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Purification and Characterization of an Extracellular Dextransucrase from *Pediococcus pentosaceus* Isolated from the Soil of North East India

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Summary

The extracellular dextransucrase produced from *Pediococcus pentosaceus*, a new isolate from the soil in Assam, India, was purified and characterized. The enzyme activity of cell-free supernatant was 3.4 U/mL and specific activity was 0.6 U/mg. The crude enzyme was purified by a single-step fractionation using polyethylene glycols of different molecular mass. The specific activity achieved was 18 U/mg with 31-fold purification by PEG 400 and 26 U/mg with 45-fold purification by PEG 1500. The molecular mass of dextransucrase determined by non-denaturing SDS-PAGE was approx. 180 kDa. The dextran formation activity of the enzyme was confirmed by activity staining. Optimum conditions for dextransucrase activity were: pH=5.4, reaction temperature 30 °C, 5 % sucrose and 20 mM sodium acetate buffer. A concentration of 1 mM $MgCl_2$ and 6 mM $CaCl_2$ enhanced dextransucrase activity by 5 and 150 %, respectively. The chaotropic agent urea (7 M) and chelating agent EDTA (1 mM) resulted in the residual enzyme activity of 98 and 80 %, respectively. The organic solvents such as ethanol (50 %), DMSO (90 %), acetone (50 %) and acetonitrile (20 %) decreased the dextransucrase activity by 80, 91, 94 and 80 %, respectively.

Key words: dextransucrase, polyethylene glycol, SDS-PAGE, activity staining, *Pediococcus pentosaceus*

Introduction

Dextransucrase (EC 2.4.1.5), the extracellular enzyme belonging to glycoside hydrolase family 70, is of immense industrial importance owing to its dextran- and prebiotic oligosaccharide-synthesizing properties (*1*). Dextrans can be used as viscosifying, stabilizing, emulsifying, sweetening, gelling or water-binding agents in food production as well as in the non-food industries (*2*,*3*). This enzyme is generally produced by lactic acid bacteria, *viz. Leuconostoc* and *Streptococcus* (*4*). *Pediococcus*, another lactic acid bacterium, may also produce dextransucrase (*5*). However, not much work has been done on the dextransucrase production by *Pediococcus*. This enzyme cleaves its substrate sucrose to release glucose and fructose. The free glucosyl moieties are polymerized to form a homopolysaccharide dextran. Dextran has numerous industrial and medical applications (*6*). 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 (*3*). Polyethylene glycol (PEG) fractionation is an effective, rapid and single-step purification method (*4*). The extracellular dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 was purified using polyethylene glycol (PEG 400) fractionation giving specific enzyme activity of 9.2 U/mg in a single step (*4*). The dextransucrase from *Leuconostoc dextranicum* NRRL B-1146 was purified using the same protocol, which gave specific activity of 4.5 U/mg (*7*).

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It has been reported that dextransucrase exists in either single or multiple forms having molecular mass in the range of 64–245 kDa (*8–14*). Dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 purified with PEG 400 showed multiple protein bands on SDS-PAGE with a prominent band corresponding to the size of 180 kDa (*4*). However, the same enzyme showed a single band on non-denaturing native PAGE (*15*). This result confirmed the multimeric entity of this dextransucrase, which remains in single molecular form in the native state and separates into multiple bands when denatured by boiling, SDS or 2-mercaptoethanol. Metal ions Ca^{2+}, Mg^{2+}, Fe^{2+} and Co^{2+} have been reported to stimulate the activity of extracellular dextransucrase from *Leuconostoc mesenteroides* strains, *viz.* NRRL B-1299, NRRL B-512F and NRRL B-1146 (*7,16–18*). Urea displayed deactivating effect on glucansucrase from *Leuconostoc dextranicum* B-1146 (*3*). EDTA, a chelating agent, caused potent inactivation of dextransucrase from *Leuconostoc mesenteroides* NRRL B--1299 (*16*). The effect of organic solvents on the glucansucrase activity was investigated and it was observed that different organic solvents, *viz.* dimethyl sulphoxide (DMSO) and bis(2-methoxyethyl) ether (MEE) inactivated dextransucrase to different extents (*19*). In the present study, purification of dextransucrase from *P. pentosaceus* isolate was carried out by polyethylene glycol fractionation. The dextransucrase enzyme activity was identified and confirmed by periodic acid Schiff (PAS) staining and the approximate molecular mass was determined. The reaction conditions for the enzyme activity were optimized. The effects of various salts, chaotropic agents and organic solvents on the enzyme activity were studied.

Materials and Methods

Culturing of Pediococcus pentosaceus

P. pentosaceus (GenBank accession number EU569832) isolate from the soil sample collected from a sugarcane field in Assam (near Guwahati, India) was screened for its dextransucrase activity (*20*). The isolate was maintained as a stab culture in modified MRS agar medium (containing 2 % sucrose by mass per volume) at 4 °C and subcultured every 2 weeks (*21*). For the development of inoculum, a loopful of culture from modified MRS agar stab was transferred to 5 mL of enzyme production medium as described by Tsuchiya *et al.* (*22*). This enzyme production medium consisted of (in % by mass per volume) sucrose 2, yeast extract 2, K_2HPO_4 2, $MgSO_4{\cdot}7H_2O$ 0.02, $MnSO_4{\cdot}4H_2O$ 0.001, $FeSO_4{\cdot}7H_2O$ 0.001, $CaCl_2$ 0.001, NaCl 0.001 and the pH was adjusted to 6.9 (*22*). The *P. pentosaceus* culture was incubated at 25 °C and 180 rpm for 12 h.

Production of dextransucrase from P. pentosaceus

One percent of the broth was again inoculated to 100 mL of enzyme production medium in a 250-mL Erlenmeyer flask and incubated for 16 h at 25 °C under shaking at 180 rpm. A volume of 1 mL of broth was withdrawn and centrifuged at 10 000×*g* for 10 min at 4 °C. The cell-free supernatant was analyzed for enzyme activity and protein concentration. All experiments were performed in duplicates for the accuracy of the results.

Enzyme activity and protein concentration assay

The assay of dextransucrase was conducted in 1 mL of a reaction mixture containing 20 mM sodium acetate buffer (pH=5.4), 5 % sucrose and 20 μL of cell-free supernatant as the enzyme source. The reaction mixture was incubated at 30 °C for 15 min. The enzyme activity was analyzed by estimating the reducing sugar following the Nelson-Somogyi method (*23,24*). The absorbance was recorded at 500 nm in a UV-VIS spectrophotometer (Varian, model Cary 100, Agilent Technologies, Palo Alto, CA, USA). The protein content of the cell-free supernatant and other purified protein samples were estimated by the method of Lowry *et al.* (*25*) using BSA as standard.

Purification of dextransucrase with PEG fractionation

For purification of dextransucrase, the cell-free supernatant was subjected to polyethylene glycol fractionation. Different percentages of the ice cold polyethylene glycols of various molecular mass (PEG 200, PEG 400 and PEG 1500) were added to 50 mL of cell-free supernatant. PEG 200 and PEG 400 were used in the range of 25–50 % and PEG 1500 was used in the range of 10–30 %. They were incubated at 4 °C for 12 h to allow the dextransucrase to fractionate. The mixture was centrifuged at 13 200×*g* for 30 min at 4 °C to separate the fractionated dextransucrase. The obtained soft pellets were dissolved in 1 mL of 20 mM sodium acetate buffer (pH= 5.4). The enzyme samples were subjected to dialysis using 5-kDa cut-off membranes. Purified enzyme samples were analyzed for enzyme activity and protein content, and used for further biochemical characterization.

Non-denaturing SDS-PAGE analysis of purified dextransucrase

SDS-polyacrylamide gel electrophoresis was performed with a vertical slab mini gel unit (Bio-Rad Laboratories, Hercules, CA, USA) using 1.5 mm thick gels (*26*). The dextran-synthesizing activity of dextransucrase was detected by conducting non-denaturing SDS-PAGE on 7.5 % gels (*27*). Two gels were run simultaneously under identical conditions. The loading dye buffer contained 0.0625 M Tris-HCl buffer (pH=6.8), 2.3 % (by mass per volume) SDS, 10 % (by mass per volume) glycerol and 0.05 % (by mass per volume) bromophenol blue, but it did not contain β-mercaptoethanol. The enzyme sample was mixed with 5× sample buffer in the ratio of 4:1. The heat denaturation step was omitted. The sample was loaded on 7.5 % acrylamide gel and the electrophoresis was carried out using running buffer (200 mM glycine, 0.1 % SDS, 50 mM Tris-HCl, pH=8.3) with a current of 2.5 mA per lane.

Identification of dextransucrase by periodic acid Schiff staining

After the completion of denaturing SDS-PAGE, one gel was stained with Coomassie Brilliant Blue dye for 40 min to fix the protein bands and destained by repeated washing with a solution of methanol, acetic acid and water in ratio of 30:10:50. Broad range protein marker (29–205 kDa) purchased from Bangalore Genei, Bangalore, India,

was used as standard. The other gel was subjected to SDS removal by incubating in sodium acetate buffer (20 mM sodium acetate, pH=5.4, 0.3 mM $CaCl_2$ and 0.1 % Tween 80) at 4 °C for 30 min. Then the gel was incubated in sodium acetate buffer (20 mM sodium acetate, pH=5.4, 0.3 mM $CaCl_2$) supplemented with 10 % sucrose at 30 °C for 48 h. Following incubation, the gel was washed once with a solution of methanol/acetic acid (50:10) in water for 30 min, then with water for 30 min, and incubated in a periodic acid solution (1 % periodic acid and 3 % acetic acid) at 30 °C for 45 min. After the periodic acid treatment, the gel was washed with water for 2 h with several changes. The gel was then stained with 15 mL of Schiff reagent composed of 0.5 % (by mass per volume) basic fuchsin, 1 % sodium bisulphite and 0.1 M HCl, until the magenta colour band within the gel matrix appeared, for confirmation of dextransucrase activity (*4*).

Optimization of conditions for assay of dextransucrase activity

The reaction conditions for dextransucrase were optimised. The effects of sucrose concentration, temperature, pH and ionic strength on dextransucrase activity were studied. The enzyme purified by 25 % PEG 400 having a specific activity of 18 U/mg was used. To study the effect of different concentrations of sucrose, the reaction was carried out in 1-mL mixture, containing 20 μL of enzyme (0.24 mg/mL) and various concentrations of sucrose, from 1–15 % (29.2–438 mM) in 20 mM sodium acetate buffer, pH=5.4. The reaction mixture was incubated at 30 °C for 15 min in a water bath and the activity was determined by estimating the released reducing sugar, as described earlier. To study the effect of pH, the dextransucrase activity assay was conducted at different pH values ranging from 4.2 to 6.0. The effect of different ionic strength of the buffer was studied by using various molar concentrations of the sodium acetate buffer (pH=5.4) ranging from 10–500 mM. Dextransucrase activity assay was conducted at different temperatures ranging from 20–40 °C to study the effect of reaction temperature.

Effect of salts, chaotropic and chelating agents on the activity of dextransucrase

The effects of salts, *viz.* $CaCl_2$ (0–10 mM), $MgCl_2$ (0–6 mM), chaotropic agent urea (0–7 M) and chelating agent EDTA (0–2.5 mM) on the activity of dextransucrase were studied. The purified dextransucrase with protein concentration of 0.24 mg/mL and specific activity of 18 U/mg was used. The enzyme assays were carried out in 1-mL reaction mixture containing salt or the chaotropic agent, sucrose (5 %) in 20 mM sodium acetate buffer (pH=5.4) and 20 μL of the enzyme. The enzyme activity was determined as described in the previous section. The effect of urea was studied by incubating the enzyme with urea at 30 °C for 30 min. The aliquots (20 μL) were taken and assayed for residual enzyme activity, as described in the previous section.

Effect of organic solvents on activity and stability of dextransucrase

The effect of dimethysulphoxide (DMSO), ethanol, acetone and acetonitrile on purified dextransucrase from *Pediococcus pentosaceus* was studied. A mass of 0.75 mg of lyophilized dextransucrase was dissolved in 50 % ethanol, 90 % DMSO, 50 % acetone and 20 % acetonitrile in 1 mL. An equal amount of enzyme dissolved in 1 mL of 20 mM sodium acetate buffer (pH=5.4) was taken as control. The residual enzyme activity was estimated as described in the previous section.

Results and Discussion

Purification with polyethylene glycol

The enzyme activity of the cell-free supernatant of *P. pentosaceus* was 3.4 U/mL and protein concentration was 5.9 mg/mL, giving a specific activity of 0.6 U/mg. The purification of crude enzyme by polyethylene glycol fractionation resulted in significant increase of the dextransucrase activity. The results of purification are presented in Table 1. The fractionation with 33 % (by volume) PEG 200 gave a maximum specific activity of 8.9 U/mg with 15-fold purification and 2.9 % overall yield. The fractionation with 25 % (by volume) PEG 400 gave the maximum specific activity of 18 U/mg with 31-fold purification and 8.5 % overall yield. The fractionation with 10 % (by volume) of PEG 1500 (60 %, by mass per volume) gave the maximum specific activity of 26 U/mg with 45--fold purification and 4.5 % overall yield. In the present study, PEG 1500 at lower concentration gave the best purification results. The purification of *P. pentosaceus* dextransucrase by PEG 400 gave more than double the specific activity in a single step when compared to purification of the enzyme from other strains (*4*,*7*,*12*).

Identification of dextransucrase and its molecular mass determination

The dextransucrase obtained after PEG purification was run on two identical non-denaturing SDS-PAGE gels

Table 1. Purification of dextransucrase from *P. pentosaceus* by fractionation with polyethylene glycols of different molecular mass

PEG	V / mL	Enzyme activity / U/mL	Total units	Overall yield / %	γ(protein) / mg/mL	m(total protein) / mg	Specific activity / U/mg	Purification fold
Crude	50	3.4	170	–	5.9	295	0.6	–
33 % PEG 200	1.6	3.1	5	2.9	0.35	0.6	8.9	15
25 % PEG 400	3.3	4.4	14.5	8.5	0.24	0.8	18	31
10 % PEG 1500	1.5	5	7.5	4.5	0.2	0.3	25.9	45

for *in situ* activity detection. One of the gels was incubated in 5 % sucrose for 48 h and stained with periodic acid Schiff (PAS) reagent. The location of activity staining was determined with a Coomassie Brilliant Blue stained gel as a control. A single magenta colour band appeared on the PAS-stained gel, which confirmed the presence of dextran formed on polyacrylamide gel (Fig. 1). The presence of magenta colour activity band indicated that the purified dextransucrase was active. The comparison of activity staining and gels stained with Coomassie Brilliant Blue identified the presence of dextransucrase and showed its approximate molecular mass of 180 kDa. The presence of other bands on the gels may be due to: (*i*) its existence in multiple molecular forms, as reported for dextransucrase from other strains (*4*,*15*), (*ii*) contaminating proteins or non-dextransucrase proteins, or (*iii*) proteolysed fractions of dextransucrase.

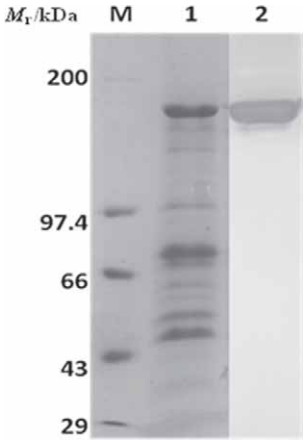

Fig. 1. Identification of PEG-purified dextransucrase from *P. pentosaceus*. Lane M: protein molecular mass marker (myosin from rabbit muscle 200 kDa, phosphorylase b 97.4 kDa, bovine serum albumin 66 kDa, ovalbumin 43 kDa, carbonic anhydrase 29 kDa), lane 1: non-denaturing SDS-PAGE with Coomassie Brilliant Blue staining, lane 2: non-denaturing SDS-PAGE with periodic acid Schiff staining of the formed dextran

Optimization of reaction conditions for dextransucrase activity

The effect of sucrose concentration on dextransucrase activity was studied by using various sucrose concentrations in the assay mixture, between 0.1 and 15 % (Fig. 2). The results showed that saturation of enzyme activity was reached at 5 % (Fig. 2a). It was observed that 5 % sucrose concentration gave the maximum enzyme activity of 4.4 U/mL and specific activity of 18 U/mg, which corroborates the findings on *Leuconostoc mesenteroides* NRRL B-640 (*28*), but deviates from the results of dextransucrases from *Leuconostoc mesenteroides* NRRL B-512F (*18*) and *Leuconostoc dextranicum* NRRL B-1146 (*7*). The effect of temperature on dextransucrase activity showed maximum at 30 °C (Fig. 2b). The enzyme activity gradually decreased after 30 °C. This result is supported by the findings that optimum temperature for dextransucrase activity from *Leuconostoc mesenteroides* B-512F is in the range of 30–35 °C (*29*).

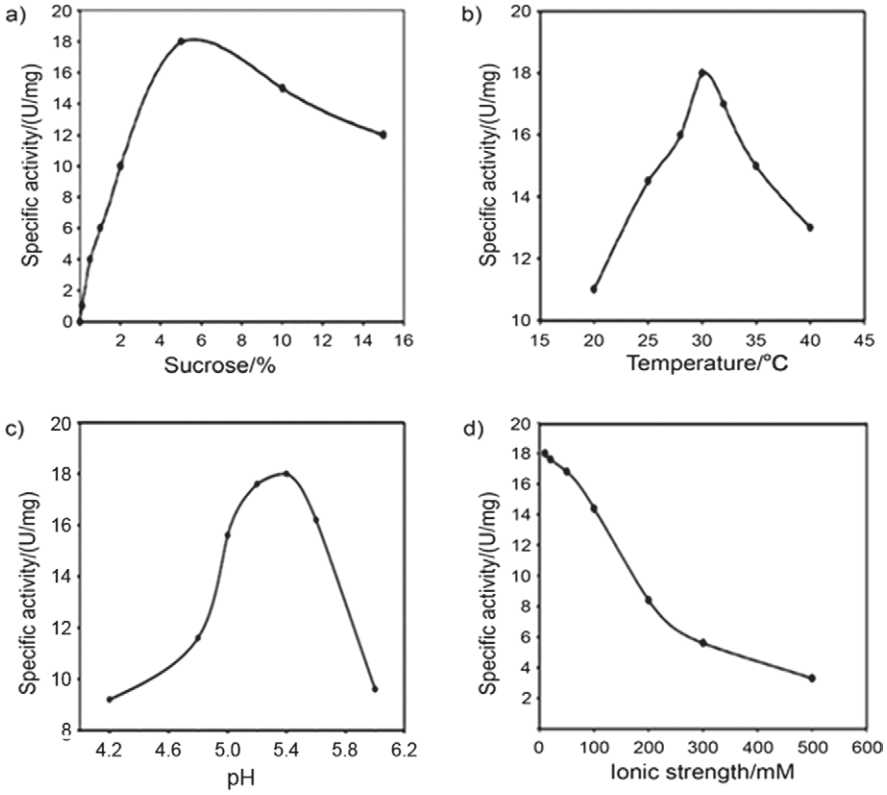

Fig. 2. Effect of: a) sucrose concentration, b) temperature, c) pH and d) ionic strength on dextransucrase activity from *P. pentosaceus* assayed in sodium acetate buffer

The effect of pH on dextransucrase activity showed the maximum at pH=5.4 (Fig. 2c). A 6 % reduction in the activity was observed at pH=5.2 and 11 % at pH=5.6. At pH=4.2, the activity was reduced to about 50 % and at pH=4.8, 39 % of the original activity was lost. The optimum pH=5.2–5.4 of dextransucrase was also observed for the strains *Leuconostoc mesenteroides* NRRL B-512F (*18*), NRRL B-640 (*28*) and *Leuconostoc dextranicum* NRRL B--1146 (*7*). The effect of ionic strength on dextransucrase activity showed that 10–20 mM was the optimum buffer concentration (Fig. 2d). Beyond 50 mM, the decrease in enzyme activity was rapid. The loss of enzyme activity at 200 mM was 50 % and at the concentration of 300 mM, the activity was about 33 %.

Effect of salts on the activity of dextransucrase

The effect of salts on the activity of dextransucrase from *P. pentosaceus* was studied as they are believed to affect the water structure of enzymes, thus affecting their solubility and activity (*30*). The effect of divalent metal ions Mg^{2+} and Ca^{2+} on dextransucrase from *P. pentosaceus* was studied. Mg^{2+} and Ca^{2+} salts stabilize the catalytic activity of enzymes by stabilizing the three-dimensional protein structure (*18*). The Mg^{2+} ions exhibited a marginal increase in the enzyme activity of dextransucrase. The enzyme activity increased by 5 % at 1 mM $MgCl_2$ (Fig. 3a). However, beyond 1 mM $MgCl_2$, the activity drastically decreased. At 6 mM $MgCl_2$, 25 % of the initial activity was lost. The Ca^{2+} ions significantly enhanced the enzyme activity of dextransucrase, which increased by 150 % at 6 mM $CaCl_2$ (Fig. 3b). Similar results were reported for *Leuconostoc mesenteroides* B-512F dextransucrase (*18*). The Ca^{2+} ion has been reported to be associated with the catalytic sites of dextransucrases (*31*).

Effect of chaotropic and chelating agents on the activity of dextransucrase

Urea inactivated dextransucrase at all concentrations ranging from 1 to 7 M. With an increase in the concentration of urea, there was a rapid decrease in enzyme activity. The enzyme lost 26, 48, 64, 72, 82, 88 and 98 % of its activity in the treatment with 1, 2, 3, 4, 5, 6 and 7 M urea, respectively (Fig. 4a). Similar pattern of activity loss was observed in glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 (*7*), where 5 M urea completely inactivated the glucansucrase. The addition of EDTA inhibited the dextransucrase activity from *P. pentosaceus*. Inactivation of 10 % was obtained with 0.5 mM EDTA and 75 % inactivation was obtained with 1 mM EDTA (Fig. 4b). The saturation was reached at 1.5 mM EDTA with about 20 % residual activity.

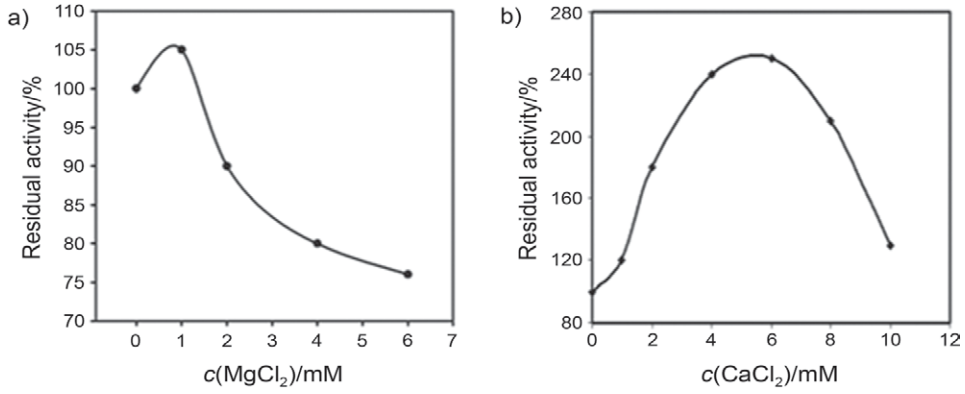

Fig. 3. Effect of: a) $MgCl_2$ and b) $CaCl_2$ on dextransucrase activity

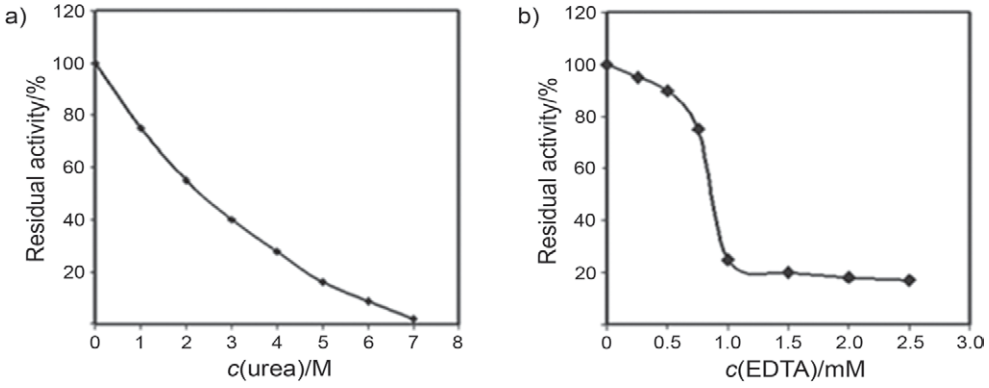

Fig. 4. Effect of: a) urea and b) EDTA on dextransucrase activity from *P. pentosaceus*. Dextransucrase (specific activity of 18 U/mg) in 20 mM sodium acetate buffer (pH= 5.4) was incubated with various concentrations of urea at 30 °C for 30 min, the aliquots (0.1 mL) were taken and the enzyme activity was estimated as described in Materials and Methods

Effect of organic solvents on the activity of dextransucrase

The behaviour of dextransucrase from *Leuconostoc mesenteroides* NRRL-B-512F in various organic solvents was studied by Girard and Legoy (*32*). This study was conducted to gain some insight in the solubility, activity and stability of acceptors in organic solvents, which may improve the interactions between the acceptors and enzymes, facilitating the acceptor reactions. The influence of various organic solvents on the stability of dextransucrase from *P. pentosaceus* was investigated. The enzyme activity loss in (%) ethanol 50, DMSO 90, acetone 50 and acetonitrile 20 was 80, 91, 94 and 80 %, respectively (Fig. 5). It has been suggested that the modifications in the enzyme structure by the organic solvents led to the activity loss (*32*).

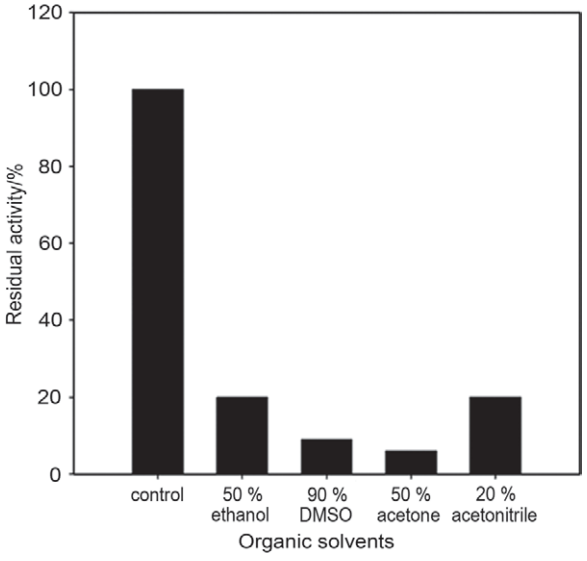

Fig. 5. Effect of different organic solvents on dextransucrase activity from *P. pentosaceus*. Dextransucrase (specific activity of 18 U/mg) in 20 mM sodium acetate buffer (pH=5.4) was incubated at 30 °C along with the organic solvents. The enzyme activity was estimated as described in Materials and Methods

Conclusions

Polyethylene glycol (PEG) fractionation method was used to purify dextransucrase from *P. pentosaceus*. A final concentration of 10 % PEG 1500 resulted in dextransucrase with maximum specific activity of 25.9 U/mg and 45-fold purification in a single step, whereas 25 % PEG 400 resulted in maximum specific activity of 18 U/mg with 31-fold purification. However, PEG 400 showed better yield than PEG 1500. The dextran-synthesizing activity of the purified enzyme was identified and confirmed by activity staining. The denaturing SDS-PAGE of the purified dextransucrase determined its approximate molecular size to be 180 kDa. The optimization of reaction conditions revealed that 5 % sucrose, 30 °C reaction temperature, 10–20 mM ionic strength and pH= 5.4 of the buffer were optimum for dextransucrase activity. The enzyme activity was enhanced by Mg^{2+} and Ca^{2+} ions, whereas urea, EDTA and organic solvents caused the loss of dextransucrase activity. In view of the ever increasing demand for dextrans and oligosaccharides, it is important to further purify and characterize the dextransucrase from *P. pentosaceus*.

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