological cells [20-25]. But so far there are few reports on the immobilization of enzyme on loofah sponge. In this paper, the cellulose in loofah sponge was activated by periodate oxidation to generate aldehyde groups in order to obtain a suitable carrier for covalent immobilization of enzyme. The lipase (EC 3.1.1.3) subsequently was immobilized on activated loofah sponge by Schiff's base reaction between the aldehyde groups in carrier and the amino groups in lipases. The aldehyde groups of cellulose in activated loofah sponge were confirmed by Fourier transform infrared spectroscopy and chemical analysis. The enzymatic characteristics, stabilities and reusability of immobilized lipase were investigated and compared with free lipase.

MATERIALS AND METHODS

1. Materials

Loofah sponge, supplied by a local farm, was cut into discs of about 1 cm height and 7 cm diameter. The loofah-sponge discs were soaked in boiling water for 30 min, washed thoroughly with tap water to remove impurities, left in distilled water for 24 h, dried overnight at 50 °C, then stored in a desiccator. Lipase from porcine pancreas in a powder form was purchased from Sigma (St. Louis, MO, USA). *p*-nitrophenyl palmitate (*p*-NPP) was obtained from Fluka (Ronkonkoma, NY, USA). All other chemicals used were of analytical grade from several trademarks.

2. Oxidation of Loofah Sponge with Sodium Periodate

0.5 g of loofah sponge was soaked in 100 ml of sodium periodate solution (10 mg/ml) in 0.1 M acetic buffer (pH 4.0). The mixture

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was placed in the dark and stirred at ambient temperature for 10, 20, 40, 60, 120, 180, 240, 300 and 360 min. The oxidized loofah sponge was then washed with ice-cold distilled water several times to remove the oxidant and used for the immobilization of lipase without drying.

3. Determination of Aldehyde Groups

The aldehyde group content of oxidized loofah sponge was measured according to the method described in literature [26]. The aldehyde groups were selectively oxidized to carboxyl groups by sodium chlorite and the yielded carboxyl groups reacted with calcium acetate to release an equivalent amount of the weaker acid, which was titrated by standardized sodium hydroxide solution using phenolphthalein as indicator. The aldehyde group content was calculated by deducting the carboxyl group content of the original oxidized loofah sponge.

4. Immobilization of Lipase on Oxidized Loofah Sponge

The lipase solution (1.0 mg/ml) was prepared by dissolving enzyme in phosphate buffer solution (PBS: 0.05 M, pH 7.5). The above-mentioned oxidized loofah sponge was incubated in lipase solution (ratio 1 : 60, w/v) with gentle stirring for 1.5 h at room temperature. Then the lipase immobilized loofah sponge was taken out and washed thoroughly with physiological solution until no enzymatic activity was detected in the washing solution. The liquid remnant and washing solutions were pooled for assaying residual enzyme activity and protein concentration. The schematic representation of preparation of oxidized loofah sponge and immobilized lipase is shown in Fig. 1.

5. Determination of Immobilized Protein Amount

Protein concentration was determined by the method of Bradford [27] with bovine serum albumin as the standard protein. The amount of bound protein was indirectly calculated from the difference between the amount of protein used for immobilization and the amount of protein present in liquid remnant and washing solutions after immobilization.

6. Measurement of Lipase Activity

The activities of free and immobilized enzymes were analyzed spectrophotometrically by measuring the increment in the absorption at 410 nm caused by the release of *p*-nitrophenol because of the hydrolysis of *p*-NPP [28]. The reaction mixture which was incubated at 37 °C consisted of 7.7 ml of PBS (50 mM, pH 7.5) containing 0.4% (w/v) Triton X-100, 0.1% (w/v) gum arabic and 0.1 ml of free lipase solution (1.0 mg/ml) or 0.1 ml of above PBS as well as 10 mg of immobilized lipase. The reaction was initiated by adding 0.2 ml of *p*-NPP substrate solution (40 mM) prepared with 2-propanol followed by incubating for 5 min at 37 °C, and then the reaction was terminated by adding 4 ml of Na_2CO_3 solution (0.1 M). The resultant mixture was centrifuged to obtain supernatant. The amount of released *p*-nitrophenol in supernatant was measured by a UV spectrophotometer (Shimadzu UV-1201, Japan) at 410 nm. The lipase activity was calculated from standard calibration curve of *p*-nitrophenol. One unit (U) of lipase activity was defined as the amount of enzyme necessary to liberate 1 μM *p*-nitrophenol per minute under described experimental conditions. The retention activity was defined as the ratio of the activity of the immobilized lipase to the activity of the same amount of free lipase.

7. Effect of pH and Temperature on the Lipase Activity

The effect of buffer pH on free and immobilized lipase activities

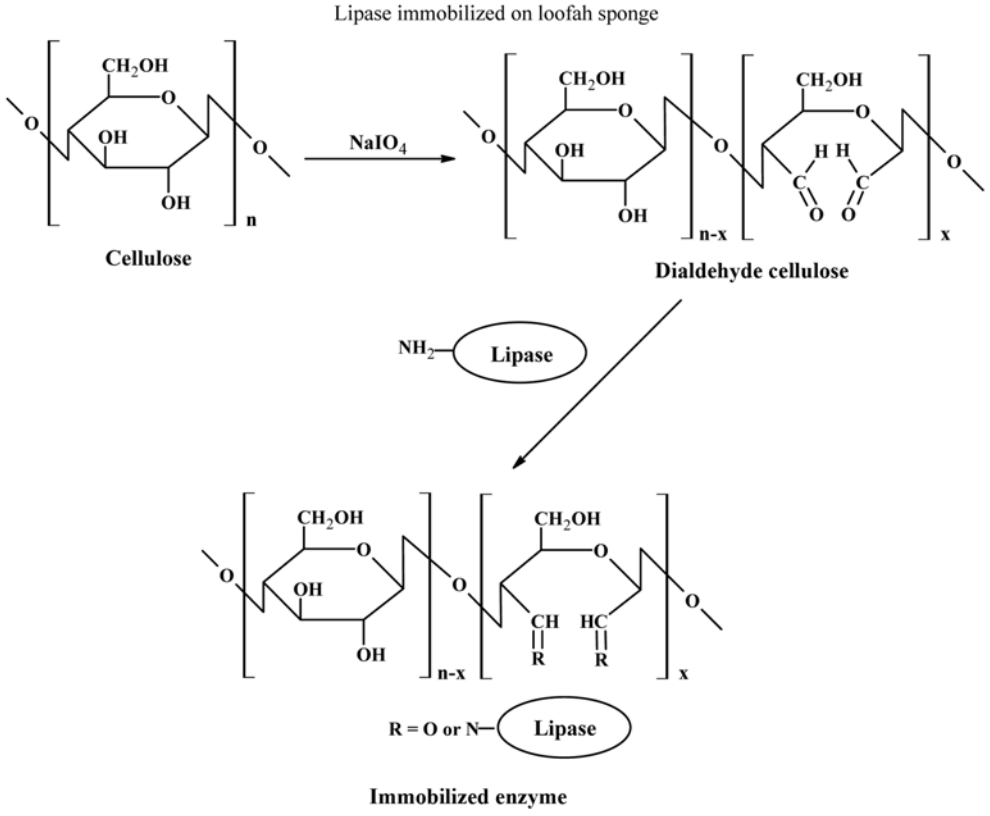

Fig. 1. Schematic illustration for preparation of the oxidized loofah sponge and immobilized lipase.

was estimated with reaction mixtures containing 50 mM of PBS or Gly-NaOH buffer at different pH values (5.5-10.0) at 37 °C. The effect of temperature on both lipase activities was determined at different temperatures in the range of 20-60 °C in 50 mM of PBS (pH 7.5) under the same assay conditions. The activity of free and immobilized lipases at optimal pH and optimal temperature was considered as 100% for their respective reaction.

8. Stability and Reusability of the Immobilized Lipase

For assessment of thermal stability, both free and immobilized lipases were incubated in substrate-free PBS (50 mM, pH 7.5) at 60 °C for 3.5 h. The remaining activities of the enzymes were periodically measured according to the above described method. The activity of free and immobilized lipases without incubation at 60 °C was regarded as 100% for their respective reaction. For investigation of storage stability, both free and immobilized lipases were preserved in a refrigerator at 4 °C for 24 days. The remaining activities of samples were also measured periodically. The activity of free and immobilized lipases without preservation at 4 °C was considered as 100% for their respective reaction. The reusability of the immobilized lipase was evaluated in a successive catalysis cycle process. After each reaction, the immobilized lipase was removed and thoroughly washed with 1% Triton X-100 and 10% 2-propanol in PBS (50 mM, pH 7.5) for the next use. It was then reintroduced into fresh reaction medium. The residual activity was determined as described above and expressed as percentages of its residual activity compared to the initial activity. The activity of immobilized lipase in the first catalysis was recognized as initial activity.

RESULTS AND DISCUSSION

1. FTIR Spectra of Loofah Sponge

The FTIR spectra of loofah sponge and oxidized loofah sponge are shown in Fig. 2. Compared with loofah sponge, the characteristic stretching vibration absorption band of carbonyl groups at 1,738 cm^{-1} obviously increased in FTIR spectrum of oxidized loofah sponge. This result confirmed that the aldehyde groups were introduced into loofah sponge by periodate oxidation.

2. Influence of Oxidation Time on Lipase Immobilization

The influence of oxidation time on lipase immobilization was evaluated by determining aldehyde group content, amount and relative activity of immobilized lipase. As shown in Table 1, the aldehyde group content rose with increasing oxidation time and reached a

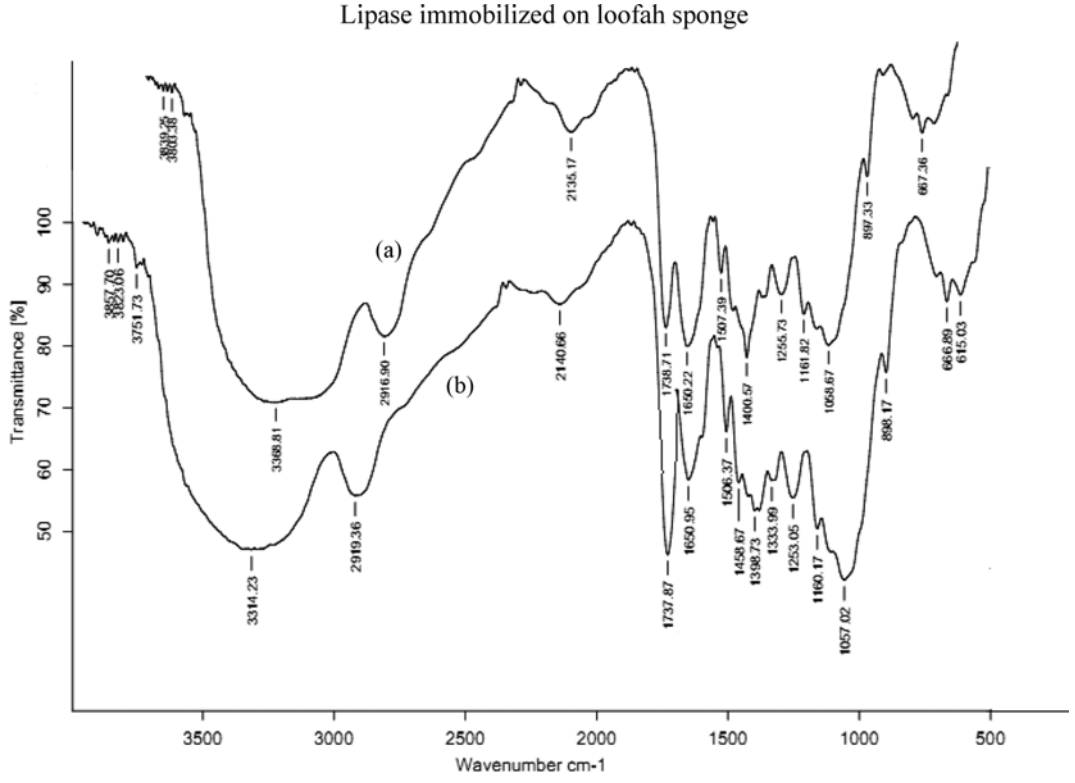

Fig. 2. FTIR spectra of loofah sponge and oxidized loofah sponge.

Table 1. Influence of oxidation time on lipase immobilization

	Oxidation time (min)								
	10	20	40	60	120	180	240	300	360
Aldehyde group content (μM/g)	10.5	15.4	49.6	79.3	134.5	159.7	172.2	176.3	178.1
Amount of immobilized lipase (mg/g)	4.2	5.1	6.5	7.5	10.3	11.9	12.7	12.9	13
Relative activity (%)	32.4	52.6	73.1	84.9	100	99.1	93.8	85.7	76.8

maximum value at about 240 min, since then it kept almost no variety. The amount of immobilized lipase exhibited similar change trend as aldehyde group content. The relative activity of immobilized lipase increased with increasing oxidation time and the peak value was observed at about 120 min; afterwards, it decreased with further increasing oxidation time. It might be attributed to that the excessive increase of aldehyde group content led to more multipoint covalent immobilization, which might have largely inhibited conformational flexibility of enzyme and reduced retention activity of immobilized enzyme [29]. So, the 120 min of oxidation time was chosen for preparing oxidized loofah sponge.

3. Properties of Immobilized Lipase

The effect of pH on the activity of free and immobilized lipases is presented in Fig. 3. The optimal pH for free and immobilized lipases was approximately 7.5, which indicated that no large conformational change occurred in process of lipase immobilization. Furthermore, we found that the optimal pH range of the immobilized lipase was broader than that of the free lipase. It was probably due to the rigid conformation of immobilized lipase, which limited the transition of enzyme conformation with the change of pH.

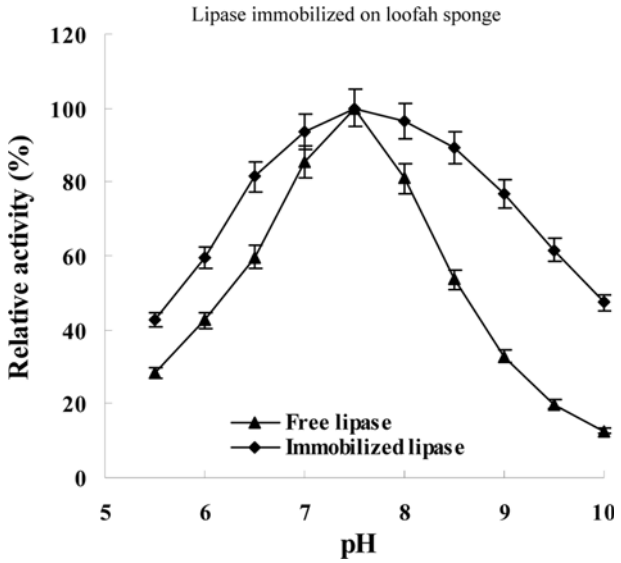

Fig. 3. Effect of pH on activity of free and immobilized lipases.

The influence of temperature on the activity of free and immobilized lipases is shown in Fig. 4. The optimal temperature of the immobilized lipase was 40 °C, which was 3 °C higher than that of the free lipase. It was identical to most literatures; the immobilized enzyme often exhibits higher optimal temperature than its free counterpart. The covalent attachment of the lipase on oxidized loofah sponge by Schiff's base reaction could decrease the conformational flexibility of the enzyme molecule. Thus, the immobilized lipase was much more stable than its free counterpart at higher temperature. In addition, the influence of the temperature on free lipase seemed to be relatively higher than on immobilized lipase, so the optimal temperature range of immobilized lipase was slightly broader.

4. Stabilities and Reusability of Immobilized Lipase

The results for thermal stability of the free and immobilized lipases are in Fig. 5. The immobilized lipase showed better thermal stability than free lipase at all time periods. The free lipase lost almost all its initial activity after heat treatment for 3.5 h at 60 °C, while the immobilized lipase exhibited significant resistance to thermal inactivation and maintained above 50% of its initial activity under the same condition. The enzyme immobilized on carrier often restricts its freedom to undergo drastic conformational changes, thus resulting in increasing thermal stability of enzyme towards heat denaturation.

The results for storage stability of free and immobilized lipases are in Fig. 6. The free enzyme completely lost its initial activity within 10 days, whereas the immobilized lipase only lost about 10% of its initial activity during the same period, and it retained more than 48% of its initial activity after 24 days of storage period. This result indicated that the immobilized lipase exhibited improved storage stability over free lipase. In general, the enzyme is unstable during storage in solution and its activity reduces gradually by time. This decrease in enzyme activity can be interpreted as a time-dependent natural

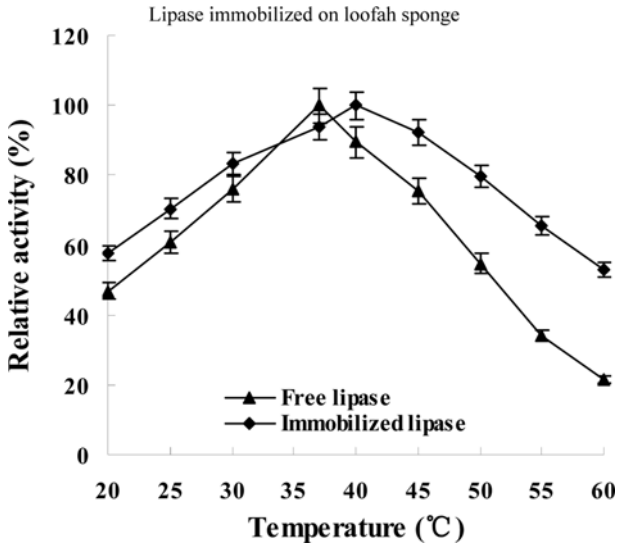

Fig. 4. Influence of temperature on activity of free and immobilized lipases.

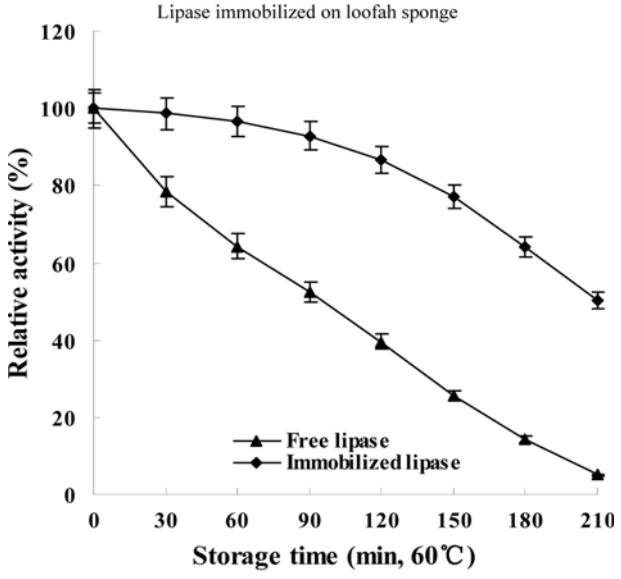

Fig. 5. Thermal stability of free and immobilized lipases.

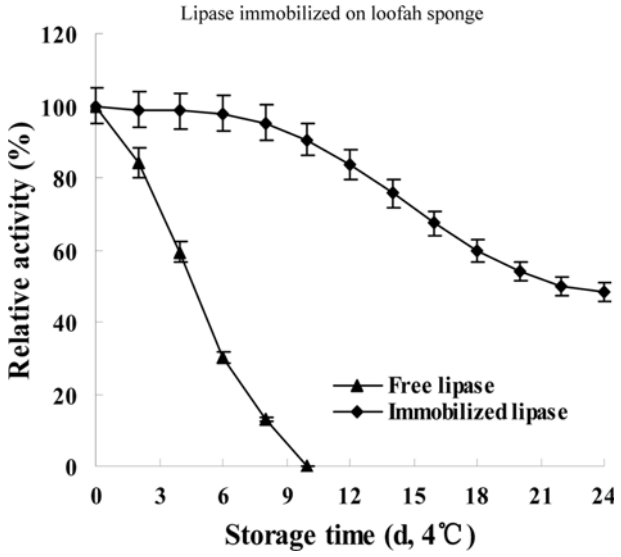

Fig. 6. Storage stability of free and immobilized lipases.

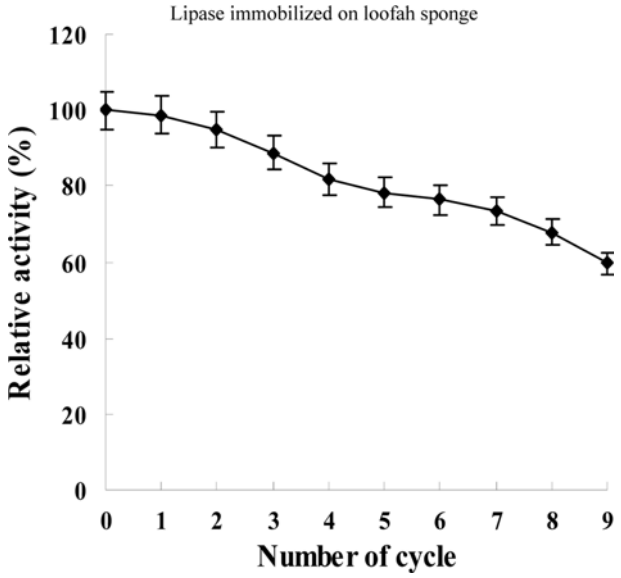

Fig. 7. Reusability of the immobilized lipase.

loss of enzyme activity. It can be prevented to a significant degree by immobilization.

The main advantage of immobilized enzyme is the easy separation and reusability. The effect of repeated use on activity of immobilized lipase is illustrated in Fig. 7. The activity of immobilized lipase decreased gradually during continuous operation process, and the immobilized lipase retained 59.6% of its initial activity after nine reuses. The loss of activity for immobilized enzyme in repeated use process is a common phenomenon [30] that can be attributed to the inactivation of the enzyme caused by the denaturation of protein.

CONCLUSION

Cellulose in loofah sponge was first oxidized by sodium periodate to produce aldehyde groups, and then oxidized loofah sponge was employed as carrier for covalent immobilization of lipase. Loofah sponge immobilized lipase was successfully obtained by Schiff's base reaction between aldehyde groups of cellulose in oxidized loofah sponge and amino groups of lipase. Lipase immobilized on oxidized loofah sponge exhibited excellent thermal stability, storage stability and reusability. Lipase immobilized on loofah sponge by periodate oxidation makes a cheap immobilized biocatalyst for future industrial applications.

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