



***Pseudomonas cepacia* Lipase Immobilized onto Chitosan-Coated Activated Carbon: An Efficient Catalyst for Transesterification Enantiomer Resolution of (R,S)-1-Phenyl-3-buten-1-ol†**

PING XUE, WEIGUANG SU*, WEIJING YE, TIANSHENG ZHAO and XIAOYONG LAI

State Key Laboratory Cultivation Base of Natural Gas Conversion, Ningxia University, Yinchuan, P.R. China

*Corresponding author: Fax: +86 951 2062323; Tel: +86 951 2062835; E-mail: weiguangsu@nxu.edu.cn

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Pseudomonas cepacia lipase (PSL) immobilized onto chitosan-coated activated carbon (CS-AC) with specific surface area of 798 (m²/g), average pore diameter of 2.13 nm and pore volume of 0.43 cm³/g was synthesized by a simple adsorption method. The immobilized lipase PSL/CS-AC catalyst exhibited enhanced catalytic activity for resolving racemic 1-phenyl-3-buten-1-ol compared to free PSL and PSL immobilized on sole activated carbon or chitosan. The enantiomeric excess value (ee_s) of (S)-1-phenyl-3-buten-1-ol and the enantiomeric excess value (ee_p) of (R)-1-phenyl-3-buten-1-ol acetate reached 99 and 94 %, respectively with the reaction substrate conversion of 51 %. PSL/CS-AC immobilized lipase exposed the more surface active sites resulting in the higher catalytic performances. The preparation of the support for immobilizing lipase is low cost and the reaction conditions are moderate and environmentally friendly. The immobilized lipase PSL/CS-AC catalyst possessed of high thermal stability, reusability and storage stability, it could be a potential chiral catalyst for enantiomer resolution reactions.

Keywords: *Pseudomonas cepacia* lipase, (R,S)-1-Phenyl-3-buten-1-ol, Immobilized lipase, Chitosan-coated activated carbon.

INTRODUCTION

Enantiomerically pure alcohols and esters are important intermediates for synthesizing fine organic chemicals and widely used in the pharmaceuticals, agrochemicals and chemical engineering^{1,2}. Optically active 1-phenyl-3-buten-1-ol and its substituted derivatives are important precursors for synthesis of enantiomerically pure natural compounds and biologically active molecules, such as tetrahydrofuran, α,β -unsaturated lactones, antitumour macrolides, alkaloids, chiral epoxy alcohols, β -hydroxy carbonyl compounds and optically pure 1,3-diols, *etc.*³⁻⁵. Efficient transesterification resolution of racemic alcohols is one of the most important reactions to produce optically pure alcohols and esters. In comparison with classical chemical methods such as preferential crystallization, chromatographic separation, biocatalytic processes are broadly accepted as good options to prepare optically pure alcohols⁶. Lipase is one of the important biocatalysts and widely used in the synthesis and resolution of high optically pure chiral compounds^{7,8}, which is mainly based on its high efficiency, stereoselectivity, mild reaction condition and environment friendly, *etc.* However, free lipase is difficult to be widely used in industry because of its insufficient stability, easy deactivation

as well as hard recovery and re-use. In this case, immobilization of lipase can availablely overcome these applied limits^{9,10} and it has been a hotspot in lipase catalysis.

Usually, the properties of immobilized enzyme depend on the nature of the support¹¹. Hence, it is important to develop an efficient support to improve enzymatic properties of immobilized enzyme. Activated carbon (AC) is one of the most promising supports for enzyme immobilization because of its large surface area, high adsorption efficiency¹²⁻¹⁴. However, the enzyme with a benign surrounding microenvironment cannot be provided by simple physical adsorption between enzyme and activated carbon due to low surface OH groups density on activated carbon. Therefore, many investigations have focused on the surface modification of activated carbon with organic functionalities to improve enzymatic properties^{15,16}. Chitosan (CS), the amino polysaccharide has received much attention because of its low toxicity, good biocompatibility, physiological inertness, remarkable affinity to protein and easy availability of its reactive amino and hydroxyl groups^{17,18}. Chitosan was used as a modified material to improve the surface biocompatibility and increase the surface functional groups density of the activated carbon.

In the present work, the surface of activated carbon was modified by the natural polymer chitosan with a physical

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coating method, which was denoted as CS-AC. The lipase (*Pseudomonas cepacia* lipase, PSL) was successfully immobilized on the CS-AC and used for transesterification resolution of (R,S)-1-phenyl-3-buten-1-ol to obtain high-purity chiral single enantiomers. The immobilized lipase PSL/CS-AC showed the highest catalytic activity and enantioselectivity compared with free PSL and PSL immobilized on sole activated carbon or chitosan. The enantiomeric excess value of (S)-1-phenyl-3-buten-1-ol and (R)-1-phenyl-3-buten-1-ol acetate reached 99 and 94 %, respectively at 51 % substrate conversion. Furthermore, the PSL/CS-AC catalyst possessed of higher thermal stability, re-usability and storage stability.

EXPERIMENTAL

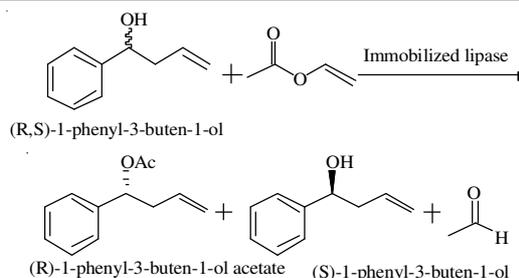
Activated carbon, obtained from Aladdin (Shanghai, China). Chitosan (DACe ≥ 90 %) was obtained from Haidei Bei Marine Biological Engineering Co., Ltd. (Jinan, China). *Pseudomonas cepacia* Lipase (PSL) was supplied by Amano (USA). (R,S)-1-Phenyl-3-buten-1-ol was purchased from Sigma-Aldrich Shanghai Pvt. Ltd. (Shanghai, China). Heptane, isooctane, cyclohexane, toluene, benzene, acetonitrile, vinyl acetate and other analytical reagents were purchased from Kelong Chemicals (Chengdu, China). All chemicals and enzymes were used without any further purification.

Preparation and characterization of chitosan coating-modified activated carbon: The activated carbon was modified by chitosan in order to improve its surface biocompatibility. Chitosan was dissolved in 1 % (w/w) aqueous acetic acid solution at a concentration of 0.1 g/100 mL at 50 °C. Dry activated carbon particles (0.5, 1.0 and 1.5 g) were added to chitosan solution and the resulting mixture was shaken for 1-2 h. After added 0.1 N sodium hydroxide, flocculent precipitate appeared from chitosan solution, the precipitate was obtained by filtration and washed with distilled water to remove residual sodium hydroxide. The chitosan-coated activated carbons CS-AC were dried overnight in a vacuum oven at 60 °C.

The surface area and pore size distribution were derived from the N₂ adsorption-desorption isotherms (Micromeritics ASAP-2010). Surface morphology of CS-AC was observed under a scanning electron microscope (SEM, JSM-7500F/QUANTAX).

***Pseudomonas cepacia* lipase (PSL) immobilization on CS-AC:** *Pseudomonas cepacia* lipase was immobilized onto the CS-AC as follows: 0.1 g CS-AC was dispersed into 10 mL isooctane followed by the addition of 30 mg of PSL in solution. The mixture was shaken in a water bath for 4 h at 30 °C. The resulting products were separated, washed with 10 mL of isooctane and then dried under vacuum, denoted as PSL/CS-AC.

Transesterification resolution of (R,S)-1-phenyl-3-buten-1-ol: 100 mg of immobilized lipase PSL/CS-AC or 30 mg of free lipase was firstly placed in a 25 mL apparatus, followed by the addition of 10 mL of heptane, 92 μ L of vinyl acetate and 0.25 mmol of (R,S)-1-phenyl-3-buten-1-ol. Then the apparatus was oscillated in a 130 rpm water bath shake bed at the reaction temperature. The principle of transesterification resolution of (R,S)-1-phenyl-3-buten-1-ol catalyzed by lipase is shown in the following equation:



Reaction process and enantiomeric excess (ee) were monitored by periodic withdrawal of clear liquid samples from the reaction mixture which were analyzed by high performance gas chromatography (HPGC) (FL9790II, Fuli Analysis Instrument Co., Ltd., China) equipped with Cyclosil-B (Agilent technologies, China). The conversion (C, %) was calculated from the enantiomeric excess of the substrate (ee_s, %) and product (ee_p, %) based on the following formulas:

$$C (\%) = \frac{ee_s}{ee_s + ee_p} \quad (1)$$

$$ee_s (\%) = \frac{[S]_s - [R]_s}{[S]_s + [R]_s}, ee_p (\%) = \frac{[R]_p - [S]_p}{[R]_p + [S]_p} \quad (2)$$

In the formulae, [S]_s and [R]_s denotes the concentration of (S)-1-phenyl-3-buten-1-ol and (R)-1-phenyl-3-buten-1-ol, respectively, also [R]_p and [S]_p denotes the concentration of (R)-1-phenyl-3-buten-1-ol ester and (S)-1-phenyl-3-buten-1-ol ester, respectively.

RESULTS AND DISCUSSION

Characterizations of CS-AC: N₂ adsorption-desorption isotherms of activated carbon and CS-AC with different mass ratios were showed in Fig. 1. With increasing chitosan amount, m (chitosan): m (activated carbon) from 1:15 to 1:5, the adsorption capacity of CS-AC composite gradually decreased (1:5, 1:10, 1:15 mean the mass ratio of chitosan and activated carbon). The surface morphologies of activated carbon with and without coating(s) were obtained by SEM (Fig. 2). The surface of uncoated activated carbon had a rough and highly porous structure. However, the surface of chitosan-coated activated carbon was greatly improved and much smoother than uncoated activated carbon.

Comparing the catalytic activity of immobilized lipase and free lipase: The catalytic activities of transesterification resolution of (R,S)-1-phenyl-3-buten-1-ol as a function of reaction time over the free and immobilized lipase were shown in Fig. 3. It indicated that immobilized lipase PSL/CS-AC exhibited much higher catalytic activity comparing with the free lipase. The conversion of (S)-1-phenyl-3-buten-1-ol reached 50 % on PSL/CS-AC after 12 h, however the conversion was only 6.5 % on free lipase. It was worthy to mention that the enantioselectivity of (S)-1-phenyl-3-buten-1-ol was very high on PSL/CS-AC. After reaction 18 h, the enantiomeric excesses (ee_s) of (S)-1-phenyl-3-buten-1-ol were maintained at 99 %, thereby higher optical purity substrate (S)-1-phenyl-3-buten-1-ol could be obtained. However, the enantiomeric excesses (ee_s) was only 9.0 % on free lipase after 24 h.

Free PSL was well dispersed during immobilization, which decreases the chance of free enzyme convergence in

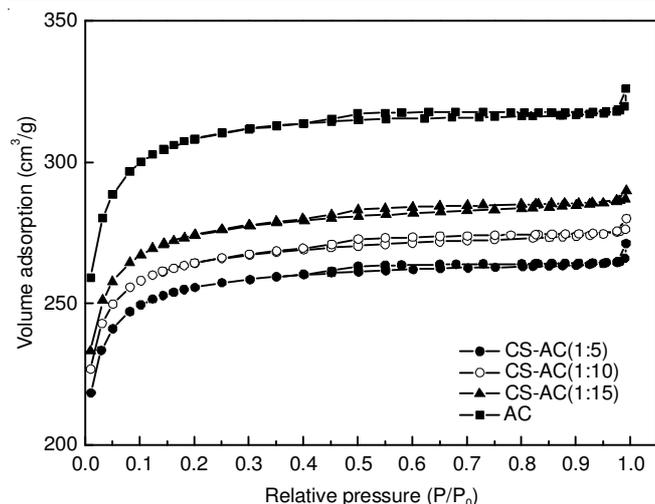


Fig. 1. N_2 adsorption-desorption isotherms of activated carbon and CS-AC

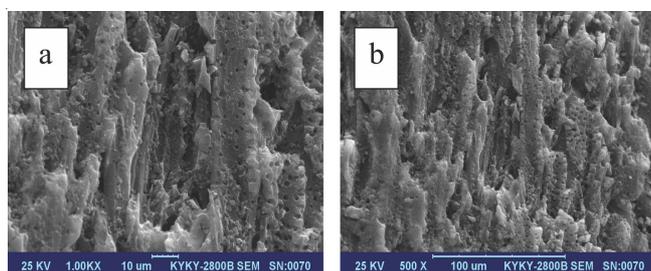


Fig. 2. SEM images of activated carbon (a) and CS-AC (1:10) (b)

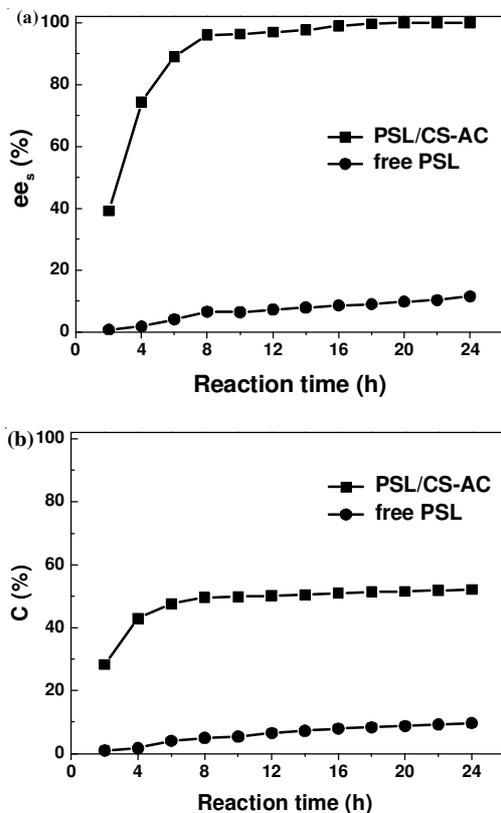


Fig. 3. Compared catalytic performance of PSL/CS-AC and free PSL

organic solvents. Comparing with the free PSL, the exposed active sites on immobilized PSL were much more, increasing the accessibility for substrate to the active site of enzyme and

enhancing the activity of immobilized enzyme. A second possibility was that the conformation of lipase changed during the course of the CS-AC preparation, which possibly made the lipase adopt a more active conformation.

Comparing the catalytic activity of immobilized lipase on different supporters: Fig. 4 displayed the supporter effect on catalytic activity of immobilized lipase. The results showed that the enzyme activity of PSL/CS-AC was higher than that of PSL/CS and PSL/AC. For the immobilized lipase PSL/CS-AC catalyzing transesterification resolution of racemic 1-phenyl-3-buten-1-ol, when reaction temperature was 40 °C and reaction time was 6 h, the ee values of (S)-1-phenyl-3-buten-1-ol and (R)-1-phenyl-3-buten-1-ol acetate reached, respectively 90.1 and 98.1 % with the reaction substrate conversion of 47.9 %. It demonstrated that chitosan could improve the surface biocompatibility of activated carbon and provide a suitable surface for enzyme immobilization. Therefore, chitosan may have a widely potential application in preparation of the required functional carriers.

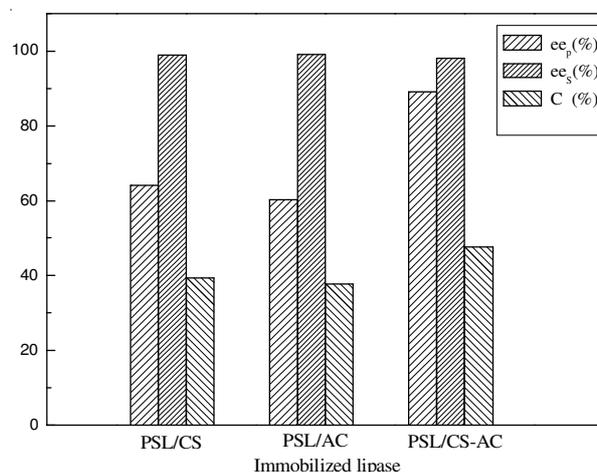


Fig. 4. Compared catalytic performance of immobilized lipase on different supporters

Recycle reusability of immobilized lipases PSL/CS-AC:

The major and important purpose of immobilized lipases is in the design of reusable biocatalysts. Hence, we also investigated the durability of PSL/CS-AC in the repeated catalyzed transesterification of (R,S)-1-phenyl-3-buten-1-ol. After each run, the immobilized lipase was washed several times with heptane and was reintroduced into fresh reaction medium for another assay run. These procedures were repeated up to several times. Fig. 5 showed that the ee, and ee_p values were only slightly decreased during 10 consecutive operations at 40 °C under the same reaction time of 18 h. These results indicated that PSL/CS-AC could retain high enantioselectivity and catalytic performance in the process of reuse and the recycle reusability of PSL/CS-AC was good. However, it was still not ideal because the activity of immobilized lipase was decreased with increasing the recycle number, which may be due to the leakage and deactivation of partial lipase resulting from long-playing mechanical stirring under the continuous reactions.

Comparing the thermal stability of free PSL and immobilized lipases PSL/CS-AC: The transesterification of (R,S)-1-phenyl-3-buten-1-ol catalyzed by PSL/CS-AC in a

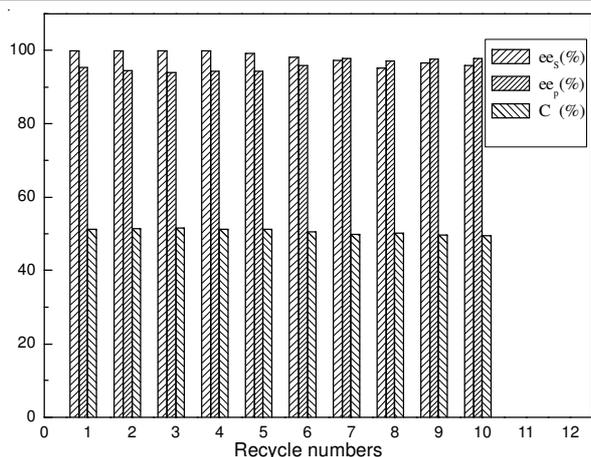


Fig. 5. Reusability of PSL/CS-AC

temperature range from 40 to 85°C was investigated in Table-1 and the highest activity of PSL/CS-AC was obtained at 40 °C. The thermal stability of the free and immobilized lipase was studied under the same temperature range. The enantiomeric excess of (S)-1-phenyl-3-buten-1-ol and (R)-1-phenyl-3-buten-1-ol acetate reached, respectively 63.2 and 96.8 % with the reaction substrate conversion of 40.0 % when the reaction temperature was 85 °C. However, both conversion and enantiomeric excesses (ee_s) were only 0.8 % on the free lipase. It was concluded that the thermal stability of PSL could be greatly improved through the immobilization on CS-AC.

Reaction temperature (°C)	Lipase	ee _s ^a (%)	ee _r (%)	C (%)
40	Free PSL	1.7	99.9	1.7
	PSL/CS-AC	90.1	98.1	47.9
70	Free PSL	1.1	99.9	1.1
	PSL/CS-AC	79.1	95.8	45.2
80	free PSL	0.9	99.9	0.9
	PSL/CS-AC	70.0	97.7	41.6
85	Free PSL	0.8	99.9	0.8
	PSL/CS-AC	63.2	96.8	40.0

^aReaction conditions: reaction time 6 h.

Storage stability of immobilized lipases PSL/CS-AC:

Enzyme is generally stored at 0-4 °C to maintain the stability of its nature conformation. However, enzyme is inevitably exposed to external environment and loses activity rapidly in industrial application. Thus the storage stability of immobilized enzyme has always been of interest. As shown in Table-2, immobilized lipase PSL/CS-AC could remain high catalytic performance and enantioselectivity after storing for 90 days at room temperature, the enantiomeric excesses of (S)-1-phenyl-3-buten-1-ol and (R)-1-phenyl-3-buten-1-ol acetate were still sustained at 94.1 and 99.7 %, respectively with the reaction substrate conversion of 47.4 %.

Storage days (d)	ee _s ^a (%)	ee _r (%)	C (%)
1	99.9	94.9	51.3
90	94.1	99.7	47.4

^aReaction conditions: reaction temperature 40 °C, reaction time 18 h.

Conclusion

In summary, we have successfully developed an efficient method for the transesterification resolution of racemic 1-phenyl-3-buten-1-ol by PSL immobilized on chitosan-coated activated carbon. Chitosan was used as a modified material to improve the surface biocompatibility and increase the surface functional groups density of the activated carbon. The prepared immobilized lipase PSL/CS-AC catalyst exhibited much enhanced catalytic activity for resolving racemic 1-phenyl-3-buten-1-ol to the corresponding optically pure (S)-1-phenyl-3-buten-1-ol compared with the free lipase PSL and PSL immobilized on single activated carbon or chitosan. The ee_s and ee_r reached 99 and 94 %, respectively with the reaction substrate conversion of 51 %. Moreover, the immobilized lipase PSL/CS-AC catalyst possessed of high thermal stability, reusability and storage stability.

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