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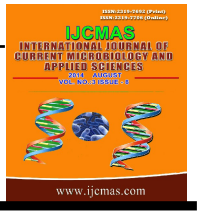

Original Research Article

Immobilization techniques for *P. aculeatum* dextranase

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A B S T R A C T

The dextranase purified from *P. aculeatum NRRL-896* (α-I,6-glucan 6-glucano-hydrolase, EC 3.2.1.11) was immobilized on different carriers were used by different techniques including: physical adsorption, Entrapment, Covalent binding and Cross-linking. The organic carriers such as natural polymers polysaccharides (i.e. CMC, Chitosan, Alginate, Oyster mushroom stem and Activated charcoal), synthetic polymers (i.e. Polyacrylamide gel and Hydroxyapatite) and inorganic carriers (i.e. Bentonite and Silica gel) were used as matrixes for dextranase immobilization techniques.Immobilization of *P. aculeatum* dextranase immobilized by physical adsorption indicated that bentonite had the highest activity 263.74 units/g carrier, immobilization yield 74.88 %, specific activity 19989.44 units/100 mg protein, amount of isomaltose produced 1.12 mg/ml, and retained about 67 % of its original activity, followed by treated and untreated silica gel. While, immobilization by entrapment on alginate beads had the highest specific activity (22472.43 units/100 mg protein) followed by polyacrylamide gel (17048.46 units/100 mg protein), then hydroxyapatite treated and untreated (14549.0, 11621.40 units/100 mg protein). Highest immobilization yield 59.89 %, amount of isomaltose produced 1.14 mg/ml, and activity retained 79.0 % of its original compared to the previous carriers were also observed.Immobilization by covalent binding on chitosan had the highest activity 234.16 units g^{-1} carrier, immobilization yield 61.04 %, and specific activity (18143.33 units/100 mg protein) compared to the free enzyme and activated charcoal. While immobilization on chitosan retained 54.4 % of its original activity compared to that immobilized on activated charcoal 44.6 %.Immobilization by cross-linking on CMC treated via ultrasonicator had the highest activities 143.69 units/ g carrier, specific activity (18066.15 units/100mg protein) and the immobilization yield was 48.82 %, and the free ones isomaltose produced was also higher 0.51 mg/ml compared to the free enzyme 0.38 mg/ml.Oyster mushroom stem was used as a new technique for the immobilization of *P. aculeatum* dextranase by four different carrier forms including: native stem un-treated, glutaraldehyde, cyanogen bromide (CNBr), and carbodiimide. The low immobilization yield (30.03 %) and immobilized enzyme activity (34.88 units/g carrier) were obtained from physical adsorption on oyster stem without any treatment; the amount of isomaltose was 0.53 and 0.13 mg/ml for free and immobilized dextranase, respectively. The immobilization yield of dextranase immobilized on oyster mushroom stem by Cross-linking technique with glutaraldehyde was 76.77 % and the immobilized dextranase activity was 321.59 U/g carrier. The specific activity of the immobilized dextranase was 27051.66 units/100mg protein, and the amount librated from dextran analyzed was 1.67 mg/ml. The *P. aculeatum* immobilized dextranase by covalent binding on stem modified by either CNBr or carbodiimide indicated that the highest enzyme activity, yield, amount of isomaltose and activity retained were obtained with carbodiimide being 137.49 units/g carrier, 54.31 %, 1.08 mg/ml and 80.3 % compared to that immobilized with CNBr and free enzyme. Comparative studies between the immobilization techniques of *P. aculeatum* dextranase indicated that the immobilization technique on oyster mushroom stem by physical adsorption with cross-linking via glutaraldehyde had the higher overall performance and was used for further investigation.

K e y w o r d s

Immobilization techniques, *P. aculeatum* dextranase

Introduction

Dextran formation creates processing problems in raw sugar factory operations and leads to a decline in sugar recovery, translating into economic losses for the

sugar industry. In addition to processing problems, dextran has a significant impact on the resulting market of the final processed product. Raw sugar purchase prices offered to raw sugar factories by buyers from sugar refiners include specific price penalties related to the level of dextran in raw sugar.

An alternative method for industrial enzyme applications is enzyme immobilized on a solid support. There are different methods to immobilize enzymes (Bickerstaff, 1996): covalent attachment to a solid support, entrapment in a gel matrix, adsorption onto an insoluble matrix, and intermolecular cross-linking of the enzyme to form an insoluble matrix.

Advantages of immobilization are the re-use of the enzyme, continuous output of products, control of the reaction rate by regulating flow rate, easy stoppage of the reaction for the facile removal of the enzyme, non-contamination of the product, and increasing enzyme stability.

Disadvantages of immobilization are often reduced volumetric activity of the enzyme (Licvonen, 1999) and mass transfer problems.

Dextranase isolated from *Penicillium funiculosum* and *P. lilacinum* could be immobilized on porous, silanized-silica beads and a phenol-formaldehyde resin. The commercial dextran of relatively low molecular weight ($2x10^6$) was degraded by immobilized dextranase, with the formation of reducing sugars, but with little decrease in viscosity, while soluble dextranase caused rapid loss of viscosity, but only a slight increase in reducing sugar (Karl *et al.*, 1982). While dextranase (1,6-α-D-glucan 6-glucanohydrolase, EC3.2.1.11) from *Penicillium aculeatum* culture has been immobilized on a bentonite support. The matrix-bound enzyme could be stored as acetone-dried powder or as a suspension in acetate buffer, pH 5.6, for about three weeks at 4 °C without any loss of activity. There was no change in the enzyme specific activity by immobilization and the enzyme yield was 0.1—0.6 mg/g bentonite matrix (Madhu and Prabhu, 1985_a). Immobilization of industrial *endo*-dextranase from *Chaetomium erraticum* (Novozymes A/S) could be made by using various techniques, i.e. adsorpting on bentonite (Montmorillonite), hydroxyl-apatite and Streamline DEAE. Highest activity loads could be achieved using bentonite and hydroxyapatite ($12{,}000Ug^{-1}$; $2900Ug^{-1}$, respectively) without a significant change of the apparent Michaelis–Menten constant K_m (Frank and Hans-Joachim, 2007). The immobilization of dextranase from *Penicillium aculeatum* to bentonite ratios above 0.5:1 (w/w) resulted in a high conservation of activity upon adsorption. Furthermore, dextranase could be used in co-immobilizates for the direct conversion of sucrose into isomalto-oligosaccharides (e.g. isomaltose). Yields of co-immobilizates were 2–20 times that of basic immobilizates, which consist of dextransucrase without dextranase (Frank *et al.*, 2008).

The dextransucrase from *Leuconostoc mesenteroides* and dextranase from *Penicillium lilacinum* were co-immobilized by encapsulating soluble dextransucrase and dextranase covalently attached to Eupergit C in alginate (beads, fibers, and capsules) and used to produce isomaltooligosaccharides from sucrose. They found that the enzymes were co-immobilized. The alginate capsule co-immobilization was done in the presence of soluble starch and resulted in a high immobilization yield (71%), and the enzymes retained their activities during 20

repeated batch reactions and for a month in storage at 4°C (Zehra and Aziz, 2010).

Penicillium lilacinum dextranase was immobilized covalently onto Eupergit C and was used for the production of isomalto-oligosaccharides. Immobilization resulted in 90% relative activity, and the immobilized enzyme showed no decrease in activity for 20 batch reactions (Yakup and Aziz, 2007). Rogalski *et al.,* (1997) reported that the two highly purified enzymic fractions of dextranase (1,6-α-D-ghtcan 6-glucanohydrolase, EC 3.2.1.11) from *Penicillium notutum* have been immobilized on silanised porous glass modified by glutaraldehyde, carbodiimide and bis-oxirane binding, and that the immobilization on alkylamine glass through the process of glutaraldehyde coupling proved to be the best of the methods studied. *A. subolivaceus* dextranase produced by submerged fermentation on dextran was efficiently immobilized by two methods of entrapment and covalent bonding with cross-linking. Cross-linking within BSA in the presence of glutaraldehyde gave immobilization yield (66.7%) and highest specific activity (1.56 U /g protein^{-1}) compared with the other carriers used. The immobilized dextranase remained stable for longer periods of time and also at higher temperatures as compared to the free enzyme.

The bacterial dextranase has been immobilized on zirconia coated alkylamine glass through the process of glutaraldehyde coupling; and the immobilized enzyme preparation exhibited 62 % of the initial enzyme activity with a conjugation yield of 18 mg/g support (Ramesh *et al.,* 1987).

Immobilized enzymes have been receiving a great deal of attention (Gerhartz, 1990). In applied research, the value of solid-phase enzymes lies in their potential as specific, re-usable, non-contaminating catalysts, often of increased stability, for use in industrial processes (Powell, 1990).

Carrier-bound water insoluble dextranase has potential for application in commercial production of clinical dextran in obtaining isomaltose and higher isomaltooligomers and also in continuous removal of dextran from infected diffusive sugar juices (Madhu, 1985). The expected qualities which could be most useful in this regard would be the improved thermal stability and the ease of recovery, both factors being of possible importance in the economic viability of such a scheme. Organic and inorganic supports have been employed in the immobilization of dextranase (Ramesh and Singh, 1980). Some of these matrices (cellulose derivatives, nylon) are not convenient in large scale operations in enzymic reactors because of their low stability and poor resistance to microbial attack. Therefore, the attachment of enzymes to porous glass beads is of commercial interest (Weetall, 1969). Such beads are chemically, mechanically and thermally resistant. They are also resistant to microbial attack and the carrier does not change its configuration over an extensive pH range nor under various solvent conditions and is therefore easier to use in continuous systems. The enzymic preparations thus obtained are inexpensive, easy to manufacture and possess a long working life span. Moreover, the porous glasses can be regenerated by heating or boiling in acid to remove organic materials adsorbed on them.

Therefore, this study aimed to use different immobilization techniques of *P. aculeatum NRRL-896* dextranase on different carriers as a trial to solve sugarcane factories problems study the characterization of the immobilized dextranase.

Materials and Methods

3,5-dinitrosalysilic acid reagent, dextran (M.W. 40,000 Da) , 3-Amino-propyl-triethoxy-silane (3-APTES), glutaraldehyde, cross-linking monomer (*N*,*N*, methylene-*bis*-acrylamide), cyanuric chloride ($CNCL_2$), and thionyl chloride ($SOCL_2$), cyanogen bromide, carbodiimide were purchased from Sigma Company, England.

The organic carriers such as natural polymers polysaccharides (i.e. Carboxy Methyl Cellulose (CMC), Chitosan, Alginate, Oyster mushroom stem and Activated charcoal), synthetic polymers (i.e. Polyacrylamide gel and Hydroxyapatite) and inorganic carriers (i.e. Bentonite and Silica gel) used for immobilization methods techniques, were obtained from Pharmacia, Sweden. Oyster mushroom stem was obtained from Mushroom Production, Cultivation and Utilization Special Unit – National Research Center – Dokki – Egypt.

Methods

Dextranase activity

Dextranase activity was assayed by a modification method described by Webb and Spencer-Martin (1983) as follows: Reaction mixture containing 2 ml of 2.0 % dextran in acetate buffer (0.05 M, pH 5.0) and 10 µl of dextranase in a total volume of 3 ml was incubated at 50 °C for 10 minutes. Reaction was stopped by adding 2 ml of 3,5-dinitrosalicylic acid color reagent and the absorbance of the reaction mixture was read at A_{540} by T80+UV/VIS spectrometer PG Instruments Ltd .

One unit of dextranase is defined as the amount of enzyme, which liberates one µmole of isomaltose (measurement as maltose) in 1 minute under the described conditions.

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 different concentrated maltose (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. 1).

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).

Immobilization techniques of *P. acule*atum *NRRL-896* dextranase

Preparation of carriers via ultrasonicator

The ultrasonication processors is a well-established method for particle size reduction in dispersions and emulsions and are used in the generation of nano-size material supports (Taurozzi *et al.,* 2010), dispersions because of the potential in the deagglomeration and the reduction of primaries indicating the mechanical effects of ultrasonic cavitation. The physical effects of cavitation are being used in a top-down generation of nano-particles. Where,

particles are reduced in size by the forces of cavitations (Fig. 2).

The carriers (i.e. silica gel, hydroxyapatite, and CMC) were treated with ultrasonicator waves (Ultrasons J. P. SELECTA, 100 Watt, 220 Volt and o.45 Ampere).

Transmission Electron Microscopy

The morphology of used carriers were examined by Transmission Electron Microscopy (TEM) using freeze-fraction replica method (JED 1230, JEOL Ltd., Tokyo, Japan) (Nobuo, 2008).

Adsorption technique

On Bentonite carrier

Adsorption on bentonite support (inorganic carrier and natural mineral) was used after treating with cyanuric chloride $CNCL_2$, thionyl chloride $SOCL_2$ and glutaraldehyde; then Transmission Electron Microscopy (TEM) image was taken for the shape particles size of bentonite.

Four hundred mg of untreated bentonite via ultrasonicator was stirred with 1 ml enzyme (531.65 units) pH 4.5 overnight at 4°C. The support was removed by cooling centrifuging and washed twice with 0.1M acetate buffers pH 4.5. The supernatant and the washings were analyzed for protein and dextranase activity. Activity remaining on the support or adsorbed was calculated from the difference in activities before and after treatment of the enzyme.

The dextranase was adsorbed on bentonite at pH 4.5 and was not eluted. Further experiments were carried out to optimize the conditions for insolubilization.

Finally, the sample was suspended in the same buffer (pH 4.5) and stored at 4°C for about three weeks for enzymatic studies by the method of Madhu and Prabhu (1985_a), Frank and Hans-Joachim (2007). The immobilization yield was calculated by the following equation:

$$\text{Immobilization yield (\%)} = \frac{\text{Immobilized enzyme}}{\text{Enzyme added} - \text{Unbound enzyme}} \times 100$$

Adsorption with cross-linking technique: On silica gel carrier

One g of dry silica gel was mixed with 15 % 3-Amino-propyltriethoxysilane (3-APTES) in 20 mL acetone and incubated at 50 ˚C for 2 h with constant mixing. The activated silica gel was then washed with water and dried at 60 °C for 2 h. Glutaraldehyde was modified in aqueous solution at 64 ˚C for 20 min; the activated silica gel was suspended in 0.05 M phosphate buffer (pH 7.0). Thereafter, the modified glutaraldehyde was added to the silica suspension and the suspension was filtered after stirring at 20 °C for 2 h. The activated silica gel produced was then washed with 0.05 M phosphate buffer (pH 4.5) and resuspended in the same buffer according to Dong *et al.,* (2006). One mL of dextranase (518.37 units) solution was added to the activated silica gel suspension and stirred at 20 °C for 2 h. The immobilized dextranase was recovered by filtration, washed with water, and resuspended in 0.05 M phosphate buffer (pH 5.5). The product was set aside for storage at 4 °C.

To reduce the toxicity of the glutaraldehyde, glutaraldehyde was heated in aqueous solution at different temperatures and times, as described by Park *et al.,* (2001). The specific conditions used for this process were governed by the design of the experiment. In this study, 3-Amino-propyltriethoxysilane 15 % (w/v) was used

as the silanization reagent for subsequent silanization of silica gel surfaces, dissolved in acetone and tested for their ability to silanize a silica gel surface.

Entrapment technique:
On Polyacrylamide gel carrier

The polymerization mixture contained 10.0 ml polyacrylamide 10 % (w:v), and 8.0 ml of acetate buffer (0.05 M, pH 5.5), then 2 % of *N*,*N*, methylene-*bis*-acrylamide was added as cross-linker monomer. The catalyst system added to the polyacrylamide mixture consisted of: 0.02 ml of tetramethyl-ethylenediamine (TEMED) and 0.01 g of ammonium persulphate dissolved in 0.2 ml of distilled water.

After polymerization, the gel was washed with acetate buffer (0.05 M, pH 5.5), then 2.0 ml of the enzyme dextranase solution (571.42 units) was added, cut into l x l x l mm fragments and kept in the same buffer at 4°C to remove the unbound enzyme as was described by Mohamed *et al.,* (1999).

On Alginate beads carrier

An alginate beads was used to immobilize dextranase through the entrapment technique as described by Frank and Hans-Joachim (2007) as follows:

First step: Beads formation: One gram of alginate beads is treated with 10 ml of 200 mM Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3-sulfonat) in 20 mM borate buffer pH 9.0 for 2 h at room temperature in a rotating device (10 rpm), then beads are washed 5 times with 25 mM calcium acetate buffer pH 4.5.

Second step: The activated alginate beads was contacted with dextranase solution 3137.43 Units gm^{-1} carrier in 20 mM calcium acetate pH 4.5 for 2 h at 35 °C and the residual dextranase activity in the solution was assayed.

On Hydroxyapatite carrier
Untreated carrier with ultrasonicator

Five gram of hydroxyapatite (500 nm particle size) was used as carrier, and 50 ml of sodium acetate buffer solution (0.05 M) pH 5.4 were added to a mixture of 50 mg albumin (albumin used as stabilizer) according to the method described by Doaa and wafaa (2009).

The mixture was homogenized by using ultrasonicator (Ultrasons J. P. SELECTA, 100 Watt, 220 Volt and o.45 Ampere) to enlarged the carrier surface, image was taken by Transmission Electron Microscopy (TEM), then 5 ml of dextranase solution (4531.62 unit) was added.

Treated carrier with ultrasonicator

Five gram of hydroxyapatite (200 nm particle size) was used as a carrier with 50 ml of 0.05 M sodium acetate buffer solution (pH 5.4), 50 mg albumin was added to the mixture (albumin used as stabilizer) according to the method described by Doaa and wafaa (2009). The activated hydroxyapatite was treated via ultrasonicator. Image was taken by Transmission Electron Microscopy (TEM), and then 5 ml of dextranase solution (4531.62 units) was added to the mixture.

Covalent binding technique
On Chitosan

One gram of chitosan in 25 ml of 0.01 M HCl containing 2.5%, (v:v) glutaraldehyde was shaken for 2 h at 30°C. The solubilized chitosan was precipitated by the addition of 1 ml of 0.1 M NaOH. The precipitate was

collected by filtration and washed with distilled water to remove the excess glutaraldehyde. The wet chitosan (untreated via ultrasonicator) was mixed with 2.5 ml of the dextranase solution containing 599.82 units/g carrier. After being shaken for 1 h at 4°C, the unbound enzyme was removed by washing with distilled water. The immobilization yield and the enzyme activity were determined as described above (Mohamed *et al.,* 1999).

Covalent binding with cross-linking technique

On Activated charcoal carrier

Immobilization of dextranase by covalent binding was achieved by cross linking between the enzyme and activated charcoal throughout glutaraldehyde according to the method of Mutloab and Mohammed (1980).

Charcoal support was pre-activated with glutaraldehyde, react with different degrees with the terminal amino residues of the enzyme protein.

Dextranase was immobilized by using activated charcoal as biomaterial support. Two grams of activated charcoal (untreated with ultrasonic) was added in a 100 ml Pyrex beaker containing 2 ml enzyme solution (638.84 units/g carrier). The mixture was stirred over-night at room temperature (35 °C) using a magnetic stirrer. The charcoal mixture was centrifuged and finally decanted and was thoroughly washed with phosphate buffer pH 4.5 until no dextranase enzyme activity was detected in the washings. The adsorbed charcoal was dried and kept in desiccator's.

After the completion of reaction, the reaction mixture containing immobilized enzyme was centrifuged. Charcoal binding the enzyme was dried and again checked for activity.

Cross-linking technique

On Carboxy Methyl Cellulose (CMC) carrier

Untreated carrier with ultrasonicator

Five gram of Carboxy Methyl Cellulose (CMC) as a carrier was shaked in 50 mL tris–HCl buffer (0.01 mole, pH 5.5) containing 2.5 % glutaraldehyde at room temperature for 2h. The carrier was filtered off and washed with distilled water to remove the excess of glutaraldehyde. The size and morphology of the particles carrier were investigated by TEM.

The activated carrier was incubated with 10 mL of tris-HCl buffer containing 2 mL of enzyme (531.47 units). After being shaken for 2h at room temperature, the unbounded enzyme was removed by washing with distilled water until no protein or activity was detected in the wash (Maysa *et al.,* 2010).

Treated carrier with ultrasonicator

Five gram of CMC was shaked in 50 mL tris–HCl buffer (0.01 mole, pH 5.5) containing 2.5 % glutaraldehyde at room temperature for 2h. The carrier was filtered off and washed with distilled water to remove the excess of glutaraldehyde, then the mixture was homogenized by using ultrasonicator, and the morphology of particles carrier was investigated by TEM.

The carrier was incubated with 10 mL of tris-HCl buffer containing 2 mL of enzyme (531.47 units). After being shaken for 2h at room temperature, the unbounded enzyme was removed by washing with distilled water until no protein or activity was detected in the wash (Maysa *et al.,* 2010).

New immobilization technique for *P. aculeatum NRRL-896* dextranase by using oyster mushroom stem (by-product) as a carrier

Oyster mushroom stem (by-product) was used as a natural carrier in the immobilization of dextranase for the first time as a trial to produce new natural support to be used.

Unmodified stem carrier

Oyster mushroom stem (5 gm) was used as a support (organic and natural material) without any treatment was stirred with 10 ml of acetate buffers 0.1M pH 4.5, then 1 ml of *Penicillium aculeatum NRRL-896* dextranase (547.65 units) was added. The support was washed with acetate buffers to remove the unbound enzyme, and then the immobilization yield was calculated.

Cross-linking technique

The purified dextranase from *Penicillium aculeatum NRRL-896* was immobilized on reactivation carbohydrate polymer (oyster mushroom stem) by 3-aminopropyl-triethoxysilane via amino groups using glutaraldehyde as follow:

Oyster mushroom stem (2 gm) was activated by the addition of 10 ml of 2.5 % glutraladehyde with occasional shaking for one hour and the excess glutaraldehyde was washed off with water. One ml of dextranase solution was added in phosphate buffer (0.05 M) pH 4.5 to 2 g of the activated support and kept overnight at 4 ˚C for coupling. At the end, the unbound enzyme was washed off with acetate buffer (0.05 M) pH 5.5 until no more enzyme activity could be detected in the wash and the immobilized enzyme was stored in the same buffer at 4 °C.

Covalent binding technique by cyanogens bromide

The activation of oyster mushroom stem by cyanogen bromide to form the reactive cyclic imidocarbonate by covalent binding was carried out as follow:

Oyster mushroom stem powder (2 gm) was treated with 2.5% aqueous 25 ml of cyanogen bromide (CNBr) at pH 5.5 for 10 min at 30 °C. One ml of dextranase was added to a flask containing 25 ml of activated stem support; the mixture was stirred magnetically for 2h, then washed as previously described.

The carrier-bound enzyme was washed copiously with distilled water and kept at 4 °C until further use.

Covalent binding technique by carbodiimide

Dextranase have been immobilized by covalent bonding on oyster mushroom stem modified by carbodiimide for the activation of the carrier –COOH groups as follow:

Oyster mushroom stem powder (2 gm) was treated with 10 ml of carbodiimide (0.05 M) pH 5.5 for 30 min at 30 °C, then 1 ml of dextranase in phosphate buffer (0.05 M) pH 5.5 was added to the activated stem support, stirred magnetically for 2h, then washed as previously described. Carrier-bound enzyme was washed copiously with distilled water and kept at 4 °C until further use.

Results and Discussion

Immobilization techniques of *P. aculeatum NRRL-896* dextranase

The dextranase purified from *P. aculeatum NRRL-896* was immobilized on different

carriers (organic and inorganic) and by different techniques.

Adsorption technique

Dextranase can be absorbed on bentonite material; the mechanisms involved in adsorption are not well understood. This method of immobilizing dextranase had the advantages of being inexpensive and chemically simple, since no reagents are required, and the process involves only a minimum of activation steps. Additionally, the enzyme is less likely to be denatured during the process of immobilization when compared with chemical methods of immobilizing enzymes. It has been speculated that the binding forces involved in adsorption are hydrogen bonds, coordinate bonds, and Van der Waal's forces.

Immobilization *P. aculeatum NRRL-896* dextranase by physical adsorption was employed on different carriers including: bentonite and silica gel (untreated and treated via ultrasonicator) carriers (Table 1). Results indicated that the immobilized enzyme prepared by adsorption on bentonite had highest activity (263.74 units g^{-1} carrier) and highest immobilization yield (74.88 %). This immobilization yield was higher than that reported by Madhu and Prabhu, (1985_a) and Frank and Hans-Joachim, (2007) being 50 %. Results in Table (2) showed that the immobilized enzyme on bentonite had the highest specific activity and amount of isomaltose 19989.44 unit/100 mg protein and 1.12 mg/ml, respectively followed by treated and untreated silica gel being 17636.11 units/100 mg protein, 0.82 mg/ml and 17390.59 units/100mg protein, 0.79 mg/ml, respectively.

Results proved that the immobilized dextranase by physical adsorption on bentonite and treated silica gel retained approximately the full activity (protein basis) and the original homogenous nature in suspension.

Results also indicated that the immobilized *P. aculeatum NRRL-896* dextranase by adsorption on bentonite retained about 67.7 % of its original activity. On the other hand, the immobilized *P. aculeatum NRRL-896* dextranase by adsorption with cross-linking on treated and untreated silica gel retained 61.2 and 57.0 % of its original activity, respectively.

The morphology of activated bentonite, activated silica gel untreated and treated via ultrasonic were obtained by using Transmission Electron Microscopy (TEM) Figures (3, 4, and 5). TEM showed that silica gel treated via ultrasonicator had nanoparticles size being 50 nm compared with untreated silica gel and bentonite being 2 and 3 µm, respectively.These results indicated that ultrasonication increased the surface area of silica gel to attach the enzyme.

Entrapment technique

Dextranase from *P. aculeatum* was immobilized by entrapment in polyacrylamide gel, hydroxyapatite and alginate beads (Tables 3 and 4). The immobilized enzyme prepared by entrapment on alginate beads had the highest specific activity (22472.00 units/100mg protein) compared to other carriers with immobilization yield about 59.89%, and retained about 78.8 % of its original activity. These results are similar to those reported by Shafei and Allam, (2010) for *Penicillium chrysogenum* dextranase immobilization and by Zehra and Aziz, (2010) for *Penicillium lilacinum* dextranase immobilization. The isomaltose produced by entrapment on alginate beads was 1.14 mg/ml higher than the free dextranase 0.68 mg/ml.

The immobilization yields of the immobilized enzyme by entrapment on polyacrylamide gel, hydroxyapatite treated with ultrasonicator and untreated were 68.84, 52.04 and 43.39 %, respectively, while the specific and retained activities were lower as compared to the alginate beads carrier, reaching to (17048.46, 38.8), (14549.00, 28.9) and (11621.40 units / 100 mg protein, 12.8 %), respectively. The lower values of dextranase activity with entrapment may be due to enzyme leakage.

This drop in the specific activity after immobilization on hydroxyapatite untreated (11621.40 units/100mg protein) may be due to diffusion limitation (i.e., resistance to diffusion of the substrate into the immobilization matrix and resistance to diffusion out of the products). On the other hand, the treated hydroxyapatite via ultrasonicator increased the specific activity of the immobilized dextranase to 14549.00 units/100mg protein compared to the free dextranase and untreated carrier.

Results of *Penicillium aculeatum NRRL-896* dextranase immobilization by entrapment in polyacrylamide were higher than that reported for other immobilized *P. funiculosum* 258 dextranase by entrapment in polyacrylamide (Mohamed *et al.,* 1999). The total amount of dextranase immobilized within polyacrylamide gel was 0.013 mg of protein per g of support.

Of the three polymers used, alginate beads is the most widely used carrier for entrapping dextranase, for it has the advantage of being non-ionic.

Result in Figures (6, 7 and 8) showed that the morphology particles size of alginate beads by Transmission

Electron Microscopy (TEM) was 10 nm compared to untreated and treated hydroxyapatite carriers via ultrasonicator being 500 nm and 200 nm, respectively.

Covalent binding technique

Data of the *P. aculeatum NRRL-896* dextranase immobilized by covalent binding (Table 5, 6) indicating that the enzyme immobilized on chitosan had the highest immobilized and specific activities (234.16 units/g carrier and 18143.33 units/100 mg protein, respectively), and the highest immobilized yield (61.04 %). Compared to that immobilized on activated charcoal by covalent binding with cross-linking, the immobilized enzyme and specific activities were 91.27 units/g carrier, 15011.58 units/100mg protein, respectively and the immobilization yield was 37.71 %. The immobilized activity for *P. aculeatum* dextranase was higher than those reported by Madhu and Prabhu (1985_a) 150 units/g bentonite matrix for *P. aculeatum* or by Kuboki *et al.,* (1985) 106 units/g hydroxyapatite for *P. fimiculosum*

Results also indicated that the immobilized *P. aculeatum NRRL-896* dextranase on chitosan retained 54.4 % of its original activity compared to that immobilized on activated charcoal by covalent binding being 44.6 % and to those obtained by Mohamed *et al.,* (1999) 63 % from *P. fimiculosum* 258, by Sugiura and Ito, (1974) 30 % from *P. funiculosum*, and by Sugiura and Ito, (1975) 30 % from *Brevibacterium fuscum.*

The amount of isomaltose produced was approximately the same by these two covalent binding techniques 0.60 and 0.63 mg/ml for chitosan and activated charcoal, respectively.

On the other hand, Gottschalk and Jaenicke (1991) reported that immobilization of dextranase by covalent binding (as the present case) would lead to a decrease in the

flexibility of the enzyme molecule. Also, it could be elucidated that the substrate had a low accessibility to the enzyme active site. Consequently, the maximum rate of the reaction catalyzed by the immobilized enzyme was lower than the free form.

These results are in agreement with El-Tanash *et al.,* (2011) who reported that immobilization by covalent binding using a cross-linking agent (glutaraldehyde) probably increases the local surface area, which contributes to minimizing the steric effect around the active site of the immobilized enzyme and similar also to those reported by Abdel-Naby *et al.,* (1999) during immobilization of *P. funiculosum* dextranase.

These results indicated that the immobilized dextranase by covalent attachment on chitosan improved the immobilization dextranase, since it does not leach out.
The morphology of activated chitosan and activated charcoal were measured by using TEM and the particles size were 15 µm and 100 nm, respectively as shown in Fig. 9 and 10.

Cross-linking technique

The cross-linking immobilization technique of dextranase on Carboxy Methyl Cellulose (CMC) is shown in Table (7). The results showed that, the treated CMC via ultrasonicator had the highest activity 143.69 units/g carrier, the highest immobilization yield 48.82 % and retained about 44.2 % of its original activity compared to the untreated ones.

Results also indicated that the immobilized enzyme on treated CMC (Table 8) had the highest specific activity 18066.15 units/100mg protein and highest amount of isomaltose 0.51 mg/ml compared to the untreated being 16524.00 units/100g protein and 0.49 mg/ml, respectively.

The amino groups in CMC were reacted with glutaraldehyde (GDA) as a cross-linking agent and then dextranase was bounded to them. The reaction of CMC with GDA is similar to those have been made by Juang and Min-Yun (2005) & Gamze and Senay (2007).

The TEM of CMC untreated and treated carrier was shown in Figures (11 and 12). The particles size were about 350 and 130 nanometer, respectively.

New immobilization technique for *P. aculeatum NRRL-896* dextranase by using oyster mushroom stem (by-product) as a carrier

Adsorption technique

Oyster mushroom stem was used as a new technique for the immobilization of *Penicillium aculeatum NRRL-896* dextranase by four different carrier forms including: native stem untreated, glutaraldehyde, cyanogen bromide, and carbodiimide. Properties of the immobilized forms of the enzyme were investigated and compared with the free enzyme.

The purified dextranase from *Penicillium aculeatum NRRL-896* immobilized by physical adsorption on oyster mushroom stem (carbohydrate polymer) without any treatments.

Results in Table (9) showed that the immobilization yield was 30.03 % and the immobilized enzyme activity was 34.88 units/g carrier.

Results in Table (10) indicated that the specific activity and isomaltose amount of

the immobilized dextranase on stem decreased to 421.54 units/100 mg protein and 0.13 mg/ml, respectively, compared to the free one 14042.31 units/100 mg protein and 0.53 mg/ml, respectively. The retained activity of the immobilized dextranase by adsorption on stem unmodified was 1% of its original activity.

The morphology of oyster mushroom stem was measured by TEM. Results indicated that the particle size was 310 nm (Fig. 13).

Cross-linking technique

The purified dextranase from *Penicillium aculeatum NRRL-896* was immobilized on reactivation carbohydrate polymer (oyster mushroom stem) by 3-aminopropyl-triethoxysilane via amino groups using cross-linking by glutaraldehyde (Fig. 14).

Results in Table (11) showed that the immobilized enzyme on oyster mushroom stem modified by glutaraldehyde had higher immobilization yield 76.77 % and the immobilized dextranase activity was 321.59 units/g carrier.

Compared to the free dextranase, data in Table (12) indicated that the immobilization of dextranase on oyster mushroom stem modified by glutaraldehyde increased the specific activity to 27051.66 units/100mg protein and the amount of isomaltose was 1.67 mg/ml compared to the free dextranase 14042.31 units/100mg protein and 0.53 mg isomaltose/ml, respectively. The immobilized dextranase on oyster mushroom stem modified by glutaraldehyde retained about 88.9 % of its original activity.

Covalent binding techniques by cyanogens bromide and carbodiimide

The purified *Penicillium aculeatum NRRL-896* dextranase was immobilized by covalent binding on oyster mushroom stem (carbohydrate polymer) via cyanogen bromide (CNBr) to give the reactive cyclic imido-carbonate group to react with amino groups of lysine under mild conditions (Fig. 15) or via carboxyl groups using EDC (1-Ethyl-3- (3-dimethylamino-propyl)-carbodiimide)) for action with dextranase by covalent binding as shown in Fig. 15 and 16. Results in Table (13) showed that the immobilized dextranase on oyster mushroom stem modified with carbodiimide had the highest activity 137.49 units/g carrier and the highest immobilization yield 54.31 % compared to dextranase immobilized on stem modified with CNBr being 75.54 units/g carrier and 46.85 %, respectively.

Results also indicated that the immobilized enzyme on stem modified with CNBr and carbodiimide retained about 74.1 and 80.3 % of its original activity, respectively.

The overall performance of the free and immobilized dextranase by covalent binding on oyster mushroom stem was shown in Table (14). Results revealed an increase in the specific activity and amount of isomaltose produced by immobilization on stem modified with CNBr 19334.76 units/100mg protein and 0.98 mg/ml compared to free enzyme 14042.31 units/100mg protein and 0.53 mg/ml, respectively.

On the other hand, although the immobilized dextranase on stem modified with carbodiimide had lower specific activity 16291.11 units/100mg protein compared to that modified with CNBr, it had highest amount of isomaltose produced 1.08 mg/ml compared to the free enzyme.

The enzyme immobilized on oyster

mushroom stem treated with glutaraldehyde had the highest specific activity 27051.66 units/100 mg protein, immobilization yield 76.77 %, the amount of isomaltose produced being 1.67 mg/ml and retained about 89 % of its original activity, followed by entrapment technique on alginate beads being 22472 units/100 mg protein, 79 % and 1.14 mg/ml, respectively.

Therefore, it was concluded from the previous results that immobilized dextranase on oyster mushroom stem modified with glutaraldehyde was the best matrix (carrier) for the immobilization because it was much economics (as it is waste) so, it used for further investigation.

Table.I List of isolated of Diatoms

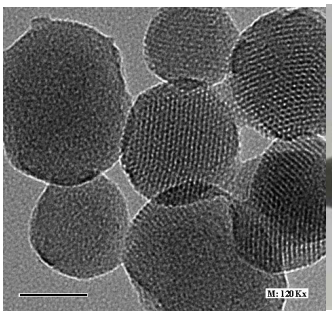


Fig. (3): Transmission Electron Microscopy image of

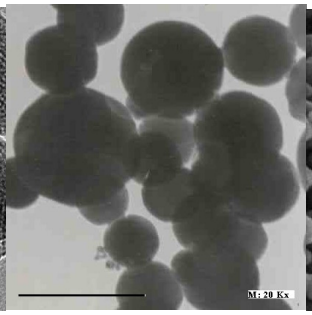
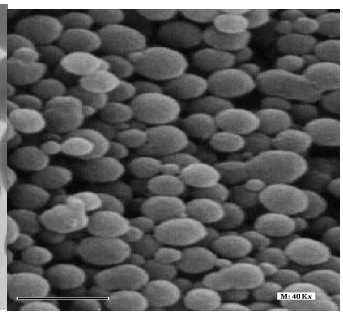


Fig. (4): Transmission Electron Microscopy image of bentonite



g. (5): ransmission Electron Microscopy image of untreated silica gel

Table.1 Immobilization of *P. aculeatum NRRL-896* dextranase by adsorption techniques:

	Immobilization techniques	Carrier	Enzyme added (U/g carrier)	Unbound enzyme (U/g carrier)	Immobilized enzyme (U/g carrier)	Immobilization yield (%)
1	Adsorption	Bentonite	531.65	179.43	263.74	74.88
2	Adsorption with cross-linking	Silica gel				
		Untreated*	518.37	193.69	83.93	25.85
		Treated*	518.37	213.06	86.43	28.31

Table (2): The overall performance of the free and immobilized dextranase by adsorption techniques:

Carrier	Form of dextranase	Protein mg/ml	Activity U/ml	Specific activity U/100mg protein	Retained activity (%)	Amount of isomaltose mg/ml
Bentonite	Free dextranase	0.037	531.65	14368.92		0.66
	Immobilized dextranase	0.018/mg carrier	359.81	19989.44	67.7	1.12
Silica gel	Free dextranase	0.037	518.37	14010.00		0.34
• Untreated*	Immobilized dextranase	0.017/mg carrier	295.64	17390.59	57.0	0.79
• Treated*	Immobilized dextranase	0.018/mg carrier	317.45	17636.11	61.2	0.82

* Untreated or Treated activated silica gel via ultrasonicator.

Table.3 Immobilization of *P. aculeatum NRRL-896* dextranase by entrapment techniques:

	Immobilization techniques	Carrier	Enzyme added (U/g carrier)	Unbound enzyme (U/g carrier)	Immobilized enzyme (U/g carrier)	Immobilization yield (%)
1	Entrapment	Polyacrylamide gel	571.42	273.64	205.00	68.84
2	Entrapment	Alginate beads	3137.43	1265.33	1121.20	59.89
3	Entrapment	Hydroxyapatite				
		Untreated*	4531.62	2128.67	1042.64	43.39
		Treated*	4531.62	1952.71	1342.06	52.04

Table.4 The overall performance of the free and immobilized dextranase by entrapment techniques:

Carriers	Form of dextranase	Protein mg/ml	Activity U/ml	Specific activity U/100mg protein	Retained activity (%)	Amount of isomaltose mg/ml
Polyacrylamide gel	Free dextranase	0.042	571.42	13605.24		0.41
	Immobilized dextranase	0.013/mg carrier	221.63	17048.46	38.8	0.86
Alginate beads	Free dextranase	0.226	3137.43	13882.43		0.68
	Immobilized dextranase	0.11/mg carrier	2471.92	22472.00	78.8	1.14
Hydroxyapatite	Free dextranase	0.328	4531.62	13815.915		0.68
• Untreated	Immobilized dextranase	0.05/mg carrier	581.07	11621.40	12.8	0.49
• Treated	Immobilized dextranase	0.09/mg carrier	1309.41	14549.00	28.9	0.82

* Untreated or Treated via ultrasonicator

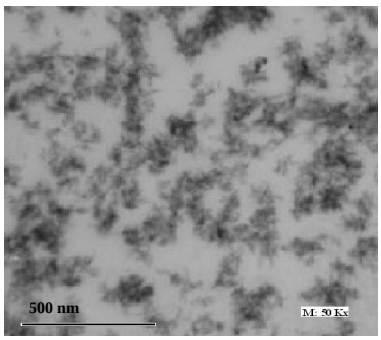


Fig. (6):
Transmission Electron Microscopy image of untreated hydroxyapatite

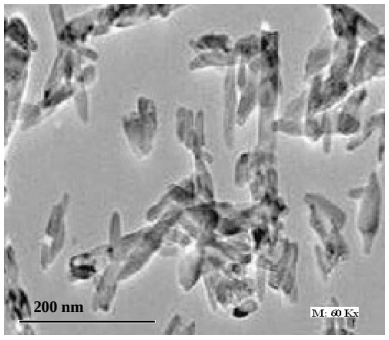


Fig. (7):
Transmission Electron Microscopy image of treated hydroxyapatite with ultrasonicator

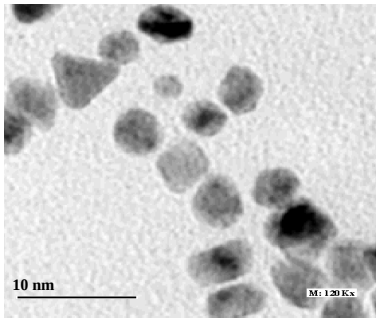


Fig. (8):
Transmission Electron Microscopy image of alginate beads

Table.5 Immobilization of *P. aculeatum NRRL-896* dextranase by covalent binding techniques:

	Immobilization technique	Carrier	Enzyme added (U/g carrier)	Unbound enzyme (U/g carrier)	Immobilized enzyme (U/g carrier)	Immobilization yield (%)
1	Covalent binding	Chitosan	599.82	216.21	234.16	61.04
2	Covalent binding with cross-linking	Activated charcoal	638.84	396.82	91.27	37.71

Table.6 The overall performance of the free and immobilized dextranase by covalent binding techniques:

Techniques	Form of dextranase	Protein mg/ml	Activity U/ml	Specific activity U/100mg protein	Retained activity (%)	Amount of isomaltose mg/ml
Covalent bonding on chitosan	Free dextranase	0.043	599.82	13949.30		0.42
	Immobilized dextranase	0.018/mg carrier	326.58	18143.33	54.4	0.60
Covalent binding with cross-linking on activated charcoal	Free dextranase	0.046	638.	3887.83		0.44
	Immobilized dextranase	0.019/mg carrier	285.	5011.58	44.6	0.63

Table.7 Immobilization of *P. aculeatum NRRL-896* dextranase on CMC by cross-linking technique:

Technique	Carrier	Enzyme added (U/g carrier)	Unbound enzyme (U/g carrier)	Immobilized enzyme (U/g carrier)	Immobilization yield (%)
Cross-linking	CMC untreated	531.47	284.22	106.69	43.15
	CMC treated	531.47	237.14	143.69	48.82

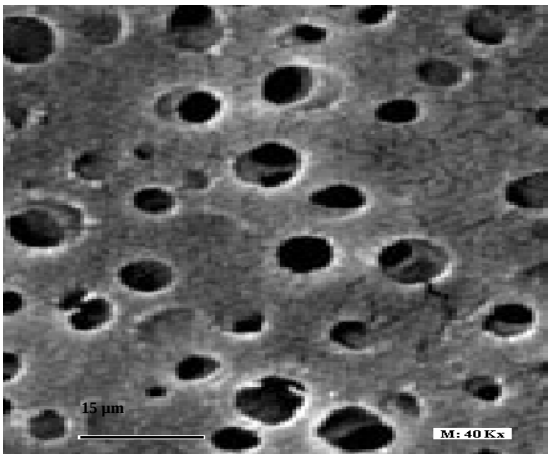


Fig. (9): Transmission Electron Microscopy image of chitosan

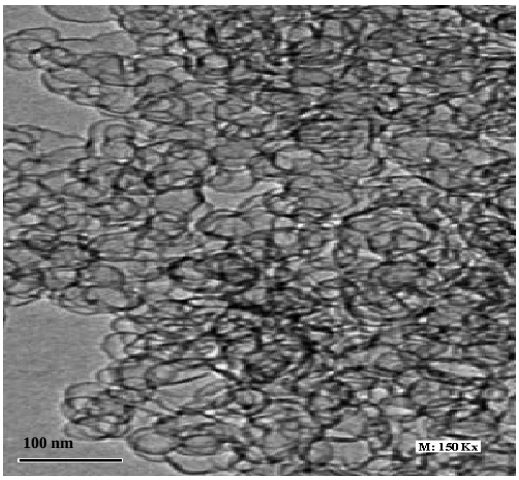


Fig. (10): Transmission Electron Microscopy image of activated charcoal

Table.8 The overall performance of the free and immobilized dextranase on CMC by cross-linking technique:

Technique	Form of dextranase	Protein mg/ml	Activity U/ml	Specific activity U/100mg protein	Retained activity (%)	Amount of isomaltose mg/ml
Cross-linking on CMC	Free dextranase	0.038	531.47	13986.05		0.38
• Untreated*	Immobilized dextranase	0.015/mg carrier	247.86	16524.00	46.6	0.49
• Treated*	Immobilized dextranase	0.013/mg carrier	234.86	18066.15	44.2	0.51

*Treated and untreated via ultrasonicator

The TEM of CMC untreated and treated carrier was shown in Figures (11 and 12). The particles size were about 350 and 130 nanometer, respectively.

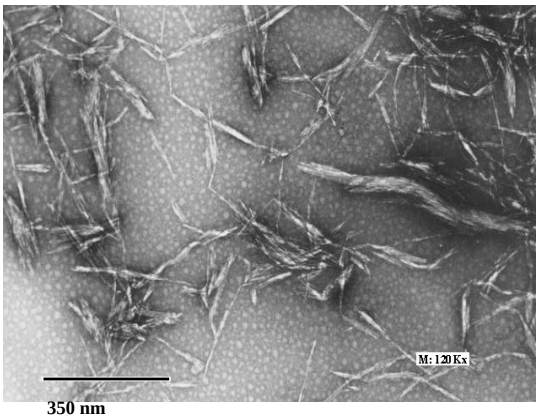


Fig. (11): Transmission Electron Microscopy image of untreated CMC

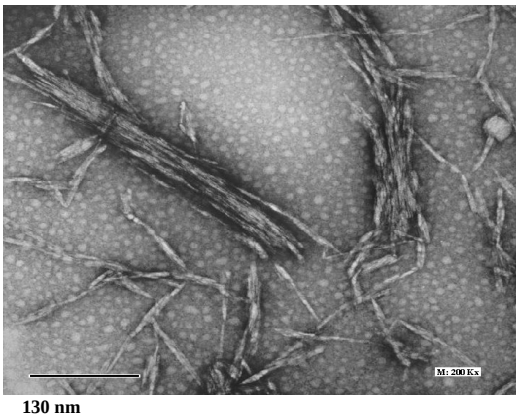


Fig. (12): Transmission Electron Microscopy image of treated CMC with ultrasonicator

n NH
mu

Carrier	Enzyme added (U/g carrier)	Unbound enzyme (U/g carrier)	Immobilized enzyme (U/g carrier)	Immobilization yield (%)
Oyster mushroom stem	547.65	431.52	34.88	30.03

Results in Table (10) indicated that the specific activity and isomaltose amount of the immobilized dextranase on stem decreased to 421.54 units/100 mg protein and 0.13 mg/ml, respectively, compared to the free one 14042.31 units/100 mg protein and 0.53 mg/ml, respectively. The retained activity of the immobilized dextranase by adsorption on stem unmodified was 1% of its original activity.

Table.10 The overall performance of free and immobilized dextranase by adsorption technique on oyster mushroom stem:

Form of dextranase	Protein mg/ml	Activity U/ml	Specific activity U/100mg protein	Retained activity (%)	Amount of isomaltose mg/ml
Free dextranase	0.039	547.65	14042.31		0.53
Immobilized dextranase on oyster stem	0.013/mg carrier	5.48	421.54	1	0.13

The morphology of oyster mushroom stem was measured by TEM. Results indicated that the particle size was 310 nm (Fig. 13).

Fig.13 Transmission Electron Microscopy image of oyster mushroom stem
Cross-linking technique:

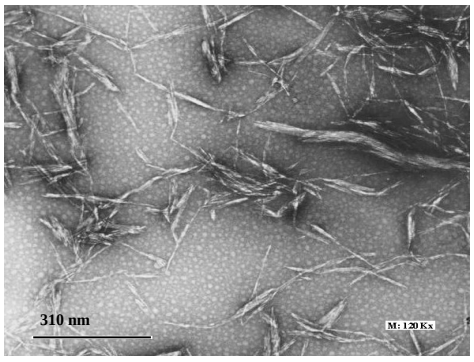


Fig.14 Cross-linking of enzyme at amino group by glutaraldehyde

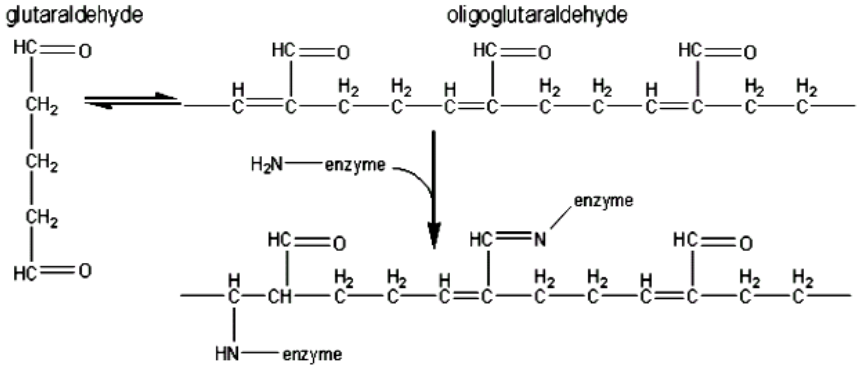


Table.11 Immobilization of *P. aculeatum NRRL-896* dextranase on oyster mushroom stem by cross-linking via amino groups, using glutaraldehyde

Carrier	Enzyme added (U/10g carrier)	Unbound enzyme (U/g carrier)	Immobilized enzyme (U/g carrier)	Immobilization yield (%)
Oyster mushroom stem modified by glutaraldehyde	547.65	128.74	321.59	76.77

Table.12 The overall performance of free and immobilized dextranase by cross-linking technique on modified oyster mushroom stem via amino groups using glutaraldehyde

Form of dextranase	Protein mg/ml	Activity U/ml	Specific activity U/100mg protein	Retained activity (%)	Amount of isomaltose mg/ml
Free dextranase	0.039	547.65	14042.31		0.53
Immobilized dextranase modified with glutaraldehyde	0.018/mg carrier	486.93	27051.66	88.9	1.67

Fig.15 Covalent binding of enzyme at amino group by cyanogen bromide

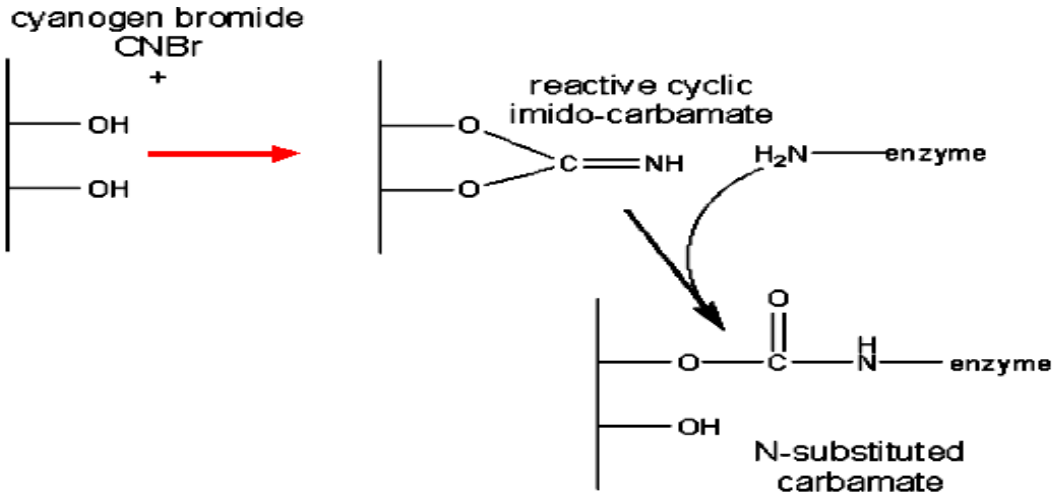


Fig.16 Binding of dextranase on activated oyster mushroom stem (carboxyl groups) by carbodiimide

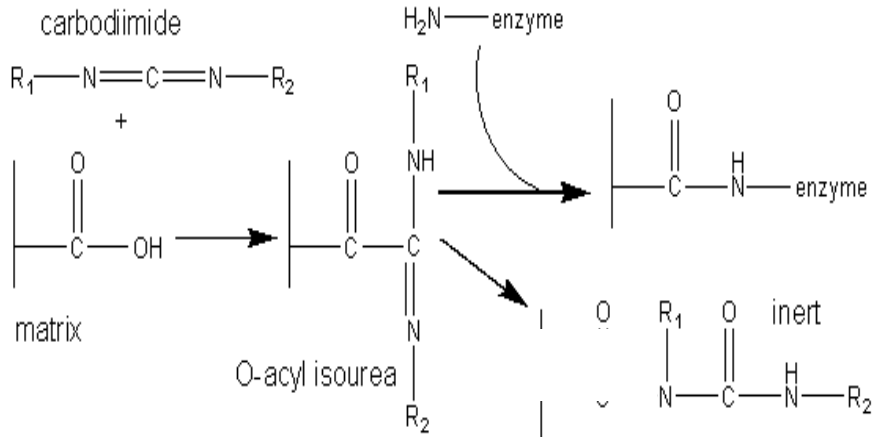


Table.13 Immobilization of *P. aculeatum NRRL-896* dextranase by covalent binding on oyster mushroom stem modified by cyanogen bromide (CNBr) or carbodiimide:

	Carrier	Enzyme added (U/10 g carrier)	Unbound enzyme (U/g carrier)	Immobilized enzyme (U/g carrier)	Immobilization yield (%)
1	Stem modified by CNBr	547.65	386.42	75.54	46.85
2	Stem modified by carbodiimide	547.65	294.48	137.49	54.31

Table.14 The overall performance of the free and immobilized dextranase by covalent binding on oyster mushroom stem modified by cyanogen bromide (CNBr) or carbodiimide:

Techniques	Form of dextranase	Protein mg/ml	Activity U/ml	Specific activity U/100mg protein	Retained activity (%)	Amount of isomaltose mg/ml
	Free dextranase	0.039	547.65	14042.31		0.53
Covalent binding on stem modified with CNBr	Immobilized dextranase	0.021/mg carrier	406.03	19334.76	74.1	0.98
Covalent binding on stem modified with carbodiimide	Immobilized dextranase	0.027/mg carrier	439.86	16291.11	80.3	1.08

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