Modified Pectin as Imprinting Substrate to Immobilize Pectinase *via* Both Adsorption and Crosslinking

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A broad-spectrum substrate-imprinted adsorption and crosslinking double immobilized pectinase (SDP) was prepared using a universal modified pectin obtained through enzymatic hydrolysis as the imprinting substrate. Its structure was characterized by infrared spectroscopy, circular dichroism, and scanning electron microscopy. The results showed that 1) cross-linking increased the Schiff base in SDP, 2) immobilization barely changed the secondary structure such as α-helix and β-sheet of SDP, and 3) adhesives were evenly distributed on the surface after immobilization. Studies on the enzymatic properties of SDP showed that the substrate imprinting significantly improved heat resistance and neutralization resistance of SDP. For example, the relative activity of SDP at 35 to 75 °C and at pH 4.4 to 6.5 was 5% and 15% more than that of the adsorption and crosslinking double immobilized pectinase (DP), respectively. In addition, after 8 cycles of use, the relative enzyme activity of SDP still reached 39.5%. Moreover, use of SDP decreased the cation demand in whitewater by 10% compared with DP. Overall, the use of a broad-spectrum substrate for imprinting to obtain SDP provides a new idea and method for using pectinase in in complex systems such as juice clarification and wastewater treatment.

Keywords: Pectinase; Immobilized; Substrate-imprinted; Whitewater treatment

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INTRODUCTION

Pectinase is a hydrolytic enzyme that can degrade pectic substances and primary constituents of cell walls into basic monomeric units. It is used frequently in various industries for fruit juice extraction, water and wastewater treatment, and paper bleaching (Esawy *et al.* 2013). The activity of free pectinase (FP) decreases after repeated use or under high temperature, as well as in strongly acidic or alkali conditions, which limits its industrial application. Using immobilization techniques such as adsorption, embedding, covalent and ionic bonding, and crosslinking can improve its stability and reuse ability (Wu *et al.* 2014; Ramirez *et al.* 2016). However, enzymes are often randomly immobilized using these methods, resulting in reduction or loss of enzyme activity (Liu *et al.* 2010). Therefore, it is necessary to find new immobilization methods to reduce the adverse effects on enzyme activity during immobilization, protect enzyme active center, and allow enzyme reuse in specific environments.

Substrate imprinting is a technique developed based on molecular imprinting technology. It could change the conformation of enzymes *via* interaction between enzymes and substrates. Imprinting substrates on enzymes could avoid inactivation of enzymes

during immobilization. Molecular imprinting technology was originally used as an artificial biotemplate technique to mimic antibody-antigen interactions to construct template polymers with specific selective recognition sites (Chaput *et al.* 2012; Rehman *et al.* 2014; Gaio *et al.* 2017). Later, it was used to imprint and immobilize enzymes. Ohmiya *et al.* (1975) obtained glucoamylase with high activity, stability, and repeatability by first immobilizing it onto polyacrylic acid nitride in the presence of a substrate and then cross-linking the multimeric-enzyme complex with hydroxylamine. Compared with glucoamylase, industrial pectinase is an enzyme with low substrate specificity. When imprinted with a single substrate, the retained enzyme activity can only be directed to a single substrate, and the activity of the non-imprinted pectinase in the process of enzyme immobilization will still be affected (Kashyap *et al.* 2001; Wu *et al.* 2013). Studying the immobilization of broad-spectrum enzymes by selecting a universal substrate has important significance for improving the catalytic performance of industrial composite enzymes and their commercial application prospects.

In this paper, modified pectin was selected as a universal substrate for pectinase immobilization, and it was used to immobilize pectinase *via* the substrate imprinting technique. The obtained immobilized enzyme was subjected to structural tests using Fourier transform infrared spectroscopy (FT-IR), circular dichroism spectra (CD spectrum), and scanning electron microscopy (SEM), and the properties of the immobilized enzyme and its application in whitewater were examined. This technology has broad application prospects for improving the catalytic performance of pectinase to meet the needs of current large-scale production.

EXPERIMENTAL

Preparation of Adsorption-Crosslinking Double Immobilized Pectinase (DP)

Adsorption-crosslinking double immobilized pectinase (DP) was prepared following the below steps. Chitosan powder (4.0 g, deacetylation degree ≥ 95%) was dissolved in 100 mL of 5% (v/v) acetic acid solution. The chitosan-acetic acid solution was added dropwise into 200 mL mixture of 3 parts 2 M NaOH and 1 part absolute ethanol. After incubation at room temperature for 3 h, the liquid was removed by vacuum filtration, and the products were washed to neutrality to obtain chitosan microspheres with a diameter of 2 mm. The chitosan microspheres were crosslinked with 0.05% glutaraldehyde solution for 1 h and washed with distilled water to remove residual glutaraldehyde. The prepared wet cross-linked chitosan microsphere carriers were added into 20 mL of pectinase solution (1 g/L, pectin hydrolase, 10000 U/g) and incubated at 25 °C for 3 h to allow adsorption. The mixture was filtered and washed thoroughly, and the products were stored at 4 °C after lyophilization.

Preparation of Substrate-Imprinted Adsorption-Crosslinking Double Immobilized Pectinase (SDP)

Substrate-imprinted adsorption-crosslinking double immobilized pectinase (SDP) was prepared using the following steps. First, 50 mL of orange pectin substrate S (5 g/L, galacturonic acid, ≥ 74%) was mixed with 1L of pectinase solution (1 g/L, *ibid*) and incubated at 50 °C in a water bath for certain period of time. The mixture was centrifuged for 15 min at 8000 rpm, and the supernatant was freeze-dried. The modified pectin (5 mg) was mixed with 20 mL of pectinase solution (1 g/L, *ibid*). All of the prepared wet cross-

linked chitosan microsphere carriers were added into 20 mL of pectinase solution (1 g/L, *ibid*) and incubated at 25 °C for 3 h to allow adsorption. The mixture was filtered and washed thoroughly, and the products were stored at 4 °C after lyophilization.

Structural Characterization of Immobilized Pectinase

The samples were characterized using a Hitachi Regulus 8220 scanning electron microscope (Tokyo, Japan) at voltage of 5.0 kV. In addition, the samples were prepared as KBr tablets and tested at a Nicolet 10 Fourier transform infrared spectrometer (Massachusetts, America) at 500 to 4000 cm^{-1}. These samples were examined using a Chirascan circular dichroism spectrometer (Shanghai, China) with the scanning range of 300 to 190 nm.

Determination of Enzyme Properties

Effects of temperature and pH on the activities of the three enzymes (FP, DP, and SDP)

To explore the effect of temperature on the activity of the three enzyme samples, their activity was measured at pH 4.0 for 1 h at different temperatures (35 °C to 75 °C). The activity of pectinase at its optimal temperature was defined as 100%, and used to calculate the relative enzyme activity (%) at other temperatures.

To explore the effect of pH on the activity of the three enzyme samples, their activity was measured at 50 °C for 1 h at different pH (pH 3.0 to 6.5). The activity of pectinase under the optimal pH was defined as 100% and used to calculate the relative enzyme activity (%) under other pH conditions.

To explore the thermal stability of the three enzyme samples, three pectinase enzymes were incubated at pH 4.0 and different temperatures (20 °C to 80 °C) for 1 h, and the pectinase activity was measured at 50 °C. The enzyme activity at 20° C was 100%, and the relative enzyme activity (%) at other temperature conditions was calculated.

Determination of kinetic parameters of the three enzymes (FP, DP, and SDP)

To determine kinetic parameters of the three enzymes, their activity was measured at 50 °C under pH 4.0 for 1 h at different substrate concentrations and used to calculate K_m of the three enzymes using Lineweaver-Burk double reciprocal method. Michaelis constant (Km) means the concentration of substrate (S) when the enzymatic reaction reaches half the maximum speed (Vm).

Application of the Three Pectinases (FP, DP, and SDP) in Whitewater Treatment

A certain amount of pectinase was mixed with 50 mL of papermaking whitewater and incubated at 50 °C for 1 h. The galacturonic acid content in papermaking whitewater was measured using the DNS method. The pH and conductivity of the papermaking whitewater was measured using a pH meter and conductivity meter, respectively. The cation requirement was determined using the titration method with a standard cationic titration solution PDADMAC (charge density: 1000 μeq/L) and a PCD streaming current detector.

RESULTS AND DISCUSSION

Structural Characterization of SDP

The infrared spectra of the imprinted substrate modified pectin as well as DP and SDP are shown in Fig. 1a. DP exhibited a stretching vibration of -C=O at 1640 cm^{-1}, which is related to the formed Schiff base. In contrast, the corresponding peak was shifted to 1590 cm^{-1} in SDP with increased intensity. The imprinting substrate MP showed a strong O-H absorption peak at 3300 cm^{-1}-3480 cm^{-1}, a vibration absorption peak of CH-in methylene at 1730 cm^{-1}, and a strong-C=O stretching vibration peak at 1730 cm^{-1}, all of which were weakened or not present in SDP. This may be due to the fact that the structure of SDP tends to be stable at the presence of the imprinting substrate, which facilitates the cross-linking reaction and results in an increased Schiff base. The disappearance and significant decrease of these characteristic peaks of MPs in immobilized SDP after immobilization indicates that the imprinting substrate has been removed and the enzyme activity center is released (Silva *et al.* 2012; Bustamante-Vargas *et al.* 2015; Chauhan *et al.* 2015; Chakraborty *et al.* 2016; Sojitra *et al.* 2017).

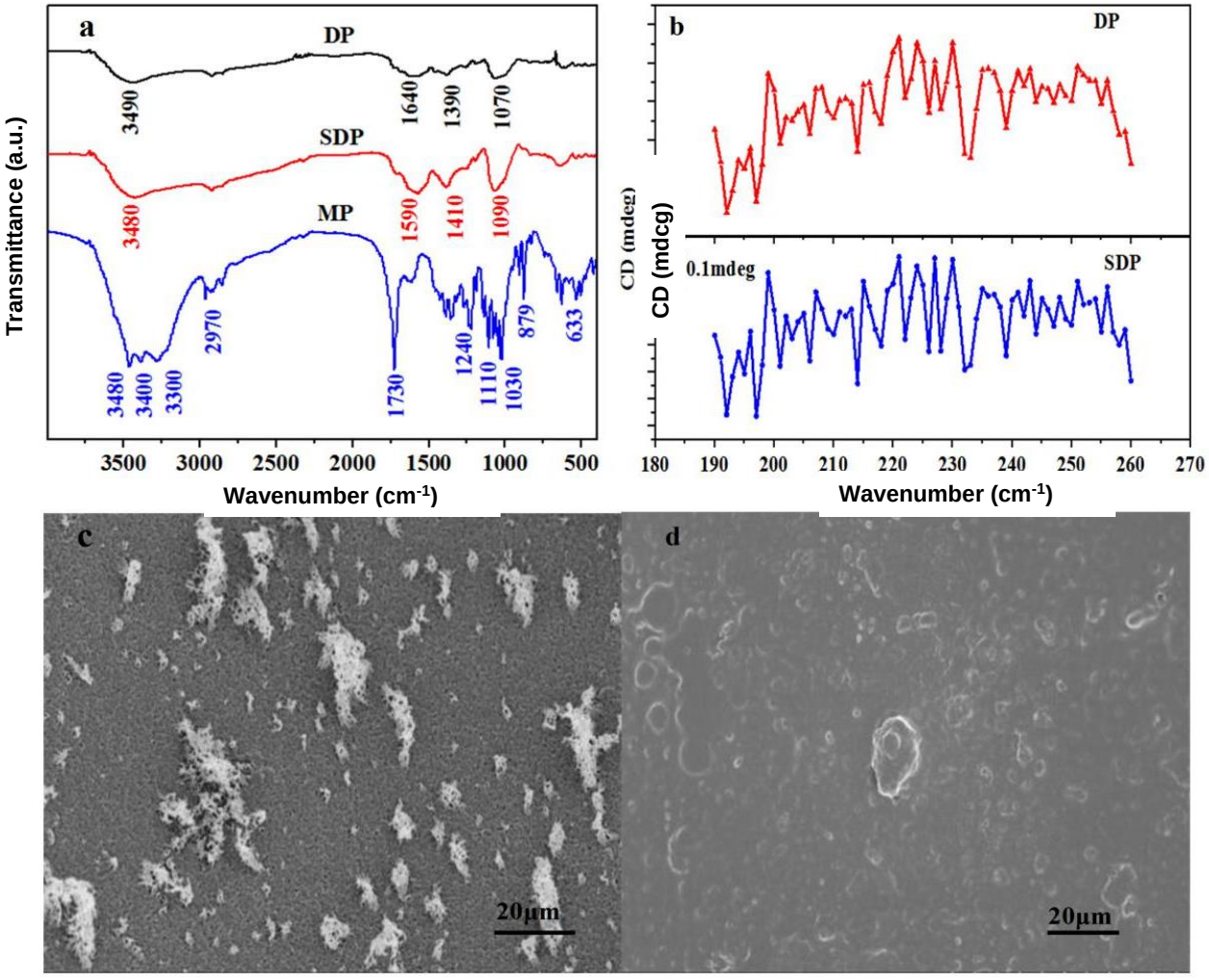

Fig. 1. Infrared spectra of SDP, DP and MP (a), CD spectra of SDP and DP (b), and SEM images of DP (c) and SDP (d)

The circular dichroism (CD) spectra of DP and SDP are shown in Fig. 1b. The immobilization process caused a conformational change in the secondary structure of the enzymes, showing great fluctuations in the range of 190 nm to 260 nm. The value of SDP increased by only about 5% compared with that of DP, suggesting that SDP and DP have similar alpha-helix and beta-sheet structures. This may be related to the catalytic and binding groups in the active center and other essential group outside the active center (tryptophan, *etc*.). When substrate binds to these groups, the active center of the enzyme is protected, and its structure is stabilized. In addition, the crosslinking increases the content of Schiff base, which is consistent with the results obtained by infrared spectral analysis (Bahrami and Hejazi 2013; Rehman *et al*. 2013).

Figures 1c and d show the SEM images of chitosan microsphere after addition of DP and SDP, respectively. In Fig. 1c, it is clear that there are many unevenly distributed adhesives on the surface of microspheres. This may be because the pectinase is immobilized on the surface of the DP-adsorbed microspheres, forming enzyme clusters with various sizes. In Fig. 1d, the adhesives on the surface of the SDP-adsorbed microspheres are evenly distributed and have morphology different from that shown in Fig. 1c, indicating that substrate imprinting before immobilization changes the molecular structure of pectinase (Wang *et al*. 2013) and subsequently alters the morphology of adhesives on the surface of the carrier. Taking together, the increased Schiff base in SDP found in the infrared and CD spectra and the evenly distributed adhesives on the surface of SDP-adsorbed microspheres in SEM images clearly show that the contact area between the immobilized enzyme and the imprinting substrate was increased.

Enzymatic Properties of SDP

To explore changes in the enzymatic properties of SDP, the ability of FP, DP, and SDP to hydrolyze pectin was determined at pH 4.0 and different temperature conditions or at 50 °C and different pH conditions using citrus pectin S as the substrate. The thermal stability of FP, DP, and SDP was measured. The results in Fig. 2a show that the optimal temperature was 50 °C for FP and 60 °C for DP and SDP. In addition, the relative enzyme activity of SDP was slightly higher than that of DP at 35 °C to 75 °C (except at 60 °C). This result indicates that the heat resistance of DP and SDP was similar and improved compared with FP because the stabilization effect of the carrier matrix limits the conformational change of pectinase during thermal denaturation. Moreover, higher activation energy makes the substrate easily diffuse to the immobilized enzyme and bind to its binding sites. Imprinting substrate to SDP before immobilization also stabilizes the physicochemical properties of SDP and increases its rigidity, resulting in a slightly lowered heat resistance of SDP than DP (Mai *et al.* 2016; Rajdeo *et al.* 2016). However, long-term incubation at higher temperatures can decrease enzyme activity or even inactivate the enzyme; proteins denature at a certain temperature, which ultimately affects their enzymatic activity (Mendes *et al.* 2011).

The results in Fig. 2b show that the optimal pH of FP, DP, and SDP was pH 3.5, and the trend of relative enzymatic activity at different pH was similar for these three enzymes, although FP and DP had higher relative activity only at pH 3.0 to 4.0, while SDP maintained a relatively high relative activity at pH 3.0 to 6.5. Specifically, the relative activity of SDP at pH 4.5 to 6.5 was 15% higher than that of DP (Lei *et al.* 2007). Changes in pH affect the structure and charge of the enzyme activity center as well as the matrix (Rehman *et al.* 2014). Because the structure of the immobilized enzymes is stable, they are

less affected by pH of the reaction solution. Because SDP is subjected to substrate imprinting before immobilization, the remaining activity of SDP after immobilization is high. In addition, the stable chemophysical properties and enhanced rigidity of the active center of SDG improve its resistance to acidic and basic environments.

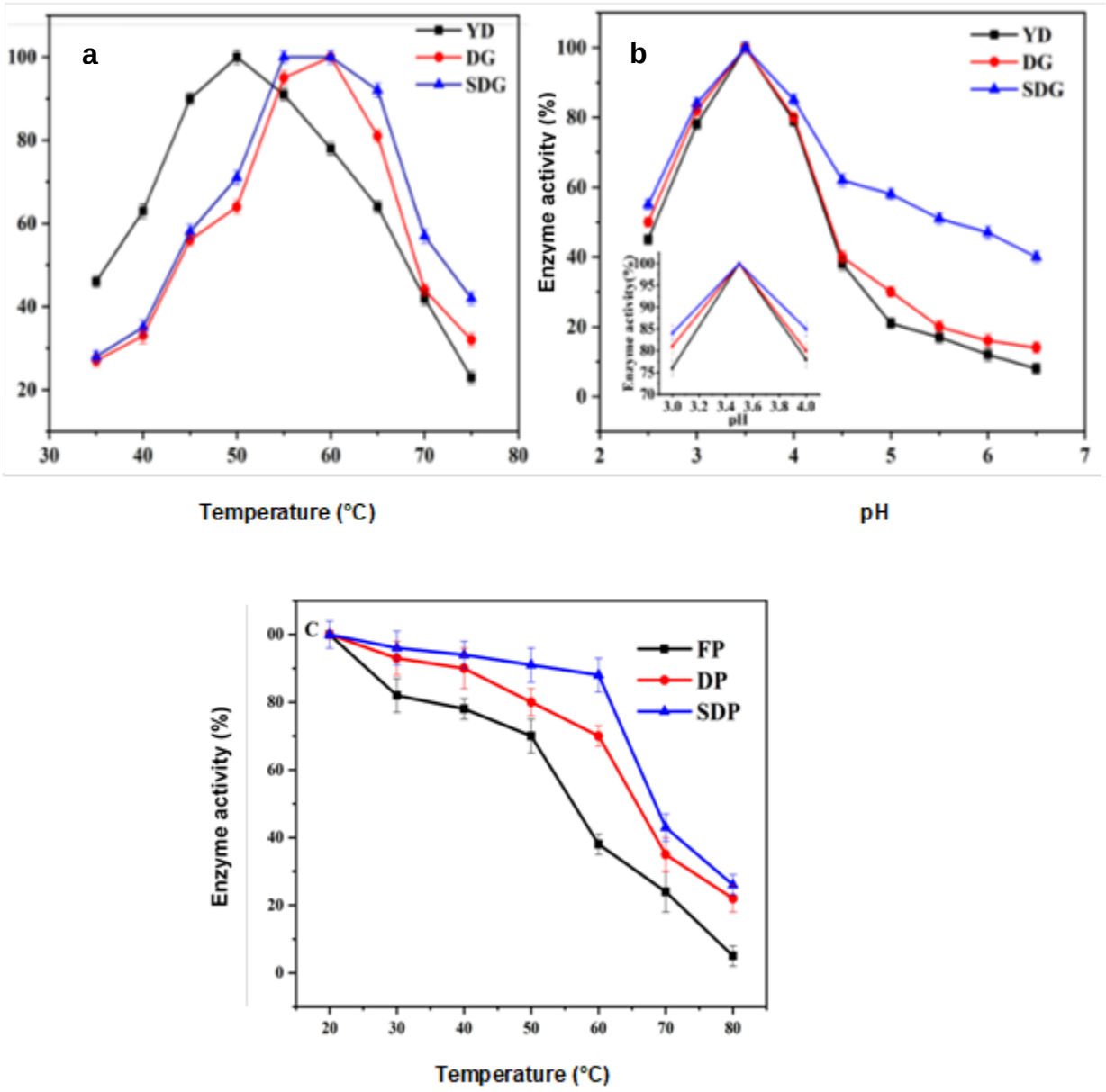

Fig. 2. Effects of temperature (a) and pH (b) on the activity of FP, DP, and SDP; and effects of FP, DP, and SDP on thermal stability (c)

The thermal stability of FP, DG, and SDG was determined using citrus pectin S as a substrate. The results are shown in Fig. 2c. The relative activity of FP was maintained at 60% or higher at 20 to 50 °C. DP and SDP maintained high stability at 20 to 60 °C and 20-65 °C, respectively. With the increase of temperature, the activities of three enzymes decreased to different degrees. However, the thermal stability of SDG was always higher

than that of FP and DG, because the high temperature causes the enzyme molecule to be thermally denatured, and the substrate imprinting process enhances the rigidity of the enzyme molecule and improves the thermal stability of the enzyme molecule.

To explore changes in substrate affinity of SDP, the activity of the three pectinases was determined at 50 °C and pH 4.0 with different substrate concentrations and used to calculate their K_m using the Lineweaver-Burk double reciprocal method. As shown in Fig. 3a, b, and c, the K_m values of FP, DP, and SDP were 9.585 mg/mL, 8.901 mg/mL, and 7.854 mg/mL, respectively. The reduced K_m value of SDP indicates that the affinity of SDP to pectin was increased. This may be due to the catalytic ability of pectinase to pectin after substrate imprinting to the enzyme increases the concentration of the intermediate product, making it tend to dissociate into enzyme and product and resulting in a smaller K_m. In addition, the enzyme is activated due to the induction of the imprinting substrate, leading to increased affinity of the enzyme to the substrate (Lei and Bi 2007; Liu *et al.* 2008).

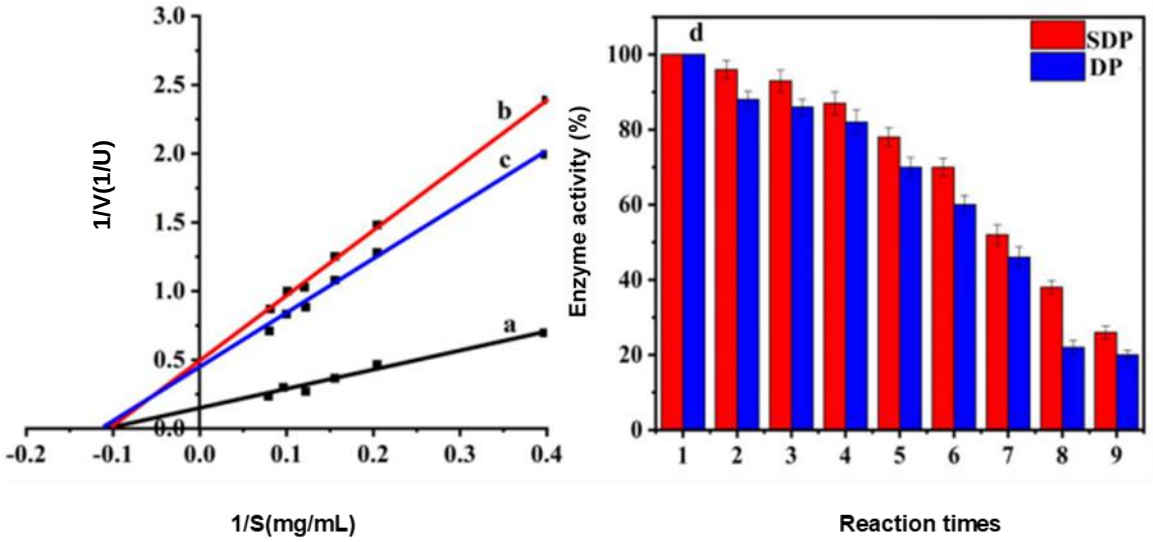

Fig. 3. Lineweaver-Burk double reciprocal plot of YP (a), DP (b) and SDP (c) as well as the activity recovery of DP and SDP after multiple uses (d)

Figure 3b shows the operational stability of DP and SDP using citrus pectin S as the substrate. As the number of usage increased, the enzyme activity of SDP and DP decreased. At the 8 and 9 cycles of use, the relative activity of DP decreased to 21.5% and 19.6%, respectively, while that of SDP decreased to 39.5% and 27.6%, respectively. The decrease in activity is due to both enzyme inactivation and enzyme leakage after repeated use (Sardar and Gupta 2005). These results indicated that substrate imprinting increases the time of pectinase reutilization, which may be due to tighter cross-linking of enzyme molecules with the chitosan carrier, thus avoiding excessive enzyme leakage during repeated use. These results are consistent with the infrared spectral results showing increased Schiff base content in SDP.

Effect of SDP on Pectin Substances in Whitewater

The parameter changes of paper making whitewater and the galacturonic acid content were measured. As shown in Table 1, treatment with the three pectinases had almost no effect on the pH of papermaking whitewater. After FP treatment, the

conductivity of whitewater increased from 3720 to 3730 μS/cm. After the DP and SDP treatments, the conductivity of whitewater decreased, which may be because the conductivity of free pectinase dissolved in whitewater increased the total conductivity of the whitewater. In contrast, DP and SDP can be recycled after whitewater treatment, so that the total conductivity of the whitewater was reduced. Moreover, because SDP is protected by substrate imprinting, it had higher activity than DP. Thus, the conductivity of whitewater after SDP treatment was lower than that after DP treatment (Carrin *et al.* 2000; Esawy *et al.* 2013; Chauhan *et al.* 2015; Ma *et al.* 2016).

Table 1. Effect of Pectinase Treatment on the Properties of Whitewater

	pH	Conductivity (μS/cm)	Demand for Cation (μeq/L)	Galacturonic Acid Content (mg/L)
Whitewater	8.20	3720	905	0
FP	8.22	3730	655	65.4
DP	8.12	3570	320	84.6
SDP	8.10	3490	305	93.1

The effect of SDP on controlling pectin in whitewater was the best. SDP treatment reduced the cation demand of whitewater from 905 μeq/L to 305 μeq/L. DP treatment reduced the cation demand for whitewater from 905 μeq/L to 320 μeq/L.

The galacturonic acid content in whitewater after FP, DP, and SDP treatment was 65.4 mg/L, 84.5 mg/L, and 93.1 mg/L, respectively, which was consistent with the decrease of cation demand in whitewater. The galacturonic acid content after SDP treatment was 10% more than that after DP treatment, indicating that the active center of SDP was protected by substrate imprinting. In addition, imprinting with universal substrate (modified pectin) improved the substrate selection spectrum of the enzyme. Compared with DP, the substrate selection range of SDP was broader, which led to higher catalytic performance of SDP than DP to degrade pectin in whitewater and reduced its demand for cation (Li *et al*. 2007; Bustamante-Vargas *et al*. 2015).

CONCLUSIONS

1. The results show that the enzymatic properties of SDP were significantly improved due to the protective effect of substrate imprinting. Under the same conditions, the relative enzyme activity of SDP compared to DP was increased by 5% and 15%, respectively, at 35 to 75 °C and pH 4.4 to 6.5. SDP was more stable during operation with activity recovery being 39.5% and 27.6% after 7 and 8 cycles of use, respectively.
2. The universal imprinting of the substrate improved the catalytic performance of the industrial composite enzyme system, so that the anionic waste in the papermaking whitewater was significantly reduced, and the efficiency of immobilizing pectinase was improved.
3. Overall, the use of a broad-spectrum substrate for imprinting provides a new idea and a new method for immobilizing enzymes. It is proposed that SDP will play a huge role in fruit juice clarification and sewage treatment, and has broad application prospects.

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