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Immobilization of pectinase on oxidized pulp fiber and its application in whitewater treatment

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ABSTRACT

Modified pulp fiber was originally used as a new type of carrier for pectinase immobilization. Pulp fiber was oxidized by sodium periodate to produce aldehyde groups for covalently binding with amino groups of pectinase. Results showed that the enzymatic activity of immobilized pectinase on pulp fiber reached 65 $\mu g\, g^{-1}\, min^{-1}$ when immobilization pH value, temperature and time were of 7.0, 20 °C and 15 min, respectively. The immobilized pectinase showed higher thermo stability in a wider temperature range of 40–70 °C than its free type and its optimal pH shifted from 8.0 to 8.8. Furthermore, the immobilized pectinase exhibited good operational stability. When employed in whitewater treatment of papermaking industry, it still efficiently decreased the cationic demand after operating repeatedly for six batches. The results obtained demonstrate a promising route to prepare available, cheap and biodegradable carrier for immobilizing enzymes with potential application in wastewater treatment in papermaking industry.

1. Introduction

The uses of enzymes are increasingly being exploited for industrial applications due to high selectivity, mild reaction conditions and environmentally friendly process (de Carvalho, 2011). However, the practical application of free enzymes is often hampered by low operational stability and difficulties in recycling (Idris & Bukhari, 2011; Sheldon, 2007). Immobilized enzymes provide various advantages over free enzymes including rapid termination of reactions, facile separation of the enzymes and products from reaction media, repeated or continuous usage and simplification of the design of the reactors (Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007; Sheldon, 2007). Due to these properties, immobilized enzymes have been successfully applied in many areas, such as heterogeneous biocatalysis, selective adsorption, controlled release of protein drugs, analytical device and solid phase protein industries (Cao, 2005).

The properties of the carrier materials have an overwhelming influence on the performance of the immobilized enzyme system. Many attempts have been made for the development of desirable support materials to achieve high catalytic activity and enzyme loading (Huang, Yu, & Xu, 2008; Sheldon, 2007). A variety of synthetic and natural organic polymers, as well as inorganic materials, have been explored for immobilization purpose. Of particular interest is the use of cellulose and their derivatives. Cellulose is the most abundant natural, renewable and biodegradable polymer in the world. Its long chains and loose mesh structure make it accessible for macromolecular infiltration and adsorption. Apart from that, there are a great number of hydroxyl groups in cellulose which impart cellulose chemical variability with high donor reactivity. Cellulosic materials are generally hydrophilic, insoluble in water, stable to chemicals, nontoxic, inexpensive and biodegradable, which makes them useful supports for enzyme immobilization (Klemm, Heublein, Fink, & Bohn, 2005).

To broaden its application, researchers have modified cellulose in many ways. Among the chemical modification methods, periodate oxidation is highly specific by cleaving the C2—C3 bond of the anhydroglucose unit and resulting in two aldehyde groups (Kim, Kuga, Wada, Okano, & Kondo, 2000; Kristiansen, Potthast, & Christensen, 2010; Princi et al., 2004). The aldehyde groups can couple with primary amines via a facile Schiff base reaction (Kim & Kuga, 2001; Maekawa & Koshijima, 1991). Thus enzymes would be immobilized on the cellulosic carrier. By using this method, several kinds of natural cellulosic materials, including cotton yarn (Nikolic et al., 2010), bagasse (Varavinit, Chaokasem, & Shobsngob, 2001) and cotton gauze bandage (Seabra & Gil, 2007), have been modified and successfully applied for enzyme immobilization. Pulp fiber, another form of cellulosic material, is readily available in

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papermaking and can be made into diverse shapes. Since pulp fiber is the main composition in the papermaking system, the immobilized enzyme on pulp fiber can be discharged into pulp directly after its activity decreases to some ineffective extent, thereby further reducing operating cost. However, till now no efforts have been made for the application of pulp fibers in enzyme immobilization.

As one of the most important industries, pulping and papermaking industry provides paper products for our daily use (Žarković, Todorović, & Rajaković, 2011). However, it also generates large quantities of by-products. In particular as a result of alkaline treatment steps, pectic compounds are released from the fiber structure into the effluent, being a major contributor to a phenomenon known as anionic trash. Usually, the level of dissolved anionic trash is identified in terms of cationic demand. Microbial pectinase has been used as an efficient biocatalyst in papermaking to reduce cationic demand in peroxide-bleached mechanical pulp (Liu, Li, Li, He, & Zhao, 2010; Ricard, Orccotoma, Ling, & Watson, 2005). It could decompose anionic pectin or polygalacturonic acid which account for much of the cationic demand in peroxide-bleached mechanical pulp. However, major limitations of free enzyme used in papermaking industry are the expensive process and the low operational stability of the biocatalyst (Sheldon, 2007; Mateo et al., 2007).

Our previous researches showed that immobilized pectinase on cross-linked chitosan beads may significantly lower the cationic demands of polygalacturonic acid solution (Liu et al., 2010). In order to further improve the activity/stability but lower the cost of the immobilized pectinase system for practical application in waste water clean-up, a new immobilization method by using modified pulp fiber was developed in this research. Pulp fiber was, for the first time, used as carrier for enzymatic immobilization in this research. The modification process of pulp fiber via oxidization was evaluated. The immobilization procedure and the properties of immobilized pectinase on pulp fiber were also investigated for the first time.

2. Materials and methods

2.1. Materials

Bleached kraft softwood pulp board from a Chinese mill was used. The pulp board was dispersed thoroughly in a disintegrator, screened by Dynamic Drainage Jar to remove fines passing 200 mesh, and then washed three times with distilled water. The prepared fibers with a moisture content of around 70% and α-cellulose content of 88.2% were stored at 4–5 °C. Commercially available pectinase Novozym 51019 was supplied by Novozymes (Denmark). Pectin from citrus peel was purchased from Sigma–Aldrich (USA). Sodium periodate, glycerol and all other chemicals were purchased from local market, of analytical grade and used without further purification. Whitewater samples were provided by a Chinese Paper Mill producing alkaline peroxide-bleached mechanical pulp. The whitewater was sampled after bleaching tower and stored at 4 °C. Buffer solutions used were 0.05 mol L^{-1} phosphate ($KH_2PO_4 + Na_2HPO_4$).

2.2. Oxidation of pulp fibers with sodium periodate ($NaIO_4$)

1 g (oven dry) pulp fibers were immersed in 50 mL $NaIO_4$ aqueous solutions of various concentrations (2 g L^{-1}/8 g L^{-1}/25 g L^{-1}). The pH value was adjusted to below 4 using sulfuric acid. The mixture was then stirred in the absence of light at 45 °C. The reaction was terminated by removal of $NaIO_4$ and addition of 40 mL 0.1 mol L^{-1} glycerol. Then the oxidized fibers were washed with deionized water several times. This oxidized sample was used for immobilization of pectinase without drying. All the oxidation tests were performed in duplicate.

2.3. Effects of reaction conditions on the oxidation rate of pulp fibers

Three different $NaIO_4$ concentrations (2 g L^{-1}, 8 g L^{-1}, and 25 g L^{-1}) were utilized to evaluate the effect of $NaIO_4$ concentration on the oxidation rate of pulp fibers. Different lengths of oxidation time were employed to study the effect of oxidation time on the oxidation rate of pulp fibers.

The $NaIO_4$ consumption was determined by UV absorbance at 290 nm. The formation of soluble fragments, as a result of cellulose destruction (i.e. cellulose chain scission caused by subsequent reaction, not by oxidation itself), was determined by measuring the weight loss of oxidized fibers by applying the direct gravimetric method (Janjic et al., 2009; Koblyakov, 1989).

2.4. Determination of aldehyde group contents

The aldehyde content present in the oxidized fiber was measured according to the method described in literature (Clift & Cook, 1932; Parkinson & Wagner, 1934). Oxidized fiber (0.3000 g) was added into a conical flask of 50 mL, followed by adding 10 mL deionized water and 10 mL sodium bisulfate (4.4 g L^{-1}). After being mixed, the flask was transferred to a gas bath rotary shaker under 180 r min^{-1}. The reaction was protected from light and lasted for 1 h at 25 °C. Then, after adding 0.5 mL starch indicator into the mixture, titration was immediately carried out using standard solution of I_2/KI (0.1000 mol L^{-1}). Blank test was conducted as the same except for adding oxidized fiber. The aldehyde group content was calculated according to the following formula:

$$\text{aldehyde group content (mmol g}^{-1}) = \frac{(v_0 - v)c}{m}$$

v_0 is the consumption of I_2/KI in blank test, mL; v is the consumption of I_2/KI, mL; c is the concentration of I_2/KI solution, mol L^{-1}; and m is the mass of sample, g.

2.5. Immobilization of pectinase on oxidized fiber

The oxidized fiber was immersed in 25 mL of buffer solution (pH 7.0) in a conical flask of 100 mL, and then 25 mL enzyme solution diluted by 400 times was added while stirring. The mixture was incubated in a gas bath rotary shaker with constant shaking at 20 °C. Subsequently, the fiber was filtered to remove unbound enzyme and washed with 300 mL distilled water. The filtrate and the washings were collected for protein measurements. The resultant immobilized pectinase on fiber was stored in refrigerator at 4 °C. All immobilizing tests were performed in duplicate.

2.6. Assay of protein loading

The amount of pectinase coupled onto the oxidized fibers was estimated from the initial protein amount present in the enzyme-coupling solution subtracting the final total protein amounts present in the remaining coupling solution and the washing solution by means of a mass balance, following the UV absorption method (John, 2002). The immobilization efficiency of the protein was defined as the amount of protein per gram of the support.

2.7. Assays of catalytic activities of the free and immobilized enzymes

The activity of free and immobilized pectinase was measured by assaying the amount of reducing sugars. One unit of enzyme activity was expressed as the amount of enzyme required to release 1 μg of galacturonic acid per min as quantified by the dinitrosalicylic acid method (Miller, 1959).

The activity of free pectinase was determined by adding 1.0 mL of pectinase solution (diluted 200 times) into 5.0 mL of 5 g L^{-1} pectin and 4.0 mL buffer (pH 8.0). The activity of immobilized pectinase was determined by adding 0.3 g of immobilized pectinase into 5.0 mL of 5 g L^{-1} pectin and 5.0 mL buffer (pH 8.0). The mixture was incubated in a water bath at 48 °C for 30 min.

2.8. Assays of the free and immobilized enzyme stabilities

In order to evaluate the pH stability of the enzyme, free or immobilized pectinase was added into separate vials containing pectin and buffer at different pH values from 6.0 to 9.5. The reaction mixture was incubated for 30 min at 48 °C and the enzymatic activity was measured. The relative activity was expressed as the ratio of the retained activity to the maximal activity of the enzyme.

In order to assess the thermal stability of the enzyme, free or immobilized pectinase was added into separate vials containing pectin and buffer (pH 8.0). The reaction mixture was incubated for 30 min at various temperatures from 38 to 68 °C and the enzymatic activity was measured. The relative activity was expressed as the ratio of the retained activity to the maximal activity of the enzyme.

To examine the reusability of immobilized pectinase, the enzyme was repeatedly used for depolymerizing pectin (in typical reaction system mentioned in Section 2.8) over 7 reaction cycles. The enzyme was retrieved after being used, then washed with deionized water and added into a fresh reaction mixture. The relative enzyme activity was calculated as the ratio of the enzyme amount required to release 1 μg of galacturonic acid per min in given batch to its original activity.

2.9. FT-IR analysis of the immobilized enzyme

The IR spectra of all the samples were measured with a Fourier transform IR (FT-IR) spectrometer (FT-IR 2000, PE). The KBr disk method was adopted to conduct the FT-IR measurements. The sample was mixed with KBr powder, ground well and prepared to KBr disks. The spectra were recorded at an average of 32 scans in the standard wavenumber range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1}.

2.10. Treatment of whitewater with pectinase

50 mL of whitewater was stirred for 5 min and maintained at 55 °C. Then 1.5 g of immobilized pectinase or 56 μL concentrated pectinase was added at a stirring rate of 100 rpm. The cationic demand was measured at intervals. Cationic demand was measured using the Mütek Particle Charge Detector (PCD-04) and titrator (PCD-T). The samples were diluted with deionized water and then titrated with 0.0001 N polydiallyl dimethyl ammonium chloride (poly-DADMAC).

To evaluate the operational stability of the immobilized enzyme in whitewater, the whitewater treatment test by the enzyme was performed repeatedly over 6 batches (20 min per batch). Between cycles the immobilized enzyme was filtered from the whitewater mixture, washed twice with deionized water and then added to an untreated bath of whitewater solution (50 mL).

3. Results and discussion

3.1. Oxidation of pulp fibers

Pulp fibers were oxidized by $NaIO_4$ to form dialdehyde cellulose fibers. Since $NaIO_4$ concentration and oxidation time are two major factors in the oxidation of pulp fibers, their effects on the reaction were investigated by determining the $NaIO_4$ consumption and carbonyl group content.

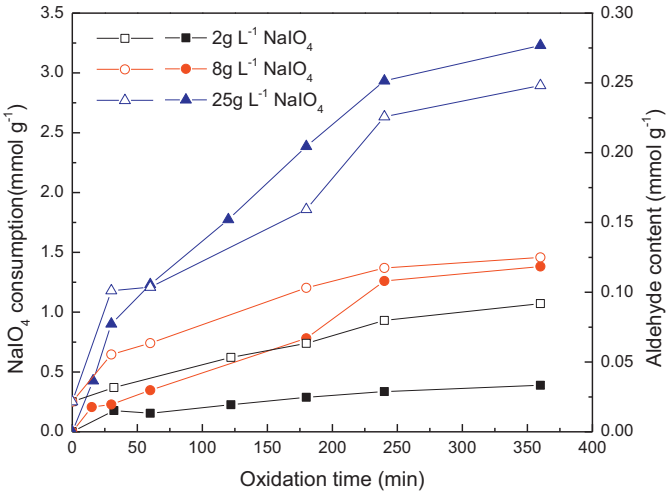

Fig. 1. Time courses of the oxidization of fiber pulp with different concentrations of $NaIO_4$. Full symbols (■,●,▲) refer to $NaIO_4$ consumption and open symbols (□,○,△) refer to aldehyde group formation.

The rate of $NaIO_4$ consumption in oxidization of pulp fibers is shown as the slopes of the curves in Fig. 1. The reaction proceeded through two distinct phases. At the first phase, the rate of $NaIO_4$ consumption was relatively high, but then it decreased at the second phase. The slopes almost tended to zero after 4 h when low $NaIO_4$ dosages were adopted. When $NaIO_4$ dosage increased to 25 g L^{-1}, its consumption rate significantly rose. Calvini, Conio, Princi, Vicini, and Pedemonte (2006) revealed that at least three reactions occurred simultaneously during the oxidation, including a random oxidation in the amorphous region of cellulose at very high rate, a second slow reaction attributed to the oxidation on the surface of crystallites and a very slow reaction possibly due to the oxidation of the crystalline cores. As the oxidation proceeded, the amount of unoxidized amorphous region decreased leading to the oxidation rate falling.

Fig. 1 also displays that aldehyde group content of the modified pulp fibers significantly increased by prolonging the oxidation time. Increase in $NaIO_4$ concentration also resulted in a dramatic increase in the rate of oxidation. High concentration of $NaIO_4$ might promote IO_4^- permeation into fibers so that the oxidation occurred in crystalline region to generate more aldehyde groups. For instance, after a 240-min reaction, the aldehyde group content of fiber oxidized by 25 g L^{-1} $NaIO_4$ was 0.23 mmol g^{-1}, which was nearly three times higher than that of the one oxidized by 2 g L^{-1} $NaIO_4$. Notably, there was a large difference in quantity between the consumption of $NaIO_4$ and the formation of aldehyde and the difference became greater for higher $NaIO_4$ concentration. This might be related to the fact that much $NaIO_4$ was depleted by degrading cellulose to fragments and ineffective decomposition. The formation of soluble fragments was demonstrated by measuring the weight loss of oxidized fibers. The weight loss of oxidized fibers by 8 g L^{-1} $NaIO_4$ varied from 0.35% to 5.3% during 6 h and was more pronounced for higher $NaIO_4$ charge (not shown). Moreover, the oxidized fibers became stiff and brittle if being oxidized for a long period as described by Princi et al. (2004). Oxidized pulp fibers by 25 g L^{-1} $NaIO_4$ for 30 min had relatively high aldehyde content and low weight loss, which were used as supports for subsequent immobilization of pectinase.

3.2. Optimization of the immobilization procedure

Immobilized pectinase was prepared by covalently bonding the pectinase in buffer to the oxidized fibers. In this process, the free

Table 1
Effect of temperature on immobilization. Reactions were carried out in buffer pH 8.0 for 60 min and the pectinase was diluted 200 times.

Temperature (°C)	Protein loading ($mg\,g^{-1}$)	Enzymatic activity ($\mu g\,g^{-1}\,min^{-1}$)
4	3.2	66
10	5.0	68
20	5.4	70
30	8.7	65

amino groups of pectinase reacted with aldehyde groups on the oxidized fibers, forming a covalent Schiff base. In order to achieve high catalytic activity and enzyme loading of the immobilized pectinase, the influence of immobilization conditions were investigated and the immobilization procedure was optimized. The immobilization efficiency was evaluated by analyzing the biocatalyst activity and the amount of pectinase coupled onto the oxidized fibers (the protein loading).

3.2.1. *Effect of temperature on immobilization*

By varying temperature, the influences of immobilization temperature on the enzymatic activity of pectinase and protein loading were investigated. As illustrated in Table 1, protein loading on oxidized fiber increased with increasing the immobilization temperature within the test ranges, while the enzymatic activity reached the highest at 20 °C. Further increasing the temperature above 20 °C led to a decline in enzyme activity. This may be due to that enzyme molecules went into the carriers' inner after the active sites of the carriers were saturated and the enzyme molecules could not efficiently perform the catalysis capability for the limitation of substrate diffusion. Meanwhile, high protein loading might lead to enzyme denaturation by protein–protein interactions and/or distortion of protein molecules (Jiang, Long, Huang, Xiao, & Zhou, 2005). Taking into account both the protein loading and activity of the immobilized enzyme, immobilization temperature of 20 °C was considered as the best condition.

3.2.2. *Effect of time on immobilization*

As described in Fig. 2, protein loading rose substantially during the first 15 min to $0.3\,mg\,g^{-1}$. For the next 6 h, it experienced a slow increase to approximately $0.47\,mg\,g^{-1}$, possibly due to that the amount of available aldehyde group decreased. As for the enzymatic activity of the immobilized pectinase, it reached maximum about $65\,\mu g\,min^{-1}\,g^{-1}$ in about 15 min and then almost stayed constant. The slight decline of the enzymatic activity might be caused

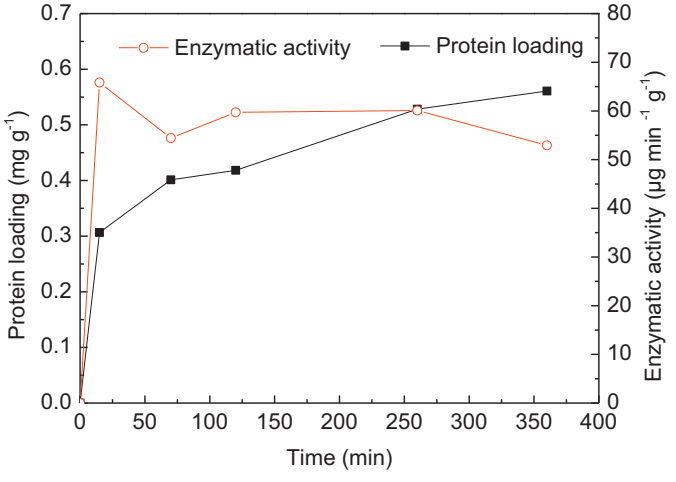

Fig. 2. Effects of immobilization time on protein loading and enzyme activity. Reactions were performed in buffer pH 7.0 at 20 °C.

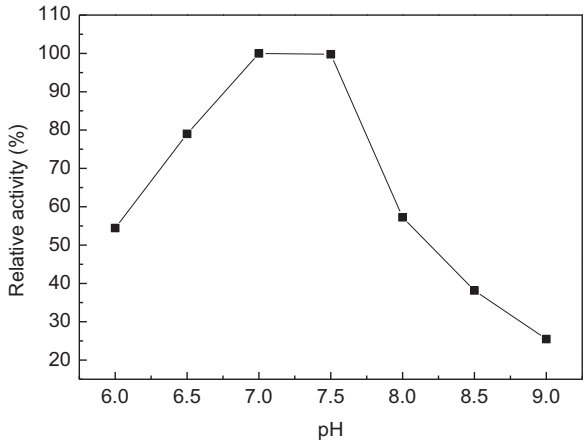

Fig. 3. Effect of immobilization pH on relative activity of the biocatalyst. Reactions were carried out at 20 °C.

by the coverage of active center by excessive pectinase resulting lower accessibility of the substrate to the active site (Jiang et al., 2005). On top of that, further incubation may lead to multiple linkages between enzyme and oxidized fiber (Pedroche et al., 2007) changing the conformation of the enzyme or undermining the active center to be inactive.

3.2.3. *Effect of pH on immobilization*

To study the effect of pH on the efficiency of immobilization, pectinase was diluted with buffer at different pH from 6.0 to 9.0.

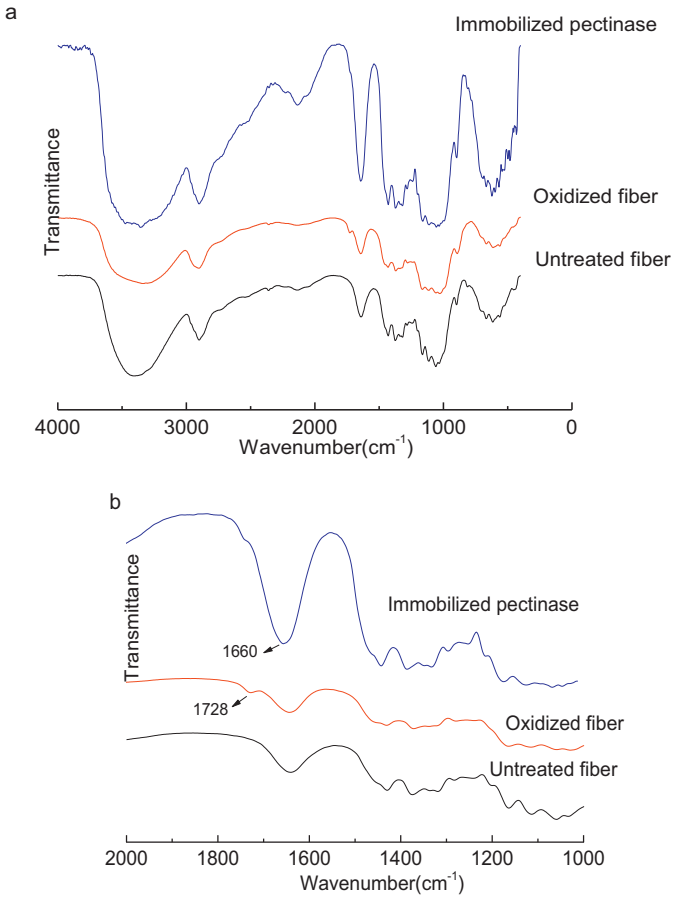

Fig. 4. FT-IR spectra of the untreated fiber, oxidized fiber and fiber carrying the immobilized pectinase in the ranges of 0–4000 cm^{-1} (a) and 1000–2000 cm^{-1} wavenumbers (b).

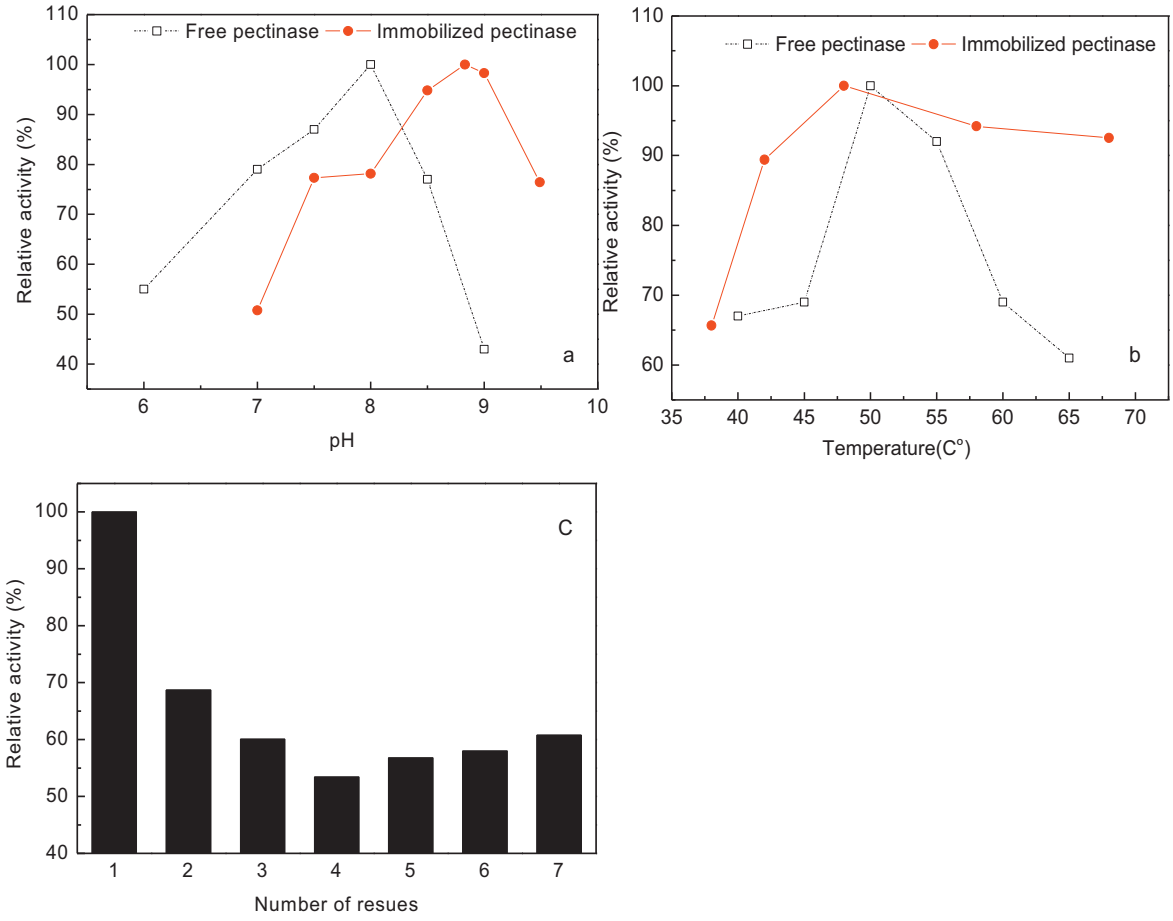

Fig. 5. The pH (a), thermo (b) and reuse stability (c) of the immobilized pectinase.

Fig. 3 shows that higher enzymatic activities were achieved in slightly basic solutions with pH of 7.0–7.5. This may result from the fact that a slightly basic condition was beneficial for the Schiff base reaction as well as favored the attachment of the enzyme in a more active conformation (Seabra & Gil, 2007).

3.3. *Infrared analysis of the original fiber, oxidized fiber and immobilized pectinase*

Fig. 4 demonstrates the IR spectra of the original fiber, oxidized fiber and oxidized fiber carrying pectinase. After oxidation with $NaIO_4$, the characteristic absorption band of the oxidized fibers clearly appeared at 1728 cm^{-1} due to the stretching vibration of the C=O double bond of the aldehyde group. Then after coupling with pectinase, the characteristic absorption band of the Schiff's base C=N bond at 1660 cm^{-1} was formed, which was covered by the absorption band of absorbed water at 1640 cm^{-1}. The results of the infrared spectroscopic analysis confirmed that the Schiff base (C=N double bond) formed between the aldehyde group provided by oxidized fiber and the amide group provided by pectinase.

3.4. *Stabilities of the immobilized pectinase on oxidized pulp fiber*

Limited biocatalyst stability is still a major challenge in large-scale applications (Rosche, Li, Hauer, Schmid, & Buehler, 2009). Improved stability is also essential for immobilized enzymes which determines both the performance and cost benefit of a biocatalyst in industrial application. To provide a comprehensive assessment of the immobilized pectinase on oxidized pulp fiber, the pH, thermo and operational stabilities of both the free and immobilized enzymes were comparatively studied.

Investigation of the pH stability of the enzymes was performed in buffer solutions with pH values ranging from 6.0 to 9.0 for free pectinase (diluted 200 times) and pH 7.0–9.5 for immobilized pectinase at 48 °C. The effects of the pH on the relative activity of enzymes are presented in Fig. 5a. It can be seen that pectinase immobilized on oxidized fiber had its optimal pH shifted toward the alkaline value (8.8) compared with its free counterpart (8.0). This shift could be attributed to the different concentrations of charged particle species between the domain of the immobilized pectinase and that in the bulk solution. Fibers were negatively charged in aqueous solution, which increased the concentrations of protons around due to electrostatic interactions. Owing to these interactions, the local pH around the catalytic site was lower so as to necessitate a higher pH value to counteract this effect (Buchert, Tamminen, & Viikari, 1997).

Fig. 5b shows the thermo stabilities of the free (diluted 200 times) and immobilized pectinase. It can be seen that the immobilized pectinase remained high activity above 90% in a broader range of temperature than the free solution, which indicated a higher resistance to thermal inactivation of the protein because strong bonds between the enzyme and the pulp fiber were created. Free pectinase activity decreased to 70% at 60 °C while immobilized pectinase remained 90% of its original activity at 68 °C. Therefore, immobilized pectinase on pulp fiber may maintain high activity in the practical environment of papermaking processes (50–60 °C).

The reuse stability of immobilized enzymes was of particular importance for economical use of an enzyme. As displayed in

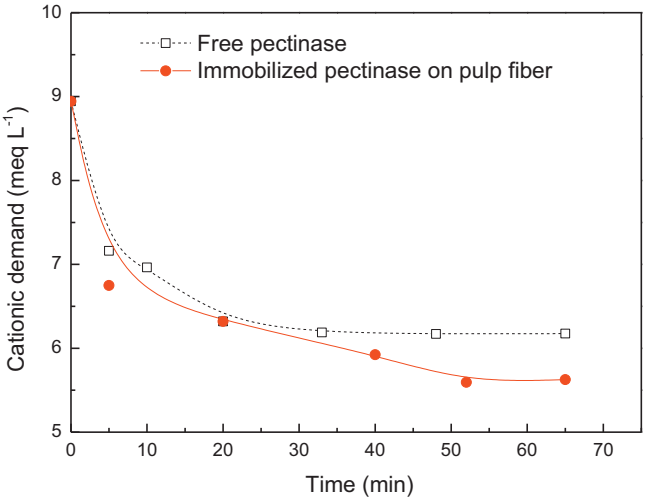

Fig. 6. Time course of cationic demand of whitewater treated with free pectinase and immobilized pectinase on pulp fiber.

Fig. 5c, immobilized pectinase retained 60% of its original activity after 7 cycles. The significantly improved reuse stability of the immobilized enzyme indicated that the enzyme protein molecule immobilized on oxidized pulp fiber was quite stable under typical assay conditions used.

3.5. Application of pulp fiber-immobilized pectinase in treatment of whitewater

To investigate the effect of immobilized pectinase on reducing cationic demand of whitewater in papermaking, the time course of pectinase-mediated whitewater treatment was followed (Fig. 6). As displayed in Fig. 6, with equivalent dosage, immobilized pectinase on pulp fiber and free pectinase had a similar effect on reducing cationic demand of whitewater during the first half an hour. However, the former could decrease the cationic demand to a greater level with prolonged reaction time. After a 30-min reaction period, free pectinase became ineffective whereas the immobilized pectinase on pulp fiber maintained its catalytic activity observed from a continuous decrease of cationic demand. In addition, we further evaluated the operational stability of the immobilized pectinase for treatment of whitewater in an industrial scale by checking the reaction efficiency after repeatedly uses of the immobilized enzyme. As shown in Fig. 7, the catalytic capability of the immobilized pectinase to decrease cationic demand was weakened obviously after the first 2 cycles. However, the cationic demand of the whitewater in the following batches was decreased to an almost constant value. The results confirmed the practical reuse capability of the biocatalyst in a given papermaking industrial environment, and indicated that pectinase covalently coupled on oxidized pulp fiber could be continuously employed to treat whitewater in papermaking industry with good performance.

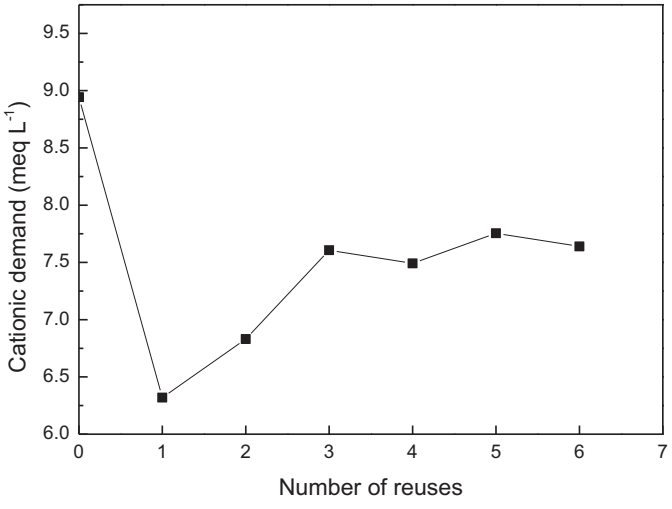

Fig. 7. Operational stability of the immobilized pectinase in whitewater treatment.

4. Conclusions

The findings reported herein demonstrated for the first time that pulp fiber, an available and easily activated cellulosic material, was modified by a facile oxidization reaction and efficiently covalent-linked with pectinase. Compared with free pectinase, pectinase coupled on oxidized pulp fiber exhibited better stability and performance in lowering cationic demand of whitewater as well as good reusability. In addition, biodegradable oxidized pulp fiber can be reused in papermaking after the final use of enzyme for several batches. It was superior to other carrier synthetic materials previously used since they cannot be utilized and therefore worsen contamination problems in papermaking process. Accordingly, it is suggested that pectinase immobilized on oxidized pup fiber is suitable for industrial application in papermaking industry.

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