Synergistic degradation of arabinoxylan by free and immobilized xylanases and arabinofuranosidase

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Abstract

Effective degradation of hemicellulose is of utmost importance in a wide variety of applications in bioindustry. Five endoxylanases from different glycoside hydrolase families and microorganisms were tested with an arabinofuranosidase, Araf51A, for the hydrolysis of insoluble wheat arabinoxylan, which is a structural component of hemicellulose. The optimized combination was XynZ/Xyn11A/Araf51A with a loading ratio of 2:2:1, and the value of degree of synergy increased with the increase of Araf51A proportion in the enzyme mixture. Afterwards, selected enzymes were immobilized on commercial magnetic nanoparticles through covalent bonding. Both free and immobilized enzymes showed a similar conversion to reducing sugars after hydrolysis for 48 h. After 10 cycles, approximately 20% of the initial enzymatic activity of both the individual or mixture of immobilized enzymes was retained. A 5.5-fold increase in the production of sugars was obtained with a mixture of enzymes immobilized after 10 cycles in total compared with free enzymes. Importantly, a sustainable synergism between immobilized arabinofuranosidase and immobilized endoxylanases in the hydrolysis of arabinoxylan was demonstrated.

Keywords: Synergism; Xylanase; Arabinofuranosidase; Enzyme immobilization; Hemicellulose

1. Introduction

Hemicelluloses include a number of polysaccharides, of which xylans are the second most ubiquitous in nature. In recent years, biodegradation of hemicellulose has attracted a lot of attention in the industry, such as for the conversion of biomass to biofuels and value-added chemicals, enhancing the efficiency of delignification and the bleaching process in the pulp industry [1].

Arabinoxylans are the main form of hemicellulose in plant cell walls, especially in cereal grains such as wheat. They consist of a xylan backbone with arabinose residues linked to its O-2 or O-3 positions [2]. The breakdown of xylan backbone by xylanase(s) is not efficient because of the presence of the substituted arabinose. Therefore, the removal of the arabinose side chains by supplementing with arabinofuranosidase (AFases) is a common method used to increase the efficiency of hemicellulose degradation. The cooperative activity between AFase and xylanase has been used to synergistically increase the release of soluble saccharides during the hydrolysis of arabinoxylan [3,4]. For example, Yang and coworkers recently reported a bifunctional AFase from glycoside hydrolase (GH) family 51 that has the activity of both arabinofuranosidase and β -xylosidase. It worked synergistically with endoxylanase XynBE18 in the degradation of wheat arabinoxylan, increasing the production of sugar about 3-fold [5].

Many enzymes have been investigated and developed in an insoluble or immobilized form for academic interest and industrial use, owing to their advantages, such as enabling continuous usage, improved stability, activity and selectivity [6,7]. There are both physical and chemical ways to immobilize enzymes on various supports,

which have been well summarized in previous reports [8–10]. For the application in xylan degradation, immobilization of xylanase has been investigated onto varied supports, such as multi-walled carbon nanotubes [11], synthesized polymer [12], nanoporous gold [13], and resin [14]. Recently, the use of magnetic nanoparticles as supports are of particular interest because they possess both the advantages of the simple separation process of magnetic materials and the high surface area and mass transfer resistance of nanosized materials [15–19]. An increased thermal and pH stability and good reusability of the insoluble form of the enzyme was observed in all the studies compared with the corresponding soluble enzymes. **However, enzymes immobilized on nano-sized materials are yet to be fully utilized on an industrial scale.**

Furthermore, almost all the research on the synergism between enzymes has been conducted with free enzymes [20]. Because the basic characteristics of enzymes might be altered upon immobilization onto solid supports, it is also important to investigate the synergism between immobilized enzymes. Tsai et al. observed 2-fold improvement of sugar production when co-immobilizing endoglucanase CelA and exoglucanase CelE onto CdSe-ZnS core-shell quantum dots for hydrolysis of phosphoric-acid-swollen cellulose [21]. **However, little has been studied and there is a need for the development of a recyclable and synergistic enzymatic system for (hemi)cellulose degradation. Therefore, we studied the degradation of arabinoxylan by enzymes in insoluble form, especially focusing on the change in synergism due to the recycling process.**

In the present study, we have explored the potential synergistic action of different enzyme formulations, free and immobilized enzymes, in hemicellulose degradation. Five xylanases, XynZ from *Clostridium thermocellum*, belonging to GH10, and Xyn11A and Xyn10B from *Thermobifida fusca*, belonging to GH11 and GH10,

respectively, and XlnB and XlnC from *Streptomyces lividans*, all belonging to GH11 were tested for their effectiveness in arabinoxylan degradation. The two endoxylanases (XynZ and Xyn11A) that showed the highest conversion to reducing sugars among the five xylanases were selected and investigated together with a family 51 arabinofuranosidase, Araf51A, from *C. thermocellum*. Subsequently, three enzymes were separately immobilized on commercial magnetic nanoparticles through covalent bonding. The immobilized enzymes were successfully recycled 10 times. Continued synergism between the enzymes immobilized on magnetic nanoparticles after 10 cycles was illustrated using arabinoxylan hydrolysis as a model system.

2. Materials and Methods

2.1 Substrates and enzymes

Insoluble wheat arabinoxylan was purchased from Megazyme (P-WAXYI, Wicklow, Ireland). It consists of 36% arabinose, 51% xylose, 6.5% glucose, 4.4% mannose, and 1.6% galactose. The magnetic nanoparticles decorated with N-hydroxysuccinimide groups (190±20 nm in diameter) were purchased from Tamagawa Seiki. Co., Ltd (Japan).

The details of the expression and purification of the five xylanases used in this study have been described in our previous work [22]. Arabinofuranosidase, Araf51A, was prepared as follows. The genomic DNA of *C. thermocellum* was obtained from National Institute of Technology and Evaluation Biological Resource Center. An open reading frame encoding AraF was amplified from the genomic DNA of *C. thermocellum* using Phusion DNA polymerase with the primer combination of 5'-CATATGAAAAAAGCCAGAATGACCGTTGACA-3' and

5'-CTCGAGTTTACCTATCCGAATTACATTCCAAGAGGCT-3'. The amplified gene fragment was cloned into pBluescript II, digested with NdeI/XhoI, and then inserted into the cloning site of pET22 to construct an expression plasmid of AraF. *E. coli* BL21 (DE3) was transformed with the expression plasmid of AraF. The fresh transformant was seeded into 40 mL of Luria–Bertani medium supplemented with ampicillin (100 mg/L) and grown overnight with shaking (180 rpm) at 37 °C. The overnight culture was transferred into 1 L of Terrific Broth medium supplemented with ampicillin (100 mg/L). Cells were grown with shaking (180 rpm) at 37 °C until the OD₆₀₀ reached a value of 0.8. The culture was then cooled to 27 °C, supplemented with Isopropyl β-D-1-thiogalactopyranoside (0.5 mM), and incubated with shaking (180 rpm) for 48 h. The *E. coli* cells were then harvested by centrifugation (4500 ×g), washed with 100 mL of 50 mM potassium phosphate (pH 7.4), and resuspended in 100 mL of lysis buffer and treated with 10 mg lysozyme (Wako Pure Chemicals) and 250 U Benzonase (Novagen) in the presence of 10 mM MgCl₂ for 30 min at 4 °C. The resultant spheroplasts were collected by centrifugation (7000 ×g), frozen in liquid nitrogen and stored at –80 °C. Before use, the spheroplasts were thawed in 50 mL of ice-cold lysis buffer and disrupted by sonication in an ice-cold water bath. After removing the cell debris by centrifugation (10,000 ×g), cell-free extracts were applied for His-tag affinity chromatography using an AKTA Prime plus system (GE Healthcare) equipped with HisTrap FF crude columns (GE Healthcare) at 4 °C. After the affinity chromatography, recombinant AraF was further purified by anion-exchange chromatography using HiTrap Q HP column (GE Healthcare).

2.2 Enzyme immobilization on magnetic nanoparticles

The immobilization method followed the protocol provided by the manufacturer with minor modifications. Briefly, 1 µg/µL of the enzyme solution was prepared with immobilization buffer, which was 25 mM HEPES-NaOH (pH 7.9). Then the nanoparticles were washed with 50 µL of methanol and immobilization buffer successively, the liquid phase was removed after centrifugation. The immobilization buffer (50 µL) and the prepared enzyme solution (50 µL) were added into a small tube and reacted at room temperature for 1 h and then incubated at 4 °C for 20 h with gentle rotating. After the reaction, the supernatant was removed by centrifugation. To mask the residue carboxyl groups on the nanoparticles, 250 µL of 1 M ethanolamine solution (pH 8.0) was added into the tube and reacted at 4 °C for 20 h with gentle rotating. After that, the enzyme-immobilized nanoparticles were washed three times with 50 mM phosphate buffer (pH 7.0), and resuspended in 200 µL phosphate buffer for further hydrolysis experiments. The amount of bound protein was determined by a bicinchoninic acid assay (BCA assay, Thermo Fisher Scientific). The **immobilization** efficiency was calculated using the following equation:

Immobilization efficiency (%) = Total amount of **immobilized** protein/Total amount of added protein ×100%

2.3 Enzymatic hydrolysis and sugar analysis of hydrolysates

For the assay of free enzymes, the selection of proper endoxylanases and optimization of the loading ratio together with arabinofuranosidase was performed. **The hydrolysis experiments were performed with 0.25 wt% of the arabinoxylan at 50 °C in 2 mL of sodium phosphate buffer (50 mM, pH 7.0) and placed in a rotary shaker (TAITEC MBR-022UP, Japan) at 1000 rpm.** In each tube the total enzyme loading was

kept at 100 nM. For the binary mixtures, each enzyme loading was 50 nM, and for the ternary mixture, the enzymes were mixed at a specific ratio, but the total concentration was kept at 100 nM. After hydrolysis, the produced reducing sugars were analyzed by the dinitrosalicylic acid method (DNS assay) with xylose as the standard [23]. Briefly, 100 μ L of reaction solution was mixed with 100 μ L of DNS reagent, which containing 1.3M DNS, 1M potassium sodium tartrate, and 0.4 N NaOH, and incubated at 99 °C for 5 min. The reducing sugars were quantified colorimetrically at an absorbance of 540 nm. The soluble sugars were analyzed by high-performance liquid chromatography (HPLC) with a Shodex sugar KS-801 column (8.0 $\times$ 300 mm, Showa Denko Co., Tokyo, Japan) and a refractive index detector at 80 °C with HPLC-grade water as the eluent at a flow rate of 1 mL/min.

For investigation of the enzymes immobilized on nanoparticles, the saccharification experiments were performed using the same conditions, but with a final volume of 500 μ L. Correspondingly, for the binary mixture each enzyme loading was 50 nM; for the ternary mixture, XynZ, Xyn11A and Araf51A were mixed at an optimized ratio of 2:2:1, which was obtained from the results of the free enzymes, with the total concentration kept at 100 nM. After hydrolysis, the concentrations of the reducing sugars were analyzed by using tetrazolium blue (TZ) assay [24]. After hydrolysis for 48 h, the enzymes immobilized on magnetic nanoparticles were separated using a magnet then washed with phosphate buffer three times. After that, a new substrate was introduced to the system. All the hydrolysis experiments were performed in triplicates. Values are averages of results from three independent trials; error bars indicate the standard deviation values.

2.4 Calculation of the degree of synergy (DS)

The calculation of the degree of synergy (DS) was performed using the equation:

$$DS = Y_{1+2+3}/(\alpha Y_1 + \beta Y_2 + \gamma Y_3),$$

where α , β and γ correspond to the molar ratios of the enzymes. Y_{1+2+3} , indicates the yield of reducing sugars by the three enzymes working simultaneously, whereas Y_1 , Y_2 and Y_3 indicate the yield of the reducing sugars achieved by each enzyme working individually. For all cases, single enzymes or combination of enzymes, the enzyme loading was kept at 100 nM.

3. Results and Discussion

3.1. Enzymatic hydrolysis of insoluble wheat arabinoxylan by free xylanases and free arabinofuranosidase

Five endoxylanases, XynZ from *C. thermocellum*, Xyn11A and Xyn10B from *T. fusca*, XlnB and XlnC from *S. lividans*, were selected to investigate their performances in the hydrolysis of insoluble wheat arabinoxylan. Afterwards, the concentration of the reducing sugars was measured by DNS assay. The reducing sugars we measured here indicate the total amount of sugars including the monomeric and oligomeric forms, which were estimated by DNS method. As shown in Fig. 1, XynZ hydrolyzed the highest amount of reducing sugars (1.5 mM) from the substrate after 24 h, followed by Xyn11A (1.4 mM). The high conversion of substrate to reducing sugars achieved by XynZ or Xyn11A, might be the result of their different structural features. XynZ was purified from *C. thermocellum*, which is an anaerobic bacterium, producing lignocellulose hydrolyzing enzymes in the form of cellulosomes. The full length of XynZ includes a feruloyl esterase domain, a cellulose-binding module (CBM) from

family 6 and GH10 xylanase domain. We have only expressed the xylanase catalytic domain of XynZ, since it has higher activity against insoluble birchwood xylan than that of intact enzyme [25]. Conversely, Xyn11A has a special CBM, so-called XBM, that can bind to both cellulose and xylan, thus increasing the interactions between xylan and enzymes [26]. Our previous works also showed that these two endoxylanases are highly active in the hydrolysis of pretreated bagasse [22,27].

Interestingly, xylanases from different families showed different patterns in enzymatic saccharification of insoluble wheat arabinoxylan. All the xylanases from family 11, which are Xyn11A, XlnB and XlnC, produced more reducing sugars in the initial stage of hydrolysis than the xylanases from family 10, such as Xyn10B and XynZ. It has been suggested that GH10 xylanase has a greater affinity to highly branched xylan, whereas GH11 xylanases preferably cleave in the unsubstituted regions of the xylan backbone [28,29]. Thus, at the beginning of the hydrolysis process, GH 11 xylanase acts on the unsubstituted sites and the concentration of reducing sugars would not change as much even if the time period was extended. Similar observation was reported by using xylanases from these two families to degrade wheat bran [30].

Surprisingly, arabinofuranosidase, Araf51A, from *C. thermocellum*, showed little conversion to the reducing sugars under the described conditions. Taylor and co-workers have previously demonstrated that Araf51A is an intracellular enzyme that shows relatively lower activity on polymeric wheat arabinoxylan than on an artificial substrate, PNP-Araf, and short soluble oligosaccharides [31].

Meanwhile, the hydrolytic performance of xylanase(s) together with arabinofuranosidase, with a loading ratio of 1:1, for the hydrolysis of arabinoxylan was also evaluated. First, binary combination, that is each individual xylanase combined

with Araf51A, were tested. In general, all the combinations displayed a higher conversion to the reducing sugar than the single enzymes. In particular, the combination of XynZ and Araf51, and Xyn11A and Araf51A, had the highest production of reducing sugars, approximately 2 mM after 24 h hydrolysis of arabinoxylan. This proves that the addition of Araf51A is benign to the action of xylanase. Moreover, the concentration of reducing sugars converted by ternary mixture of enzymes (loading ratio of 1:1:1) was quantified to select the best combination. As our previous data has already shown the synergistic effect between GH10 and GH11 xylanases in the hydrolysis of hemicellulose content in pretreated bagasse [22], in the present experiments we only combined family 10 and 11 xylanases with arabinofuranosidase, with the aim of achieving the highest conversion to sugar. As expected, after addition of xylanases from different GH families, the concentration of the reducing sugars produced by the enzyme mixture was improved. Among these, not surprisingly, the combination of XynZ and Xyn11A with the addition of Araf51A resulted in the highest amount of reducing sugars. Therefore, XynZ, Xyn11A, as well as Araf51A were selected to conduct further experiments.

3.2. Synergism between free enzymes, XynZ, Xyn11A and Araf51A

Because of its complex structure, lignocellulosic biomass, in most cases, needs a consortium of enzymes to degrade collaboratively. The synergism between enzymes is a reflection of the degree of collaboration between two or more enzymes during the degradation of a substrate. The degree of synergy (DS) is a direct and quantitative way of evaluating the cooperation between enzymes. The value of DS is calculated by dividing the observed activity of a consortium of enzymes with the sum of the activities of the same enzymes, but working separately. If the DS value is greater than one, it

means that synergism exist between the enzymes. Conversely, if DS is less than one, it indicates there is no synergy between the enzymes and they are probably competing with each other for binding sites on the substrate.

As shown in Fig. 1, with the addition of Araf51A, more sugars were released, compared with the amount of reducing sugars produced by each enzyme working individually. For example, when used alone, XynZ and Xyn11A produced 1.5 mM and 1.4 mM reducing sugars, respectively, after 24 h hydrolysis. With the addition of Araf51A, the amount of reducing sugars increased to 2.1 mM and 1.8 mM, respectively (Fig. 1). This suggests that a synergistic effect exists between endoxylanase and arabinofuranosidase.

3.2.1 Impact of synergism on soluble sugars production

The soluble sugars released from the hydrolysis of arabinoxylan by individual or combined enzymes after 24 h were analyzed by HPLC. Note that higher polymerized oligomers have not been detected (Fig. 2). Smaller polymeric products were generated in the hydrolysis of arabinoxylan by XynZ, which belongs to GH10, whereas the products liberated by the action of Xyn11A were mostly xylotri- and xylobiose. This observation is in good agreement with a previous report [30]. A small amount of arabinose was detected when only Araf51A was used for the hydrolysis of arabinoxylan. But when XynZ and Xyn11A were supplemented with Araf51A, 0.53 mM of arabinose was released. Conversely, higher concentration of soluble xylose and short xylo-oligomers were released after mixing xylanases with Araf51A. In particular, the production of xylotri- and xylobiose increased by 42% and 50%, respectively, compared with the sum of those sugars produced by XynZ and Xyn11A

individually. Earlier studies have reported that there are soluble products generated from the action of xylanases on arabinoxylan, such as xylobiose with arabinose, linked at the non-reducing end (A^2X) from GH10 xylanases, and xylotriose with arabinose linked at the central xylose (XA^3X) from GH11 xylanases [29]. Consequently, these are the ideal substrates for Araf51A; by the action of Araf51A, these short soluble oligomers can be digested into arabinose and release xylotriose and xylobiose simultaneously. Besides, with the debranching action of Araf51A, more xylan backbone was exposed to the xylanase, and in turn, more xylose and short xylo-oligomers could be detected.

3.2.2 Effect of enzyme ratio on synergism between free enzymes

Because of the high efficiency of XynZ and Xyn11A in the hydrolysis of arabinoxylan together with Araf51A, different molar ratios of these three enzymes were tested to investigate the relationship between the enzyme loading and the conversion to reducing sugars (Fig. 3A). The ratios of XynZ, Xyn11A and Araf51A were chosen as 1:1:2, 1:1:1 and 2:2:1, respectively, but the total loading was kept constant at 100 nM. A similar amount of reducing sugars was obtained when XynZ, Xyn11A and Araf51A, were mixed at different ratios. The mixture of enzymes at a ratio of 2:2:1 converted slightly more sugars than the enzymes loaded at a ratio of 1:1:2 or 1:1:1. As mentioned before, Araf51A catalyzes the α -1,2- or α -1,3-arabinofuranosyl residues of the side chain, as a debranching enzyme, liberating a smaller amount of sugars than xylanase, which is a depolymerizing enzyme. More sugar could be generated with a higher proportion of xylanases. The degree of synergy between xylanases and arabinofuranosidase was also affected by the enzyme-loading ratio (Fig. 3B). The DS value increased with the increase of the arabinofuranosidase proportion in the enzyme

mixture. As shown in Fig. 3B, the ratio of XynZ/Xyn11A/Araf51A=1:1:2 resulted in the highest DS. However, a high DS value did not correspond to a higher reducing sugars concentration. The addition of less xylanase resulted in less degradation of the xylan main chain. Therefore, because of the high conversion to reducing sugars, subsequent experiments were conducted at a XynZ/Xyn11A/Araf51A ratio of 2:2:1.

3.3. Immobilization of hemicellulases on magnetic nanoparticles

There are physical and chemical ways to immobilize enzymes on solid support. Physical adsorption, based on hydrophobic and van der Waals interactions, is generally characterized as weak binding methods, and the enzyme(s) could not stably fix on the support [10]. Conversely, the enzyme immobilization through chemical reaction is strong that proteins are covalently bound to appropriately modified supports through functional side groups exposed on protein surface. By far, the reactive groups coupling with amine-containing molecules are the most common choice for protein immobilization. Solid supports surface covering suitable functional group, such as N-hydroxysuccinimide ester, epoxy, aldehyde or sulfonyl chloride, can readily interact with amino group of proteins to form amide bonds [32].

Three enzymes were immobilized individually on commercial magnetic nanoparticles using covalent bonding. Using commercial succinylated magnetic nanoparticles with an average diameter of 200 nm, the enzyme was readily conjugated using a common activation chemistry method; the N-hydroxysuccinimide (NHS) activated carboxylate group primarily coupled with α -amines at the N-terminals and the ϵ -amines of lysine side chains [32]. Consequently, a new and stable amide linkage was created. As shown in Table 1, the amount of enzymes bound on the magnetic

nanoparticles was different. Immobilization efficiency of Xyn11A was 44.8%, whereas only 22.4% and 16.4% of XynZ and Araf51A was bound, respectively. One possible reason might be the difference in the isoelectric point (pI) of the enzymes. Because the activated carboxylate groups make the nanoparticles negatively charged after hydrolysis, a closer proximity could be formed if the enzyme is positively charged at the given pH. In the present study, XynZ, Xyn11A and Araf51A have an isoelectric point of 5.2, 9.5 and 5.4, respectively. Thus, only Xyn11A is positively charged in the buffer solution at a pH of 7.9. Because of the electrostatic adsorption between the positively charged protein and negatively charged carboxyl group, more Xyn11A was bound. Johnsson and colleagues have compared the immobilization of one small protein with a high pI of 9.3, ribonuclease A, and one medium-sized protein with low pI of 5.1, *Staphylococcus aureus* protein A, on an NHS activated gold surface. The highest amount of protein was immobilized when the solution pH was lower than the pI of the protein, which reveals the importance of electrostatic attraction forces in the immobilization process [33]. Xyn11A is the smallest of the three enzymes. Thus, with a protein loading mass of 50 µg, more Xyn11A was added in the immobilization solution; as a result, more Xyn11A was available for immobilization.

The enzymes in the free and immobilized form were characterized. The Fourier transform infrared spectroscopy (FTIR) results confirmed the successful immobilization of the enzymes on the nanoparticles (Fig. S1). In the case of free enzymes, the characteristic bands at approximately 1650 and 1541 cm^{-1} were assigned to Amide I and II (data not shown), respectively, which are the two prominent vibration bands of the protein backbone [34]. In the last step of the immobilization, the unreacted carboxyl groups were masked with ethanolamine to form an amide bond. The difference between

the nanoparticles reacted with ethanolamine and enzyme was also demonstrated by FTIR. After the immobilization of XynZ on the nanoparticles, the Amide I band at 1650 cm^{-1} was clearly observed and the Amide II band shifted to 1604 cm^{-1} , whereas these bands were not observed in the case of the magnetic nanoparticles masked with ethanolamine. Similar observations were made in the case of Xyn11A and Araf51A.

Thereafter, the performance of the free enzyme and the enzyme immobilized on nanoparticles in the hydrolysis of insoluble arabinoxylan was investigated (Fig. 4). After hydrolysis for 48 h, comparable amounts of reducing sugars were obtained using the free enzymes and immobilized enzymes, although the initial stage of the conversion by the immobilized enzymes was slow (Fig. S2). The reason might be the slower rate of substrate diffusion in the conjugated system owing to steric hindrance from the solid support [35]. It is also worth mentioning that the immobilized enzyme showed a similar trend to the free enzyme in the hydrolysis process, whereby xylanase from GH11 converted a higher amount of sugars in the initial stage, whereas xylanase from GH10 gradually degraded the insoluble substrate. This result indicated that enzymes immobilized on nanoparticles have the potential to be used in the hydrolysis of (hemi)cellulosic biomass, with similar features as the free enzymes, and with the advantage of being reused.

3.4 A recyclable and synergistic enzyme system for hemicellulose degradation

The recyclability of the immobilized enzymes was evaluated by using it multiple times. After the first hydrolysis, the immobilized enzymes were separated using a magnet, washed, and a fresh substrate was introduced. As illustrated in Fig. 5A, 10 cycles of hydrolysis were performed. Since the concentration of arabinose was below

the detection limit of TZ assay used in this study, the amount of reducing sugars produced by immobilized Araf51A alone could be negligible. We thus set the value of Y_3 as zero for the calculation of DS. Not surprisingly, in all cases, for individual or a mixture of immobilized enzymes, the highest conversion was achieved in the first cycle. An apparent loss of activity was observed after each cycle, with the largest reduction after the first cycle. The amount of reducing sugars produced by immobilized XynZ, immobilized Xyn11A and mixture of immobilized enzymes dropped by 58%, 42% and 35%, respectively, after the first cycle, probably owing to the disassociation of weakly bound enzymes. Only the strongly bound enzymes were retained after the first cycle. After 10 cycles, the amount of reducing sugars produced by the individual enzymes, XynZ and Xyn11A, fell to 21% and 25% of the amount of sugars produced in the first cycle, respectively; the amount of reducing sugars produced by the mixture of immobilized enzymes was 42% of that from the first cycle. This drop in the conversion to sugar might be because of both dissociation and deactivation of the enzymes. Jordan and co-workers covalently immobilized commercial cellulase on magnetic nanoparticles and recycled cellulase six times until the activity decreased to 10% of initial cycle. Moreover, they observed a noticeable drop of 47.5% of enzyme activity after the first cycle [36,37].

The degree of synergy (DS) between immobilized xylanases and arabinofuranosidase after 48 h of hydrolysis of insoluble wheat arabinoxylan were calculated (Fig. 5B). After ten times recycling of the immobilized enzymes, the DS value increased from 1.9 (cycle 1) to 3.4 (cycle 10). As shown in Fig. 5A, the conversion of reducing sugars by each individual enzyme, through ten cycles, was decreased, especially from cycle 1 to cycle 5, and there was a substantial loss in the

conversion of reducing sugars for the immobilized XynZ. However, the concentration of reducing sugars released by the collaborative action of the enzymes in the mixture did not decrease as much, probably because of the synergism between the enzymes, which were still active. Based on the equation of DS, in which synergism is a function of the ratio of reducing sugars converted by enzyme mixture and individual enzyme components, the value of DS would increase mathematically with the large decrease of the denominator, as the significant loss in reducing sugars by XynZ, from cycle 1 to cycle 5. Nevertheless the synergism between arabinofuranosidase and xylanases improved the production of the reducing sugars through all ten cycles. This indicates the potential application of enzymes immobilized on inorganic solid supports for the synergistic degradation of hemicellulose.

In most studies, the hydrolysis experiments with free enzymes can only be performed once, owing to the poor recoverability of the free enzymes. However, with the same initial enzyme loading, enzymes immobilized on nanoparticles could be recycled several times. Fig. 5C shows the comparison of the total amount of sugars produced by free enzymes after one cycle and by the enzymes immobilized on nanoparticles after ten cycles. The free XynZ and Xyn11A produced 2.0 and 1.6 mM sugars, respectively, after 48 h hydrolysis of arabinoxylan. Correspondingly, with the same enzyme loading, immobilized XynZ and Xyn11A produced 6.2 and 7.9 mM sugars, respectively, after 10 cycles. The mixture of free enzymes produced 2.7 mM reducing sugars after hydrolysis of insoluble arabinoxylan for 48 h, whereas the corresponding mixture of immobilized enzymes produced a 5.5-fold increase of reducing sugars after 10 cycles.

In summary, clear synergism was observed between endoxylanases from GH10 and

GH11, and arabinofuranosidase in the hydrolysis of insoluble arabinoxylan. More importantly, continued synergism was observed between the same enzymes after they were immobilized on magnetic nanoparticles. Because of the recyclability, a 5.5-fold increase in the amount of sugar was obtained after 10 cycles compared with free enzymes. These remarkable improvements in the production of sugars demonstrate that immobilized enzymes can be used to reduce enzyme loadings and lower the production costs in industries that use enzymes. **Although the technology of enzyme recovering through nanoparticle immobilization is not mature enough to be transferred into production scale, the presenting recyclable and synergistic enzyme system could provide as a reference for the cost-effective enzyme utilization study.**

4. Conclusions

Two purified recombinant endoxylanases, XynZ and Xyn11A, were shown to degrade insoluble arabinoxylan with a high conversion to reducing sugars. The selected xylanases worked synergistically with arabinofuranosidase and converted the highest amount of sugars at a ratio of XynZ:Xyn11A:Araf51A=2:2:1. After immobilization of the enzymes on magnetic nanoparticles through covalent bonding, the immobilized enzymes were recycled 10 times. Continued synergism between the enzymes immobilized on magnetic nanoparticles after 10 cycles was illustrated. Our results indicate efficient utilization of the interaction between arabinofuranosidase and xylanases for the degradation of natural polymers, which can be used to lower the production costs in industries that utilize enzymes.

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Figure captions

Fig.1 Determination of reducing sugar concentration after 1, 3, 6, 10, and 24 h

hydrolysis of arabinoxylan by individual or combinations of endoxylanases, XynZ, Xyn11A, Xyn10B, XlnB and XlnC, with arabinofuranosidase, Araf51A. The total enzyme loading was kept at 100 nM with working volume of 2 mL. For the binary mixtures, each enzyme loading was 50 nM, and for the ternary mixture, each enzyme loading was 33 nM. The concentration of reducing sugars was measured by DNS assay with xylose as standard. The error bars represent the standard deviations from three independent trials. Asterisk symbol (*) represents the concentration of reducing sugars converted by Araf51A alone was below the detection limit of DNS assay.

Fig. 2 The concentration of soluble sugars after 24 h hydrolysis of arabinoxylan by individual or mixed enzymes, XynZ, Xyn11A and/or Araf51A, measured by HPLC. The total enzyme loading was kept at 100 nM with working volume of 2 mL. For the ternary mixture, each enzyme loading was 33 nM. The error bars represent the standard deviations from three independent trials.

Fig. 3 (A) The optimization of the loading of enzymes mixture for the hydrolysis of arabinoxylan. Total enzyme loading was 100 nM in 2 mL working volume. The enzymes were loaded as the ratios shown below. The concentration of reducing sugars was measured by DNS assay with xylose as standard. Asterisk symbol (*) represents the concentration of reducing sugars converted by Araf51A alone was below the detection limit of DNS assay. (B) The degree of synergy (DS) of xylanases and arabinofuranosidase for hydrolysis of arabinoxylan under different enzyme-loading ratios.

Fig. 4 Reducing sugar concentration after 48 h hydrolysis of arabinoxylan by free and

immobilized enzymes. The total enzyme loading was kept at 100 nM with working volume of 500 μ L. For the mixture of enzymes, enzyme-loading ratio was XynZ/Xyn11A/Araf51A=2:2:1. The concentration of reducing sugars was measured by TZ assay with xylose as standard. The error bars represent the standard deviations for three independent trials. Asterisk symbol (*) represents the concentration of reducing sugars converted by Araf51A alone was below the detection limit of TZ assay.

Fig. 5 (A) The amount of reducing sugars converted by individual or a mixture of immobilized enzymes in the hydrolysis of arabinoxylan for 48 h after 10 cycles. For immobilized Araf51A, the detection limit of TZ assay in this study (0.08 mM) was shown. (B) The degree of synergy (DS) of xylanases and arabinofuranosidase for hydrolysis of arabinoxylan after 48 h. The white bar represents the DS of free enzymes; the grey bars represent the DS values of enzymes immobilized on nanoparticles after 10 cycles. (C) The amount of reducing sugars converted by free enzymes (white bar); and sum of sugar production converted by immobilized enzymes after 10 cycles (gray bar). The total enzyme loading was kept at 100 nM with working volume of 500 μ L. For the mixture of enzymes, enzyme-loading ratio was XynZ/Xyn11A/Araf51A=2:2:1. The concentration of reducing sugars was measured by TZ assay with xylose as standard. The error bars represent the standard deviations from three independent trials. Asterisk symbol (*) represents the concentration of reducing sugars converted by Araf51A alone was below the detection limit of TZ assay.

Table**Table 1** Immobilization efficiency of proteins on the magnetic nanoparticles and properties of the enzymes

Enzyme	Immobilization efficiency (%)	Theoretical isoelectric point	Molecular weight (kDa)
XynZ	22.4 ± 2.2	5.2	38.0
Xyn11A	44.8 ± 6.2	9.5	36.4
Araf51A	16.4 ± 5.8	5.4	57.7

Figure 1

Figure 2

Figure 3

(A)

(B)

Figure 4

Figure 5