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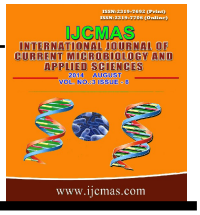

Original Research Article

Characterization of Free and Immobilized *P.aculeatum* NRRL-896 Dextranase

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Keywords

Characterization; Free dextranase; Immobilized dextranase;

A B S T R A C T

The characterization of free and immobilized dextranase on oyster mushroom stem by cross-linking technique with glutaraldehyde were studied. The optimum pH of the immobilized dextranase was shifted to a higher value (5.5) from pH 4.5, which was the optimal for the free enzyme, the immobilized dextranase was significantly stable over wide range of pH (4 to 8.0) than the free enzyme (pH; 4 to 6.5). This result indicated that the immobilized dextranase was more resistant to pH changes and could be used industrially.The optimal reaction temperature of the immobilized dextranase was shifted from 45 °C for the free dextranase to 55 °C and the thermal stability ranged from (35 - 65 °C) compared to (35 - 45 °C) for the free dextranase. This indicated that the immobilization process improved the thermal stability of dextranase relative to free enzyme. Dextranase activity of both free and immobilized enzyme did not change in the present of different sugars (sucrose, glucose, fructose, mixture of (sucrose + glucose + fructose) except that of mannitol and starch were 78.5 and 91.8 % residual activity, respectively. Metals ions ($CoCl_3$, $MnCl_2$, $PbCl_2$, $CuCl_2$, $AlCl_3$ and $MgCl_2$,) tested had no effect on dextranase activity except Fe^{+2}, Fe^{+3} and Hg^{+2} had 24.2, 28.1 and 51.8 % residual activity, respectively. However, aluminum chloride had slightly inhibitory effect with 97% residual activity and retained 73.4 % residual activity of the purified dextranase, while with cobalt Co^{+3} retained 78.5% of dextranase activity. Only Mn^{+2} and Mg^{+2} had stimulatory effect on dextranase activity. Linweaver Burk plots showed that K_m of the immobilized dextranase was (86.43 ± 0.1 mg mL^{-1}) via glutaraldehyde with appreciably higher values than that the free dextranase (52.13±0.02 mg mL^{-1}). While V_{max} (1/slope) of the immobilized enzyme (2.98 ± 0.03 µmole min^{-1}) is equally to the free dextranase (2.97±1.02 µmole min^{-1}).

Introduction

The immobilization of *Penicillium aculeatum* dextranase on a bentonite support did not change its specific activity and the enzyme yield was 0.1-0.6 mg/g bentonite matrix. In the presence of sucrose, thermal stability of the immobilized enzyme was high and the bound enzyme could be used for about six cycles (Madhu and Prabhu, 1985). While the immobilized dextranase from *Penicillium* was stable after the third

cycle and no appreciable decrease of immobilized enzyme activity was observed for more than ten cycles. The immobilized enzyme was able to maintain a good yield of reducing sugars (1.3–1.5 mg ml^{-1}) as high as 81% of the initial catalytic activity after 12 cycles. The operational stability of *P. funiculosum* 258 dextranase immobilized on chitosan by covalent binding appears to be more stable than *P. aculeatum* dextranase immobilized on bentonite (Madhu, 1985).

On the other hand, *Penicillium notutum* dextranase immobilized on silanised porous glass, showed a catalytic activity at wider pH and temperature ranges than those of the free enzymes. They were also characterized by a relatively high affinity to the substrate and good storage stability (Rogalski *et al.*, 1997). Also the immobilized dextranase from *Penicillium funiculosum* 258 prepared by covalent binding on chitosan using glutaraldehyde had the highest activity, and retaining 63% of its original specific activity. Compared with the free dextranase, the immobilized enzyme exhibited: higher pH optimum, optimal reaction temperature and energy of activation, Michaelis constant, improved thermal stability and higher values of deactivation rate constant. The immobilized enzyme retained about 80% of the initial catalytic activity even after being used for 12 cycles (Mohamed *et al.*, 1999).

The optimum conditions of *Penicillium lilacinum* dextranase were not affected by immobilization, and that the optimum pH and temperature for free and immobilized enzyme were 4.5–5.5 and 30–35 ̊C, respectively. Immobilized enzyme was more stable at low and high pH and high temperatures, and could be used for the production of isomaltooligosaccharides, since the immobilization efficiency is high (90%) and the immobilized enzyme retains its activity for 20 days without any decrease (Yakup and Aziz 2007)

The co-immobilized dextransucrase from *Leuconostoc mesenteroides* and dextranase from *Penicillium lilacinum* were used to produce isomaltooligosaccharides from sucrose, gave high yields (70 and 64%), but enzymatic activities decreased by 74 and 99%, respectively, after a month in storage at 4 °C (Zehra and Aziz 2010). While, Shafei and Allam (2010) stated that the optimal pH of the partially purified lipase from *Penicillium chrysogenum* was 7.5, the optimum temperature was 35 °C. At 60 °C, and the immobilized enzyme retained 62.79% of its activity.

Aspergillus subolivaceus dextranase which was immobilized on bovine serum albumin (BSA) with a cross-linking agent had the highest activity and considerable immobilization yield (66.7%). The optimum pH of the immobilized enzyme was shifted to pH 6.0 compared with the free enzyme (pH 5.5) at 60 °C, the thermal and pH stability are significantly improved. The calculated K_m of the immobilized dextranase (14.24 mg ml^{-1}) is higher than that of the free dextranase (11.47 mg ml^{-1}), while V_{max} of the immobilized enzyme (2.80 U µg $protein^{-1}$) is lower than that of the free dextranase (11.75 U µg $protein^{-1}$). The immobilized enzyme was able to retain 76% of the initial catalytic activity after 5.0 cycles (El-Tanash *et al.*, 2011).

Cheetham and Richards (1973) compared the properties of insolubilized forms of bacterial dextranase with those of the enzyme in solution and found that increasing temperature stabilized the insolubilized forms while, whilst pH optima and action patterns had no significant changes. Kinetic properties and pH or temperature optima of immobilized enzymes can also frequently be manipulated to favors specific applications, and enzyme stability is often enhanced upon

immobilization (Klibanov, 1979). Immobilization can also reduce the conversion time because very high enzyme concentrations can be used and recovered (Pitcher, 1980). The immobilized dextranase from bacteria on zirconia coated alkylamine glass. K_m exhibited a decline in value compared to the soluble enzyme while V_{max} remained unaltered. E_a of the enzyme was decreased upon conjugation. The soluble enzyme had its optimal pH at 5.4 while the alkylamine conjugated dextranase was optimally active at pH range 5.2-6.2 (Ramesh and Singh, 1980).

Therefore, this work was initiated to study the combination of characterization and stability of the free and immobilized dextranase produced from *P. aculeatum* NRRL-896.

Materials and Methods

Free and immobilized *P. aculeatum* NRRL-896 dextranase on oyster stem mushroom obtained from National Research Center (NRC) by adsorption and covalent bond techniques were used, 3,5-dinitrosalysilic acid reagent, ammonium sulphate, dextran (M.W. 40,000 Da) were purchased from Sigma Company, England.

Dextranase activity

Dextranase activity was assayed by a modification method described by Webb and Spencer-Martin (1983).

Protein determination

Protein was determined according to the method described by Lowry *et al.* (1951)

Determination of maltose

Maltose was enzymically assayed by the procedure described by Yoshio *et al.*, (2000) as follows: 0.9 ml of reagent (sodium phosphate buffer, pH 4.5) was added to 1.0 ml of maltose with different concentrations (0.0, 0.25, 0.5, 0.75 and 1.0 mg/ml distilled water). The absorbance was measured at A_{540} after 3 min reaction at 37 °C (Fig. 14). The amount of maltose was calculated by the following formula:

$$\text{Maltose (mg/ml)} = \frac{(E_s - E_b)}{(E_{st} - E_b)} \times 1.0$$

Where, E_s: is absorbance's of the reaction mixtures of the sample.

E_{st}: is absorbance's of the maltose standard.

E_b: is absorbance's of the distilled water

1.0: represents the concentration of the maltose standard solution (mg/ml).

Immobilized dextranase activity

Immobilized dextranase activity was assayed by using dextran (10 mL, 2 %, w/v) in calcium acetate buffer (25 mM, pH 5.5) was reacted with agitation at 200 rpm with 1.5 g of immobilized dextranase (136.72 units) at 50 °C for 60 min in a water bath. Reaction mixture (2.0 mL) was added to distilled water (8.0 mL) and boiled for 10 min with (1 ml) of 3,5 dinitrosalysilic acid color reagent to inactivate the enzyme **(Yakup and Aziz, 2007).**

Optimum pH for immobilized dextranase

Optimum pH for 1.5 g immobilized dextranase (136.72 units) was determined by changing separately the conditions of activity assays from 3 to 8 pH and the dextran concentration was 2.0 % (w/v). Buffer solutions of potassium phosphate pH

from 3.5 to 5.5 were used according to the method described by (Yakup and Aziz, 2007).

Optimum temperature for immobilized dextranase

Optimum temperature for 1.5 g immobilized dextranase (136.72 units) was determined by changing separately the conditions of activity assays from 5 to 70 °C at pH 5.5 according to the method described by (Yakup and Aziz, 2007).

Effect of different sugars on immobilized dextranase

Immobilized dextranase activity was assayed in the presence of 1% (weight/volume) of different sugars (Sucrose, Glucose, Fructose, mixture of (Sucrose + glucose + fructose), Mannitol and Starch) + 2% dextran (MW 40.000 Da), and incubated for 30 min at 45 °C according to the method described by Doaa and wafaa, (2009).

Effect of different metals on immobilized dextranase

Immobilized dextranase activity was assayed in the presence of various metal ions 1 mM concentrations of ($FeCl_2$, $FeCl_3$, $CoCl_3$, $MnCl_2$, $PbCl_2$, $CuCl_2$, $AlCl_3$, $MgCl_2$, and $HgCl_2$) then incubated at 45 °C for 10 min, according to the method described by Doaa and wafaa, (2009).

Operational and storage stabilities

Operational stability was carried out by repeating 20 batch experiments using the method described by **Yakup and Aziz, (2007).** Storage stability was estimated for a period of 20 days by determining the activity and the immobilized enzyme was then kept in a refrigerator at 4 °C.

Determination the half-life ($t_{1/2}$) of dextranase

The half-life of the enzyme activity ($t_{1/2}$) which corresponds to the time necessary for the residual enzyme activity to decrease to 50 % of its initial value was calculated as described by El-Tanash *et al.,* (2011) from the following equation:

$$t_{1/2} = 0.693/K_d \quad \text{.....(1)}$$

Where: K_d is deactivation rate constant

The relationship between the rate of reaction precedes and its temperature was determined by the Arrhenius Equation as follows:

$$K = Ae^{-Ea/RT} \quad \text{.....(2)}$$

Where: K is the rate coefficient, the pre-exponential factor A is assumed to be independent of temperature, E_a is activation energy, R is the gas constant (8.317 x 10^{-3} kJ $mol^{-1}K^{-1}$), and T the temperature (in kelvin).

Taking the natural logarithm of this equation gives:

$$\text{Ln } K = -E_a/RT \quad \text{.....(3)}$$

Results and Discussion

After the immobilization of the purified *P. aculeatum* dextranase on oyster mushroom stem by cross-linking technique. The characterizations of the immobilized dextranase were studied and compared to the free one.

Optimum pH and stability of immobilized dextranase

The optimum pH of immobilized dextranase was carried out over a various pH ranges (3-8) Fig. (3). The obtained results indicated that the optimum pH of the immobilized dextranase was shifted to a higher value (5.5) from pH 4.5, which was the optimal for the free enzyme. These effects may be depended on the ionic change environment

around the active site of the enzyme as a result of the immobilization process (Krajewska *et al.,* 1990).

This change in optimal pH from 4.5 to 5.5 for free and immobilized dextranase, respectively, which in turn may be attributed to the loss of amino groups from the enzyme upon immobilization since glutaraldehyde coupling involves the amino groups of the enzyme for cross-linking technique. This result is in agreement with that reported by Ramesh and Singh (1980), who revealed that the shift in pH was from 5.5 to 6.0 for the free and immobilized dextranase, respectively.

Results also is agreed with those reported by Yakup and Aziz (2007), who study the immobilization of *Penicillium lilacinum* dextranase by covalently onto Eupergit C as a support, and is in agreement with that found by El-Tanash *et al.*, (2011) for *Aspergillus subolivaceus* dextranase being pH 5.5 and 6.0 for free and immobilized dextranase, respectively.

The pH stability of immobilized dextranase (Fig. 4), showed that the activity is shifted to alkaline range and significantly stable over wide range of pH (4 to 8.0) than free enzyme (pH; 4 to 6.5). This result means that immobilized dextranase would be more resistant to pH changes and could be used industrially. This effect may have been caused by the micro-environmental pH of the carrier (Cao, 2005). On the other hand, immobilized dextranase from *P. lilacinum* is more stable at low pH (3.5–4.0) and higher pH (6.0–7.5) ranges (Yakup and Aziz 2007).

Optimum temperature and thermal stability of immobilized dextranase

The optimal reaction temperature of the immobilized dextranase was studied over a wide range of temperature 5 – 70 °C (Fig. 5). The results indicated that the optimal reaction temperature was shifted from 45 °C for the free dextranase to 55 °C for the immobilized enzyme. This higher value indicated that the applied immobilization procedure by cross-linking on stem contributed to greater stability.

This result is in agreement with that reported by (Abdel-Aziz *et al.*, 2007, Erhardt and Jördening, 2007, Hoster *et al.*, 2001, Arnold *et al.,* 1998).

This optimum temperature may be applicable in sugar industry as the ambient juice temperature in many factories is 32.2°C, where most applied dextranases showed low activity at the same temperatures (Eggleston and Monge, 2005).

In this respect, it may be great interest to apply the immobilized dextranase to overcome dextran problems at lower temperature especially in the crystallization process.

Results in (Fig. 6) showed that the thermal stability of free dextranase ranged from 35 to 45 °C and from 35 to 65 °C for immobilized dextranase, which indicated that the immobilization process improved the thermal stability of dextranase relative to free enzyme. For example, the free dextranase was completely inhibited after 30 min incubation at 70 °C, while the immobilized form retained 49.6 % of its original activity after the same treatment.

The thermal stability of the immobilized dextranase also was increased as it could withstand heat treatments up to 65 °C for 30 min while the free enzyme could withstand heat treatments only up to 45°C for 30 min. The immobilization technique with cross-linking improved the thermal stability of the

immobilized enzyme. The increase in thermal stability probably results from the prevention of conformational inactivation of the enzyme and the steric shielding that minimizes attack by reactive solutes (Ramesh and Singh 1980).

It could be also attributed to the restricted conformational change of dextranase upon immobilization, as suggested by Martinek *et al.,* (1980) and Klibanov, (1979). Yakup and Aziz, (2007) who found that the immobilization of *Penicillium lilacinum* dextranase enhanced the thermal stability than that of the free enzyme by lost about 6 to 16% of its activity at 55 and 60 °C, respectively, and retained 99 and 94% of its full activity at the same temperatures

Effect of different sugars on immobilized dextranase:

Results in Table (1) showed the effect of different sugars (sucrose, glucose, fructose, mixture of (sucrose + glucose + fructose), mannitol and starch) in presence of 2 % dextran (40,000) on the immobilized dextranase. Results indicated that there is no effect of adding these different sugars on dextranase activity of both free and immobilized enzyme except that of mannitol and starch decreased the activity of free dextranase to 78.5 and 91.8 %, respectively. The immobilized dextranase retained 100% of its activity in the presence of different sugars.

Sugarcane juices have high levels of glucose, fructose, and particularly sucrose. Mannitol could be present in sugarcane (formed mainly from *Leuconostoc mesenteroides*) which affect only free dextranase (Eggleston, 2002).

This result confirms the capability of applying immobilized dextranase in sugar factories, with no effect on its activity from sugars presented in sugarcane juices. These short and long chain carbohydrate sugars could potentially affect the activity of free dextranase if applied in sugar factories than immobilized dextranase.

So, this study was focused on overcoming dextran problems with new immobilized dextranase and overcoming accompanied problems which might face practical application of the enzyme like presence of high amount of mannitol. These results are in agreement with Doaa and wafaa, (2009).

Effect of different metals on immobilized dextranase

The effect of different metals on immobilized dextranase (Table 2) showed that metals had no effect on dextranase activity except that of Fe^{+2}, Fe^{+3} and Hg^{+2} were markedly inhibited the immobilized dextranase activity by 24.2, 28.1 and 51.8 %, respectively, On the other hand, Pb^{+2}, Cu^{+2}, Hg^{+2} ions inhibited the activity of free enzyme to 37.6, 48.4 and 31.2 %, respectively.

Aluminum chloride had slightly inhibited the immobilized dextranase to 92%, however retained 73.4 % residual activity of the purified dextranase. Moreover, cobalt chloride Co^{+3} retained 78.5% of free dextranase activity. Only Mn^{+2} and Mg^{+2} had stimulatory effect on dextranase as they retained 100 % of the original activity, this result is coincide with the results that reported by Doaa and wafaa, (2009) for dextranase of *Streptomyces* and highest from immobilized *Streptomyces Anultus* dextranase on hydroxyabatite.

It could be concluded that the application of immobilized *P. aculeatum* dextranase in sugarcane factory is very effective since it

could be actively work in the present of some metals and hence any dextran form in this temperature will auto-hydrolyzed by the enzyme. Application of this immobilized dextranase will overcoming dextran formation in sugar processing especially that this immobilized dextranase is energy saving and succeeded on laboratory scale. Immobilized dextranase may improve sugar industry and its processing by increasing sugar recovery as well as sugar purity will be reflected on the price of produced sugar and hence the economy.

K_m and V_{max} of immobilized dextranase

Michaelis constant (K_m) and maximum reaction velocity (V_{max}) of free and immobilized dextranase were estimated under optimal pH and temperature (Fig. 7).

K_m of the immobilized dextranase (86.43 ± 0.1 mg mL^{-1}) via glutaraldehyde was appreciably higher than the value of free dextranase (52.13±0.02 mg mL^{-1}), (Fig. 7). However, the difference was not as significant as in the case of immobilized dextranase via glutaraldehyde, While V_{max} (1/slope) of the immobilized enzyme (2.98 ± 0.03 µmole min^{-1}) is equally to that of free dextranase (2.97±1.02 µmole min^{-1}).

The increase in K_m values can be explained on the basis that the enzyme is immobilized not only on the surface but also within the particles. Similar results have been reported by (Madhu and Prabhu 1985).

The activation energy (E_a) for free and immobilized dextranase:

The activation energy value determined from the Arrhenius plots (Fig. 8) for free and immobilized dextranase were linear, and the activation energy of immobilized dextranase was lower (16.4±1.02 KJ mol^{-1}) than that of free enzyme (32.8±1.64 KJ mol^{-1}).

The activation energy (E_a) of the immobilized dextranase exhibited a decline as compared to the soluble enzyme indicating an increase in stability and efficiency of the enzyme upon immobilization as was reported by Rogalski *et al.,* (1997).

The half-life time ($t_{1/2}$) of immobilized dextranase:

The half-life time of the enzyme activity ($t_{1/2}$), which corresponds to the time necessary for the residual enzyme activity to decrease to 50 % of its initial value was calculated from the following equation:

$$t_{1/2} = 0.693/K_d$$

Results indicated that the immobilized dextranase was more thermostable than the free enzyme. The calculated half-life for thermal deactivation at 55°C was 2.08 and 19.36 h for the free and immobilized enzyme, respectively.

Properties of oyster mushroom stem immobilized dextranase:

The *P. aculeatum* immobilized on oyster mushroom stem by physical adsorption with cross-linking via glutaraldehyde retained about 52.3 % of its original activity after 30 min and 70 °C incubation, while the free dextranase was completely inhibited after the same conditions. (Table 3).

The optimal pH of the immobilized dextranase was shifted to a higher value (5.5) from 4.5, which was the optimal for the free enzyme. These effects may be dependent on the ionic environment around the active site of the enzyme as was reported by Ramesh and Singh (1980).

The optimal reaction temperature was shifted from 45 °C for the free enzyme to 55 °C for the immobilized dextranase (Table 3). This higher value for the immobilized dextranase indicated that the applied immobilization technique (physical adsorption on oyster mushroom stem with cross-linking) contributed to greater stability. Using Arrhenius plots, the calculated values of the activation energy (E_a) were 32.8±1.64 kJ mol^{-1} and 16.4±1.02 kJ mol^{-1} for the free and immobilized dextranase, respectively. The low value of the activation energy of the immobilized dextranase could be attributed to diffusion limitation at different treated temperatures. The calculated value of the kinetic constant K_m of the immobilized dextranase (86.43 ± 0.1 mg ml^{-1}) was higher than that of the free enzyme (52.13±0.02 mg ml^{-1}), while V_{max} for dextran (M.W. 40,000 Da) of the immobilized dextranase (2.98±0.03 µmole min^{-1}) was similar to that of the free enzyme (2.97±1.02 µmole min^{-1}).

This increase in the K_m after the immobilization revealed that the enzyme was immobilized not only on the surface but also within the stem particles as was reported by Madhu and Prabhu, (1985).

Fig.1 Standard curve obtained with various dilution of maltose solution

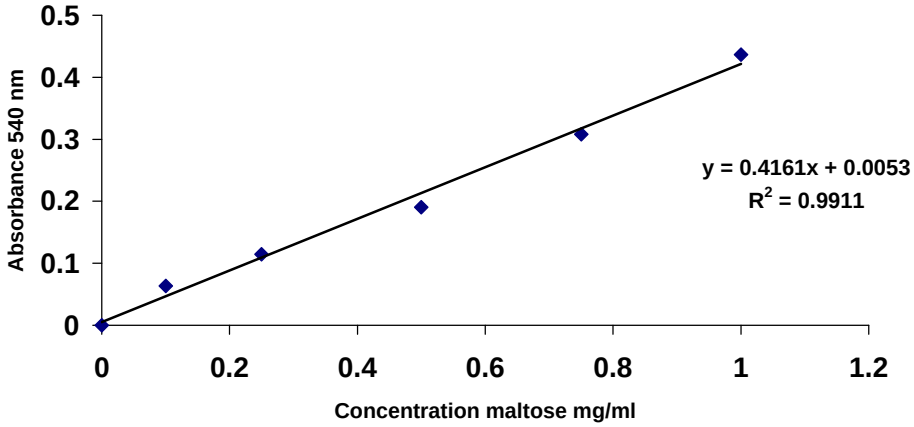

Fig.2 Optimum pH on free and immobilized dextranase, enzyme concentration 1.98 u/ml, dextran as substrate 1.0 % (w/v), reaction temperature 45 ˚C and reaction time 30 min.

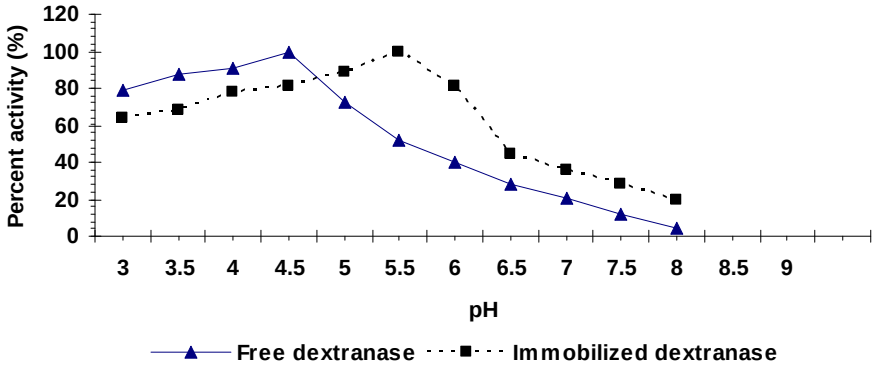

Fig.3 pH stability on free and immobilized dextranase, enzyme concentration 1.98 u/ml, dextran as substrate 1.0 % (w/v), reaction temperature 45 ˚C and reaction time 30 min.

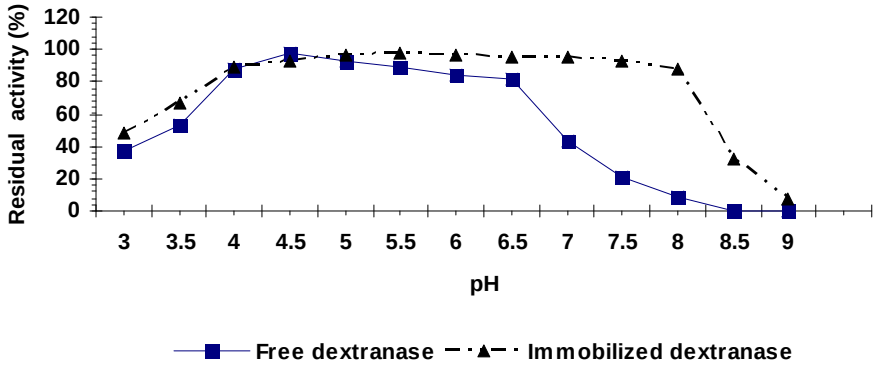

Fig.4 Optimum temperature of free and immobilized dextranase, enzyme concentration 2.34 u/ml, dextran as substrate 1.0 % (w/v), with 20 % sucrose, (0.025 M acetate buffer pH 5.0) and reaction time 30 min.

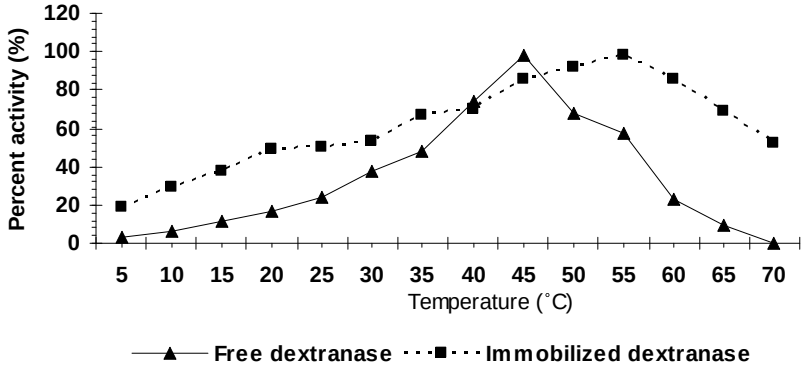

Fig.6 Thermal stability of free and immobilized dextranase, enzyme concentration 2.34 u/ml, dextran as substrate 1.0 % (w/v), with 20 % sucrose, (0.025 M acetate buffer pH 5.0) and reaction time 30 min.

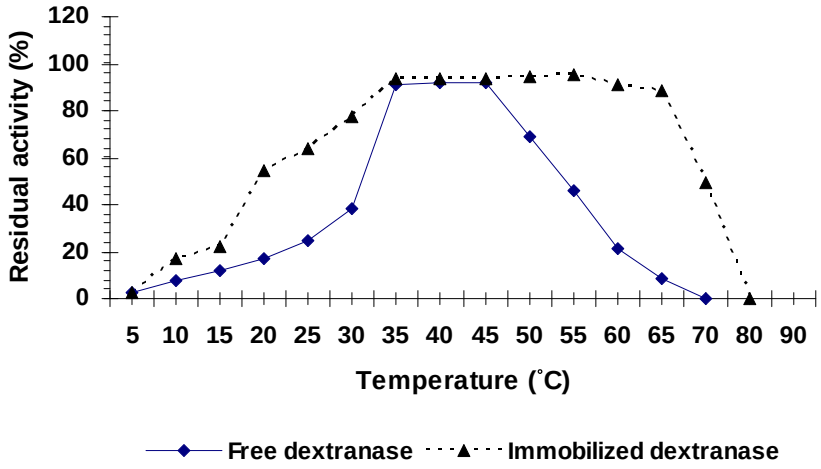

Table.1 Effect of different sugars on free and immobilized dextranase

Sugars 1% (w/v)	Free dextranase (Residual activity %)	Immobilized dextranase (Residual activity %)
Sucrose	100.0	100.0
Glucose	100.0	100.0
Fructose	100.0	100.0
Sucrose + glucose + fructose	100.0	100.0
Mannitol	78.5	100.0
Starch	91.8	100.0

Table.2 Effect of different metals on dextranase activity:

Metals (1mM)	Free dextranase (Residual activity %)	Immobilized dextranase (Residual activity %)
Control	100	100
Ferrous Chloride ($FeCl_2$)	6.2	24.2
Ferric Chloride ($FeCl_3$)	9.6	28.1
Cobalt Chloride ($CoCl_3$)	78.5	86.9
Manganese Chloride ($MnCl_2$)	100.0	100.0
lead Dichloride ($PbCl_2$)	37.6	73.6
Copper Chloride ($CuCl_2$)	48.4	80.3
Aluminium Chloride ($AlCl_3$)	73.4	92.0
Magnesium Chloride ($MgCl_2$)	100.0	100.0
Mercuric Chloride ($HgCl_2$)	31.2	51.8

Fig.7 Lineweaver- Burk plot of free and immobilized dextranase.

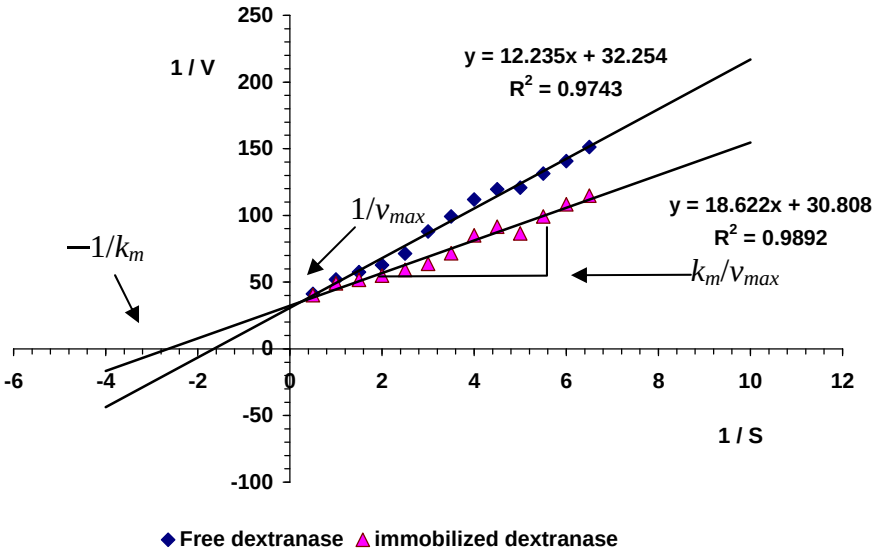

Fig.8 Arrhenius plots for the activation energy of free and immobilized *P. aculeatum* dextranase

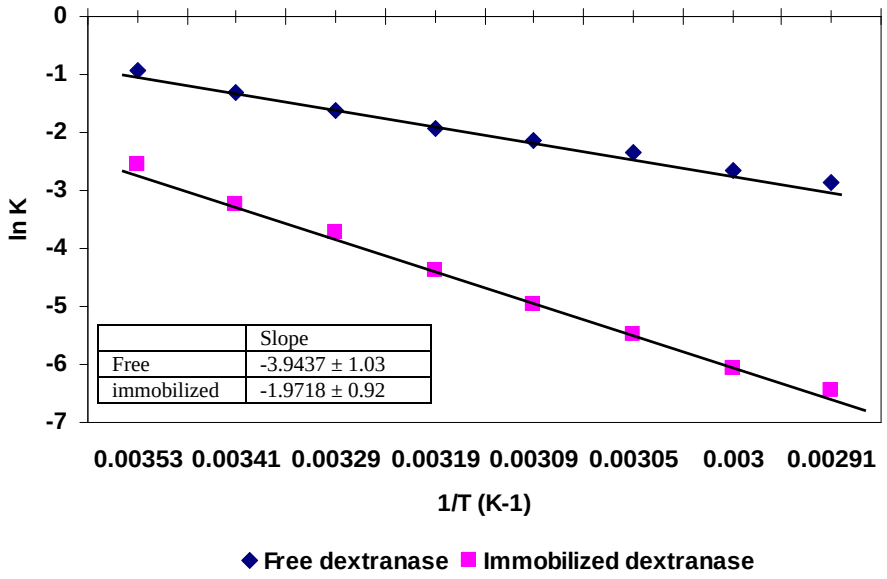

Table.3 Properties of free and immobilized dextranase on stem carrier

Property	Free dextranase	Immobilized dextranase
Specific activity (unit/100mg protein)	14042.31	27051.66
Optimum pH of the reaction	4.5	5.5
pH stability	Stable from 4.0 to 5.5	Stable from 4.0 to 8.0
Optimum reaction temperature (°C)	45	55
Thermal stability with 20% sucrose	Stable upto 45 °C for 30 min	Stable upto 65 °C for 30 min
Activation energy (E_a) (kJ/mol)	32.8±1.64	16.4±1.02
Half-life value $t_{1/2}$ at 55 °C (h)	2.08	19.36
K_m value (mg/ml) for dextran (40,000)	52.13±0.02	86.43 ± 0.1
V_{max} (µmole min^{-1})	2.97±1.02	2.98 ± 0.03

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