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# Immobilization of *Aspergillus niger* Xylanase on Chitosan Using Dialdehyde Starch as a Coupling Agent

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**Abstract** Dialdehyde starch (DAS) was used as a novel coupling agent to prepare chitosan carrier to immobilize the xylanase from *Aspergillus niger* A-25. Compared with glutaraldehyde-cross-linked chitosan (CS-GA) and pure chitosan beads, the DAS-cross-linked chitosan (CS-DAS) beads exhibited the highest xylanase activity recovery. The DAS adding amount and cross-linking time in CS-DAS preparation process were optimized with respect to activity recovery to the values of 1.0 g (6.7% *w*/*v* concentration) and 16 h, respectively. The optimum temperature of both the CS-DAS- and CS-GA-immobilized xylanase was observed to be 5 °C higher than that of free enzyme (50 °C). The CS-DAS-immobilized xylanase had the highest thermal and storage stability as compared to the CS-GA-immobilized and free xylanase. The apparent $K_m$ and $V_{max}$ values of the CS-DAS-immobilized xylanase were estimated to be 1.29 mg/ml and 300.7 μmol/min/mg protein, respectively. The CS-DAS-immobilized xylanase could produce from birchwood xylan high-quality xylo-oligosaccharides, mainly composed of xylotriose, as free xylanase did. The proposed CS-DAS carrier was more advantageous over the CS-GA or pure chitosan carrier for xylanase immobilization application.

**Keywords** Dialdehyde starch · Xylanase · Chitosan · Immobilization

## Introduction

Xylan is the major polysaccharide of the hemicellulose fraction of plant cell walls, accounting for as much as 30% of the dry weight of some plant tissues. Bioconversion of xylan to useful materials always needs xylanase to hydrolyze β-1,4-linkage of xylan backbone. One of the most valuable hydrolysis products, xylo-oligosaccharides has received more and more attention due to its function of proliferating beneficial bifidobacteria in human intestine [1]. For xylo-oligosaccharides production, immobilization

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of xylanase on solid support can exhibit many advantages, including reuse of enzyme, ease of product separation, improvement of enzyme stability, and continuous operation in packed-bead reactors, all these resulting in cost reduction.

Various carriers were reported to be applied to xylanase immobilization, such as chitosan [2], magnetic latex beads [3], reversibly soluble–insoluble polymer Eudragit S-100 [4, 5] and Eudragit L-100 [6], chitosan–xanthan hydrogel [7], Duolite A147 pretreated with glutaraldehyde [8], cellulose acetate membrane grafted with acrylamide [9], and multi-walled carbon nanotubes [10]. As chitosan is remarkably hydrophilic, biocompatible, and easy to be linked with enzymes and to be modified owing to the presence of abundant hydroxyl and amino groups, it has been widely used as immobilization carrier not only for xylanase [2, 7], but also for other enzymes [11–14]. Meanwhile, some new treatments to chitosan carrier were continuously carried out to improve its immobilization efficiency and also to enhance its acidic and alkali stability. Chang et al. [15] have made chitosan–$ZrO_2$ composite beads by adding $ZrO_2$ powders to chitosan/acetic acid slurry. Cheng et al. [16] have used tris(hydroxymethyl) phosphine (THP) instead of glutaraldehyde (GA) as a coupling agent to prepare THP-activated chitosan beads. Chiou et al. have developed two agents to treat chitosan: one is carbodiimide used for activating the hydroxyl groups of chitosan [17], and the other is genipin, a natural cross-linker of plant origin, used for chitosan cross-linking [13].

In this work, a novel coupling reagent, dialdehyde starch (DAS) as shown in Fig. 1 is introduced to chitosan activation procedure. DAS has the properties of hydrophilicity, biocompatibility, and structure flexibility. It contains a lot of –CHO groups like GA, and by Schiff's base reaction of its –CHO group with chitosan or enzyme's $–NH_2$ group, it can easily cross-link covalently with both chitosan and enzyme. Here, both DAS and GA were used as coupling reagent in chitosan support to immobilize xylanase from *Aspergillus niger*, and the properties of the two immobilized xylanases were compared in terms of their catalytic activity, stability, and kinetic parameters.

## Materials and Methods

### Chemicals

DAS (oxidation degree≥90%) and birchwood xylan were purchased from Sigma-Aldrich. Chitosan with a degree of deacetylation of ≥95% was purchased from Jinan Haidebei Marine Bioengineering Co., LTD., China. The standard xylo-oligosaccharides mixture was kindly provided by Dr. Z. Ai of China Agricultural University. All other chemicals used were of analytical grade or higher purity.

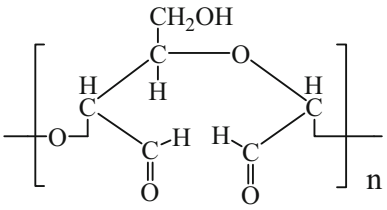


**Fig. 1** The structure of DAS

## Enzyme Production

*A. niger* A-25 (from Henan Agricultural University, China) was cultured at 30 °C, 220 rpm for 76 h in 300-ml shake flasks with each containing 50 ml of culture medium. The components of the culture medium were as follows (*w*/*v*): wheat bran 2%, smashed corncob 2%, glucose 0.1%, ammonium oxalate 2%, and Tween-80 0.15%, pH 6.0. After cultivation, the culture was centrifuged at 10,000×*g*, 4°C for 10 min, and the resulting supernatant was used as xylanase solution.

## Preparation of Chitosan Carriers

For chitosan beads preparation, an amount of chitosan powder was fully dissolved in 8% *w*/*v* acetic acid to get a slurry with 2.5% *w*/*v* chitosan final concentration. After neutralization with NaOH, the viscous solution was placed in a vacuum dryer for 3 h to remove air bubbles and then sprayed dropwise through a syringe, at a constant rate, into 1.5% *w*/*v* $(NaPO_3)_6$ solution with a magnetic stirrer. The chitosan was then solidified to microspheric beads, and those uniform-sized (about 1.5 mm in diameter) were collected and transferred to the another fresh $(NaPO_3)_6$ solution. Let stand there overnight, and the chitosan beads (designated as CS) were finally placed in 0.2 M acetate buffer (pH 5.6).

For GA coupling reaction, 1 ml of 50% *w*/*v* GA and 15 ml of 50% *v*/*v* ethanol solution were added to 1.0 g chitosan beads such that the final concentration of GA was 3.1% *w*/*v*. The mixture was gently shaken at 70 °C for 12 h, and the beads were then collected and washed alternately with hot and cold deionized water to remove excess GA. This GA-activated chitosan beads (CS-GA) were kept in 0.2 M acetate buffer (pH 5.6). As for DAS-coupling reaction, all procedures were the same as above except that 1.0 g DAS replaced 1 ml GA. The resulting DAS-activated chitosan beads were designated as CS-DAS.

## Enzyme Immobilization and Activity Determination

One milliliter of xylanase solution (393 IU/ml) was mixed with 4 ml of 0.2 M acetate buffer (pH 5.6). To the mixture was added 1.0 g of differently prepared chitosan carriers (CS, CS-GA, and CS-DAS) separately. After shaking on a water bath shaker (140 rpm) at 30 °C for 8 h, unbound enzymes were removed by washing with deionized water.

For free enzyme determination, 0.1 ml appropriately diluted enzyme, and 0.1 ml of 2.0% *w*/*v* birchwood xylan in 0.2 M acetate buffer (pH 4.6) were incubated at 50°C for 15 min. The reducing sugars liberated were determined with dinitrosalicylic acid reagent [18]. One unit of enzymatic activity (IU) was defined as the amount of enzyme required to release 1 μmol of xylose equivalent per minute. The immobilized enzyme assays were carried out in the same way, except that 0.1 ml deionized water was substituted for above 0.1 ml enzyme solution, and an amount of chitosan beads (0.02 g) was added to the reaction mixture.

## Determination of Apparent $K_m$ and $V_{max}$

The same amount of free and immobilized xylanase (20 mg immobilized xylanase, or equivalent 0.145 mg free xylanase, estimated by the method of Bradford [19] using bovine serum albumin as a standard protein) was incubated with various concentrations of birchwood xylan (1–14 mg/ml, pH 4.6) at 50 °C for 5 min, and each initial rate of reaction was determined. The apparent $K_m$ and $V_{max}$ values were calculated according to Lineweaver–Burk plot.

### Saccharification of Birchwood Xylan by Free and CS-DAS-Immobilized Xylanase

Sixty unit of enzymatic activity xylanase (equivalent to 0.15 ml enzyme solution for free xylanase and 0.25 g beads for CS-DAS-immobilized xylanase) was mixed with 5 ml of 2.0% *w*/*v* birchwood xylan in 0.2 M acetate buffer (pH 4.6), making final xylanase concentration 0.6 IU/mg substrate. The saccharification reaction mixture was incubated at 40 °C for 6–12 h under shaking (140 rpm) condition. To determine the products of the enzymatic hydrolysis, high-performance liquid chromatography (HPLC) analysis was carried out using a Kromasil 100-5 $NH_2$ column (150×4.6 mm). Acetonitrile/water (75:25, *v*/*v*) was pumped as the eluent at 1.0 ml/min using a Dionex P680 pump. The temperature of the analysis was 30°C, and the detector was a Shodex RI-101 refractive index unit.

## Results and Discussion

### Immobilization of Xylanase on Differently Prepared Chitosan Carriers

Under the aforementioned immobilization conditions optimized beforehand using CS-GA carrier, the *A. niger* xylanase was bound separately with the CS, CS-GA, and CS-DAS carriers. After intense washing, the activity of both the unbound enzyme and immobilized enzyme was determined. The results are presented in Table 1 and show that the CS-DAS carrier had the highest activity recovery of 60.8% while the CS carrier had the lowest one of 44.8%. It was clear that immobilization on chitosan through covalent bonding as displayed in the CS-DAS and CS-GA beads had a considerably better efficiency than through physical adsorption as displayed in the CS beads.

Another fact noteworthy is that the detected xylanase (i.e., the addition of activity of unbound and immobilized xylanase) of CS (331 IU/g) and CS-GA (332 IU/g) was lower than that of CS-DAS (352 IU/g). However, the situations for CS and CS-GA were quite different. In CS immobilization system, due to weak adsorption force, more xylanase formerly adsorbed onto CS carrier was finally lost through beads washing step, leading to the lower detected xylanase. While in CS-GA immobilization system, CS-GA probably had the same binding force as CS-DAS, inferred from nearly the same protein coupling rate (data not shown) and similar unbound xylanase, but the xylanase bound on CS-GA carrier could not display the same activity like that on CS-DAS carrier, also leading to the lower detected xylanase. We can further deduce that, being a long polymer chain which can form a stretched out space at the surface of chitosan microspheres, DAS was more favorable than GA in maintaining the configuration of enzyme molecule and also in enhancing accessibility of substrate to active site of the immobilized enzyme. Hence, DAS might play a role of spacer arm and could reduce the steric hindrance in the immediate vicinity of

**Table 1** Immobilization of *A. niger* xylanase on differently prepared chitosan carriers.

| Carrier | Added enzyme (IU/g) | Unbound enzyme (IU/g) | Immobilized enzyme (IU/g) | Activity recovery (%) |
|---|---|---|---|---|
| CS | 393 | 155 | 176 | 44.8 |
| CS-GA | 393 | 119 | 213 | 54.2 |
| CS-DAS | 393 | 113 | 239 | 60.8 |

enzyme molecules in a better manner than GA. Consequently, CS-DAS carrier displayed higher activity than CS-GA did, although with nearly the same protein loading amount.

## Effect of DAS Concentration and Cross-Linking Time on Activity Recovery of Immobilized Enzyme

Taking into account the preparation procedure of the CS-DAS carrier, DAS concentration and cross-linking time in coupling reaction could be the two influential factors on immobilization efficiency of the carrier. Hence, to the above-mentioned coupling system varying amounts of DAS (0.6, 0.8, 1.0, 1.2, and 1.4 g, corresponding to a final DAS concentration of 4.0, 5.3, 6.7, 8.0, and 9.3% *w*/*v*, respectively) were added, and at the same DAS concentration different cross-linking time was carried out. Each resulting CS-GAS beads was applied to immobilizing the xylanase, and the activity recovery of immobilized enzyme was detected.

Figure 2a shows that the activity recovery of immobilized enzyme was almost unchanged at DAS adding amount of 0.6–1.0 g. When DAS adding amount exceeded 1.0 g, the activity recovery tended to decrease, suggesting that overmuch aldehyde groups introduced by DAS on the surface of spheres may lead to excessive cross-linking between DAS and enzyme, resulting in an increase of substrate diffusional resistance and then the drop of enzyme activity. This is in agreement with the observation of Chang and Juang [15] that when treated with higher concentration of GA, chitosan beads displayed slightly lower immobilized enzyme activity.

The effect of cross-linking time in CS-DAS beads preparation (still with 1.0 g DAS added) on enzyme activity is shown in Fig. 2b, indicating that the activity recovery kept a high level at a wide range of cross-linking time of 12–20 h with the highest recovery of 63% being at 16 h. Hence, a DAS amount and cross-linking time of 1.0 g (6.7% *w*/*v* concentration) and 16 h, respectively, were selected as the optimal conditions for the CS-DAS beads preparation.

## Properties of Free and Immobilized Enzymes

The optimal reaction temperature for both the CS-DAS- and CS-GA-immobilized xylanase was found to be 55°C, 5°C higher than that of the free xylanase, yet immobilization did not

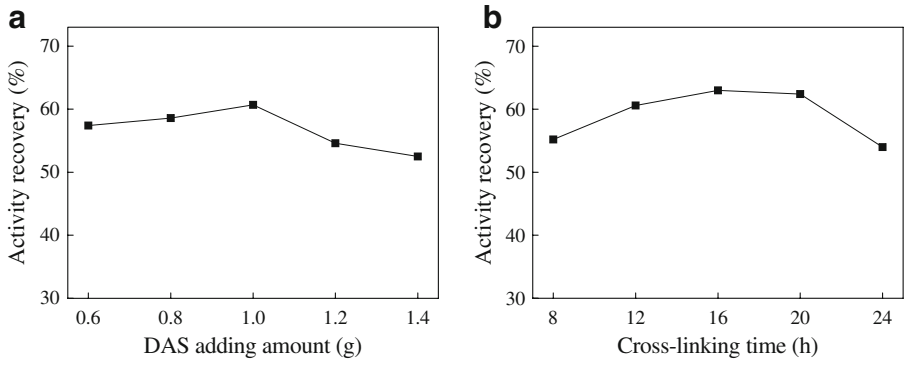


**Fig. 2** Effects of DAS adding amount (**a**) and cross-linking time (**b**) on activity recovery of immobilized enzyme. Cross-linking time in (**a**): 12 h; DAS adding amount in (**b**): 1.0 g

have any effect on xylanase optimum pH, all three enzymes exhibiting maximal activity at pH 4.6.

Although immobilization of xylanase had a very marginal effect on its pH stability (data not shown), immobilization had significant effect on xylanase thermal stability and storage stability as shown in Fig. 3. After 60 min of incubation at 50°C, the CS-DAS- and CS-GA-immobilized and free xylanase retained 78.5%, 64.4%, and 56.0% of initial activity, respectively, and the half-life of the three at 55°C was 30, 17, and 10 min, respectively, indicating that both CS-DAS and CS-GA immobilization improved the thermal stability of xylanase, and the CS-DAS-immobilized xylanase was more stable than the CS-GA one toward heat denaturation. In storage experiment, after 75-day storage at 4°C, the CS-DAS and CS-GA-immobilized xylanase retained 89% and 80% of its initial activity, respectively, whereas the free xylanase kept only 60% of its initial activity, showing that CS-DAS immobilization had a more remarkable stabilizing effect toward storage than CS-GA immobilization.

The apparent $K_m$ value for birchwood xylan of the free and CS-DAS- and CS-GA-immobilized xylanase was determined as 1.14, 1.29, and 1.33 mg/ml, respectively, while the apparent $V_{max}$ value was 310.3, 300.7, and 224.1 μmol/min/mg protein, respectively. Both immobilized xylanases had a higher $K_m$ and a lower $V_{max}$ value compared to the free counterpart. The CS-DAS-immobilized one had a slightly lower apparent $K_m$ and noticeably higher apparent $V_{max}$ than the CS-GA-immobilized one, hinting that due to introduction of polymer GAS, the immobilized xylanase was likely to reduce its conformational change during immobilization procedure and/or weaken the sterical impediments near enzyme molecules resulting in an enhanced accessibility of substrate to enzyme active site.

## Saccharification of Birchwood Xylan by Free and CS-DAS-Immobilized Xylanase

The birchwood xylan was hydrolyzed by the same amount of the free and CS-DAS-immobilized xylanase separately, and the hydrolysate was analyzed by HPLC to determine its sugar components. The result shows that xylan hydrolysis by the CS-DAS-immobilized xylanase may proceed at the same speed and the same mode as by the free xylanase, as evidenced by nearly the same HPLC profiles of the two hydrolysates (Fig. 4, only presenting HPLC for the CS-DAS-immobilized case). It is clear that xylotriose is a predominant product with a small amount of xylobiose and much less xylose after 6 h of

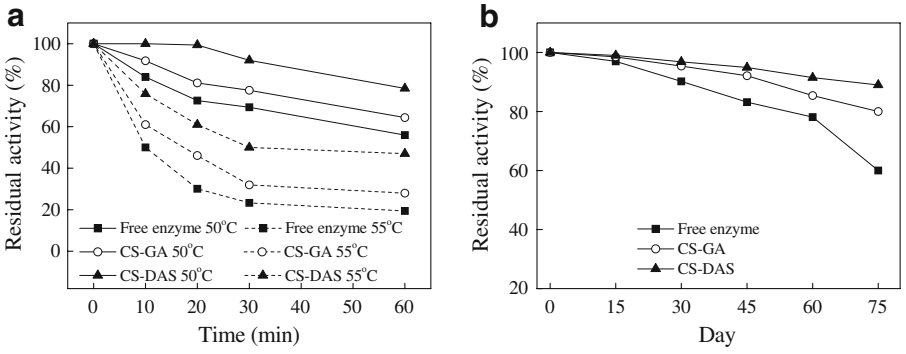


**Fig. 3** Thermal stability and storage stability of free and immobilized xylanases. **a** Thermal stability at 50°C and 55°C. **b** Storage stability at 4°C

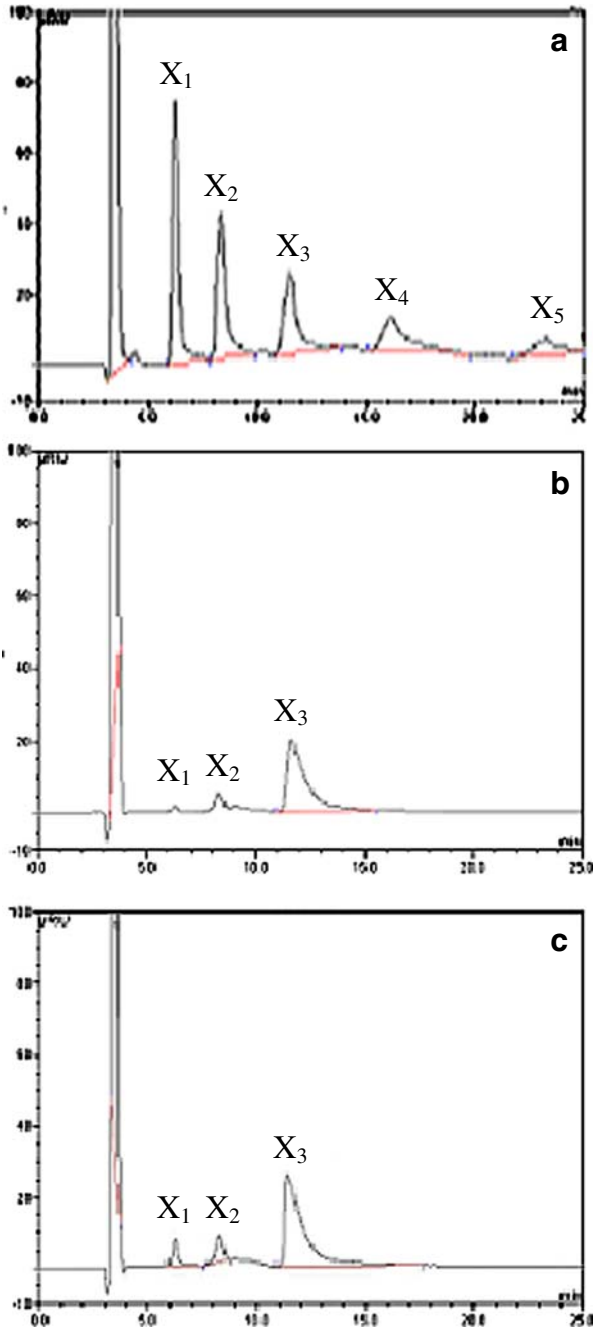


**Fig. 4** HPLC chromatograms of the hydrolysis of birchwood xylan by the CS-DAS-immobilized xylanase. **a** The standard xylo-oligosaccharides mixture. $X_1$ xylose, $X_2$ xylobiose, $X_3$ xylotriose, $X_4$ xylotetraose, $X_5$ xylopentaose. **b** Xylan solution after 6 h of hydrolysis. **c** Xylan solution after 12 h of hydrolysis

hydrolysis. Upon long-term hydrolysis (12 h), the content of all three sugars has increased, without any new products emerging. Therefore, the CS-DAS immobilization does not affect the substrate–cleavage mode of the native xylanase, a mode favorable for xylo-oligosaccharides production.

## Conclusions

DAS, a polymer rich in aldehyde groups, was used as a novel coupling agent to prepare chitosan carrier to immobilize the xylanase from *A. niger* A-25. Among CS-DAS, CS-GA, and CS carriers, the CS-DAS carrier exhibited the highest activity recovery. Moreover, the CS-DAS-immobilized xylanase had the highest thermal and storage stability. The apparent $K_m$ and $V_{max}$ values of free and immobilized xylanases suggested that the CS-DAS immobilization make an improvement on maintenance of the native conformation of enzyme and/or on accessibility of macromolecular substrate of xylan to enzyme active site, as compared to the CS-GA immobilization. The CS-DAS-immobilized xylanase could produce from birchwood xylan high-quality xylo-oligosaccharides, mainly composed of xylotriose, as free xylanase did. The proposed CS-DAS carrier was more advantageous over the CS-GA or pure chitosan carrier for xylanase immobilization application.

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