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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.

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

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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 $MgCl_2$ 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:

$$\text{Immobilization efficiency}(\%) = \text{Total amount of immobilized protein} / \text{Total amount of added protein} \times 100\%$$

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 μL of reaction solution was mixed with 100 μL of DNS reagent, which containing 1.3 M DNS, 1 M 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