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Immobilization of dextranase by *Aspergillus penicillioides* NRC 39 and its properties

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Dextranase from *Aspergillus penicillioides* NRC 39 was immobilized using different carriers by various techniques including physical adsorption, covalent binding, ionic binding and entrapment. Immobilization of dextranase by covalent binding using 2% glutaraldehyde on prawn shell as carrier produced a high immobilization yield 87.4%. Comparison between the free and immobilized enzyme showed that immobilized enzyme on prawn shell produce highest immobilization yield at pH 6.0 and 40°C after 30 min of the reaction. Immobilized enzyme loses its activity when heated at 70°C for 40 min. The Km of free and immobilized enzyme was 15.8 and 17.4 mg/ml, respectively while V max of the free and immobilized enzyme was 28.5 and 23.8 U/mg protein, respectively.

Key words: Dextranase, immobilization, *Aspergillus penicillioides*.

INTRODUCTION

Dextran is a long chain carbohydrate polymer (1,6-glucosidic linkages), synthesized from glucose by the enzyme dextransucrase (EC 2.4.1.5). Various bacteria, fungi and other organisms are capable of producing dextran as an exopolysaccharide (Khalikova et al., 2005).

Dextranases [(1-6) X-D-glucan-6-glucanohydrolases; EC 3.2.1.11] are a group of hydrolytic enzymes that specifically hydrolyze the (1 - 6) linkages in dextrans (Abdel-Naby et al., 1999). Differences in molecular weight of dextran are of significant commercial interest in drug formulations, vaccines, cryoprotectants and stabilizers in the food industries, cosmetic products and as separating gels in research studies (Khalikova et al., 2005). Specific molecular weight fractions of dextran generated by dextranase are used to restore blood volume in patients suffering from shock as a result of severe blood loss (Mehvar, 2000).

Dextran is involved in dental plaque formation, so dextranases are used in the manufacture of dentifrices as an additive for presentation of dental carries (Kuboki et al., 1985) Dextranases also have other important industrial applications since these enzymes can depolymerise various troublesome microbial dextran deposits and reduce viscosity in sugar process. Dextran can be modified by dextranases to be used in many biotechnological applications.

Immobilized enzymes find broad application in industry, biotechnology, biomedicine and analytical chemistry (Yagiz et al., 2007 and Camacho et al., 2007). Generally, immobilized enzymes show better thermal and pH stabilities and are easier to separate, can be reused and their effect appears to be more suitable for practical applications (Ye et al., 2007).

Various techniques have been developed for enzyme immobilization, including adsorption to insoluble materials, entrapment in polymeric gels, encapsulation in membranes,

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cross linking with bifunctional reagent, or covalent linking to an insoluble carrier (Gomez et al., 2008; Hector et al., 2013).

Dextranase from *Penicillium funiculosum* 258 was immobilized on chitosan using glutaraldehyde for covalent binding. Comparison with the free and immobilized dextranase, the immobilized enzyme exhibited a higher pH optimum, optimal reaction temperature and thermal stability (Abdel-Naby et al., 1998).

Tanash et al. (2011) illustrated that *Aspergillus subolivaceus* dextranase could be immobilized on several carriers by entrapment and covalent binding with cross-linking. Dextranase immobilized on BSA with a cross-linking agent showed the highest activity .The optimum pH, temperature of the reaction and thermal stability were significantly improved by the immobilization process.

In the present study, *Aspergillus penicillioides* NRC 39 was immobilized on different carriers using different methods of immobilization including, physical adsorption, covalent binding, ionic binding and entrapment. The properties of both free and immobilized enzyme were compared.

MATERIALS AND METHODS

Microorganism

Aspergillus penicillioides NRC 39 was obtained from the culture collection of the Chemistry of Natural and Microbial Products Department at the National Research Centre, Dokki, Cairo, Egypt.

Culture medium and cultivation

The culture medium for enzyme production was prepared (Abdel-Nabyet al., 1998) as follows: (g/l) dextran with molecular weight (70000) (10.0), yeast extract (2.0), $NaNO_3$ (10.0), K_2HPO_4 (4.0), $MgSO_4$. 7 H_2 O(0.2), KCl (0.2) and $FeSO_4.7H_2O$ (0.01) (Pleszczynska et al., 1997).

Two discs (6 mm in diameter) from 7 days old cultures were transferred to 250 ml Erlenmeyer conical flasks each containing 50 ml fermentation medium. The inoculated flasks were incubated on a rotary incubator shaker at 180 rpm for 7 days at 28-30°C. At the end of incubation period, cultures were centrifuged at 8000 rpm. The cell free supernatant was used as a crude enzyme for further determinations.

Assay of dextranase activity

Dextranase activity was determined according to Miller (1959). The standard dextranase assay mixture contained 1 ml of 2% dextran in 0.1 M acetate buffer (pH 5.0) and 0.5 ml of suitably diluted enzyme solution. After 30 min incubation at 50°C the reducing sugars formed were analyzed by DNS method. One unit of enzyme activity was defined as the amount of enzyme that converts one micromole of isomaltose per minute reaction under the described condition.

Immobilization method

Physical adsorption

One gram of the carriers was inoculated 1 ml of enzyme from *A. penicillioides* NRC 39 (Abdel- Naby et al., 1998).

Covalent binding

One gram of the carriers was treated with 2 ml of 2.5% (v/v) glutaraldehyde for 2 h at 30°C. Washed with distilled water to remove the excess glutaraldehyde. The wet weight of carriers were mixed with 1 ml of enzyme solution and incubated overnight at 4°C. The unbounded enzyme was washed with distilled water (Abdel-Naby et al., 1998).

Ionic binding

One gram of cation was exchanged with acetate buffer (pH 5.0) or anion exchanger with phosphate buffer (pH 7.0). The carriers were incubated with 1 ml of enzyme solution at 4°C for 12 h (Abdel- Naby et al., 1998).

Entrapment immobilization

In agar and agarose

Ten milliliters of different concentrations of agar and agarose solutions (2.0, 2.5, 3.0 and 4.0 %) were mixed with 1.0 ml enzyme solution. The mixture was quickly solidified at 40°C, cut into small fragments and washed with 0.2 M acetate buffer (pH 5.0) to remove the unbounded enzyme (Wood Ward, 1985).

In calcium alginate

Ten milliliters of different concentration of calcium alginate (2.0, 2.5, 3.0 and 4.0%) were mixed with enzyme solution. One milliliter enzyme solution was added to 10 ml alginate, enzyme mixture was made into beads by dropping sodium alginate solution into 0.15 M of calcium chloride, the beads (0.5-1.0 mm diameter) were left for 2 h before collecting and washed with 0.1 M acetate buffer (pH 5.0), the unbounded enzyme was removed by washing with distilled water (Bicherstaff, 1997).

RESULTS AND DISCUSSION

Immobilization of dextranase obtained from *A. penicillioides* NRC 39

The culture filtrate from the optimized medium with the fungus was partially purified with ammonium sulphate which produced 1.1 fold of purification and specific activity, 8.2 U/mg protein while 70% acetone produced specific activity, 14.2 U/mg protein and 2.8 fold of purification.

Immobilization by physical adsorption

Immobilization of dextranase from *A. penicillioides* NRC 39 by physical adsorption was employed on different carriers including alumina, foam, chitin, loafacylinderica, prawn shell, pumice, sawdust, natural sponge and synthetic sponge. Results in Figure 1 show that prawn shell produced the highest immobilization yield (81.9%) with immobilization activity (113.3 U/g carrier) while alumina produced the lowest immobilization yield

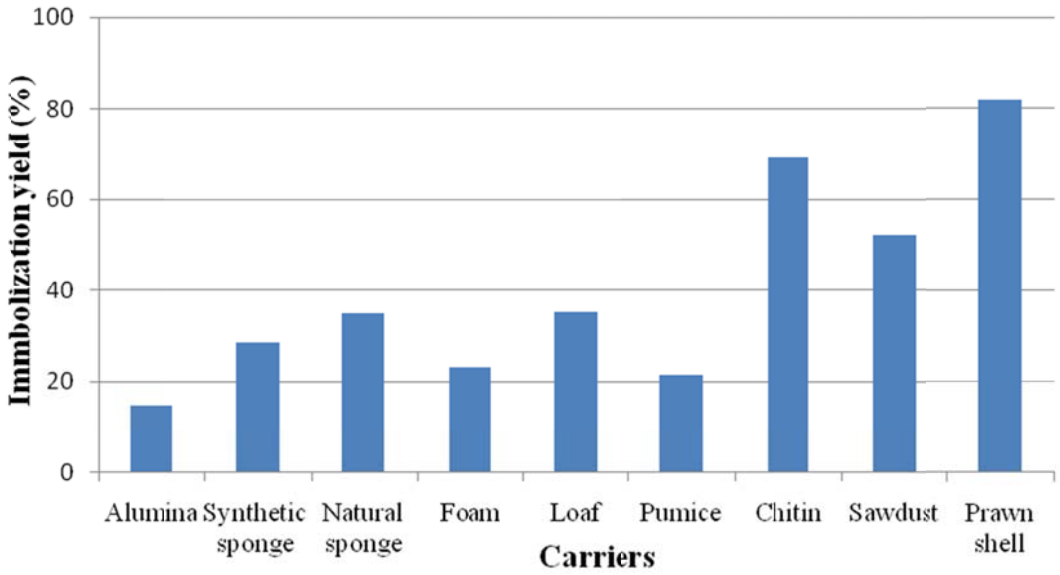

Figure 1. Immobilization of dextranase from *A. penicillioides* NRC 39 by physical adsorption.

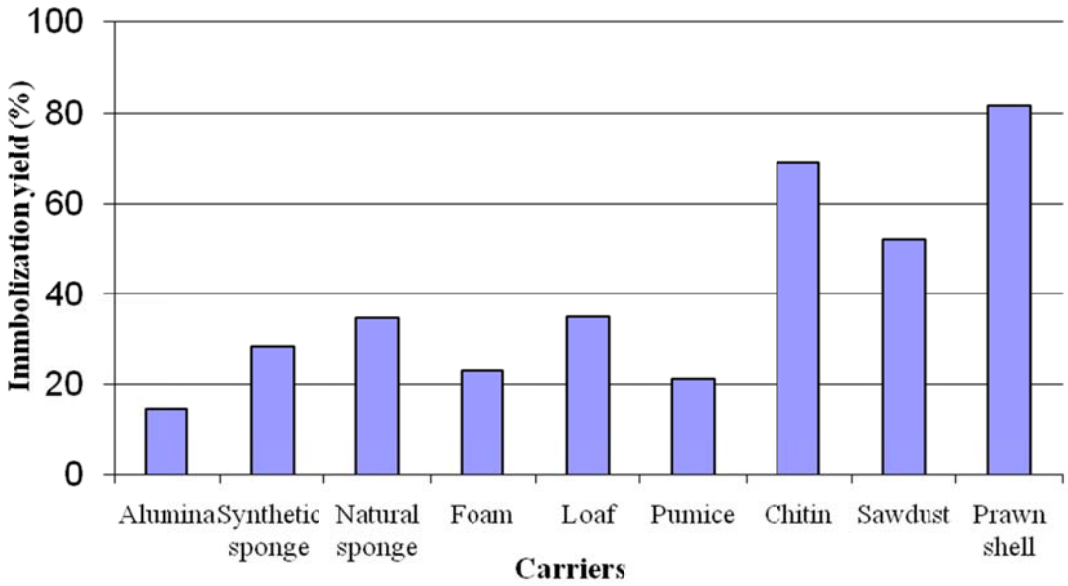

Figure 2. Immobilization of dextranase from *A. penicillioides* NRC 39 by covalent binding.

(14.7%), other carriers produced moderate to low immobilization yields. Abdel-Nabyet al. (1998) proved that the immobilized enzymes prepared by physical adsorption had the highest activity.

Immobilization by covalent binding

Results in Figure 2 showed that enzyme immobilization by covalent binding at prawn shell produce high immobilization yield (87.4%) with immobilization activity (124.2 U/g carrier). This high loading efficiency for the immobilization by covalent binding could be due to the formation of stable cross linking between the carrier and the enzyme through a spacer group which increased the local surface area of the carrier and reduced the steric hindrance around the active site of the enzyme molecule (Siso et al., 1990). These results were similar to those of Abdel-Naby et al. (1998) who reported that immobilization of *P. funiculosum* dextranase produced good immobilization

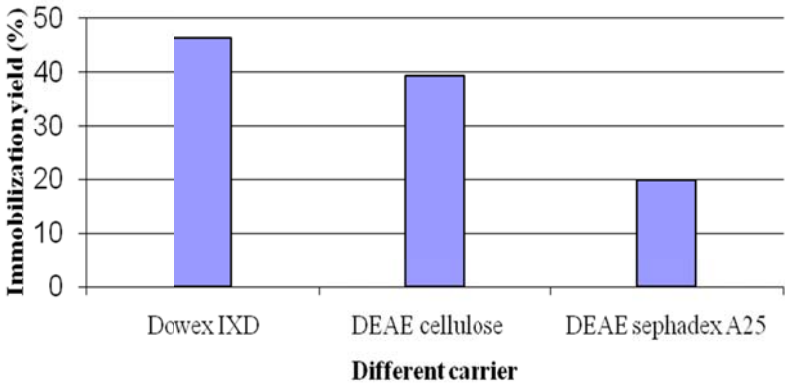

Figure 3. Immobilization of dextranase by ionic binding.

yield by covalent binding. Immobilizing enzymes by covalent binding result in an increase of the enzyme rigidity, which is commonly reflected by increase in the stability toward denaturation (Gottschalk and Jaenicke, 1991).

The presence of chitin, together with other polysaccharides, form fibrils of different lengths, depending on the species and the cellular location. In this work, a homopolymer was used with a broad spectrum of distribu-tion in the biosphere, being formed in the shells of crustaceans, such as crab, shrimp and lobster (Prasad et al., 2005).

Immobilization by ionic binding

The data illustrated in Figure 3 indicated that the highest enzyme yield (46.3%) was obtained using Dowex. On the other hand, the lowest immobilization yield (19.9%) was obtained by immobilizing the enzyme on sephadex, this inhibition may be due to the involvement of the fixation process to the active site of the enzyme (Galvez-Marisal and Lopez-Munguia, 1991).

Immobilization by entrapment

Immobilization of dextranase using different concentrations of agar, agarose and sodium alginate were examined. The results in Figure 4 show that 2% agarose produce the highest immobilization yield and immobilization decreased with increasing concentration of the carriers. These results agreed with those of Siso et al. (1990) who illustrated that decrease in yield with increase in carrier concentration might be due to the decrease in porosity of the gelmatrix which caused diffusion limitation for the substrate.

Properties of free and immobilized enzyme

pH of the reaction

The effect of different pH values of the reaction for free and immobilized enzyme on production of extracellular dextranase were investigated at the pH range of 4.0 to 7.0. Results in Table 1 show that the immobilized dextranase retain maximum activity at pH 6.0 while pH 5.0 was the optimal for the free enzyme. These results were the same as those of Galvez-Mariscal and Lopez-Munguia (1991) who found that the enzyme productivity of *Paecilomyces lilacinus* ranged from pH 5.4 to 7.0. Tanash et al. (2011) found that the optimum pH for activity of the immobilized enzyme of *A. subolivaceus* was shifted to pH 6.0 as compared to the free enzyme (pH 5.5). Shao-ying et al. (2013) found that the highest free dextranase activity was observed under the optimal reaction conditions of pH 5.5. These effects may be dependent on the ionic environment around the active site of the enzyme. Yakup and Aziz (2007) reported that the immobilization efficiency of dextranase was very high at pH 5.3.

Temperature of the reaction

Activity of free and immobilized dextranase was determined by incubation at different temperatures ranging from 20 to 80°C. Results in Table 2 show that the maximum dextranase activity was achieved at 40°C for immobilized enzymes, and at 50°C for free enzymes, increasing temperature above this range adversely affected the enzyme activity which lost 40% of activity at 80°C. The loss of enzyme activity might be due to low multiplication rate of the fungus biomass which ultimately decrease the enzyme production (Subasioglu and Cansunar, 2010).

Abdel Naby et al. (1999a) on the other hand reported that the optimal reaction temperature of dextranase from *Penicillium funiculosum* 258 was shifted from 60 for free to 80°C for the immobilized enzyme. Tanash et al. (2011) found that the optimal temperature of the reaction of dextranase from *A. subolivaceus* resulted at 60°C for both free and immobilized enzyme. This higher value of the optimal reaction temperature for the immobilized enzyme indicated that the applied immobilization procedure (covalent binding) contributed to greater

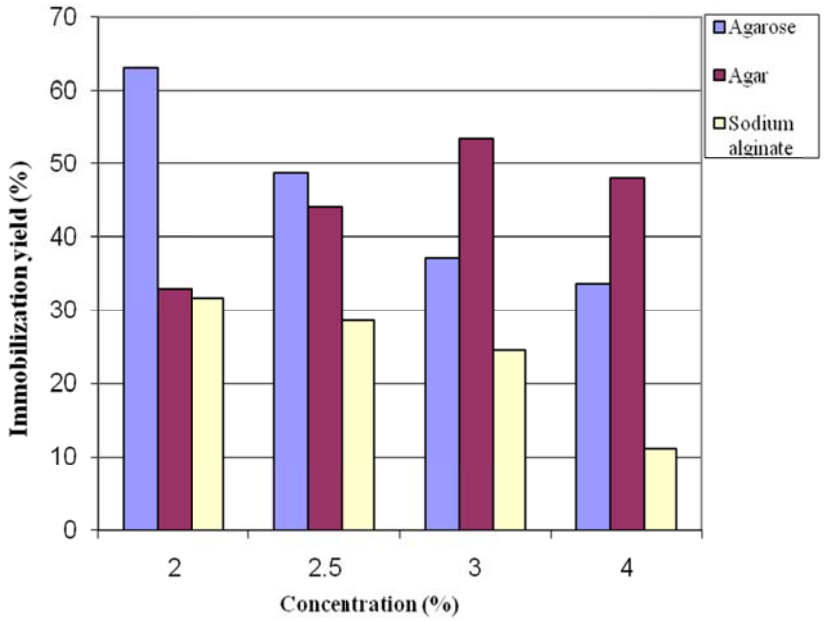

Figure 4. Immobilization of dextranase by entrapment.

Table 1. Effect of different pH values.

pH value	Relative activity (%)	
	Free enzyme	Immobilized enzyme
4	92.3	98.4
4.6	98.6	98.8
5.0 (control)	100	100
5.5	93.7	110.4
6	81.8	115.3
6.5	79	99.7
7	70	95.8

Table 2. Effect of different temperatures on the enzyme activity.

Temperature (°C)	Relative activity (%)	
	Free enzyme	Immobilized enzyme
20	34.1	87.2
30	63.9	103.2
40	84.3	112.1
50(control)	100	100
60	88.2	102.8
70	78.1	98.5
80	57.7	79.4

stability. Shao-ying et al. (2013) found that optimum temperature for free enzyme was 60°C.

Time of the reaction

The activities of the free and immobilized dextranase were assayed after incubation at various incubation times (15-90 min). As indicated in Figure 5 both the free and immobilized enzyme had maximum activity after 30 min. Increasing incubation time led to reduction in enzyme activity. Multiple fixation of the enzyme to matrix would also lead to a decrease in the activity due to the decrease in the catalytic activity (BicKerstaff, 1997).

Thermal stability

This experiment was designed to determine the effect of temperature on the activity of free and immobilized dextranases. Thermal stability of free and immobilized dextranases were investigated when incubated in the absence of substrate, at different temperatures ranging from 30 to 60°C, each for 10 to 60 min. The results illustrated in Figures 6 and 7 indicated that, immobilized enzymes was more stable than the free enzyme when heated at 30°C, the immobilization process protected the enzyme against heat inactivation. Immobilized enzyme lose its activity when heated at 70°C for 40 min. Whereas, the free enzyme was inactivated at a temperature of 60°C when heated for 40 min. Yakup and Aziz (2007) showed that *Penicillium lilacinum* dextranase thermal activity was enhanced by immobilization, soluble enzyme lost about 6 and 16% of its activity at 55 and 60°C, whereas immobilized enzyme retained 99 and 94% of its full activity at same temperature. In general, an immobilized enzyme is more stable than the free enzyme (Abdel-Naby et al., 1999a). This may be due to mass transfer resis-tance of the substrate into the carrier which particularly shows with a high molecular weight substrate like dextran.

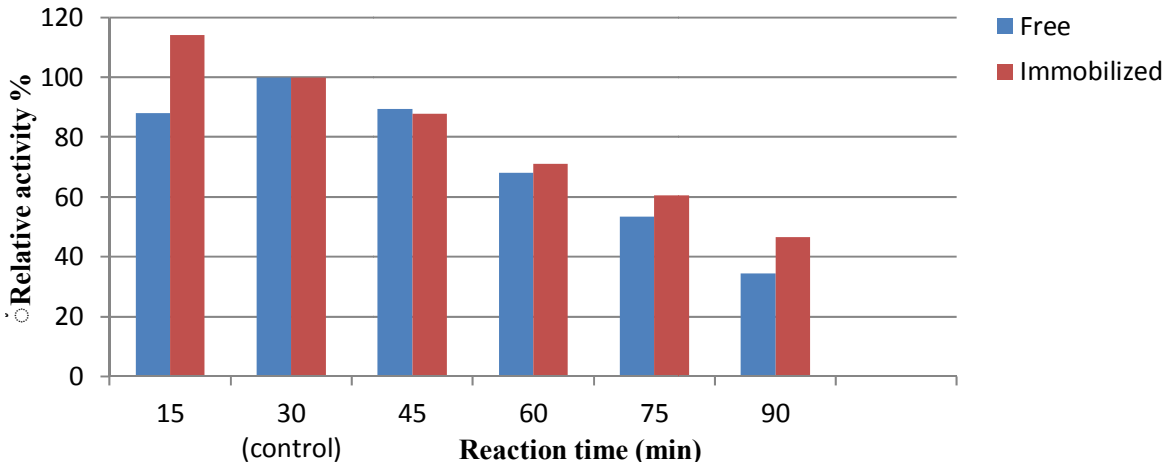

Figure 5. Effect of time of the reaction.

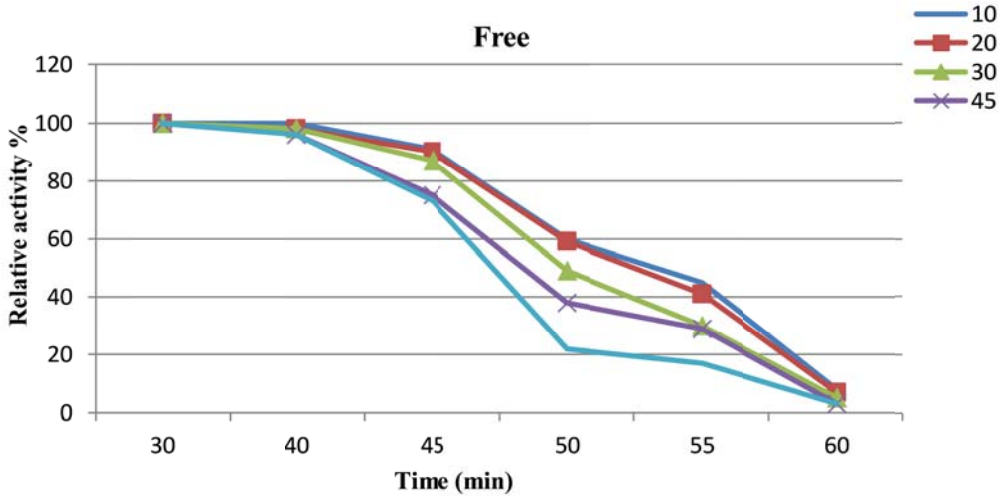

Figure 6. Thermal stability of free dextranase by *Aspergillus penicilliodies* NRC 39.

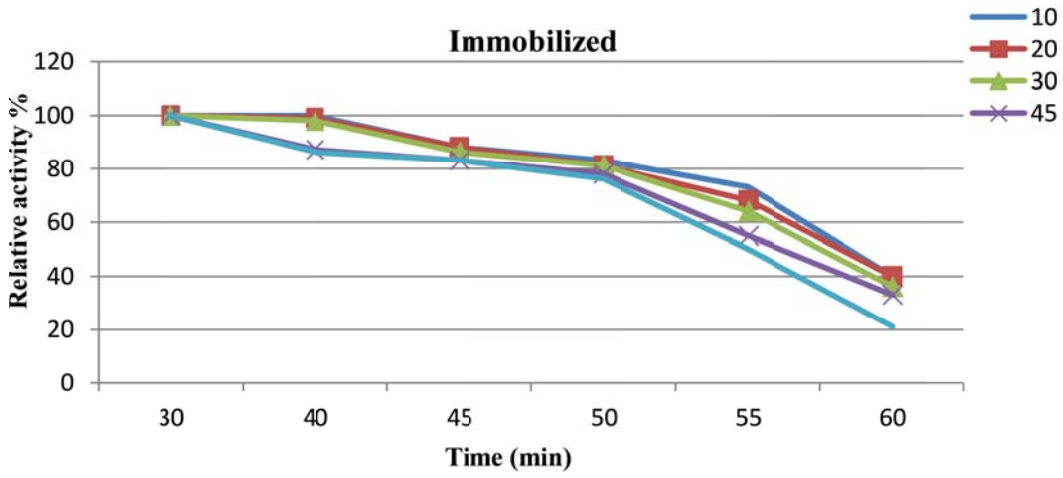

Figure 7. Thermal stability of immobilized dextranase by *Aspergillus penicillioides* NRC 39.

Table 3. Effect of metal salts on the activity of free and immobilized dextranase.

Different metal salts	Relative activity (%)	
	Free	Immobilized
Magnesium chloride	78.1	181.2
Zinc sulphate	85.3	80.2
EDTA	160.8	185.5
Cysteine Hcl	104.8	148.4
Copper sulphate	73.4	97.2
Cobalt chloride	97.2	90.6

Metal ions

Results in Table 3 indicated that relative activity of free enzyme increased in the presence of EDTA, while other metal ions decreased dextranase activity. On the other hand, EDTA, magnesium chloride and cysteine HCl increased relative activity of immobilized dextranase, other metal ions showed low activity. The results suggested that immobilization protected the enzyme against the inhibitory effects of some metal ions and inhibitors. These results are in agreement with those reported for other enzymes (Abdel-Naby et al., 1999a). The latter authors reported that the glycosylation of the enzyme formed a stable covalent bond that led to achievement of resistance against chemicals. The significant low activity of free enzyme may be attributed to direct contact between metal ions and the active site of enzyme. However, in immobilized enzyme, the fibrous porous structure play a role in protection due to the time required for these metal ions (Cu^{2+}, Hg^{2+} and Fe^{3+}) to diffuse to the carrier surface to reach the active site of enzymes (Abdel-Naby et al., 1999b). These results indicate the partial protection of the enzyme by immobilization is in agreement with those reported for other immobilized enzymes (Kimura et al., 1989).

Determination of Km and V max

Linweaver-Burk plots of the free and immobilized enzyme gave Km of 15.8 and 17.4 mg/ml, respectively. The V max of the free and immobilized enzyme were 28.5 and 23.8 U/mg protein, respectively.

The increase of the Km value after the immobilization may be due to mass transfer resistance of the substance into the immobilization matrix and to low substrate accessibility to the enzyme active site. Increasing the Km value of other enzymes after the immobilization has been reported by Abdel-Naby et al. (1999a). On the other hand, fixation of the enzyme on the immobilization matrix would lead to decrease in the flexibility of the enzyme molecule, which is commonly reflected by a decrease in the catalytic activity as reported by Erarslan et al. (1996). Consequently, the maximum reaction rate of the immobilized enzyme was lower than that of the free enzyme.

Conclusion

Prawn shell was a suitable carrier for immobilized dextranase from *A. penicillioides* NRC 39 by covalent binding using 2% glutaraldehyde resulting in the highest immobilization yield (87.4%). Immobilized enzyme exhibited a higher activity at optimum pH, temperature and time of reaction. Dextranase thermal activity was enhanced by immobilization. Protection of enzyme against inhibitory effects of some metal ions and inhibitors was indicated by immobilization. Km and V max of free and immobilized enzyme was determined.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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