Scientia Iranica, Vol. 9, No. 1, pp 39–46

SCIENTIA
IRANICA

Kinetic Studies of Dextranase Produced by Mutant of *Penicillium lilacinum* in Batch Culture

M. Shamsolahrar*, A. Kheirolomoom[1] and M. Farshbaf Dadjour[2]

In this paper, dextranase produced by a high dextranase-producing mutant strain of *Penicillium lilacinum* was purified and was considered for kinetic investigation. It has been shown that the optimum pH and temperature for enzyme activity were 4.5 and 55°C, respectively. The enzyme was stable at pH of 5.5-6 and temperatures up to 60°C during 45 min incubation. Under optimal conditions, the kinetic parameters of K_m and $V_{\max}$ were obtained as 5.78 mg/ml and 0.379 μmole maltose monohydrate/min, respectively. The effects of the most likely inhibitors present in the juice were also studied and it was found that glucose, sucrose, raffinose, galactose and dextran competitively inhibit the enzyme activity, while mixed-type inhibition was observed by maltose, pectin and galactronic acid. Finally, the enzyme activity was improved by addition of glycerol, sorbitol and egg albumen. The results were in good agreement with the experimental data obtained for the existing conditions in Bisotoon Sugar Factory.

INTRODUCTION

Dextranase [$\alpha-1$, 6-D-glucan-6-glucanohydrolase E.C. 3.2.1.11] is an enzyme produced by several microorganisms such as *Aspergillus, Penicillium, Fusarium, Chaetomium* and bacteria [1-4]. This enzyme hydrolyses the glucosidic $\alpha - 1,6$ bonds of dextran and, therefore, it can be used in sugar production processes to remove or reduce the destructive effects of dextran-contaminated juices [2,5].

To use the enzyme properly, the optimal conditions of reaction are required. Therefore, to reach the goal, kinetic information of the enzyme and its reaction characteristics were studied. Igarashi et al. had conducted similar studies on dextranase produced by *Streptococcus* species [1]. In another report, Sugiura investigated the hydrolyzing action of dextranase on the dextran and studied the effect of molecular weight of the substrate on K_m and $V_{\max}$ of the enzymes produced by *Brevibacterium fuscum* and *Penicillium funiculosum* [4]. Consequently, he reported that K_m and $V_{\max}$ values of dextranase produced by *B. fuscum* decrease with an increase in molecular weight of dextran; however, both values for dextranase produced by *P. funiculosum* were constant. He also reported the effect of immobilization on the dextranase activity [6]. In [7], the inhibition type of several substrate analogues on the enzyme produced by these microorganisms has been investigated. Kinetic models of this hydrolysis reaction and the role of affecting parameters have also been presented elsewhere [6,8,9].

In this research, dextranase produced by an improved strain of *P. lilacinum* at Iranian Research Organization for Science and Technology (IROST) was considered in order to obtain the optimum conditions for proper application. Kinetic parameters were obtained and the effects of several inhibitors on the enzyme activity were studied. Furthermore, to reduce the instability effects of enzyme dilution, which usually happens upon the enzyme application in sugar factories, the effects of the addition of stabilizing agents such as polyolic, proteinic and polymeric compounds were investigated [10,11].

MATERIALS AND METHODS

Dextran was purchased from Sankyo Company. All of the other materials used in this study were of analytical grade.

*. *Corresponding Author, Iranian Research Organization for Science and Technology (IROST), Tehran, I.R. Iran.*
1. *Sharif University of Technology, Department of Chemical Engineering, Tehran, I.R. Iran.*
2. *Zakaria Pharmaceutical Company, Department of Research and Development, Tabriz, I.R. Iran.*

Microorganism and its Maintenance

A high dextranase producing mutant of *P. lilacinum* was used throughout this research. Strain improvement of *P. lilacinum* ATCC 10114 was performed in the biotechnology department of IROST by physical and chemical mutation methods (UV, NTG). Selection was based on a random screening method. Produced strains were stored as lyophilized samples.

Production and Partial Purification of Dextranase

Enzyme was produced extracellulary by the microorganism in the shaking flask at 250 rpm. Mycelium of fungi were grown in a modified culture with the following composition in weight percent: dextran, 0.8; K_2HPO_4, 0.2; KH_2PO_4, 0.1; NH_4Cl, 1.5; KCl, 0.5 and C.S.P., 1.3 at pH of 5.5. After incubation for 72 – 90 hours at 30°C, the fermentation was terminated and the mycelium were separated by filtration. Then, the filtrate containing dextranase was partially purified by ion exchange chromatography on DEAE-sephadex G-200 (fine particles) and concentrated using an ultra filter with a 30 kD MW cut off membrane. A single band with high purity was observed on SDS-PAGE around 40 kD indicating that the enzyme was pure enough for kinetic studies. The isoelectric point of the partially purified enzyme was found to be 5.5.

Assay of Dextranase Activity

The substrate solution was prepared by dissolving blue-dextran in 10 mM McVain buffer with pH of 5.5 at a concentration of 20 mg/ml. An aliquot of 20 μl of the enzyme solution was added to 200 μl of the substrate solution. The reaction was carried out at 37°C for 10 min. The enzyme activity was measured by the aqueous two phase separation method [12]. The enzyme activity was calculated on the base of maltose monohydrate as equivalent to the amount of the reducing sugar released. One unit enzyme activity was defined as the amount of dextranase producing 1 μmol of maltose monohydrate in 1 min under the above-stated conditions. Furthermore, the enzyme activity was determined by a viscometer and Brigel-Muller equipment in Bisotoon Sugar Factory by which viscosity and filterability of the dextran-contaminated juices were measured before and after the enzymatic treatment.

Effect of pH and Temperature on Dextranase Activity

The enzyme was incubated in test tube at different pH values and constant room temperature; and different temperatures at constant pH of 5.5 for a period of 4[illegible] min. Then, an aliquot of 20 μl sample was taken from each test tube and added to the reaction mixture and the enzyme activity was measured for each sample.

Inhibitory Effects of Saccharides

To study the inhibitory effect of various saccharides on dextranase activity in sugar syrup of the Sugar Factory, the effects of the glucose, sucrose, raffinose galactose, dextran, maltose, pectin and galactronic acid were considered. Different concentrations of these compounds were added to the reaction mixture separately and the reaction was started by the addition of the enzyme.

Effect of Polyolic, Proteinic and Polymeric Compounds on Dextranase Activity

To improve the dextranase stability against the dilution which occurs upon its application in Sugar Factory, different compounds of polyolic, proteinic and polymeric compounds such as sorbitol, egg albumen, glycerol, gelatin, PVP, PEG and BSA at different concentrations were added to the enzyme solution. The effect of these compounds on dextranase were determined by measuring the initial rate of the reactions.

RESULTS AND DISCUSSION

Determination of K_m and V_{max}

Michaelis constant and the maximum velocity of the enzyme were calculated from Lineweaver-Burk plot by determining the initial velocities of enzymatic reactions at different concentrations of the substrate. According to the results obtained, K_m and V_{max} were determined as 5.78 mg/ml and 0.379 μmole maltose monohydrate/min, respectively (Figure 1).

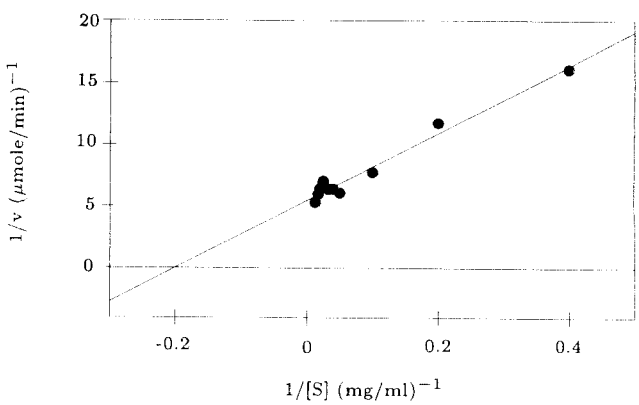

Figure 1. Lineweaver-Burk plot for different concentrations of blue dextran.

Effect of pH on Dextranase Activity and Stability

Inactivation and modification of enzymes due to extremes of pH is a common well-documented phenomenon in protein studies. The degree of inactivation can range from minor conformational changes to irreversible inactivation, depending on the incubation conditions. The important factor at either pH extreme is that once far away from a protein isoelectric point, electrostatic interactions between like charges within the protein molecule result in a tendency to unfold. Also, residues that are buried in a protein interior in non-ionized form can be ionized only if the protein unfolds.

Therefore, for investigation of pH stability, the enzyme was incubated at various pH values in the range of 4 to 8 at room temperature for one hour and the activity was monitored. Figure 2 shows that the enzyme is stable at pH range of 5.5 to 6.0 which is close to the isoelectric point of the enzyme found to be equal to 5.5. Increase in activity observed for other pH values may be due to the effect of pH on the dissociation constant of a particular group in the active center of the enzyme [8]. Figure 3 illustrates the effect of pH on the enzyme activity. A sharp increase in activity starts at pH of 4 and the highest activity is observed at pH of 4.5. Before and after the pH of 4.5, the activity sharply decreases which might be due to the denaturation of the enzyme. Therefore, the optimum pH activity was found to be 4.5.

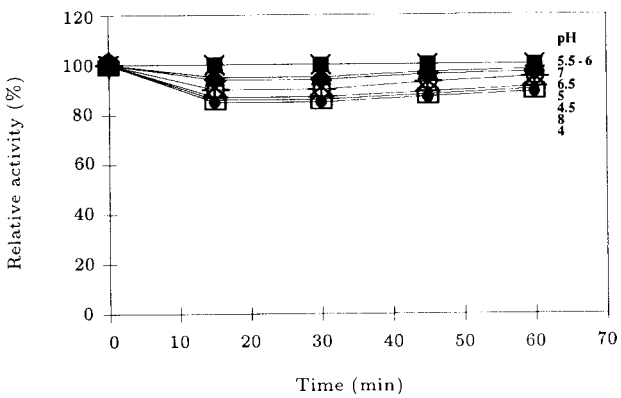

Figure 2. Effect of pH on enzyme stability at 25°C.

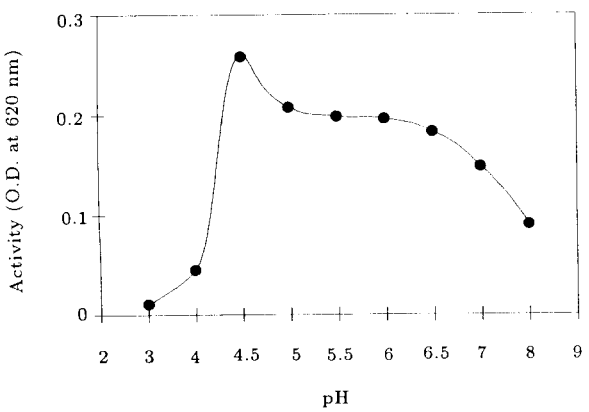

Figure 3. Effect of pH on enzyme activity at 50°C.

Effect of Temperature on Dextranase Activity and Stability

Thermal inactivation is perhaps the most frequently encountered and most thoroughly investigated mode of enzyme inactivation. Thermal stability was examined by incubation of the enzyme at different temperatures. As is shown in Figure 4, the enzyme retains 90% of its activity at temperatures of up to 60°C during 45 min incubation. According to Figure 5, the optimum temperature for the enzyme activity was also obtained in the range of 50–60°C.

It has to be mentioned that the same optimal conditions have been reported for dextranase produced by different sources [3,4,6]. It is obvious that the enzyme is stable within its active range and this determines the optimal conditions of the reaction. These results, which

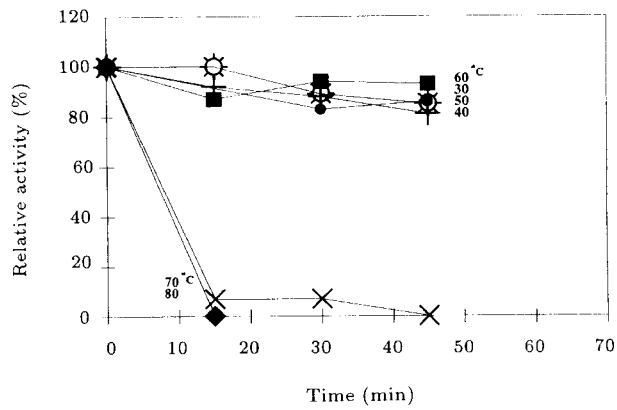

Figure 4. Effect of temperature on enzyme stability at pH of 5.5.

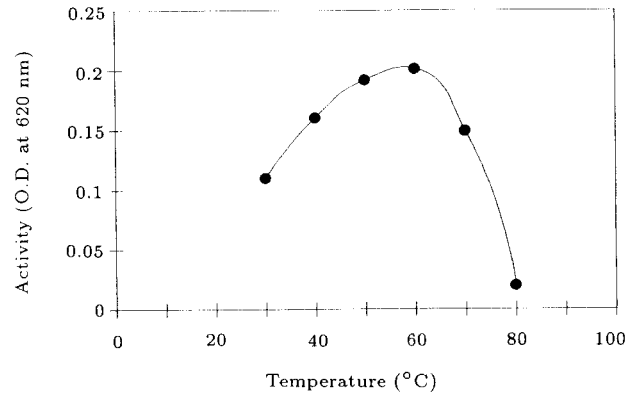

Figure 5. Effect of temperature on enzyme activity at pH of 5.5.

well match with the experimental data obtained in the Bisotoon Sugar Factory, offer optimum conditions for industrial usage [5].

Inhibition Study

Inhibition of enzyme within the juice could be one of the most important problems that might arise in industrial application. To deal with this problem, the effect of such inhibitors on enzyme activity was investigated. According to the composition of beet juice, most likely existing inhibitors, e.g., glucose, galactose, sucrose, dextran, pectin, galactronic acid, raffinose and maltose were considered in this study. Figures 6a to 6h present Lineweaver-Burk plot of different concentrations of inhibitors. It was deduced that glucose, sucrose, raffinose, galactose and dextran have a competitive inhibition on the enzyme activity and maltose, pectin and galactronic acid exhibit a mixed type inhibition. Among the inhibitors studied, pectin and galactronic acid had a strong effect on the enzyme activity; they inhibited the enzyme completely over the concentration of 0.1%. Mamuro et al. have reported the same results for dextranase produced by *Penicillium funiculosum* [7].

As shown in Figures 7a to 7h, comparing the amount of K_m for substrate with the amount of K_i for each inhibitor, it was demonstrated that K_i of each inhibitor was much higher than K_m of substrate. Therefore, it was deduced that their inhibitions are minor at high concentrations of the substrate. Consequently, there would be no problem in applying the enzyme to the contaminated juices with a high concentration of dextran. However, this inhibitory effect would be significant when the contamination of juice, caused by the dextran content, decreased due to enzymatic cleavage. Thus, an optimum reaction time should be considered in industrial usage [5].

Effect of Stabilizing Agents

Another factor that may severely affect the yield of the enzymatic reaction is dilution of the enzyme. Since the enzymes usually lose their activity at dilute conditions, the effects of stabilizing agents were investigated using polyolic, proteinic and polymeric compounds [11,13]. These reagents may stabilize the enzymes at specific concentrations and inactivate at other ranges. Therefore, the effects of several concentrations of sorbitol, egg albumen, glycerol, gelatin, PVP, PEG and BSA on the enzyme activity were determined. As shown in Figures 8a and 8b, sorbitol and egg albumen improved the activity of the enzyme to 60% at a factor of 0.7% and glycerol (Figure 8c) with a concentration of 0.3% stimulated the activity by 20%. PVP, PEG and BSA (Figure 8d-8f) had no significant effect on the activity over a broad range of the concentrations used Gelatin (Figure 8g) decreased the activity in the range of 0.3-2%. According to the results, it was declared that sorbitol, glycerol and egg albumen increased the enzyme activity up to 50%. These compounds probably preserve the active conformation of the enzyme Substances such as glycerol or sorbitol have a high content of hydroxyl groups; therefore, they are capable of forming multiple hydrogen bounds and acting like the solvent water. These reagents cause preferential hydration, a thermodynamic phenomenon resulting in a microscopic phase separation between protein and solvent as well as an increase in the surface tension of water [10,11,13].

By such formulation, it is possible to increase the yield of the enzymatic reaction and reduce the reaction time as well as the amount of required enzyme. However, in spite of thermal stabilization of other enzymes by such compounds [10], there was no considerable effect on the thermal stability of dextranase by them.

Figure 9 shows the effect of enzyme on the contaminated juice of Bisotoon Sugar Factory at different concentrations of dextran, which has been monitored by a viscometer. As is obvious, high viscous juice loses its viscosity due to the enzymatic cleavage of dextran during 20 min. This enzymatic treatment of juice also reduces the filterability factor successfully. These results reveal the successful application of the enzyme solution for industrial conditions (Figure 10) [5].

CONCLUSION

Dextranase produced by a high producing mutant of *P. lilacinum* was considered for kinetic investigation. Effect of pH and temperature on the enzyme activity and stability was studied and it was shown that the enzyme is stable at a pH range of 5.5 to 6, while it is active at pH values of 4 to 5. Optimum temperature for enzyme activity was also obtained in the range of 50-60°C and it was shown that the enzyme retains 90% of its activity under temperatures up to 60°C in 45 min. Therefore, it was concluded that optimum pH and temperature for proper application of the enzyme is 5 and 55°C, respectively. Under these optimum conditions, K_m and V_{max} values of the enzyme were determined as 5.78 mg/ml and 0.379 μmole maltose monohydrate/min, respectively. Inhibition studies demonstrate that glucose, sucrose, raffinose, galactose and dextran have a competitive inhibition on the enzyme activity, while maltose, pectin and galactronic acid exhibited a mixed type inhibition. Comparing the amount of K_m for substrate with the amount of K_i for each inhibitor, it was shown that K_i of each inhibitor was much higher than K_m of the substrate. Therefore, it was deduced that there would be no problem in applying the enzyme in contaminated juices with a high concentration of

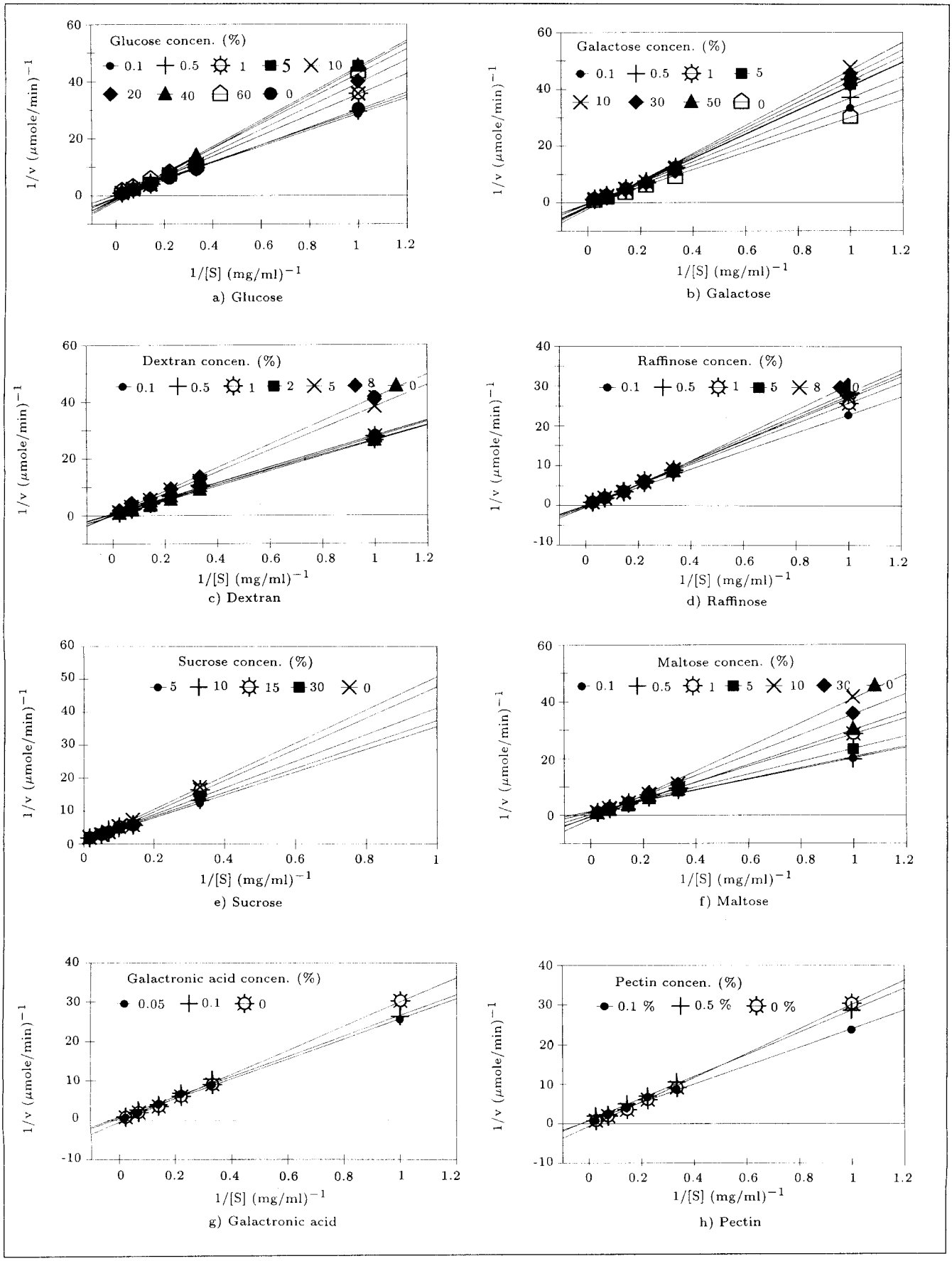

Figure 6. Linewcaver-Burk plots for different concentrations of inhibitors.

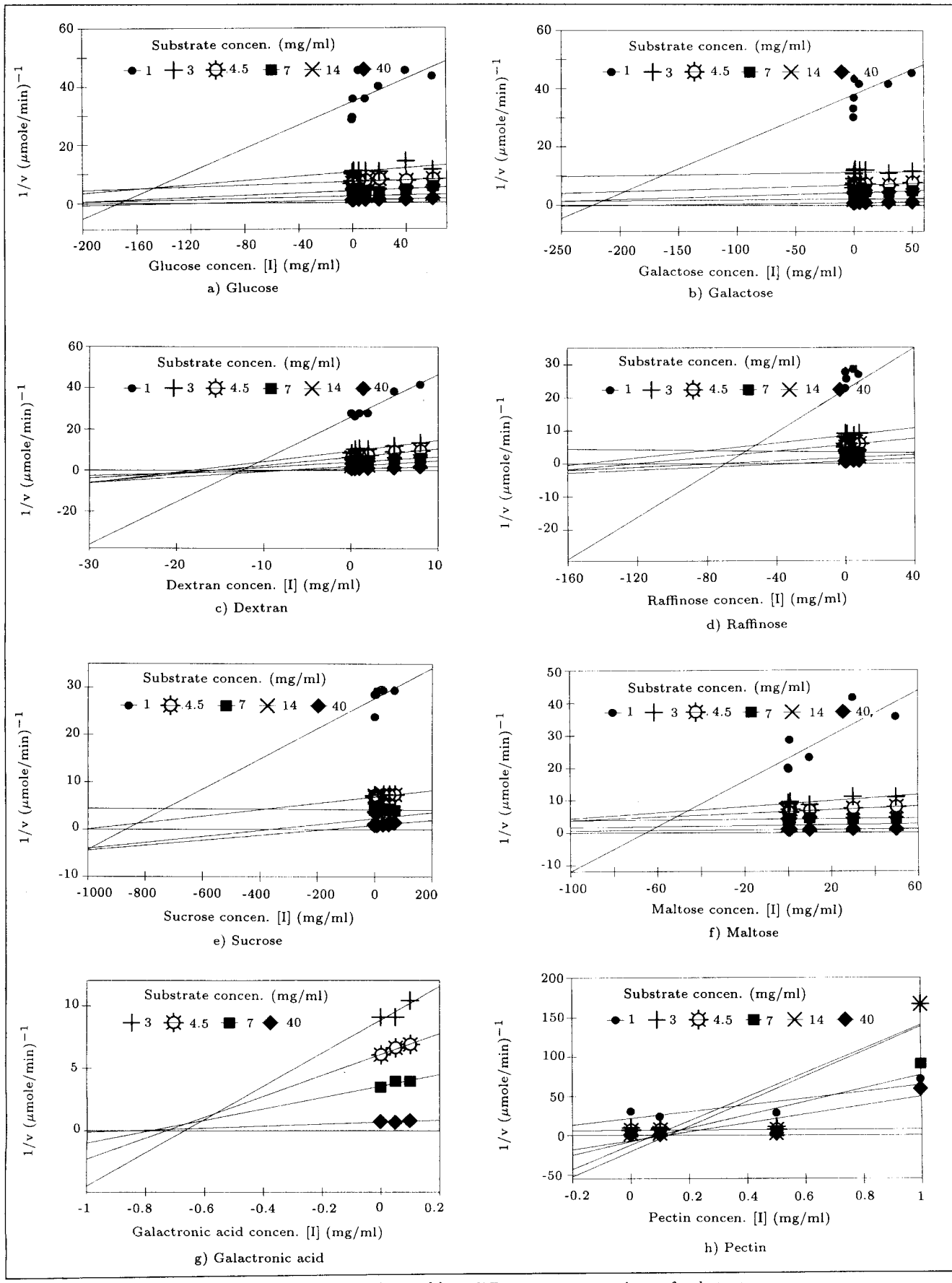

Figure 7. Plots of 1/v vs. [I] at different concentrations of substrate.

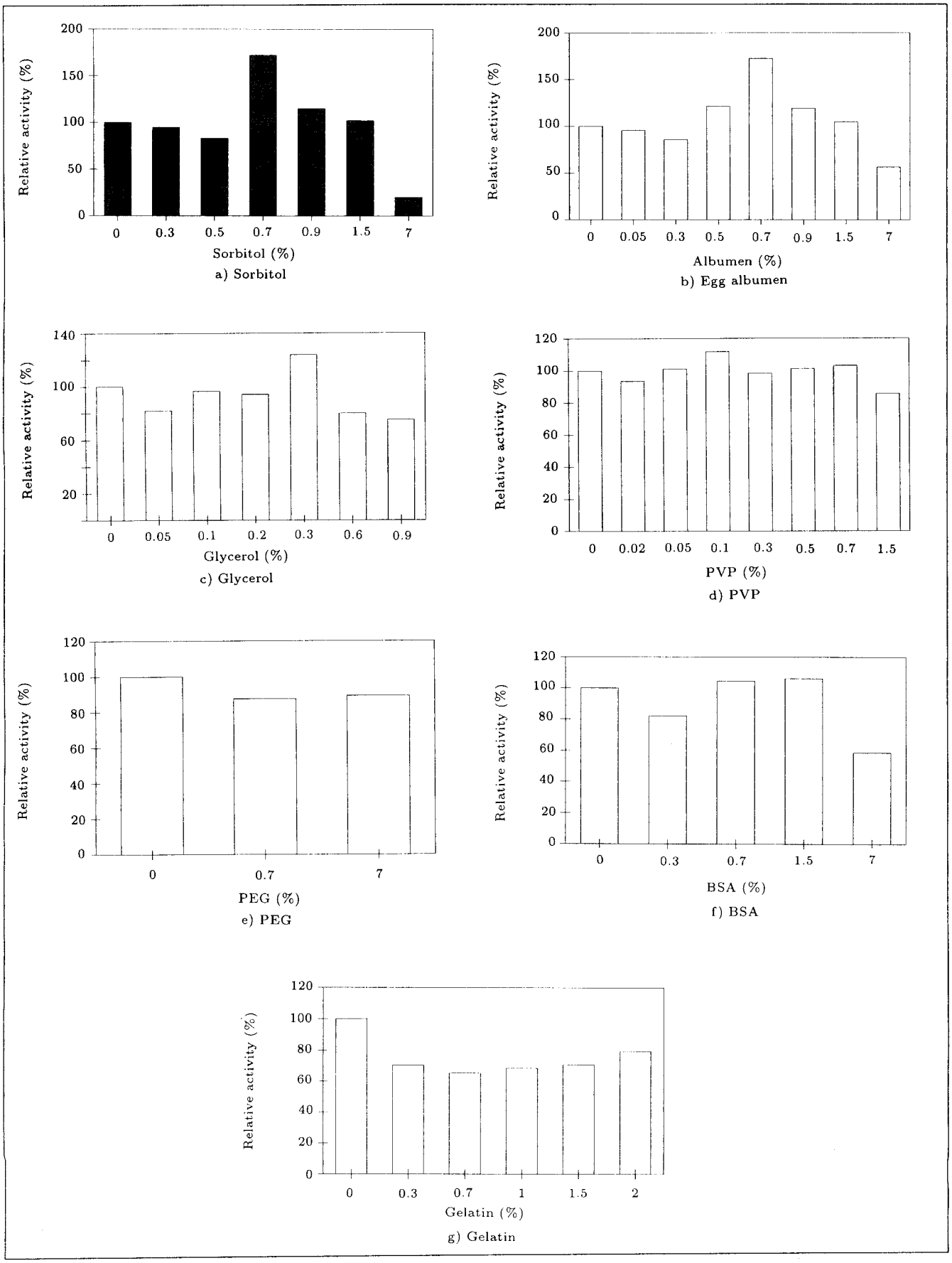

Figure 8. Effect of different concentrations of stabilizing agents on the enzyme activity.

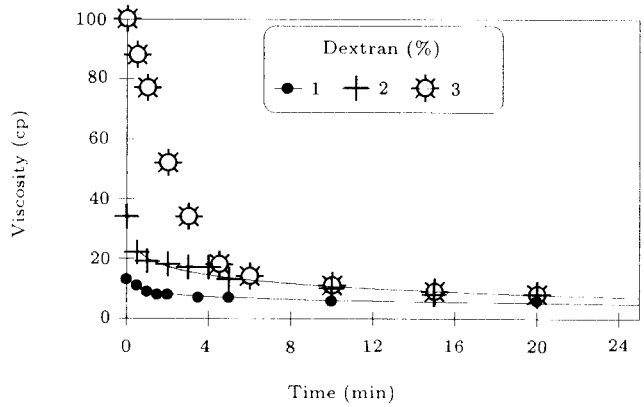

Figure 9. Effect of enzyme treatment on viscosity of the contaminated juice at different concentrations of dextran.

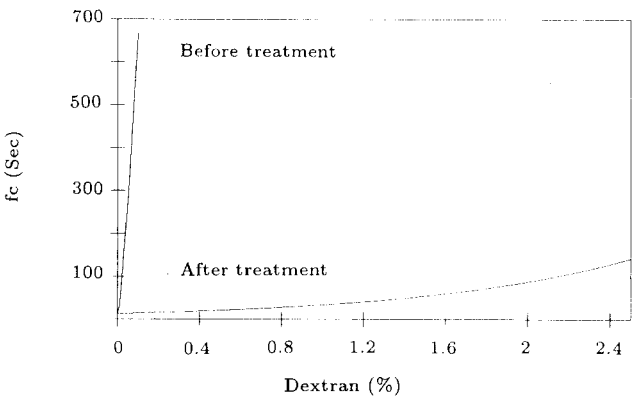

Figure 10. Effect of enzyme treatment on the filterability of the contaminated juice at different concentrations of dextran.

dextran. Finally, the effects of several stabilizing agents were investigated to improve the enzyme activity and it was shown that sorbitol, glycerol and egg albumen stimulate the enzyme activity at concentrations of 0.7, 0.3 and 0.7%, respectively.

ACKNOWLEDGMENT

The authors wish to express their gratitude to Dr. Moazami, the head of the biotechnology department of IROST and also to Mr. Bahmani, manager of Bisotoon Suger Factory for their sincere cooperation in conducting this research.

REFERENCES

1. Igarashi, T. and Yamamoto, A. et al. "Charaterization of the dextranase purified from *Streptococcus mutants*ingibritt", *Microbiol. Immunol.*, **36**(9), pp 969-976 (1992).
2. Koenig, D.W. et al. "Use of dextranase produced by *Lipomyces starqiy*", *J. Am. Soc. Sugar Cane Technol.*, **8**, pp 112-116 (1988).
3. Staat, R.H. and Schachtele, C.F. "Analysis of the dextranase activity produced by an oral strain of *Bacteroides ochraceus*", *J. Dent. Res.*, **55**(6), pp 1103-1110 (1976).
4. Sugiura, M. and Ito, A. "Studies on dextranase VII. The kinetic parameters of *Brevibacterium fuscum* dextranase and molecular properties of the digestion products", *Chem. Pharm. Bult.*, **23**(7), pp 1532-1536 (1975).
5. Shams, M. and Farshbaf, M., *Control of Dextran by Dextranase in Sugar Production Process*, Iranian Sugar Industries Publication, **128**(23), pp 54-59 (1998).
6. Wheatley, M.A. and Moo-Young, M. "Degradation of polysaccharides by endo- and exoenzymes: Dextran-dextranase model systems", *Biotech. and Bioeng.*, **19**, pp 219-233 (1977).
7. Sugiura, M. and Ito, A. "Studies on dextranase III. Action patterns of dextranase from *Penicillium funiculosum* on substrate and inhibition on hydrolysis reaction by substrate analogues", *Chem. Pharm. Bult.*, **22**(7), pp 1593-1599 (1974).
8. Greulich, K. and Ludwig, H. "pH dependence of dextranase-catalysed hydrolysis of dextran", *J. Chem. Research (S)*, Issue 1, pp 30-31 (1979).
9. Suga, K. et al. "Enzymatic breakdown of water insoluble substrates", *Biotech. and Bioeng.*, **17**, pp 185-201 (1975).
10. Graber, M. and Combes, D. "Effect of polyols on fungal alpha-amylase thermostability", *Enzyme Microb. Technol.*, **11**, pp 673-677 (Oct., 1989)
11. O'Fagain, C. et al. "Maintenance of enzyme structure: Possible methods for enhancing stability", *Process Biochemistry*, December Issue, pp 166-171 (1988).
12. Mattison, B. et al. "A simple method involving aqueous two phase separation system for activity determination of enzymes hydrolysing mocromolecular substrates", *Anal. Let.*, **73** (B10), pp 851-860 (1980).
13. Jaqess, P.A., *Stabilized Liquid Enzymatic Compositions*, U.S. Patent 5,356,800 (Oct. 18, 1994).