African Journal of Biotechnology Vol. 9(31), pp. 4954-4965, 2 August, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Characterization of xylose reductase from *Candida tropicalis* immobilized on chitosan bead

Yanqiu Su, Wei Li, Weihua Zhu, Rongqing Yu, Baojing Fei, Tao Wen, Yi Cao and Dairong Qiao*

Microbiology and Metabolic Engineering Key Laboratory of Sichuan Province, College of Life Science, Sichuan University, Chengdu 610064, China.

Accepted 7 June, 2010

A xylose reductase (XR) with high activity and dual coenzyme activity from *Candida tropical* was purified to homogeneity by Ni^{2+}-chelating column, and immobilized on chitosan bead. Studies on free and immobilized XR systems for determination of optimum temperature, optimum pH, thermal stability, pH stability, operational stability, and kinetic parameters were carried out. Free and immobilized XR showed higher activity at 45 and 50°C, respectively. The optimum pH for free and immobilized XR were 4.5, but immobilized XR had higher activity with a broader pH range of 4.0-6.0. Thermal and pH stability of immobilized XR were higher than that of free XR. The residual activity of immobilized XR was about 40% after 7 cycles of batch operation. The Km value of free XR was 30.3 mM, and that of immobilized XR was 20.1 mM, which indicated that the affinity of xylose was increased for immobilized XR. The immobilized XR activity was stimulated by $MnSO_4$, and inhibited by NaCl, βME,Glu. In addition, catalytic efficiency with NADH as cofactor of immobilized XR was better enhanced than free XR. It is the first report on immobilizing XR with chitosan, with a relative high activity.

Key words: Xylose reductase, crosslinked chitosan bead, immobilization, catalytic property, NADH, NADPH.

INTRODUCTION

Xylitol is a naturally occurring five-carbon sugar alcohol, which is found generally in various fruits and vegetables. Xylitol has been highly valued by the pharmaceutical and food industries, among others, due to its high sweetening power, anti-carcinogenic property, and possibilities for uses in diabetic food products. Three methods have been used to produce xylitol, including biochemical extraction, chemical transformation and bioconversion. At present, xylitol is manufactured on an industrial scale by chemical hydrogenation process requiring metal catalysts and extreme reaction condition from hemicellulose-derived xylose. However, the chemical process is not a cost-effective method for the mass production of xylitol because of its high cost of purification with a xylitol yield that is as low as 50-60% (Chung et al, 2002). Alternatively, there has been a great demand of research interests in microbial production of xylitol. In comparison to chemical hydrogenation, many advantages of microbial xylitol production are the possibility of using industrial side-streams as raw material, and fewer by-product, gentle reaction condition as well as simpler production procedure (Jeppson et al., 1999). During microbial xylitol production, D-xylose assimilation in microorganism involves xylose reduction to xylitol via xylose reductase (XR, EC 1.1.1.21), and the chief process is the traditional microbial fermentation, which generally shows many advantages. But the cost of purification of xylitol from broth is still high. There is only one catalyzed reaction from xylose to xylitol. If the enzyme in this reaction immobilized on a suitable support is directly applied in catalytic reaction, this will reflect characteristics of high-performance, faster and lower

*Corresponding author: E-mail: geneium@scu.edu.cn. Tel: +86 28 85412842.

Abbreviations: XR, Xylose reductase; **ALR,** aldose reductase; **AKR,** aldo-keto reductase; **NADH,** nicotinamide adenine dinucleotide; **NADPH,** nicotinamide adenine dinucleotide phosphate; **XDH,** xylose dehydrogenase; **IPTG,** isopropyl-α-D-thiogalactopyranoside; **PBS,** phosphate buffered saline; **BSA,** bovine serum albumin; **EDTA,** ethylenediaminetetraacetic acid.

power consumption for bioconversion. Binding of enzymes on a solid support is an advantageous modification of their application, which can increase their thermostability, operational stability, and recovery. Other advantages are obtained as well, including better operational control, flexibility of reactor design, and ease of product recovery without catalyst contamination (Liaw et al., 2008).

In xylose-assimilating yeast and fungi, XR catalyzes the reduction of xylose for xylitol at the first step of xylose metabolism, which not only shows D-xylose specificity, but also has the affinity of other aldose substrate, such as D-glyceraldehyde, L-gum aldose and D-ribose (Lee, 1998). Based on sequence and structural similarities, XRs were categorized into the aldose reductase (ALR) family, a member of the aldo-keto reductase (AKR) superfamily (Jez and Penning, 2001). XRs can be classified into a major group of nicotinamide adenine dinucleotide phosphate (NADPH)-specific enzymes (ms-XR), and another, smaller group of enzymes (ds-XR) that may utilize both NADPH and nicotinamide adenine dinucleotide (NADH) with physiological catalytic efficiencies according to cofactor specificity (Mishra and Singh, 1993). Ds-XRs prefer the reduction of NADPH to NADH generally (Verduyn et al., 1985; Verduyn et al., 1985; Ho et al., 1990), and may be in favor of NADH in the *Candida parapsilosis* (Lee et al., 2003). NADH is less costly, more prevalent in the cell, and more stable than NADPH (Jeffries and Shi, 1999). From the perspective of practical application, XR depended on the coenzyme NADH alone or dual coenzyme specificity shows higher application value. Xylitol is oxidized to xylulose by xylitol dehydrogenase (XDH) in second step during xylose metabolism, whose cofactor is NADH only (Watanabe et al., 2007). Dependence on the coenzyme NADPH alone for XRs are in favor of accumulating xylitol, but it may result in the intercellular redox imbalance, and impede xylose metabolism. Due to the different coenzyme specificity between NADPH-preferring XR and NAD+-dependent XDH, the yield of xylitol may be influenced at last when cell growth is inhibited in a minimal medium containing D-xylose as a sole carbon source. Therefore, making greater use of coenzyme NADH for XR is very important. In addition, catalytic efficiency is another significant parameter in xylitol production. However, the catalytic efficiencies of all the reported XRs from microorganisms, including *Pichia stipitis* (Verduyn et al., 1985), *C. parapsilosis* (Lee et al., 2003), *Neurospora crassa* (Rawat and Rao, 1997), *Candida tenuis* (Klimacek et al., 2003), and *C. tropicalis* (Yokotama et al., 1995; Oh and Kim, 1998) were not very high.

Xylose reductase gene (*xyl1*) has been cloned from the strain *C. tropicalis* SCTCC 300249, and expressed heterologously in *Escherichia coli* BL21 (DE3). The XR showed dual coenzyme specificity and most importantly the highest catalytic efficiency among the XRs characterized so far (Zhang et al., 2009). In this study, calcium alginate, alginate/chitosan microcapsul, chitosan and polyacrylamide gelatum were utilized to immobilize XR, and the optimized solid support was defined by comparing activity.

The present study investigates suitable conditions as well as the effect of immobilization on the XR catalytic characteristic. Chitosan bead was used as a support for XR immobilization, and the stabilities of the immobilized XR towards pH, temperature, reuse and utilization rate of cofactor NADH were enhanced. These results imply that XR immobilized on chitosan is sutiable for further development of producing xylitol in the industry.

MATERIALS AND METHODS

Xylose reductase gene, enzyme cofactors and plasmids

E.coli BL21(DE3) was used as host strains for gene expression. pMD18-T (TaKaRa, Japan) was used as a cloning vector, and the expression vector used in this work was pET32a (Novagen, USA). *C. tropicalis* xylose reductase gene was isolated from plasmid pMD18-T (TaKaRa, Japan). Enzyme cofactors (NADH, NADPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade or higher.

Expression and purfication of xylose reductase

E.coli BL21 (DE3) with plasmid pET32a-*XYL1* was inoculated into Luria Bertani (LB) medium containing 50 µg/mL ampicillin and grown at 37°C. When OD600 reached 0.6-0.8, isopropyl-α-D-thiogalactopyranoside (IPTG) was added to a differentl concentration of 0, 0.1, 0.3, 0.5, 0.7, 1 mM and incubation was continued for 1-5 h at 26, 30, 34, 37°C. The final concentration of IPTG, time and temperature for induce were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). According to the above conditions, *E.coli* cells were inoculated, inducted, harvested by centrifuging at 10000 rpm for 1 min and resuspended in phosphate buffered saline (PBS), and were then lysed by sonication. The supernatant and lysate pellet fractions were separated by centrifugation at 12000 rpm for 10 min. The supernatant was subsequently loaded onto Ni^{2+}-chelating column (Amersham), and bound proteins were then eluted from the column by a gradient of 20 to 200 mM imidazole in PBS buffer at a flow rate of 100 ml h^{-1}. The purfied fusion was dialyzed against PBS for desalting. All of the fractions flowing through the column were analyzed by SDS-PAGE. The recombinant XR, which carried an artificial sequence of 43 amino acids, was cleaved with enterokinase at 37°C for 24 h. Then the reaction mixture was loaded onto the Ni^{2+}-chelating column again. The purified protein was obtained from the flow through Ni^{2+}-chelating column, and frozen in 10% glycerol at -70°C.

Activity assay of free and immobilized XR

Xylose reductase activity was monitored spectrophotometrically by measuring the oxidation of NAD(P)H in a spectrophotometric cuvette at 340 nm at room temperature. Unless otherwise indicated, the assay volume of 3 ml contained 1.9ml 100 mM potassium phosphate buffer (pH 7.4), 0.3ml 1.5 mM NAD(P)H, 0.6ml 200 mM D-xylose and 0.1µl of enzyme solution. This reaction mixture was allowed to stand for 1 min to eliminate the endogenous oxidation of NAD(P)H. The reaction was started by the addition of 0.1 ml of enzyme solution. One unit of xylose reductase activity was defined as the amount of enzyme that can oxidize one micromole of

NAD(P)H per minute. Specific activity was expressed as units of the enzyme per mg of protein. Protein concentration was measured by the Bradford (1976) method with bovine serum albumin (BSA) as the protein standard.The results presented show the means of triplicate assays.

Screening of immobilization mthods for XR

The four methods of calcium alginate gels embedding (Walter et al., 2003), alginate/chitosan microcapsul (Lu et al., 2007), chitosan crosslinked with glutaraldehyde (Gamze and Senay, 2007) and polyacrylamide gelatum emdedding (Liaw et al., 2008) were used to immobilized XR, respectively, according to the method previously reported. The best method will be screened by determining the activity of the immobilized XR in aqueous medium with standard method already mentioned.

Preparation of chitosan beads and immobilization of XR

Preparation of chitosan beads and immobilization of XR on chitosan bead were performed by the procedure of Akku et al. (2003) with some modifications. Some parameters in the test were measured by one-factor experimental design. 1-5 g of chitosan flakes were added into 95-99ml of distilled water and suspended by magnetic stirring for 10min. Then 1-5 ml of glacial acetic acid was added, and mixing continued for 2 h at room temperature. All the final concentraion range of chitosan and acetic acid were 1-5% (m/v and v/v). The solution thus obtained was stored at room temperature and was used within 1 week of preparation. NaOH was dissolved in 3.0% (v/v) methanol solution, and the concentration of NaOH was 2, 3, 4, 5 and 6% (m/v). The solution was mixed and used for the solution of coagulating. Chitosan solution was added dropwise into the coagulation solution at room temperature by pumping the chitosan solution (1.5 ml min^{-1}) through the internal tube of an injector. The beads were allowed to cure for 1, 1.5, 2, 2.5 and 3 h and washed three times with distilled water. Freshly prepared beads were incubated in different concentration of glutaraldehyde solution (4, 5, 6, 7, 8%; w/v) in 0.1 M phosphate buffer (pH 7.0) for 2, 4, 6, 8 and 10 h at 30°C. The reinforced beads were washed several times by distilled water to abscise glutaraldehyde on the surface of chitsoan bead. Finally chitosan bead was added in the XR solution (50, 100, 150, 200, 250 U g^{-1}, w/w). The suspension was kept at 30°C with slight stirring for 1 h, and at 4°C for different time of 4, 8, 12, 16 and 20 h for adsorption. Then it was washed with sodium chloride until no protein was detected. Finally, the beads were dried in a vacuum incubator at room temperature and stored at 4°C. Each reported value was the mean of three experiments at least, and the standard deviation was within ±5%.

The optimum temperature and pH value of free and immobilized XR

The temperature activity profiles of free and immobilized XR was measured in the range of 30-65°C by standard method with NADPH as cofactor. This was determined at indicated temperatures in 0.1 M phosphate buffer (pH 7.0). Enzyme activity as a function of pH was determined at 30°C in 0.1 M phosphate buffer over the pH range 1.5-8.0 by standard method using NADPH as cofactor. The results of pH, temperature of the medium are presented in a normalized form, with the highest value of each set being assigned the value of 100% activity.

The thermal and pH stability of free and immobilized XR

The thermal stability of free and immobilized XR was ascertained by measuring the residual activity of enzyme exposed to various temperatures (45-80°C) in 0.1M phosphate buffer (pH 7.0) for 1 h, and then determining the residual activity at optimum reaction temperature. Activity of samples was performed at optimum conditions. The pH stabilities of the free and immobilized XR were assayed by immersing the samples in the phosphate buffer (0.1 M, pH 7.0) in the pH range of 1-6 at 30°C for 1 h, and then determining their activities. Activities of samples were performed at optimum conditions.

The operational stability of free and immobilized XR

Operational stability of the immobilized XR was determined for 7 successive batch reactions. After each reaction run, the immobilized XR preparation was removed and washed with 0.1 M phosphate buffer (pH 7.0) to remove any residual substrate on the chitosan beads. Then, they were reintroduced into fresh reaction medium and enzyme activities were detected at optimum conditions.

Kinetics assay of free and immobilized XR

Kinetics parameters for xylose were obtained from the initial velocity at the constant level of NADPH (0.15 mM) using xylose as the varied substrate. All of the measurements were performed at optimal pH and optimal temperature.

The effect of chemical on specific activity of free and immobilized XR

The free and immobilized XR were in the presence of several chemicals (0.1 mM) for 1 h.Then the sepcific activity of free and immobiliaed XR were measured, including $MgCl_2$, $MnSO_4$, $ZnCl_2$, $CaCl_2$, $NiCl_2$, $FeCl_3$, NaCl, KCl, EDTA, $CuSO_4$, βME, Glu, dithiothreitol (DTT), Cys and BSA.

RESULTS

XR fusion protein expression and purification

pET32a (+) was used to transform *E.coli* BL21 (DE3) to obtain high level expression of the *xyl1* gene encoding XR. The studies indicated that the highest expression of XR protein was obtained after induction for about 4 h by 0.5 mM of isopropyl-b-D-thiogalactoside at a temperature of 30°C (Figure 1 A, B, C) when the OD_{600} reached to 0.6. The fusion XR with an enterokinase cleavage site immediately upstream to XR was expressed abundantly, which accounted for nearly 80% of the total cellular protein in a soluble form (Fig. 1D). The purified soluble fusion XR on SDS-PAGE was approximately 57 kDa (Figure 2), which is consistent with its theoretical molecular weight. By means of other purification procedure, a mass of purified protein was obtained, which would be used for immobilization test below.

Screening of immobilization methods for XR

All the efficiency of the four methods had high residual activity which was above 70% (Table 1). In the four

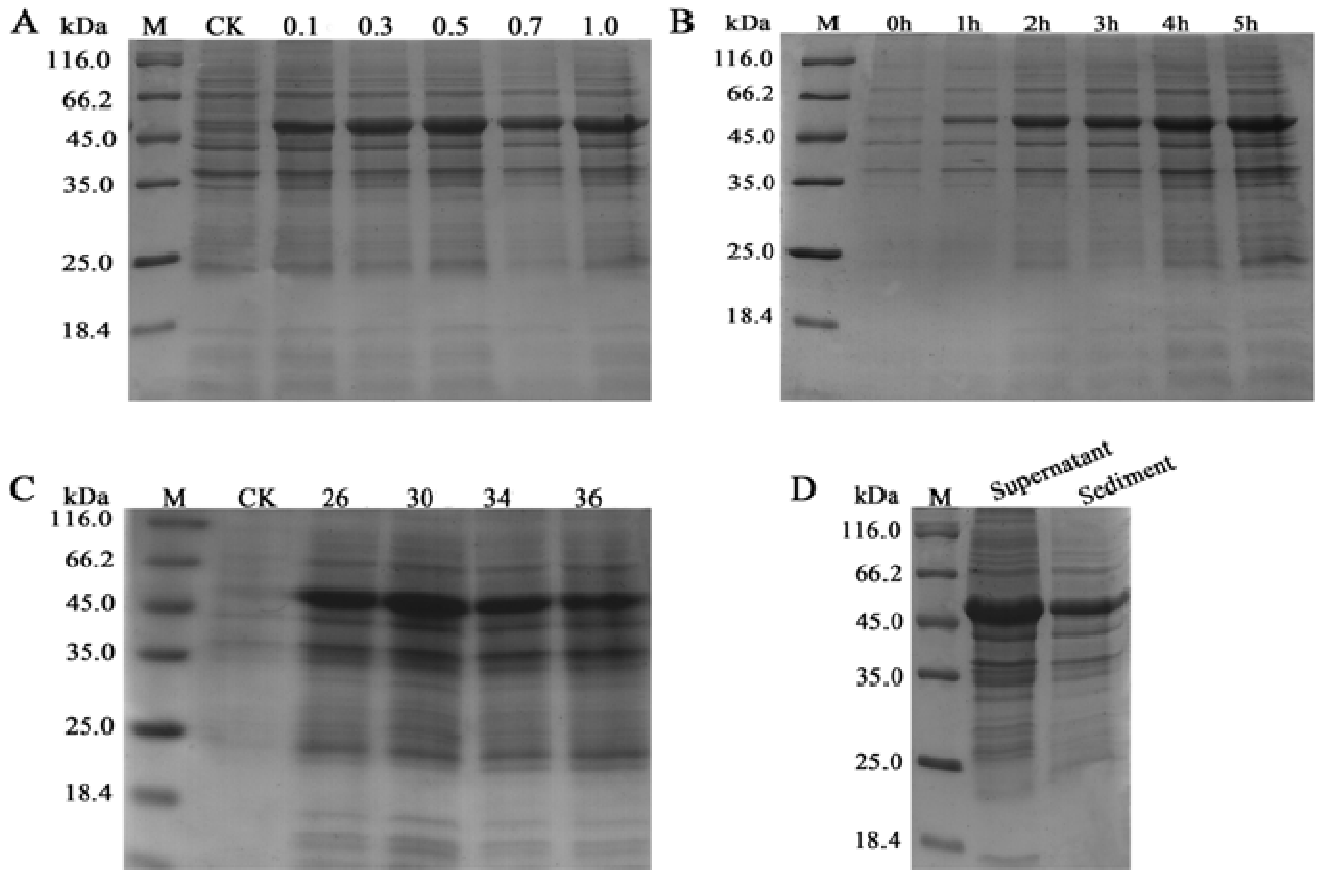

Figure 1. SDS–PAGE analysis of XR on the inducation condition. (A) When OD600 reached 0.6-0.8, the final IPTG concentration of 0, 0.1, 0.3, 0.5, 0.7, 1 mM were added and incubation was continued for4 h at 30℃; (B) After adding IPTG for final concentraion of 0.5 mM, the time of inducation were kept for 1-5 h at 30℃; (C) The temperature of 26, 30, 34 and 37℃ were set for inducation of 4 h with 0.5 mM IPTG final concentration; (D) The expression amount of XR cellular protein in a supernatant and sediment was compared using SDS-PAGE.

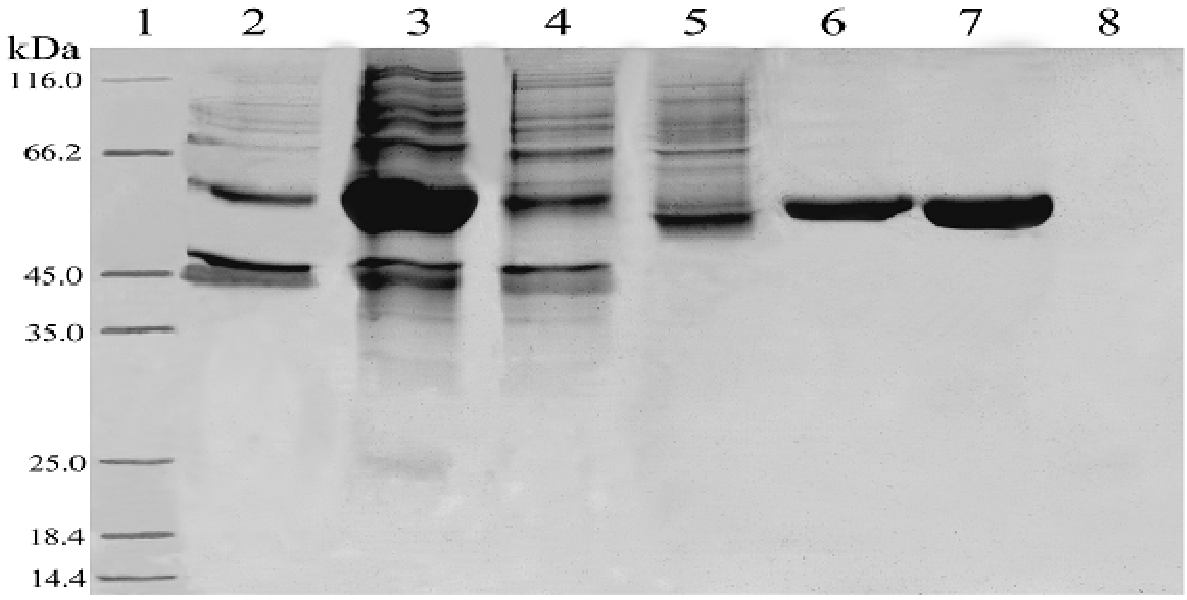

Figure 2. SDS-PAGE analysis for the purification of XR-His_6-tagged fusion protein. Lane 1: the molecular weight maker; 2, sedimentum; 3, supernatant; 4, the flow through during purification; 5, 6, 7, 8, protein eluted from the column by elution buffer containing 20, 50, 100 and 200 mM imidazole.

Table 1. Comparison of efficiency with four different immobilization methods of xylose reductase.

Types	Specific activity of free XR (λ/Umg^{-1}) [a]	Specific activity of immobilized XR (λ/Umg^{-1})[a]	Residual activity (r/%)
Alcium alginate gels embedding	124.39	89.58	72.0
Alginate/chitosan microcapsul	124.39	91.39	73.48
Chitosan crosslinked with glutaraldehyde	124.39	94.23	75.76
Polyacrylamide gelatum emdedding	124.39	94.75	76.17

[a]According to the method reported previously, XR were immobilized with four methods respectively; the activity of the immobilized XR was detected with standard method using NADPH as cofactor; specific activity of free XR was taken as 100%.

methods, the residual activities of chitosan crosslinked with glutaraldehyde and polyacrylamide gelatum emdedding were higher than other methods. Taking into account the industrial cost and the safety of the food industry, chitosan crosslinked with glutaraldehyde was selected to immobilize XR.

Optimisation of XR immobilisation in chitosan

As presented in Figure 3, the optimum condition of chitosan bead is as shown below: four grams of chitosan flakes were added into 97 ml distilled water and suspended by magnetic stirring for 10 min. 3ml of glacial acetic acid was then added and mixed. Chitosan solution was added dropwise into the coagulation solution (2% NaOH, 3% CH_3OH) at room temperature by pumping the chitosan solution (1.5 ml min^{-1}) through the internal tube of an injector. The beads were allowed to cure for 1.5 h and washed three times with distilled water. Freshly prepared beads were incubated in glutaraldehyde solution (6%) for 10 h at 30°C. The reinforced beads were washed with distilled water until the glutaraldehyde was removed. Finally chitosan beads were mixed with XR solution (100 U g^{-1}), and inoculated for 1 h with slight stirring at 30°C, and kept at 4°C for 12 h, and finally washed with PBS to remove free XR. Then, the beads were dried in a vacuum incubator at room temperature and stored at 4°C.

The effect of immobilization on catalytic properties of xylose reductase

The optimum temperature and thermal stability of free and immobilized XR

The temperature dependence of the activities of the free and immobilized XR were studied in the temperature range 30-60°C in 0.1M (pH 7.0). Tempe-rature profiles of free and immobilized XR are shown in Figure 4. Optimum temperature for free XR was found at about 45°C. It is often observed that immobilized XR showed higher activity at a wide range of temperatures 45-55°C, and the loss of the activity of immobilized XR was lower than that of the free XR for high temperatures.

Thermal stability was investigated by incubating free and immobilized XR at temperatures ranging from 45-80°C for 1 h and then determining the activity at optimum reaction temperature. The effect of temperature on stability of free and immobilized XR were illustrated in Figure 5. All the free and immobilized XR had the highest stability at 45°C. However, the free XR activity decreased significantly when temperature was higher than 45°C. The residual activity of the free XR was lower than 20% at 70°C. At 45-70°C, the activity of immobilized XR decreased slightly, and all the residual activity were more than 50%. These results suggested that the thermostability of immobilized XR became significantly higher than that of free XR at higher temperature.

The optimum pH and pH stability of free and immobilized XR

The pH dependence of the immobilized XR activities was compared with that of the free enzyme in the pH range of 1.5–8.0 at 30°C, and the results are given in Figure 6. The optimum pH values for free and immobilized XR were the same at pH 4.5. But the activity of immobilized XR was higher than free XR in a broader pH range 4.0-6.0. Therefore, the relative activity of the immobilized lipase was improved in a broad pH range compared with the free one.

The pH stabilities of immobilized and free XR were compared by immersing in buffer solutions of different pH for 1 h at room temerature, and then the activity at its optimum pH was determined. It can be found from Figure 7 that the residual activities of free and immobilized XR were highest at pH 5.0. When pH value was more than or less than 5.0, the stability of free XR decreased noticeably, the residual activity of immobilized XR was more than 60%. The specific activity of immobilized XR was still 79.778 U mg^{-1} when the pH value was 2.5. Therefore, it is shown that the immobilized XR have better pH stability.

Reusability of immobilized XR

Reusability of immobilized enzyme is one important para-meter. The effect of repeated use on activity of immobilized

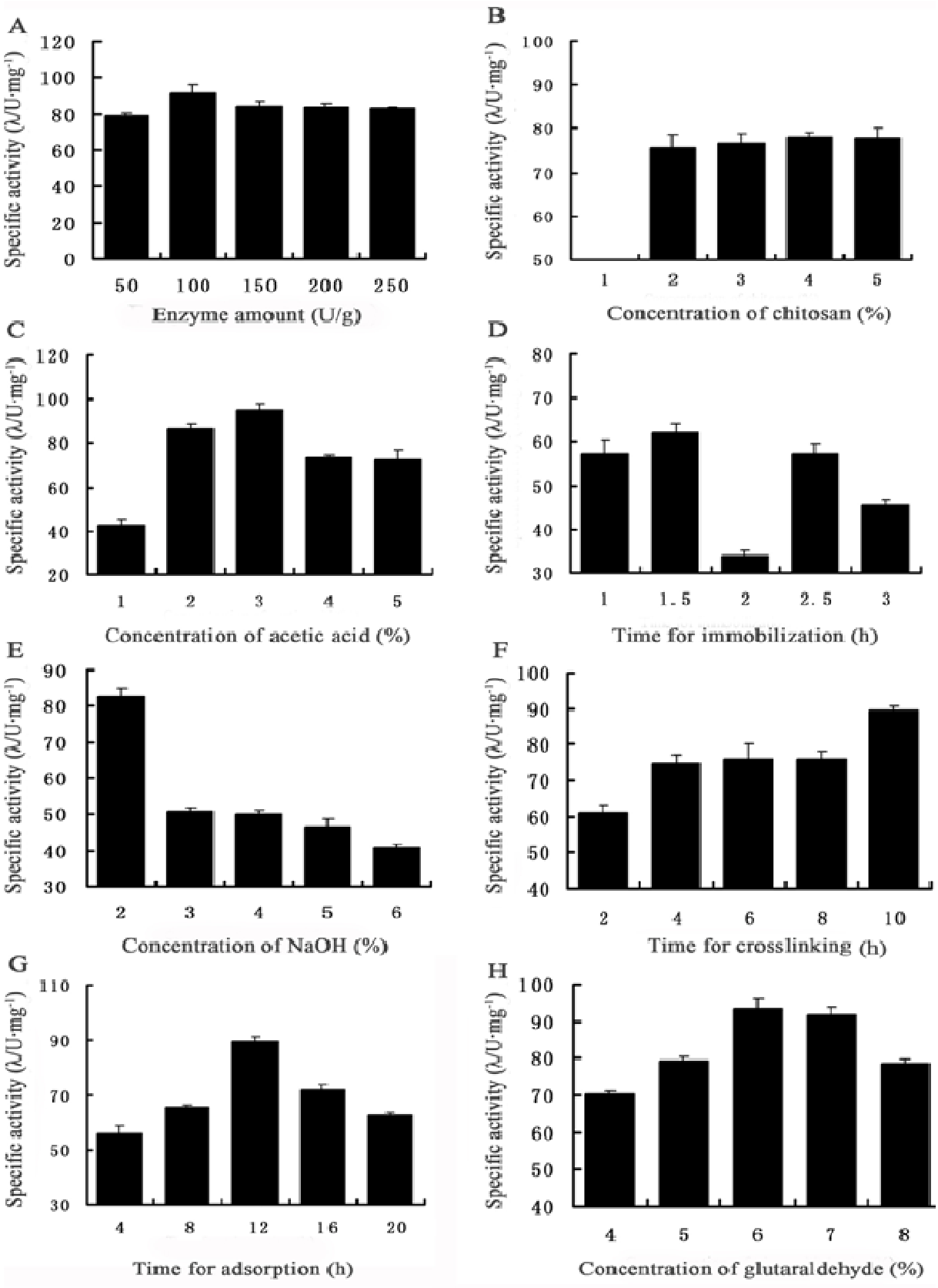

Figure 3. Optimisation of XR immobilisation in chitosan. Eight parameters of immobilisation were measured by one-factor experimental design and the procedure was performed in accordance with Akku (2003) method. Each reported value was the mean of three experiments at least, and the standard deviation was within ±5%.

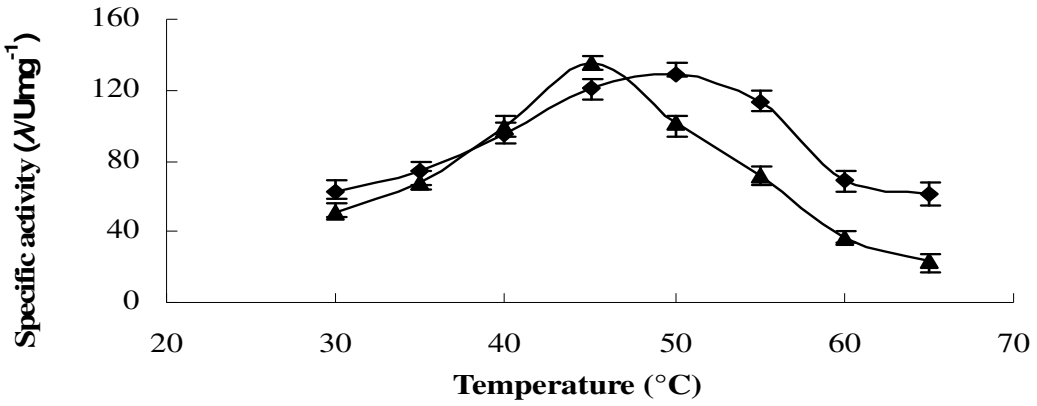

Figure 4. Effects of temperature on the free (▲) and immobilized XR (♦).The activity of free and immobilized XR was assayed with NADPH as cofactor incubated from 30 to 65℃ in 0.1 M phosphate buffer (pH 7.0) by standard method.

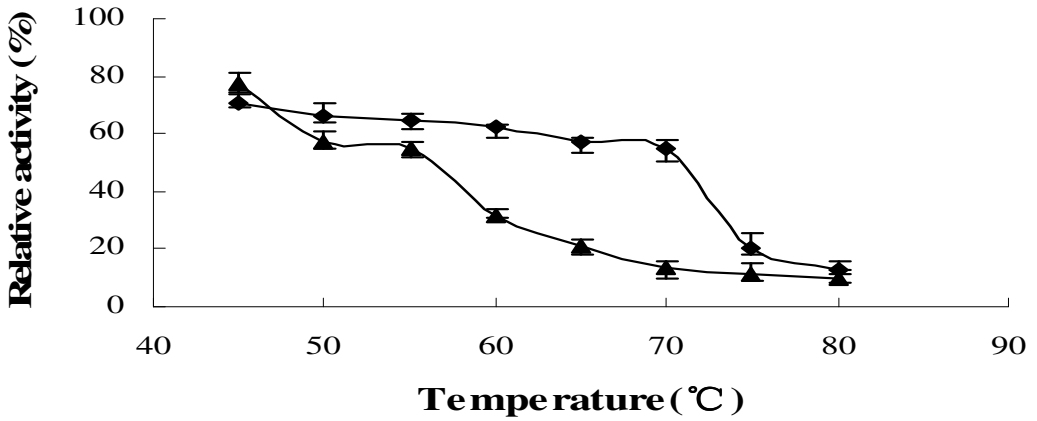

Figure 5. The thermal stability of the free (▲) and immobilized XR (♦). The thermal stability was investigated by keeping free and immobilized XR in the 0.1 M phosphate buffer (pH 7.0) at temperatures ranging from 45 to 80℃ for 1 h. Then the residual activity was determined with NADPH at 30℃ in the 0.1 M phosphate buffer (pH 7.0); specific activity of free XR was taken as 100%.

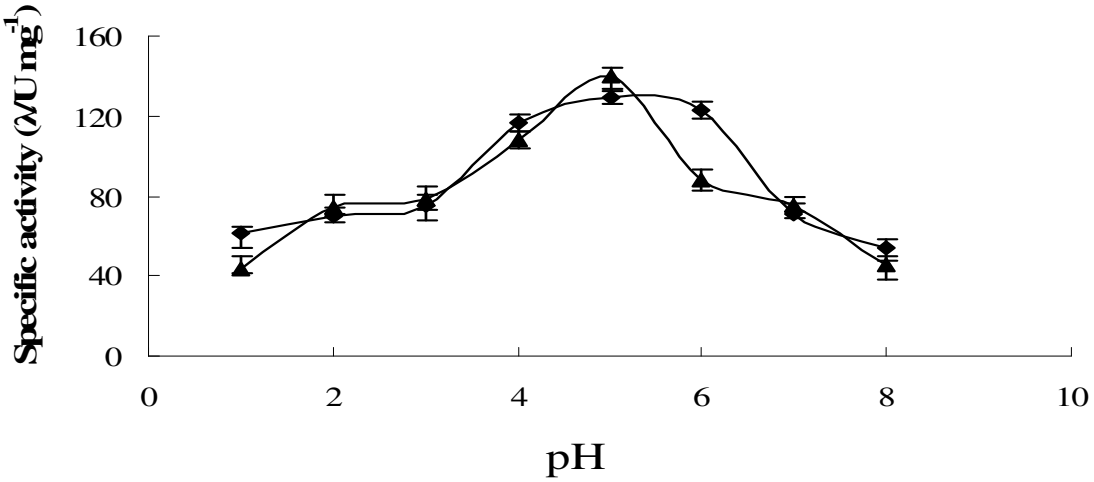

Figure 6. Effects of pH on the free (▲) and immobilized XR (♦). Activity assays pH ranged from 1.5 to 8.0, employing NADPH as cofactor at 30℃ in the 0.1M phosphate buffer (pH 7.0).

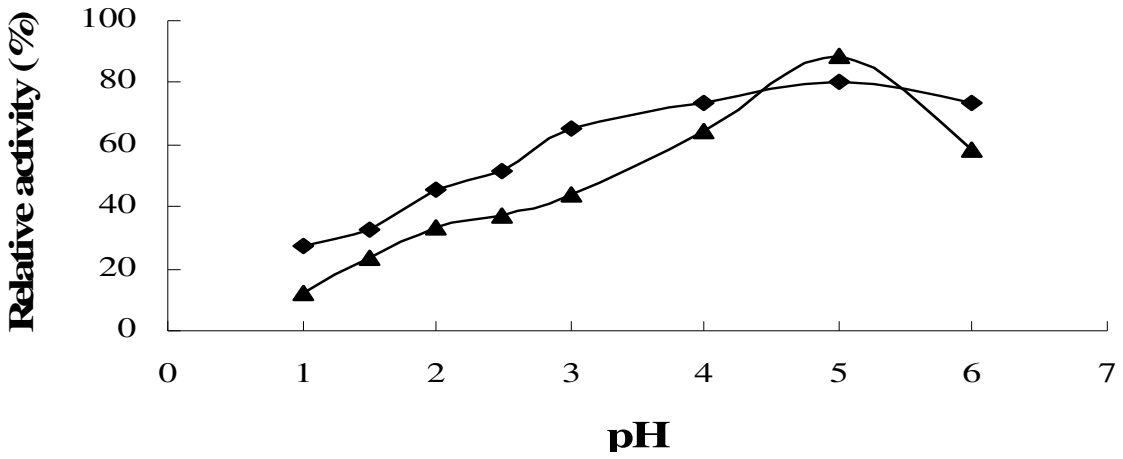

Figure 7. pH stability of the free (▲) and immobilized XR (♦). The pH stability was ascertained by measuring the residual activity of enzyme exposed to various pH (1.0–6.0) buffer solution for 1 h. Activities of samples were performed with NADPH at 30℃ in the 0.1M phosphate buffer (pH 7.0). Specific activity of free XR was taken as 100%.

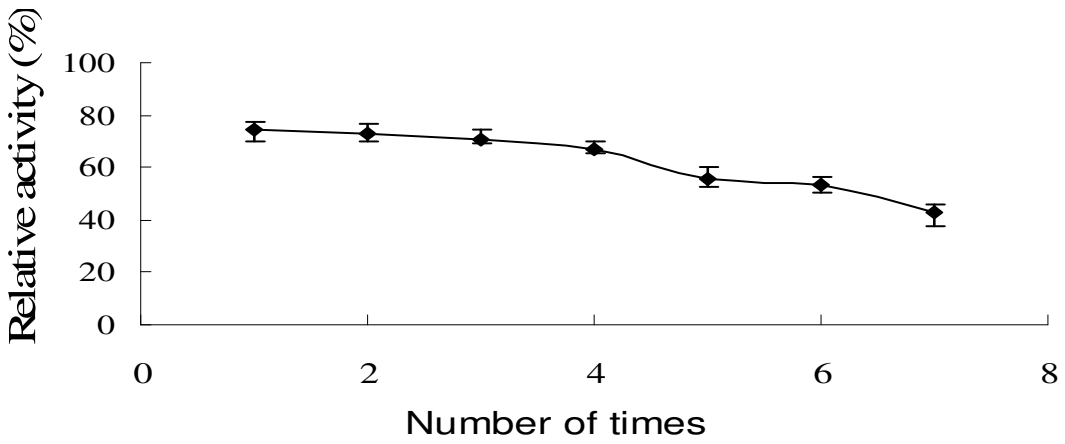

Figure 8. Reusability of immobilized XR. The activities of immobilized XR were detected repeatedly with NADPH at 30℃ in the 0.1M phosphate buffer (pH 7.0). The specific activity of immobilized XR in the first run was taken as 100%.

XR is shown in Figure 8. The XR immobilized on the chitosan beads retained the similar specific activity as the initial within four reuses. After that, the specific activity decreased gradually. However, the residual activity of immobilized XR was still 42.64% after 7 reuses. The properity of immobilized XR brought an advantage over using free XR.

Kinetics assay

Kinetics of free and immobilized XR was investigated at various concentrations (25-150 mM) of xylose as a substrate. These datas were plotted using the Lineweaver-Burk plot (Figure 9) and the apparent Km was calculated from the graphs. The Km value of free XR was 30.3 mM (Table 2), and that of immobilized XR was 20.1 mM. The former was found to be higher than that of the latter.This decrease in apparent Km value indicated that the affinity of substrate for immobilized XR was increased compared to the free XR.

The effect of chemicals on the specific activity of free and immobilized XR

The impacts of several chemicals on the activity of free and immobilized XR were also evaluated. The acivity for free and immobilized XR was stimulated by Cys and BSA, but inhibited strongly by $ZnCl_2$, $FeCl_3$, $CuSO_4$, DTT. Especially, $FeCl_3$ had mightly inhibited the activity of free and immbilized XR, and made immobilized XR activity

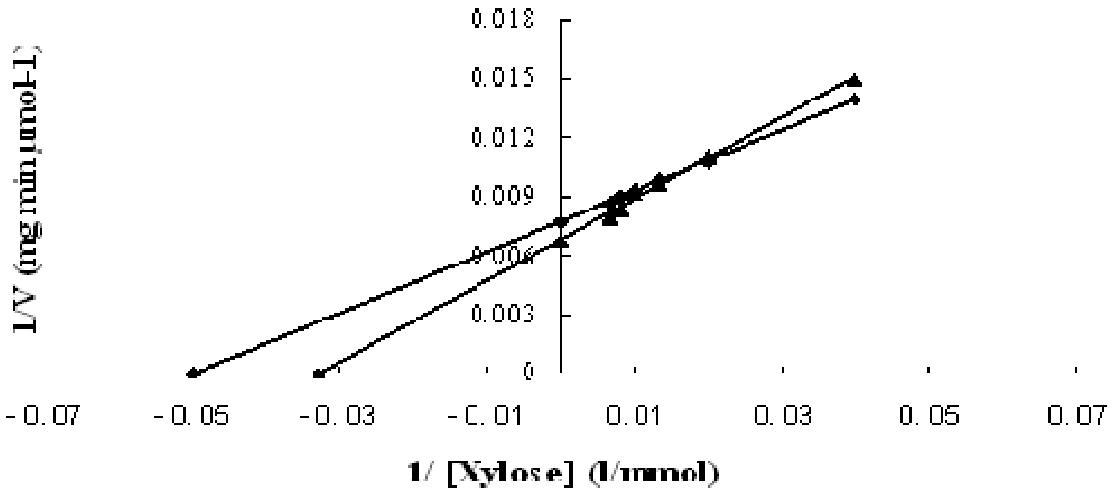

Figure 9. Kinetic parameters of the free (▲) and immobilized XR (♦).Using xylose as a variable, the kinetics was determined by assaying the XR activities with NADPH at 30°C.

Table 2. Kinetic parameters for free and immobilized XR.

Types	*Vmax* (U/mg of protein) [a]	*Km* (mmol)
Free XR	147.06	30.3
Immobilized XR	129.87	20.1

[a] The specific activity of XR in this work was carried out at optimal pH and temperature, and with NADPH as a cofactor.

chose to 0 U mg^{-1}. The other nine chemicals had shown lower influence on the activity of free XR, but some chemicals during the nine chemicals enhanced XR activity distinctly such as $MnSO_4$, and others reduced XR activity obviously with NaCl, βME. Figure10 showed the effect of part of the fifteen chemicals on the immobilized XR.

The effect of immobilization on cofactor (NADH) availability of XR

Determining by means of standard method with NADH as cofactor, the activity of free XR was found to be 2.19 U mg^{-1}, and that of immobilized XR was 37.58 U mg^{-1}. The XR immobilized on chitosan beads exhibited specific activity which was about 17.15 fold higher than that of free XR (Table 3).

DISCUSSION

Chitosan (poly-*β* (1→4)-2-amino-2-deoxy-d-glucose) is the product of deacetylation of chitin. In a linear polysaccharide, there are a great quantity of hydroxyl and amino groups, which make the physical and chemical properties of chitosan (e.g. porosity, degradability) easily modified in mild conditions (Paul and Sharma, 2000). In addition, chitosan is an inexpensive, inert and hydrophilic support, and is thus attractive for enzyme immobilization. Except for using chitosan as immobilizing materials, there are also many other solid supports, such as calcium alginate, polyacrylamide cellulose and so on. Among the various available matrices for immobilization, alginate is a natural biopolymer extracted from brown alga, and in the presence of divalent cations like Ca^{2+} alginates can form hydrogel. This method for making hydrogel is simple and does not require drastic conditions, and calcium alginate can be accepted as food additives with regard to food applications (Prashanth and Mulimani, 2005). However, the gels is instable, soft and even dissolve in the solution containing multivalent anions or high concentration of electrolyte, which limits its application. Chitosan is used to have a modification of the calcium-alginate beads' surface. The treatment with chitosan in media could improve the properties of calcium-alginate gel, but the residual activity of XR on the gel is not very high in the work, and it demands a complicated procedure for forming alginate/ chitosan microcapsul. Polyacrylamide gel is a synthetic polymeric gels derived from acrylamide and has better mechanical strength, semipermeability, unreactiveness, and no physical chemical reaction with the enzyme, as well as less loss of specific activity among others (Liaw et al., 2008). Nevertheless, N, N-methylene bisacrylamide shows nerval toxicity, and thus is not suitable to be applied in the food industry. Although the residual activity of XR immobilized on this support was high as well as the chitosan, it was not used as immobilization matrix (Table 1). Comparing with the three former materials, chitosan exhibits a unique set of characteristics such as bio-compatibility, biodegradability to harmless products, non-toxicity, physiological inertness,

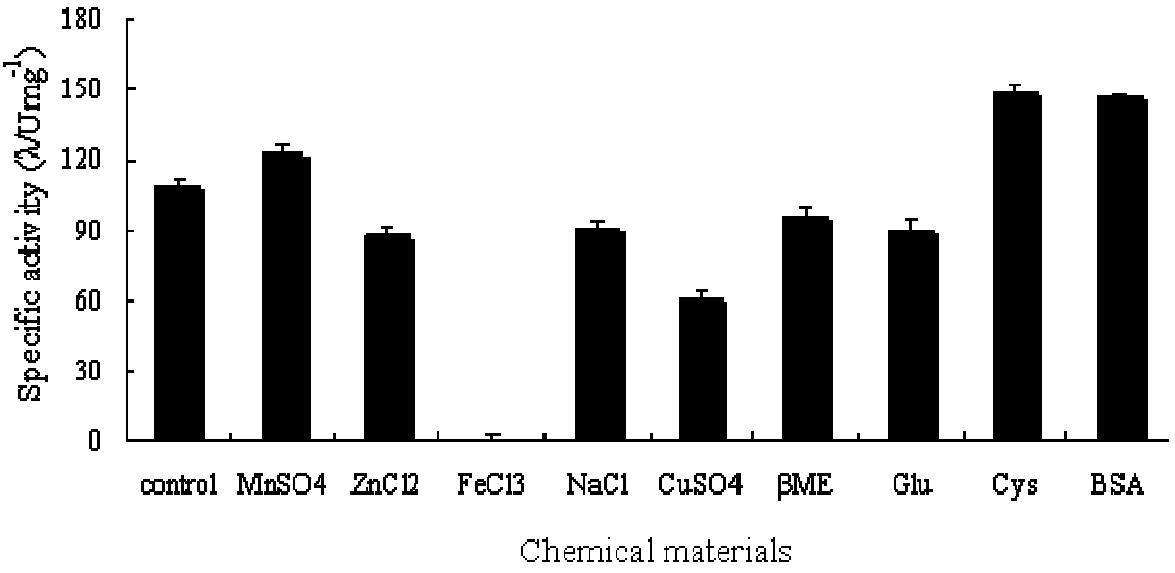

Figure 10. Effect of chemical materials on the immobilized XR activity. In the presence of several chemicals (0.1 mM) for 1 h, the sepcific activity of free and immobiliaed XR were measured under standard condition.

Table 3. The availability of cofactor NADH for XR.

Types	Total protein (μg/100 μl)	Specific activity (U/mg of protein)[a]	Availability (fold)
Free XR	3.5	2.19	1
Immobilized XR	3.5	37.57	17.15

[a] The specific activity of XR in this work was carried out with NADH as a cofactor at optimal pH and temperature.

hydrophilicity, remarkable affinity to proteins, and high mechanical strength (Altun and Cetinus, 2007). These significant biological and chemical characteristics make chitosan a desirable biomaterial for XR immobilization, which might provide an optimal microenvironment for XR to maintain relatively high biological activity (75.76%) and stability (Table 1).

After immobilizing XR on chitosan bead, the optimum temperature range was extended and thermostability of immobilized XR becomes significantly higher than that of free XR at higher temperature (Figures 4 and 5). The reason is that the conformational flexibility of the enzyme was affected by immobilization. Immobilization of XR into chitosan beads caused an increase in enzyme rigidity which is commonly reflected by enhancing the stability towards denaturation by raising the temperature. In addition, an immobilized matrix is supposed to preserve the tertiary structure of an enzyme, and it protected the enzyme from conformational changes causing effects of the environment (Altun and Getinus, 2007).

Although enzymes showed an optimum pH of 4.5, the immobilized enzyme has a broader pH range. At the same time, the acid stablity of immobilized XR was improved in the lower acid concentration of high activity (Figures 6 and 7). Firstly, chitosan belongs to a polycationic support. Secondly, chitosan beads could be positively charged at lower pH because there are a number of available amino groups on cross-linked chitosan beads; hydrogen ions in the enviroment could bind to free amino groups, and the number of bound hydrogen ions is reduced, resulting in a shift of solution pH to acidic side significantly. At the same time, it leads to the change of the local concentration of proton near the enzyme molecules. In addition, when XR binds to the support, the support has a protecting effect at the lower pH at which the enzyme deactivation takes place. For these reasons, acidic microenvironment can change the optimum pH and pH stability of XR. A similar result about pH for other enzyme immobilized on chitosan was also observed in literatures (Ye et al., 2006; Vaillant et al., 2000; Huang et al., 2007).

Characterization of their operational stabilities is very important, when comparing performance of immobilized biocatalysts and intending for preparative or industrial use. The residual activity of XR immobilized on chitosan bead was about 40% after 7 cycles of batch operation,

which allows the continuous use of this enzyme in industrial application (Figure 8). The activity loss could be related to the inactivation of the enzyme caused by the denaturation and leakage of protein from the support's surface because of reutilization (Huang et al., 2007). Cross-linking with glutaraldehyde decreased the stereo-specific blockade of chitosan bead surface, and positive charge on the chitosan accelerated the transfusion from substrate to enzyme active center, which resulted in increasing XR immobilized on chitosan and substrate, and degrading of Km value (Figure 9).

It is prominent that chemicals generally influence the catalytic activity of all kinds of enzyme. In this work, we made use of a number of chemcals from many researches to investigate their effects on the activity of mmobilized and free XR. In agreement with eariler data (Lee et al, 1998), our results indicate that Gu^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Ni^{2+} and ethylenediaminetetraacetic acid (EDTA) have influence on the activity of XR. Sulfhydryl compounds (including DTT), the best reductant, kept the active enzyme in a reduced state. The addition of sulfhydryl compound increased the enzyme activity for *C. parapsilosis* by 13, 21, 29, and 43%, which has been reported in the Jung-Kul Lee's study (Lee et al, 1998). Moreover, the enzyme activity depending on sulfhydryl compound has been reported for several reductases purified from *C.tennuis* (Neuhauser et al., 1997) and *pig lens* (Branlant, 1982). However, only Cys showed the stimulatory effect on the XR activity from *C.tropicalis* and other compounds had inhibitory effect including DTT, cys and βME in our work. At the same time, the effect of Fe^{3+} on XR activity was also studied. Our results indicated that Fe^{3+} ion caused a significant inhibition of *C.tropicalis* XR, whose activity was closed to 0 Umg^{-1} (Figure 10). Therefore, it is very important to guard against of Fe^{3+} pollution from industrial production.

NADH is more prevalent in the cell, less costly, more stable and suitable for a variety of extracellular catalytic reactions than NADPH. Furthermore, XR enzyme with dual coenzyme specificity or a preference for NADH may improve the efficiency of xylose conversion. In this study, the XR cloned from *C. tropicalis* depend on two cofactor comparing with XR from *Candida* (Yokoyama et al., 1995), the activity ratio of NADH/NADPH was very low before immobilization (Zhang et al., 2009), XR could be observed to have a higher affinity for NADPH. After immobiling on the chitosan bead, the utilization ability of NADH was enhanced significantly for 17.15 fold (Table 3). Therefore, we can add two cofactor into the reaction solution or broth so as to increase the activity and reduce prodution cost.

In conclusion, immobilization of XR on chitosan beads has been successfully carried out. The chitosan beads prepared with crosslinking solution were treated with glutaraldehyde for stability in both alkaline and acidic media. These results indicate that immobilized XR comparing with the free enzyme have a higher substrate affinity, and improve stability of various parameters, such as temperature, pH and reuse. In addition, the immobilized enzyme also the higher availability of NADH. Therefore, the modified chitosan is an ideal intelligent polymer support to immobilize XR in various industrial applications.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (NSFC 30740055, 30771312, 30871321), the R&D Infrastructure and Facility Development Program from the Ministry of Science and Technology of the People's Republic of China (2005DKA21208-7) and the Foundation of Sichuan Province, China (04NG002-006-1).

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