Performance and Kinetic Study of Xylan Hydrolysis by Free and Immobilized *Trichoderma* Xylanase

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Abstract. Enzyme immobilization is essential for enhancing the stability and reusability of enzymes in various industrial processes. To improve its feasibility, efficient yet simple immobilization techniques were required to be explored with respect to enhance overall catalytic efficiency and/or operational performance. This study investigates the enzymatic hydrolysis of beechwood xylan using free and immobilized xylanase in a batch reaction system at 60°C, which was considered as damaging temperature for most enzymatic protein. Xylanase immobilization was carried out by direct adsorption of xylanase onto dry zeolite, which resulted in 92.70% efficiency. The experimental variables used in this study include pH levels (pH 5.0 and 6.0) and xylan concentrations at 1.00% (w/v), 1.50% (w/v), 2.00% (w/v), and 2.50% (w/v). The optimal process variables for xylan hydrolysis by free enzyme and immobilized enzyme were found to be at pH of 5.0 and xylan concentration of 2.50% (w/v). At the optimum condition, the concentration of xylose obtained at 40 min of hydrolysis was 4.82 mg/mL and 5.68 mg/mL for free and immobilized enzymes, respectively. The saturation constants (K_m) were determined to be 19.12 mg/mL and 42.03 mg/mL for free and immobilized enzymes, respectively, while the maximum rates (v_{max}) were 1.92 mg/(mL.min) and 1.21 mg/(mL.min) for free and immobilized enzymes, respectively. The results of this study suggest that xylanase immobilization could improve enzyme stability at higher processing temperatures without changing its response to surrounding pH.

Keywords: Xylanase, Immobilization, Kinetic.

1 Introduction

Xylan is a complex polysaccharide that is abundantly found in plant cell walls. It is composed of a linear chain of xylose units connected by β-1,4 glycosidic linkages. Xylan is considered as the second most abundant polysaccharide in nature, after cellulose. Paper pulp may consist of xylan around 10 % weight [1]. It provides structural support to plants and plays a crucial role in the strength and rigidity of cell walls. The hydrolysis of xylan has been applied to the production of xylo-oligosaccharides and

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xylose, which are healthy dietary fibers and the main ingredients in xylitol production, respectively.

Xylanase is an enzyme that catalyzes the hydrolysis of xylan, breaking it down into smaller sugar units. It belongs to the glycoside hydrolase family and specifically acts on the β-1,4 glycosidic linkages between xylose units in the xylan polymer. Xylanases are produced by various organisms, including microorganisms (bacteria, fungi, and yeasts), protozoans, molluscs, and ruminant animals [2]. Xylanase can be classified into different types based on their mode of action. Endo-xylanase randomly cleaves the internal β-1,4 glycosidic bonds within the xylan chain, resulting in the generation of shorter xylan fragments or xylo-oligosaccharides. On the other hand, exo-xylanase or β-D xylosidase acts on the ends of the xylan chain, sequentially releasing xylose units from the non-reducing end or removing xylose residues from the reducing end of the xylan molecule. Xylanase has been widely used in several industries, i.e., food and beverage, pulp and paper, textile, biofuel, and animal feed [3].

Enzyme immobilization could solve practical problems in industrial enzymes usage such as its lifetime, performance stability at higher temperature and/or substrate concentration during the hydrolysis, and difficulty for enzyme recovery that could bring better economic and technical feasibility of related process [4]. Many immobilization techniques have been used for enzyme immobilization, including entrapment [5], cross-linking [6], adsorption [7] and covalent bonding [8]. Among those techniques, adsorption is the simplest and inexpensive [9]. Xylanase immobilization through direct adsorption on zeolite has been rarely studied. A previous study conducted by Sutrisno et al. successfully immobilized 156.5 mg of xylanase per mg of zeolite by stirring 0.1 g of dried and HCl-activated zeolite with 5 mL xylanase solution at room temperature and 100 rpm for 3 h [10]. However, the evaluation of performance under various process variables (pH, temperature, and substrate concentration), as well as the assessment of activity changes after immobilization, were not investigated. This could overlook the negative impact of enzyme immobilization such as higher mass transfer resistance for the substrate and alteration of enzyme molecular conformation, thus its catalytic ability. Hence, this study evaluated the performance of immobilized xylanase in contrast to the free xylanase to hydrolyse xylan. The effect of pH and xylan concentration on xylose generation was carried out systematically at moderately high temperature (60°C) and fixed reaction time to prove the performance stability of immobilized enzyme at higher temperature. Michaelis-Menten kinetic was also explored to provide valuable insights on the immobilization effect towards catalysis performance of xylanase.

2 Experimental

2.1 Immobilization of xylanase

Zeolite (Lot no. 96096: molecular sieve, type 13X, pore diameter: 4 Å, Sigma Aldrich, USA) was heated in an oven at 105°C for 5 hours to remove any moisture,

which can hinder enzyme immobilization on its active adsorption site. Endo-1,4-β-Xylanase M1 from *Trichoderma viride* (1500 U/mL, Megazyme, USA) was diluted with citrate buffer (0.1 M, pH 5.3) to obtain a xylanase solution with an enzymatic activity of 375 U/mL. Immobilization was achieved by mixing 6 mL of the diluted enzyme solution with 12 g of zeolite at 4°C for 24 hours. After immobilization, the solid phase was separated by centrifugation at 4000 rpm and 4°C, followed by several rinses with citrate buffer (0.1 M, pH 5.3). The liquid phase was pooled to determine the enzyme content using a Bradford assay kit (Proteomics grade, Amresco, USA). It was found that the immobilization reached 92.70% of the enzyme (expressed as bovine serum albumin-equivalent) relative to the enzyme mass in the diluted xylanase solution.

2.2 Hydrolysis of xylan

Xylanase hydrolysis was performed for both the free and immobilized formulations at the same activity density (1.45 U/mL of substrate). The pH effect was assessed by testing the catalyst's ability to hydrolyze 1% (w/v) xylan dissolved in citrate buffer with pH 5.0 or 6.0 at 60°C for 40 min until equilibrium was reached (data not shown). The concentration of xylose formed was then analyzed using the 3,5-dinitrosalicylic acid (DNS) assay [11]. The analysis was conducted in triplicates using a UV-Vis spectrophotometer at 540 nm.

To evaluate the substrate-dependent kinetics, xylan concentration was varied at 1.00% (w/v), 1.50% (w/v), 2.00% (w/v), and 2.50% (w/v), which corresponded to 10 mg/mL, 15 mg/mL, 20 mg/mL and 25 mg/mL, respectively. Xylan was let to hydrolyse at 60°C and the optimum pH for a duration of 40 min. The Michaelis-Menten equation (equation 1) was employed to determine the reaction rate of xylose production.

$$v = \frac{v_{max} \times [S]}{K_m + [S]} \tag{1}$$

where v expressed in mg xylose/(mL.min) is the increase of xylose concentration during reaction time, [S] is xylan concentration expressed in mg xylan/mL, v_{max} and K_m are parameter kinetics which are maximum rate expressed in mg xylose/(mL.min) and saturation constant expressed in mg xylan/mL, respectively.

3 Results and Discussion

3.1 Effect of pH

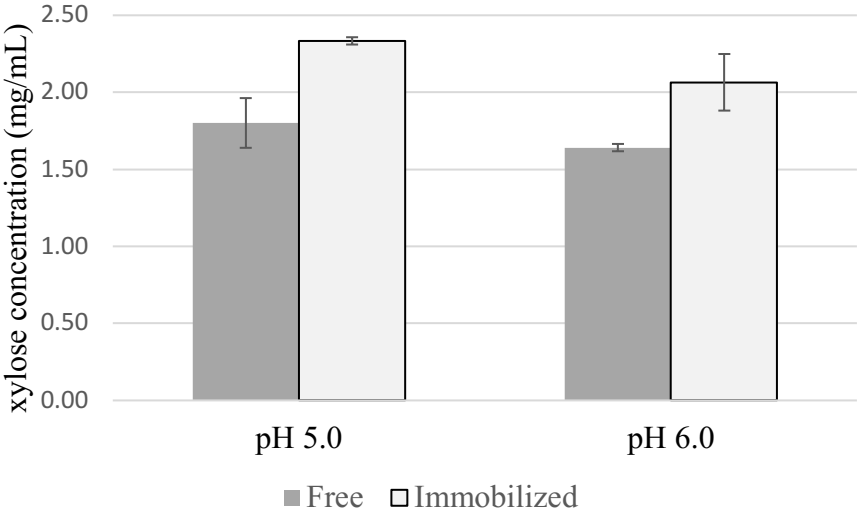

Fig. 1. The profile of xylose concentration from hydrolysis of 1% w/v xylan by different xylanase system at 60°C and pH 5.0 or 6.0 for 40 min.

As depicted in Figure 1, both the free and immobilized xylanase demonstrated superior performance at pH 5.0 compared to pH 6.0. This observation aligns with the optimal pH range specified by the enzyme producer, which falls within the pH 4.5-5.0 range [12]. Similar to other enzymatic reaction systems, pH plays a crucial role in determining the three-dimensional protein structure, which in turn influences the accessibility of the substrate to the active reaction site. Additionally, the activity of the reaction site itself may be affected as the amino acids in the enzyme can have different charges at different pH values. Interestingly, the pH increase in both systems led to a comparable decrease in xylose concentration of approximately 0.2 mg/mL. This suggests that the response of enzyme molecules to pH changes remained unchanged upon immobilization.

3.2 Substrate-dependent kinetic analysis

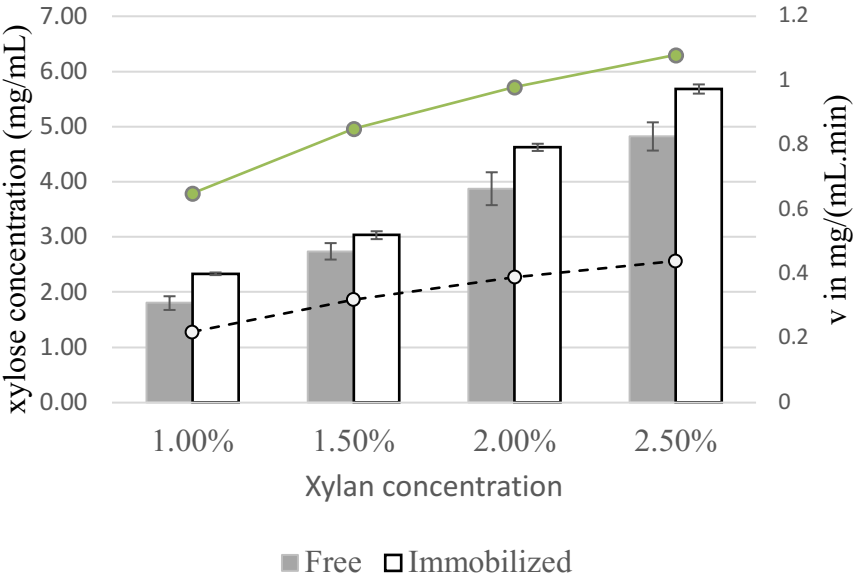

Fig. 2. The profile of xylose concentration from hydrolysis of varying xylan concentration by different xylanase system at 60°C and pH 5.0 for 40 min. The dots representing xylose formation rate (v, mg/mL.min).

Figure 2 illustrates the variation in xylan concentration and its impact on the catalytic performance of both free and immobilized xylanase. It is evident that both catalyst systems exhibit substrate-dependent behaviour, wherein higher substrate availability leads to increased product generation. To further elucidate this behavior, Michaelis-Menten kinetic was employed. The kinetic parameters (K_m and v_{max}) for xylan hydrolysis were determined for both free and immobilized xylanase (Table 1).

Table 1. Parameter kinetics of xylan hydrolysis at 60°C and pH 5.0.

Catalyst	K_m mg/mL	v_{max} mg/(mL.min)	R^2
Free xylanase	19.12	1.92	0.99
Immobilized xylanase	42.03	1.21	0.99

Based on the kinetic parameter comparison, it can be observed that the immobilized xylanase exhibited higher K_m and lower v_{max} values in contrast to the free xylanase. This suggests that the immobilized xylanase system requires a larger amount of substrate to achieve a faster reaction and has a lower maximum reaction rate. This can

be attributed to the more limited accessibility of the substrate to reach the active site of the enzyme in the immobilized system. While the only factor limiting substrate accessibility in the free enzyme system is the enzyme structure itself, substrate flow restriction through the pores can also occur in the immobilized xylanase system.

Although the reaction rate was slower for the immobilized xylanase, the data demonstrate a higher concentration of product when the immobilized xylanase was employed compared to the free xylanase. This can likely be ascribed to the ability of the porous zeolite matrix to protect the enzyme from direct heat exposure, which may otherwise result in heat denaturation. This observation is supported by the specification data of the enzyme used in this study, which indicates a temperature stability lower than 55°C for free enzyme [12].

4 Conclusion

This study provided a brief evaluation of the performance and kinetics of free and immobilized xylanase. The immobilization of xylanase did not alter the enzyme's behaviour with respect to pH. However, it introduced some mass transfer resistance, potentially limiting the substrate's access to the active site of enzyme molecules. Nevertheless, immobilization demonstrated promise in maintaining enzyme stability at higher processing temperatures compared to the free enzyme system. Further investigation into the reusability and performance stability of immobilized xylanase is warranted to validate its full range of beneficial features.

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