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Immobilization of α-amylase from *Exiguobacterium* sp. DAU5 on Chitosan and Chitosan-carbon Bead: Its Properties

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Abstract Glutaraldehyde was used as a cross-linking agent for immobilization of purified α-amylase from *Exiguobacterium* sp. DAU5. Befitting concentration of glutaradehyde and cross-linking time is the key to preparation of cross-linking chitosan beads. Based on optimized immobilization condition for α-amylase, an overall yield of 56% with specific activity of 2,240 U/g on chitosan beads and 58% with specific activity of 2,320 U/g on chitosan-carbon beads was obtained. The optimal temperature and pH of each immobilized enzyme activity were 50°C and 50 mM glycine-NaOH buffer pH 8.5, respectively. Those retained more than 75 and 90% of its maximal enzyme activity at pH 7.0-9.5 and after incubation at 50°C for 1 h, respectively. In addition, the immobilization product showed higher organic-solvent tolerance than free enzymes. The mode of hydrolyzing soluble starch revealed that the α-amylase possessed high hydrolyzing activity. These results indicate that chitosan is good support and has broad application prospects of enzyme immobilization.

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Keywords α-amylase · chitosan bead · chitosan-carbon bead

Introduction

Starch is one of the most abundant polymers in nature, while α-amylase can decompose starch into glucose units by hydrolyzing on α-1,4-glycosidic bonds. α-Amylases (EC 3.2.1.1) are widely found in animals, plants, and microorganisms (Vihinen and Mantsala, 1989), which are classified into the glycosyl hydrolases families 13 and 57 (Henrissat, 1991; Henrissat and Bairoch, 1996; Davies et al., 2005; Stam et al., 2006). The 3-dimensional structure of α-amylase, which is consisted of an eight-stranded α/β barrel and contained the highly conserved regions: the catalytic center, the substrate- and calcium biding domains, is extremely preserved in different living-organisms (Svensson, 1994; Machius et al., 1995; Janecek, 1997; Strobl et al., 1998; MacGregor et al., 2001; Pujadas and Palau, 2001; Stam et al., 2006). Because the outcome of the α-amylase's spacious distribution in the overall kingdom, these display the high degree of sequence variability analyzed by phylogenetic relationships (MacGregor et al., 2001; Stam et al., 2006).

α-Amylases can be used in a number of industrial processes, such as starch processing, brewing, biofuel, textile, baking, and detergent making (Pandey et al., 2000; Gupta et al., 2003: Kandra, 2003). α-Amylases, as an important glycoside hydrolase, represent the largest groups of industrial enzymes and constitute approximately 25-33% of the world market for the industrial enzymes at present (Saxena et al., 2007). However, many of the commercially available enzymes do not withstand harsh conditions during the industrial processes. Thus discovery and development of new amylases suitable for industrial demands are practically significant. Immobilized enzymes could provide a number of advantages as compared to the free enzyme, such as increasing the pH and temperature ranges of enzyme activity and stability. Thus, immobilized enzymes have been widely used in many industry areas, which are food

production, pharmaceuticals, and other biologically important fine products (Dodia et al., 2008).

In the past few years, many studies have discussed immobilization of various enzymes by different supports and methods, such as immobilization of acid protease on activated carbon (Ganesh Kumar et al., 2010), and immobilization of β-xylosidase on chitosan and gelatin, etc (Smaali et al., 2009). Chitosan, which is partly acetylated or non-acetylated forms of chitin, is presented in exoskeletons of the mycelial, fungi, insects, and crustaceans (Pelletier and Sygusch, 1990). Chitosan serves as a non-toxic and harmless immobilization support of biological macromolecules, it has lots of advantages for the industrial application, e.g. a cheap price, a high porousness, an outstanding hydrophilicity, and low sterichindrance to enzyme (Chang et al., 2008). The hydroxyl (-OH) and amino (-NH_2) groups of chitosan could be linked and cross-linked enzymes using glutaraldehyde to prevent liquefaction in acidic conditions (pH <2) (Srere and Uyeda, 1976). The amino groups could be personally reacted with glutaraldehyde to make aldehyde groups and the hydroxyl groups could be activated by using epoxy reactants, followed by oxidation to make reactive aldehyde glyoxal groups (Adriano et al., 2008).

Previously, we reported properties of an organic-solvent tolerant α-amylase from *Exiguobacterium* sp. DAU5 (Chang et al., 2013). Subsequentially in this study, we reported immobilization of α-amylase from *Exiguobacterium* sp. DAU5 on chitosan and chitosan-carbon beads for improving enzymatic properties. To improve the cost-effectiveness practicability of α-amylase in many biotechnological processes. The immobilized α-amylase from *Exiguobacterium* sp. DAU5 showed higher pH, thermal stabilities, and organic-solvent-tolerance than free enzymes, and also showed high activity toward soluble starch.

Materials and Methods

Materials. Chitosan, activated carbon, and soluble starch were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Glutaraldehyde, 25% solution was purchased from Junsei Chemical Co. (Tokyo, Japan). All other reagents were of the highest grade available.

Preparation of cross-linking chitosan beads. 2.5% (w/v) chitosan solution was prepared by dissolving 0.25 g of chitosan powder in 10 mL of 2% (v/v) acetic acid solution and stirred for 2 h. The viscous solution was centrifuged for 15 min at 6,000 rpm to remove air bubbles, and then sprayed drop-wise through a syringe, at a constant rate, into 7.5% NaOH and 95% ethanol in a volume ratio of 4:1 to solidified bead form. The beads were placed in the NaOH-ethanol solution at 4°C overnight. The prepared beads were washed with deionized water until the chitosan beads were neutral. The chitosan-carbon beads were also prepared in the similar manners, added the activated carbon/ chitosan mixture (1% [w/v] activated carbon and 2.5% [w/v] chitosan solution).

The optimal cross-linking condition for preparation of chitosan beads and chitosan-carbon beads was determined by various glutaraldehyde concentrations and cross-linking times. Each bead (1 g) with 30 mL of 50 mM sodium phosphate buffer (pH 6.5) was placed with various conditions in each 250 mL flask. Each solution was shaken at 180 rpm and 37°C and then washed thoroughly by 50 mM sodium phosphate buffer (pH 6.5).

Enzyme immobilization. The optimal pH and time for immobilization were determined with various buffers and times. The cross-linked chitosan beads or chitosan-carbon beads (each 0.2 g) were incubated with purified enzyme (800 U) in a shaker at 150 rpm and 20°C for immobilization. The efficiency of immobilization was evaluated in terms of activity yield and specific activity as follows:

$$\text{Immobilization yield (\%)} = A_{imm}/A_{tot} \times 100$$
$$\text{Specific activity (U/g-bead)} = A_{imm}/W_{bead},$$

where A_{imm} is the number of units detected in the after immobilization and washing; A_{tot} is the total number of units added to the support during the immobilization reaction; W_{bead} is the weight of chitosan-carbon beads for immobilization reaction.

Enzyme assay. The assessment of α-amylase activity by measuring the amount of reducing sugar contents, determination of reducing sugar concentration using 3,5-dinitrosalicylic acid (DNS) method, as described by Bernfeld (1951). The activity was quantitatively determined by estimating reducing groups released from soluble starch. Reaction mixtures (300 μL) consisted of 10 μL purified α-amylase and 290 μL 50 mM glycine-NaOH buffer (pH 8.5) containing 1% soluble starch and incubated the mixtures at 40°C for 10 min. 700 μL DNS was added to stop the reaction, and transferred to boiling water bath for 10 min. After cooling, the absorbance of mixture was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugars from soluble starch per min under the assay conditions. Maltose was used as a standard. The protein concentration was determined by bovine serum albumin as a standard, the method described by Lowry et al. (1951).

Activities of the immobilized α-amylase were similarly determined by the above condition. Reaction mixture containing 300 μL of 1% soluble starch in 50 mM glycine-NaOH buffer (pH 8.5), and a given amount of immobilized enzyme was incubated at 50°C for 5 min, 700 μL of DNS was added to stop the reaction, and then transferred the reaction mixture to boiling water bath for 10 min. After cooling, the absorbance of the mixture was measured at 540 nm.

Effect of pH and temperatures on enzyme activity. To determine the effect of various pH, 50 mM sodium phosphate (pH 6.5–8.0), and 50 mM glycine-NaOH (pH 8.5–11.0) were used. The pH stability on activity of immobilized α-amylases was tested by incubating the enzyme at different pH (6.5–10.0) for 2 h on ice. The remaining activity was assaying under the standard assay conditions.

The effect of temperature on activity of α-amylases was determined at various temperatures in the range of 25–60°C. Thermostability was tested by incubating immobilized enzyme at

different temperatures (25 and 60°C) for 1 h, and then added substrate, reaction at different temperatures for 10 min. The remaining activity was measured under the standard assay conditions.

Effects of chemical reagents on enzyme activity. The effect of organic solvents on the free enzyme activity was determined by reaction mixture containing 40% (v/v) of dimethylsulfoxide (DMSO), dimethylformamide (DMF), methanol, acetone, acetonitrile, 1-butanol, chloroform, DMF, DMSO, and ethanol. Residual activity was measured under the standard condition. Controls for each set were also carried out simultaneously.

The effect of organic solvents on the immobilized enzyme determined by reaction mixture containing 40% (v/v) various organic-solvent. Residual activity was measured under the standard condition.

Study on hydrolysis products of soluble starch. Immobilized α-amylases were used for the hydrolysis of soluble starch, and the hydrolysis products were analyzed by thin-layer chromatography (TLC). Reaction mixtures (100 μL) consisted of 50 mM glycine-NaOH buffer (pH 8.5) containing 1% soluble starch and immobilized α-amylases, reaction was carried out at 50°C. Within 6 h, different reaction time period of hydrolysis products were detected by TLC plate. 2 μL reaction mixture was loaded onto silica gel 60 plate (Marck), every reaction mixture was loaded onto each plate lane, and the chromatographed with 1-butanol-acetic acid-water (6:3:2, v/v/v) the products were detected by staining the plate with aniline-diphenylamine reagent (4 mL of aniline, 4 g of diphenylamine, 200 mL of acetone, and 30 mL of 85% phosphoric acid) and baked it at 180°C for 5 min. 1 μg of various maltooligosaccharides was loaded as standard.

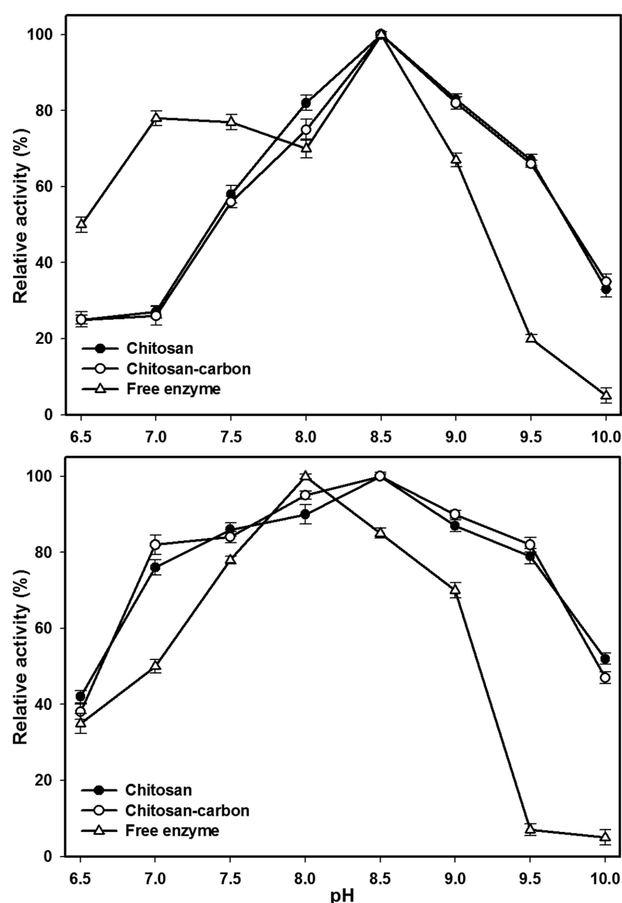

Fig. 1 Optimal pH (A) and pH stability (B) on the free and immobilized enzyme. The experiments were performed in triplicates. Free enzyme, (Chang et al., 2013); chitosan beads, this study; chitosan-carbon beads, this study. The each immobilized enzyme activity, which was the highest enzyme activity level at the above standard enzyme assay condition, was set as 100% (0.861 mmol maltotriose/ mg immobilized bead/min).

Results

Optimization of α-amylase immobilization on chitosan and chitosan-carbon beads. The effect of glutaraldehyde concentration during enzyme immobilization is shown in Supplementary Fig. 1A. Different glutaraldehyde concentrations (0.5–3%) were used in the optimization of cross-linking conditions. Maximal enzyme activities were observed when concentrations of 1.5 and 1% glutaraldehyde were used in the cross-linking of chitosan and chitosan-carbon beads, respectively, and subsequent enzyme activity assays were thus carried out with α-amylase immobilized with 1.5% glutaraldehyde for chitosan beads and 1% glutaraldehyde for chitosan-carbon beads. Supplementary Fig. 1B, which shows the effects of cross-linking times on immobilized enzyme activity, shows that maximal α-amylase activity was achieved with a cross-linking time of 1 h for both chitosan and chitosan-carbon beads. Chitosan beads were thus subsequently prepared for immobilization by 1 h of cross-linking with 1.5% glutaraldehyde and chitosan-carbon beads with 1% glutaraldehyde. After the beads were washed exhaustively with dH_2O. Two kinds of beads were shaking at 150 rpm, with purified α-amylase suspended in buffers of various pH (5.0–7.0) (Supplementary Fig. 2A), and it was found that the chitosan-immobilized and chitosan-carbon-immobilized enzymes exhibited maximal activities when immobilized at pH 9.0 and pH 8.5, respectively. The effect of immobilization time on enzyme activity is shown in Supplementary Fig. 2B, in which enzyme activity can clearly be seen to increase with increasing immobilization times of up to 12 h, after which no further increase in activity is observed. Based on these findings, subsequent immobilizations of α-amylases were carried out for at least 12 h. Using on the optimal pH and time required for immobilization described above, the enzyme activities and immobilization yields of immobilized α-amylase were determined for different amounts of purified enzyme immobilized on 0.2 g of each bead type. The activity of α-amylase immobilized on chitosan (Supplementary Fig. 3A) and chitosan-carbon (Supplementary Fig. 3B) beads was found to increase with increasing enzyme amount. Based on the immobilization yields and the enzyme activities measured, 800 U α-amylase and 0.2 g chitosan or chitosan-carbon beads were used for subsequent immobilizations. This combination result in immobilization yields and specific activities

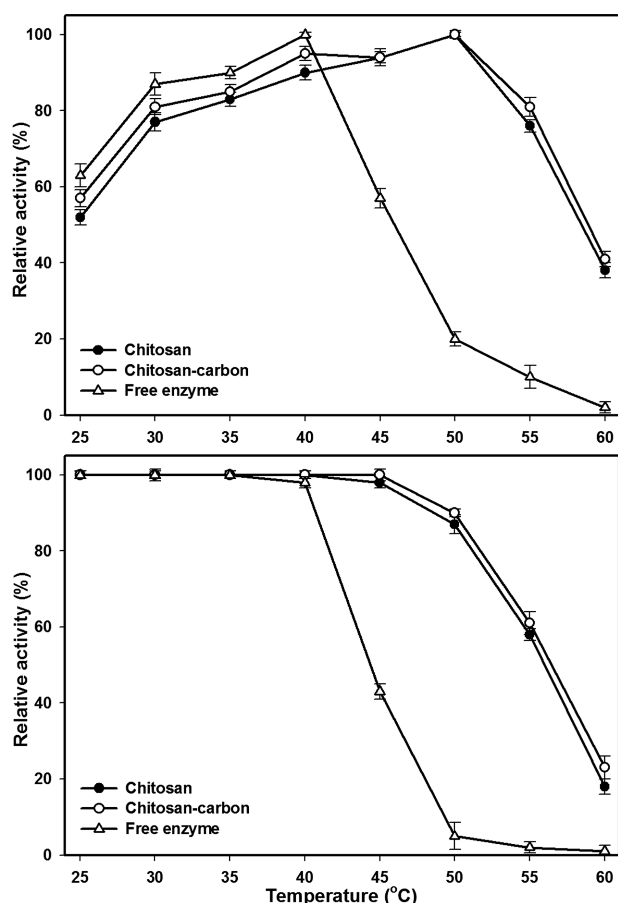

Fig. 2 Optimal temperature (A) and temperature stability (B) on the free and immobilized α-amylases. The experiments were performed in triplicates. Free enzyme, (Chang et al., 2013); chitosan beads, this study; chitosan-carbon beads, this study. The each immobilized enzyme activity, which was the highest enzyme activity level at the above standard enzyme assay condition, was set as 100% (0.861 mmol maltotriose/ mg immobilized bead/min).

of 56% and 2,240 U/g, respectively, for chitosan beads (Supplementary Fig. 3A) and 58% and 2,320 U/g, respectively, for chitosan-carbon beads (Supplementary Fig. 3B).

The effect of pH and temperature on the free and immobilized α-amylase. The optimal pH and pH stability of free and immobilized α-amylases were assessed. The free and immobilized α-amylases exhibited the highest enzyme activity at pH 8.5 (Fig. 1A). Compared with the free enzyme, immobilized α-amylases were more stable over a broad pH range (7.0–10.0), retaining ≥75% and ~50% of their maximum activities at pH 7.0–9.5 and pH 10.0, respectively (Fig. 1B). The optimal temperature of the free and immobilized α-amylases was assessed under standard assay conditions at 25–60°C. The optimal temperature of free α-amylase exhibited at 40°C, while the enzymes immobilized on chitosan and chitosan-carbon beads exhibited maximal activities at 50°C. The free α-amylase activity dramatically decreased at temperature ≥45°C, while the immobilized α-amylases retained more than 75% of their maximal activities ≤55°C (Fig. 2A). The

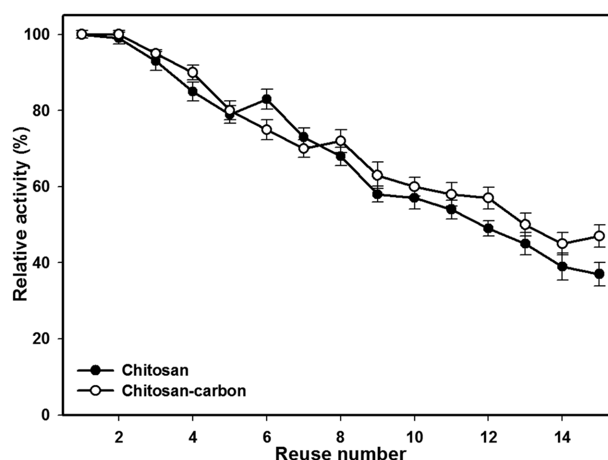

Fig. 3 Reuse effect on the enzyme activity of immobilized α-amylases. The experiments were performed in triplicates. The each immobilized enzyme activity at beginning of the experiments was set as 100% (0.861 mmol maltotriose/ mg immobilized bead/min).

temperature stability of free and immobilized α-amylase was determined by pre-incubation at various temperatures for 1 h (Fig. 2B). The immobilized enzymes were found to retain ≥90% those activity at 25–50°C and ≥55% those activity at 55°C, while the free enzymes exhibited <7% those activity at 50°C.

The effect of reuse number on the immobilized α-amylase activity. In an assessment of the reusability of immobilized α-amylases, the residual enzyme activity levels were similar in the chitosan- and chitosan-carbon-immobilized α-amylase. After enzyme activity assays, the beads were separated from the unbound reaction components by centrifugation, and were subsequently washed with 50 mM glycine-NaOH buffer (optimal pH) before the next reaction. The residual activity of the immobilized α-amylases after reuse is shown in Fig. 3. Immobilized α-amylases were found to have a higher capacity for reusability than the free enzyme (data not shown), the chitosan- and chitosan-carbon-immobilized enzyme were found to retain ≥70% of their activity after eight uses.

The effect of organic solvents on the free and immobilized α-amylase. The effects of various organic solvents (DMSO, DMF, methanol, acetonitrile, ethanol, acetone, 1-butanol, and chloroform) on α-amylase activity are shown in Table 1. Amylase activity in the absence of organic solvents (control) was considered 100% activity. Chloroform, DMSO, DMF, ethanol, and methanol were found to inhibit amylase activity by at least 50%; while acetone, acetonitrile, and 1-butanol were found to have weaker inhibitory effect, inhibiting amylase activity by ≤30%. Under the experimental conditions used in the assessment of organic solvent effects on amylase activity, the immobilized enzyme activities on chitosan and chitosan-carbon beads were compared to the enzyme activity of the free enzyme. The immobilized enzymes exhibited higher activities than the free enzyme in the presence of acetone. The α-amylase immobilized to chitosan and chitosan-carbon beads exhibited 110% and 115% relative activities, respectively.

Table 1 Effects of organic solvents on the activities of free and immobilized α-amylases

Reagent	Relative activity (%)		
	Free enzyme	Chitosan Bead	Chitosan-Carbon Bead
Control	100	100	100
Acetone	83±1.7	111±2.1	117±2.7
Acetonitrile	77±2.2	90±2.7	86±1.9
n-Butanol	80±1.5	83±1.2	88±2.1
Chloroform	16±2.3	36±1.9	35±2.0
DMF	32±2.0	42±1.6	45±2.3
DMSO	43±1.1	55±1.6	53±1.3
Ethanol	59±1.5	71±1.9	68±2.3
Methanol	65±1.5	69±2.0	67±1.3

Reaction mixtures contained 40% (v/v) organic solvent, and residual enzyme activity was measured under standard assay conditions.

Fig. 4 TLC of the hydrolysis products from soluble starch by immobilized α-amylase. The reaction was carried out at 50°C. Maltooligosaccharides from glucose (G_1) to maltoheptaose (G_7) were used as standard markers (M).

The products of starch hydrolysis by α-amylase. The products of the hydrolysis of soluble starch substrate by immobilized α-amylases, were detected by TLC (Fig. 4). After 5 min of hydrolysis, the first product to appear was maltotriose, after which maltose and maltopentaose appeared as the reaction continued. After allowing hydrolysis to proceed for 3 h, the final products detected were various maltooligosaccharides, of which maltotriose and maltopentaose were detected at the highest levels, and glucose at the lowest level.

Discussion

We previously characterized an organic-solvent-tolerant α-amylase from *Exiguobacterium* DAU5 (Chang et al., 2013). The α-amylase gene consists of 1,545 bp encoding 514 amino acids of 57 kDa belonging to the glycosyl hydrolase family 13. To assess the properties of immobilized α-amylase, the enzyme was immobilized on chitosan and chitosan-carbon beads in this study.

The cross-linking of chitosan beads using low concentrations of glutaraldehyde results in poor mechanical strength; however, high concentrations of glutaraldehyde may denature enzymes. The appropriate concentration of glutaraldehyde used in cross-linking is thus keys as it will determine the loading capacity of the support beads as well as the immobilized enzyme activity. The highest enzyme activity levels were observed following 1 h of cross-linking with 1.5 and 1% glutaraldehyde for chitosan and chitosan-carbon beads, respectively. Different α-amylase enzymes have different isoelectric points, and thus the net negative charge of the enzyme depends on the pH. Buffers of different pH were thus assessed for their effects on α-amylases immobilization, and it was found that maximal enzyme activity is achieved when purified α-amylase is cross-linked with chitosan beads, 50 mM glycine-NaOH buffer (pH 9.0) and chitosan-carbon beads, 50 mM glycine-NaOH buffer (pH 8.5) for 12 h, respectively.

An assessment of the pH effect on enzyme activity revealed that compared to the free enzyme, immobilized α-amylase has a broader pH range: immobilized α-amylases retained more than 55% of their original activities at pH 7.5–9.5. The pH stability of immobilized α-amylases was also broader than the free enzyme: ≥75% and ~50% of the maximum activity was retained at pH 7.0–9.5 and pH 10.0, respectively. α-Amylase immobilized on calix[4]arene derivative from *Saccharomyces cerevisiae* showed the maximum activity of the immobilized enzyme was shifted 0.5 pH unit toward the basic region. The maximum activity of the free enzyme was at pH 7.0, but the immobilized enzyme was at pH 7.5 (Vessar et al., 2015). Amylase immobilized on an arabic gum-chitosan bead from *Hylocereus polyrhizus* showed the optimum pH of immobilized enzyme was shifted up. The optimum pH of free enzyme was at pH 6.0, but the immobilized enzyme was pH 7.0 (Amid et al., 2014). α-Amylase immobilized on chitosan bead and amberlite MB-150 bead from soybean (*Glycine max*) showed the optimum pH was at pH 8.0 and 7.0, respectively, but free enzyme showed an optimum pH of 5.5 (Kumari and Kayastha, 2011). It might be because of the modification on the enzyme as well as formation of covalent bonds between the α-amylase and the carrier (Vessar et al., 2015).

The temperature effect on enzyme activity was assessed, and it was found that the optimal temperature was 40°C for free α-amylases and 50°C for immobilized α-amylases. Immobilized α-amylases exhibited more thermo-stability than free α-amylases: after pre-incubation at 50°C, free α-amylases retained little activity (7% of maximum activity), while immobilized α-amylases retained 92% of their original activity. α-Amylase immobilized on calix[4]arene derivative from *S. cerevisiae* showed that maximum activity for free enzyme and immobilized enzyme was found at 40 and 60°C, respectively. The critical temperature for the free enzyme is 60°C, because above 60°C it losses more than 90% activity, whereas highest activity is shown by immobilized enzyme at same temperatures (Veesar et al., 2015). Amylase immobilized on an arabic gum-chitosan bead from *H. polyrhizus* showed that maximum activity for free enzyme and immobilized enzyme was found at 60 and 70°C, respectively (Amid et al., 2014). Both and

α-Amylase immobilized on chitosan bead from soybean (*Glycine max*) showed the optimum temperature was at 70°C, whereas it was 75°C for enzyme immobilized on amberlite MB-150 (Kumari and Kayastha, 2011). Enzyme immobilization was generally found to have a protective effect on α-amylase enzymes exposed to extreme conditions, which may lead to enzyme deactivation. This can in part be explained by the immobilization affecting the conformational flexibility of the enzyme: immobilization is known to increase enzyme rigidity, which may result in increased stability and thus increased resistance to denaturation due to temperature elevation (Abdel-Naby, 1993).

The tolerance to organic solvents and the higher capacity for reusability of immobilized α-amylase compared with free α-amylases make these immobilized enzymes an attractive research focus. In the presence of various organic solvents (40%), only partial inactivation of the immobilized enzyme was observed. Compared to the free enzyme, the immobilized α-amylases furthermore exhibited a higher capacity for reusability. Chitosan- and chitosan-carbon-immobilized enzymes were shown to retain 70% of their maximum activity after six and ten uses, respectively. α-Amylase immobilized on calix[4]arene derivative from *S. cerevisiae* was shown to retain 70% of its maximum activity after 5 uses and above 50% enzyme activity after 15 uses, respectively (Veesar et al., 2015). Amylase immobilized on an arabic gum-chitosan bead from *H. polyrhizus* could be maintained 90.3± 0.01% of the enzyme activity for up to 14 days (Amid et al., 2015).

In the present study, chitosan and chitosan-carbon beads were cross-linked with glutaraldehyde to prepare them as carriers for purified α-amylases immobilization. Under immobilization and assay conditions shown to yield optimal immobilized α-amylase activity, an overall immobilized α-amylases yield of 56% and a specific activity of 2,240 U/g were measured for chitosan beads and an overall immobilized α-amylases yield of 58% and a specific activity of 2,320 U/g were measured for chitosan-carbon beads obtained. These findings are of great significance for industrial applications such as bioremediation of industrial wastewater contaminated with organic solvents.

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