Preparation of Immobilized Enzymes on Pulp Fiber with the Layer-by-layer Self-assembly Technique and its Application in Whitewater Treatment

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Anionic pectic substances in whitewater are harmful to the papermaking process and product quality. To diminish their negative effect, immobilized pectinase was prepared to treat pectic compounds in whitewater. Pectinase was immobilized on pulp fiber *via* a layer-by-layer assembly technique using polyethyleneimine. The activity of the immobilized enzyme varied nearly linearly, from 2876 U/g to 4838 U/g, until the number of layers reached four. The properties of the bound enzymes and their free counterparts were investigated. After being fixed on the pulp fiber, the optimum pH of the enzymes shifted from 4.4 to 4.0 and the optimum temperature increased by 5 °C to 50 °C. It was shown that the V_m value was slightly reduced for the immobilized enzymes, while the K_m value noticeably decreased. Furthermore, the immobilized enzyme was tested in papermaking whitewater treatment. The four-layer immobilized enzymes lowered the whitewater cationic demand by 18% and 30% at pH values of 7 and 4, respectively. The immobilized pectinase could potentially be employed to treat wastewater in the papermaking industry.

Keywords: Immobilized; Fiber; Layer-by-layer; Papermaking; Whitewater; Polyethyleneimine

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INTRODUCTION

As environmental pollution threatens the planet, finding environmentally friendly processes and materials has become crucial. Enzymes are increasingly used in industrial applications because of their effectiveness, environmental friendliness, and mild reaction conditions. Pectinase has demonstrated effectiveness in decomposing anionic pectic compounds in peroxide-bleached mechanical pulp, which lowers the cationic demand (Thornton 1994; Ricard *et al.* 2005; Liu *et al.* 2012). To improve the operational stability, recyclability, and performance, enzymes are generally fixed on a carrier (Bolivar *et al.* 2016). Immobilized biocatalysts have been utilized in a number of applications, including heterogeneous reactions, controlled release, adsorption, and analytical instruments (Jesionowski *et al.* 2014).

The layer-by-layer (LbL) self-assembly method has proven to be useful in immobilizing enzymes. Typically, the LbL process involves the alternate buildup of opposite-charged materials on matrices through electrostatic interactions. This methodology provides a valuable route for enzyme immobilization because it is able to preserve the native structure and enzyme activity. Several types of enzymes (*e.g.*, glucose oxidase, hydrogen peroxide, tyrosinase, lipase, *etc*.) have been immobilized using the LbL

approach with control of the composition (Ping and Hsieh 2010; Han *et al.* 2013; Barsan and Brett 2016).

Polyethyleneimine (PEI) has been deemed effective at immobilizing enzymes (Karimpil *et al.* 2012; dos Santos *et al.* 2014). Additionally, PEI is a commonly used chemical in the papermaking industry, which means that using PEI-immobilized pectinase to treat whitewater from papermaking will not introduce pollutants to the current papermaking process. In the previous study by the authors, pectinase was immobilized on pulp fiber with PEI. The resulting bound enzyme exhibited good enzymatic properties and effectively decreased the cationic demand of whitewater from the papermaking process (Wu *et al.* 2014).

To obtain immobilized pectinase with an increased tunable activity and stability, the LbL self-assembly technique was utilized. The PEI and pectinase were alternately adsorbed onto pulp fiber to obtain multilayer immobilized pectinase. The properties of the immobilized pectinase were characterized. Moreover, the obtained bound enzyme was used to treat whitewater to evaluate its ability to reduce cationic demand. This study elucidated the potential utilization of the LbL technique in preparing immobilized pectinase, using pulp fiber as a support, for whitewater treatment in the papermaking industry.

EXPERIMENTAL

Materials

Bleached kraft softwood pulp board provided by Shanying Paper Industry (Maanshan, China) was dispersed, screened, and washed with deionized water to obtain pretreated pulp fibers as described previously. Novozym 863 was provided by Novozymes (Tianjin, China). Polyethyleneimine (MW 70 kDa, 50% (w/v)) and pectin were purchased from J&K (Beijing, China). All of the other chemicals were of analytical grade. Simulated whitewater samples (pH 7, cationic demand 8.7 mmol/L) were supplied by a Chinese paper mill, stored at 4 °C and used after filtration.

Methods

Layer-by-layer adsorption of PEI and pectinase

One gram (oven dry weight) of pulp fibers was immersed in 100 mL of 0.01% (w/v) PEI aqueous solution and stirred for 30 min at 25 °C. After adsorption, the pulp fiber was filtered and washed with deionized water to remove the excess PEI. The PEI-fiber was dispersed in 100 mL of 0.25% (v/v) pectinase solution, and the mixture was stirred at 25 °C for 30 min. The fibers were then separated from the unbound enzyme by filtration and rinsed with deionized water to attain one layer of immobilized pectinase.

After that, the fibers were again alternately immersed in PEI solution and pectinase solution. This procedure was repeated to obtain immobilized enzymes with different numbers of layers.

Variation of ζ-potential of the pulp fiber in the layer-by-layer adsorption process was monitored using Fiber Potential Analyzer (AFG Analytic GmbH, Germany). The amount of PEI and pectinase adsorbed on pulp fiber was calculated based on the content of nitrogen measured using vario MACRO cube (Elementar, Germany).

Assays of the pectinase activity

The free/immobilized pectinase activity, pH, and thermal stabilities of the enzymes were assessed as previously described. The enzyme activity was calculated based on dinitrosalicylic acid (DNS) method using pectin as the substrate. One unit (1 U) was defined as the amount of enzyme required to release 1μg of galacturonic acid per minute. Effects of pH and temperature on the activities of free/immobilized enzyme were determined by measuring the activities in different pH values or at different temperatures. The immobilized enzyme was recycled by filtration after the catalytic reaction, washed and repeatedly tested for enzymatic activity using a fresh pectin solution over 6 times to evaluate its reusability.

Determination of the kinetic parameters

One milliliter of free pectinase (or 0.6 g of immobilized pectinase) was added to 5 mL of pectin with different concentrations in a 0.05 mol/L buffer (pH of 4.4). The total volume was 10 mL, which included the buffer. The reaction was performed at 45 °C. The initial rate of the reaction was measured.

Treatment of the whitewater with pectinase

The pH values of the simulated whitewater samples were approximately 7 and adjusted when necessary. The pectinase dosage was approximately 4000 U/L of whitewater. One hundred milliliters of whitewater were incubated and stirred at 50 °C for 5 min before the pectinase was added. Samples were taken from the mixture and the cationic demand was determined at intervals (5 min, 10 min, 20 min, 30 min, 60 min) using a Mütek Particle Charge Detector PCD-04 (BTG, Egham, UK) by titration with poly-(diallyl dimethyl ammonium chloride) (poly-DADMAC) as described previously.

RESULTS AND DISCUSSION

Immobilization of the Pectinase on Pulp Fiber through the Layer-by-layer Self-Assembly Technique

Figure 1a illustrates the changes in the enzymatic activity of the multilayer pectinase immobilized on the pulp fiber with different numbers of layers. It was observed that the immobilized enzyme activity increased nearly linearly with the number of enzyme layers, until the fourth layer. The enzymatic activity increased with the number of enzyme layers as more enzyme molecules were adsorbed, from 2876 U/g to 4838 U/g. This implied that each layer of enzyme was adsorbed onto the carrier homogeneously and the effect of the diffusion limitation was not important (Caruso and Schüler 2000). However, when the enzyme was further adsorbed onto the four-layer enzyme with PEI as the outer layer, a decrease in the activity was detected. It was also seen that further adsorption of PEI on the four-layer enzyme caused a decrease in the activity. This phenomenon might have been because the diffusion limitation effect increased as the number of layers increased. Moreover, protein–protein and PEI interactions and/or the distortion of enzyme molecules may have caused enzyme denaturation (Seabra and Gil 2007).

The Variation of ζ-potential of the pulp fiber in the layer-by-layer adsorption process is depicted in Fig. 1b. The ζ-potential of pulp fiber in aqueous solution was around -41 mV, which was attributed mostly to dissociation of carboxyl groups. After positively charged PEI was adsorbed, the ζ-potential of the carrier increased to approximately 20 mV.

Then the ζ-potential changed to -31 mV when pectinase was adsorbed on PEI-fiber. As PEI and enzyme were further alternately fixed, the ζ-potential turned to almost zero when PEI formed the outer layer while 32 mV when enzyme was the outer layer. This indicated the stepwise deposition of PEI and pectinase on pulp fiber (Caruso and Schüler 2000). The amount of PEI and enzyme adsorbed on pulp fiber was quantified, and the results are shown in Table 1. As the layer number increased PEI loading and enzyme loading increased. The difference in increase amount might be attributed to the roughness of the layer underneath (Guzmán et al. 2017).

Table 1. Change of PEI and Enzyme Loading of the Immobilized Enzyme on Fiber with Layer Number

Layer number	PEI loading (mg per gram fiber)	Enzyme loading (mg per gram fiber)
1	0.0049	0
2	0.0049	0.16
3	0.0071	0.16
4	0.0071	0.35
5	0.011	0.35
6	0.011	0.63
7	0.013	0.63
8	0.013	0.80

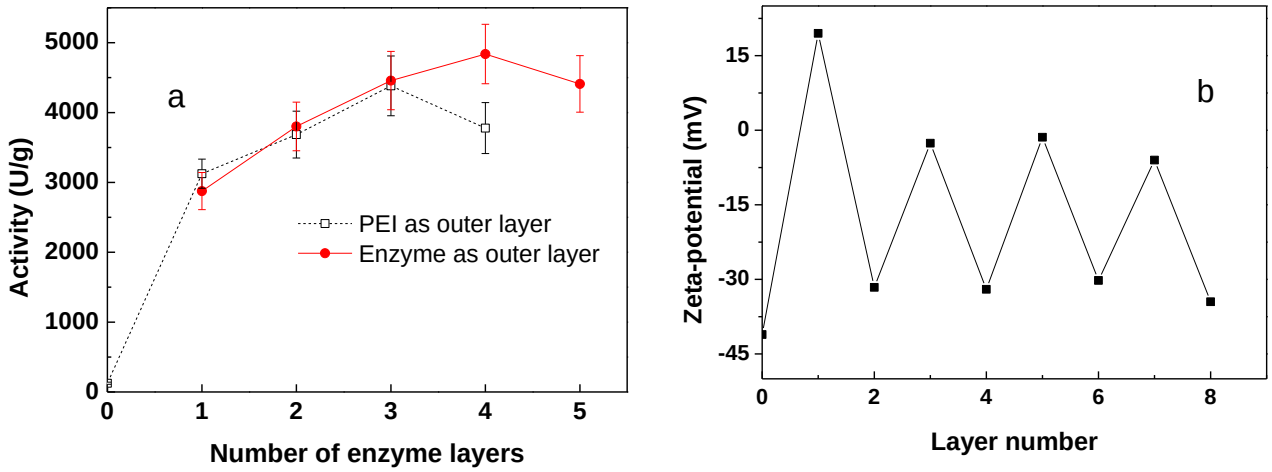

Fig. 1. Change in the enzymatic activity of the enzyme immobilized on the pulp fiber with different numbers of layers (a) and change of zeta-potential as a function of layer number. The odd layer numbers correspond to PEI deposition and the even layer numbers to enzyme deposition (b)

It has been demonstrated that one of the advantages of immobilizing enzymes using the LbL self-assembly technique is that immobilized enzymes with different activities can be obtained as the number of enzyme layers varies (Xing *et al.* 2007). This work showed that immobilized enzymes on pulp fiber carriers with a tunable activity could be obtained. In the rest of the study, the four-layer immobilized pectinase with enzyme as the outer layer was utilized.

pH and Thermal Stability of the Free and Immobilized Pectinases

Figure 2a presents the influence of the pH on the enzymatic activity. The free pectinase reached its maximum activity when the pH value was 4.5, while the optimum pH value for the four-layer fixed enzyme on the pulp fiber shifted towards the acidic value of 4.0. This was in agreement with the results of other researchers, where enzymes attached to positively charged supports (as in the present case) had lower optimum pH values after immobilization (Abdel-Naby 1999). The pulp fiber had a positive charge after adsorbing PEI in an aqueous solution, which resulted in a decrease in the proton concentration because of electrostatic interactions. Therefore, a lower pH in the bulk solution was required to counteract this effect.

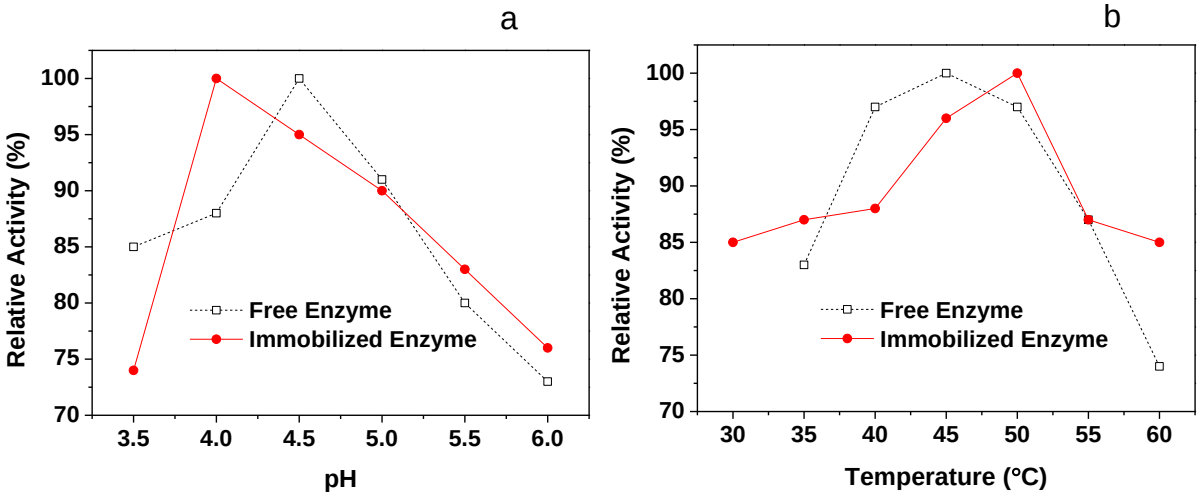

Fig. 2. Effect of the pH (a) and temperature (b) on the activity of the free pectinase and four-layer pectinase immobilized on the pulp fiber

The effect of the temperature on the enzyme activity is depicted in Fig. 2b. After immobilization on pulp fiber *via* the LbL method, the optimum temperature increased by 5 ℃ to 50 ℃. This is advantageous for practical applications because the temperature of the whitewater in the papermaking process is approximately 50 ℃. Additionally, the high activity (above 80%) of the immobilized enzyme could be maintained over a larger temperature range than its free counterpart. When the temperature increased to 60 ℃, the activity of the free enzymes decreased to below 75%. In contrast, the immobilized enzymes maintained about 85% of their maximum activity. The enhancement of the stability was probably caused by the increased rigidity of the enzyme after attachment to the carrier, which to some extent made the enzyme less susceptible to temperature changes (Talbert and Goddard 2012).

Kinetic Results

The linear nature of the plots indicated that both the enzymes followed the Michaelis-Menten kinetics in the pectin concentration range discussed (Krajewska *et al.* 1990). The calculated kinetic parameters (maximum reaction rate V_m and Michaelis–Menten constant value K_m) are listed in Table 1. Compared with the free enzyme, the immobilized enzyme exhibited slightly lower V_m values. Generally, the K_m values of enzymes increase after immobilization, probably as a consequence of the increased diffusion limitation and structural changes to the enzyme. Interestingly, a clear decrease in the K_m value was observed in this study. Guedidi *et al.* (2010) obtained similar results, and

found that trypsin and urease immobilized on porous membranes using the LbL method exhibited decreased K_m values compared with their free forms. This phenomenon might have been explained by the increase in the local enzyme concentration around the carrier because enzymes gathered on the carrier during the LbL immobilization process. Apart from that, the positive charge of the support might have been beneficial for adsorption of the substrate, and thereby facilitated the catalytic reaction.

Table 2. Kinetic Parameters of the Free and Immobilized Enzymes

	K_m	V_m
Free enzyme	9.76	0.75
Immobilized enzyme	2.40	0.67

Reusability of the Immobilized Pectinase

Reusability of the four-layer immobilized pectinase was evaluated, and the results were displayed in Fig. 3. The catalytic activity of the enzyme decreased to 88% and 23% after the second and third reuse cycle, respectively. After the third batch, the activity of the fixed enzyme almost remained constant. Over 20% of initial activity was retained after the enzyme was reused for 6 cycles.

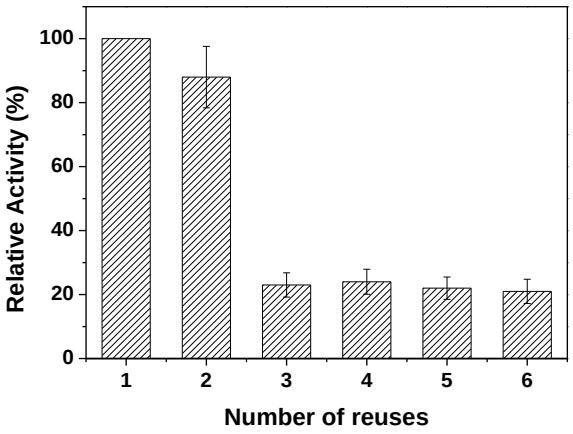

Fig. 3. Reusability of the four-layer pectinase immobilized on the pulp fiber

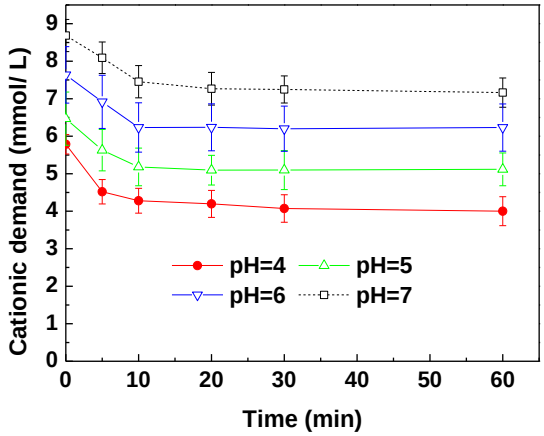

Fig. 4. Cationic demand over time of the whitewater with different pH values treated with

Application in Whitewater Treatment

Immobilized pectinase on the pulp fiber obtained *via* the LbL method was utilized to treat whitewater from a papermaking process to evaluate its effect on the cationic demand. Figure 4 shows the effect of immobilized enzyme in reducing the cationic demand of the whitewater with different pH values. When the whitewater pH value was adjusted from 7 to 4, the initial cationic demand of the whitewater was lower since some of the carboxylic groups could not effectively dissociate into anionic groups (Hubbe *et al.* 2012). As the pH value of the whitewater was decreased, the enzyme had better performance at reducing the cationic demand since the optimum pH value for the immobilized pectinase was approximately 4. When pH value was raised to 7 the cationic demand was decreased by nearly 18%, while it was lowered by 30% at pH 4. Additionally, it was evident that under the conditions, the cationic demand decreased rapidly during the first 10 min and nearly reached a plateau. After that, the cationic demand decreased slightly. The time it

took for the cationic demand to plateau was shorter than that in previous reports (Wu *et al.* 2013), in which other immobilization methods were applied. This could reduce the reaction time for practical applications.

CONCLUSIONS

1. Pectinase was immobilized on pulp fiber *via* the LbL technique using PEI. The enzymatic activity of the immobilized enzymes increased with an increase in the number of layers (until four layers), from 2876 U/g to 4838 U/g.
2. After being fixed on the pulp fiber, the optimum pH of the enzyme shifted from 4.4 to 4.0. Also, the immobilized enzyme reached its highest activity at a temperature of approximately 50 ℃, which was 5 ℃ higher than that of the free form. This meant the immobilized enzyme was better adapted to practical whitewater environments. The immobilized enzyme on pulp fiber maintained over 20% of its original activity after being used for 6 batches. Moreover, the V_m value slightly decreased for the immobilized enzyme, while the K_m value decreased noticeably.
3. The immobilized enzyme was tested in the treatment of papermaking whitewater. The results showed that the four-layer immobilized enzyme lowered the whitewater cationic demand by 18% and 30% at pH values of 7 and 4, respectively, and a dosage of 4000 U/L after 10 min.

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