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Immobilization of *Thermomyces lanuginosus* Xylanase on Aluminum Hydroxide Particles Through Adsorption: Characterization of Immobilized Enzyme

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Xylanase plays important roles in a broad range of industrial production as a biocatalyst, and its applications commonly require immobilization on supports to enhance its stability. Aluminum hydroxide, a carrier material with high surface area, has the advantages of simple and low-cost preparation and resistance to biodegradation, and can be potentially used as a proper support for xylanase immobilization. In this work, xylanase from *Thermomyces lanuginosus* was immobilized on two types of aluminum hydroxide particles (gibbsite and amorphous $Al(OH)_3$) through adsorption, and the properties of the adsorbed enzymes were studied. Both particles had considerable adsorptive capacity and affinity for xylanase. Xylanase retained 75% and 64% of the original catalytic activities after adsorption to gibbsite and amorphous $Al(OH)_3$. Both the adsorptions improved pH and thermal stability, lowered activation energy, and extended lifespan of the immobilized enzyme, as compared with the free enzyme. Xylanase adsorbed on gibbsite and amorphous $Al(OH)_3$ retained 71% and 64% of its initial activity, respectively, after being recycled five times. These results indicated that aluminum hydroxides served as good supports for xylanase immobilization. Therefore, the adsorption of xylanase on aluminum hydroxide particles has promising potential for practical production.

Keywords: Xylanase, gibbsite, amorphous $Al(OH)_3$, adsorption, catalytic performance

Introduction

Enzymes are attractive biocatalysts, a fact attributed to their high efficiency under ambient conditions. Therefore, their applications are important in industrial productions [7, 32, 45]. Despite their intrinsic stability, the enzymes are often inactivated under extreme reaction conditions; for example, conformational changes or other transformations in their chemical structure. Additionally, the utilization of natural enzymes has other processing difficulties such as the issues on reuse of enzymes and product contamination, which enormously limit their industrial application [17]. Therefore, it is extremely important to improve the stability of the enzyme and to make it reusable during consecutive treatment cycles. One of the pathways to solve the issue is to immobilize enzymes on solid surfaces, which enhances the recoverable and stable heterogeneous biocatalysts [3, 24].

Different immobilization methods have been established in previous literature, such as adsorption to solid carriers, entrapment into polymers, cross-linking on biofunctional materials, or covalent binding to solid supports [13, 41]. Among the proposed methods, adsorption to solid carriers would be more economic since the process is straightforward, inexpensive, and with high remaining activity. Furthermore, the carrier could be repeatedly reused [18].

Inorganic supports provide better stability characteristics, and have been applied to various enzymes [40, 48]. Aluminum hydroxide has many advantages for being used

as support material based on previous research [29, 31]: resistant to biodegradation; high surface areas and properly functionalized surfaces; low cost.

Xylanases, a kind of carbohydrase, have a global market of about $200 million [11], and hence have attracted considerable research interest due to their application in various industrial processes, such as animal feed digestion, waste treatment, energy generation, production of chemicals, and paper manufacture [10, 19]. Because of the industrial potential of xylanases, their immobilization for industrial application has been addressed [26, 31, 42]. Various supports under extreme conditions have been reported in the literatures. However, these are often costly and/or not easily available. For instance, chitosan, a commonly used support for xylanase immobilization [9, 28], is priced at $107/50 g (Sigma-Aldrich, China); anionic exchange resin, used for the adsorptive immobilization of xylanase [25], is priced at $150/100 g (Sigma-Aldrich, China); or Eudragit S-100 (a product of Rohm, Germany) [2], priced at $ 35/50 g in China. Such high prices limit their application. On the other hand, the complex production process of immobilized enzymes would result in their inefficient application as well as the increase of cost, such as through covalent binding. To covalently immobilize enzymes on a pre-existing support material is very complex. In most cases, the support needs activating and the reaction conditions are usually important, which would slow down the immobilization rate [13]. Therefore, a simply prepared immobilization method would have great advantage for the application of xylanase in the future.

In the current study, the applicability of aluminum hydroxide particles as supports for xylanase immobilization through adsorption was investigated, aiming to establish an immobilization method for xylanase with a simple preparation procedure and with low cost. Xylanase from *Thermomyces lanuginosus* was immobilized on two types of aluminum hydroxide particles (gibbsite and amorphous $Al(OH)_3$) through adsorption. The adsorption characteristics and the catalytic performances of the adsorbed xylanases, including activity, kinetics, stability, and reusability of the adsorbed enzyme, were studied.

Materials and Methods

Chemicals

In order to maintain the optimal activity of the enzyme, all experiments were conducted in NaOA(sodium acetate) buffer (0.05 M, pH 5.3), with the exception of the pH stability studies. Xylanase (purified from *Thermomyces lanuginosus*) was supplied by Sigma Aldrich. The substrate for enzyme activity assay, xylan from beechwood, was supplied by Sigma Aldrich.

Preparation of Aluminum Hydroxide Particles

The aluminum hydroxides can be classified into two types according to the crystalline order: crystalline and amorphous [44]. Different types of aluminum hydroxides have different microstructure, which may have significant effect on the enzyme loadings and activity limitation for the adsorption. In this study, gibbsite (in a crystalline type) and amorphous $Al(OH)_3$ (in an amorphous type) were synthesized and used as supports. The gibbsite was synthesized by the method of Kyle *et al.* [21], and amorphous $Al(OH)_3$ by the method of Manning and Goldberg [27].

Both supports were passed through a 250 μm sieve and characterized by X-ray diffraction. The specific surface area (determined by BET method) of gibbsite and amorphous $Al(OH)_3$ was 21.41 and 52.18 m^2/g, respectively; and the point of zero charge (PZC, determined by salt titration method) was 8.3 and 8.1 for gibbsite and amorphous $Al(OH)_3$, respectively. The sieved particles had an average diameter of approximately 200 μm, determined on an Adsorption instrument (Micromeritics, ASAP2020) [46]. Prior to experiment, the particles were washed with buffer by shaking for 6 h, centrifuged (12,000 ×*g*, 30 min), and then freeze dried.

Adsorption of Xylanase to Particles

The adsorption was operated according to Wu *et al.* [46]. Each particle stock suspension was prepared by sonicating 500 mg of particle in 100 ml of buffer. In sterilized plastic centrifuge tubes, an aliquot of each particle stock solution (1 ml) was mixed with various amounts of 100 μg/ml xylanase stock solutions and buffer, to give final concentrations of xylanase that ranged from 8 to 85 μg/ml, and the total volume was set to be 5 ml. The mixed suspensions were then shaken at 200 rpm for 3 h at 30°C for adsorption procedure. After the adsorption was finished, the suspensions were centrifuged (12,000 ×*g*, 10 min), and the enzyme-particle complexes were washed twice with buffer. The washed enzyme-particle complex was regarded as adsorbed xylanase. Xylanase concentrations in the supernatant and washing buffers were assayed by Bradford's method using BSA as a standard. The adsorbed xylanase concentration was calculated through mass balance.

The adsorption parameters, maximum adsorption capacity (a_{max}) and binding affinity (K), were determined by fitting to the Langmuir equation:

$$y = a_{max}Kx/(1+Kx)$$

where y and x are the concentrations of adsorbed and free xylanases respectively.

Determination of Catalytic Performance of Free and Adsorbed Xylanases

Adsorption of xylan and xylose to the particles. To study the influence of particle adsorption on enzyme activity, which may

change substrate and product concentrations in the reaction [43], the adsorption of particle on the substrate and the product was tested.

Approximate amounts of substrate and product to those in enzyme activity determination were used. First, 5 ml of 1% xylan (substrate)/0.3 mg/ml xylose (product) was mixed with 5 mg of particles and gently shaking for 5 min at 50°C. Then the mixture was centrifuged (12,000 ×*g*, 10 min), and the xylan/xylose concentration in the supernatant was measured. The amount of adsorbed xylan/xylose was calculated according to the differences between the concentration before and after adsorption.

Catalytic activities of free and adsorbed xylanases. The enzyme activity was determined by the DNS-stopping method [4]. The assay mixture that consisted of free or adsorbed xylanases and 1% (0.333 mM) xylan in buffer, in total volume of 5 ml, was incubated at 50°C for 5 min. After that, the xylose concentration was determined. International units (U/mg proteins) were used to express the xylanase activity, where 1 unit was defined as the amount of enzyme that releases 1 μmol of xylose per minute at pH 5.3 at 50°C. Denatured enzymes were used as controls.

Kinetic parameters of free and adsorbed xylanases. The kinetic parameters K_m and V_{max} were determined by measuring the xylanase activity over a 0.3–4% (0.1–1.3 mM) range of initial xylan concentrations with approximately 50 μg free/adsorbed xylanases. The parameter values were obtained by fitting to the Michaelis-Menten equation: $V = V_{max} S/(K_m + S)$
where V is the xylanase activity and S is the xylan concentration.

pH and thermal stabilities of free and adsorbed xylanases. Free/adsorbed xylanases were incubated under different pH conditions (3.0–8.0) in phosphate/citrate buffer for 1 h at 25°C to study their pH stability. The enzyme activity at each pH was measured in the corresponding pH. The relative activity (%) was determined by calculating the ratio of the enzyme activity at various pH to that at pH 5.0 (standard conditions).

Free/adsorbed xylanases were incubated at various temperatures (4–90°C) in 0.05 M NaOA(sodium acetate) buffer, pH 5.3, for 1 h to study their thermal stability. The enzyme activity at each temperature was measured at the corresponding temperature. The residual activity (%) was determined by taking the enzyme activity at the optimum temperature as 100%. Meanwhile, the activation energy (E_a) of free/adsorbed xylanase was calculated from the slope of the Arrhenius plot (regression of logarithm of reaction rate versus reciprocal of absolute temperature) [35]. $E_a = -\text{Slope} \times R$, where R is 8.314 J mol^{-1} k^{-1}.

Lifespan of free and adsorbed xylanases. The lifespan test was conducted by periodically (on 0–15 days) determining the residual activities, as defined above, of free/adsorbed xylanase during 15 days of incubation at 25°C.

The half-life ($t_{1/2}$) of free/adsorbed xylanase was calculated according to the equation: $t_{1/2} = \ln 2/k_i$

k_i was calculated from the equation [5]: $\ln A_t = \ln A_0 - k_i t$
where A_0 is the initial activity and A_t the activity after a time t (day).

Reusability of Adsorbed Xylanases

The reusability study was carried out according to previous studies [15, 20]. The adsorbed xylanases were assayed for 8 cycles of 5 min each. For each cycle, 5 ml of 1% xylan in buffer was mixed with adsorbed xylanases and incubated for 5 min at 50°C. At the end of the reaction, the adsorbed xylanases were recovered by centrifugation (12,000 ×*g*, 10 min) and resuspended in freshly prepared xylan to start a new run. The concentration of xylose in the supernatant in each assay was measured. The activity of the adsorbed xylanase in the first cycle was considered 100%.

Statistical Analysis

Each treatment had three independent replicates for all experiments. Comparisons between means were performed at the 5% probability level with a Fisher's least significant difference (LSD) test ($p < 0.05$), using the statistical package SPSS16.0. Linear and non-linear curve fittings were conducted using the statistical package Origin 8.5.

Results and Discussion

Adsorption of Xylanase to the Particles

In order to evaluate the support adsorptive capacity, the adsorption isotherms for xylanase on particles were determined by following the Langmuir model (Fig. 1). Through fitting analysis, the maximum capacity (a_{max}) values were found to be 31.85 and 22.41 μg/mg for adsorption by gibbsite and amorphous $Al(OH)_3$, respectively. The binding affinity (K) values were 40.99 and 49.43 L/mg, respectively, much greater than those of the aluminum hydroxide adsorption on other enzymes such as laccase [1] (a_{max} = 6.18 μg/mg, K = 12.51 L/mg). These results indicated

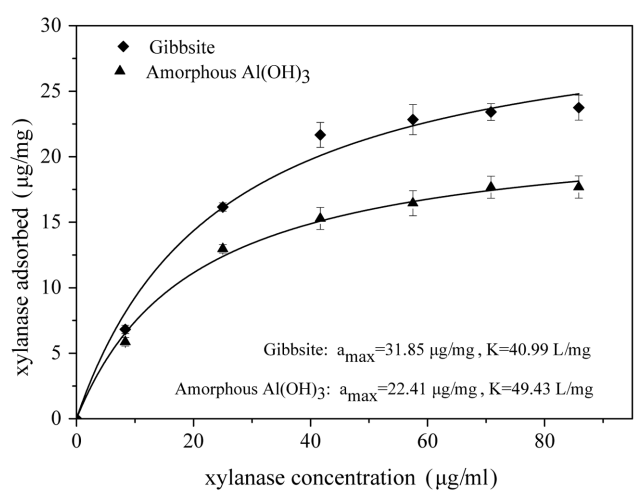

Fig. 1. Adsorption isotherms of xylanase on minerals during 3 h at 30°C.

Bars are standard errors (n = 3).

a high adsorptive capacity and strong binding of gibbsite and amorphous $Al(OH)_3$ for xylanase.

At the experimented pH 5.3, the enzyme would be negatively charged since the PZC of *Thermomyces lanuginosus* xylanase (3.7−4.1) [6, 8] is lower than 5.3, whereas the aluminum hydroxides would be positively charged as their PZC is larger than 5.3. Thus, electrostatic interaction would occur between the positive particles and the negative xylanases and plays an important role in the binding between enzyme and particle [39]. In addition, other interactions such as van der Waals force, hydrophobic force, or hydrogen binding [18, 37], as well as the particle size of aluminum hydroxides [16, 36], would also be important mechanisms for the adsorption of enzyme by the particles.

Influence of Particle Adsorption on the Activity and Kinetics of Xylanase

Adsorption of xylan and xylose to the particles. During the testing time (5 min), no statistically significant difference ($p < 0.05$) in the concentration of xylan or xylose before and after adsorption was detected (data not shown), indicating that the particles did not affect the substrate/product concentrations Thus, the catalytic reaction kinetics with xylan must be attributed entirely to interactions with xylanase.

Activities and kinetics of free and adsorbed xylanases. In this study, the product (xylose) concentrations in reactions catalyzed by free and adsorbed xylanases were investigated over a range of xylanase amounts (15−80 μg free/adsorbed xylanases) (Fig. 2A). When xylanase was adsorbed on the aluminum hydroxide particles, its catalytic activity was reduced but still remained at a high level. Calculated from the slopes of each fitting line, xylanase retained 75% and 64% of the original activity after adsorption to gibbsite and amorphous $Al(OH)_3$, respectively. These activities were higher than some previous results, such as *Aspergillus niger* xylanase immobilized on chitosan beads (45% activity recovery) or on glutaraldehyde-activated chitosan beads (54% activity recovery) [9]. *Aspergillus tamari* xylanase covalently immobilized on Duolite A 147 retained 54% of its original specific activity [15], and *Aspergillus niger* xylanase A immobilized on Fe_3O_4-coated chitosan magnetic nanoparticles had a recovery activity of 56% [26], lower than our results. There were numerous studies that obtained better achievements than ours as well; for example, *Thermomyces lanuginosus* xylanase recovered 78% of initial activity after immobilization on nanoporous gold [47].

It was assumed that the decrease of enzyme activity after

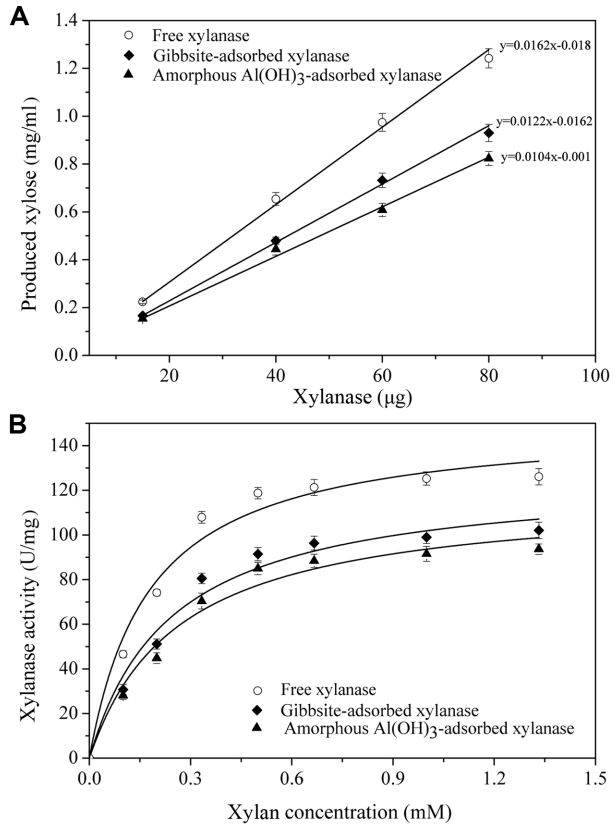

Fig. 2. Enzyme activities (**A**, determined over a range of xylanase amount) and kinetics (**B**) of free and adsorbed xylanases.

Bars are standard errors ($n = 3$).

adsorption might be due to an enzyme conformational change or steric hindrance of the substrate to the enzyme's active site [38]. The adsorption of aluminum hydroxides resulted in losses of xylanase activities from 25% to 36%, among which a large proportion of the activities were retained, implying that catalysis inhibition caused by adsorption was not sufficient to severely destroy the enzyme's conformation or active site. This might because xylanase is a "hard" protein, whose structure is strong enough to defend the severe deformation of its tertiary structure, and the enzyme's active site was still properly oriented to expose to the substrate after conformational changes [22].

Kinetics results showed that both adsorbed xylanases had higher K_m values and lower V_{max} values versus free xylanase (Fig. 2B, Table 1). The K_m values of xylanase increased by a factor of 1.36 and 1.47 upon adsorption to gibbsite and amorphous $Al(OH)_3$, respectively, indicating a reduced enzyme-substrate affinity after xylanase was

Table 1. Michaelis-Menten kinetic parameters (K_m and V_{max}), activation energy (E_a), and half-life ($t_{1/2}$) values for free and adsorbed xylanases.

Xylanase state	K_m (mmol/l)	V_{max} (U/g)	E_a (kJ/mol)	$t_{1/2}$ (days)
Free xylanase	0.1795	150.95	51.77	6.17
Gibbsite - xylanase	0.2433	126.65	29.39	8.92
Amorphous $Al(OH)_3$ - xylanase	0.2632	118.28	28.67	9.95

adsorbed. The V_{max} values of xylanase decreased after adsorption to particles, with 84% and 78% values of the free enzyme for the xylanases adsorbed onto gibbsite and aluminum $Al(OH)_3$, respectively, suggesting a lower hydrolytic rate in reactions catalyzed by adsorbed xylanases than that of free xylanase. Moderate increased K_m values and decreased V_{max} values upon immobilization have been reported; for example, *Aspergillus tamarii* xylanase immobilized on the surface of ion-exchange cross-linked polystyrene (Duolite A147) showed an increase of 47% in K_m value and a decrease of 29% in V_{max} value relative to its free form [15]. The reduction in the affinity to the substrate and the decreased catalytic activity of xylanase after adsorption might be due to a lower possibility of generating enzyme-substrate complexes resulted by the enzyme conformational change, or the lower accessibility of the substrate to the active sites caused by the increased diffusion limitation [34]. In any case, the adsorbed xylanases exhibited lower specificity constants compared with the free counterpart.

Stability of Free and Adsorbed Xylanases

pH and thermal stabilities of free and adsorbed xylanases. One main purpose of enzyme immobilization is to enhance its stability to resist different deactivating forces.

Broader pH profiles of adsorbed xylanases, as compared with that of the free xylanase, were observed in the pH range of 3–4 and the range of 7–8 (Fig. 3A). The immobilized xylanases were able to retain up to 24%–29% and 11%–16% more of the relative activities than the free enzyme at pH 3 and 8, respectively, indicating an elevated pH stability of xylanase after adsorption to the particles. This elevated pH stability would be advantageous for the immobilized xylanase in industrial application. Similarly, other reparted immobilized xylanases rendered broader pH stability than the free enzymes, such as *Aspergillus niger* xylanase immobilized on Fe_3O_4-coated chitosan magnetic nanoparticles that had enhanced stability over pH range of 3–9 [26]. Reasons to explain the increase in pH stability of the immobilized enzymes might be that the immobilization supports protect the enzyme from denaturation under extreme pH conditions [30]. Unchanged pH stability of xylanases after immobilization was also reported, such as *Streptomyces olivaceoviridis* E-86 xylanase non-covalently immobilized on Eudragit S-100 [2], or *Aspergillus niger* xylanase covalently immobilized on alginate beads [35]. In contrast to this study, some immobilized xylanases exhibited decreased pH stability after immobilization. For instance, at pH 6.5–10, *Thermotoga maritima* xylanase B immobilized

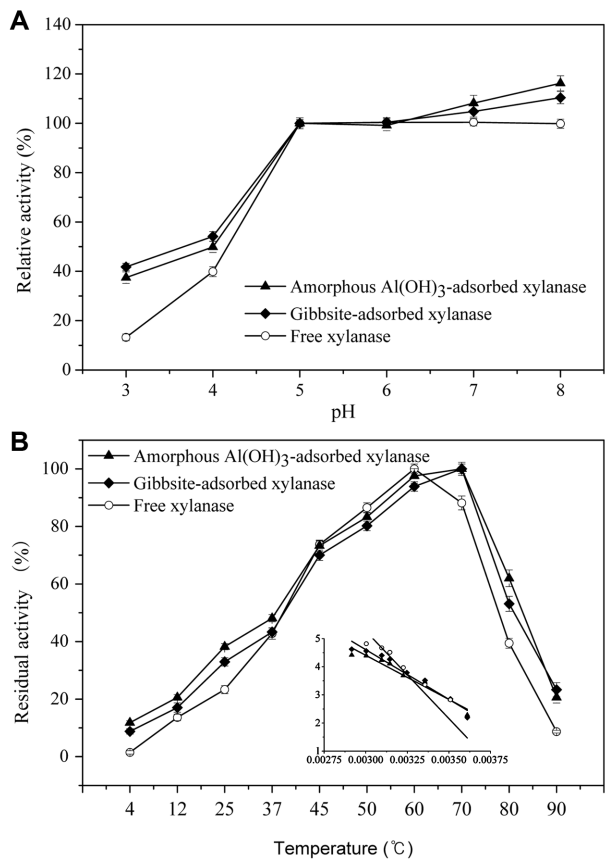

Fig. 3. Relative activities of free and adsorbed xylanases after incubation at various pH (**A**) and residual activities after incubation at various temperatures (**B**) for 1 h.

Bars are standard errors (n = 3).

on metal-chelate Eupergit C 250L showed lower stability than its free form [23].

Optimum temperature values of 60°C and 70°C were recorded for free and adsorbed enzymes, respectively (Fig. 3B). A similar increased displacement of optimum temperature for immobilized enzymes was previously observed [26], which implied the protection of enzyme from heat inactivation [15, 20]. The temperature range of the adsorbed xylanases was broader than that of the free xylanase. Free xylanase was stable at 45°C–70°C, whereas the adsorbed xylanases were stable at 45°C–80°C (Fig. 3B), indicating an enhanced thermal stability of xylanase after adsorption to the particles. The enhancement in thermal stability might be due to enzyme rigidity [33]. Similar to these observations, xylanases immobilized on some other supports also exhibited enhanced thermal stability versus free xylanase, such as *Bacillus pumilus* xylanase immobilized on glutaraldehyde-activated aluminum oxide pellets [31]. Moreover, it was reported that immobilized xylanases had similar thermal stability compared with their free forms, such as xylanase NS50014 immobilized on chitosan [28].

Activation energy (Ea) was analyzed (Arrhenius plot inserted in Fig. 3B, values presented in Table 1). The regression equations for free, $Al(OH)_3$-adsorbed, and gibbsite-adsorbed xylanases were $y = -6227x + 24$ ($R^2 = 0.88$), $y = -3449x + 15$ ($R^2 = 0.98$), and $y = -3535x + 15$ ($R^2 = 0.96$), respectively. Ea of xylanase was greatly reduced after adsorption, with 57% and 55% values of the free xylanase for those adsorbed on gibbsite and amorphous $Al(OH)_3$, respectively, implying a lower energy input need for starting the enzymatic reaction. Thus, the adsorption was beneficial to higher catalytic efficiency in xylanase-catalyzed reactions. Pal and Khanum [35] also proposed that *Thermomyces lanuginosus* xylanase immobilized on glutaraldehyde-alginate beads significantly lowered its Ea from 50.65 to 37.33, with 26% loss of Ea value after immobilization. Covalent immobilization of *Aspergillus tamari* xylanase on Duolite A also resulted in a 13% decrease of Ea of the enzyme [15]. In contrast, *Scytalidium thermophilum* xylanase non-covalently immobilized on Eudragit L-100 showed an increase of 8% in Ea value as compared with the free form [14].

In general, the adsorption method preserved xylanase efficiently in a wider pH/temperature range, which is important for its industrial application.

Lifespan of free and adsorbed xylanases. During the incubation period (25°C for 15 days), the adsorbed xylanases exhibited higher residual activities than the free xylanase (Fig. 4A). At day 15, free xylanase lost more than 90% of its original activity, whereas $Al(OH)_3$-adsorbed and gibbsite-adsorbed xylanases showed 3.7 and 3.4 times higher residual activities than that of the free enzyme, respectively. In addition, adsorption extended the lifespan of xylanase. Xylanase adsorbed on gibbsite and amorphous $Al(OH)_3$ showed 1.45- and 1.61-fold half-lives of the free xylanase, respectively (Table 1). These results indicated the enhanced storage stability and activity sustainability of xylanase after adsorption to the particles, in agreement with previous results that immobilization definitely appeared to hold the enzyme in a stable position. For instance, results of Nagar, *et al*. [30] showed that *Bacillu pumilus* xylanase physically adsorbed on wheat bran retained 14% more residual activity than the free enzyme after 14 weeks' storage at 4°C. *Thermomyces lanuginosus* xylanase immobilized on functionalized magnetic nanoparticles exhibited 25% more residual activity than its free counterpart after 90 days' storage at 4°C [42].

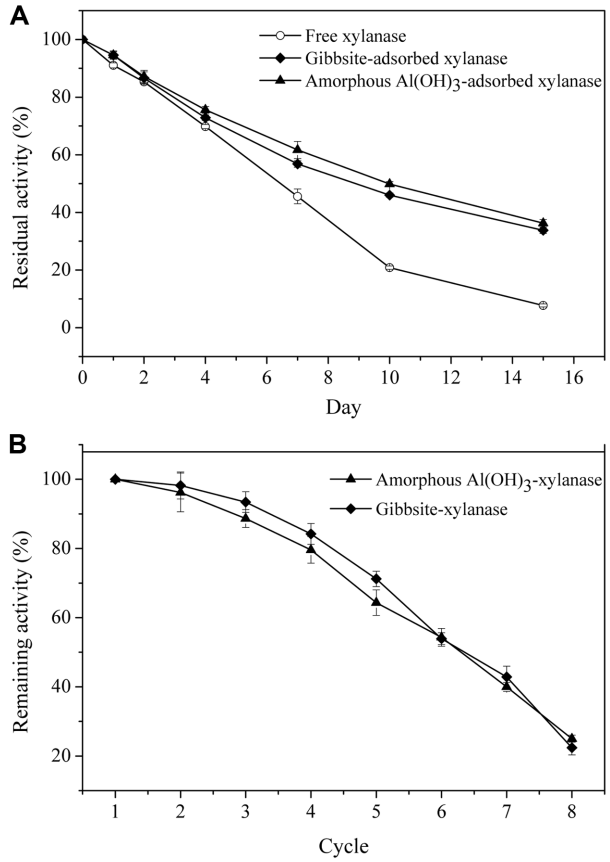

Fig. 4. Residual activities of free and adsorbed xylanases during incubation (**A**) and remaining activities of adsorbed xylanases after cycling use (**B**).
Bars are standard errors ($n = 3$).

Reusability of Adsorbed Xylanases

The reuse of enzyme is an important factor when taking its cost-effectiveness into consideration for commercial applications. Immobilization of enzymes may allow their reuse in many cycles of the reaction, decreasing the process costs. The reusability of immobilized xylanase in this study was high during the initial stage (Fig. 4B). The adsorbed xylanase showed considerable operational stability and retained an average of 91% and 68% enzyme activity after 2nd and 4th repeated cycles, respectively. However, because of the disadvantage of the adsorption method, in that it is not covalently bound and the enzyme might be leached from the support in an aqueous medium [41], more activity was lost with 54% and 41% activity after recycling five and six times, respectively. After eight recycle uses, only an average of 24% of initial activity was retained.

The high reusability of immobilized xylanase has been observed previously but the extent varied dependent on the enzyme species, support characteristics, and the kind of interactions between them. For example, the xylanase from *Bacillus pumilus* strain MK001 entrapped into gelatin or adsorbed on chitin only had less than 20% of initial activity after reuse of five cycles [20], much lower than our results. *Streptomyces olivaceoviridis* xylanase immobilized on Eudragit S-100, which retained 81% of the original activity after four cycles of use [2], exhibited similar operational stability to our results. Immobilization of *Thermomyces lanuginosus* SSBP xylanase using Eudragit S-100 retained 62% of initial activity after six reuses [12], a little more than our results. Some immobilized xylanases showed greater reusability than our results, such as *Aspergillus niger* immobilized on glutaraldehyde-alginate beads that retained 85% of its initial activity after being used for five cycles [35].

In general, the xylanase immobilized on aluminum hydroxide particles showed considerable reusability. Xylanase adsorbed on gibbsite and amorphous $Al(OH)_3$ retained 71% and 64% of the initial activity, respectively, even after five recycles.

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