IMMOBILIZATION OF LIPASE ON CHITOSAN BEADS FOR REMOVAL OF PITCH PARTICLES FROM WHITEWATER DURING PAPERMAKING

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Pitch deposits originating from alkaline peroxide bleaching of mechanical pulps can seriously decrease the runnability of the paper machine when efforts have been made to increase the reuse of process water. In order to degrade pitch particles present in whitewater, lipase was immobilized on chitosan beads using a binary method. The operational stability of the immobilized lipase and its efficacy for treating whitewater were also preliminary studied. The results showed that the highest activity of immobilized lipase was achieved by using 0.5% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for activation and 0.0025% glutaraldehyde for cross-linking chitosan. The immobilized lipase also exhibited very good operational stability, and the pitch particles present in whitewater could be reduced by 66.8% after treatment with the immobilized lipase.

Keywords: Chitosan; Immobilized lipase; Pitch deposits; Whitewater; Papermaking

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INTRODUCTION

With closing of paper mill water circulation, dissolved and colloid substances (DCS) tend to accumulate in the whitewater. Lipophilic extractives (commonly called "pitch particles" or "pitch agglomerates") originating from alkaline peroxide bleaching of mechanical pulps are considered especially troublesome substances (Yu and Deng 2004; Boegh *et al.* 2001). The triglycerides in the pitch particles can degrade product quality and impair the production process when they are deposited on exposed parts of paper machines, such as on wires or hydrofoils. Therefore, the removal of pitch particles is always a key problem in modern paper mills. The traditional strategy for reducing these troublesome substances is by using cationic polyelectrolytes for flocculation and viscosity reducers such as talc, which cause extra consumption of chemicals. However, due to the tremendous increase in the awareness regarding the effects of pollution from the pulp and paper industry, the demand is high to replace those traditional processes with "green" processes involving microorganisms and enzymes. They not only provide an economically viable alternative, but are also environmentally friendly (Pakarinen *et al.* 2012; Long *et al.* 2011; Kaur *et al.* 2010).

Free lipase has been shown to be effective in degrading pitch components and decreasing the size of colloidal particles (Rundlof *et al.* 2002). However, free enzymes

dissolved directly in water not only can contaminate the paper, but are also difficult to recover. Compared to free enzymes, immobilized enzymes are more rigid and resistant to environmental changes. Their thermal, operational stability, and recoverability were therefore increased greatly (Bayramoglu *et al.* 2011; Zhao *et al.* 2011; Liu *et al.* 2010; Busto *et al.* 2006). Recently, several methods for immobilizing lipase on different supports have been presented in the literature (Yi *et al.* 2009; Liu *et al.* 2010, 2011, 2012). The results of these studies demonstrated that the immobilized lipase has higher activity and operational stability than free enzyme and primarily showed promising application potential in the papermaking industry. But until now, very few reports have been published on the application of immobilized lipase in treatment of the pitch deposits in whitewater from papermaking. This work extends the preliminary application of the immobilized lipase to treatment of whitewater and examines their effect on reducing the amount of pitch present in whitewater.

EXPERIMENTAL

Materials

Chitosan (MW 1×10^6 Da, degree of deacetylation 95%) was generously provided by Yuhuan Ocean Biochemical Co., Ltd. (Zhejiang, China). Alkaline lipase from *Thermomyces lanuginosus* was purchased from Novozymes (Denmark); the activity and protein content of free lipase were 42 U/mL^{-1} and 92 $\mu g/mL^{-1}$, respectively. Bovine serum albumin (BSA) used as the standard for protein assay was purchased from Sigma-Aldrich (USA).

The whitewater samples were donated by a southern China paper mill producing high-brightness mechanical specialty grades from peroxide-bleached softwood (mason pine) TMP. All the samples used in experiments were brought back on the same day after their collection and then stored in a refrigerator at 4 °C. 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC) was purchased from Aladdin Reagent Co., Ltd. (China). All other chemicals were of analytical grade and used without further purification.

Preparation of Cross-Linked Chitosan Beads

The swollen chitosan beads were prepared according to the literature (Cetinus and Oztop 2003; Hung *et al.* 2003). The main procedure can be described as follows: first, the chitosan solution was prepared by dissolving 2 g of chitosan flakes in 100 mL of 5% (v/v) acetic acid. Then, the resulting solution was dropped into 200 mL of 2.0 M NaOH/20% ethanol through a needle (diameter, 1.2 mm). The chitosan gelled spheres were formed instantaneously. After hardening, the chitosan beads were separated and washed with deionized water until the filtrate became neutral. The diameter of the wet beads was approximate 1.8 to 2.0 mm.

Immobilization of Lipase on Chitosan Beads

The detailed procedure for immobilizing of lipase on chitosan was carried out according to the literature (Hung *et al.* 2003; Ting *et al.* 2006).

Method A

Three grams of chitosan beads were treated with 10 mL of 0.5% (w/v) EDC solution and mixed for 30 min. Then 5 mL of 0.1% (v/v) lipase was added into the achieved chitosan-EDC mixture at 25 °C and pH 8.0. After 1 h, the beads were filtered and washed three times in deionized water to remove unbound enzymes.

Method B

Three grams of chitosan beads were mixed with 10 mL of 0.0025% glutaraldehyde for 30 min, and 5 mL of 0.1% (v/v) lipase was added into the cross-linking chitosan beads at 25 °C and pH 8.0. After 1 h, the beads were filtered and washed three times in deionized water to remove unbound enzymes.

Method C

Three grams of chitosan beads were treated with 10 mL of 0.5% (w/v) EDC solution and mixed for 30 min, then 5 mL of 0.1% (v/v) lipase was added into the achieved chitosan-EDC mixture at 25 °C and pH 8.0. After 1 h, the beads were filtered and added into 10 mL of 0.0025% glutaraldehyde. After 30 min, the supernatant was removed, and 5 mL of 0.1% (v/v) lipase was added into the beads at 25 °C and pH 8.0. After 3 h, beads were washed three times in deionized water to remove unbound enzymes.

Method D

Three grams of chitosan beads were mixed with 10 mL of 0.0025% glutaraldehyde for 30 min, and 5 mL of 0.1% (v/v) lipase was added into the cross-linking chitosan beads at 25 °C and pH 8.0. After 1 h, the beads were filtered and added into 10 mL of 0.5% EDC solution for 30 min. Then, the supernatant was removed, and 5 mL of 0.1% (v/v) lipase was added into the beads at 25 °C and pH 8.0. After reaction for 3 h, the beads were washed three times in deionized water to remove unbound enzymes.

Lipase Activity

The activity of lipase was assayed by titrating the fatty acid produced in the hydrolysis of olive oil. The substrate consisted of 4% (w/v) polyvinyl alcohol and olive oil (volume ratio 3:1). Four mL of substrate and 6 mL of phosphate buffer solution (50 mM, pH 8.5) were mixed and placed in a bath vessel maintained at 55 °C; then 0.5 g immobilized lipase was added and stirred sufficiently. After reacting for precisely 15 min, 15 mL of ethanol was added to terminate the reaction. Then the produced fatty acid was titrated using 0.05 M NaOH. Similarly, a blank experiment with 0.5 g more of beads without immobilized lipase was carried out using the assay procedure above. According to the amount of alkali consumed, the produced amount of fatty acid was calculated. One unit (U) of lipase was defined as the amount of enzyme required to release 1 μmol of free fatty acid per min under the assay conditions.

Protein Assay

Protein content was estimated by the method of Bradford, using Bio-Rad protein dye reagent concentrate. Bovine serum albumin was used as the standard (Bradford 1976).

Treatment of Whitewater with Chitosan-Immobilized Lipase

500 mL of whitewater was stirred for 5 min and maintained at 55 °C; then 6460 U/L of immobilized lipase was added with a stirring rate of 100 rpm for 15 min. The size of pitch particles in whitewater before and after treatment with immobilized lipase was determined by using a Malvern-Sizer laser diffraction particle size analyzer. The turbidity of whitewater samples was conducted using a 2100 AN turbidity meter (Hach).

The depositable pitch contents of whitewater samples were measured according to TAPPI method RC 324. In this method, two stainless steel plates were vibrated rapidly in a sample of whitewater for 30 min at 55 °C and pH 7.5. The plates were then removed and kept in a drying oven at 105 °C for 120 min. The net weight gain of the plates was used as an index of the amount of depositable pitch present in the whitewater.

RESULTS AND DISCUSSION

Activities of Immobilized Lipase from Different Methods

The protein loading and activities (including specific activities) of immobilized lipases on chitosan by four methods were measured, and the results are listed in Table 1. The different immobilization methods showed obvious effects on the activity of chitosan-immobilized lipase. The highest activity of immobilized lipase was achieved by method C, in which the activity and total protein loading of immobilized lipase were 64.6 U/g-chitosan and 253.7 μg/g-chitosan, respectively. The specific activity reached 254.6 U/mg-protein. The highest activity and protein loading for immobilized lipase achieved by method C might be due to more effective ways for lipase molecules to immobilize onto chitosan. In method A, the lipase molecule was immobilized on the hydroxyl groups of chitosan by activation with EDC. The immobilization reaction in method B occurred by means of the amino groups of chitosan by cross-linking with glutaraldehyde. However, it is valuable to point out that in methods C and D, the lipase molecule can be immobilized not only on the amino groups, but also on the hydroxyl groups of chitosan, which was termed as a binary immobilization method. Additionally, the higher activity of the binary-immobilized lipase prepared by method C than that prepared by method D might be due to the different deactivation degree of enzymes cross-linked to the chitosan during different immobilization procedures.

Table 1. The Activities and Protein Loading of Immobilized Lipase from Different Methods

Method of immobilization	Total protein loading (μg/g-chitosan)	Lipase activity (U/g-chitosan)	Specific activity (U/mg-protein)
Method A	81.7	14.2	173.8
Method B	117.3	15.6	133.0
Method C	253.7	64.6	254.6
Method D	220.3	48.3	219.2

Kinetic Studies

In addition to investigation of activity and protein loading for immobilized lipase, the corresponding kinetic parameters were also studied. The kinetic parameters of Michaelis-Menten on hydrolysis of olive oil by free and immobilized lipase were calculated after changing the substrate concentration. The results are listed in Table 2. The K_m of immobilized lipase was 19.8%, which was approximately 6-fold higher than that of free lipase. As is known, immobilization might alter the structure, especially the active site of enzyme, and can also reduce the accessibility of enzyme to substrate. Then, the resulting K_m value of lipase will therefore increase (Hung *et al.* 2003). Normally, the actual hydrolytic activity of enzyme can be expressed by V_{max}. From Table 2, it can be seen that the V_{max} of immobilized lipase (2500 U/g-chitosan) was significantly higher than the corresponding value of free lipase (46.7 U/mL). This probably originated from ineffective adsorption of free enzyme to substrate. Similar results for lipase immobilized on chitosan support can also be found in the literature (Ting *et al.* 2006).

Moreover, the results show that a higher affinity of lipase to chitosan's active sites caused the corresponding E_a value (47.3 kJ/mol) to be about 18 percent lower than the corresponding E_a value (57 kJ/mol) of free enzyme. Similar results for free and immobilized lipase were also reported recently (Chiou and Wu 2004). This is probably due to the fact that the process of immobilization can reduce the number of active sites of enzyme to substrate, which would increase K_m, but decrease the E_a.

Table 2. Activation Energy (Ea) and Kinetic Parameters for Free and Binary-Immobilized Lipase

Types	E_a (kJ/mol)	V_{max} (U/g-chitosan or ml)	K_m (%)
Binary-immobilized lipase	47.3	2500.0	19.8
Free lipase	57.0	46.7	3.2

Reusability of the Immobilized Lipase

Normally, the temperature of whitewater from paper machines varies from about 50 to 60 °C. Coincidently, based on a previous study, it was found that the highest activity of immobilized lipase can be obtained if the temperature of solution is controlled at approximately 55 °C. That is to say, enzymatic hydrolysis of the triglycerides to

remove pitch deposits in whitewater would save more energy than other treatment methods do. Ideally, if the enzyme can be well recycled and kept at relatively high enzymatic activity, then this method will be wonderfully promising in whitewater treatment. Therefore, the immobilized lipase was reused eight times to investigate the operational stability. From Fig. 1, it can be clearly observed that after being used repeatedly for four batches, the immobilized lipase retained approximately >75% of its original activity. And after being used for eight batches, about 60% residual activity of the immobilized lipase was found. Although some loss of enzyme occurred during reuse, the enhanced operational stability of chitosan-immobilized lipase made it an excellent biocatalyst for potential use in whitewater treatment in the papermaking industry.

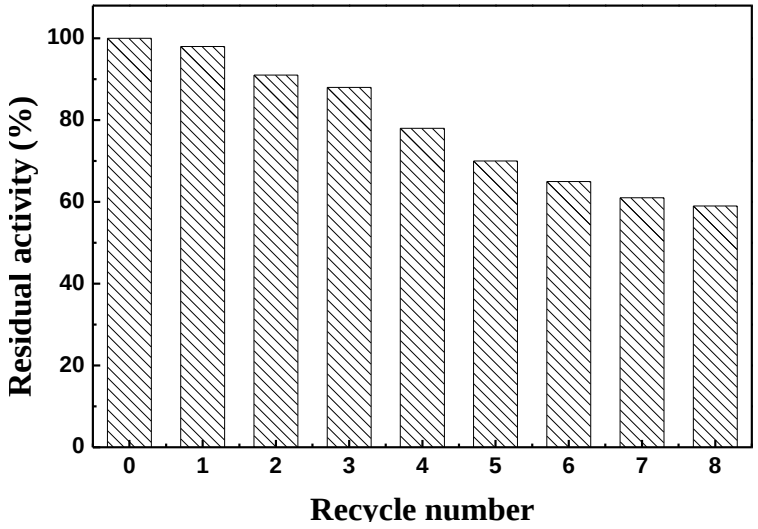

Fig. 1. Operational stability of the immobilized lipase in olive oil treatment at 55 °C and pH 8.5

Effect of the Immobilized Lipase on Hydrolyzing Pitch Particles Present in Whitewater

In order to check whether the immobilized lipase would be effective in the treatment of whitewater or not, the effect of the immobilized lipase on removing the pitch particles from whitewater was preliminary investigated. As seen in Table 3, the immobilized lipase resulted in an appreciable reduction in average particle size, turbidity, and pitch deposit contents. Especially, the pitch contents in whitewater could be reduced by 66.8%. This is as expected, due to strong catalytic performance of the immobilized lipase to triglycerides in the pitch deposits in whitewater. Also, it is obvious from Fig. 2 that the particle size of pitch decreased significantly, from 1000 to 100 μm, and the distribution of particle size was also proportionally decreased. This was mainly because the pitch particles of small size were hydrolyzed by the lipase and disappeared. The pitch particles of large size were also hydrolyzed and became small particles in whitewater. Pitch deposits of larger size are more easily deposited on exposed parts of the paper machine and can cause a more serious deposition problem than pitch deposits of small size do. Therefore, the reduction in the size of pitch particles in whitewater was responsible for decreasing the deposition problem on the paper machine.

For chitosan-immobilized lipase, on the one hand, the chitosan might absorb the pitch particles directly; on the other hand, the triglycerides in the pitch also could be hydrolyzed by immobilized lipase. Moreover, compared to free lipase, we hypothesize that higher enzyme activity can be maintained during the process of hydrolysis because non-effective adsorption of enzyme to pitch particles and inactivation of enzyme induced by heavy metal ions in whitewater could be alleviated by immobilizing enzyme onto chitosan beads.

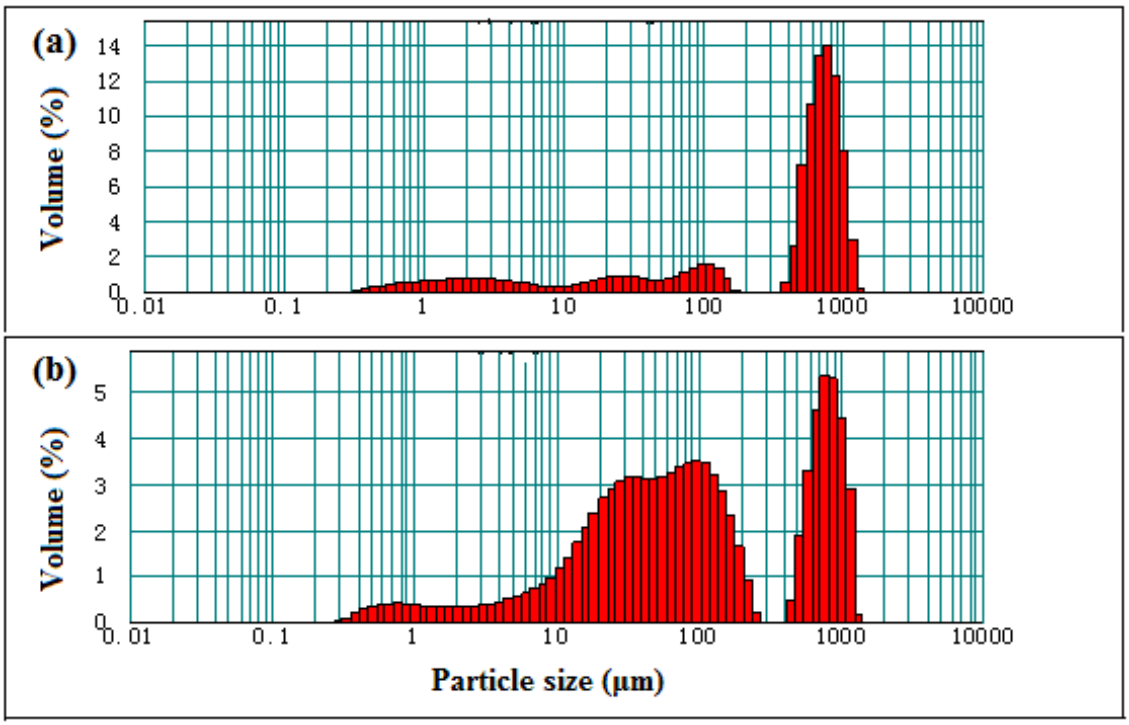

Fig. 2. Particle size distribution of pitch deposits in whitewater before (a) and after (b) treatment with immobilized lipase

Table 3. Effect of Immobilized Lipase on Eliminating the Pitch Particles in Whitewater

	Average particle size(μm)	Turbidity (NTU)	Pitch content of whitewater (g)	Efficiency of pitch removal (%)
Original whitewater	552.0	101.0	0.3961	—
Whitewater after treatment	276.0	80.8	0.1316	66.8

CONCLUSIONS

1. In this work, lipase was immobilized to chitosan beads by a binary method. The activity and specific activity of immobilized lipase were 64.6 U/g-chitosan and 254.6 U/mg-protein, respectively.
2. About 60% residual activity of the immobilized lipase was found after eight applications.
3. The immobilized lipase was very effective in degrading pitch particles in whitewater. The present work indicated that the immobilized lipase can be used to develop a bioreactor for large-scale use in whitewater treatment in the papermaking industry.

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