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Purification and Immobilization of Dextransucrase from Immobilized *Lactobacillus acidophilus* ST76480.01

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Production of dextran by immobilized *Lactobacillus acidophilus* ST76480.01was intensively investigated. Adsorption was the most suitable immobilization technique for production of dextran and Luffa pulp was the preferred matrix recording (4.74 U/mg protein) of dextransucrase activity and (5.28 mg/ml) of dextran yield. During semi continuous cultivation, adsorbed cells on Luffa pulp retained about 84% of dextransucrase activity and 26% of dextran yield respectively at the 8th run. The fraction obtained at 65% ammonium sulphate saturation was the richest in its protein content contributing about 4.05% of the total recovered protein, and the highest recovery of dextransucrase activity, about 3.34 % of the culture supernatant. By immobilizing of dextransucrase on chitosan, the highest dextransucrase activity (5.06 U/mg protein) was obtained, achieving 4.4 fold increase than that of entrapping one in alginate, and 2.8 fold of the free enzyme. The enzyme showed a high thermal stability and retained about 60% and 33% of its original activity when heated for 30 min and 60 min respectively at 80°C. $MnCl_2$, $CaCl_2$ and KCl enhanced the activity for both free and immobilized enzymes, while ions, as $HgCl_2$, $BaCl_2$, $CuSO_4$ and EDTA, showed different degrees of inhibition of the tested dextransucrase activity.

Key words: Dextran, Dextransucrase, Purification, Immobilization.

Dextran is an extracellular bacterial polymer of d-glucopyranose with predominantly α(1→6) linkage in the main chain and a variable amount of α-(1→2), α-(1→3), α-(1→4) branched linkages[1]. Dextransucrase is the enzyme responsible for dextran production. Its action leads the formation of α (1-6) bonded linear dextran chains and release of fructose in the broth. Subsequently, different medium compositions and fermentation conditions have been used [2].

The use of immobilized cells for dextransucrase production has been reported. Several gel materials of synthetic or natural origin were used for microbial cell entrapment mainly in the form of beads. The most popular examples are entrapment into calcium alginate beads as reported by El-Sayed *et al*[3] to produce dextransucrase in three sequential cycles of semi continuous fed-batch fermentations. While Qader *et al*[4] studied the immobilization of cells of *Leuconostoc mesenteroides* KIBGE HA1 on acrylamide for the continuous production of dextran at various substrate concentrations and temperatures and also the immobilized cells for commercial production of dextran were reused. Also cell adsorption on different supports has been used successfully for immobilization. They have the advantages of porosity, ease of recovery and reuse[5]. It was shown that alginate beads immobilized dextransucrase could be used for the production of leucrose and isomalto- oligosaccharides with some success, that is, the activity of the enzyme in alginate beads decreased in every repetitive batch reaction due to the formation of dextran which cannot diffuse out of the beads[6]. The extracellular dextransucrase has been also immobilized on DEAE Sephadex A-50, Bio-Gel P2, cellulose acetate membrane, dextran-

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based resins using an affinity interaction, polysulfone hollow fiber and epoxy-activated acrylic polymers with different textural properties[7]. The most obvious benefits of immobilization are the capability of reusing the microorganisms, prolonged metabolic activities, protection of the organisms from inhibitory compounds or metabolites and easier separation of the products from the biocatalysts. Immobilization also facilitates the use of dense cell populations without alteration of the rheological properties of the suspending medium, thus higher rates of products biosynthesis may be obtained[8].

Purification methods such as salt or alcohol precipitation, fractionation by polyethylene glycol, ultrafiltration, chromatography and phase partitioning have been successfully employed for purification of dextransucrase from different strains of lactic acid bacteria[9,10].

MATERIALS AND METHODS

Microorganism

The strain used throughout this study was isolated from fermented vegetables in Egypt, identified as *Lactobacillus acidophilus* ST76480.01 The nucleotide sequences were analyzed with the BLAST database[11]. The sequence was deposited at GenBank with accession No ST76480.01, as was previously described[12].

Fermentation Medium Composition

For dextran production, the culture was grown in the broth medium previously optimized by Plackett and Burman statistical design with the following composition: (g/l): Sucrose, 150.0; bacto-peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 10.0; $MnCl_2.H_2O$, 0.01; NaCl, 0.01; $CaCl_2$, 0.05, this medium was modified from[13], pH was adjusted to 8.3 before sterilization at 121ºC for 15 minutes.

Maintenance of Culture and Inoculum Preparation

The isolated culture designated as *Lactobacillus acidophilus* ST76480.01 was streaked on medium agar slants and kept for incubation at 30±2ºC for 24 hr. These slants were kept at 4ºC for further experiments and were sub cultured monthly.

Cultivation Conditions

Fifty ml aliquot of the medium was dispensed in 250 ml Erlenmeyer flasks. Each flask was inoculated with 5ml bacterial suspension, obtained from 48 hours old slant culture, the flasks were then incubated at 30ºC for 48 hours. Unless and otherwise mentioned all experiments were carried at 30ºC under static conditions for 48 hours. For semi continuous production of dextran in flasks, the fermentation media were decanted at the end of 48 hrs incubation period, and fresh media were added under aseptic conditions.

Growth and Dextran Preparation

For recovery, After 48 hours, culture medium was precipitated using equal volume of chilled ethanol, shaken vigorously, centrifuged at 10,000 rpm for 15 minutes and the supernatant was decanted. Purified dextran was dried under vacuum over calcium chloride at 30ºC. The dextran yield was calculated on dry weight basis[14].

Determination of viscosity

Dextran solution (5%) was used as a stock solution for viscosity measurement at 25°C using an Ostwald Viscometer[15].

Estimation of Total Protein and Dextransucrase Standard Assay

Total protein was determined by the Lowry's method using bovine serum albumin as a standard[16]. Dextransucrase activity was determined by measuring the reducing sugar released from sucrose[17]. "One unit of enzyme activity was defined as the enzyme quantity that converts 1.0 milligram of sucrose into fructose and dextran in 1.0 hour under standard conditions" [18].

Immobilization of *Lactobacillus acidophilus* ST76480.01 cells by Entrapment in Ca-alginate and Agar-Alginate

Cells were entrapped in 4% calcium alginate gel beads as described by Eikmeier *et al.*[19], 4% gel solution (w/v) was prepared by dissolving 4 g Na-alginate in 90 ml distilled water (for preparation of alginate beads) or 2g agar and 2g alginate (for agar-alginate beads) in 90 ml distilled water, then autoclaved at 108ºC for 10 min. Ten ml cell suspension was added to the sterile gel solution. Ten ml of the gel-bacterial cell mixture were drawn with the aid of a sterile syringe, and allowed to drop through a hypodermic needle into a cross-linking solution (100 ml of 2% $CaCl_2$ solution in 250 ml Erlenmeyer flask) to obtain spherical beads (about 3mm in diameter). The beads were left in calcium chloride solution for one hour

and then washed several times with sterile distilled water. Beads resulted from 10ml alginate were added to 50 ml sterile medium in a 250 ml Erlenmeyer flask. The flasks were incubated on a rotary shaker (120 rpm) at 37°C.

Entrapment in Agar

The gel was prepared by dissolving 2g agar in 80 ml water. After sterilization, about 20 ml cell suspension was added and mixed well (at about 45.50°C). 10 ml of this mixture were aseptically poured into a Petri-dish. After solidification, the gel was cut with a sterile cutter into small cubes of about 0.5 cm in length that was subsequently transferred to 50 ml of the cultivation medium.

Immobilization of *Lactobacillus acidophilus* ST76480.01cells by Adsorption

Two ml cell suspension was added to the Erlenmeyer flasks containing 50 ml sterilized culture medium and sponge cubes (about 20 cubes), Luffa pulp, pumice and clay particles (about 20 particles). The flasks were incubated in a rotary shaker (120 rpm) at 37°C.

Partial Purification of Dextransucrase Enzyme produced by Immobilized *Lactobacillus acidophilus* ST76480.01 cells

The crude dextransucrase enzyme was obtained by conducting several fermentation processes with *Lactobacillus acidophilus* ST76480.01under the optimum cultural condition studied before. The whole culture filtrate was centrifuged at 7000 rpm at 4ºC and the clear supernatants were used for fractional precipitations. Several protein fractions were obtained by adding different concentrations of ammonium sulphate separately to a certain volume of the clear crude enzyme solution. After precipitation each fraction was dissolved in water and dialyzed against distilled water in refrigerator [20]. The protein content as well as dextransucrase activity were determined as mentioned before.

Immobilization of Dextransucrase by Adsorption

To fulfill enzyme immobilization, about 0.1 g (dry basis) of each of the supports as active charcoal, silica gel, carboxy methyl cellulose and chitin, were stirred in 0.06M phosphate buffer pH 8 in refrigerator for about 2 hours, with 5mg of the enzyme protein, in a total volume of 5 ml. the solid supports were then removed by centrifugation at 4000 rpm for 10 min in a refrigerated centrifuge, washed well with distilled water to remove the free enzyme. Washing was finally ended with 0.06M phosphate buffer pH 8, the adsorbed protein was calculated by the difference of protein taken for immobilization and protein left in combined liquor of washings[21].

Immobilization of Dextransucrase by Entrapment in Ca-alginate Beads

This method was carried out by Fraser and Bickerstuff[22]. 4% of sodium alginate was prepared by dissolving sodium alginate in distilled water then; 1 ml of the enzyme solution was mixed with 20 ml of alginate solution. The alginate-enzyme mixture was drawn with syringe and allowed to drop into 100ml of 0.15 M $CaCl_2$ as cross-linking solution. The flow was continued until desired numbers of beads have been formed. The beads were left for further 20-30 min before collecting using large filter paper, and then washed. The dextransucrase activity was estimated by resuspending the beads in the buffer containing the substrate. The immobilized enzyme was washed and assayed for enzyme activity after removing the beads.

Characterization of Free and Immobilized Dextransucrase Activity

The fraction purified by 65% ammonium sulphate was selected. The effects of enzyme concentration, sucrose concentration, temperature, incubation period, pH and on dextransucrase activity were studied. Thermal stability of free and immobilized enzyme preparations was studied at pH 8. Identical portions of each preparation were preheated separately in absence of the substrate for a period of 20, 30and 60 min. at different temperatures (40, 50, 60, 80°C). In each case, a control was carried out using inactivated enzyme solution. The effect of some metal ions on the activity of the free and immobilized enzyme was also studied under optimum conditions.

RESULTS AND DISCUSSION

Production of Dextran Polymer and Dextransucrase by Immobilized *Lactobacillus acidophilus* ST76480.01cells

Different gel materials namely; Na-alginate, agar-alginate, and agar were used for entrapment of *Lactobacillus acidophilus* ST76480.01cells .The gel-cell beads (or cubes) were used to inoculate 50 ml production medium. Results

shown in (Fig 1) indicated that immobilization by entrapment using alginate as the gel material showed the highest dextransucrase activity (4.27 U/mg protein), dextran production (3.22mg/ml) and medium viscosity (2.38) , but lower than those of free cultures (4.46 U/mg protein, 4.12 mg/ml and 1.89 respectively). Furthermore, different support materials namely; spong, Luffa pulp, pumice and clay particles were used for adsorption of *Lactobacillus acidophilus* ST76480.01 cells. Cells adsorbed on supports were used as previous to inoculate 50ml of production medium. Results showed that the optimal dextransucrase activity (4.74 U/mg protein), dextran production (5.28 mg/ml) and medium viscosity (2.6) were obtained by the use of Luffa pulp as a support, even more than that of free cells.

Scanning electron microscopy (SEM) of immobilized *Lactobacillus acidophilus* ST76480.01 showed an irregular pattern of the microorganism inside the pores of Luffa pulp as shown in (Fig. 2).

In practical utilization of bacterial cells entrapped in gel matrix, diffusion of essential nutrients, oxygen transfer, physical and chemical properties of the gel and immobilization procedure are the important factors affecting microbial metabolism and efficiency of the system. However, the fluctuation in the amount of dextran produced by *Lactobacillus acidophilus* ST76480.01cells entrapped in various gel matrices was attributed to variation in aeration and diffusion of nutrient in the immobilized cell system[23].

Indeed, immobilization of dextran from immobilized cells of *Leuconostoc mesenteroides* KIBGE HA1 using acrylamide as a support was studied by Qader *et al*[24] showing high conversion rate of sucrose to dextran by free cells with reference to immobilized cells, however El-Sayed *et al.,*[3] showed that cultures of free cells of *Leuconostoc mesenteroides* produced about 18% more total enzymatic activity than immobilized cells in calcium alginate beads, but about 64% less than immobilized cells on Celite R630. It is expected that larger amounts of enzymatic activity than measured are immobilized inside the alginate-coated Celite R630 and calcium alginate beads due to the mass transfer limitation conferred by the dextran product formed there in.

Semi Continuous Production of Dextran by Adsorbed *Lactobacillus acidophilus* ST76480.01 cells

Adsorption was the most suitable immobilization technique for production of dextran and the Luffa pulp was the preferred matrix. The effect of reusing adsorbed *Lactobacillus acidophilus* ST76480.01cells on the production of dextran using Luffa pulp as a support was investigated. For this purpose, cultures containing Luffa pulp were incubated for 48 hr in the production medium. At the end of each reuse, medium was decanted and fresh medium was added. Results in (Table 1) showed that by reusing cells adsorbed on Luffa pulp, a slow cell leakage was observed up to the 8th run, while protein content, dextransucrase, relative viscosity and dextran production decreased from the 1st run and continuo to decrease by repeated use till the 8th run.

Cycles of semi continuous operation (144 h) of immobilized cells produced more than three

Table 1. Semi Continuous Production of Dextran by Adsorbed *Lactobacillus acidophilus* ST76480.01 cells on Luffa pulp

Number of reuse	Final pH	Protein content (mg/ml)	Dextransucrase activity (U/mg protein)	Increase in medium viscosity %	Dextran production (mg/ml)
0	0.6	1.80	4.74	2.6	5.28
1	0.6	1.79	4.64	1.89	4.50
2	0.6	1.78	4.56	1.73	4.34
3	0.6	1.77	4.47	1.62	3.78
4	0.65	1.76	4.44	1.62	3.62
5	0.65	1.75	4.42	1.59	3.40
6	0.7	1.72	4.27	1.54	3.28
7	0.7	1.68	4.13	1.51	3.04
8	0.8	1.64	3.96	1.35	1.92

times as much dextran as free cells during one cycle (24 h) as reported by Elsayed *et al.,*[25]. Another investigation found by Qader *et al*[24] who estimated that the yield of dextran from free cells is higher than the immobilized cells of *Leuconostoc mesenteroides* (PCSIR-4) on acrylamide gel at 10% sucrose concentration. The free cells stop producing dextran after 144 hours, while immobilized cells continued to produce dextran after 480 hours.

Purification of Dextransucrase using Fractional Precipitation with Ammonium Sulphate

The crude dextransucrase enzyme preparation was obtained by conducting several fermentation processes with free *Lactobacillus acidophilus* ST76480.01cells under optimal cultural conditions. The combined culture supernatants were used for fractional precipitation. Several protein fractions were obtained by adding different concentrations of ammonium sulphate. The results in (Fig. 3) indicated that the fraction obtained at 65% ammonium sulphate saturation was the richest in its protein content (7.351 mg protein) contributing about 4.05% of the total recovered protein, and the highest recovery of dextransucrase activity (28.49 U) reaching about 3.34% of the culture supernatant. This fraction was selected for studying properties of the enzyme.

Chludzinski *et al.,*[20] used ammonium sulphate for precipitating dextransucrase from cultures of *Streptococcus. mutans* strain 6715. In contrast of this study, Robyte and Walseth[26] found that ammonium sulphate was completely ineffective in precipitating dextransucrase from culture supernatant concentrate of *Leuconostoc mesentroides* NRRL-512F cultures.

Optimization of Conditions for Assay of Dextransucrase Activity

The reaction conditions for free dextransucrase were optimized. The effects of enzyme concentration, sucrose concentration, temperature, incubation period and pH, on

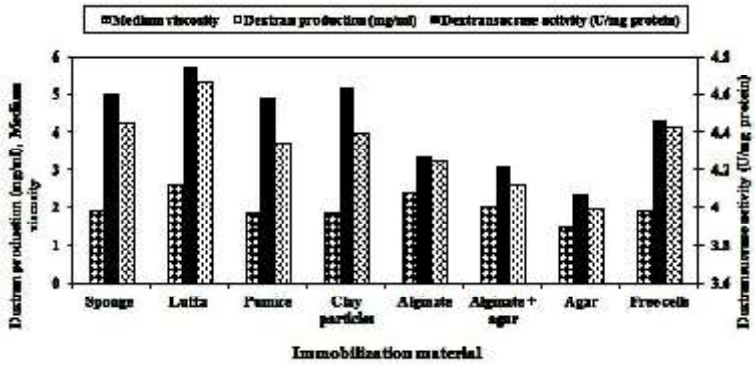

Fig 1. Production of Dextran Polymer and Dextransucrase Enzyme by Immobilized *Lactobacillus acidophilus* ST76480.01 cells

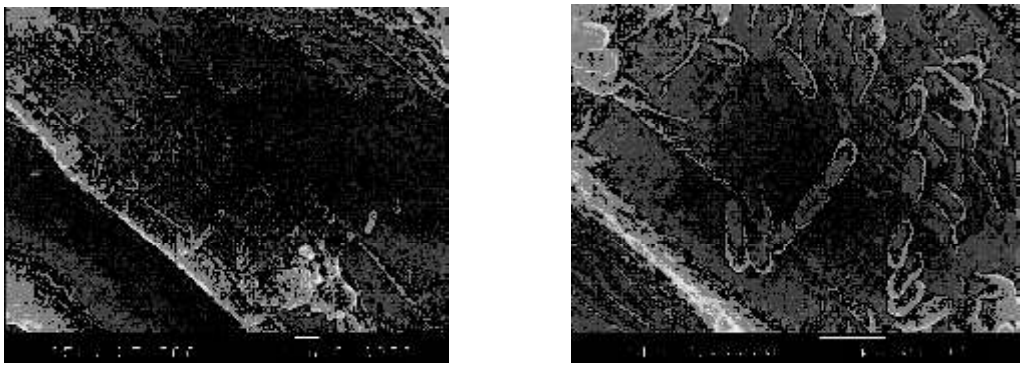

Fig 2. Scanning Electron Microscope Photographs showing *Lactobacillus acidophilus* ST76480.01 cells Adsorbed on Luffa pulp

dextransucrase activity were studied (Data not shown). The results showed that a parallel relationship occurred between the enzyme concentration and dextransucrase activity. The maximum specific activity (1.59 U/mg protein) was obtained at an enzyme protein concentration 1.05 mg/ reaction mixture. The results also indicated that a parallel relationship between substrate concentration and dextransucrase activity. The maximum dextransucrase activity was obtained at a substrate concentration of 10 mg/reaction mixture and the dextransucrase activity at this point reached (1.60 U/mg protein). The effect of temperature of the reaction on the activity of the enzyme indicated that 35ºC was the optimal reaction temperature for dextransucrase enzyme (1.7 U/mg protein). Loss of activity was observed at temperatures in excess of' that temperature, which is most likely due to thermal inactivation. The optimum temperature is similar to that reported for the dextransucrase activity of *Leuconostoc mesenteroides*[27]. The influence of the pH of the reaction on the activity of the enzyme was studied in the range of 3.6 to 8.0 (acetate buffer, 0.02M, and phosphate buffer, 0.02M). The results indicated that the optimum pH value was 8.0 giving 1.8 U/mg protein dextransucrase activity. This was contradicted with that found by other results that reported that the optimum pH=5.2–5.4 for dextransucrase of *Leuconostoc mesenteroides* NRRL B-512F[28] and *Leuconostoc dextranicum* NRRL B—1146[29].

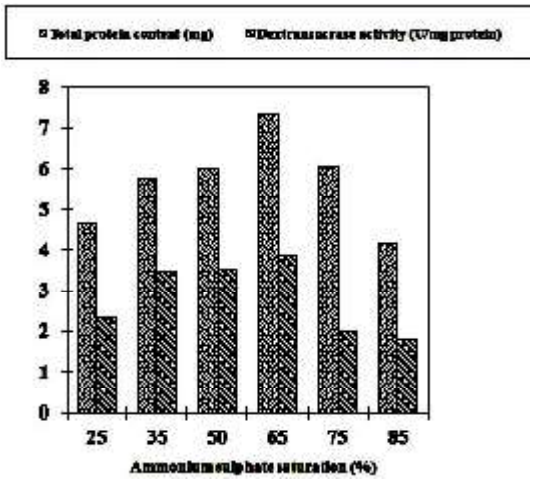

Fig. 3. Purification of Dextransucrase produced by *Lactobacillus acidophilus* ST76480.01 cells using Fractional Precipitation with Ammonium Sulphate

Immobilization of Partially Purified Dextransucrase by Physical Adsorption and Entrapment

The carriers of tricalcium phosphate, active charcoal, carboxy methyl cellulose, silica gel and chitosan were used for the adsorption process and alginate with 4% concentration for the entrapment process. In each case, retained protein and dextransucrase activity for the immobilized enzymes were assayed. The highest activity (5.06 U/mg protein) was observed with the immobilized enzyme on chitosan as shown in (Fig 4). On the other hand the lowest activity was observed with the entrapped enzyme in alginate (1.15 U/mg protein). Comparatively, the immobilized enzyme on chitosan was 4.4 fold of that of entrapped one in alginate, and 2.8 fold of the free enzyme.

Tanriseven and Dog[30] studied immobilization of dextransucrase in alginate fiber

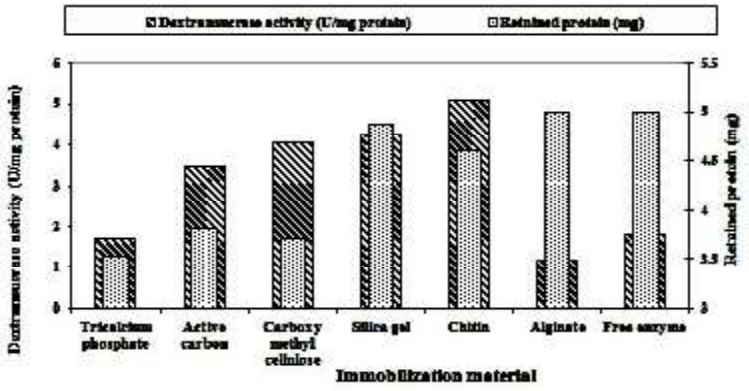

Fig. 4. Immobilization of Partially Purified Dextransucrase using Different Supports.

and in alginate beads; the first was superior to that in alginate beads in terms of immobilization yield and repetitive use. Several other workers studied immobilization of dextansucrase[31;4].

Thermal Stability of the Partially Purified Free and Immobilized Dextransucrase

The thermal stability of the partially purified enzyme was studied at pH 8. Identical enzyme solutions in phosphate buffer (0.02M) were preheated separately at different temperatures (40, 50, 60, 70 and 80°C) for various periods (20, 30 and 60 min). In each case, controls were carried out using inactivated enzyme solutions. The results in (Fig 5) showed that by exposing the enzyme to 40°C, the activity is slightly decreased. A further increase in temperature, led to a further decrease in stability of the enzyme. A treatment temperature of 80°C, led the enzyme to retain 60% and 33.17% of the original activity after 30 min and 60 min of exposure, respectively for free enzyme and 47.38% and 29.39% of the original activity after 30 min and 60 min of exposure for the immobilized one.

Qader *et al*.[7] found that maximum stability of immobilized dextransucrase was achieved at 25 ºC with respect to time. Also, De Segura *et al*.[32] found a notable stabilization effect at 30ºC was observed as a consequence of immobilization. After a fast partial inactivation, the dextransucrase immobilized on Eupergit C 250L maintained more than 40% of the initial activity over the following 2 days.

Effect of Some Metal Ions on the Activity of Partially Purified Free and Immobilized Dextransucrase

The enzyme activities were measured under optimum conditions. The results in (Fig 6) revealed that $MnCL_2$, $CaCL_2$ and KCL enhanced the activity for both free and immobilized enzymes. Among them, $MnCL_2$ and $CaCL_2$ showed 12.76 and

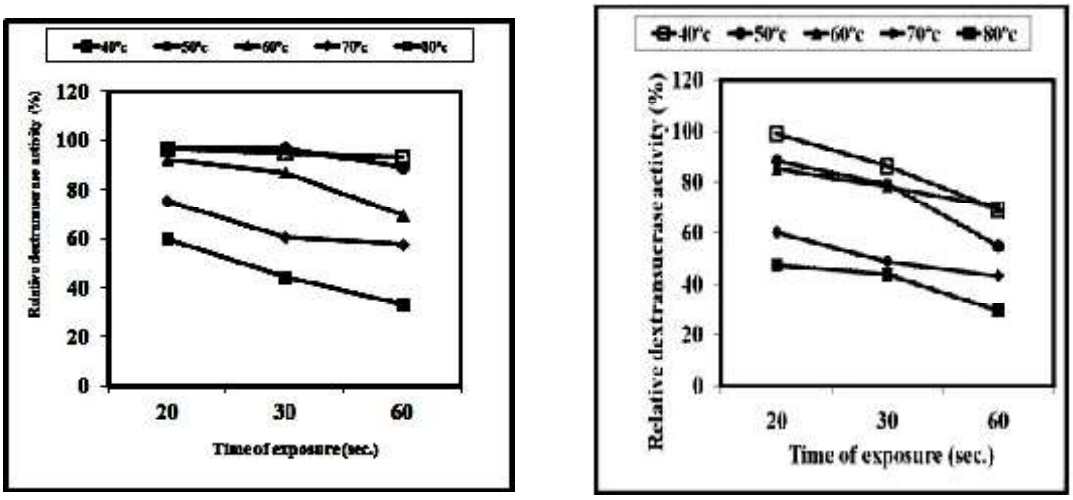

Fig 5. Thermal Stability of Partially Purified Free Dextransucrase (A) and Partially Purified Immobilized Dextransucrase (B).

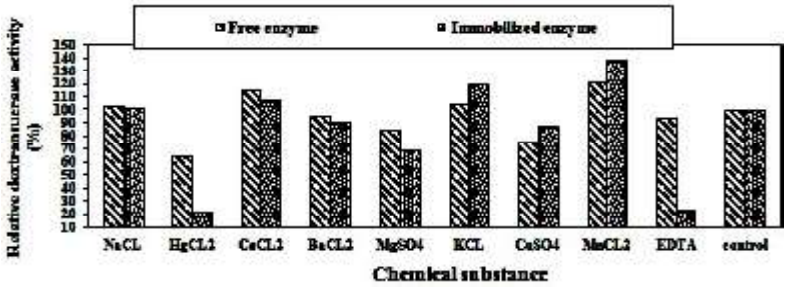

Fig 6. Effect of Some Metal Ions on the Activity of Free and Immobilized Partially Purified Dextransucrase.

16.67 % increase in activity of the free enzyme, respectively while $MnCL_2$ and KCL showed 37.47 and 18.73% increase of immobilized enzyme. Some other ions, as $HgCL_2$, $BaCL_2$, $CuSO_4$ and EDTA, showed different degrees of inhibition of the tested dextransucrase enzyme preparations. Among them $HgCL_2$ and $CuSO_4$ showed 34.44 and 24.44% inhibition of free enzyme, respectively and 78.23 and 12.85% inhibition of immobilized enzyme. On the other hand, EDTA and $MgSO_4$ showed 76.71 and 24% inhibition of the immobilized enzyme. It was generally noticed that in many cases the effect of ions was higher on immobilized enzyme preparation. This could be referred to accumulation of these substances on the surface of the support forming a concentration gradient which increased the effect of these ions on the enzyme. Chang *et al*[33] added $CaCl_2$ and NaN_3 to increase the stability of the enzyme. Metal ions as Mg_{2+} and Ca_{2+} salts stabilize the catalytic activity of dextransucrase from *P. pentosaceus* by stabilizing the three-dimensional protein structure while EDTA, a chelating agent, caused potent inactivation of dextransucrase, as reported by Patel *et al*[34].

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