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Lignin from bamboo shoot shells as an activator and novel immobilizing support for α-amylase

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ABSTRACT

This study examined the feasibility of α-amylase activation and immobilization, using lignin from bamboo shoot shells (BSS). Our results demonstrated that BSS lignin is an excellent α-amylase activator and it elevated α-amylase activity more than two-fold at a concentration of 5 mg/ml. For immobilization of α-amylase via adsorption, BSS lignin was incubated in an α-amylase solution (5 mg/ml) for 20 min, and the maximum specific activity, amount of loaded protein and activity recovery were 92.4 U/mg, 19.0 mg/g and 111%, respectively. In contrast to its free counterpart, immobilized α-amylase improved the catalytic efficiency and storage stability, under comparable working conditions (temperature and pH). Regarding its convenient usage, immobilized enzyme can be suspended in advance, but a suspension incubated at 60 °C should be used within 30 min. The residual activity after 14 re-uses remained at a reasonable level (53.2%). In conclusion, this study reveals a novel support for enzyme immobilization.

1. Introduction

Enzymes are biological catalysts that facilitate complicated chemical processes under optimum conditions; however, large-scale industrial application of enzymes has not yet been achieved due to their considerably high cost, difficult recovery and recycling and low stability. Utilization of immobilized enzymes not only circumvents these problems but also has additional advantages, such as the ability to repeatedly re-use a single batch of enzyme and to better control the catalytic period (Hanefeld, Gardossi, & Magner, 2009; Brady & Jordaan, 2009; Singh, Singh, Suthar, & Dubey, 2011). Enzyme immobilization methods basically include adsorption onto insoluble materials, entrapment in a polymeric matrix, encapsulation and covalent linking to an insoluble carrier (Tüzmen, Tülden, & Denizli, 2012; Gashtasbi, Ahmadian, & Noghabi, 2014; Torabizadeh, Tavakoli, & Safari, 2014). Among these methods, adsorption is the most common, easiest to perform and oldest protocol. In addition, when necessary, adsorption allows the recovery of free enzyme and support material due to its reversibility (Tüzmen et al., 2012; Gashtasbi et al., 2014; Torabizadeh et al., 2014).

Amylases are one of the most significant hydrolase enzymes, accounting for more than one-quarter of the entire global enzyme market (Shukla & Singh, 2016). α-Amylase belongs to the amylase family, which catalyzes the hydrolysis of α-1,4-glycosidic linkages in starch and other related carbohydrates and has a variety of potential applications, such as starch saccharification, fermentation, baking, textiles and pharmaceuticals (Eslamipour & Hejazi, 2016; Richardson, Tan, Frey, Callen, Cabell, Lam, & Miller, 2002; Sajedi, Naderi-Manesh, Khajeh, Ranjbar, Ghaemi, & Naderi-Manesh, 2004; Homaei & Saberi, 2015). Previous research has revealed that α-amylase can be immobilized with various solid supports, such as poly(hydroxyethyl methacrylate-glycidyl methacrylate) membranes (Bayramoğlu, Yilmaz, & Arical, 2004), organofunctionalized glass beads (Kahraman, Bayramoğňlu, Kayaman-Apohan, & Güngör, 2007), poly(2-hydroxyethyl methacrylate) and poly(styrene-2-hydroxyethyl methacrylate) microspheres (Tümtürk, Aksoy, & Hasirci, 2000), DEAE-cellulose resin (Singh & Kayastha, 2014), gold nanorods (Homaei & Saberi, 2015) and others. Most of these support materials are synthetic polymers and are unsafe for use in the food industry due to the possibility of support leakage. Therefore, natural, non-toxic and inexpensive immobilized supports for α-amylase have attracted considerable attention. For example, chitin could be used as an immobilizing support for many enzymes and was extensively reviewed by Krajewska (2004). To be a good enzyme immobilizing support, the solid material must be insoluble in water, capable of

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enzyme accommodation and stabilization, resistant to the enzyme and mechanically stable (Kahraman et al., 2007). A good support material that is suitable for large-scale application should be easily available, inexpensive and pliable, so that it can be molded into a variety of forms (Jesionowski, Zdarta, & Krajewska, 2014).

Lignin is a common component of insoluble dietary fibre that is found in many different food by-products, such as sugarcane bagasse, bean dregs, sweet potato residue, sunflower seed shells and aged bamboo shoots (Li, Sun, Xu, & Sun, 2012a; Li, Sun, Xu, & Sun, 2012b). Natural lignins are environmentally friendly, biodegradable, non-toxic and even possess certain bioactive properties, such as antioxidant capacity and resistance to UV radiation (Li et al., 2012b; Ugartondo, Mitjans, & Vinardell, 2008). They have also been shown to improve many properties of plastic and edible films, and in particular, their barrier properties (Acosta, Chávez, Ramírez-Wong, Bello-Pérez, Ros, Millán, & Osuna, 2015). In addition, it has been demonstrated that lignin can activate pancreatic α-amylase and lipase (Zhang, Cui, Yin, Sun, & Li, 2013; Zhang, Xiao, Yang, Wang, & Li, 2014). Interestingly, lipase immobilized on cellulose/lignin beads was more active and stable than was lipase immobilized on pure cellulose beads (Park, Kim, Kim, Yu, & Kim, 2015). Moreover, Zdarta, Klapiszewski, Wysokowski, Norman, Kolodziejczak-Radzimska, Moszyński, and Jesionowski, (2015) demonstrated that a chitin/lignin composite could potentially serve as a lipase-immobilizing support. Furthermore, there is extensive evidence supporting the use of lignin and its derivatives as a sorption-active material (Harmita, Karthikeyan, & Pan, 2009). Given the previous findings, we assumed that natural insoluble lignin can serve as a potential candidate material for the immobilization of enzymes via adsorption; however, this hypothesis has not yet been tested. With a firm basis in the preparation and characterization of lignin powder from bamboo shoot shells (BSS) (Gong, Xiang, Ye, & Zhao, 2016), the current study examined its activating effects and assessed its feasibility as an α-amylase support. The immobilizing conditions were optimized and the resultant immobilized enzyme was extensively characterized, including its catalytic properties, stability and re-usability.

2. Materials and methods

2.1. Materials

Reagent-grade α-amylase (from porcine pancreas, EC 3.2.1.1) was purchased from Sigma-Aldrich Chemical Company (United States), while food-grade α-amylase from *Bacillus subtilis* was purchased from Beijing Aoboxing Biotechnology Co., Ltd (China). The reagent-grade *a*-amylase was used in the experiments to evaluate the activating capacity of BSS lignin. Considering the practical value of this study to the enzyme industry, food-grade α-amylase was used in the trials of enzyme immobilization. According to their optimal working parameters provided by their suppliers, the reagent-grade and food-grade *a*-amylases were suspended in PBS buffers with pH of 6.9 and 6.5, respectively, and then applied in the following tests. After 5 min of gentle stirring, clear enzyme solutions were obtained from the supernatants after centrifugation (4000 rpm, 5 min). Soluble potato starch and 3,5-dinitrosalicylic acid were purchased from Sinopharm Chemical Reagent Co., Ltd (China). Lignin from bamboo (*Dendrocalamus Latiforus*) shoot shells (BSS) was prepared in our laboratory (Gong et al., 2016). All reagents were of analytical grade, and water was doubly distilled.

2.2. Determination of lignin promotion activity on free α-amylase

The promotion activity of lignin on α-amylase was assessed according to a previously reported procedure with minor modifications (Zhang et al., 2013). Reagent-grade α-amylase was used in this experiment. Solutions of α-amylase (1.0 U/ml) and starch (2.5 mg/ml) in 0.02 M pH 6.9 PBS buffer were prepared. A series of lignin suspensions was prepared by suspending the lignin in the same buffer and varying the final concentrations from 1.0 to 5.0 mg/ml. An aliquot of the α-amylase solution (0.75 ml) was mixed with 0.5 ml of the lignin suspension in a 10-ml plastic tube and incubated at 37 °C for 10 min. Then, 0.75 ml of starch solution preheated at 37 °C was added, and the tube was further incubated at 37 °C for 5 min. Once time had elapsed, DNS reagent (1.5 ml) was added and the tube was immediately transferred to a boiling water bath and kept for 5 min to inactivate α-amylase. After cooling to room temperature, the absorbance of the solution (A_{sample}) in the tube was recorded at 540 nm on a Shimadzu UV–visible spectrophotometer (UV, Shimadzu Co., Japan). An enzyme control (A_{enzyme}) was conducted by replacing the lignin suspension with PBS buffer. The lignin control (A_{lignin}) was performed by replacing the α-amylase solution with PBS buffer to remove the effects of lignin on the absorbance of the final solution. The promotion ratios (%) were calculated, using the following formula: $(A_{sample} - A_{lignin} - A_{enzyme})/A_{enzyme} \times 100$.

Furthermore, the promotion ratios of the three reaction models, with different mixing sequences of lignin suspension, enzyme and starch solutions, were evaluated. For the first model (denoted as L + E + S), lignin, enzyme and starch were simultaneously added to the reactor. For the second model (denoted as (L + E) + S), lignin and enzyme were mixed for 10 min, and then, starch was incorporated. For the third model (denoted as (L + S) + E), lignin and starch were mixed for 10 min prior to the addition of enzyme.

2.3. Immobilization of α-amylase with lignin

Commercial crude α-amylase from *Bacillus subtilis* was used in this experiment. To immobilize the enzyme, 1 g of BSS lignin powder was suspended in 200 ml of the supernatant obtained earlier, and the suspension was incubated at 30 °C for 30 min with continuous stirring to allow the adsorption of the enzyme onto the lignin particles (Veesar, Solangi, & Menon, 2015). Afterwards, the mixture was centrifuged (4000 rpm, 15 min) and the solid residues (immobilized α-amylase) were washed in triplicate with 200 ml of PBS buffer (0.01 M pH 6.5). The immobilized enzyme was lyophilized and stored at −18 °C prior to characterization. The protein amount in the resultant supernatant was determined, using Bradford's method, with bovine serum albumin as a reference (Eslamipour & Hejazi, 2015). The amount of α-amylase protein immobilized by the lignin particles was determined, by measuring the decrement of α-amylase in the liquid part of the suspension. To determine its activity, equivalent volumes (1 ml) of immobilized enzyme (5 mg/ml) and soluble starch (10 mg/ml) suspensions in pH 6.5 0.01 M PBS buffer were mixed and incubated at 60 °C for 5 min. One unit of enzyme activity was defined as the amount required to generate 1 μmol of maltose within 1 min. The specific activity was calculated as the amount of maltose (μmol) generated by 1 mg of enzyme protein per min. The immobilization efficiency is reflected in the recovery of the enzyme activity and was expressed as the percentage of activity, which was calculated by the quotient of the amounts of immobilized enzyme by free enzyme multiplied by 100.

2.4. Characterization of immobilized α-amylase

Immobilized α-amylase was characterized through the determination of its optimal catalysis conditions, kinetic parameters, storage stability and operation stability. The optimal temperature and pH conditions for catalysis were determined by comparing the relative activities under varied temperatures (50–85 °C) and pH (5.0–

8.5). The relative activity (%) was the percentage relative to the highest activity obtained for a specific enzyme under different temperature or pH conditions. The kinetic parameters, Michaelis constant (K_m) and maximum velocity (V_{max}) were determined, using a Lineweaver-Burk plot. To determine the storage stability, the effects of pH (3–9), temperature (ambient and 4 °C) and time (60 days) on the residual activity were evaluated. The operation stability against the incubation time (120 min, incubation stability) and the number of re-use cycles (14, re-usability) were assessed in terms of residual activity. The residual activity (%) was expressed relative to the percentage of enzyme activity without treatment. To assess re-usability, immobilized enzyme was collected at the end of each cycle as a solid residue from the working solution via centrifugation. The corresponding resultant supernatant was used to determine the residual enzyme activity of the previous cycle. Prior to the next cycle, the recovered enzyme was washed with pH 6.5, 0.01 M PBS buffer to remove any attached substrate.

2.5. Statistical analysis

All data are expressed as the mean values ± standard deviation of three experimental replicates. Statistical analyses were performed using SPSS v19.0 software (SPSS, Inc., Chicago, IL, USA). Statistically significant differences were determined, using one-way ANOVA, followed by Duncan's multiple-range test with a significance level of 5% ($p < 0.05$).

3. Results and discussion

3.1. Activating α-amylase by BSS lignin

Fig. 1 shows the promotion ratios of BSS lignin to α-amylase across varying concentrations of lignin ranging from 1 to 5 mg/ml. The promotion ratio rapidly increased with increasing lignin concentrations. At a lignin concentration of 5 mg/ml, the promotion ratio reached 220%. Even at a low lignin concentration (1 mg/ml), the promotion ratio reached 31.1%. This confirmed that BSS lignin is a potentially excellent α-amylase activator. Although previous research has implied that the massive hydroxyl groups on the surface of soluble lignin molecules could promote the protonation of glucosides and, in turn, favour the formation of glycosyl-enzyme intermediates (Zhang et al., 2013), research on the mechanisms of insoluble lignin particles is scarce. Interestingly, the present study revealed that the promotion ratio of the enzyme was highly dependent on the addition sequence of the solution or suspension involved (Fig. 1II). There was no significant difference between the promotion ratios of the (L + E) + S (210%) and (L + S) + E (254%) models; however, they were significantly lower than that of the L + S + E model (335.8%) at a lignin concentration of 5 mg/ml. However, due to a lack of solid evidence, we assumed that the small size ($D_{0.5}$ = 110.41 μm) and large specific surface area (2.085 m^2/g) of fine BSS lignin particles with coarse surfaces significantly contributed to this difference (Supplementary material Fig. S1). More specifically, in the L + S + E model, the simultaneous adsorption of the enzyme and starch molecules in the surface pits (average volume 0.00002 cm^3/g and average diameter 3.588 nm) of lignin particles substantially enhanced their contact and, in turn, promoted the reaction (Singh, Rezac, & Pfromm, 2009). However, in the (L + E) + S and (L + S) + E models, the surface of BSS lignin was occupied by enzyme or starch molecules first and there was no space available for the late-comers. In this context, the surface pits of lignin particles may provide a better environment for α-amylase to react with starch molecules than that of bulk solution. The subsequent enzyme immobilization results revealed that BSS lignin had a strong adsorption capacity for α-amylase protein.

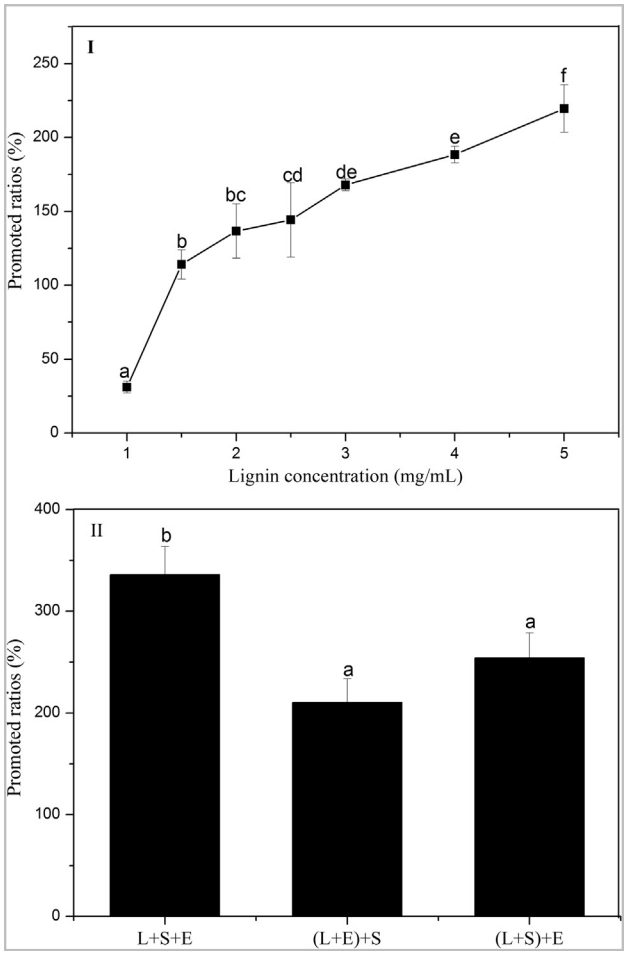


Fig. 1. The dependence of the promoted ratios of α-amylase on the concentration of BSS lignin (I) and working modes (II). L + E + S: Lignin, enzyme and starch were simultaneously added to the reactor; (L + E) + S: Lignin and enzyme were first mixed for 10 min and then starch was incorporated; (L + S) + E: Lignin and starch were first mixed for 10 min prior to enzyme addition. Data bearing different lowercase letters are significantly different ($p < 0.05$).

3.2. Immobilizing α-amylase onto BSS lignin

In the present study, α-amylase was immobilized onto BSS lignin, using an adsorption approach. The immobilization efficiency, in terms of specific activity, amount of protein loaded and activity recovery, was investigated over the incubation time (10–60 min) (Fig. 2I) and across varying concentrations of α-amylase (2–7 mg/ml) (Fig. 2II). An effect of the incubation time was observed with an enzyme concentration of 5 mg/ml. The results showed that the adsorption of enzyme protein onto BSS lignin occurred during the early stages of incubation and reached equilibrium at 20 min. Similar trends were observed for the specific activity and activity recovery. Thus, the maximum values of the specific activity (92.4 U/mg), amount of protein loaded (19.0 mg/g) and activity recovery (111%) were obtained at an incubation time of 20 min. Fig. 2II shows that both the amount of protein loaded and the specific activity increased with increasing enzyme concentrations from 2 to 5 mg/ml; however, they remained constant with further increases (up to 7 mg/ml). In contrast, the activity recovery initially remained stable across enzyme concentrations in the 2–5 mg/ml range and then decreased dramatically with further increases. This

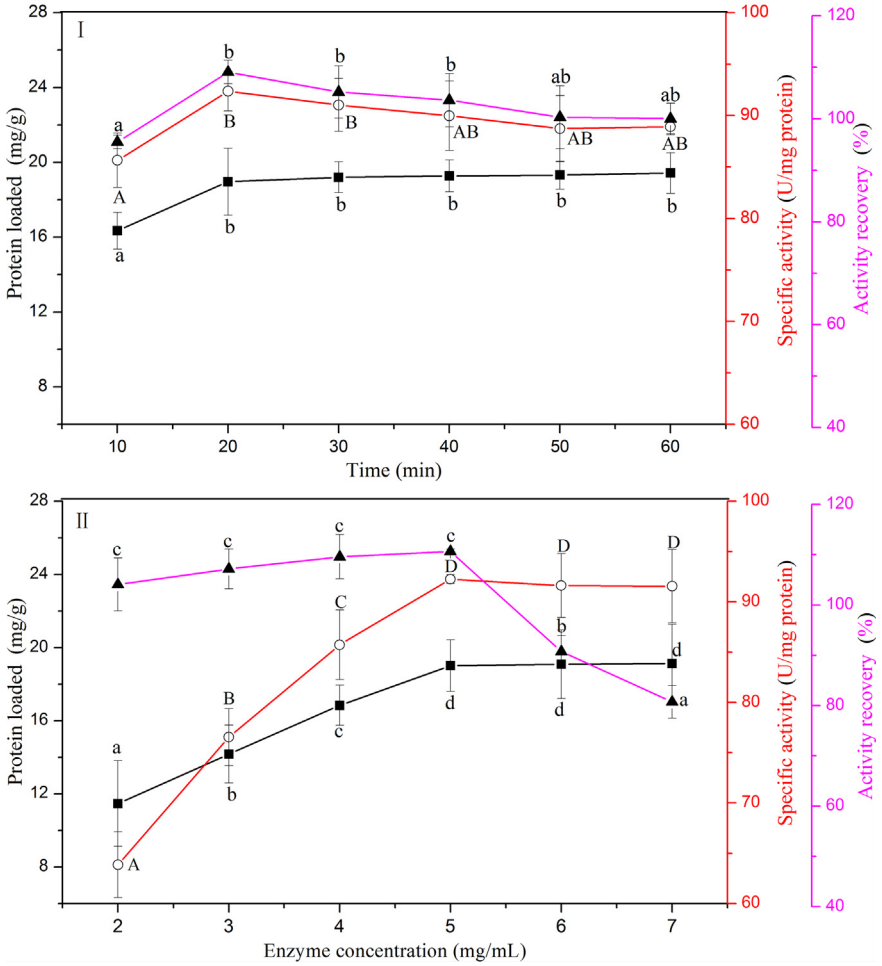


Fig. 2. The dependence of specific activity (○), protein load (■) and activity recovery (▲) on the incubation time (I) and enzyme concentration (II) of immobilization. Data bearing different lowercase or capital letters in the same curve are significantly different ($p < 0.05$).

indicated that all of the available adsorption sites on the surface of lignin were fully occupied by the enzyme protein when the amount of protein loaded reached 19.0 mg/g. Further increases in enzyme supply inevitably brought about a decrease in enzyme recovery. It is worth noting that, surprisingly, in some instances the activity recovery in this experiment surpassed 100%. This was a rather rare exception encountered during enzyme immobilization. The enzyme activation effects of lignin support observed above should be the initiator of this exception. On the other hand, the more common α-amylase supports, such as cellulose resin and glass beads, did not present any activating effects (Tümtürk et al., 2000; Singh & Kayastha, 2014).

3.3. Characterizing α-amylase immobilized onto BSS lignin

3.3.1. Optimal pH and temperature for catalysis

The relative activities of immobilized and free α-amylases with varying pH values and temperatures during catalysis are illustrated in Fig. 3. Either against temperature (Fig. 3I) or pH (Fig. 3II), the enzyme activity plots of immobilized and free amylases were rather superimposed. Hence, no influence of the immobilization on the optimal working temperature or pH could be highlighted. In other words, the immobilized and free α-amylases shared an optimal temperature range of 60–65 °C and pH point of 6.5. Previous results demonstrated that α-amylase immobilized onto DEAE cellulose possessed the same optimal catalysis parameters for temperature and pH as its free enzyme counterpart (Kikani, Pandey, & Singh, 2013).

3.3.2. Kinetic parameters

The Lineweaver-Burk plots corresponding to the free and immobilized enzymes were generated at a pH of 6.5 and 60 °C. Their derivative K_m and V_{max} values were also calculated and are presented in a separate table in Fig. 4, showing that the K_m value of immobilized enzyme (21.0 mg/ml) was less than half that of the free enzyme (48.2 mg/ml). This indicated that the immobilization greatly enhanced the enzyme-substrate affinity. A previous study found that the non-covalent interactions involved in immobilization between lignin and enzyme may induce a slightly positive distortion in the enzyme structure (Singh & Kayastha, 2014). These interactions may inhibit the occurrence of massive changes in enzyme conformation which in turn cause inactivation of the enzyme (Shukla & Singh, 2016). In the present study, the activating effect of the lignin support on α-amylase activity may be another contributing factor to the decrease in K_m induced by the immobilization (Hayashi, Talukder, Takeyama, Wu, Kawanishi, & Shimizu, 2003) However, immobilized α-amylase onto BSS lignin (250 U/mg) had a significantly lower V_{max} value than its free counterpart (333 U/mg). The decrease in enzyme V_{max} induced by immobilization was initiated by the effects of steric hindrance imposed by the support, which inhibited the diffusion of the substrate into the enzyme active site and contact between them

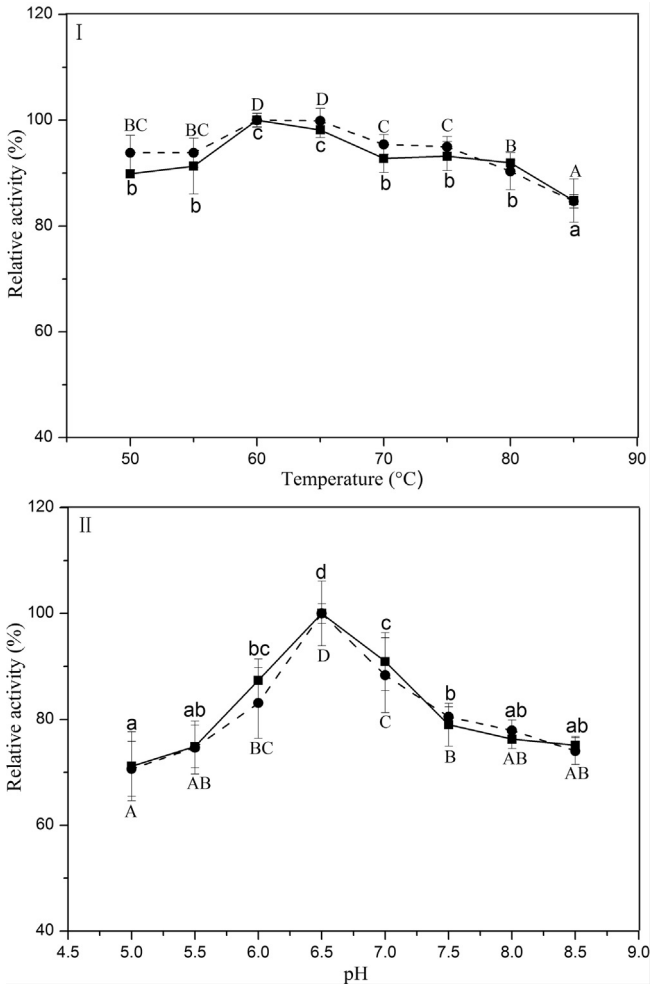
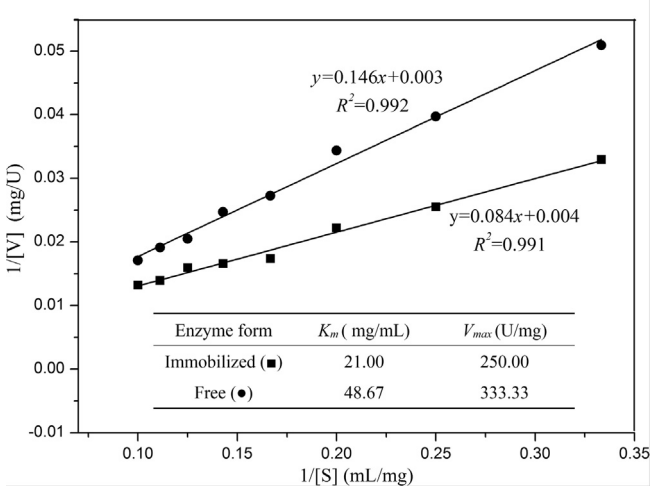


Fig. 3. Effects of temperature (I) and pH (II) on the activities of free (●) and immobilized (■) enzymes. Data bearing different lowercase or capital letters in the same curve are significantly different ($p < 0.05$).



Enzyme form	K_m (mg/mL)	V_{max} (U/mg)
Immobilized (■)	21.00	250.00
Free (●)	48.67	333.33

Fig. 4. Lineweaver-Burke double reciprocal plots of free (●) and immobilized (■) enzymes and their kinetic parameters.

(Eslamipour & Hejazi, 2016). Furthermore, as mentioned above, the non-covalent interactions between lignin and immobilized enzyme favoured the stability of the enzyme but decreased its flexibility, which is essential for substrate binding (Singh & Kayastha, 2014). The fact that the immobilization decreased the V_{max} of the enzyme has been widely observed in various studies of enzyme immobilization (Singh & Kayastha, 2014; Eslamipour & Hejazi, 2016; Cao, Huang, Li, Xu, Wu, Li, & Zong, 2016). In this context, immobilization onto BSS lignin is a double-edged sword for the catalytic efficiency of α-amylase, e.g., there was a positive effect on K_m but an adverse effect on V_{max}. However, immobilization onto BSS lignin favoured the catalytic efficiency of α-amylase in terms of V_{max}/K_m (Cao et al., 2016); the V_{max}/K_m ratio of immobilized enzyme (11.9) was much higher than that of the free (6.85).

3.3.3. Storage stability

It is widely known that enzyme solutions are easily broken down by some microorganisms during storage, especially at ambient temperatures and neutral pH. For this reason it is dangerous to store the immobilized enzyme described in the current study at its optimal pH (i.e., 6.5). In principle, the decay of the enzyme solution could be effectively inhibited by lowering its pH. To determine if this is possible with the immobilized enzyme in this study, its residual activity was assessed by varying the pH of the storage buffer from 3 to 9. Unfortunately, both free and immobilized enzymes were badly damaged when the pH of storage buffer fell below 5 (Fig. 5I), which denied our attempt to store the immobilized enzyme at a decreased pH.

In addition, enzymes are not stable in solution and their activity slowly declines during storage (Oktay, Demir, & Kayama-Apohan, 2015). The extent of instability was highly dependent on the storage temperature, but the dependency profile varied across enzymes. Common sense suggests that storing enzymes at lower temperatures would extend the shelf life of enzymes by preserving the enzymatic activity; however, this is not always the case. For example, α-amylase immobilized onto silica particles exhibited comparable stability when stored at 4 °C and ambient temperature (Lim, Macdonald, & Hill, 2003), while α-amylase immobilized onto gold nanorods stored at room temperature decayed at a higher rate than when stored at 4 °C (Homaei & Saberi, 2015). Therefore, selection of the optimal temperature is of much more importance for storing enzymes in a safe and economic way. In the current study, the stability of the immobilized enzyme stored at room temperature and 4 °C in PBS buffer (pH 6.5, 0.01 M) was assessed up to 60 days.

The results revealed that the activity of both the free and immobilized enzyme decreased with longer storage durations (Fig. 5II, III) when stored at either room temperature or 4 °C. In addition, the inactivation rate of the immobilized enzyme was lower than that of the free enzyme when both were stored at either room temperature or 4 °C for 60 days. Irrespective of its form, enzymes stored at 4 °C are more stable than those stored at room temperature. When stored at room temperature, significant differences in stability were occasionally observed between the immobilized and free enzyme during the first 20 days of storage. As the storage time progressed, the differences became larger and increasingly more significant. By the end of the storage period, the free enzyme was completely inactive, while the immobilized enzyme retained forty percent of its original activity. When stored at 4 °C significant differences in activity between the immobilized and free enzyme emerged on the tenth day and continued until the end of storage. On the 60th day of storage, the residual activities of the free and immobilized enzymes were 30.3% and 72.6%, respectively. The immobilization of α-amylase onto BSS lignin significantly improved the stability of the enzyme during storage and storage at 4 °C yielded better results than that at ambient temperatures. The stabilization of α-amylase through immobilization using other supports, such as DEAE-cellulose resin (Singh & Kayastha, 2015), magnetic nanoparticles (Eslamipour & Hejazi, 2016) and ZnO

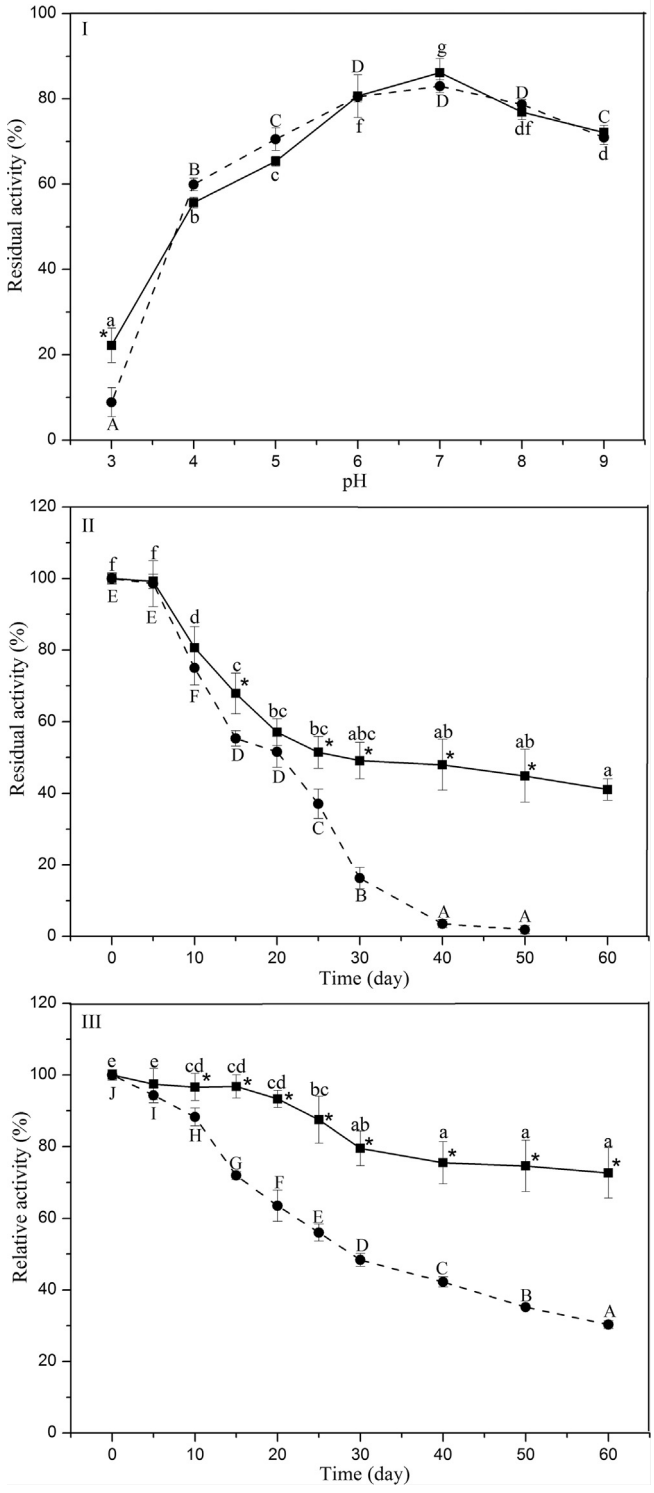


Fig. 5. The stabilities of free (●) and immobilized (■) enzymes against storage pH (I) and duration at room temperature (II) and 4 °C (III). Data bearing different lowercase or capital letters in the same curve are significantly different ($p < 0.05$). Data bearing asterisk (*) in the curve of immobilized enzyme are significantly different from the values corresponding to free enzyme at the same pH or time.

nanoparticles (Antony, Balachandran, & Mohanan, 2016) has also been recently demonstrated. In addition to the impact of denaturation via conformational changes on the destabilization of enzymes during storage, contaminating microbes may also have an effect, in particular, when storing at room temperature for long periods of time. In the present study, regardless of the storage temperature, the suspensions of immobilized enzyme remained clear throughout the entire storage period, while free enzyme suspensions became turbid during the latter storage period. These changes in clarity were due to the presence of microbes in the suspension. After incubation at 25 °C for 10 days, plate count revealed that the bacterial development in immobilized enzyme suspensions ($(3.10 \pm 0.23) \times 10^3$ cfu/ml) was significantly reduced in contrast to that in free enzyme suspensions ($(9.80 \pm 0.94) \times 10^5$ cfu/ml) (Supplementary material Fig. S2). This suggested that BSS lignin possibly has antimicrobial activity, as have lignins from other sources (Medina et al., 2016; Dong, Dong, Lu, Turley, Jin, &Wu, 2011). Medina et al. (2016) reported that the permeability of the cell wall to gram-negative bacteria and the resistance to β-lactamase convert lignin into a potential antibiotic.

3.3.4. Operation stability

For the practical application of immobilized enzymes, the stability of the suspension during incubation and the re-usability of the enzyme are of great importance when considering matters of convenience and cost. Unexpectedly, for incubation times exceeding 60 min, the immobilized enzyme was less stable compared to

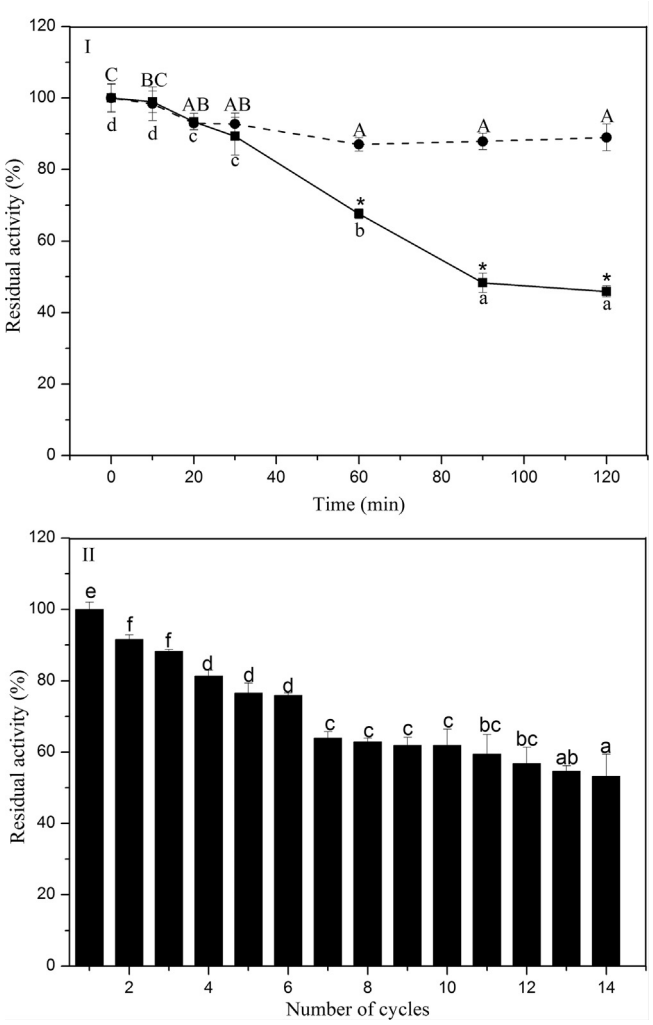


Fig. 6. Incubation stabilities (I) of free (●) and immobilized (■) enzymes and re-usability (II) of immobilized enzyme. Data bearing different lowercase or capital letters in the same curve are significantly different ($p < 0.05$). Data bearing asterisk (*) in the curve of immobilized enzyme are significantly different from the values corresponding to free enzyme at the same time.

the free enzyme (Fig. 6I). This implied that the contact between α-amylase and BSS lignin without substrate at an elevated temperature (60 °C) for a long time (>60 min) may have allowed excessive hydrogen bonding and hydrophobic interactions between the protein chain and the phenolic structure of lignin, which generates non-productive enzyme sites and hence damages the immobilized enzyme (Ximenes, Kim, Mosier, Dien, & Ladisch, 2010). Fortunately, when incubation did not exceed 30 min, the residual activity of both the free and immobilized enzymes approached 90% and there were no significant differences between them. Based on these findings, usage of the immobilized enzyme could be facilitated by preparing the suspension in advance, incubating at 60 °C and restricting the incubation time to 30 min.

The greatest advantage of immobilized enzymes over their free counterparts lies in their re-usability, which makes the enzyme-assisted process more economical (Gashtasbi et al., 2014). However, many studies have demonstrated, without exception, that the activity of immobilized enzyme decreases over a number of re-use cycles; however, the extent of these decreases varies (Gashtasbi et al., 2014; Torabizadeh et al., 2014; Kikani et al., 2013). Fig. 6II clearly illustrates that the residual activity of the immobilized enzyme in the present study gradually decreased as the number of re-use cycles increased (up to a maximum of fourteen). In detail, after ten re-use cycles, the residual activity fell to 62%, and after fourteen cycles, the residual activity fell to 53%. This result is comparable to those obtained using α-amylase immobilized on calix[4]arene, which yielded residual activities of 62% and 53% with ten and fifteen re-use cycles, respectively (Veesar et al., 2015). The decrease in residual activity of immobilized enzymes can be attributed to the denaturation and desorption of the enzyme protein from the support (Kikani et al., 2013; Singh & Kayastha, 2014).

4. Conclusion

As a valuable but under-exploited natural resource, lignin, which is only inferior to cellulose, is one of the most abundant naturally existing polymers in the world. This study has confirmed BSS lignin activation of α-amylase and is the first to examine its feasibility as an immobilizing support for this enzyme. The activating effects of BSS lignin can be partially ascribed to its coarse surface, in which numerous pits provide an optimal location for stable contact between the starch and enzyme. Through adsorption, α-amylase from *Bacillus subtilis* can be efficiently immobilized on BSS lignin particles with a maximum protein load of 19.0 mg/g, which was achieved by running the immobilizing process for 20 min with a free enzyme concentration of 5 mg/ml. Under these conditions, a maximum recovery of activity was also obtained (111%). In contrast with its free counterpart, the immobilized enzyme was characterized by improved overall catalytic efficiency and storage stability under comparably optimal temperature and pH conditions. In addition, the immobilized α-amylase lignin also presented reasonably good re-usability. However, as a support to α-amylase, BSS lignin is not without some limitations. The most prominent among them lies in its inferior incubation stability at 60 °C when the incubation time exceeds 30 min. In conclusion, this study has revealed a naturally derived novel support for enzyme immobilization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.02.017.

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