Production, purification, identification and characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640

A Thesis Submitted in Partial Fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY
in
Biotechnology

by

Ravi Kiran Purama

to the

Department of Biotechnology
Indian Institute of Technology Guwahati

September 2007

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Dedicated to

My Parents,
brother and sister

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India under the guidance of Dr. Arun Goyal.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

September, 2007. Ravi Kiran Purama

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

CERTIFICATE

It is certified that the work described in this thesis entitled "Production, purification, identification and characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640" by Mr. Ravi Kiran Purama for the award of degree of Doctor of philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

September, 2007.

Arun Goyal
Associate Professor
(Supervisor)

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

CERTIFICATE OF COURSE WORK

This is to certify that Mr. Ravi Kiran Purama has satisfactorily completed all the courses required for the Ph.D. degree program. These courses include

BT 601	Analytical Biotechnology
BT 602	Basic Biotechnology
BT 604	Enzymology
BT 605	Gene Therapy
BT 607	Plant Biotechnology
BT 608	Microbial Biotechnology

Mr. Ravi Kiran Purama was admitted to candidacy of PhD degree program after he successfully completed his Ph.D. qualifying examination in June 2005.

Dr. Siddartha Sankar Ghosh
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Course	Course Name	Credit	Grade
BT 604	Enzymology	6	AB
BT 605	Gene Therapy	6	AB
BT 608	Microbial Biotechnology	6	AA
BT 601	Analytical Biotechnology	6	BB
BT 602	Basic Biotechnology	6	AA
BT 607	Plant Biotechnology	6	AA

Cumulative Performance Index: (CPI): 9.33

September, 2007

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ACKNOWLEDGEMENTS

I express my profound gratitude and indebtness to my thesis supervisor Dr. Arun Goyal who guided me beyond the realm of research and taught me a way of life. Without his meticulous planning, incisive thinking and cogent advice this work could not have been assumed the form it is in today. Discussions with him have aided a long way in structuring and cohering the thoughts that lay random and unfocussed. Here I would take delight in acknowledging that he has helped me arm myself with the metaphor for perception of present thesis by developing the qualities of hard working, independence and self reliance. His suggestions, criticisms and constant encouragement helped me immensely to achieve this target. His true scientific spirit has helped me a lot during my research work. My everlasting gratitude goes to him.

I am grateful to Dr. Goyal for finding time out of his commitments to make himself available for thoughtful discussions at odd hours of the day. Interacting with him has whetted my intellectual appetite and opened new alleys of thought. At this juncture, I indeed feel fortunate to have come across a 'Guru' who inspires a quaint respect, personifying a philosophy of highest tradition which transcends beyond the text books to the sublime, comprehensively encompassing diverse facets of life.

I am extremely thankful to Dr. Goyal for his valuable suggestions, helpful discussions, encouragement and appreciation throughout the work. He has been a ceaseless source of inspiration and an epitome of patience during the phase I was struggling for tangible results. I remain grateful to him for all that he did for me.

I express my sincere thanks to Prof. A. T. Khan, Dr. A. Ramesh and Dr. L. Sahoo for their earnest suggestions and corrections for the nourishment of the work.

I feel pleasure in thanking Dr. G.K. Saini, Dr. K. Pakshirajan, Dr. V.V. Dasu, and Dr. R. Chaturvedi for their informative guidance, admonition, valuable suggestions and discussions and enthusiastic support during the work.

My sincere thanks are due to other faculty member of the department for their help, encouragement and providing facility for carrying out the experiments.

I remain thankful to my labmates Avishek Majumder, Shadab Ahmed, Dr. Mrs. Sangeeta Bharali Dutta, Deepmoni Deka, Gurutej Singh, Mayur Agrarwal, Biswanath Mahanty, Siva Kumar Solleti, Satish Kumar Singh, Gopinath Packirisamy and Atul Kumar Singh for the cheerful atmosphere in lab and for help in every aspect through out the research work.

Hearty thanks to Babu, Raju, Vishnu, Vijay, Mannem, Hariharanath, Nagaraju, Hariprasad, Sivasankar and Anna, for making my stay at IIT Guwahati memorable. Thanks to Chandramauli, Siva, Rajendra and many others I cannot mention here, with whom I have shared beautiful moments at IIT Guwahati. Life emerged out its shackles in the vegetative milieu of IIT Guwahati.

Sharing the Lab facilities and Instruments with some persons has been an affable pleasure, which will remain memorable to me. Each one a great soul, made the rigor of research appear so much more exciting!

I am thankful to Mr. Sharan, Nurul, Prarthana, Anita, Lepakshi and Rashmi for technical help and assistance. I sincerely thank the other staff members Mrs. Bandana and Krishna of Biotechnology Department.

Finally, no words would suffice to express my feelings for my parents, brother and sister consistently encouraging and advising me throughout my educational career and whose love and support have so luxuriously continued to enrich my life.

Ravi Kiran Purama
September 28, 2007

Synopsis

Introduction

Microbes produce an array of exopolysaccharides by various extracellular and cell bound enzymes. Microbial exopolysaccharides play major role in protection of the producer cells from unfavourable conditions, serve as nutrients in trophic metabolism of cells and also in interaction of cells with other organisms. They are produced mainly by hand in hand action of glycosyl, -hydrolases and -transferases. Dextransucrases (E.C. 2.4.1.5) are extracellular enzymes produced by lactic acid bacteria of the genus *Leuconostoc, Streptococcus* and *Lactobacillus.* The dextransucrases have been included in glycoside hydrolase family 70 based on their sequence homologies (http://afmb.cnrs-mrs.fr/CAZY/). *Leuconostoc* species is the major contributor of dextransucrase in industry. Dextransucrase catalyses the synthesis of glucose polymer called dextran from sucrose and liberates fructose.

$$\underset{\text{(Sucrose)}}{nC_{12}H_{22}O_{11}} \longrightarrow \underset{\text{(Dextran)}}{(C_6H_{10}O_5)_n} + \underset{\text{(Fructose)}}{nC_6H_{12}O_6}$$

There are several strains of *Leuconostoc mesenteroides* available and many of them have been extensively studied. However, there are still strains which are never explored and yet they may possess dextransucrase with higher activity and resulting in a type of dextran that may find potential use in industry. The dextran is produced on industrial scale by dextransucrase for its applications in pharmaceutical, food, agriculture and fine chemical industries. Isomalto-oligosaccharides synthesized as an acceptor products with dextransucrase acts as prebiotics. These isomalto-

oligosaccharides displayed the selective growth promotion of intestinal bacteria such as Lactobacilli and Bifidobacterium which beneficially affect host health. The iron and sulfate oligosaccharides displayed the potential of dental plaque control. The type of functional oligosaccharides is based on the type of linkage pattern of dextan synthesized by dextransucrase. There is an increasing interest to develop new oligosaccharides for application as prebiotics. Several strains of *Leuconostoc mesenteroides* have been extensively studied, however, there are no reports available on production of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. This strain produces a linear dextran with 97% α(1→6) glycosidic linkage, which makes it highly soluble. There is no information on nutrient and growth conditions of the strain and the dextransucrase produced by it. Therefore in the present study *Leuconostoc mesenteroides* NRRL B-640 was chosen for dextransucrase production. The microorganism was studied for nutrient, micronutrient and growth conditions requirement. The dextransucrase was purified and biochemically characterized. The functional characterization of dextransucrase elaborated by *Leuconostoc mesenteroides* NRRL B-640 was carried out.

Present work

The present investigations are carried out on the "Production, purification, identification and characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640". The thesis work comprises 8 Chapters. Chapter 1 is the General Introduction where the literature described is related to the characteristics of microorganism; the dextran produced and its applications; production, purification methods and properties of dextransucrase from Leuconostoc strains. It also contains a brief review of recombinant dextransucrase from different Leuconostoc strains.

The second chapter describes the characteristics of the microorganism *Leuconostoc mesenteroides* NRRL B-640. The antibiotic resistance, carbohydrate fermentation profile, plasmid profile and sucrose hydrolyzing activity or polysaccharide synthesis activity of *Leuconostoc mesenteroides* NRRL B-640 were determined. The strain was tested for susceptibility to thirty antibiotics using agar disc diffusion test. The antibiotic tests were performed using commercially available antibiotic octodiscs. The strain was resistant to the antibiotics co-trimazine, norflaxacin, norfloxacin and vancomycin and was sensitive to amoxycillin, bacitracin, carbenicillin, cephalothin, cephatoxamine, chloramphenicol, clindamycin, linomycin, oxytetracyclin and tetracyclin. The ability of the Leuconostoc strain to degrade and ferment carbohydrates with the production of acid was tested. The microorganism was tested for its ability to ferment 15 carbohydrates. It utilized fructose, glucose, mannose, melibiose, sucrose and trehalose and did not show any activity towards arabinose, lactose, mannitol and rhamnose. It was able to weakly ferment galactose and xylose. The plasmid profile of the strain was determined. *Leuconostoc mesenteroides* B-640 also showed a single faint band showing that it contained a single plasmid of approximate size 23kb. Polyacrylamide gel electrophoresis was used for *in-situ* detection of enzyme activity to characterize dextransucrase production by dextran-producing Leuconostoc strain. This study, however, was carried out to see if strain produced a similar or different glucan pattern that could be used to distinguish among the glucansucrase producing strains. It showed a faint band on Coomassie Brilliant Blue staining and produced one glucan-synthesizing activity band. The strain B-640 showed a single activity band of dextransucrase corresponding to the approximate size, 180 kDa.

The third Chapter is on optimization of dextransucrase production and its assay conditions. The aim of the study was to optimize the media composition and culture conditions for obtaining the maximum yields of dextransucrase. The optimum culture conditions for dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 were investigated. The dextransucrase production was studied in the temperature range, 22 to 30°C under shaking and static flask conditions. The optimum temperature for dextransucrase production was 25°C. The shake flask culture gave higher enzyme activity by 20% than the static flask culture. The dextransucrase activity of *Leuconostoc mesenteroides* NRRL B-640 was compared with *Leuconostoc mesenteroides* NRRL B-512F. The results showed that the maximum dextransucrase activity achieved by *Leuconostoc mesenteroides* NRRL B-640 at 25°C under shaking flask culture, was 15% higher than that the enzyme activity given by *Leuconostoc mesenteroides* NRRL B-512F grown at 23°C under the static flask culture. The conditions for maximum enzyme activity were optimized. The effect of sucrose concentration on the enzyme activity was studied with varying sucrose concentration between 0.1-10% final concentrations. The results showed that it follows the classical Michaelis-Menten kinetics and the saturation reached at 5% sucrose. The purified dextransucrase gave a maximum of 28.4 U/mg specific activity and K_m of 17.8 mM. The purified enzyme was maximally active with in the temperature range of 30-35°C. The enzyme activity drastically decreased after 35°C and it decreased by 35% at 40°C. The maximum enzyme activity was observed at pH 5.4. A 15% reduction in activity was observed on either side of the optimum pH 5.4 for maximum activity. The enzyme was stable below the pH 5.2, up to pH 4.6 and retained 85% of the initial enzyme activity. The effect of ionic strength on dextransucrase activity showed that

the dextransucrase activity was stable with in the range of 10-500 mM sodium acetate buffer pH 5.4.

The Chapter 4 describes the effect of nutrients on dextransucrase production. The nutrient effects on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 were studied using 'one-variable-at-a-time' approach. Interestingly, the increase in dextransucrase activity was significant (3-fold), from 4.8 U/ml to 15 U/ml with sucrose concentration increase from 2% to 7% in the enzyme production medium. The doubling of yeast extract concentration in the enzyme production medium from 2% to 4% resulted, 10% higher enzyme production. The increase in K_2HPO_4 from 2 to 3% gave 15% higher enzyme activity. Other nitrogenous sources like peptone and beef extract separately, enhanced the enzyme activity by 15%. Tween 80 (0.2%, v/v) enhanced dextransucrase production by 11%. Effect of micronutrients displayed a 10% increased dextransucrase production by individual addition of $MgSO_4$, $MnSO_4$ or NaCl. The present results show that nutrient requirements are not only species specific, but also strain specific. This strain required higher K_2HPO_4 and sucrose for higher enzyme production. *Leuconostoc mesenteroides* NRRL B-640 gave higher enzyme production under the experimental conditions as compared to most studied strains.

The Chapter 5 is devoted to the statistical approach to production of dextransucase. The aim was to enhance the dextransucrase production and determine the interaction between various medium components using response surface methodology. Statistical-based experimental designs were applied to optimize the production of dextransucrase by *Leuconostoc mesenteroides* NRRL B-640 using software package Minitab 15. Six medium components were examined for their significance on enzyme production using Plackett-Burman factorial design. The

medium components sucrose, yeast extract, K_2HPO_4, peptone, beef extract and Tween 80 were selected in their two levels of -1 and +1 according to the factorial design of Plackett-Burman. The effect of these medium components on enzyme activity and specific activity were determined. Sucrose, yeast extract, K_2HPO_4 and beef extract were found to significantly affect the enzyme production process. The combined effect of these nutrients on dextransucrase production was studied using a 2^4 full-factorial central composite design, having the four medium components at five levels of -2, -1, 0, +1, and +2. All other media components were kept at their middle level. A second-order polynomial was established to identify the relationship between the enzyme output and the four medium components. An R^2 (regression coefficient) of 0.96 and 0.91 was obtained for dextransucrase activity and specific activity respectively, explaining 96% and 91% variability in the response. The optimal concentration of variables for maximum dextransucrase production were sucrose, (30 g/l); yeast extract (18.9 g/l); K_2HPO_4 (19.4 g/l) and beef extract (15 g/l). The maximum enzyme activity and specific activity by predicted model was 10.9 U/ml and 0.64 U/mg. The maximum enzyme activity and specific activity obtained experimentally were 10.7 U/ml and 0.68 U/mg, respectively which, were in perfect agreement with the predicted values of the model.

The Chapter 6 reports the dextransucrase production by batch fermentation. The aim was to determine the effect of aeration and pH on dextransucrase yield and activity from *Leuconostoc mesenteroides* NRRL B-640 and to develop a model for its production in batch fermentation. Dextransucrase is obtained by fermentation of *Leuconostoc mesenteroides* NRRL B-640 using sucrose as carbon source. The production of dextransucrase in high yields is of great importance for dextran biosynthesis. There are no reports on dextransucrase production from *Leuconostoc*

mesenteroides NRRL B-640 using batch fermentation which was investigated in the present study. The production of dextransucrase was maximum at 1.5 vvm aeration rate. There was no significant effect of controlled pH runs on the enzyme activity as compared to the uncontrolled runs. The maximum specific growth rate increased with decrease in the pH of fermentation from 7 to 6 of controlled pH runs. Maintaining higher phosphate concentration (0.3 M) in the medium also did not have significant effect on the enzyme activity as compared to the control run with 0.11 M K_2HPO_4. The dextransucrase production was dependent on the cell growth. The mathematical models were developed for cell growth and enzyme activity which gave the best fits of the experimental data.

The Chapter 7 describes the purification, identification and confirmation of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. The extracellular dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 was purified using polyethylene glycol fractionation (PEG) and gel-filtration. The cell free extract was subjected to fractionation by PEG-200, 400 and 1500. The 10% (w/v) PEG-1500 gave dextransucrase with maximum specific activity of 23 U/mg with 40 fold purification in a single step. The purified enzyme showed multiple molecular forms on SDS-PAGE however, the same sample showed a single band on non-denaturing native-PAGE. The purified dextransucrase fractions obtained from PEG-1500, confirmed the presence of dextran, when run on SDS-PAGE under non-denaturing gels for *in-situ* activity detection by Periodic Acid Schiff staining. The activity bands corresponded to the native and active form of the purified dextransucrase of approximately, 180 kDa molecular size, when compared to the bands that appeared on the SDS-PAGE gels stained with Coomassie Brilliant Blue. No bands appeared after staining the activity of dextransurase on non-denaturing SDS-PAGE gels with raffinose, which excluded

the presence of fructosyltransferases. Further purification of 10% PEG-1500 purified dextransucrase by gel-filtration using Sephacryl S200HR column gave dextransucrase with specific activity of 35 U/mg with 61 fold purification.

The last Chapter 8 is devoted to the characterization of purified dextransucrase and dextran from *Leuconostoc mesenteroides* NRRL B-640. Thermo-stability experiment was carried out by incubating the enzyme at different temperatures for 15 min and the aliquots were assayed at 30°C using 5% sucrose in 20 mM Sodium acetate, pH 5.4. Dextransucrase was stable at lower temperatures (10-30°C) but rapidly lost the enzyme activity at temperature higher than 30°C. The addition of divalent cations such as $CaCl_2$, $CoCl_2$ and $MgCl_2$ had an enhancing effect on the dextransucrase activity. 22% higher dextransucrase activity was obtained by 4 mM $CoCl_2$. EDTA had an inhibitory effect and 50% of the dextransucrase activity was lost by the addition of 1 mM EDTA. Urea denatured the enzyme and caused 50%, 70%, 90% and 98.5% loss of activity in 40 min when treated with 1M, 2M, 3M and 4M concentrations, respectively. The storage stability of enzyme was determined at various temperatures. The enzyme lost 98% activity with in 4 days at 30°C but, retained 75% of activity at 4°C after 14 days and retained 95% of activity at -20°C after 14 days. Various stabilizers were screened to stabilize dextransucrase against the activity losses. Amongst Tween 80, glycerol, PEG-8000, dextran (500 kDa) and glutaraldehyde, Tween 80 provided the maximum stability at all temperatures. In the presence of Tween 80 the enzyme lost only 10% activity up to 24h but, it lost 60% activity with out any stabilizer at 30°C. The dextran produced by dextransucrase of *Leuconostoc mesenteroides* NRRL B-640 was purified and characterized. The purified dextransucrase (1%, v/v) with specific activity of 23 U/mg was added to 5% sucrose solution in 20 mM sodium acetate buffer (pH 5.4) containing 0.3 mM $CaCl_2$

and incubated at 30°C for 24h. Dextran was recovered by repeated alcohol (60%) precipitation. The characterization of dextran was carried out by optical rotation, FT-IR, ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) techniques. Optical rotation of +96.1 was observed at sodium D-line (c 0.5, 25°C). The FT-IR spectrum was used to investigate the functional groups of purified polysaccharide. The band in the region of 3400 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide. The band in the region of 2930 cm^{-1} was due to C-H stretching vibration and the band in the region of 1639 cm^{-1} was due to carboxyl group. The absorption peak at 906 cm^{-1} indicated the existence of α-glycosidic bond. The main characteristic peaks found in the FT-IR spectra of dextran at 1154, 1103 and 1020 cm^{-1} are due to valent vibrations of the C-O and C-C bonds and deformational vibrations of the CCH, COH and HCO bonds. The band at 1154 cm^{-1} is assigned to valent vibrations of C-O-C bond and glycosidic bridge. The peak at 1103 cm^{-1} is due to the vibration of the C-O bond at the C-4 position of glucose residue. The presence of peak at 1020 cm^{-1} is due to the great chain flexibility present in dextran around the α(1→6) glycosidic bonds. The dextan synthesized by purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 showed six prominent ^{13}C-NMR resonance peaks at 97.867, 71.563, 73.563, 69.660, 70.335 and 65.706 ppm which is characteristic of linear dextran. The branching contributes additional resonances to the ^{13}C-NMR spectra of dextrans in the anomeric region (95 -105 ppm) and the 80–85 ppm, which was absent in the ^{13}C-NMR of dextran from *Leuconostoc mesenteroides* NRRL B-640 dextran. The major resonance in the anomeric region occurred at 97.46 ppm showing that the C-1 is linked. An equally intense signal at 65.70 indicated that the C-6 is also linked. All the results showed that the dextran generated by dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 is a linear dextran with α-D(1→6) glycosidic bonds.

CONTENTS

Chapter 1

General Introduction

Microbes produce an array of exopolysaccharides, which form a biofilm around the cells facilitating attachment of the cells to surface, colonization and providing protection against unfavourable conditions. Xanthan, alginate, pullulan, dextran, alternan, levan and inulan are some of the examples of exopolysaccharides. Dextran, alternan, levan and inulan are produced by a group of bacteria belonging to *Lactobacillus* family. These bacteria utilize the sucrose as an inducer for the extracellular enzymes which synthesize these polymers. The extracellular enzyme breaks the sucrose to glucose and fructose, where the glucose gets polymerized to dextran or alternan while fructose is used as energy source by the exocellular or cell membrane bound enzymes or *vice versa* in the case of fructose polymer (levan or inulan) synthesis. The dextransucrases (also called glucansucrases or glucosyltransferases) have been included under glycoside hydrolases and based on their sequence homologies grouped into 110 families (http://www.cazy.org/fam/acc_GH.html). The enzyme dextransucrase belongs to glycoside hydrolase family 70. The dextransucrase consists of N-terminal two-third catalytic domain and C-terminal glucan-binding domain containing a series of direct repeating units (Funane *et al.* 1998). It is a member of class glucosyltransferases and has been named as

α(1→6) D-glucan: D-fructose 2-glycosyltransferase [EC 2.4.1.5]. This enzyme catalyzes the synthesis of glucose polymer, dextran, from sucrose according to the equation.

$$\underset{\text{(Sucrose)}}{nC_{12}H_{22}O_{11}} \longrightarrow \underset{\text{(Dextran)}}{(C_6H_{10}O_5)_n} + \underset{\text{(Fructose)}}{nC_6H_{12}O_6}$$

The energy required for the condensation of glucose residues is derived from the hydrolysis of sucrose. Sucrose is the only known inducer for the synthesis of dextransucrase (Neely and Nott 1962). The extracellular dextransucrases are synthesized by microorganisms belonging to families Lactobacillaceae and Streptococcaceae, and especially by the genera *Lactobacillus, Leuconostoc* and *Streptococcus*

The bacterium *Leuconostoc mesenteroides* belonging to the family Lactobacillaceae is the most commonly used microorganism for the production of dextrnasucrase and the dextran. This bacterium has been isolated from sucrose-rich food preparations, materials related to sucrose production (like juices from pressed cane) and soil from the cane fields and vicinity of distilleries. It is a gram positive, non-motile, non-spore forming bacterium with spherical or lenticular cells forming pairs or chains. The gram-positive *Leuconostoc mesenteroides* NRRL B-512F, which synthesizes the extracellular homopolysaccharide dextran, is an extensively used organism for the industrial production of dextransucrase. In addition, this strain secreted only a single dextransucrase with very low amount of levansucrase (Jeanes *et al.* 1948). Thus, dextran can be produced commercially, almost exclusively from the bacterial strain *Leuconostoc mesenteroides* NRRL B-512F. The dextransucrase has gained commercial importance because of dextran produced it has applications in pharmaceutical, food, agricultural and fine chemical industries.

1.1 Culturing of microorganism

Various media for the maintenance of *Leuconostoc mesenteroides* cultures have been reported (Jeanes *et al.* 1948; Hehre 1941; Stacey 1942; Hehre 1946; Jeanes 1965; Lawford *et al.*1979; El-Sayed *et al.* 1990a; De Man *et al.* 1960; Bhatnagar *et al.*1985; Goyal and Katiyar 1996). The organism can be stored and maintained in sucrose rich media (Hehre 1941; Stacey 1942; Hehre 1946; Bhatnagar *et al.*1985), which are also used for isolation of *Leuconostoc mesenteroides*. Jeanes in 1965 described two different media for the maintenance of stock cultures. *Lactobacillus* MRS medium is traditionally used for maintaining all the organisms belonging to family Lactobacillaceae (De Man *et al.* 1960). This medium is also used for long-term preservation of *Leuconostoc mesenteroides*. For short duration, the culture can be stored in enzyme production medium as described by Tsuchiya *et al*. 1952). El-Sayed *et al*. (1960a) described a tomato-tryptone medium for culture maintenance. Although, there are many reports suggesting different media for maintenance of the *Leuconostoc mesenteroides* NRRL B-512F, but there is only one report available on the influence of maintenance medium on enzyme production (Goyal and Katiyar 1996). They discovered a modified MRS medium for maintenance of culture that gave higher production of dextransucrase when the culture was transferred to enzyme production medium.

The lactic acid bacteria of the genera *Leuconostoc* and *Lactobacillus* are known to produce glucansucrases (Monchois *et al.* 1999; Kralj *et al.* 2004; Tieking *et al.* 2005). Glucansucrases from *Leuconostoc* genus are inducible enzymes, which are induced in the presence of sucrose (Neely and Nott 1962). The organisms of *Leuconostoc* genus are micro-aerophilic. Limited information is available on antibiotic susceptibilities and carbohydrate fermentation behavior of glucansucrase

producing Leuconostoc strains (Kelly *et al.* 1986; Holt *et al.* 2001). It was found that several Leuconostoc strains used in dairy and wine industries were resistant to vancomycin (Orberg and Sandine 1984; Holt *et al.* 2001). The type of glucansucrase elaborated by a strain can be identified by the type of glucan produced depending upon the type of substrate utilized. The sucrose hydrolyzing activity of Leuconostoc strains was analyzed by the synthesis of polysaccharide which was detected as activity bands within a polyacrylamide gel using a Periodic acid Schiff staining protocol (Leathers *et al.* 1997). There are few reports on the presence of plasmid DNA from Leuconostoc strains (O'Sullivan and Daly 1982; Orberg and Sandine 1984). The plasmids possessing genes for lactose utilization (David *et al.* 1992), citrate utilization (Vaughan *et al.* 1995) and for bacteriocin (Fremaux *et al.* 1995) have been identified.

1.2 Production of dextransucrase

The production of dextransucrase in sucrose broth cultures of *Leuconostoc mesenteroides* NRRL B-512F and its role in dextran synthesis was thoroughly explored (Hehre 1941; Koepsell and Tsuchiya 1952; Tsuchiya *et al.* 1952). A large number of reports followed on the production of dextransucrase under different culture conditions using different production media using batch, fed-batch, semi-continuous and continuous processes (Lawford *et al.* 1979; Landon and Webb 1990; Lazic *et al.*1993; Ajongwen and Barker 1993; Alsop 1983; Koepsell and Tsuchiya 1952; Brown and McAvoy 1990; El-Sayed *et al.*1992; El-Sayed *et al.*1990a; El-Sayed *et al.*1990b; El-Sayed *et al.*1990c; Lopez and Monsan 1980; Kobali and Reilly 1980; Monsan and Lopez 1981; Monsan *et al.*1987; Tsuchiya *et al.* 1952; Robyt and Walseth 1979; Paul *et al.* 1984; Veljkovic *et al.*1992; Pennell and Barker 1992). These reports showed that dextransucrase production varies with the culture

conditions. It was observed that higher yield of dextransucrase was obtained with fed-batch fermentation techniques (Ajongwen and Barker 1993; Brown and McAvoy 1990. The most important parameters for dextransucrase production are pH (Lazic *et al.* 1993; Alsop 1983; Tsuchiya *et al.* 1952; Veljkovic *et al.* 1992) and temperature (Alsop 1983; Tsuchiya *et al.* 1952). The maximal enzyme production occurred when the fermentation was carried out at constant pH of 6.7 and 23°C (Alsop 1983; Tsuchiya *et al.* 1952). Corn steep liquor and yeast extract served as very good sources of nitrogen for the growth of *Leuconostoc mesenteroides* NRRL B-512F (Tsuchiya *et al.* 1952). Yeast extract also provided other essential trace elements. The yield of enzyme was also affected significantly by, the type of yeast extract used. Voluminous information is available on increasing the enzyme yield by the bacterium (Lawford *et al.* 1979; Landon and Webb 1990; Lazic *et al.*1993; Ajongwen and Barker 1993; Alsop 1983; Koepsell and Tsuchiya 1952; Brown and McAvoy 1990; El-Sayed *et al.*1992; El-Sayed *et al.*1990a; El-Sayed *et al.*1990b; El-Sayed *et al.*1990c; Lopez and Monsan 1980; Kobali and Reilly 1980; Monsan and Lopez 1981; Monsan *et al.*1987; Tsuchiya *et al.* 1952; Robyt and Walseth 1979; Paul *et al.* 1984; Veljkovic *et al.*1992; Pennell and Barker 1992). Sporadic reports are available on the effect of other micro and macronutrients on the production of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F (Behravan *et al*. 2003; Tsuchiya *et al.* 1952; Robyt and Walseth 1979; Goyal and Katiyar 1996; Goyal and Katiyar 1997; Ajongwen and Barker 1993; Lopretti 1999; Paul *et al*. 1984; Santos *et al*. 2000; Ul-Qader *et al*. 2001).

Tsuchiya *et al.* (1952) studied the effect of certain growth conditions on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-512. They found that the optimum temperature was 25°C and that 2% sucrose was optimum for

maximum production of dextransucrase by *Leuconostoc mesenteroides*. They observed that the high amount of phosphate was required for maintaining the pH in the production stage of the cultures and NaOH or KOH could serve the purpose. Robyt and Walseth (1979) reported a 2-fold increase in enzyme production by the addition of 0.005% of calcium chloride to the medium of *Leuconostoc mesenteroides* NRRL B-512F. Lawford *et al*. (1979) reported dextran biosynthesis and dextransucrase production by continuous culture of *Leuconostoc mesenteroides* NRRL B-512F. Under glucose or maltose-limited conditions no enzyme production was detected. Cote and Robyt (1982) produced two extracellular α-D glucans L and S from *Leuconostoc mesenteroides* NRRL B-1355 on sucrose containing medium. On the basis of structural and physical properties and endo-dextranase treatment, glucan S was named as alternan, which contained α-D-glucose units with alternate α(1→6) and α(1→3), glycosidic linkages. The fraction L was similar to *Leuconostoc mesenteroides* NRRL B-512F dextran with 95% α(1→6) and 5% α(1→3) linkages.

El-Sayed *et al.* (1990b) used three types of Celite (diatomaceous earth material) R648, R633 and R630 to immobilize *Leuconostoc mesenteroides* and studied the production of dextransucrase and dextran. Enzyme production with the immobilized cell cultures was significantly affected by both the pore diameter and particle size of the supporting material. Immobilized cells using Celite R648 showed highest enzyme activity followed by R633, calcium alginate coated R630, and calcium alginate beads. A layer of calcium alginate coating over Celite R630 improved mechanical stability, increased soluble dextransucrase production and decreased the cell leakage from the highly porous support (El-Sayed *et al.*1990b). Dextran yield was higher in immobilized cell cultures than that obtained from cell-free culture under similar conditions. El-Sayed *et al*. (1990c) studied dextransucrase

production by *Leuconostoc mesenteroides* immobilized in calcium alginate beads in three sequential cycles of semi-continuous fed batch fermentation. Each cycle consisted of 24h fed-batch dextransurase production period followed by another 24h fed batch dextran production period. In dextransucrase fed-batch semi-continuous fermentation of immobilized cells, the total enzyme activity was 1.6 times greater than that produced by free cells. This increase in enzyme productivity with immobilized cells, required three times longer operating time than with the free cells. Three times higher dextran was produced by immobilized cells than free cells. El-Sayed *et al*. (1990a) produced dextransucrase by *Leuconostoc mesenteroides* immobilized in calcium alginate beads in batch and fed-batch fermentations. Batch fermentation in a bubble column reactor the immobilized cells showed 93% of dextransucrase activity of free suspended cells. They also studied the enzyme production by the immobilized cells under continuous sucrose feeding (5 g/l) and in batch operation in the air-lift bioreactor. In the continuous sucrose feeding culture, the dextransucrase activity was higher than the ordinary batch operation.

Barker and Ajongwen (1991) reported high yield of dextransucrase in a fed batch fermentation. The cultures grown in non-aerated fed-batch fermentation and aerated fermentation had the same cell population density and were different only in the enzyme activity of 21.9 and 17 U/ml, respectively. Different commercial grades of yeast extract significantly affected in the final cell concentration in the broth and also the enzyme yield. Veljkovic *et al.* (1992) studied the effect of oxygen transfer rate on extracellular dextransucrase production by *Leuconostoc mesenteroides* in batch fermentation without pH control. They reported that oxygen transfer rate of about 1 mmol/l/h was optimum for both the cell growth and the dextransucrase production. Barker *et al.* (1993) produced dextransucrase by non-aerated fed batch and continuous

fermentation and purified by continuous ultracentrifugation and ultrafiltration, and used it in a bioreactor separator stage for the biosynthesis of dextran.

Lazic *et al*. (1993) studied dextran production under anaerobic and aerobic culture conditions with and without pH control. They obtained maximum dextran yield when the oxygen transfer rate in the bioreactor was equal to the maximum uptake rate of the organism and pH at 5.5. They attributed this to the higher production, stability and activity of dextransucrase. Kim and Robyt (1994) studied the properties of *Leuconostoc mesenteroides* NRRL B-512 FMC constitutive dextransucrase and showed that the three sugars glucose, fructose and sucrose resulted in the synthesis of the same amount of cell-associated dextransucrase. The purified enzyme had a molecular size of 184 kDa as determined by SDS-PAGE.

Shamala and Prasad (1995) added extracts of wheat bran and dibasic potassium phosphate to the normally used yeast extract medium. They studied the production of low and high viscosity dextran by *Leuconostoc dextranicum* FPW-10 and compared with the standard strain *Leuconostoc mesenteroides* NRRL B-512F under un-aerated liquid surface fermentation and shake flask conditions, using optimized medium composition (Shamala and Prasad 1995). They found that wheat bran was a complex nutrient substrate containing amino acids and vitamins and increased the yield of dextran from 18 to 38 g/100 g sucrose in a newly formulated wheat bran extract (WBE) medium. They further showed that WBE and dibasic potassium phosphate form a complete nutrient medium for dextran production by *Leuconostoc*. On increasing the concentration of K_2HPO_4 from 0.1 to 1% in WBE medium, the yield increased from 12 to 38 g/100 g sucrose, with a concomitant increase in the enzyme dextransucrase.

Goyal and Katiyar (1995) studied dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F in under static and shake flask culture conditions at varied temperature. The optimum temperature supporting maximum enzyme production was 23°C. Maximum enzyme production was reported when fermentation in bioreactor in the controlled pH range of 5-6 was carried out (Lazic *et al*. 1993). Alsop (1983) reported that at higher temperature of fermentation, the inactivation of enzyme was rapid at pH 6.1-6.7 causing lower enzyme yield. The decrease in enzyme activity in all cultures, at the end of fermentation was attributed to the extreme sensitivity of dextransucrase to unfavorable pH conditions of broth (Goyal *et al.* 1995). Static flask culture gave higher dextransucrase production as compared to shake flask culture (Goyal *et al.* 1995).

Goyal and Katiyar reported that *Leuconostooc mesenteroides* NRRL B-512F maintained in modified MRS medium gave maximum specific activity of 1.2 U/mg (Goyal and Katiyar 1996). In the maintenance media described by Stacey (1942) and Bhatnagar *et al.* (1985) the high sucrose content resulted in lower enzyme specific activities (Goyal and Katiyar 1996). The maintenance medium described by Jeanes (1965) did not give higher enzyme production, possibly due to the lack of certain micronutrients. Low sucrose maintenance media induced higher enzyme activity than the higher sucrose media. It was repeatedly found that transferring the culture from modified MRS medium to other media produced enzyme with lower activity and that the reverse transfer to modified MRS medium resulted in an increase in enzyme activity (Goyal and Katiyar 1996).

Goyal and Katiyar (1997) studied the effect of certain nutrients on the production of dextransucrase from *Leuoconostoc mesenteroides* NRRL B-512F by taking the enzyme production medium described by Tsuchiya *et al*. (1952) as control.

They observed that increase in the concentration of sucrose from 2 to 4% increased the activity of dextransucrase. The broth became viscous with increase in sucrose concentration due to concomitant formation of dextran. The enzyme production was dependent on the concentration of yeast extract and K_2HPO_4 with 1.5% yeast extract and 2.5% K_2HPO_4 the enzyme activity increased by 49% as compared to the control. The enzyme production decreased with an increase in the concentration of yeast extract above 1.5% at all K_2HPO_4 concentrations. This was contradictory to the reports of Tsuchiya (1952) and Ajongwen and Barker (1993). The addition of peptone and beef extract separately in presence of yeast extract resulted in 30% increase of the enzyme activity (Goyal and Katiyar 1997).

The addition of Tween 80 to the medium resulted in an increase in the dextransucrase production and increase was concentration dependent (Goyal and Katiyar 1997). Umesaki *et al*. (1977) and Sato *et al*. (1989) reported 30 fold increase in the enzyme activity in presence of 0.1% Tween 80. They proposed that the change in fatty acid composition of the membrane of bacterial cells, when grown in presence of Tween 80, was probably responsible for enhanced production of dextransucrase. Mg^{+2} ions had no effect on the growth rate of the bacterium but played a significant role in the signal peptide processing of the secretary proteins. Magnesium sulphate was not included in the enzyme production medium of Tsuchiya *et al.* (1952) during the study. $MgCl_2$ and NaF enhanced the activity of dextransucrase. The activity increased by 25% with 100 µM NaF and by 10% with $MgCl_2$. Shimura and Onisi (1978) reported binary effect of NaF with *Streptococcus mutans,* favouring more formation of soluble dextran caused by 10 fold increase of enzyme activity.

Chellapandian *et al*. (1998) studied the production and properties of dextransucrase from *Leuconostoc mesenteroides* IBT-PQ isolated from Pulque, a

traditional Aztec alcoholic beverage prepared from agave juice containing sucrose as the main carbon source. 87% of the enzyme activity was associated with the cells. The opitmization of the culture medium by Box-Behnken method (Box and Behnken 1960) gave sucrose 30 g/l, yeast extract 20 g/l and K_2HPO_4 25 g/l and with all other trace elements as constant gave maximum glucosyltransferase activity of 2.2 U/ml and good biomass production. Kitaoka and Robyt (1998) studied fermentation conditions to obtain maximum dextransucrase yield from *Leuconostoc mesenteroides* B-512FMC in a jar fermenter. At constant concentration of glucose (34 g/l) and nitrogen source (3.8 g/l yeast extract and 3.8 g/l bactopeptone) and pH 6.0, the enzyme production was maximum. On keeping the pH and nitrogen source unchanged and doubling the amount of glucose, it was observed that the entire glucose was not consumed even after 60h of fermentation. This was attributed to the limiting source of nitrogen and formation of acids from the glucose.

Smith and Zahnley (1999) reported the production of dextransucrase by wild-type *Leuconostoc mesenteroides* in media containing sugars other than sucrose. They observed that *Leuconostoc mesenteroides* produced the inducible enzymes, glucosyltransferases (GTfs) and fructosyltransferases (FTfs) that synthesized dextran and levan from sucrose. The wild strains of *Leuconostoc mesenteroides* produced low, but detectable GTf activity when grown on glucose, maltose or melibiose instead of sucrose. This was in agreement with the report of Dols *et al.* (1998) who showed that strain B-1299 produced GTfs when grown with glucose or fructose instead of sucrose as the sole carbon source. The glucosyltransferase activity with sucrose medium was 5 to 10 fold higher than with other sugars. Most of the activity in cultures grown in non-sucrose medium was cell-associated and less than 20% enzyme activity was observed in the supernatant.

Lopretti *et al.* (1999) studied the influence of nitrogen/carbon ratio on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-512F using sucrose as substrate and also other complementary sugars and measured the enzyme concentration through activity and radiotracer tests with [^{14}C] labeled phenylalanine. They observed slowing down of enzyme synthesis and lower fermentation time with the addition of short pulses of nitrogen source. They also demonstrated that galactose was able to yield the same amount of enzyme as sucrose, but with a different production rate. Maltose favored the synthesis of dextransucrase, whereas lactose inhibited the enzyme production.

Santos *et al.* (2000) studied the production of dextransucrase, dextran and fructose from sucrose, using *Leuconostoc mesenteroides* NRRL B-512F, by batch fermentation in a bioreactor and reported optimum temperature and pH of 35ºC and 5.5, respectively. The cell dry weight reached 5.8 g/l and the enzyme activity reached 81.5 DSU/ml (one DSU was defined as the amount of enzyme that converted 1 mg of sucrose in 1 hour under reaction conditions of temperature 30°C and pH, 5.0, where 1DSU=1/21 U, according to the international definition of enzyme activity). Although, the growth of the bacterium and dextran and fructose production remained unchanged at 20-35°C, the enzyme activity decreased with increase in temperature. They found that pH 6.7 was ideal for growth but the enzyme activity was much higher at pH 5.5. They also observed the cell growth was positively affected by aeration, but the enzyme activity decreased. The enzyme activity increased with sucrose concentration up to 40 g/l of sucrose. These finding were in agreement with earlier reports (Ajongwen and Barker 1993; Goyal *et al.*1995).

Ul-Qadar *et al.* (2001) studied the effect of medium composition on enzyme activity, total protein, cell growth and pH on a newly isolated strain of *Leuconostoc*

mesenteroides (PCSIR-3) and compared with *Leuconostoc mesenteroides* NRRL B-512F. Rodrigues *et al*. (2003) studied the effect of phosphate concentration on the production of dextransucrase and cell propagation by *Leuconostoc mesenteroides* NRRL B-512F in a semi continuous shake flask culture. They used 0.3 M K_2HPO_4 instead of 0.1 M of the standard medium and reported increase in biomass and enzyme activity. Under standard medium conditions, maximum enzyme activity of 28 DSU/ml and a maximum biomass of 4.2 mg/ml were obtained.

Kim and Robyt *et al*. (2003) produced purified dextransucrase from a constitutive mutant of *Leuconostoc mesenteroides* NRRL B-512FMC, in glucose medium and studied the dextran molecular size and degree of branching as a function of sucrose concentration, pH and temperature of the reaction. Behravan *et al.* (2003) used different concentrations of molasses and wheat-bran extract, after filtration and pH adjustment for growing *Leuconostoc mesenteroides* NRRL B-512F. The cultures were incubated at 25°C to study effect of cheap local sources of carbohydrate and nitrogen. They found that with 20% sugar-beet molasses containing 9.4 g of sucrose, the dextran yield was high. They also reported that the initial pH 7.5 gave maximum dextran yield. Keeping K_2HPO_4 constant, the optimum yeast extract concentration was 0.5% (w/v) for the maximum dextran yield. Substitution of yeast extract with wheat bran extract only marginally reduced dextran production. Michelena *et al.* (2003) studied the advantages of micro-aerophilic system over the non-aerated culture system. They found that the fermentation in a 5 l vessel and aeration rate of 0.15 vvm produced dextransucrase with an activity of 127 DSU/ml.

Purama and Goyal (2007a) showed that optimum temperature for dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640 was 25°C. The shaking flask culture gave higher enzyme activity by 20% than the static flask

culture. They also reported that *Leuconostoc mesenteroides* NRRL B-640 at 25°C under shaking flask culture, gave 15% higher than that the enzyme activity given by *Leuconostoc mesenteroides* NRRL B-512F grown at 23°C under the static flask culture.

Dextransucrase genes from different strains of *Leuconostoc* sp. have been cloned and expressed (Monchois *et al.* 1996; Monchois *et al.* 1998; Funane *et al.* 2000; Kim *et al.* 2000; Kim *et al.* 2002; Bozonnet *et al.* 2002; Neubauer *et al.* 2003; Kubik *et al.* 2004). Neubauer *et al.* (2003) isolated the gene encoding the dextransucrase DsrD from the industrial strain *Leuconostoc mesenteroides* Lcc4 by PCR using degenerate primers, recognizing conserved regions present in other dextransucrase encoding genes from *Leuconostoc* sp. by southern blot analysis of total genomic DNA (Neubauer *et al.* 2003). They did the molecular characterization of dextransucrase DsrD and conducted comparative study on expression analysis of DsrD of *Leuconostoc mesenteroides* Lcc4 in homologous and heterologous *Lactococcus lactis.* They substantiated the assumption that the DsrD is translocated through the bacterial cell membrane and secreted in to the culture medium, by the gel assay of sucrose grown cell supernatant. They found a 165 kDa protein on gel corresponding to dextransucrase activity. They compared the N-terminal protein sequence of the DsrD with the gene sequence and found that the starting N-terminal amino acid was at position 43 of the gene sequence. They proposed that N-terminal of 42 amino acids of deduced sequence acted as signal peptide that was cleaved during translocation of the dextransucrase to the extracellular location. They also observed a faint band at 160 kDa protein and predicted it to be the proteolytic product of 165 kDa protein. They conducted batch-fermentation at controlled pH 6.7, using glucose and sucrose separately as sole carbon sources, to study the induction effect of sucrose on

dextransucrase production. The induction results displayed 10 to 15 fold higher levels of transcripts as well as the enzyme activity with sucrose fed culture than that with glucose fed culture. Sucrose at 20 g/l was sufficient to ensure the induction of enzyme synthesis. Increasing sucrose concentration did not proportionately increase the enzyme synthesis.

A plasmid pNZDSF was derived from pNZ124 by cloning the dsrD including 499 nucleotide upstream promoter region. This plasmid was transferred to the non-exopolysaccharide producing host strain *Lactococcus lactis* MG 1363. The ability of *Lactococcus lactis*/pNZDSF to produce dextran was tested on agar plate assay of cells grown on GM17 medium supplemented with 30 g/l sucrose. They also studied the batch-fermentation of recombinant *Lactococcus lactis* separately on glucose and sucrose supplemented GM17 medium and inferred that there was no change in the dextransucrase activity on sucrose fed cells. This indicated that sucrose was not involved in dextransucrase induction in *Lactococcus lactis* where as it does in *Leuconostoc* sp. However, the dextransucrase activity produced by *Lactococcus lactis* was five fold lower than that found in homologous strain *Leuconostoc mesenteroides*.

Malten *et al*. (2005) carried out the recombinant transfer of dextransucrase DsrS gene of *Leuconostoc mesenteroides* and studied the expression in *Bacillus megaterium*. They constructed a recombinant plasmid and incorporated the dextransucrase gene under the xylose-inducible gene expression system containing *Bacillus megaterium* ribosomal binding site. They produced an extracellular dextransucrase by recombinant plasmid expression. Elimination of the extracellular protease increased extracellular DsrS concentrations by a factor of 4 and stabilized the recombinant protein. They observed that most of the dextransucrase was cell-associated.

1.3 Purification of dextransucrase

The enzyme dextransucrase catalyzes the synthesis of exopolysacchairde, dextran from sucrose. The dextran has been extensively exploited in pharmaceutical industry as blood volume expander, as stabiliser in food industry and as a chromatographic medium in fine chemical industry because of their non-ionic nature and stability as described in Section 1.7.2. Majority of the dextrans are synthesized from sucrose by dextransucrase secreted mainly by bacteria belonging to genera *Leuconostoc, Streptococcus* and *Lactobacillus*. Bulk of the information on purification of extracellular dextransucrase has been generated from Leuconostoc sp. Various methods such as precipitation by ammonium sulphate, ethanol or polyethylene glycol, phase partitioning, ultrafiltration and chromatography have been used to purify the enzyme. However, processes like ultra-filtration, salt and PEG precipitation, chromatography and phase partitioning have been standardized and successfully used for higher scale purification of the enzyme. A recombinant dextransucrase from *Leuconostoc mesenteroides* B-512F with a histidine tag has been expressed in *E. coli* cells and purified by immobilized metal ion chromatography.

Dextransucrase of *Leuconostoc* spp. is induced by sucrose, which leads to concomitant dextran synthesis during enzyme production. The presence of dextran results in aggregated forms of enzyme. In addition, dextrans make the culture supernatants viscous, making enzyme purification cumbersome. Various methods like ultra-filtration, salt and PEG precipitation, chromatography and phase-partitioning, and their combinations used for purification, are reviewed in the following text. The first attempt to purify dextransucrase involved ammonium sulfate precipitation (Hehre 1946). A series of reports appeared on various methods of purification since then. A wide variety of techniques have been used for the purification of *Leuconostoc*

mesenteroides dextransucrase (Robyt and Walseth 1979; Kobali and Reilly 1980; Monsan and Lopez 1981; Paul *et al.* 1984; Miller *et al.* 1986; Fu and Robyt 1990; Rhee and Lee 1991; Goyal and Katiyar 1994; Nigam *et al.* 2006). A recent report describes the production of recombinant dextransucrase of *Leuconostoc mesenteroides* B-512F with a histidine tag at the C-terminal using *E. coli* TOP10 cells for expression and purification of the recombinant enzyme by affinity chromatography using Ni^{2+} ion immobilized column (Moulis *et al* 2006).

1.3.1 Precipitation by salt and solvent

In 1946, E.J. Hehre obtained crude preparations of dextransucrase from *Leuconostoc mesenteroides* by ammonium sulfate precipitation. Ammonium sulfate was added to the bacterial culture and the pellet obtained after centrifugation containing the cells and enzyme was further freed from preformed polysaccharides by washing with saturated ammonium sulfate (Hehre 1946). The supernatant obtained after centrifugation was treated with an equal volume of acetate buffer and centrifuged at low temperature. The precipitate obtained was used as the enzyme preparation (Hehre 1946). Koepsell and Tsuchiya used both, alcohol and ammonium sulfate precipitation to obtain purified dextransucrase as a lyophilized dry powder with 100 U/mg specific activity from *Leuconostoc mesenteroides* B-512 (Koepsell and Tsuchiya 1952). The culture supernatant of *Leuconostoc mesenteroides* NRRL B-1299 was subjected to ammonium sulfate precipitation. The enzyme was collected, dialyzed and used as enzyme preparation that had a specific activity of 0.1 U/mg. The recovery of protein by this method was very low (Kobayashi and Matsuda 1976). The cells from culture of *Leuconostoc mesenteroides* LM_1 were centrifuged and the pellet was washed to remove any preformed dextran and was resuspended in acetate buffer,

followed by the addition of sucrose and toluene (Gupta and Prabhu 1995). The addition of sucrose induced dextransucrase secretion into the suspension. The suspension was centrifuged and the secreted enzyme was precipitated by ammonium sulfate. An enzyme activity of 2.96 U/mg was obtained that was not significant (Gupta and Prabhu 1995). Robyt and Walseth (1979) found the ammonium sulfate to be completely ineffective in precipitating dextransucrase from a cell free culture supernatant as more than 90% of the dextransucrase activity was lost when the concentration of ammonium sulfate was 80% (w/v). Purification with ammonium sulfate thus led to considerable loss of enzyme. In general ammonium sulfate did not prove to be an agent that could efficiently precipitate the dextransucrase.

Alcohol has also been used to precipitate dextransucrase. Pre-chilled ethanol was added to the cell free supernatant of *Leuconostoc mesenteroides* B-512 with an activity of 40-50 DSU, for precipitation of enzyme (Tsuchiya *et al.* 1955). The precipitate was collected by centrifugation and the resulting enzyme gave 700 DSU, where one DSU was defined as the amount of enzyme required to convert 1 mg of sucrose to dextran in 1h at 30ºC and pH 5.0, as determined by increase in concentration of fructose (Tsuchiya *et al.* 1955). The precipitation of enzyme with alcohol removed a significant amount of dextran with it. Glycerol also has been used to precipitate the enzyme dextransucrase. The addition of 50% glycerol to the culture supernatant obtained after centrifugation resulted in dextransucrase with an activity of 64 U/ml where, one unit of activity was defined as the amount of enzyme in micromole/ml that converts 1 µmole of sucrose into dextran in 1h at 30°C and pH 5.0 (Rodrigues *et al*. 2003). The major drawback of this method was that the purified enzyme contained dextran.

1.3.2. Fractionation by polyethylene glycol

Polyethylene glycol (PEG) is known to selectively precipitate proteins, which have high molecular weights or exist in aggregated forms. The large size of dextransucrase, together with its tendency to form aggregates in solution has led to the use of non-ionic hydrophilic polymer PEG for precipitation of dextransucrase. Dextransucrase from *Streptococcus mutans* was purified by PEG precipitation using PEG-400 and 6000 (Russel 1979). PEG-6000 precipitated other non-dextransucrase proteins, while PEG-400 gave higher specificity of precipitation and had an advantage of being readily removed by dialysis (Russel 1979). A simple and effective method of purification of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F, using PEG precipitation was developed (Goyal and Katiyar 1994). The PEG-400 reproducibly gave dextransucrase with specific activity of 8.7 U/mg, with 80% yield. In a typical experiment, in successive steps of fractionation of the crude enzyme using PEG-400, the specific activity increased from 0.58 to 29 U/mg, resulting in 70% overall yield after the three steps. High molecular weight PEG-20,000 has also been used to fractionate the enzyme from *Leuconostoc mesenteroides* B-512FM (Su and Robyt 1994). The final enzyme obtained after dialysis had an activity of 89 U/mg. PEG fractionation method is relatively the easier and faster procedure to obtain purified form of dextransucrase. The enzyme preparations are also devoid of contaminating enzymes like levansucrase and invertase. The major disadvantage of this procedure is the contamination of enzyme by the dextran as it most often remains associated with enzyme. PEG is also present in the purified fractions but can be completely removed by extensive dialysis (Russel 1979; Su and Robyt 1994). Dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 was purified using PEG-1500 and gel-filtration (Purama and Goyal 2007). The PEG-1500 (10%, w/v)

gave dextransucrase with maximum specific activity of 23 U/mg. The purified enzyme showed multiple molecular forms on SDS-PAGE, however, the same sample showed a single band of 180 kDa molecular size on non-denaturing native-PAGE. The purified dextransucrase confirmed the presence of dextran band, when run on SDS-PAGE under non-denaturing gels for *in-situ* activity detection by Periodic Acid Schiff staining. The activity bands corresponded to the native and active form of the purified dextransucrase of approximately, 180 kDa molecular size, that appeared on the denaturing gels stained with Coomassie Brilliant Blue. Further purification of 10% PEG-1500 purified dextransucrase by gel-filtration gave dextransucrase with specific activity of 35 U/mg with 61 fold purification (Purama and Goyal 2007).

1.3.3 Phase-partitioning

Phase-partitioning method has also been applied to purify extracellular dextransucrase. Phase partitioning occurs between dextran and PEG (Paul *et al.*1984). When sucrose is added to the culture supernatant, the enzyme synthesizes dextran in large amounts. Addition of PEG solution to a dextran-rich aqueous solution, leads to the appearance of two-phases; the top phase which is rich in PEG while the bottom one is rich in dextran. Dextransucrase preferentially goes in to the lower dextran-rich phase because of the covalent association of dextransucrase and dextran. The dextransucrase was purified by aqueous two-phase partitioning between the dextran present in the culture supernatant of *Leuconostoc mesenteroides* B-512F and polyethylene glycol (Paul *et al.*1984). PEG-1500 was added to the supernatant and dextransucrase was obtained in the dextran-rich phase in a highly purified form. Successive phase partitioning steps resulted in an enzyme preparation with specific activity of 175 U/mg and yield of 95% and the preparation was free of levansucrase

activity (Paul *et al.*1984). Otts and Day purified dextransucrase of *Leuconostoc mesenteroides* ATTC 10830 by two-phase partitioning using PEG 3350 (Otts and Day 1988)[54]. The culture supernatant was added with 10% dextran T-500 solution followed by 20% PEG-3350 addition. The upper phase, consisting of PEG, was removed and to the lower dextran rich phase PEG-3350 solution was added again. This procedure gave 95% recovery of dextransucrase with specific activity of 30 U/mg (Otts and Day 1988). Phase partitioning of PEG and preformed dextran in culture supernatant of *Leuconostoc mesenteroides* B-742 was carried out with PEG-1500 and an enzyme preparation with an activity of 83 U/mg was obtained (Remaud-Simeon 1991).

Dextran T70 or soluble native dextran from *Leuconostoc mesenteroides* NRRL B-1299 was added to culture supernatant followed by PEG-1500 (15% v/v) addition and the dextran rich phase was concentrated by centrifugation (Dols *et al.* 1998). An activity of 4.3 U/ml was obtained in the pellet, where one unit corresponds to the amount of enzyme that produces 1 µmole of fructose per minute at 30°C and pH 5.4 (Dols *et al.* 1998). Dextransucrase from cultures of *Leuconostoc mesenteroides* B-512F grown on different carbon sources was recovered by the removal of cells by passing the culture supernatant through a membrane of 0.2 µm (Quirasco 1999). The enzyme was concentrated by aqueous two-phase partitioning with 25% (w/v) PEG-1500. Dextran T70 (1.5%) was added to the supernatant produced from carbon sources other than sucrose, as dextran is only synthesized in the presence of sucrose. Of all the carbon sources used, sucrose showed formation of dextran and with maximum dextransucrase purification, with an activity of 2.4 U/mg (Quirasco 1999). The purification of dextransucrase was carried out by a four step PEG phase partitioning method using 50% (w/v) PEG-1500 from the culture

supernatant of *Leuconostoc mesenteroides* B-512F (Goulas *et al.*2004). The enzyme preparation had an activity of 62.3 U/mg, with a carbohydrate content of 233 mg/ml (Goulas *et al.*2004). Affinity phase partitioning using PEG-6000 and 400 was carried out for dextransucrase purification from *Leuconostoc mesenteroides* B-512F (Nigam *et al.* 2006). The dextran was generated by adding 5% sucrose to the culture supernatant of *Leuconostoc mesenteroides* B-512F. Three steps of repeated phase-partitioning by PEG-6000 and PEG-400 showed that the purification of dextransucrase by PEG-6000 was much greater than that obtained by PEG-400. The specific activity of 42.1 U/mg and an overall yield of 84% obtained after the third step of phase-partitioning by PEG-6000, were significantly higher than the specific activity of 23.8 U/mg and 46% overall yield by PEG-400 (Nigam *et al.* 2006). The procedures for purifying dextransucrase discussed above either had low yields or failed to remove the associated polysaccharides. For characterization of the enzyme it should be essentially free of the carbohydrate content and the yields of purification should be high. This was achieved by a combination of dextranase treatment, ion-exchange and affinity chromatography after obtaining the crude form of enzyme through ammonium sulfate or PEG precipitation.

1.3.4 Chromatography

Chromatographic separation has been used to obtain polysaccharide free enzyme. Various matrices have been used for the purification of dextransucrase. Dextransucrase has been purified using columns containing hydroxyapatite, DEAE-cellulose, DEAE-sephadex, sephadex, ultrogel AcA34. Dextransucrase binds to dextrans of various sizes. This led to the use of Sephadex gels, which have an affinity to dextran. These gels act as affinity material for the purification of the enzyme from

Leuconostoc and *Streptococcus* species. Sephadex has been found to be good affinity matrix for dextransucrase from *Streptococcus*, but poor for *Leuconostoc* (Suzuki and Kobayashi 1975; Kobayashi and Matsuda 1976). The latter is probably due to the endogenous dextran present in the *Leuconostoc* preparations. The culture supernatant of *Leuconostoc mesenteroides* B-512F was added with ammonium sulfate to a saturation of 75% after incorporating egg white albumin at a 5% concentration (Robyt and Taniguchi 1976). The addition of egg white albumin resulted in a good precipitation of dextransucrase. The precipitate obtained was subjected to chromatography on DEAE-cellulose column and the enzyme adsorbed on the column was eluted with NaCl gradient. The enzymatically active fractions were collected and re-precipitated with ammonium sulfate. This preparation was applied to DEAE-cellulose column. The active fractions obtained were pooled and then applied to a column of BioGel P-150 and dextransucrase obtained had a specific activity of 110 U/mg (Itaya and Yamamoto 1975). The culture supernatant of *Leuconostoc mesenteroides* B-512F was concentrated, and protein was precipitated with 80% (w/v) ammonium sulfate (Robyt and Taniguchi 1976). This preparation was applied to and eluted from hydroxyapatite column with a potassium dihydrogen phosphate gradient and the enzyme gave a specific activity of 3.3 U/mg. The enzyme when purified using hydroxapatite was contaminated with levansucrase, invertase and dextranase. Furthermore, the total yield of dextransucrase was also less than 10%. Therefore, the low yield, the dilution of the dextransucrase activity and the presence of contaminating enzymes restricted the use of hydroxapatite (Robyt and Taniguchi 1976). The precipitation of enzyme from culture supernatant of *Leuconostoc mesenteroides* B-512F was done using ammonium sulfate with egg albumin as co-precipitant (Lawford *et al.* 1979). The crude preparation was desalted by gel filtration

with Sephadex G-25 and applied to DEAE-cellulose column. The enzyme was eluted with a linear gradient of NaCl (Lawford *et al.* 1979). When the crude extract of enzyme was applied to DEAE-cellulose column, the dextransucrase activity could not be eluted from the column, even at very high ionic strengths (Robyt and Walseth 1979). This was because dextransucrase tends to aggregate in the presence of dextran. Most of the dextransucrase activity was found at the top one-third of the column. This could be eluted only at very high ionic strengths, and required extensive dialysis to remove the salt (Robyt and Walseth 1979). Monsan and Lopez reported a much higher specific activity and achieved the purification of dextransucrase from *Leuconostoc mesenteroides* culture supernatant by slow addition of sucrose (Monsan and Lopez 1981). This resulted in high activity of enzyme that was purified by single step using gel permeation chromatography on Ultrogel AcA34 (Monsan and Lopez 1981). This procedure yielded enzyme with a specific activity of 122 U/mg with 96.4% yield. Kobayashi *et al*. (1986) obtained enzyme precipitates from *Leuconostoc mesenteroides* culture supernatant by ethanol precipitation and centrifugation. The precipitate was put on DEAE-cellulose column and the enzyme was eluted with a NaCl gradient and the active fractions were pooled and concentrated. The enzyme was then put on Sephadex G-100 column and the active fractions collected, and a specific activity of 26 U/mg was obtained with 679-fold purification (Kobayashi *et al*. 1986)[63]. Gel-filtration chromatography of 10% PEG-1500 fractionated dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 (specific activity of 23 U/mg) using Sephacryl S200HR resulted in highly purified dextransucrase and a specific activity of 35 with a 61-fold purification was obtained (Purama and Goyal 2007b).

1.3.5 Dextranase treatment and chromatographic separation

Dextranases (EC 3.2.1.11) are enzymes, which cleave the glycosidic bonds in dextran. Dextranase enzyme is both glycosidic bond and strain specific. Dextranase is mostly obtained from fungi. Cleavage of dextran makes the enzyme purification easier as the culture broth becomes less viscous, facilitating the separation of multiple forms of enzyme. The culture supernatant of *Leuconostoc mesenteroides* B-512F concentrate was loaded on Bio-Gel A-5m column, before and after dextranase treatment (Robyt and Walseth 1979). Dextransucrase and levansucrase migrated in the void volume before dextranase treatment. After treatment however, the dextransucrase was retarded by Bio-Gel A-5m, and levansucrase still migrated in the void volume. Furthermore, the added dextranase was retarded to a greater extent than dextransucrase, thus giving a separation of dextransucrase from levansucrase and dextranase. The purified dextransucrase was obtained with an activity of 53 U/mg, recovered with 84% of the carbohydrate removed (Robyt and Walseth 1979). Chromatography on DEAE-cellulose after dextranase treatment removed most of the polysaccharide associated with the enzyme (Robyt and Walseth 1979). The traces of carbohydrate left were removed after chromatography on Sephadex G-200. Dextranase treatment was required to degrade the enzyme associated dextran prior to DEAE-cellulose chromatography. Without dextranase treatment, up to 40% of the activity failed to bind to DEAE cellulose (Robyt and Walseth 1979). Traces of dextranase present in the dextransucrase preparation from DEAE-cellulose deteriorated the flow rates during chromatography on Sephadex due to degradation of Sephadex by dextranase. It therefore becomes essential to remove dextranase completely before applying the active fractions from DEAE-cellulose to Sephadex column.

Nitrosoguanidine mutant of *Leuconostoc mesenteroides* B-512F with an activity of 3 U/ml in the culture supernatant was selected for high dextransucrase production (Miller and Robyt 1984). The crude dextransucrase was incubated with immobilized dextranase. This preparation was passed over a column of Bio-Gel A-5m. An improvised protocol involving dextranase treatment, DEAE-cellulose chromatography, affinity chromatography on Sephadex G-200 and chromatography on DEAE-Trisacryl M to obtain dextransucrase with low carbohydrate content (1-100 µg/mg protein) and relatively higher specific activity (90-170 U/mg) was developed (Miller *et al.* 1986). Levansucrase was completely removed during DEAE-cellulose chromatography and dextranase became undetectable after Sephadex G-200 chromatography (Miller *et al.* 1986). This method was also scaled up to produce gram quantities of purified enzyme. Kobayashi and Matsuda (1986) used sepharose 6B column for effective purification of dextransucrase after dextranase treatment. The culture supernatant of *Leuconostoc mesenteroides* was added with slurry of hydroxyapatite which adsorbed the enzyme. The slurry was washed to desorb the enzyme that was applied to a column of Sephadex G-100. The major volume of enzyme, which had no affinity for sephadex, was eluted in the void volume fraction and digested with dextranase. After subsequent removal of the dextranase activity by CM-cellulose column, the enzyme fraction was chromatographed on a Sepharose 6B column. The sepharose 6B column was effective for the preparation of dextransucrase with no contaminating dextran and also removed traces of urea, which was used as elutant and resulted 72.1 U/mg of specific activity (Kobayashi and Matsuda 1986). Crude enzyme preparation of *Leuconostoc mesenteroides* B-512FM was treated with crude dextranase followed by column chromatography on Bio-Gel A-5m. The purified enzyme showed a specific activity of 84 U/mg (Fu and Robyt 1990).

1.3.6 Ultrafiltration

Bulk purification of extracellular dextransucrase from *Leuconostoc mesenteroides* B-512F using ultra-filtration was attempted for commercial production of dextran (Ajongwen *et al.* 1993). Ultracentrifugation and cross-flow ultrafiltration were employed in a three-stage process to obtain dextransucrase of over 95% purity with an overall yield of 60% (Ajongwen *et al.* 1993). In the first stage, the cells were removed by continuous centrifugation at high centrifugal force. This was followed by continuous ultrafiltration using hollow fiber membrane with flat cross flow to remove soluble low M_r, impurities. The enzyme so obtained was stored with dextran as it prevented the loss of enzyme activity. Just before the use of enzyme for dextran synthesis, a second centrifugation process was carried out as the third stage process, to remove dextran (Ajongwen *et al.* 1993).

A constitutive mutant *Leuconostoc mesenteroides* B-512FMC-16, which produced 16 U/ml dextransucrase with glucose in the medium, was used to obtain dextransucrase by ultrafiltration (Kitaoka and Robyt 1998). The absence of dextran simplified the process facilitating large-scale production of highly pure dextransucrase by ultra-filtration. The process involved the addition of Tween 80 and $CaCl_2$ to the culture filtrate prior to concentration. Tween 80 dissociated inactive enzyme aggregates and produced active enzyme molecules. $CaCl_2$ caused aggregation of the enzyme molecules, which did not pass through a polysulfone ultrafiltration hollow fiber cartridge with 100 kDa cut off at a constant flow rate resulting in concentration and purification. The enzyme was concentrated from 23.50 l to 1.65 l, giving 280 U/ml with only 7% loss in enzyme activity (Kitaoka and Robyt 1998). Purified dextransucrase from *Leuconostoc mesenteroides* B-512F was obtained by passing the culture supernatant through an ultrafiltration system, equipped with a 120

kDa cut off hollow fiber cartridge (Sanchez-Gonazalez *et al.* 1999). Samples were lyophilized and resuspended in buffer, containing $CaCl_2$ and sodium azide (Sanchez-Gonazalez *et al.* 1999).

1.3.7 Purification of dextransucrase from constitutive mutants of *Leuconostoc mesenteroides*

The enzyme dextransucrase produced by *Leuconostoc mesenteroides* mutants in the presence of various sugars was studied for adsorbtion of enzyme to the sephadex column (Lawford *et al.* 1979). Partial purification of enzyme was done by ammonium sulfate precipitation and passing through a column of hydoxyapatite (Lawford *et al.* 1979). The partially purified enzyme was run through sephadex column. The enzyme produced in glucose medium more readily adsorbed on sephadex G-100 and G-200, than that produced in sucrose medium as the enzyme produced on glucose medium was free of any preformed dextran thus, eliminating the dextranase treatment and DEAE-cellulose separation. The adsorption by sephadex decreased when the glucose-produced enzyme was pre-incubated with dextrans of molecular size greater than 10 kDa, as the dextran gets associated with the enzyme and reduces the affinity of sephadex for enzyme (Lawford *et al.* 1979). The culture supernatant of constitutive mutant SH 3002 of *Leuconostoc mesenteroides* B-512F grown on fructose was, put on DEAE-Sephadex A-50 column and eluted with NaCl gradient (Funane *et al.* 1995). The active fractions were pooled and put on a Sepharose CL-6B column. A specific activity of 171 U/mg with 43.8% activity yield was obtained (Funane *et al.* 1995). Purification of the enzyme from constitutive mutants has the advantage that it has no dextran along with the enzyme. This eases the chromatographic purification procedures by eliminating dextranase treatment and DEAE-cellulose chromatography.

1.3.8 Purification of recombinant dextransucrase

Commercially available dextransucrase produced from *Leuconostoc mesenteroides* is induced by the addition of sucrose to the culture medium and this leads to enzyme preparations consisting of 99% dextran and only 1% protein. These preparations are difficult to use for the investigation of enzyme structure and catalysis. To obtain dextran free dextransucrase, recombinant dextransucrase of *Leuconostoc mesenteroides* B-742 production by expression in *Escherichia coli* DH5α cells was established and purified (Kang *et al.* 2003). The recombinant *Escherichia coli* DH5α cells were cultivated in Luria-Bertani medium containing 2% sucrose or glucose. The culture supernatant was concentrated by the addition of PEG-1500 to a final concentration of 20%. The extracellular activity was recovered in the dextran-rich phase. Dextran bound to the enzyme was removed by dextranase. The dextranase-treated enzyme was dialyzed and the dialyzate was loaded onto a DEAE-sepharose column. The enzyme from the column was eluted on a linear gradient of NaCl. The fractions with dextransucrase activity were pooled and applied to phenyl-sepharose column. The dextransucrase fraction was eluted with a linear gradient of decreasing ionic strength of sodium acetate buffer. A specific activity of 29.2 U/mg was obtained (Kang *et al.* 2003).

The recombinant dextransucrase from *Leuconostoc mesenteroides* B512F was expressed and produced in *Bacillus megaterium* that has protein secretion capacity for the production for the production of exoenzymes (Malten *et al.* 2004). It was formed in the cytoplasm and released into the growth medium via its native leader sequence. For concentrating the excreted recombinant dextransucrase from the growth medium, samples were frozen at -20°C to precipitate the protein and the enzyme was harvested by centrifugation. The induction in *Bacillus megaterium*, produced very high specific

(362 U/g) and volumetric (28,600 U/l) activity of dextran free dextransucrase (Malten *et al.* 2004). A recent report described the production of recombinant *Leuconostoc mesenteroides* B-512F dextransucrase with $(His)_6$ tag at the C-terminal and its expression in *Escherichia coli* TOP10 cells (Moulis *et al.* 2006). The recombinant enzyme was purified by affinity chromatography using Ni^{2+} ion immobilized column. A highly pure dextransucrase was obtained with a specific activity of 430 U/mg (Moulis *et al.* 2006).

1.4 Properties of dextransucase

Dextransucrase was purified by a simple fractionation procedure with PEG (Goyal *et al.*1995). Dextransucrases are highly sensitive to pH, temperature and the dilution (Miller and Robyt 1984). A purified dextransucrase from *Leuconostoc mesenteroides* B512F enzyme exhibited maximum activity at 30°C and pH 5.2 (Goyal *et al.* 1995). The dextransucrase elaborated by *Leuconostoc mesenteroides* NRRL B-640 displayed a maximum enzyme activity when assayed in the temperature range of 30 to 35°C and at pH of 5.4 (Purama and Goyal 2007a). The optimal temperature of dextransucrase from was 35°C with half-life of 137 min (Chellapandian *et al*. 1998). The dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F exhibited a K_m of 2 mM for sucrose and an apparent optimum temperature of 30˚C (Miller and Robyt 1984). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed two protein bands, both with dextransucrase activity (Robyt and Walseth 1979). The molecular weight of dextransucrase from *Leuconostoc mesenteroides* IBT-PQ isolated from Pulque on an enzyme activity gel was 166 kDa (Chellapandian 1998). Heavy metal ions such as zinc, cadmium, lead, mercury and copper ions showed varied inhibitory effect on the enzyme activity (Robyt and Walseth 1979).

Dextransucrase was inactivated by EDTA and can be reactivated with Ca^{+2} (Robyt and Walseth 1979; Kobayashi and Matsuda 1980). Dextransucrase is strongly inhibited by heavy metal ions such as Cu^{2+}, Fe^{3+}, Zn^{+2}, Cd^{2+}, Pb^{2+}, Hg^{2+} and Mn^{2+} (Kobayashi and Matsuda 1976; Robyt and Walseth 1979;).

Dextransucrase is generally associated with the dextran. The removal of associated dextran from dextransucrase resulted in the loss of enzyme activity (Robyt and Walseth 1979). Kinetic studies of the dextransucrase are affected by the presence of dextran. The dilution of the purified dextransucrase also resulted in the loss of activity (Miller and Robyt 1984). The storage stability increased with the addition of dextran with out affecting the enzyme activity. Both crude and pure enzyme preparations became heat stable by the addition of low levels of high molecular weight dextran, PEG-20,000, methylcellulose and non-ionic detergent Tween 80 (Miller and Robyt 1984). The stability further increased with increase in molecular weight of the polymer. The enzyme stability was observed as a function of pH and time of the crude and partially purified enzyme (Rodrigues *et al.* 2003). In the crude, 30% activity loss was observed in 1h at 25°C, which increased to 70 and 80 percent at -15°C and 4°C in 24h (Rodrigues *et al*. 2003). On the other hand, storage in the ultrafreezer at -80°C the loss was only 5% (Rodrigues *et al*. 2003). Partially purified dextransucrase lost of only 10% activity at 4°C in 24h, which was further reduced to 3% when stored at -15°C ((Rodrigues *et al*. 2003). Glycerol provided maximum protection to dextransurase against inactivation as compared to Tween 80, dextran and PEG-8000 (Goyal *et al.* 1995). Certain metal ions and additives have been reported to stabilize dextransucrase from some strains *Leuconostoc mesenteroides* (Kobayashi and Matsuda 1980; Goyal *et al.* 1995). Among the metals ions; Ca^{2+}, Mg^{2+}, Co^{2+} and among stabilizers, the most widely used are Triton X-100, glycerol, Tween-80,

polyethylene glycols, dextran, glutaraldehyde and polyvinyl alcohols on various enzyme systems (Kobayashi and Matsuda 1980, Miller and Robyt 1984; Goyal *et al.* 1995). The dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 has been purified to homogeneity using PEG fractionation method and was characterized (Purama and Goyal 2007a) and its assay conditions have been optimized (Purama and Goyal 2007b). The enzyme gave a maximum activity at 30°C and pH 5.4.

1.5 Structural characteristics of dextransucrase

Several workers have reported that dextransucrase exists in single or multiple forms having molecular weight in the range 64,000 – 245,000 (Fu and Robyt 1990; Goyal and Katiyar 1994; Kobayashi and Matsuda 1980, Miller *et al.* 1986; Nigam *et al.* 2006; Willemot *et al.* 1988; Purama and Goyal 2007a). The enzyme remains in an aggregated form in the presence of dextran resulting in high molecular weight. The aggregated forms disassociate into low molecular weight forms by endodextranase treatment (Kobayashi and Matsuda 1980). The purified enzyme is stable and active only in presence of dextran (Miller and Robyt 1984). Diethyl pyrocarbonate (DEP) was shown to inhibit the enzyme activity, which indicated the presence of essential histidine residues at the active site (Fu and Robyt 1988). Funane *et al.* (1993) located a second essential carboxyl group for the catalytic activity located at 30-45 amino acids away from the catalytic aspatic acid towatrd the amino-terminal end. Statistical and kinetic analyses of the inactivation of enzyme by DEP showed that two histidine residues are essential for the enzymatic activity. A lysine residue at the active site was shown to play an important role in the catalytic activity of dextransucrase (Goyal and Katiyar 1995). The presence of an essential lysine residue at the active site was shown by 2,4,6-trinitrobenzene-sulphonic acid (Goyal and Katiyar 1995), pyridoxal-5'-

phosphate (Goyal and Katiyar 1998a) and *o*-phthalaldehyde inactivation (Goyal and Katiyar 1998b) studies. Funane *et al.* (2005) found that the introduction of Lys residue at the N-terminal end of the core domain by site-directed mutagensis caused changes in the linkages pattern of glucan products thus altering their solubility. A single but non-essential cysteine residue was located near the active site of dextransucrase by chemical modification studies and amino acid analysis (Goyal *et al.* 2007). Structural-functional relationships of glucansucrases from lactic acid bacteria have been documented in the reviews (Monchois *et al*. 1999; Hijum *et al*. 2006).

1.6 Applications of oligosaccharides produced from dextransucrase

The dextransucrase activity of *Leuconostoc mesenteroides* was used in acceptor reactions with maltose in order to obtain oligosaccharides with α-1,2 glycosidic bonds (Paul *et al.* 1992; Remaud-Simeon *et al.* 1994). These compounds resist hydrolysis by the digestive enzymes in animals and human because of the configuration of their glycosidic bonds and selectively stimulate intestinal denizens like *Bifidobacterium* spp. *Lactobacillus* spp. and *Bacteroides* spp. (Valette *et al.* 1993) and suppress or unfalter the coliform and pathogenic bacteria. They are already being used as additives in animal and human nutrition (Valette *et al.* 1993; Monsan and Paul 1995; Chung and Day 2002; Naessens *et al.* 2005). Modified iron and sulfate oligosaccharides synthesized from the oligosaccharides produced from the mixed-culture fermentation of *Lipomyces starkeyi* and *Leuconostoc mesenteroides*. They inhibited the glucosyltransferase activity of *Streptococcus mutans, Streptococcus sobrinus, Eikenella corrodens, Prevotella intermedia*, and *Actinobacillus actinomycetemcmitans* thus, reducing the growth and acid productions of these oral pathogens (Seo *et al.* 2004). 1,5-Anhydro-D-fructose oligosaccharides generated by

dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F by its action on the acceptor 1,5-Anhydro-D-fructose are shown to have antioxidant properties and are used as antioxidants in the food industry (Richard *et al.* 2005).

The major applications of glucansucrases other than the generation of oligosaccharides are glucosylation of unusual saccharides such as cellobiose, acarbose, methyl hexopyranosides, alkylglucosides, alditols, aldosulose, sugar acids and salicin and conversion of hydrophobic compounds (glyco-conjugates) to hydrophilic compounds by the glucosylation procedures (Bertrand *et al.* 2006). Luteolin-30-O-α-D-glucopyranoside and luteolin-40-O-α-D-glucopyranoside are generated from the luteolin, a flavonoid by glucosylation with dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F showed increased solubility (Bertrand *et al.* 2006). Luteolin-glucopyranosides have recently been shown to exhibit anti-inflammatory, anti-allergic, anti-oxidant or antitumoural activities, conferring to these polyphenols a potential protective function against cardiovascular and coronary heart diseases and against certain forms of cancer (Bertrand *et al.* 2006). Salicin or salicyl alcohol acceptor products generated by dextransucrase form *Leuconostoc mesenteroides* B-1299 CB, B-1355C2 and BF563 showed higher ati-coagulation activity with that of their precursors (Seo *et al.* 2005). Epigallocatechin gallate glycosides were synthesized by the acceptor reaction of a glucansucrase produced by *Leuconostoc mesenteroides* B-1299CB with epigallocatechin gallate (EGCG) and sucrose exhibited antioxidant effects and also manifested a higher degree of browning resistance (Moon *et al.* 2006).

1.7 Dextrans

Dextrans are polymeric chain of glucosyl units, synthesized by dextransucrase by the transfer of D-glucosyl unit from sucrose to acceptor molecules. This polymerisation results in formation of dextran and fructosyl unit is released free. Dextrans feature substantial number of consecutive α(1→6)-linkages in their main chain, usually comprising more than 50% of the total linkages. These α-D-glucans also possess side chains, stemming mainly from α(1→3) and occasionally from α(1→4)- or α(1→2)-branched linkages. A survey of ninety-six strains of dextran producing bacteria was done to classify dextrans by their structure and properties and identify a suitable strain, for pharmaceutical industry (Jeanes *et al.* 1954). Different types of dextrans of varying size and structure are synthesized depending on the dextransucrase produced by the strain (Seymour and Knapp 1980; Robyt 1995a; Robyt 1995b; Leathers 2002).

Extensive work has been done on optimization and modification of the fermentation processes for improved production of dextran (Stacey 1942; Hehre 1946; Koepsell and Tsuchiya 1952; Hehre 1955; Jeanes *et al.* 1957; Jeanes 1965; Lawford *et al.* 1979; Alsop 1983; Barker *et al.* 1987; Landon and Webb 1990; Brown and McAvoy 1990; Lazic *et al.* 1993; Ajongwen and Barker 1993; Ajongwen *et al.* 1993; Barker *et al.* 1993). Conventional fermentation used for the production of dextran involves three phases: cell growth, enzyme production phase and dextran synthesis (Alsop 1983). Sucrose solution, fortified with required nutrients, is inoculated with the bacterial culture with little or no process control. This approach, which combines all the three phases, has the disadvantage of having transitory optimal conditions for any of these processes (Alsop 1983). Since, dextran synthesis takes place outside the cell in presence of dextransucrase, decoupling of the enzyme and dextran production

was explored for optimizing the dextran synthesis (Landon and Webb 1990). Fed batch reactor maintained at constant pH of 6.7, was used for enhancing the production of dextran (Brown and McAvoy 1990; Ajongwen and Barker 1993). Other approaches included immobilization of *Leuconostoc mesenteroides* NRRL B-512F cells on stainless steel (El-Sayed *et al.* 1992), encapsulation in calcium alginate beads (El-Sayed *et al.* 1990a; El-Sayed *et al.* 1990b; El-Sayed *et al.* 1990c), an amino-Spherosil support activated with glutaraldehyde, an alkylamine porous silica (Lopez and Monsan 1980; Kobali and Reilly 1980; Monsan and Lopez 1981) and composite agar/micro porous membrane (Lebrun *et al.* 1994). A novel approach using chromatographic bioreactor-separator was reported for the synthesis of clinical dextran by purified dextransucrase (El-Sayed *et al.* 1990c). Fructose, which is the by product of dextran synthesis was retained complexing with calcium ions on the resin used in the reactor, which facilitated the higher production of dextran having molecular weight in the range of 10,000 and 200,000 (El-Sayed *et al.* 1990c).

1.7.1 Composition of dextran

Dextran is the collective name now given to a class of polysaccharides composed exclusively of the monomeric unit α-D-glucose linked mainly by α(1→6) bonds and a variable amount of α(1→2), α(1→3) or α(1→4) bonds. Most of the recent work with dextrans has been conducted with *Leuconostoc mesenteroides* particularly with the strain NRRL B-512F. The dextran synthesized by this strain is a homopolysaccaharide containing 95% α(1→6) glucosidic linkages in the main linear chain and remaining 5% of α(1→4), α(1→3) and a very few α(1→2) branched linkages. The branches are mostly 1-2 glucose units long. The solubility of dextran occurs as a function of linkage pattern. Presence of 95% linear linkages makes this

dextran water soluble (Van Cleve *et al.* 1956), which has various uses. Dextrans synthesized by dextransucrase from other species of Lactobacillaceae, contain higher percentage of branched α(1→3) glycosidic linkages in the main chain and are thus, water insoluble. Dextrans are classified into three classes A, B and C on the basis of the percentage of 1,3-like linked anhydroglucopyranose units (AGU) as determined by periodate oxidation (Rankin and Jeanes 1954). *Leuconostoc mesenteroides* NRRL B-640 is shown to produce dextransucrase that gives highly linear and soluble dextran (Seymour 1979a; Uzochukwu 2001). Sidebotham (1974) reported that various dextrans have ^{1}H-NMR spectral resonances (C-2, C-3, C-4, C-5 and C-6) in the 3-4 ppm region and the hemiacetal C-1 resonance in 4-6 ppm region. Seymour (1979b) showed that the ^{1}H-NMR spectral region for anomeric carbon of dextran from *Leuconostoc mesenteroides* NRRL B-1355 contained a resonance at 4.95 ppm and the branched linkages contained the resonance peak at 5.3 ppm. Seymour *et al.* (1979a) employed ^{13}C-NMR spectroscopy to examine the structure of a series of dextrans and established that linear dextran has six prominent resonances. The ^{13}C-NMR spectra displayed two major regions; (a) the 95-105 ppm region, which is the anomeric region and (b) the 75-85 ppm region for dextrans branched at C-2, C-3, or C-4. ^{13}C-NMR resonances with in the 70-75 ppm region are associated with free positions at C-2, C-3 and C-4 residues. The FT-IR spectral data was useful in determining the nature of dextrans in terms of their monomeric units and their linkages (Shingel 2002). Several other repots also supported the use of FT-IR spectral data for the chatacterization of glucans showing anti-cancer properties (Cao *et al.* 2006; Liu *et al.* 2007).

1.7.2 Applications of dextran

Soluble dextrans have well documented wide applications in industry (Robyt 1986; Purama and Goyal 2005; Naessens *et al.* 2005; Moulis *et al.* 2006). The importance of soluble dextran synthesized by *Leuconostoc mesenteroides* lies in its wide applications in pharmaceuticals, food, agricultural and fine chemical industries. Dextran and its derivatives are used in medicine as blood plasma expander and blood flow improver, anti-ulcer agent, against iron deficiency anemia and in open wound healing (Robyt 1986). Its derivative dextran sulfate, has anti-coagulant properties, although less effective than that of heparin. It also inhibits viral infections. It binds to the attenuated poliovirus and interferes with its initial adsorption to susceptible cells. Sodium salt of dextransulfate inhibits AIDS virus. Dextran is used as conditioning, stabilising, preservative and bodying agent replacing natural gums (Koepsell and Tsuchiya 1952). The derivatives of dextran like Sephadex and DEAE-dextran serve as molecular sieves and are extensively used in the separation of biomolecules (Robyt 1986). It has agricultural applications too. The seeds can be coated with dextran film in which fungicides or any insecticides can be incorporated (Robyt 1986). Dextran also finds a significant use in the photographic industry (Robyt 1986). Low molecular weight dextran results erythrocyte desegregation (Robyt 1986). High molecular weight DEAE-dextran reduces cholesterol and triglycerides (Robyt 1986). Low molecular weight dextran at 5-10% has proved beneficial in preserving osmolarity, implicated in kidney, liver and cornea perfusions (Robyt 1986). Dextran has been recommended as a cryoprotective agent for human, animal and plant cells (Pellerin-Mendes *et al*. 1997). The water solubility of nalidixic acid has been improved by the substitution of the drug with dextran (Lee *et al.* 2001). Enzyme can be preserved by conjugating with dextran. Technetium conjugated dextran is used in

lymphoscintigraphy of leg, pelvic and para-aortic lymph nodes (Matsunaga *et al*. 2005). Leuconostoc cells producing exopolysaccharides are used for oil recovery (Kim *et al*. 2000; Wolf and Fogler 2004). Dextran hydrogels were used in various pharmaceutical and biomedical applications such as contact lenses, cell encapsulation for drug delivery, burn dressing and in spinal cord regeneration (Ferreira *et al*. 2005; Aumelas *et al*. 2007).

Native polyelectrolytes generated by sulphation of branched dextran have been used as anticoagulants, agents lowering cholesterol, anti-ulceratives, as anti-metatastatic agents in the treatment of sepsis, AIDS and human prostatic carcinoma (Robyt 1986). Oxidised dextan can serve as a cross-linker in the formation of gelatin microspheres, thus decreasing the ready solubility of gelatin and are used for the controlled release of drugs for an extended time periods (Cortesi *et al*. 1999). Tissue engineering scaffolds having large pores made from dextran derivatives were used to acommodate living cells and various bone morphogenic proteins. These scaffolds can allow the easy penetration and proliferation of these cells in the target sites for the tissue regeneration processes (Chen *et al*. 2005; Levesque *et al*. 2005). Magnetic iron-dextran nanoparticles coated with antiserum against an enterohemorrhagic *Escherichia coli* O157: H7 were used to isolate them from samples within 15 min with the sensitivity of 101 CFU/ml (Duan *et al.* 2005). Carboxymethyl dextran (CM-dextran) cross-linked with the glucose binding lectin concanavalin A (ConA) was developed as as D-glucose-sensor, selectively detected the D-glucose but not the L-Glucose (Zhang *et al*. 2006).

1.8 Objectives of the present study

The *Leuconostoc* species are well studied for the production of dextran owing to its applications in pharmaceutical, food, agriculture and fine chemical industries. Dextran is used as thickening agent, emulsifier, non-cariogenic sweetener in the food industry, and as protective agent of perfumes from light, heat and oxidation in cosmetic industry. Isomalto-oligosaccharides synthesized as an acceptor products with dextransucrase acts as prebiotics. These isomalto-oligosaccharides displayed the selective growth promotion of intestinal bacteria such as Lactobacilli and Bifidobacterium that beneficially affect host health.

There are several strains of *Leuconostoc mesenteroides* available and many of them have been extensively studied. However, there are strains that are never explored and yet they may possess dextransucrase with higher activity and resulting in a type of dextran that may find potential use in industry. One such strain is *Leuconostoc mesenteroides* NRRL B-640 which gives highly soluble dextran with 97% α(1→6) glucosidic linkage. There is no information on nutrient and growth conditions of the strain and the dextransucrase produced by it. No information is available on the antibiotic resistance, carbohydrate fermentation behavior, plasmid content and polysaccharide synthesis activity of *Leuconostoc mesenteroides* NRRL B-640. Therefore in the present study *Leuconostoc mesenteroides* NRRL B-640 was chosen for dextransucrase production. The microorganism was studied for nutrient, micronutrient and growth conditions requirement. The dextransucrase was purified and biochemically characterized. The structure of dextran synthesized by dextransucrase elaborated by *Leuconostoc mesenteroides* NRRL B-640 was carried out using FT-IR, ^{1}H-NMR and ^{13}C-NMR techniques.

The specific objectives of the present study are

1. Characteristics of the *Leuconostoc mesenteroides* NRRL B-640
2. Optimization of culture conditions for production of dextransucrase and its assay.
3. Effect of nutrients on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640
4. Optimization of medium composition for higher dextransucrase production using Response surface methodology.
5. Effect of pH and aeration on secretion of dextransucrase in bioreactor.
6. Purification of dextransucrase by fractionation and chromatographic methods.
7. Identification and confirmation of dextransucrase by activity staining.
8. Molecular size analysis and biochemical characterization of dextransucrase.
9. Structure determination of dextran by FT-IR, ^{1}H-NMR and ^{13}C-NMR techniques.

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Chapter 2

Maintenance and Characteristics of Microorganism

2.1 Introduction

Four different genera of lactic acid bacteria: *Streptococcus*, *Leuconostoc*, *Weissella* and *Lactobacillus* are known to produce glucansucrases (Monchois *et al.* 1999; Kralj *et al.* 2004; Tieking *et al.* 2005). Glucansucrases from *Leuconostoc* genus are inducible enzymes, which are induced in the presence of sucrose (Neely and Nott 1962). The organisms of *Leuconostoc* genus are micro-aerophilic. The extracellular glucansucrase is used for synthesis of dextran, which has numerous applications in pharmaceutical, food and fine chemical industries (Robyt 1986; Naessens *et al.* 2005; Purama and Goyal 2005). Different *Leuconostoc* strains have been grown at temperature ranging from 20 to 30°C for glucansucrase production (Purama and Goyal 2005). Despite their commercial significance relatively little is known about their antibiotic resistance, physiology and genetics of these glucansucrase producing bacteria. Limited information is available on antibiotic susceptibilities and carbohydrate fermentation behavior of glucansucrase producing Leuconostoc strains (Kelly *et al.* 1986; Holt *et al.* 2001). It was found that several Leuconostoc strains used in dairy and wine industries were resistant to vancomycin (Orberg and Sandine 1984; Holt *et al.* 2001). The type of glucansucrase elaborated by a strain can be

identified by the type of glucan produced depending upon the type of substrate utilized. The sucrose hydrolyzing activity of Leuconostoc strains was analyzed by the synthesis of polysaccharide which was detected as activity bands within a polyacrylamide gel using a Periodic acid Schiff staining protocol (Leathers *et al.* 1997). There are few reports on the presence of plasmid DNA from Leuconostoc strains (O'Sullivan and Daly 1982; Orberg and Sandine 1984). The plasmids possessing genes for lactose utilization (David *et al.* 1992), citrate utilization (Vaughan *et al.* 1995) and for bacteriocin (Fremaux *et al.* 1995) have been identified. There are no reports available on the antibiotic resistance, carbohydrate fermentation behavior, plasmid content and polysaccharide synthesis activity of *Leuconostoc mesenteroides* NRRL B-640. In the present study, three glucansucrase producing Leuconostoc strains *viz. Leuconostoc mesenteroides* B-512F and B-640 and *Leuconostoc citreum* B-742 were characterized and compared for their antibiotic sensitivities, carbohydrate fermentation profiles, plasmid profiles and glucan synthesizing activities.

2.2 Materials and Methods

2.2.1 Bacterial strains and culture conditions

All the three Leuconostoc strains, *Leuconostoc mesenteroides* strains B-512F and B-640, and *Leuconostoc citreum* strains B-742 were obtained from the Agriculture Research Service culture collection, Peoria, IL, USA. The stock cultures of Leuconostoc strains were maintained in MRS medium (DeMan *et al*. 1960) as stab at 4°C. All strains were grown at 28°C for all experiments.

2.2.2 Antibiotic sensitivity

Leuconostoc strains were tested for susceptibility to thirty antibiotics using agar disc diffusion test (Barry and Thornsberry 1980). The antibiotic tests were performed using commercially available antibiotic octodiscs containing Amoxyclav (Ac), Cephalexin (Cp), Ciproflaxacin, (Cf), Clindamycin (Cd), Claxacillin (Cx), Erythromycin (E), Tetracyclin (T), Ampicillin (A), Carbenicilllin (Cb), Cephatoxamine (Ce), Chloramphenicol (C), Co-Trimazine (Cm), Gentamicin (G), Norflaxacin (Nx), Oxacillin (Ox), Amikacin (Ak), Amoxycillin (Am), Bacitracin (B), Cephalothin (Ch), Novobiocin (Nv), Oxytetracyclin (O), Vancomycin (V), Penicillin-G (P), Tobramycin (Tb), Cephaloridine (Cr), Kanamycin (K), Linomycin (L), Methicillin (M), Norfloxacin (Nf), Oleandomycin (Ol) from Hi-media Pvt. Ltd. India. MRS medium containing 2.0% glucose as carbohydrate source with 1.8 % agar and 0.8% agar were used. The petri-plates were first prepared with MRS medium containing 1.8% agar. The test strain was seeded in MRS-soft agar (0.8% agar) and overlaid in the Petri-plate having a bottom layer of above MRS agar (1.8%). The culture plate was allowed to dry for about 2 min. The octodiscs were gently placed over the surface of the seeded plate. The Petri plates were incubated in inverted position overnight in an incubator at 28°C and were observed next day for zone of inhibition around the discs.

2.2.3 Carbohydrate fermentation

The Leuconostoc strains were tested for their ability to ferment various carbohydrates using the method of Kandler and Weiss 1986. From the overnight grown MRS broth culture containing 2% glucose as carbohydrate source, 50 µl was inoculated in 5.0 ml liquid MRS medium lacking glucose but containing Phenol red

and other test carbohydrates to give a final inoculum to medium ratio of 1% (v/v). The test media were incubated for 2 days at 28°C with out shaking. The acid production was recorded between 24-48h. The acid production was indicated by a change in colour of the phenol red indicator dye from red to yellow.

2.2.4 Plasmid isolation

The Leuconostoc strains were grown in 5 ml liquid MRS medium for 24h. The strains were screened for the presence of plasmid DNA using an alkaline lysis protocol. The cells were collected by centrifugation from 5 ml cultures and resuspended in 100 µl TGE buffer (25 mM Tris HCl, pH 8.0, 50 mM glucose and 10 mM EDTA). A lysozyme solution was added (40 µl of stock solution consisting of 50 mg/ml), and the mixture was incubated at 25°C for 60 min. Following incubation, 200 µl lysis solution (1% sodium dodecyl sulphate in 0.2N NaOH) was added, mixed three times by inversion, and incubated on ice for 5 min. 150 µl of ice-cold 5M potassium acetate buffer pH 4.8 to the lysis mixture, mixed by vortex action for 10 s and incubated on ice for 5 min. The mixture was centrifuged at 16000***g*** for 10 min in a microcentrifuge and the supernatant was transferred to a fresh tube. Supernatant was extracted with an equal volume of phenol/chloroform (50:50) and then with an equal volume of chloroform/isoamyl alcohol (24:1). The plasmid DNA was precipitated from the aqueous supernatant with two volumes of cold 100% ethanol, and the DNA pellet was collected by centrifugation at 16000***g*** for 5 min a microcentrifuge. The pellet was washed twice with 500 µl 70% (v/v) ethanol and resuspended in 35 µl of 20 mM Tris-EDTA buffer (pH 8.0). The DNA samples were examined by using agarose gel (0.8%) electrophoresis and 1xTAE (Tris base, Acetic acid, EDTA; pH 8.0) buffer for preparing gel as well as for running buffer.

2.2.5 Detection of sucrose hydrolyzing activity

In-situ activity of dextransucrase was detected on a 7.5% acrylamide gels run under SDS-non-denaturing conditions using the protocol described by Holt *et al.* (2001) with modification. The crude cell free extract samples from all four Leuconostoc strains were loaded on duplicate 7.5% acrylamide gels under SDS-non-denaturing condition. After the run, SDS was removed by incubating the gel in sodium acetate buffer (20 mM sodium acetate, pH 5·4, 0·3 mM $CaCl_2$ and 0·1% Tween 80) at 4°C 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 5% sucrose for 48h at 30°C. 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) for 45 min at room temperature. After the periodic acid treatment, the gel was washed with water for 2h with several changes. The gel was then stained with 15 ml Schiff reagent (0.5% w/v Fuchsin basic, 1% sodium bisulphite and 0·1 N HCl) until the discrete magenta bands within the gel matrix appeared, which confirmed dextransucrase activity. The other gel was stained with Coomassie brilliant blue for location of activity bands. Molecular mass marker proteins (Myosin from Rabbit Muscle, 205000; Phosphorylase b 97400; Bovine serum albumin, 66000; Ovalbumin, 43000; Carbonic anhydrase, 29000 Da) purchased from Genei, India, were used as standard for SDS-PAGE.

2.3 Results and Discussion

2.3.1 Antibiotic susceptibility of Leuconostoc strains

A standardized filter-paper disc-agar diffusion assay is used to determine the drug susceptibility of microorganisms. This method allows rapid determination of the efficacy of the drug by measuring the diameter of the zone of inhibition which results from diffusion of the agent into the medium surrounding the disc. In this procedure, the filter-paper discs of uniform size are impregnated with specified concentrations of different antibiotics and then placed on the surface of an agar plate that has been seeded with the microorganism to be tested. Following the incubation, all the plates were examined for the inhibition of growth, which is indicated by a clear zone surrounding each disc (Fig. 2.1, A-D). A control of each strain with out the antibiotic disc is also grown, showing the normal growth of the strain (Fig. 2.1, E). The susceptibility of an organism to a drug is determined by the size of this zone, which itself is dependent on variables such as: the ability and rate of diffusion of the antibiotic into the medium and its interaction with the test organism; the growth rate of the organism; the degree of susceptibility of the organism to the antibiotic. A measurement of the diameter of the zone of inhibition is made and its size is compared to that contained in a standardized chart (Fig. 2.1). Based on this comparison, the test organism is determined to be resistant or susceptible to the antibiotic. All the three Leuconostoc strains were tested for susceptibility to thirty antibiotics representing the major antibiotics. All the three strains were resistant to the antibiotics *viz.* co-trimazine, norflaxacin, norfloxacin and vancomycin (Table 2.1). All the three strains were sensitive to amoxycillin, bacitracin, carbenicillin, cephalothin, cephatoxamine, chlorampenicol, clindamycin, linomycin, oxytetracyclin and tetracyclin.

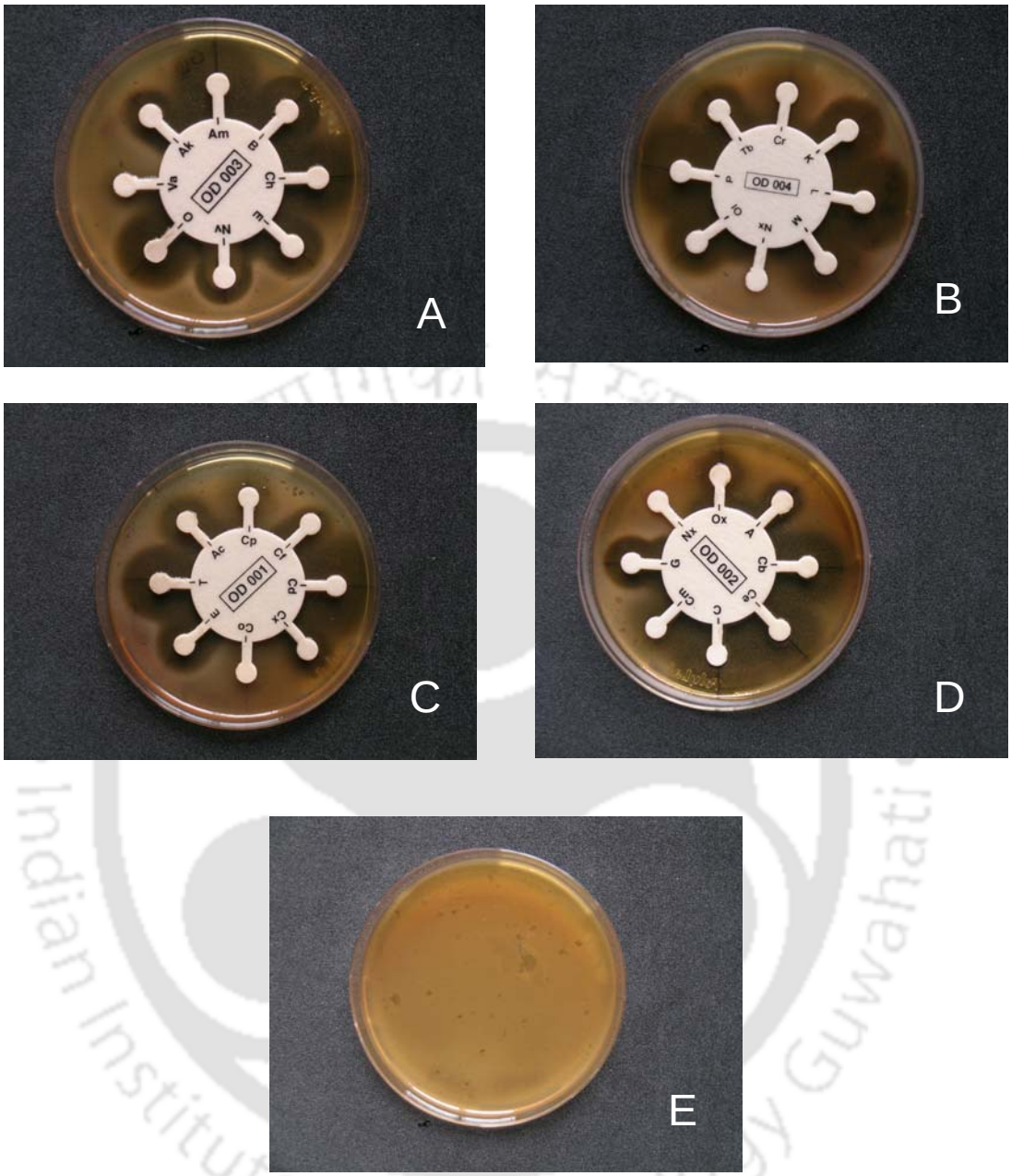

Fig. 2.1. Antibiotic susceptibility patterns of Leuconostoc strains. Figs A-D show susceptibility of *Leuconostoc mesenteroides* NRRL B-640 against different antibiotics abbreviated (as explain in Table 2.1) and Fig. 2.1E is the control showing the lawn of the grown microorganism without the antibiotic octodisc.

Table 2.1 Antibiogram of *Leuconostoc* strains using antibiotic discs on MRS medium

No.	Antibiotic	Concentration (μg)	B-512F	B-640	B-742
1.	Amoxyclav (Ac)	10	M	M	S
2.	Cephalexin (Cp)	10	M	R	S
3.	Ciproflaxacin (Cf)	10	M	R	M
4.	Clindamycin (Cd)	2	S	S	S
5.	Claxacillin (Cx)	1	M	M	M
6.	Erythromycin (E)	15	M	M	M
7.	Tetracyclin (T)	30	S	S	S
8.	Ampicillin (A)	10	M	M	M
9.	Carbenicilllin (Cb)	100	S	S	S
10.	Cephatoxamine (Ce)	30	S	S	S
11.	Chloramphenicol (C)	30	S	S	S
12.	Co-Trimazine (Cm)	25	R	R	R
13.	Gentamicin (G)	10	M	M	R
14.	Norflaxacin (Nx)	10	R	R	R
15.	Oxacillin (Ox)	5	M	M	S
16.	Amikacin (Ak)	10	R	M	R
17.	Amoxycillin (Am)	10	S	S	S
18.	Bacitracin (B)	10 Units	S	S	S
19.	Cephalothin (Ch)	30	S	S	S
20.	Novobiocin (Nv)	30	M	M	M
21.	Oxytetracyclin (O)	30	S	S	S
22.	Vancomycin (V)	30	R	R	R
23.	Penicillin-G (P)	10 Units	S	S	S
24.	Tobramycin (Tb)	10	M	M	R
25.	Cephaloridine (Cr)	30	M	R	M
26.	Kanamycin (K)	30	M	S	R
27.	Linomycin (L)	2	S	S	S
28.	Methicillin (M)	5	S	M	S
29.	Norfloxacin (Nf)	10	R	R	R
30.	Oleandomycin (Ol)	15	M	S	S

R- Resistant (0-2 mm*); M- Moderate (3-6 mm*); S- Sensitive (7-13 mm*)

*Values in millimeters are the distance of zone of inhibition of growth of microorganism.

The data were in agreement with the results of Orberg and Sandine (1984) and Kelly *et al.* (1986) who reported resistance of Leuconostoc strains to vancomycin was a common characteristic. Antibiotic such as vancomycin may be used as selective marker during gene transfer with Leuconostoc involving conjugation with other microbes of other genus that are not resistant to vancomycin. All the three strains were moderately sensitive towards claxacillin, erythromycin, ampicillin, gentamycin and novobiocin. From the data in the Table 2.1, it can be seen that the antibiotic susceptibility test of the strain B-512F is 80% similar to B-640 and 77% similar to that of strain B-742. The strains B-640 and B-742 were only 67% similar in their antibiotic susceptibility patterns.

2.3.2 Carbohydrate fermentation

The ability of all three strains to degrade and ferment carbohydrates with the production of an acid was tested. The Leuconostoc strains were tested for their ability to ferment 15 carbohydrates. The critical nature of the fermentation reaction and the activity of the indicator make it imperative that all cultures should be observed with 48 h. Extended incubation may mask acid producing reactions by production of alkali because of enzymatic action on substrates other than the carbohydrate. There is only a single report on the carbohydrate utilization by strains B-512F and B-742 (Holt *et al.* 2001). There is no report on carbohydrate fermentation patterns for the strain B-640.

All the three Leuconostoc strains used in the present study utilized fructose, glucose, mannose, melibiose, sucrose and trehalose and did not show any activity towards arabinose, lactose, mannitol and rhamnose (Table 2.2). Leuconostoc strains B-512F and B640 were able to weakly ferment galactose and xylose.

Table 2.2. Carbohydrate fermentation of *Leuconostoc* strains after 48h.

S. No.	Carbohydrate	B-512F	B-640	B-742
1.	Arabinose	-	-	-
2.	Cellobiose	-	+++	++
3.	Dextrose	+++	+++	+++
4.	Fructose	+++	+++	++
5.	Galactose	+	+++	-
6.	Lactose	-	-	-
7.	Maltose	+++	+++	+++
8.	Mannitol	-	-	-
9.	Mannose	++	+++	++
10.	Melibiose	+++	+++	+
11.	Raffinose	++	-	-
12.	Rhamnose	-	++	-
13.	Sucrose	+++	+++	+++
14.	Trehalose	+++	+++	++
15.	Xylose	+	+	-

(+++) strongly positive; (++) fairly positive; (+) weakly positive; (-) negative

Both the Leuconostoc strains B-640 and B-742 fermented cellobiose. And the raffinose was fermented by only B-512F. B-640 was unique being the only one in fermenting rhamnose. Leuconostoc strains B-512F and B-640 had nearly identical fermentation profiles, whereas, Leuconostoc strain B-742 showed quite different fermentation pattern. Most of these results of B-512F and B-742 are in agreement with those of Holt *et al.* (2001) with a few exceptions. The major exception is that they reported all the strains fermented arabinose whereas we did not observe this with any of the strain used. One possible reason could be the time period, as we reported the observation after 2 days whereas they reported the results obtained after 7 days of incubation. As mentioned earlier that the prolonged incubation might have caused production of alkali as a result of certain enzymatic action on substrates other than the carbohydrate.

2.3.3 Plasmid profile of *Leuconostoc mesenteroides* NRRL B-640

The plasmid profiles of three glucansucrase producing Leuconostoc strains were determined (Fig. 2.2). The Leuconostoc strain B-742 did not contain any plasmid and B-512F contained a single plasmid and showed a single band as reported earlier (Holt *et al.* 2001). *Leuconostoc mesenteroides* B-640 also gave a single faint band showing that it contained a single plasmid (Fig. 2.2, lane 2). The plasmids for Leuconostoc strains have been reported earlier (Orberg and Sandine 1984; David and De Vos 1987). It has been reported that the plasmids are associated with cell metabolism (David *et al.* 1992 and Vaugham *et al.* 1995) and with bacteriocin formation (Fremaux *et al.* 1995). These plasmids may find applications in expression of genes within Leuconostoc species. Structural and functional study of the Leuconostoc plasmids must be undertaken to explore their potential use.

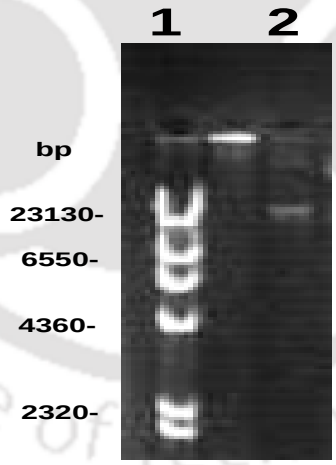

Fig. 2.2. Plasmid profile of Leuconostoc strains B-640 examined by 0.8% agarose gel electrophoresis. Lane 1: λDNA Hind III digest; lane 2: B-640 plasmid DNA.

2.3.4 Glucan synthesizing activity of Leuconostoc strains

Non-denaturing SDS-PAGE was used for *in situ* detection of enzyme activity to characterize sucrase production by dextran-producing leuconostoc strains (Leathers *et al.* 1997). This study, was carried out to see if all the Leuconostoc strains produce a similar or different glucan pattern that could be used to distinguish among the glucansucrase producing strains. All three Leuconostoc strains showed very faint bands on Coomassie Brillinat Blue staining (Fig. 2.3A). All three Leuconostoc strains tested produced at least one detectable glucan-synthesizing activity bands (Fig. 2.3B). Two Leuconostoc strains B-512F and B640 showed a common single band of 180 kDa, however, B-512F showed an extra faint band not shown by B-640 (Fig. 2.3B). Leuconostoc strain B-742 displayed the multiple activity bands compared with the other strains tested (Fig. 2.3B, lane 3). Leuconostoc strain B-742 produced three close activity bands, and two were prominent and one band was very faint (Fig. 2.3B, lane3). The results of B-742 are somewhat similar to those reported earlier (Holt et al. 2001) except that we found more intensity of the two bands. However, for strain B-512F our results were different from earlier reported (Holt et al. 2001). In the present study it was found that B-512F shows two bands corresponding to 188 kDa and 146 kDa molecular sizes (Fig. 2.3B, lanes 1 and 4) whereas, they also reported two bands but of 146 kDa and 118 kDa molecular sizes. In an earlier report also it was shown that the purified dextransucrase from strain B-512F resulted a molecular size of 188 kDa (Goyal and Katiyar 1994).

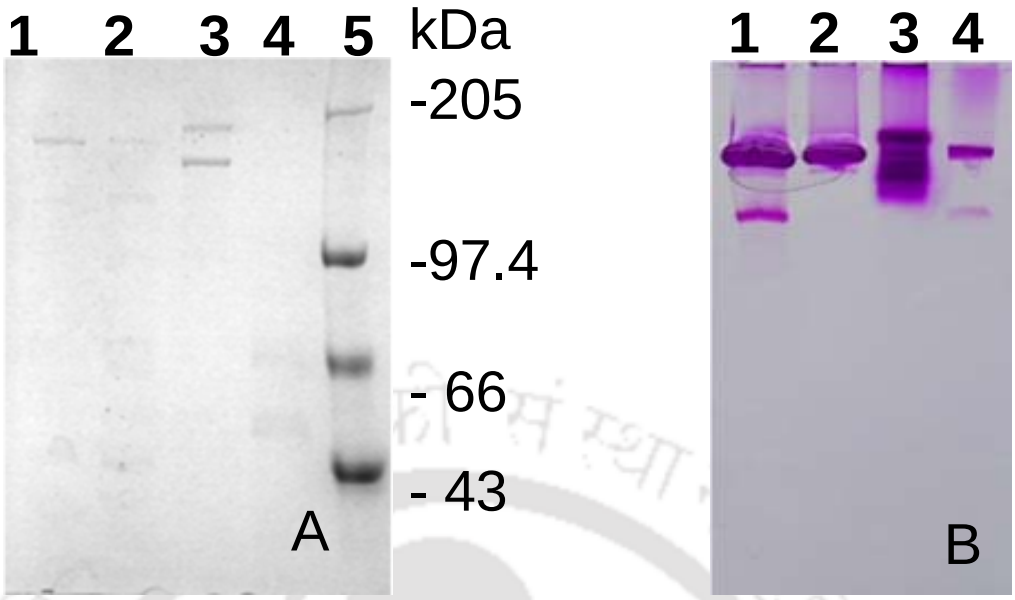

Fig. 2.3. Glucansucrase activity patterns by glucan producing Leuconostoc strains; **A)** Coomassie Brilliant Blue staining **B)** Activitiy staining using periodic acid Schiff protocol. Lanes: (1) B-512F; (2) B-640; (3) B-742 and (4) B-512F.

2.4 Conclusions

Three glucansucrase producing Leuconostoc strains *Leuconostoc mesenteroides* B-512F and B-640 and *Leuconostoc citreum* B-742 were characterized for their antibiotic resistance, carbon source utilization, plasmid profiles and glucan synthesizing activities. The antibiotic resistance, carbohydrate fermentation profiles, sucrose hydrolyzing activity or polysaccharide synthesis activity along with plasmid profiles of the dextransucrase producing Leuconostoc strain B-640 have never been reported earlier. All the three strains were resistant to the antibiotics cotrimazine, norflaxacin, norfloxacin and vancomycin. All the three strains were sensitive to amoxycillin, bacitracin, carbenicillin, cephalothin, cephatoxamine, chloramphenicol, clindamycin, linomycin, oxytetracyclin and tetracyclin. All the three strains utilized glucose, fructose, mannose, melibiose, sucrose and trehalose and did not show any activity towards arabinose, lactose and mannitol. The Leuconostoc strain B-640 was unique in fermenting rhamnose. The Leuconostoc strain B-640 possessed a single plasmid and the strain showed a single band of sucrose hydrolyzing activity by displaying the polysaccharide formation patterns in native polyacrylamide gel. The results of antibiotic resistance and carbohydrate utilization pattern will enhance understanding of these industrially significant strains and will aid in distinguishing between physiologically similar Leuconostoc strains. These data will help in finding dextransucrase producing new Leuconostoc strains, which can prove to be better strains for industrial applications.

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Chapter 3

Optimization of Dextransucrase Production and its Assay

3.1 Introduction

Dextansucrase synthesizes high molecular weight glucose polymer called dextran from sucrose. Dextran is an industrially important polysaccharide used as adjuvant, carrier, blood volume expander, emulsifier in the pharmaceutical industry, as thickening agent in the food industry and cross-linked dextran as molecular sieves used for separation of biomolecules in the laboratories (Robyt 1986; Naessens *et al.* 2005; Purama and Goyal 2005). Oligosaccharides synthesized from dextransucrase using acceptor reactions are used as neutraceuticals, stabilizers and prebiotics (Chung and Day 2002; Naessens *et al.* 2005). There is an increasing interest to develop new oligosaccharides for application as prebiotics. The most important parameters for dextransucrase production are pH, temperature and shaking or static conditions (Tsuchiya *et al.* 1952; Lazic *et al.* 1993; Goyal *et al.* 1995). Goyal *et al.* 1995 studied dextansucrase production from *Leuconostoc mesenteroides* NRRL B-512F under static and shake flask cultures at different temperatures. They reported 23°C the optimum temperature and the static flask culture conditions for maximum enzyme activity for the strain. Shamala and Prasad 1995, observed a higher yield of dextransucrase at 13°C under shaking culture flask condition by *Leuconostoc*

dextranicum FPW-10. The effect of temperature on dextransucrase production from *Leuconostoc mesenteroides* FT-045 in comparison to *Leuconostoc mesenteroides* NRRL B-512F was studied using fermentation by Cortezi *et al.* 2004. They reported 23°C as the optimum temperature for both the strains (FT-045 and B-512F). Low cost carbon and nitrogen sources like sugar-beet molasses, corn steep liquor and wheat bran extract have been successfully employed for large-scale preparation of dextransucrase by fermentation process (Tsuchiya *et al.* 1952; Behravan *et al.* 2003). The dextransucrase production from different strains by various workers has been well reviewed and documented (Naessens *et al.* 2005; Purama and Goyal 2005). There are several *Leuconostoc mesenteroides* strains available for dextransucrase production and many of them have been extensively studied (Robyt and Walseth 1979; Paul *et al.* 1984; Lopez *et al.* 1993; Goyal *et al.* 1995; Dols *et al.* 1997; Chellapandian *et al.* 1998; Santos *et al.* 2000; UL-Qader *et al.* 2001; Behravan *et al.* 2003; Cortezi *et al.* 2004; Naessens *et al.* 2005; Purama and Goyal 2005). However, there are strains which have never been explored and yet they may possess dextransucrase with higher activity and resulting in a type of dextran that may find potential use in industries. One such strain could be *Leuconostoc mesenteroides* NRRL B-640 which gives highly soluble dextran and was chosen for the present study. The aim of the present study was to explore *Leuconostoc mesenteroides* NRRL B-640 and to optimize the media composition and culture conditions for obtaining the maximum yields of dextransucrase.

3.2 Materials and Methods

3.2.1 Microorganism

Leuconostoc mesenteroides NRRL B-640 and *Leuconostoc mesenteroides* NRRL B-512F were procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA. Ingredients required for the maintenance and enzyme production media were from Hi-Media Pvt. Ltd., India. All the chemicals required for reducing sugar estimation, protein estimation and buffer preparation were of highest purity grade available commercially.

3.2.2 Sterilization and aseptic techniques

All culture media were sterilized by autoclaving at a steam pressure of 10.3 kPa (15lb/in^2), and a temperature of 121°C, for 20 min. All inoculum preparations and culture transfers were carried out under aseptic conditions using laminar air flow chambers.

3.2.3 Maintenance and sub-culturing of *Leuconostoc mesenteroides* strains

Cultures were maintained in MRS (DeMan *et al.* 1960) and modified MRS agar stabs (Goyal and Katiyar 1996) which contained (%, w/v): Glucose, 2; yeast extract powder, 0.5; beef extract and peptone, 1; dipotassium phosphate and tri-ammonium citrate, 0.2, sodium acetate, 0.5; Tween 80, 0.1 (v/v) ; $MgSO_4.7H_2O$ and $MnSO_4.4H_2O$, 0.02; and agar, 1.5. Modified MRS was prepared by substituting sucrose with glucose as a carbon source.

3.2.4 Inoculum preparation

For the development of inoculum, a loop full of culture from modified MRS agar stab was transferred to 5 ml of sterile medium described by Tsuchiya *et al.* 1952. The cultures were allowed to grow at 25°C at 200 rpm for 12h. 1% of the culture inoculum was used for the enzyme production experiments for both *Leuconostoc mesenteroides* NRRL B-640 and *Leuconostoc mesenteroides* NRRL B-512F.

3.2.5 Cell growth measurement

Cell growth of culture broth was determined by measuring optical density at 600 nm with required dilutions against a blank of the initial sterile fermentation medium. The optical density was measured using UV-Visible spectrophotometer (Varian, model Cary-100).

3.2.6 Production of dextransucrase

The enzyme was produced using the medium described by Tsuchiya *et al.* 1952 that contained (%, w/v) sucrose, 2; yeast extract, 2; dipotassium phosphate, 2; $MgSO_4.7H_2O$, 0.02; $MnSO_4.4H_2O$, 0.001; $FeSO_4.7H_2O$, 0.001; $CaCl_2.2H_2O$, 0.001; NaCl, 0.001 and the pH was adjusted to 6.9. Unless stated otherwise, all fermentations were carried out in triplicate sets of 100 ml enzyme production medium (EPM) in 250 ml Erlenmeyer flask incubated at 25°C under shaking condition at 200 rpm. The samples (5 ml) were withdrawn at indicated time intervals and centrifuged at 9,200g for 10 min at 4°C to separate the cells. The supernatant (cell free extract) was analyzed for enzyme activity and protein concentration. The dextransucrase production was compared with the more frequently studied *Leuconostoc*

mesenteroides NRRL B-512F using the same enzyme production medium at 23°C under static flask culture condition (Goyal *et al.* 1995).

3.2.7 Enzyme activity assay

The assay of dextransucrasewas carried out in 1 ml of a reaction mixture volume in 20 mM sodium acetate buffer (pH 5.4) containing 146 mM (5%) sucrose and using the cell free extract (10-20 µl) as the enzyme source. The reaction mixture was incubated at 30°C for 15 min. The enzyme activity was measured by estimating the liberated reducing sugar by the Nelson-Somogyi procedures (Nelson 1944; Somogyi 1945). Aliquots (0.2 ml), from the reaction mixture were analyzed for reducing sugar. The absorbance was measured at 500 nm wavelength using UV-Visible spectrophotometer against a blank. D-fructose was used as a standard.

3.2.8 Calculation of enzyme activity

One unit (U) of dextransucrase activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar per min. The dextransucrase activity was calculated as

$$\text{Enzyme activity (U/ml)} = \frac{\Delta A_{500} \times C \times V}{180 \times t \times v} = (\mu\text{mole/min/ml})$$

ΔA_{500} = Optical density (OD) change at 500 nm
C = 1 OD equivalent fructose concentration (mg/ml) from standard plot
V = volume of the reaction mixture (ml)
t = time of reaction (min)
180 = molecular weight of fructose
v = volume of the enzyme source (ml) for reducing sugar estimation

3.2.9 Protein determination

The protein content of the cell free extract containing dextransucrase and partially purified dextransucrase was estimated by the method of Lowry *et al.* 1951. Bovine serum albumin was used as a reference and a concentration range from 25 µg/ml to 500 µg/ml was used to plot a standard curve.

Reagents for Lowry method:

Reagent A : sodium hydroxide (0.4 g) and sodium carbonate (2.0 g) were dissolved in water and the volume made up to 100 ml.

Reagent B1 : 2% sodium potassium tartarate.

Reagent B2 : 1% copper sulfate.

Reagent C : prepared fresh by mixing 1.0 ml of reagent B1 and 100 ml of reagent A followed by addition of 1.0 ml of reagent B2.

Phenol reagent : 1 N phenol reagent.

3.2.10 Estimation of protein

To 0.2 ml of sample containing protein or BSA, 1 ml of reagent C was added. After 10 min, 0.1 ml of phenol reagent was added and mixed and the optical density (OD) was measured after 30 min at 660 nm against a blank.

The concentration of protein was calculated as follows:

$$\text{Protein Concentration (mg/ml)} = \frac{\Delta A_{660} \times C}{V} = \text{(mg/ml)}$$

C = 1 OD equivalent of BSA from standard plot
ΔA_{660} = change in absorbance of the sample
V = volume of the sample

3.2.11 Production of dextransucrase under different culture conditions

3.2.11.1 Effect of temperature

Leuconostoc mesenteroides NRRL B-640 was grown at four different temperatures 22°C, 25°C, 28°C and 30°C ± 0.2°C in the flasks as described earlier (Section 3.2.6) in an orbital shaking incubator at 200 rpm to determine the optimum incubation temperature for maximum enzyme production. The samples (5.0 ml) were withdrawn at different time intervals and centrifuged at 9,200g for 10 min at 4°C to pellet out the cells. The cell free extract was analyzed for enzyme activity and protein concentration as described earlier.

3.2.11.2 Effect of shaken flask culture

The production dextransucrase was compared under shaken flask condition with the static flask culture at 25°C ± 0.2°C in triplicate sets of 100 ml enzyme production medium in 250 ml Erlenmeyer flasks. The shaking was carried out in an orbital shaking incubator at 200 rpm. The samples (5.0 ml) were withdrawn at indicated time intervals and centrifuged at 9,200g for 10 min at 4°C to pellet out the cells. The cell free extract was analyzed for enzyme activity and protein concentration as described earlier.

3.2.12. Partial purification and characterization of dextransucrase

3.2.12.1 Purification of dextransucrase

The crude dextransucrase with (0.5 U/ml) was purified by adding ice-cold polyethylene glycol 200 to obtain a final concentration of 25% (v/v). The mixture was kept at 4°C and the dextransucrase fraction was allowed to precipitate for 12h. The fractionated dextransucrase was separated by centrifugation at 13,200g for 30 min at

4°C. The partially purified dextransucrase was subjected to dialysis using a 5 kDa cut off membrane. The partially purified dextransucrase having a specific activity of 3.2 U/mg and a concentration of 0.8 mg/ml was used for further biochemical characterization.

3.2.12.2 Effect of sucrose concentration on dextransucrase activity

The effect of sucrose concentration on the enzyme activity was studied by varying its concentration between 0.1-10% in the enzyme reaction mixture. The purified dextransucrase with 26.9 U/mg specific activity obtained after two successive steps of 10% PEG-1500 was used for this study of effect of sucrose concentration on dextransucrase activity as described in detail in the Chapter 7, Section 7.2.6, Table 7. The reaction was carried out in 1 ml mixture in 20 mM sodium acetate buffer pH 5.4 and incubated at 30°C for 15 min and activity was determined by estimating the released reducing sugar, as described earlier in Section 3.2.7

3.2.12.3 Effect of temperature on dextransucrase activity

The enzyme activity of partially purified dextransucrase with specific activity 3.2 U/mg was determined at 8 different temperatures varying from 20°C to 50°C ± 0.2°C. The enzyme reaction was carried out in 1 ml reaction mixture in 20 mM sodium acetate buffer (pH 5.4) containing 146 mM (5%) final concentration of sucrose at 30°C for 15 min. Aliquots (0.2 ml) of reaction mixture was taken for reducing sugar analysis as described earlier in Section 3.2.7.

3.2.12.4 Effect of pH dextransucrase activity

The dextransucrase activity assay was performed at 6 different pH points ranging from 4.6 to 5.6, with 0.2 unit variation. The assays were carried out in 1 ml reaction mixture containing 146 mM (5%) final sucrose concentration in 20 mM sodium acetate buffer at 30°C for 15 min. Aliquots (0.2 ml) of reaction mixture was taken for reducing sugar analysis, as described earlier in Section 3.2.7.

3.2.12.5 Effect of ionic strength on dextransucrase activity

The dextransucrase activity was determined with in a broad range of ionic strength between 10 mM-500 mM sodium acetate buffer of pH 5.4. The assays were performed in 1 ml reaction mixture containing 146 mM (5%) final concentration of sucrose at 30°C for 15 min. Aliquots (0.2 ml) of reaction mixture was taken for reducing sugar estimation as described earlier in Section 3.2.7.

3.3 Results and Discussion

3.3.1 Effect of temperature on production of dextransucrase

The optimum temperature for *Leuconostoc mesenteroides* NRRL B-640 was determined by studying the enzyme production at different temperatures under shaking condition. It was found that the lower temperatures favor the enzyme production. It was observed that 25°C gave the maximum enzyme activity (4.8 U/ml) (Fig. 3.1). The enzyme activity decreased as the temperature increased above 25°C, which might be due to the deactivation of the enzyme at higher temperatures. At 30°C there was 15% decrease in enzyme activity was observed (4.1 U/ml). The activity was lower by 5% at 22°C, which may be due to the slower growth rate of cells consequently resulting in lower enzyme production.

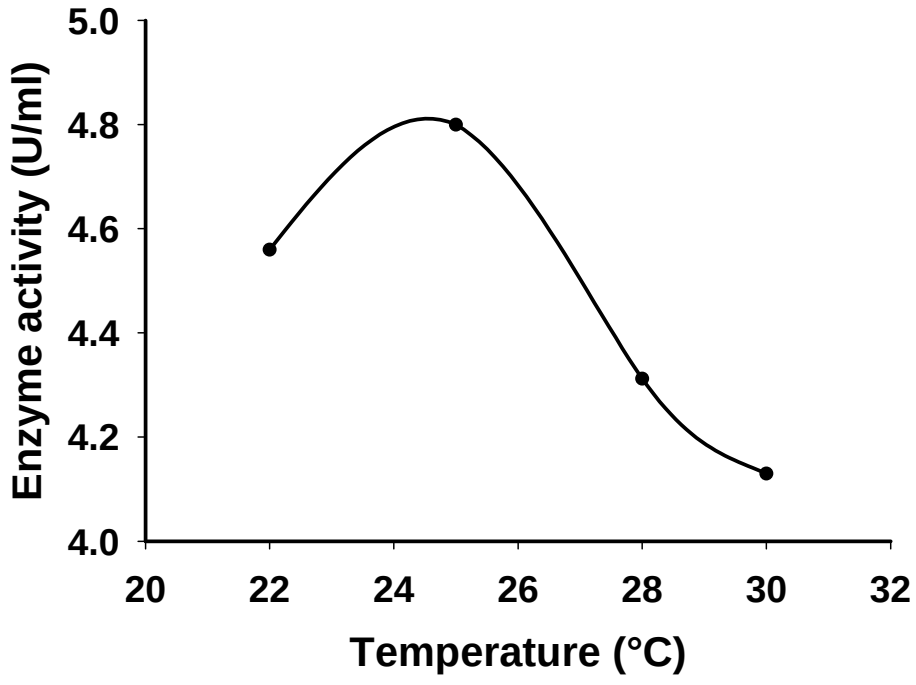

Fig. 3.1. Effect of temperature on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640. The maximum enzyme activity obtained at each temperature was plotted.

3.3.2 Effect of shaking on production of dextransucrase

The dextransucrase activity obtained by the shaking flask culture (4.8 U/ml) was significantly (20%) higher than that produced by static condition (3.8 U/ml) (Fig. 3.2). The maximum enzyme activity was observed in both when the pH of the medium reached 4.4-5.2 between 12-14h time period (after 12h). Although enzyme production under static culture started later, it extended to the longer time period, whereas under shaking condition rapid decrease in enzyme activity was observed after 12h (Fig. 3.2). During this period of rapid decrease in enzyme activity, the pH difference between the static and shaking flask culture was not that significant. However, there was a marked difference in the cell growth under shaking condition as compared to the static condition. The cell growth under static condition had a longer lag phase and the cell density was lower as compared to the culture under shaking conditions.

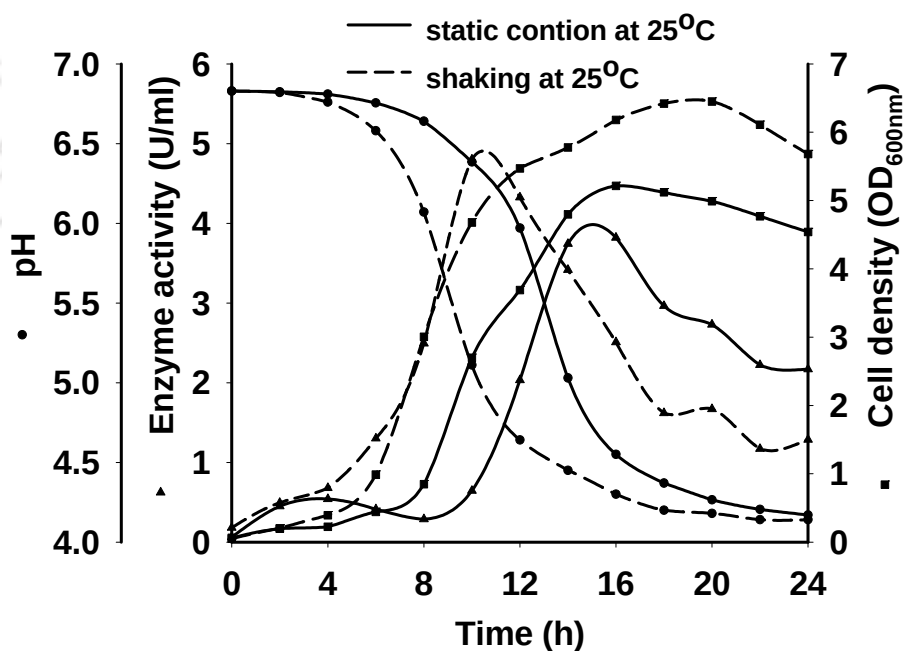

Fig. 3.2. Effect of shaken and static flask culture on dextransucrase production. The enzyme activity (▲), cell growth (■) and pH profiles (●) of *Leuconostoc mesenteroides* NRRL B-640 under shaken (----) and static flask (—) cultures are shown.

3.3.3 Comparison of dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 with *Leuconostoc mesenteroides* NRRL B-512F

Dextrasucrase production from *Leuconostoc mesenteroides* NRRL B-512F was extensively studied because of its high yielding capacity. The enzyme production from *Leuconostoc mesenteroides* NRRL B-512F was compared with *Leuconostoc mesenteroides* NRRL B-640 using the same media composition. The parameters compared are listed in Table 3.1. Goyal *et al.* 1995 reported that 23°C under static flask culture was optimum for the production dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F whereas *Leuconostoc mesenteroides* B-640 was grown at 25°C under shaking condition at 200 rpm. Surprisingly, it was found from the results that the enzyme activity (4.8 U/ml) from *Leuconostoc mesenteroides* NRRL B-640 was 15% higher than the enzyme activity from *Leuconostoc mesenteroides* NRRL B-512F (4.1 U/ml) as shown in Fig. 3.3 and Table 3.1. The cell growth obtained in both strains after 14h was similar. There was a sharp decrease in pH of the broth with *Leuconostoc mesenteroides* NRRL B-640, after 12h when the maximum activity was achieved, whereas, the pH drop was very less from the point where the maximum enzyme activity was observed with *Leuconostoc mesenteroides* NRRL B-512F (Fig. 3.3). The pH range for maximum enzyme activity for B-640 was 4.4.-5.9 whereas for B-512F it was 5.0-5.8. *Leuconostoc mesenteroides* NRRL B-512F is known to be micro-aerophilic (Tsuchiya *et al.* 1952; Dols *et al.* 1997). Since *Leuconostoc mesenteroides* NRRL B-640 gave maximum activity under shaking conditions wehereas *Leuconostoc mesenteroides* NRRL B-512F gave maximum activity under static conditions (Goyal *et al.* 1995). This shows that *Leuconostoc mesenteroides* NRRL B-640 requires more air for growth, consequently more enzyme production, so is more aerophilic than *Leuconostoc mesenteroides* NRRL B-512F.

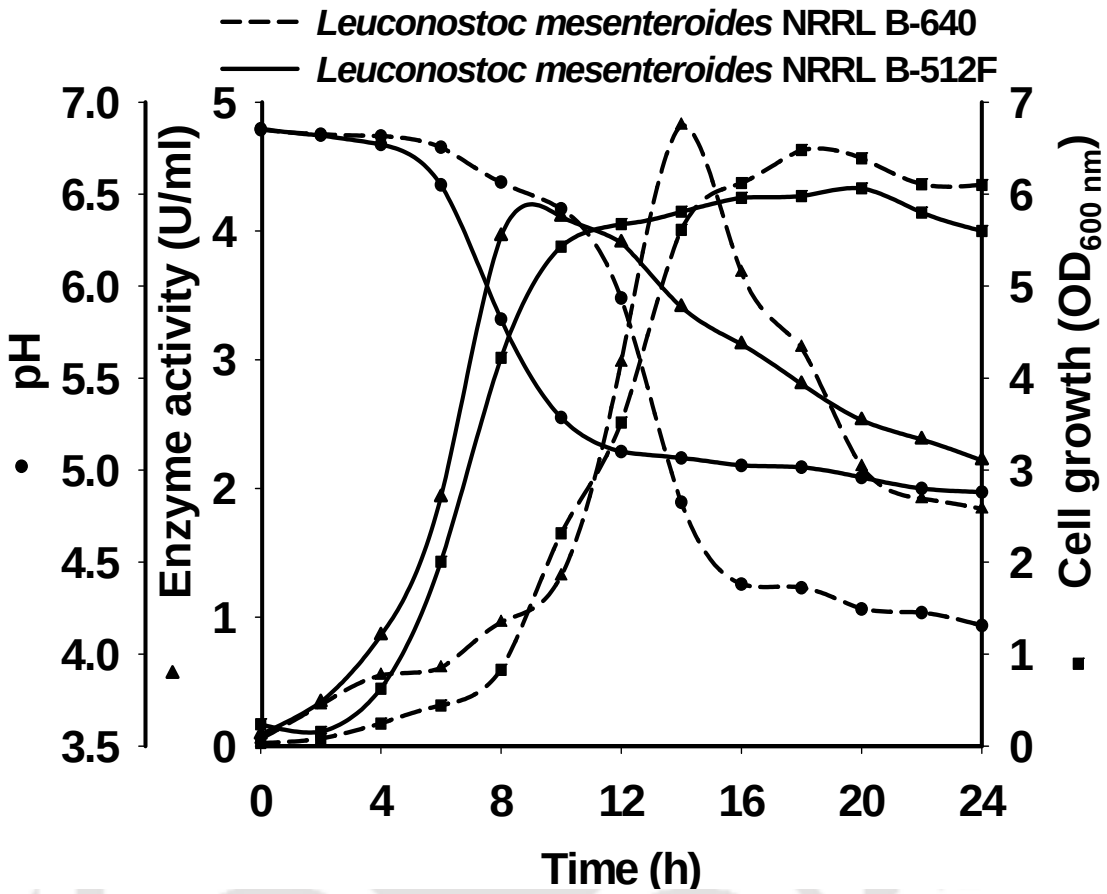

Fig. 3.3. Comparison of dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 and *Leuconostoc mesenteroides* NRRL B-512F. The enzyme activity (▲), cell growth (■) and pH change (●) in the broth by *Leuconostoc mesenteroides* NRRL B-640 (----) and *Leuconostoc mesenteroides* NRRL B-512F (—) are shown.

3.3.4 Characterization of the partially purified dextransucrase

3.3.4.1 Effect of sucrose concentration on dextransucrase activity

The effect of sucrose concentration on the enzyme activity was studied with varying sucrose concentration between 0.1-10% final concentrations. The results showed that it follows the classical Michaelis-Menten kinetics and the saturation reached at 5% (Fig. 3.4). The final sucrose concentration of 5% was considered to be the optimum concentration for the dextransucrase activity assay. The data were analysed using GraphPad Prism software. The purified dextransucrase gave V_m of 28.4 U/mg and K_m of 17.8 mM.

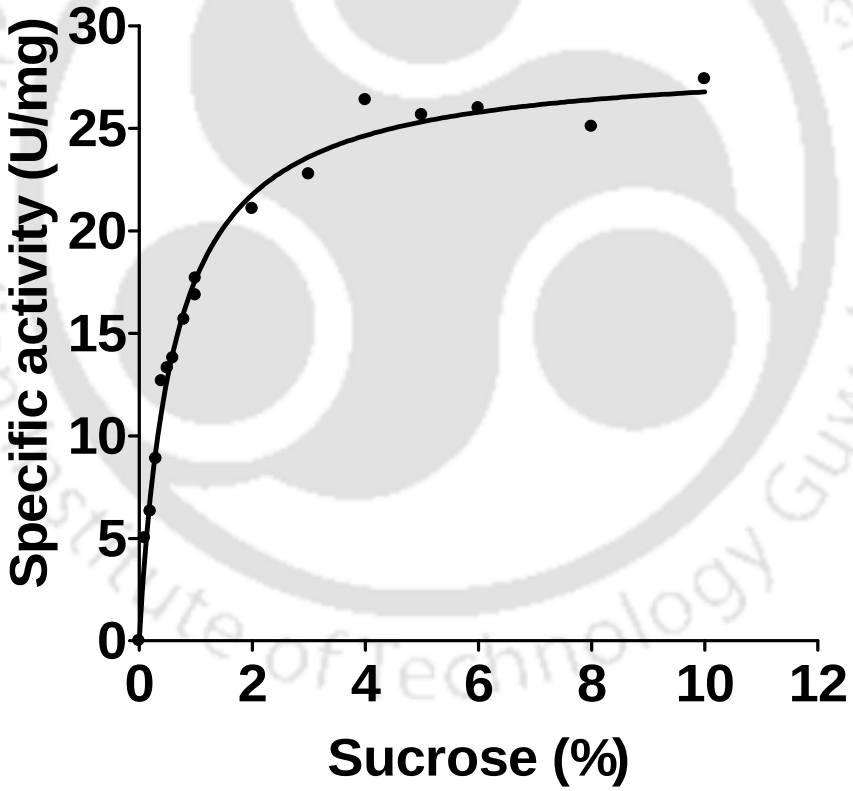

Fig. 3.4. Effect of sucrose concentration on dextransucrase activity assayed at 30°C in 20 mM sodium acetate, pH 5.4.

3.3.4.2 Effect of temperature on dextransucrase activity

The partially purified enzyme was maximally active with in the temperature range of 30-35°C with a specific activity of 3.2 U/mg at pH 5.4 in 20 mM sodium acetate buffer (Fig. 3.5). There was a fluctuation of 2% in specific activity with in this temperature range (30-35°C). The enzyme activity drastically decreased after 35°C and it decreased by 35% at 40°C.

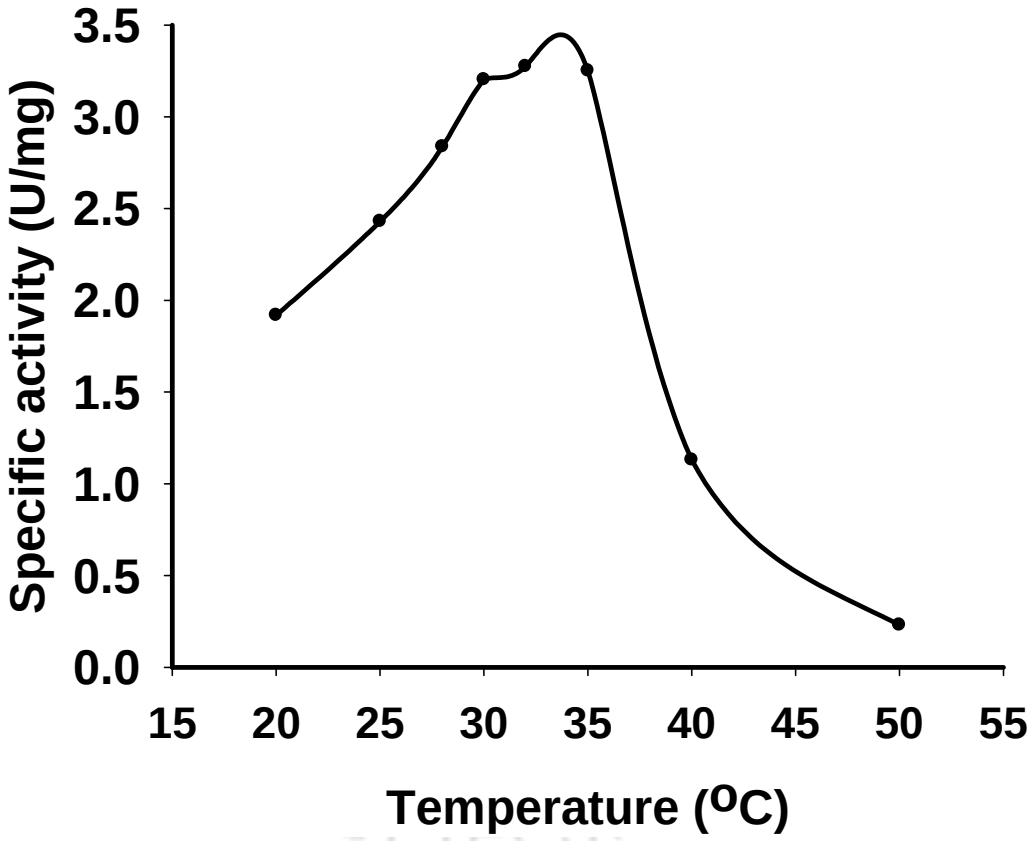

Fig. 3.5. Effect of temperature on dextransucrase activity. The enzyme activity was determined by carrying out the assay in 20 mM sodium acetate buffer (pH 5.4) containing 5% sucrose as described in Section 7.2.6.

3.3.4.3 Effect of pH on activity of dextransucrase

The maximum enzyme activity was observed at pH 5.4 with specific activity of 3.2 U/mg (Fig. 3.6). A 15% reduction in activity was observed on either side of the optimum pH 5.4 for maximum activity. The enzyme was stable below the pH 5.2, up to pH 4.6 and retained 85% of the initial enzyme activity.

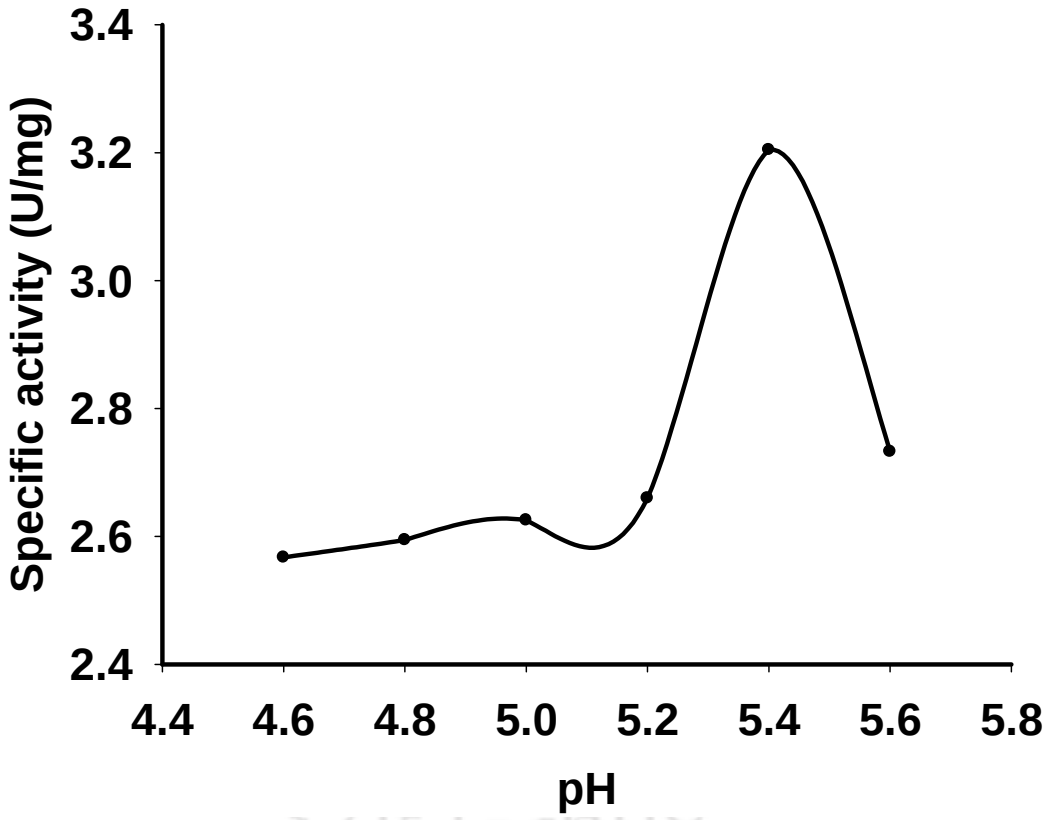

Fig. 3.6. Effect of pH on dextransucrase activity. The enzyme activity was determined by carrying out the assay in 20 mM sodium acetate buffer containing 5% sucrose as described in Section 7.2.6.

3.3.4.4 Effect of ionic strength on dextransucrase activity

The results showed that the dextransucrase activity was stable with in experimental range of 10-500 mM with a variation in specific activity between 3.4-2.5 U/mg (Fig. 3.7). However, the decrease in the enzyme activity was not significant with increase of ionic strength.

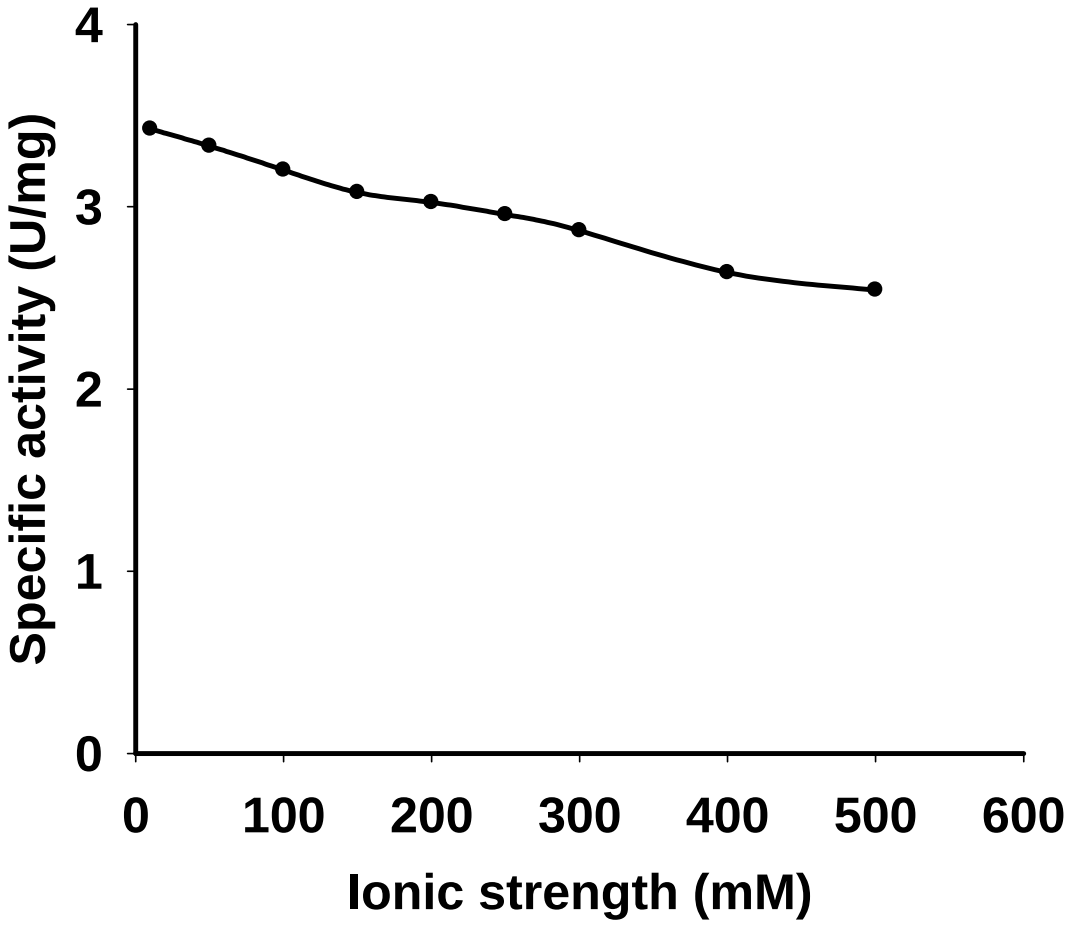

Fig. 3.7. Effect of ionic strength on dextransucrase activity. The enzyme activity was determined by carrying out the assay in 20 mM sodium acetate buffer (pH 5.4) containing 5% sucrose as described in Section 3.2.7.

3.4 Conclusions

The optimum conditions for enzyme production are species as well as strain specific. The incubation temperature and shaking or static flask conditions are the important factors for enzyme production. Most of the *Leuconostoc mesenteroides* strains were reported to produce enzyme with maximum activity in the temperature range of 20-30°C (Goyal *et al.* 1995; Dols *et al.* 1997; Santos *et al.* 2000; Cortezi *et al.* 2004). In this study, optimization of the cultural conditions for dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 was investigated. The enzyme production was favoured, when the culture was grown at 25°C and under shaking flask culture resulting in higher enzyme activity. These results are slightly different from those of *Leuconostoc mesenteroides* NRRL B-512F as it requires 23°C and static conditions for maximum enzyme activity (Goyal *et al.* 1995). It was repeatedly found from the results that the enzyme production by *Leuconostoc mesenteroides* NRRL B-640 gave 15% higher enzyme activity than the activity given by *Leuconostoc mesenteroides* NRRL B-512F. *Leuconostoc mesenteroides* NRRL B-640 dextransucrase production under standard conditions is much more efficient than with the strains NRRL B-512F and NRRL B-1299 (Dols *et al.* 1997). *Leuconostoc mesenteroides* NRRL B-640 dextransucrase gave K_m of 17.8 mM and V_m of 28.4 U/mg and a saturation concentration of 5% sucrose. The enzyme was maximally active at 30°C and at pH 5.4. Further studies on the optimization of medium composition by One Variable At a Time Approach (OVAT) and Response Surface Methodology (RSM) and effect of pH and aeration for maximizing the enzyme production from *Leuconostoc mesenteroides* NRRL B-640 using batch fermentation are described in Chapters 4, 5 and 6, respectively.

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Chapter 4

Effect of Nutrients on Dextransucrase Production

4.1 Introduction

The gram-positive *Leuconostoc mesenteroides* synthesizes the extracellular dextransucrase that is used for synthesis of dextran, which has numerous applications in pharmaceutical, food and fine chemical industries (Robyt 1986; Naessens *et al.* 2005; Purama and Goyal 2005). Oligosaccharides synthesized from dextransucrase are used as neutraceuticals, stabilizers and prebiotics (Naessens *et al.* 2005). The culture conditions and maintenance medium composition of several strains of *Leuconostoc mesenteroides* have been optimized for production of dextransucrase (Tsuchiya *et al.* 1952; Lazic *et al.* 1993; Goyal *et al.* 1995; Goyal and Katiyar 1996; Cortezi *et al.* 2004). Many of these have been studied for nutrient effect for maximizing the dextransucrase production (Tsuchiya *et al.* 1952; Barker and Ajongwen 1991; Goyal and Katiyar 1997; Dols *et al.* 1997; Dols *et al.* 1998; Chellapandian *et al.* 1998; Lopretti *et al.* 1999; Smith and Zahnley 1999; Santos *et al.* 2000; Ul-Qadar *et al.* 2001; Rodrigues *et al.* 2003; Behravan *et al.* 2003). The effect of certain nutrients on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-512F was studied (Goyal and Katiyar 1997). They reported yeast extract as good source of nitrogen for growth of the culture and higher enzyme production. The

type of yeast extract used also affects the enzyme yield. It was shown that different commercial grades of yeast extracts had significantly different effect on the final cell concentration and the enzyme yield (Barker and Ajongwen 1991). In addition to yeast extract, peptone and beef extract separately resulted in enhanced enzyme activity (Goyal and Katiyar 1997). An optimum concentration of 4% yeast extract was reported for dextransucrase production from *Leuconostoc mesenteroides* NRRL B-1299 (Dols *et al.* 1997). The influence of nitrogen/carbon ratio on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-512F was studied and a slow rate of enzyme synthesis and lower fermentation time was observed by the addition of nitrogen source, which was contradictory to the findings of another report (Lopretti *et al.* 1999).

There are several reports on effect of sucrose on dextransucrase production by various strains of *Leuconostoc* spp (Goyal and Katiyar 1997; Dols *et al.* 1997; Dols *et al.* 1998; Lopretti *et al.* 1999; Santos *et al.* 2000; Ul-Qadar *et al.* 2001; Behravan *et al.* 2003). However, dextransucrase production by wild-type *Leuconostoc mesenteroides* grown on glucose or maltose instead of sucrose has also been reported (Smith and Zahnley 1999). Behravan *et al.* (2003) used sugar-beet molasses as sucrose source and wheat bran as substitute for yeast extract. The enzyme production was dependent on the concentration of K_2HPO_4 and it increased with the increase in K_2HPO_4 concentrations (Goyal and Katiyar 1997). An increase in K_2HPO_4 from 0.1 to 0.3 M showed increased biomass and enzyme production by *Leuconostoc mesenteroides* NRRL B-512F grown in shake flask culture (Rodrigues *et al.* 2003). The low cost carbon and nitrogen sources like sugar-beet molasses, corn steep liquor and wheat bran extract for large-scale preparation of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F by fermentation process have been

reported (Behravan *et al.* 2003). Various reports on effect of micronutrients on dextransucrase production form *Leuconostoc mesenteroides* are available (Robyt and Walseth 1979; Goyal and Katiyar 1997; Dols *et al.* 1997; Ul-Qadar *et al.* 2001). The micronutrients such as $MgCl_2$, $MgSO_4$ and NaF were shown to have significant effect on the dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F (Goyal and Katiyar 1997). It was reported that Mn^{2+} ions were essential for dextransucrase production from *Leuconostoc mesenteroides* NRRL B-1299, however, increasing its concentration had no effect on enzyme activity (Dols *et al.* 1997). The presence of magnesium, manganese and calcium salts in the medium not only increased the enzyme activity but also the yield of the dextran (Ul-Qadar *et al.* 2001). There is no report available on the effect of nutrients on *Leuconostoc mesenteroides* NRRL B-640 for dextransucrase production. The aim of present study was to optimize the medium composition including macro and micronutrients for maximizing the yield of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. In the present study one variable at a time approach has been used for determining the nutritional effects on dextransucase production from *Leuconostoc mesenteroides* NRRL B-640.

4.2 Materials and Methods

4.2.1 Microorganism

Leuconostoc mesenteroides NRRL B-640 was procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA. Ingredients required for maintenance and enzyme production media were from Hi-Media Pvt. Ltd., India. All the chemicals required for reducing sugar estimation, protein estimation and buffer preparation were of high purity grade.

4.2.2 Sterilization and aseptic techniques

All culture media were sterilized by autoclaving at a steam pressure of 10.3 kPa (15lb/in^2), and a temperature of 121°C, for 20 min. All inoculum preparations and culture transfers were carried out under aseptic conditions.

4.2.3 Maintenance and inoculum preparation

The cultures of *Leuconostoc mesenteroides* NRRL B-640 were maintained in MRS (DeMan *et al.* 1960) and modified MRS (Goyal and Katiyar 1997) agar stabs. Modified MRS was prepared by substituting sucrose with glucose as a carbon source (Goyal and Katiyar 1997). A loop full of culture from an agar stab was transferred to 5 ml of sterile medium described by Tsuchiya *et al.* (1952). The cultures were grown at 25°C with shaking at 200 rpm for 12-16h. 1% of the culture inoculum was used for the enzyme production.

4.2.4 Enzyme activity assay

The assay of dextransucrase was carried out in 1 ml of a reaction mixture in 20 mM sodium acetate buffer, pH 5.4, containing 146 mM (5%) sucrose and using the cell free extract (10-20 µl) as the enzyme source (Purama and Goyal 2007). The reaction mixture was incubated at 30°C for 15 min. The assay procedure is described in Chapter 3, Section 3.2.7.

4.2.5 Effect of nutrients on dextransucrase production

The effects of various nutrients were studied by varying their concentrations in 100 ml enzyme production medium as described by Tsuchiya et al (1952) in 250 ml Erlenmeyer flask as described in Chapter 3, Section 3.2.6. The cultures were grown at

25°C with shaking at 200 rpm. 5 ml broth samples were periodically withdrawn and analyzed for dextransucrase activity as described in Section 4.2.4.

4.2.5.1 Effect of sucrose

The effect of sucrose on dextransucrase production was studied by varying its concentration from 1 to 10% in the enzyme production medium (100 ml) by keeping the concentration of other components constant. The medium as described by Tsuchiya *et al.* (1952) containing 2% sucrose was considered as control. The culture conditions were used as described in Chapter 3, Section 3.2.6.

4.2.5.2 Effect of yeast extract and K_2HPO_4

The effect of yeast extract was studied in combination with the phosphate concentration. The yeast extract concentration was varied from 1.5% to 4%, where the control flask contained 2% yeast extract and 2% K_2HPO_4 in the medium as described by Tsuchiya *et al.* (1952). The effect of phosphate on the dextransucrase production was studied by varying its concentration from 1.5% to 3%, where the control contained 2% K_2HPO_4 and 2% yeast extract. The culture conditions were used as described in Chapter 3, Section 3.2.6.

4.2.5.3 Effect of peptone and beef extract

The effects of peptone and beef extract on dextransucrase production were studied separately in addition to the presence of 2% yeast extract in the medium. The effect of peptone was studied by varying the concentration form 0.1% to 1.5%, whereas the beef extract was varied from 0.5% to 2% taking the medium as described

by Tsuchiya *et al.* (1952) as control which contained no peptone or beef extract. The culture conditions were used as described in Chapter 3, Section 3.2.6.

4.2.5.4 Effect of Tween 80

The effect of Tween 80 on enzyme production was studied by varying its concentration from 0.1 to 0.5% (v/v) in the medium. The medium as described by Tsuchiya *et al.* (1952) containing no Tween 80 served as a control.

4.2.5.5 Effect of $MgSO_4$ and $MnSO_4$ on dextransucrase production

The effects of $MgSO_4$ and $MnSO_4$ on dextransucrase production were studied separately by varying the concentrations from 0.02 to 0.06% and 0.001 to 0.005%, respectively. The medium described by Tsuchiya *et al.* (1952) containing 0.02% $MgSO_4$ and 0.001% $MnSO_4$ were taken as corresponding controls.

4.2.5.6 Effect of NaCl and $CaCl_2$

The effects of NaCl and $CaCl_2$ on enzyme production were studied separately by varying the concentration of both the salts from 0.001 to 0.005%, taking the medium described by Tsuchiya *et al.* (1952) as control that contained 0.001% of each salt in the production medium.

4.3 Results and Discussion

4.3.1 Effect of sucrose on dextransucrase production

Sucrose is the known inducer of dextransucrase (Neely and Nott, 1962). Although, some Leuconostoc strains are shown to produce dextransucrase by the medium containing sugars other than sucrose the dextransucrase levels were minimal, but when sucrose was used as substrate the enzyme activity was several fold higher in the medium (Dols *et al.* 1998). There was a steep rise (5 fold increase) in the dextransucrase production and activity from *Leuconostoc mesenteroides* NRRL B-640 from 2.9 to 15 U/ml when the sucrose concentration increased from 1% to 7% in the enzyme production medium (Fig. 4.1). Similar results were reported earlier where the enzyme production increased with increase in sucrose levels with an exception that above 4% sucrose they reported no increase in enzyme activity for other *Leuconostoc mesenteroides* strains (Goyal and Katiyar 1997; Dols *et al.* 1997; Dols *et al.* 1998; Lopretti *et al.* 1999; Smith and Zahnley 1999; Santos *et al.* 2000; Ul-Qadar *et al.* 2001; Behravan *et al.* 2003). The dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640 attained saturation at 7% sucrose. The increase in dextransucrase activity was 3-fold, from 4.8 U/ml to 15 U/ml with an increase in sucrose concentration from 2% (control) to 7% in the medium (Fig. 4.1, Table 4.1). The maximum enzyme activity of 17 U/ml was observed at 10% sucrose concentration (Fig. 4.1). As the sucrose concentration increased there was an increase in viscosity of the broth due to the subsequent formation of the exopolysaccharide dextran from the available and residual sucrose by the released enzyme, in the medium. Surprisingly, the handling of these, viscous cell free extracts were not as difficult as reported for *Leuconostoc mesenteroides* NRRL B-512F (Monsan *et al.* 1987; Goyal and Katiyar 1997) for enzyme activity determinations. This difference

might be due to higher solubility of the dextran produced by *Leuconostoc mesenteroides* NRRL B-640.

4.3.2 Effect of yeast extract and K_2HPO_4 on dextransucrase production

The dextransucrase production increased at higher yeast extract levels by *Leuconostoc mesenteroides* NRRL B-640. An increase in yeast extract concentration from 1.5% to 4% caused an increase in dextransucrase activity at all concentrations of K_2HPO_4 used (Fig. 4.2). The increase in enzyme activity at 4% yeast extract was approximately 10% when compared to the control (Table 4.1). Tsuchiya *et al.* (1952) reported the requirement of higher nitrogen sources and other nutrients for the maximal enzyme formation. These results are similar to those reported by Dols *et al.* (1997), where an increase in yeast extract concentration from 2% to 4% showed a marginal increase in dextransucrase production from *Leuconostoc mesenteroides* NRRL B-1299 and its further increase did not cause any increase the dextransucrase production. However, these results are contrary to those of Goyal and Katiyar (1997), where a decrease in enzyme production from *Leuconostoc mesenteroides* NRRL B-512F was reported with increasing the yeast extract concentration.

The increase in K_2HPO_4 concentration form 1.5% to 3% resulted in significant increase (approx. 22%) in dextransucrase activity at all yeast extract concentrations (Fig. 4.2). Similar results were reported for dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F, where the increase in enzyme activity was significantly (35%) more on increasing K_2HPO_4 concentration from 2% to 2.5% (Goyal and Katiyar 1997).

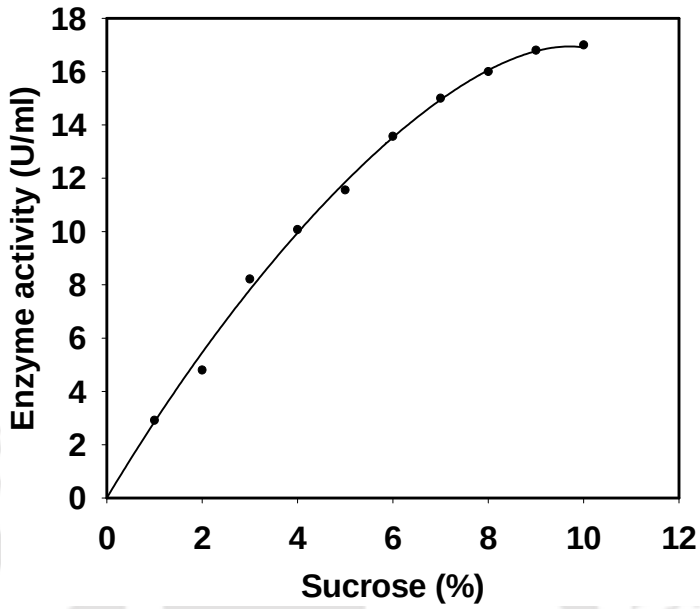

Fig. 4.1. Effect of sucrose concentration on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640. The maximum enzyme activity obtained at each sucrose concentration was plotted.

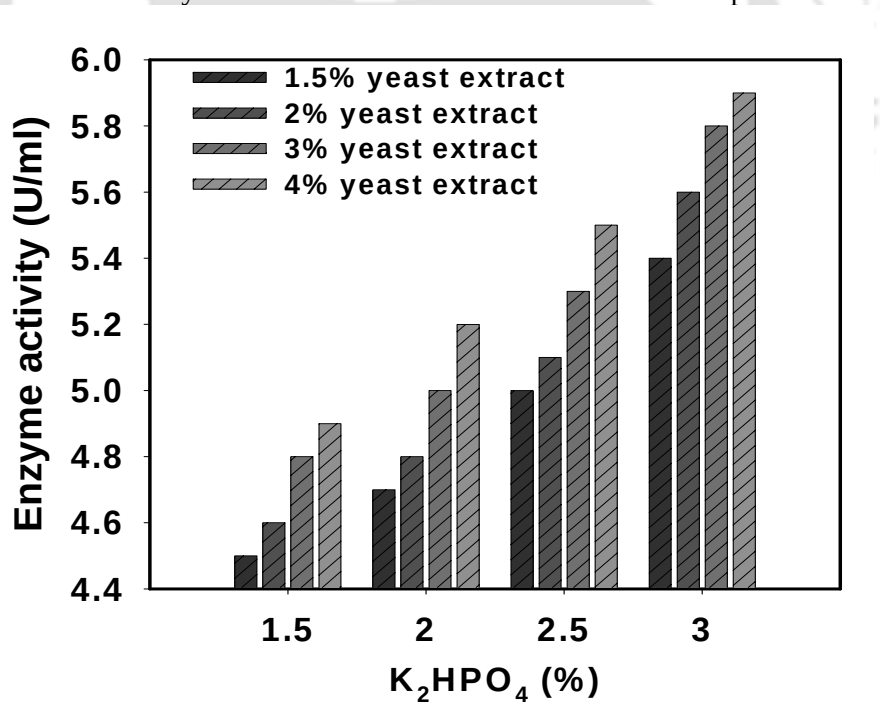

Fig. 4.2. Effect of yeast extract and K_2HPO_4 on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640. The maximum enzyme activity obtained at various combinations of yeast extract and K_2HPO_4 were plotted.

The maximum activity of 5.9 U/ml was achieved at a combination of 4% yeast extract and 3% K_2HPO_4 which was 23% higher than that of control medium (4.8 U/ml) Table 4.1. However, for large scale production of enzyme it would be economical to use a combination of 2% yeast extract and 3% K_2HPO_4 that gave 5.6 U/ml which is 17% higher (Table 4.1) than the control 4.8 U/ml (containing 2% of K_2HPO_4 and 2% of yeast extract) that will also save 2% of yeast extract.

4.3.3 Effect of peptone and beef extract on dextransucrase production

The effect of peptone on dextransucrase production was studied by varying the concentration from 0.1% to 1.5%. The addition of only 0.1% peptone to control medium showed 17% increase in dextransucrase activity (Fig. 4.3A, Table 4.1). Further increase in peptone concentration beyond 0.5%, did not favor the enzyme production, rather a decrease in enzyme production was observed (Fig. 4.3A). This might be due to effect of certain trace elements present in the peptone. Ul-Qadar *et al.* (2003) also observed higher dextransucrase production with the addition of peptone and $CaCl_2$ to the medium containing yeast extract and higher phosphate and concluded that peptone played some role in obtaining the higher enzyme levels.

The effect of beef extract on dextransucrase production was studied by varying its concentration from 0.5% to 2%. With an increase in the beef extract concentration from 0.5% to 1.5% an increase in enzyme production was observed (Fig. 4.3B). The addition of 1.5% beef extract gave 15% increase in enzyme production over control medium (Table 4.1). Further increase in beef extract concentration beyond 1.5% did not favor the enzyme production.

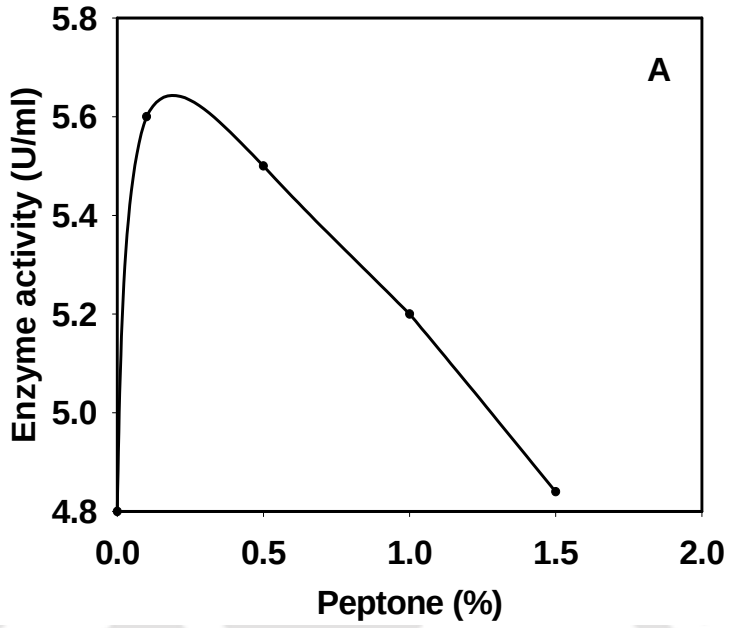

Fig. 4.3A. Effect of peptone on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640.

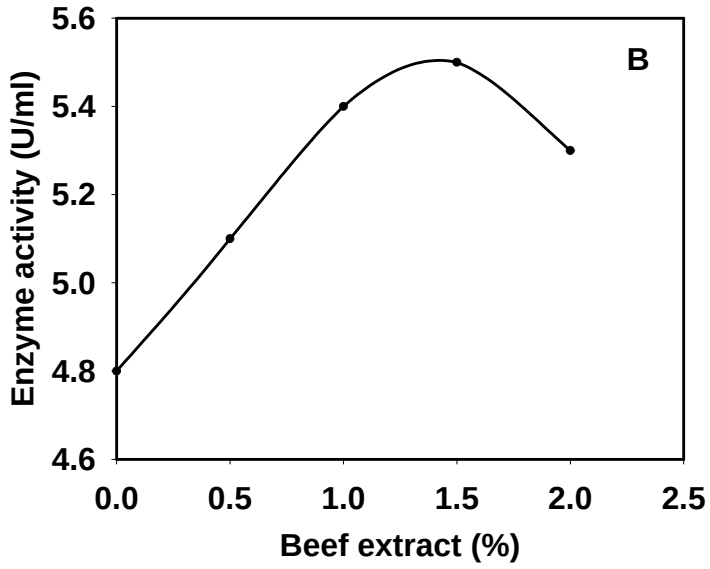

Fig. 4.3B. Effect of beef extract on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640.

4.3.4 Effect of Tween 80 on dextransucrase production

The addition of Tween 80 to the medium stimulated the production of dextransucrase. The production of dextransucrase increased with an increase in the concentration of Tween 80 (Fig. 4.4). 0.1% Tween 80 gave approximately 10% increase in the enzyme activity and the enzyme activity was saturated at higher concentrations (0.5%) of Tween 80 (Fig. 4.4). The Tween 80 did not show any effect on the *in vitro* dextransucrase activity. The results were similar to the reports of Sato *et al.* (1987) and Umesaki *et al.* (1977). They showed that addition of Tween 80 to the enzyme production medium altered the fatty acid composition of the membrane thus enhancing the secretion of the dextransucrase and its activity (Umesaki *et al.* 1977; Sato *et al.* 1987).

4.3.5 Effect of $MgSO_4$ and $MnSO_4$ on dextransucrase production

The effect of $MgSO_4$ on dextransucrase was studied by increasing the concentration in the production medium from 0.02% (0.81 mM) to 0.05% (2.43 mM) The dextransucrase activity enhanced from 4.8 U/ml to 5.3 U/ml with the increase in $MgSO_4$ concentration from 0.02% (0.81 mM) to 0.04% (1.62 mM) (Fig. 4.5A) showing a 10% increase in the enzyme production (Table 4.1). Mg^{2+} ions have been shown to increase the dextransucrase activity of *Leuconostoc mesenteroides* NRRL B-640 at 1 mM concentration. It could not be inferred that the increase in production was due to the Mg^{2+} ions as this could be due to Mg^{2+} ion effect on dextransucrase activity. However, Mg^{2+} ion have also been shown to increase the production of dextransucrase in *Leuconostoc mesenteroides* NRRL B-512F (Goyal and Katiyar 1997; Bellengier *et al.* 1997). The magnesium ions are also reported to play role in the

signal transduction by enhancing the enzyme production and its release in to the medium (Yamashita and Takehara 1989).

The effect of the $MnSO_4$ on the dextransucrase production was studied by varying the concentration from 0.001% (0.06 mM) to 0.005% (0.3 mM) and a 13% increase was observed in the enzyme production at 0.005% (0.3 mM) $MnSO_4$ concentration (Fig. 4.5B, Table 4.1). *Leuconostoc* Spp. are known to be micro-aerophilic microorganisms (Tsuchiya *et al.* 1952; Dols *et al.* 1997). Bellengier *et al.* (1997) showed the addition of Mg^{2+}, Mn^{2+} and amino acids stimulated the growth of most Leuconostoc strains. They also showed that Mn^{2+} suppressed the inhibitory effect of aeration on the growth of *Leuconostoc mesenteroides* UD-23, and suggested its protective role against oxygen toxicity on *Leuconostoc mesenteroides* UD-23. The Mn^{2+} ions showed an inhibitory effect on the purified dextransucrase activity from *Leuconostoc mesenteroides* NRRL B-640 with in the same range of concentration (0-1 mM) (data shown in Chapter 8, Section 8.3.1, Fig. 8.1D, Table 8.1). This effect clearly showed that the increase in enzyme activity was due to the increased production of dextransucrase by Mn^{2+} ions in the medium.

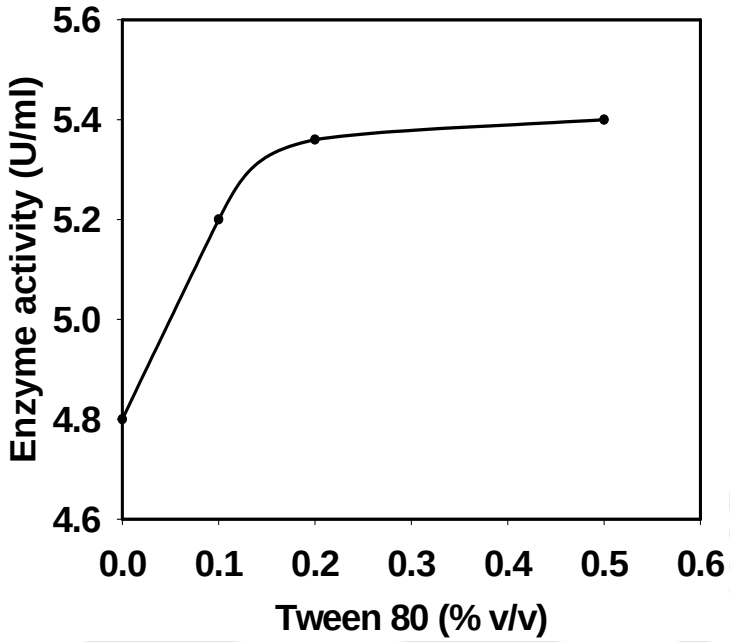

Fig. 4.4. Effect of Tween 80 on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640.

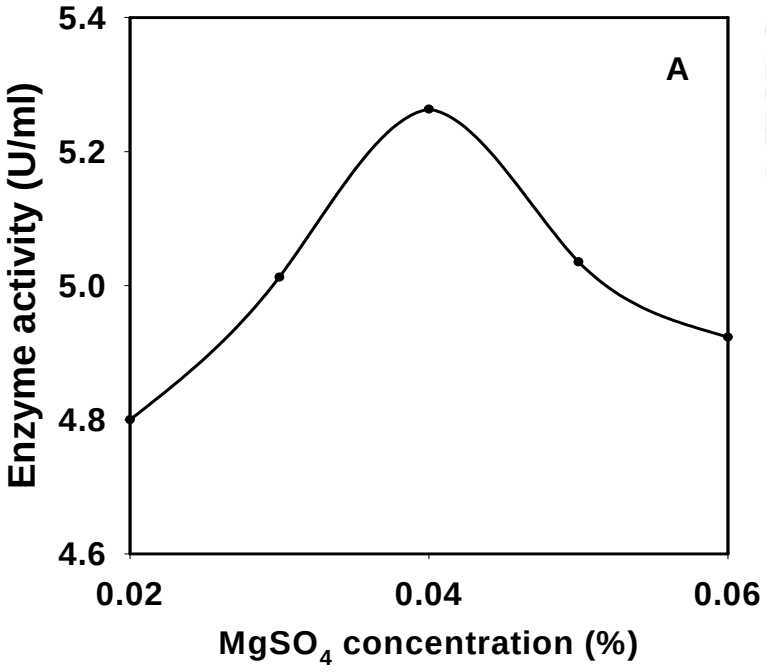

Fig. 4.5A. Effect of $MgSO_4$ on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640.

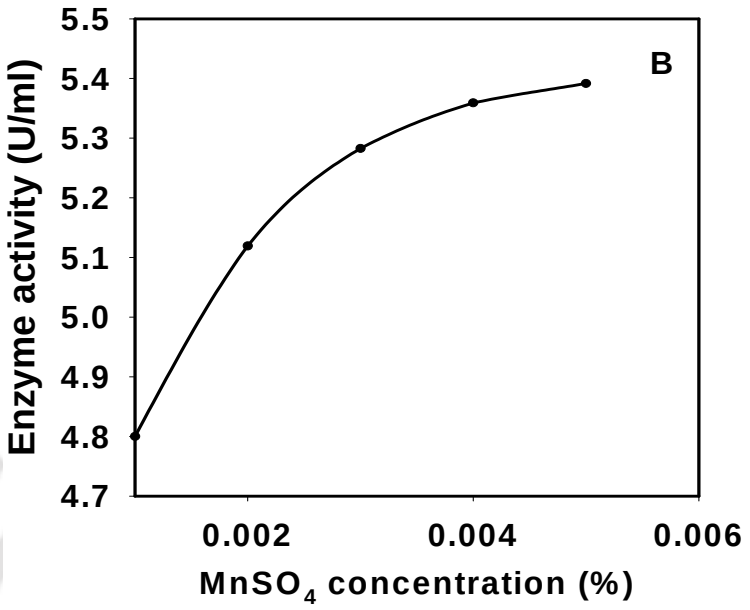

Fig. 4.5B. Effect of $MnSO_4$ n dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640.

4.3.6 Effect of NaCl and $CaCl_2$ on dextransucrase production

The effect of sodium ions was studied by increasing the concentration from 0.001% (0.17 mM) to 0.005% (0.85 mM) in the production medium. An increase of NaCl concentration up to 0.003% (0.51 mM) increased the enzyme production to 5.4 U/ml from 4.8 U/ml at 0.001% NaCl concentration and further increase rather reduced the enzyme production (Fig. 4.6A). A 12% increase in the enzyme production *Leuconostoc mesenteroides* NRRL B-640 was observed at 0.003% (0.85 mM) NaCl in the medium as compared to control that contained 0.001% (0.17 mM) NaCl (Table 4.1), though NaCl had no effect on *in vitro* enzyme activity.

The effect of $CaCl_2$ was studied by increasing the concentration from 0.001% (0.7 mM) to 0.005% (0.35 mM) production medium (Fig. 4.6B). An inhibitory effect of $CaCl_2$ on enzyme production from *Leuconostoc mesenteroides* NRRL B-640 was observed (Fig 4.6B). There was a 13% decrease in enzyme production with the increase in 5 fold $CaCl_2$ concentration (at 0.005% or 0.35 mM) as compared to the control medium (Table 4.1). However, $CaCl_2$ showed an enhancing effect (8%) on *in vitro* enzyme activity of purified dextransucrase (Chapter 8, Section 8.3.1, Table 8.1). Contrary to these results, a 2-fold increase in enzyme production from *Leuconostoc mesenteroides* NRRL B-512F was observed by the addition of $CaCl_2$ to the medium (Robyt and Walseth 1979).

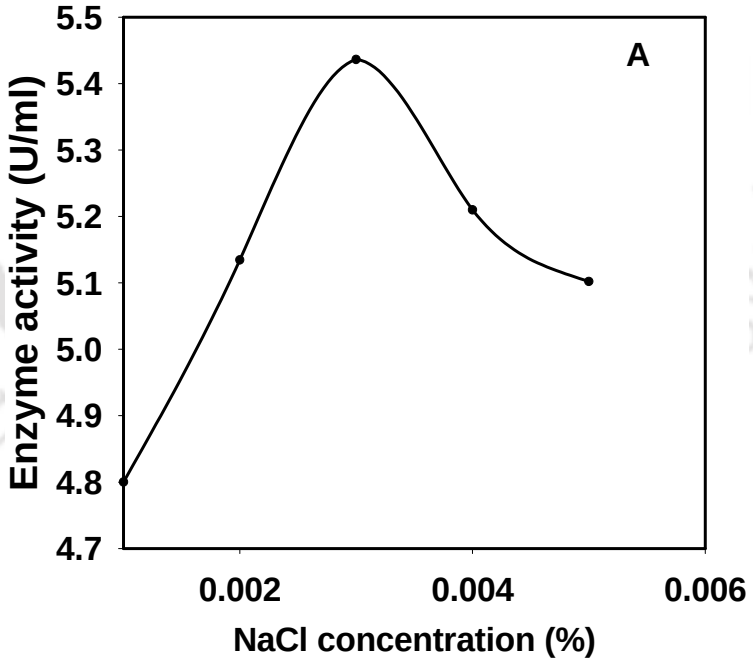

Fig. 4.6A. Effect of NaCl on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640.

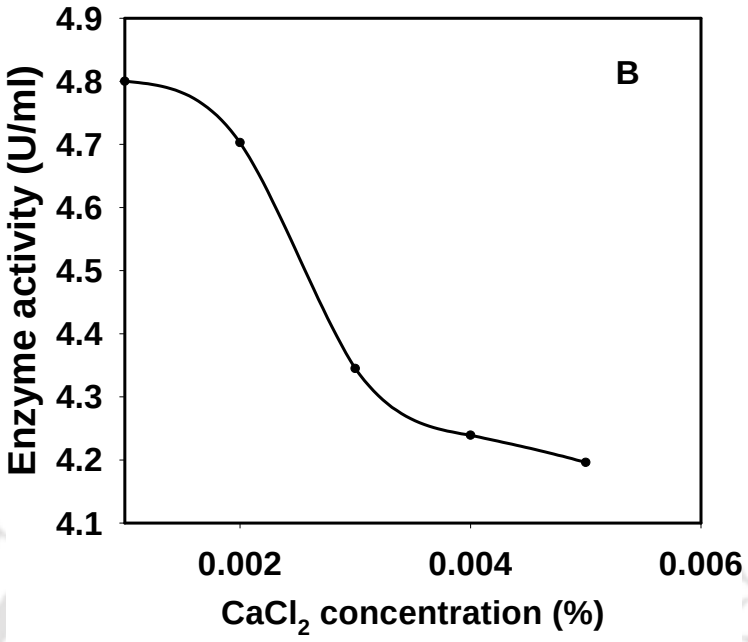

Fig. 4.6B. Effect of $CaCl_2$ on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640.

Table 4.1. Maximum activity of dextransucrase achieved at optimum concentration of nutrients. The effects of nutrients were compared with the control medium.

Nutrient (Concentration, %)	Enzyme activity (%)
Control	100
Sucrose (7%)	310
Yeast extract (4%)	110
K_2HPO_4 (3%)	117
Yeast extract (4%) + K_2HPO_4 (3%)	123
Peptone (0.1%)	117
Beef extract (1.5%)	115
Tween 80 (0.5% v/v)	112
$MgSO_4.7H_2O$ (0.04%)	110
$MnSO_4.6H_2O$ (0.005%)	113
NaCl (0.003%)	112
$CaCl_2.2H_2O$ (0.005%)	87

4.4 Conclusions

Production of dextransucrase from *Leuconostoc mesenteroides* is induced in the presence of sucrose (Neely and Nott, 1962). *Leuconostoc mesenteroides* NRRL B-640 gave an enzyme activity of 15 U/ml at 7% sucrose concentration 3 times higher than 4.8 U/ml at 2% sucrose. Tsuchiya *et al.* (1952) reported that 2% sucrose was optimum for maximum production of dextransucrase by *Leuconostoc mesenteroides* NRRL B-512. Ul-Qadar *et al.* (2001) observed a decrease in percent conversion of sucrose to dextran with increase in sucrose concentration above 1% using *Leuconostoc mesenteroides* PCSIR-3 in the fermenting media, which was due to substrate inhibition effect. The dextransucrase production increased at higher yeast extract levels by *Leuconostoc mesenteroides* NRRL B-640. Doubling the concentration of yeast extract from 2% to 4% increased the dextransucrase activity only by 10%. The increase of K_2HPO_4 from 2% to 3% in the medium gave a 17% higher enzyme activity by *Leuconostoc mesenteroides* NRRL B-640. A combination of yeast extract (4%) and K_2HPO_4 (3%) gave 23% enhanced enzyme activity (5.9 U/ml) as compared to control (4.8 U/ml). However, a combination of 2% yeast extract and 3% K_2HPO_4 giving an enzyme activity of 5.6 U/ml (17% higher than control) can be preferred for enzyme production on a large scale that will save 2% yeast extract without the loss of much enzyme activity.

The addition of 0.1% peptone to control medium in addition to 2% yeast extract showed 17% increase in dextransucrase activity from *Leuconostoc mesenteroides* NRRL B-640. *Leuconostoc dextranicum* FPW-10 produced higher dextran in the medium containing wheat bran as nitrogen source than the conventional medium containing peptone, tryptone and yeast extract as nitrogen sources (Shamala and Prasad 1995). The addition of 1.5% beef extract gave 15% increase of

dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 as compared to the control medium. However, *Leuconostoc mesenteroides* NRRL B-512F gave 25% higher enzyme activity by 2% beef extract (Goyal and Katiyar 1997). The addition of 0.5% Tween 80 to the control medium enhanced the dextransucrase activity by 12% in the culture broth. However, the increase was 25% from *Leuconostoc mesenteroides* NRRL B-512F by the addition of 0.5% Tween 80 (Goyal and Katiyar 1997). Similar results of increase in enzyme activity with Tween 80 were also reported for *Streptococcus mutans* (Umesaki *et al.* 1977; Sato *et al.* 1989).

Dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 enhanced with the increase in $MgSO_4$ from 0.02% (4.8 U/ml) to 0.04% (5.3 U/ml) showing a 10% increase. Similar results using $MgCl_2$ were reported for *Leuconostoc mesenteroides* NRRL B-512F (Goyal and Katiyar 1997). A 13% increase in the enzyme production was observed with the increase in the concentration of $MnSO_4$ from 0.001% (Control) to 0.005% from *Leuconostoc mesenteroides* NRRL B-640. Dols *et al*. (1997) reported Mn^{2+} ions to be essential for the dextransucrase production from *Leuconostoc mesenteroides* NRRL B-1299. A 12% increase in the enzyme production was observed at 0.003% NaCl as compared to the control. Surprisingly, the calcium ions displayed a negative effect on the dextransucrase production. The present results show that nutrient requirements are not only species specific, but also strain specific. These studies showed that it is essential to identify the nutrient requirements of *Leuconostoc mesenteroides* NRRL B-640 for maximum dextrasucrase production. The results of optimized nutritional effects attain importance for the production of dextransucrase on a large scale.

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Chapter 5

Statistical Approach to Dextransucase Production

5.1 Introduction

Dextran is synthesized by dextransucrases from sucrose which belongs to a class of glucosyltransferases. Glucosyltransferases are produced mainly by two bacterial genera *Leuconostoc* and *Streptococcus* (Remaud-Simeon *et al*. 2000). The major glucosyltransferases are dextransucrases, alternansucrase, mutansucrases which produce dextran, alternan and mutan respectively, which differ in their linkage pattern of the D-glucose residues (van Hijum *et al*. 2006). Dextrans are a class of homopolysaccharides composed of D-glucans with contiguous α-(1→ 6) glycosidic linkages in the main chains and α-(1→ 2), α-(1→ 3) or α-(1→ 4) branched glycosidic linkages (Robyt 1995). Dextrans have several applications in pharmaceutical, food, photochemical and fine chemical industries (Lacaze *et al.* 2007; Purama and Goyal 2005; Naessens *et al.* 2005; Robyt 1986). The low molecular weight dextrans are used as a blood plasma substitute. High molecular weight dextrans are used to increase the puffiness of bakery products (Lacaze *et al.* 2007). In addition to catalyzing the synthesis of dextran from sucrose, dextransucrase also catalyzes the transfer of a D-glucopyranosyl group from sucrose to other acceptor molecules resulting in the generation of oligosaccharides (Goulas

et al. 2004; Naessens *et al.* 2005). Maltose, isomaltose and galactose are the known acceptor molecules for dextransucrase which in presence of sucrose, synthesize oligosaccharides such as maltooligosaccharides, isomaltooligosaccharides and galactooligosaccharides, respectively (Seo *et al.* 2007). Oligosaccharides are used in food, feed, pharmaceutical or cosmetics as stabilizers, as anti-carcinogenic agents, antioxidants, immunostimulating agents and prebiotic compounds (Chung and Day 2002; Goulas *et al.* 2004; Naessens *et al.* 2005; Seo *et al.* 2007). As there are enormous applications of dextran, dextransucrase is required in higher amounts at the expense of optimum levels of nutrients.

Several authors have described the effect of nutrients and culture conditions for dextransucrase production by various *Leuconostoc* strains under flask cultures and batch fermentation (Tsuchiya *et al.* 1952; Barker and Ajongwen 1991; Goyal and Katiyar 1996; Goyal and Katiyar 1997; Santos *et al.* 2000; Behravan *et al.* 2003). All the parameters affecting the dextransucrase production reported were, using the univariate experiments (Barker and Ajongwen 1991; Goyal and Katiyar 1997; Santos *et al.* 2000; Behravan *et al.* 2003). Univariate at a time approach is time consuming and it does not account for the interactions among the medium components. The statistical approach for medium optimization is believed to be a better alternative to univariate at a time approach and have been extensively used recently (Tanyildizi *et al.* 2005; Tari *et al.* 2006; Majumder and Goyal 2007).

In the present study, the production of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 was studied by statistical approach for screening and optimization of medium components. The optimization of dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 was studied by a sequential study of factorial Plackett-Burman design followed by central composite

design (CCD). The factorial design of Plackett-Burman was used to screen the most significant factors affecting enzyme production. A central composite design (CCD) was used to identify the optimum levels of the significant variables to generate optimal response.

5.2 Materials and Methods

5.2.1 Microorganism and cultivation conditions

Leuconostoc mesenteroides NRRL B-640 was procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA. Ingredients required for maintenance and enzyme production media were from Hi-Media Pvt. Ltd., India. The culture was maintained in modified MRS (DeMan *et al.* 1961) with sucrose replaced by glucose as stab at 4°C and sub-cultured every 2 weeks. A loop full of culture from stab was transferred to 5 ml of medium as described by Tsuchiya *et al.* (1952). The cultures were grown at 25°C with 200 rpm. 1% of the culture inoculum was used to inoculate 100 ml enzyme production medium. The culture broth was centrifuged at 9,200***g*** for 10 min at 4°C to separate the cells. The cell free extract was analyzed for enzyme activity.

5.2.2 Dextransucrase activity assay

The assay of dextransucrase was carried out in 1 ml of a reaction mixture in 20 mM sodium acetate buffer, pH 5.4, containing 146 mM (5%) sucrose and using the cell free extract (10-20 µl) as the enzyme source. The reaction mixture was incubated at 30°C for 15 min. The assay procedure is described in Chapter 3, Section 3.2.7.

5.2.3 Protein determination

The total protein content of the cell free extract was estimated by the method of Lowry *et al.* (1951). Bovine serum albumin ranging from 25 µg/ml to 500 µg/ml concentration was used as a reference to plot a standard curve. The protein estimation details are given in Chapter 3, Section 3.2.9.

5.2.4 Optimization procedure and experimental design

5.2.4.1 Screening of factors affecting dextransucrase production

Plackett-Burman factorial design was employed for screening the important nutrients for dextransucrase production (enzyme activity and specific activity). Six nutrients *viz.* sucrose, yeast extract, K_2HPO_4, peptone, beef extract and Tween 80 were used to determine the key ingredients significantly affecting the dextransucrase production. Based on Plackett-Burman factorial design, each factor was examined at two levels: -1 for low level and +1 for high level, and a center point was run to evaluate the linear and curvature effects of the variables (Plackett and Burman 1946). Table 5.1 shows the Plackett-Burman experimental design with six factors under investigation as well as the levels of each factor used in the experimental design and the response. Plackett-Burman experimental design is based on the first order polynomial model:

$$Y = \beta_o + \sum \beta_i x_i \qquad (1)$$

Where, Y is the response (productivity and specific activity), β_o is the model intercept and β_i is the linear coefficient, and x_i is the level of the independent

variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. In the present work, six assigned variables were screened in twelve experimental runs in addition with three runs at their center points. Enzyme activity assays were carried out in duplicate and the averages of the dextransucrase activity and specific activities were taken as response Y_1 (Table 5.1). From the regression analysis of the variables, the factors significant at 90% level ($P < 0.1$) were considered to have significant effect on dextransucrase production and were further optimized by response surface methodology (RSM).

5.2.4.2 Central composite design (CCD) and statistical analysis

The effects of sucrose, yeast extract, K_2HPO_4 and beef extract on dextransucrase production were studied by response surface methodology. A CCD with 5 settings for each of the 4 factor levels was used to evaluate the quadratic effects and two-way interactions among these variables. A full factorial central composite design with eight star points ($\alpha = 2$) and seven replicates at the center point resulting in a total of 31 experiments which covered the entire range of combinations of variables were used to optimize the chosen key variables for dextransucrase productivity in shake flask culture.

For predicting the optimal point, a second order polynomial equation was fitted to correlate the relationship between the variables and response (dextransucrase activity and specific activity). The equation is

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \qquad (2)$$

Where, Y is the predicted response, k is the number of factor variables, β_o is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient.

The following equation was used for coding the variables

$$x_i = \frac{X_i - X_0}{\Delta X_i}, i = 1, 2, 3, \cdots, k, \quad (3)$$

Where, x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point, and ΔX_i is the step change.

Statistical analysis of the data was performed by design package DESIGN EXPERT 7.0 to evaluate the analysis of variance (ANOVA) to determine the significance of each term in the equations fitted and to estimate the goodness of fit in each case. Response surfaces were drawn for the experimental results obtained from the effect of different variables on dextransucrase production in order to determine the individual and cumulative effects of these variables and the mutual interactions between them.

5.2.4.3 Experimental validation of the optimized conditions by flask culture and bioreactor

In order to confirm and validate experimentally, the statistically optimized medium composition for dextransucrase production, triplicate fermentation runs in 250 ml culture flask (containing 100 ml medium) optimized at 25°C and 200 rpm (as described Chapter 3, Section 3.2.6) and duplicate runs in a 3 litre bench-top bioreactor (Applikon, model Bio Console ADI 1025) in batch culture experiments

(with 1.4 litre working volume of medium) optimized at 25°C, agitation speed of 200 rpm and at aeration rate of 1.5 vvm (as described in Chapter 6, Section 6.3.1) were carried out.

5.3 Results and Discussion

5.3.1 Evaluation of factors affecting dextransucrase production

Dextransucrase activity was estimated in the cell free extract of *Leuconostoc mesenteroides* NRRL B-640 culture broth at 12h, as dextransucrase production was maximum at 12h. All the assays were carried out in duplicates and the average values are reported. The data in Table 5.1 indicated that there was a wide variation of dextransucrase activity from 0.23 U/ml to 9.41 U/ml in the fifteen trials. This variation reflected the significance of factors on the enzyme activity. The analysis of regression coefficients and *t*-value of 6 ingredients for enzyme activity and specific activity are shown in Table 5.2 and Table 5.3, respectively. Sucrose, yeast extract, K_2HPO_4, and beef extract displayed a positive effect for enzyme activity where as, peptone and Tween 80 had a negative effect on enzyme activity. The variables with confidence levels greater than 90% were considered as significant. Although beef extract did not show a good confidence level for specific activity of dextransucrase (Table 5.3), it was considered as one of the variables in response surface methodology experiments.

Sucrose was significant at 99.99% confidence levels for dextransucrase production. K_2HPO_4 and yeast extract were found significant about 94% level for dextransucrase production where as, beef extract was significant at 91% level for

dextransucrase production. Peptone and Tween 80 were found insignificant with negative coefficients for enzyme activities. Peptone and Tween 80 were added at their median values for subsequent experiments. Neglecting the variables which were insignificant, the model equation for dextransucrase production can be written as:

$$Y_{\text{activity}} = 4.124 + 2.817\,X_1 + 0.563\,X_2 + 0.607\,X_3 + 0.504\,X_5 \qquad (4)$$

Sucrose, yeast extract and K_2HPO_4 were significant at 99.99%, 95.6% and 99.2% confidence levels respectively for dextransucrase specific activity. Beef extract was least significant among the six variables studied for their effect on specific activity of the dextransucrase.

$$Y_{\text{specific activity}} = 0.210 + 0.148\,X_1 - 0.022\,X_2 + 0.034\,X_3 - 0.0001\,X_5 \quad (5)$$

Where, X_1 = sucrose, X_2 = yeast extract, X_3 = K_2HPO_4 and X_5 = beef extract

On the basis of calculated *t*-values (Table 5.2), sucrose, yeast extract, K_2HPO_4 and beef extract were chosen for further optimization, since these factors significantly affected the dextransucrase production. Peptone and Tween 80 were kept at middle level.

Table 5.1. Plackett-Burman design showing 6 variables with coded values (+ or -) and the real values along with the observed results for dextransucrase production.

Run Order	Sucrose (g/l)	Yeast Extract (g/l)	K_2HPO_4 (g/l)	Peptone (g/l)	Beef Extract (g/l)	Tween 80 (g/l)	Activity (U/ml)	Specific Activity (U/mg)
1	30 (+)	10 (-)	30 (+)	1 (-)	5 (-)	1 (-)	5.89	0.40
2	30 (+)	30 (+)	10 (-)	5 (+)	5 (-)	1 (-)	7.39	0.34
3	10 (-)	30 (+)	30 (+)	1 (-)	15 (+)	1 (-)	2.78	0.10
4	30 (+)	10 (-)	30 (+)	5 (+)	5 (-)	2 (+)	6.42	0.41
5	30 (+)	30 (+)	10 (-)	5 (+)	15 (+)	1 (-)	6.89	0.26
6	30 (+)	30 (+)	30 (+)	1 (-)	15 (+)	2 (+)	9.48	0.36
7	10 (-)	30 (+)	30 (+)	5 (+)	5 (-)	2 (+)	0.96	0.05
8	10 (-)	10 (-)	30 (+)	5 (+)	15 (+)	1 (-)	2.86	0.15
9	10 (-)	10 (-)	10 (-)	5 (+)	15 (+)	2 (+)	0.18	0.01
10	30 (+)	10 (-)	10 (-)	1 (-)	15 (+)	2 (+)	5.58	0.38
11	10 (-)	30 (+)	10 (-)	1 (-)	5 (-)	2 (+)	0.62	0.02
12	10 (-)	10 (-)	10 (-)	1 (-)	5 (-)	1 (-)	0.44	0.05
13	20 (0)	20 (0)	20 (0)	3 (0)	10 (0)	1.5 (0)	6.61	0.34
14	20 (0)	20 (0)	20 (0)	3 (0)	10 (0)	1.5 (0)	5.97	0.33
15	20 (0)	20 (0)	20 (0)	3 (0)	10 (0)	1.5 (0)	6.40	0.35

Table 5.2. Statistical analysis of Plackett-Burman design showing coefficient values, *t* and *P*-value for each variable for dextransucrase activity after 12h.

Variable	Dextransucrase activity (U/ml)			
	Coefficient	*t* Stat	*P*-value	Confidence level (%)
Intercept	4.124	16.48	0.0001	99.99
Sucrose (X_1)	2.817	11.26	0.0001	99.99
Yeast extract (X_2)	0.563	2.25	0.059	94.1
K_2HPO_4 (X_3)	0.607	2.43	0.046	95.4
Peptone (X_4)	-0.008	-0.03	0.975	2.5
Beef extract (X_5)	0.504	2.02	0.084	91.6
Tween 80 (X_6)	-0.251	-1.00	0.348	65.2

Table 5.3. Statistical analysis of Plackett-Burman design showing coefficient values, *t* and *P*-value for each variable for specific activity after 12h.

Variable	Dextransucrase activity (U/mg)			
	Coefficient	*t* Stat	*P*-value	Confidence level (%)
Intercept	0.210	22.66	0.0001	99.99
Sucrose (X_1)	0.148	15.95	0.0001	99.99
Yeast extract (X_2)	-0.022	-2.45	0.044	95.6
K_2HPO_4 (X_3)	0.034	3.70	0.008	99.2
Peptone (X_4)	-0.007	-0.81	0.443	55.7
Beef extract (X_5)	-0.0001	-0.02	0.985	1.5
Tween 80 (X_6)	-0.004	-0.50	0.633	36.7

5.3.2 Optimization of medium composition for higher enzyme activity by RSM

At the end of screening experiments four nutritional factors were believed to play a significant role in dextransucrase production. A central composite design (CCD) with thirty one experiments was carried out. The respective low and high levels of each variable with the coded levels in parenthesis are given in Table 5.4 and the CCD design with dextransucrase activity and specific activity are given in Table 5.4. The results of the second order response surface model fitting in the form of ANOVA are given in Table 5.5 (for dextransucrase activity) and Table 5.6 (for specific activity). To test the fit of the model equation, the regression based determination coefficient R^2 was evaluated (Haider and Pakshirajan 2007; Liu and Wang 2007). The nearer the values of R^2 to 1, the model would explain better for variability of experimental values to the predicted values (Sayyad *et al.* 2007; Li *et*

al. 2007). The model presented a high determination coefficient (R^2 = 0.959) explaining 96% of the variability in the response (Table 5.5). The coefficients of regression were calculated and the following regression equation was obtained.

$$Y_{activity} = -9.463 + 4.115\,X_1 + 5.133\,X_2 + 7.732\,X_3 - 2.343\,X_4 + 0.487\,X_1X_2 - 0.542\,X_1X_3 + 1.368\,X_1X_4 - 0.031\,X_2X_3 - 0.745X_2X_4 + 1.030\,X_3X_4 - 0.690\,X_1^2 - 1.370\,X_2^2 - 1.949\,X_3^2 - 0.411\,X_4^2 \quad (6)$$

where, Y = response (dextransucrase activity), X_1 = sucrose, X_2 = yeast extract, X_3 = K_2HPO_4 and X_4 = beef extract in coded values.

Table 5.4. Full factorial central composite design matrix of four variables in real and coded units (Parenthesis) and the response of dextransucrase activity

Run No.	Sucrose (X_1)	Yeast extract (X_2)	K_2HPO_4 (X_3)	Beef extract (X_4)	Enzyme Activity (U/ml)	Specific Activity (U/mg)
1	10 (-1)	10 (-1)	10 (-1)	5 (-1)	3.36	0.40
2	30 (1)	10 (-1)	10 (-1)	5 (-1)	7.83	0.72
3	10 (-1)	30 (1)	10 (-1)	5 (-1)	1.65	0.16
4	30 (1)	30 (1)	10 (-1)	5 (-1)	8.01	0.44
5	10 (-1)	10 (-1)	30 (1)	5 (-1)	2.68	0.25
6	30 (1)	10 (-1)	30 (1)	5 (-1)	3.39	0.39
7	10 (-1)	30 (1)	30 (1)	5 (-1)	2.67	0.16
8	30 (1)	30 (1)	30 (1)	5 (-1)	4.80	0.27
9	10 (-1)	10 (-1)	10 (-1)	15 (1)	2.36	0.15
10	30 (1)	10 (-1)	10 (-1)	15 (1)	7.07	0.45
11	10 (-1)	30 (1)	10 (-1)	15 (1)	0.08	0.00
12	30 (1)	30 (1)	10 (-1)	15 (1)	8.02	0.37
13	10 (-1)	10 (-1)	30 (1)	15 (1)	3.13	0.20
14	30 (1)	10 (-1)	30 (1)	15 (1)	8.48	0.50
15	10 (-1)	30 (1)	30 (1)	15 (1)	0.12	0.05
16	30 (1)	30 (1)	30 (1)	15 (1)	6.73	0.30

17	0 (-2)	20 (0)	20 (0)	10 (0)	0.11	0.06
18	40 (+2)	20 (0)	20 (0)	10 (0)	12.31	0.66
19	20 (0)	0 (-2)	20 (0)	10 (0)	3.02	0.31
20	20 (0)	40 (+2)	20 (0)	10 (0)	3.96	0.13
21	20 (0)	20 (0)	0 (-2)	10 (0)	0.67	0.04
22	20 (0)	20 (0)	40 (+2)	10 (0)	1.68	0.06
23	20 (0)	20 (0)	20 (0)	0 (-2)	8.54	0.77
24	20 (0)	20 (0)	20 (0)	20 (2)	8.58	0.41
25	20 (0)	20 (0)	20 (0)	10 (0)	8.77	0.53
26	20 (0)	20 (0)	20 (0)	10 (0)	8.65	0.51
27	20 (0)	20 (0)	20 (0)	10 (0)	8.89	0.53
28	20 (0)	20 (0)	20 (0)	10 (0)	8.55	0.50
29	20 (0)	20 (0)	20 (0)	10 (0)	8.78	0.51
30	20 (0)	20 (0)	20 (0)	10 (0)	8.48	0.52
31	20 (0)	20 (0)	20 (0)	10 (0)	8.53	0.55

X_1, X_2, X_3 and X_4 are mentioned in g/l.

Table 5.5. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of dextransucrase activity

Source	SS	DF	MS	*F*-value	Prob (*P*) > *F*
Model	341.444	14	24.3889	27.07	< 0.0001
Residual (error)	14.414	16	0.9009		
Lack of fit	14.273	10	1.4273	60.83	0.0001
Pure error	0.141	6	0.0235		
Total	355.858	30			

R^2 = 0.959; CV =17.32; Adj R^2 = 0.924
SS, sum of squares; DF, degrees of freedom; MS, mean square

The statistical significance of Eq. 6 was checked by *F*-test, the results of ANOVA are shown in Table 6. The results demonstrated that the model is highly significant, and is evident from Fischer's, *F* test with a very low probability value

[(P_{model} > F = 0.0001)] (Table 5.5). A low value of coefficient of variation was observed (CV = 17.32%) which indicated precision and reliability of the experiments.

Table 5.6. ANOVA for quadratic model for specific activity of dextransucrase.

Source	SS	DF	MS	F-value	Prob (P) > F
Model	1.302	14	0.093	33.18	<0.0001 (significant)
Residual (error)	0.045	16	0.0028		
Lack of fit	0.043	10	0.0043	18.65	0.001
Pure error	0.0014	6	0.0002		
Total	1.347	30			

R^2 = 0.967; CV = 15.05; Adj R^2 = 0.938.
SS, sum of squares; DF, degrees of freedom; MS, mean square.

The model presented a high determination coefficient (R^2 = 0.967) explaining 97% of the variability in the response i.e. specific activity (Table 5.6). The coefficients of regression were calculated and the following regression equation was obtained.

$$Y_{\text{specific activity}} = -0.09631 + 0.32341\,X_1 + 0.197668\,X_2 + 0.398364\,X_3 - 0.56326\,X_4 - 0.00259\,X_1X_2 - 0.03015\,X_1X_3 + 0.043037\,X_1X_4 + 0.011796\,X_2X_3 + 0.019587\,X_2X_4 + 0.090882\,X_3X_4 - 0.0413\,X_1^2 - 0.07649\,X_2^2 - 0.11865\,X_3^2 + 0.065301\,X_4^2 \quad (7)$$

The statistical significance of Eq. 7 was checked by F-test, the results of ANOVA for dextransucrase specific activity are shown in Table 5.6. The results demonstrated that the model is highly significant, and is evident from Fischer's, F test with a very low probability value [(P_{model} > F = 0.0001)] (Table 5.6). A low

value of coefficient of variation was observed (CV = 15.05%) which indicated precision and reliability of the experiments.

Table 5.7. Results of regression analysis of a full second-order polynomial model for optimization of dextransucrase activity.

Model Term	Parameter Estimate	Standard Error	Computed *t*-value	*P*-value
Intercept	-9.463	2.7865	-3.396	0.004
X_1	4.115	1.1033	3.730	0.002
X_2	5.133	1.1033	4.652	0.0001
X_3	7.732	1.1033	7.008	0.0001
X_4	-2.343	2.2066	-1.062	0.304
X_1^2	-0.690	0.1775	-3.889	0.001
X_2^2	-1.370	0.1775	-7.720	0.0001
X_3^2	-1.949	0.1775	-10.981	0.0001
X_4^2	-0.411	0.7100	-0.579	0.571
X_1X_2	0.487	0.2373	2.055	0.057
X_1X_3	-0.542	0.2373	-2.286	0.036
X_1X_4	1.368	0.4746	2.882	0.011
X_2X_3	-0.031	0.2373	-0.132	0.897
X_2X_4	-0.745	0.4746	-1.570	0.136
X_3X_4	1.030	0.4746	2.170	0.045

Model coefficients estimated by regression analysis for each variable is shown in Table 5.7. The significance of each coefficient was determined by *t*-values and *P*-values. The larger the magnitude of *t*-test value and smaller the *P*-value indicates the high significance of the corresponding coefficient (Karthikeyan *et al.* 1996; Tanyildizi *et al.* 2005). The results revealed that K_2HPO_4 concentration (X_3) had a significant effect ($P<0.0001$) on dextransucrase production as it had largest coefficient followed by yeast extract (X_2) and sucrose(X_1). The negative coefficient observed for the beef extract indicated that decrease in its concentration can increase the dextransucrase production. Positive coefficients of X_1, X_2 and X_3 variables indicated a linear effect for the increase in dextransucrase production. Negative coefficients were observed for quadratic terms of all the four variables.

Low *P*-value of X_1 ($P < 0.001$), X_2 ($P < 0.0001$) and X_3 ($P < 0.0001$) variables for quadratic terms indicated that they are also significant. Among the interactions X_1X_2 ($P < 0.057$), X_1X_4 ($P < 0.011$), and X_3X_4 ($P < 0.045$), had positive coefficients, while X_1X_3 ($P < 0.036$), X_2X_3 ($P < 0.897$), and X_2X_4 ($P < 0.136$), had negative coefficients. The interactions of $X_1X_4 > X_1X_3 > X_3X_4 > X_1X_2$ were found to be highly significant.

The 3D Response surface plots and the 2D contour plots are the graphical representation of the regression equation generally used to visualize the relationship between the response and experimental levels of each variable and the type of interaction between the variables in order to deduce the optimum conditions (Tanyildizi *et al.* 2005; Haider and Pakshirajan 2007). Response surface plots and Contour plots are shown in Fig(s). 5.1-5.6 which depicted the interaction between two variables by keeping the other variables at their middle levels for dextransucrase enzyme activity. The maximum predicted value is indicated by the surface confined in the smallest ellipse in the contour diagram (Tanyildizi *et al.* 2005). The response surfaces having circular contour plot indicates no interaction where as, an elliptical or saddle nature of the contour plot indicates significant interaction between the corresponding variables. The Fig(s). 5.1A, 5.2A and 5.3A showing the response surface plots indicated that approximate concentration of 30 g/l sucrose in combination with 20 g/l of yeast extract, 20 g/l K_2HPO_4 and 15 g/l beef extract give high dextransucrase enzyme activity. Fig. 5.4A showing the response surface plot indicated that the concentrations of 20 g/l each of yeast extract and K_2HPO_4 will be required to give high dextransucrase activity. The response surface plots in Fig(s). 5.5A and 5.6A indicated that 20 g/l yeast extract and 20 g/l K_2HPO_4 with about 15 g/l beef extract was required to obtain the maximum dextransucrase activity. From the Contour plots of 5.1B, 5.2B and 5.4B it is evident

that there is no significant interaction exists between sucrose with yeast extract and K_2HPO_4 and yeast extract with K_2HPO_4, respectively. The elliptical contours indicated a significant interaction occurred among sucrose with beef extract, yeast extract with beef extract and K_2HPO_4 with beef extract for dextransucrase activity (Fig(s). 5.3B, 5.5B and 5.6B).

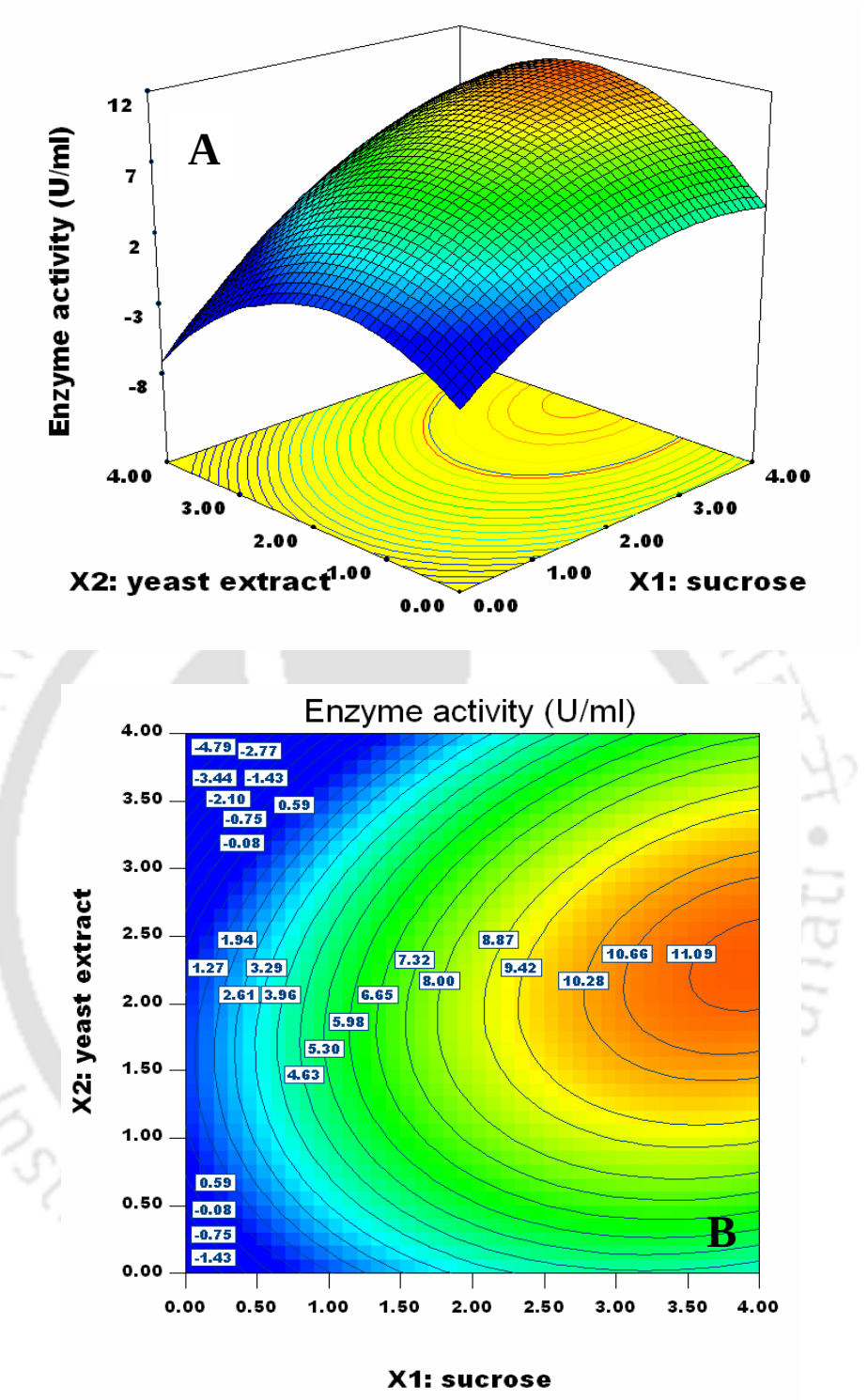

Fig. 5.1. Response surface plot (**A**) and Contour plot (**B**) of the combined effects of sucrose and yeast extract on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: K_2HPO_4 = 2 and beef extract = 1)

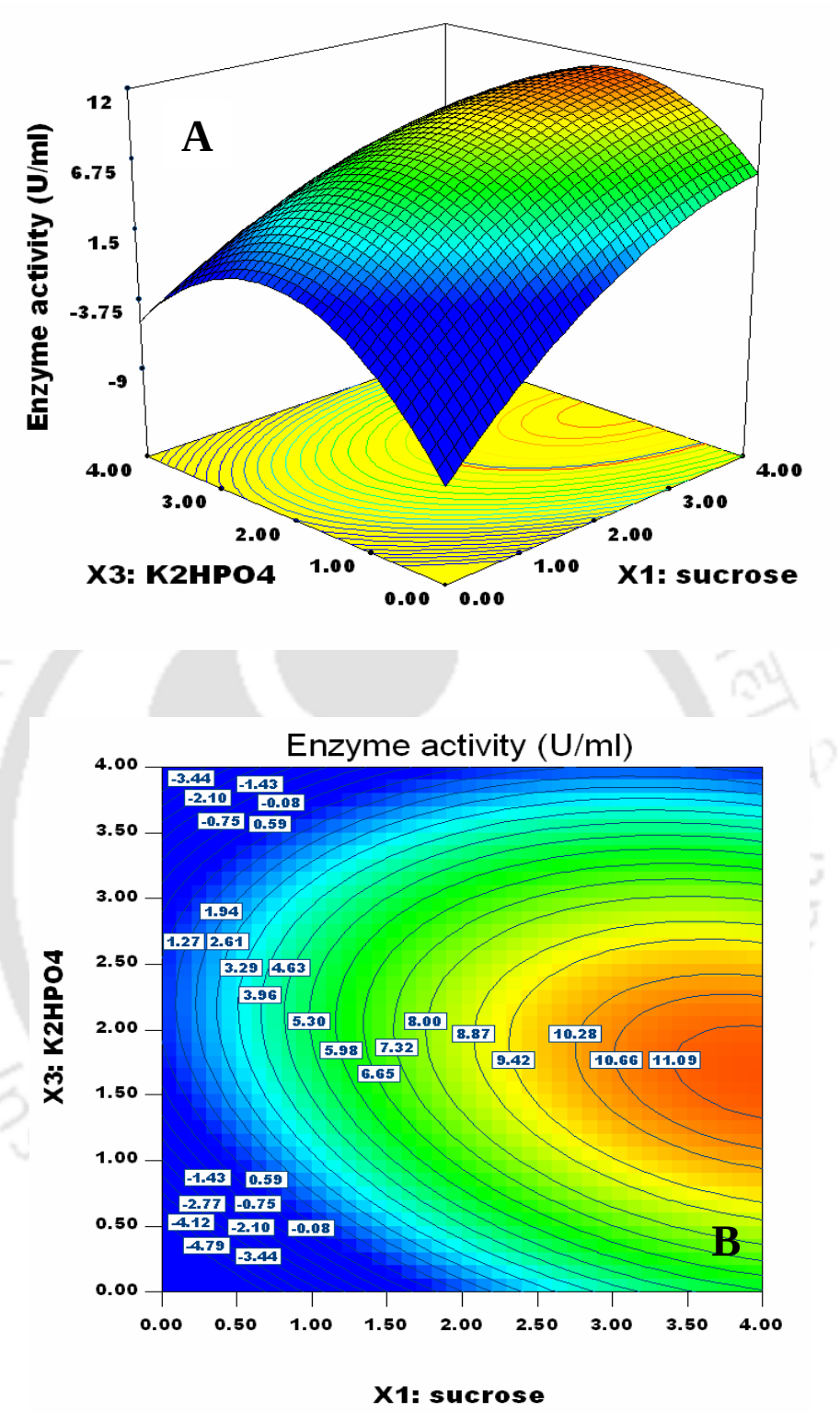

Fig. 5.2. Response surface plot (**A)** and Contour plot (**B)** of the combined effects of sucrose and K_2HPO_4 on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: yeast extract = 2 and beef extract = 1)

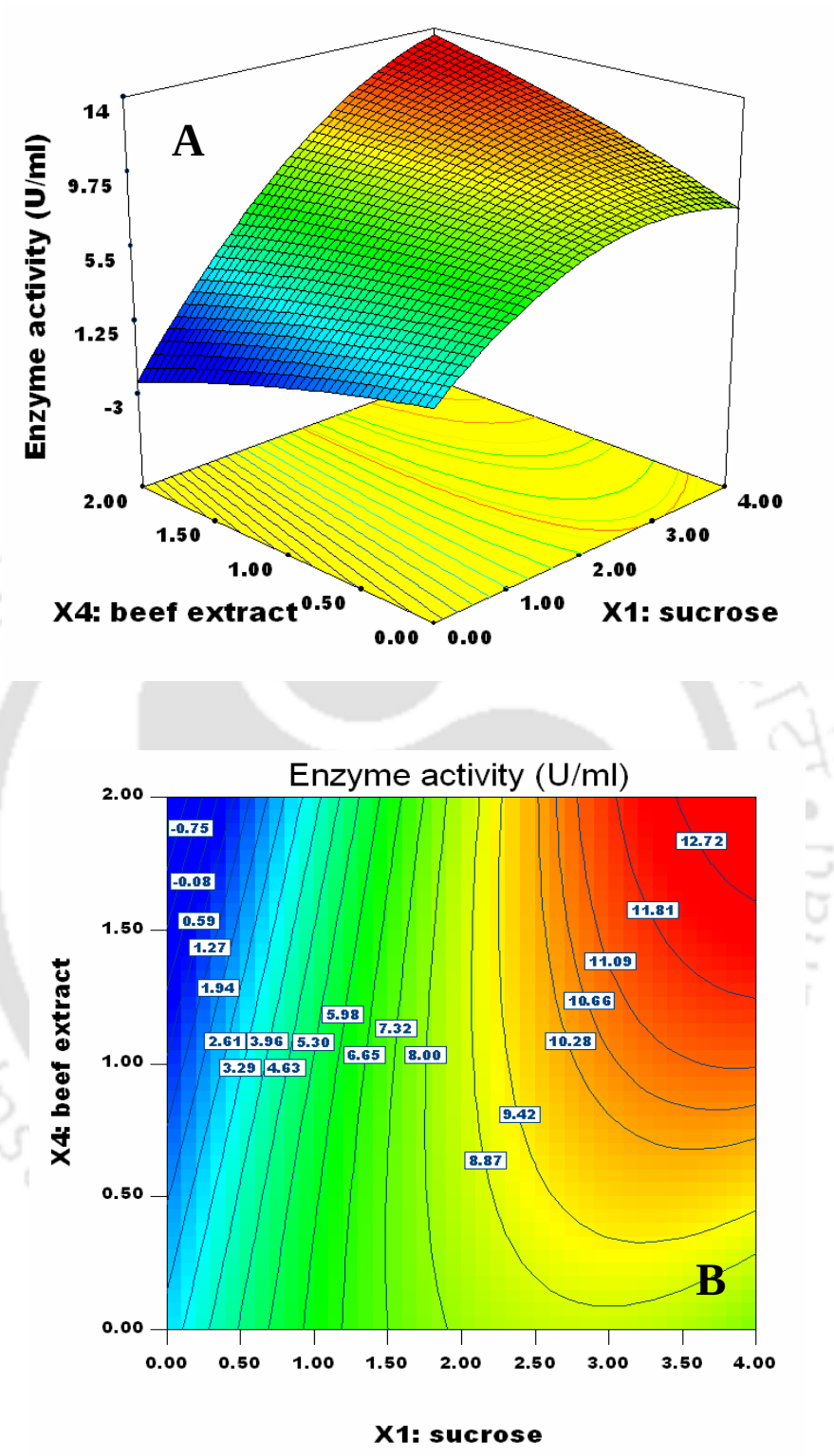

Fig. 5.3. Response surface plot (**A)** and Contour plot (**B)** of the combined effects of sucrose and beef extract on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: yeast extract = 2 and K_2HPO_4 = 2)

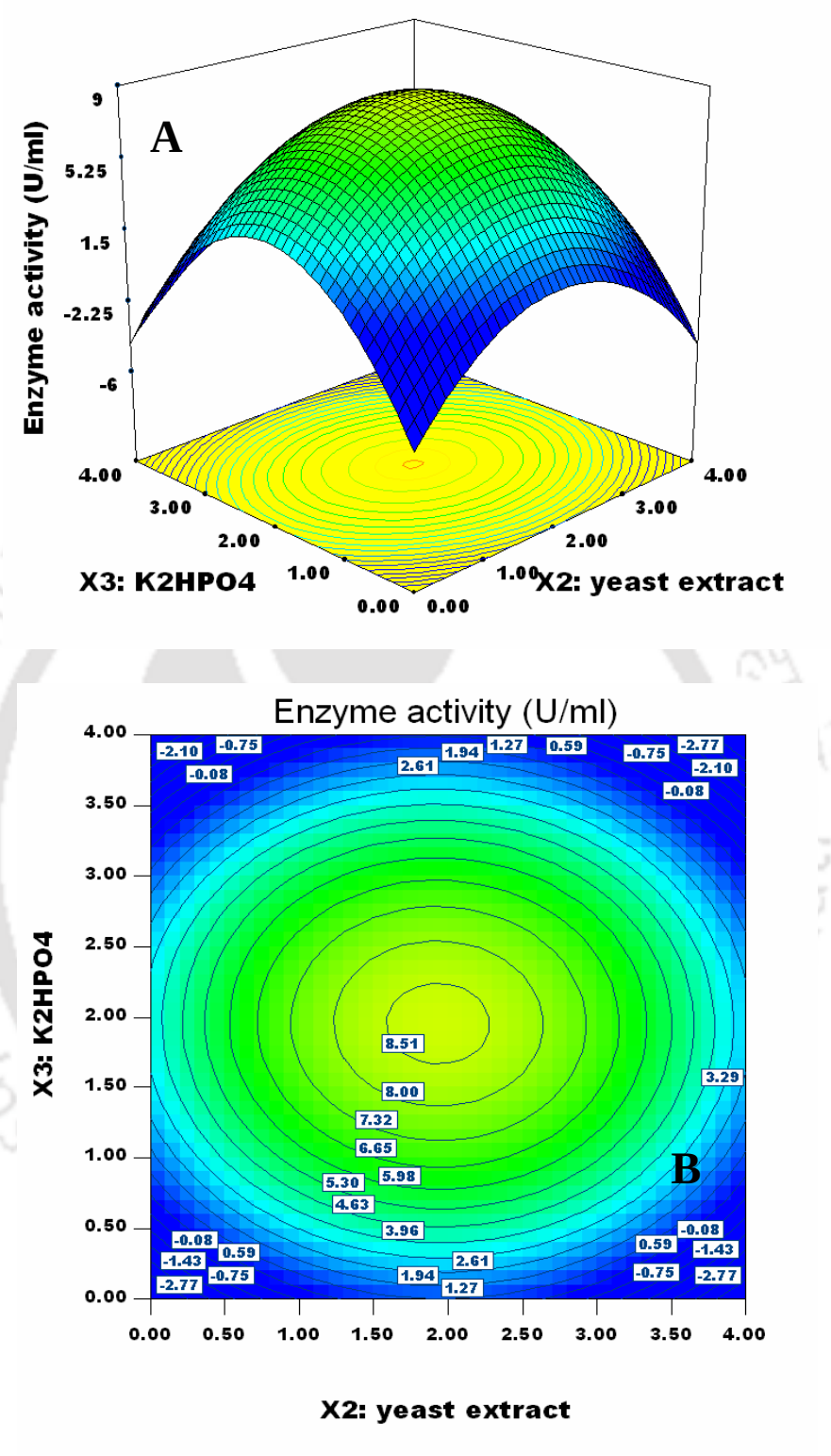

Fig. 5.4. Response surface plot (**A**) and Contour plot (**B**) of the combined effects of yeast extract and K_2HPO_4 on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: sucrose = 2 and beef extract = 1)

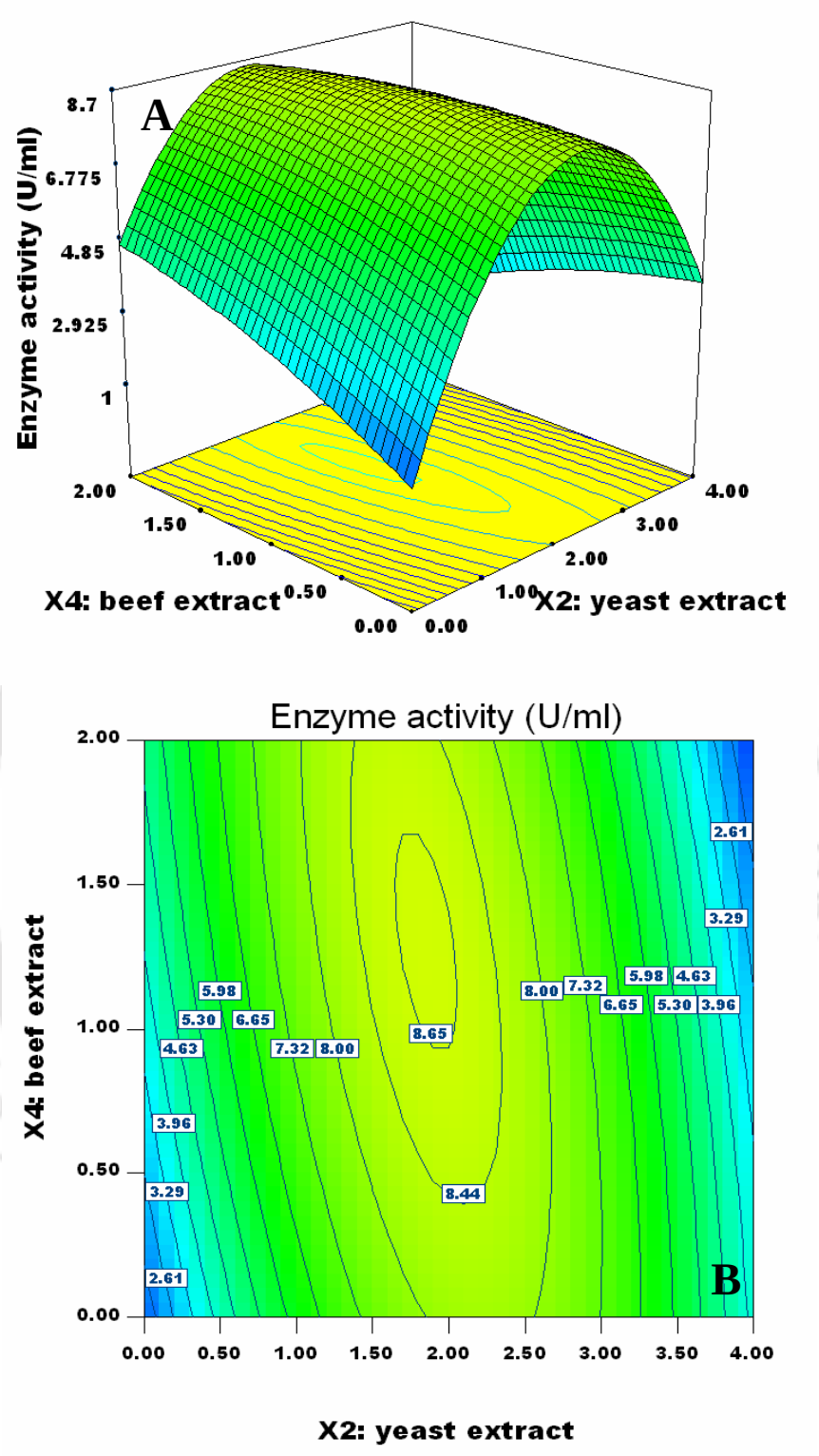

Fig. 5.5. Response surface plot (**A)** and Contour plot (**B)** of the combined effects of the combined effects of yeast extract and beef extract on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: sucrose = 2 and K_2HPO_4 = 2)

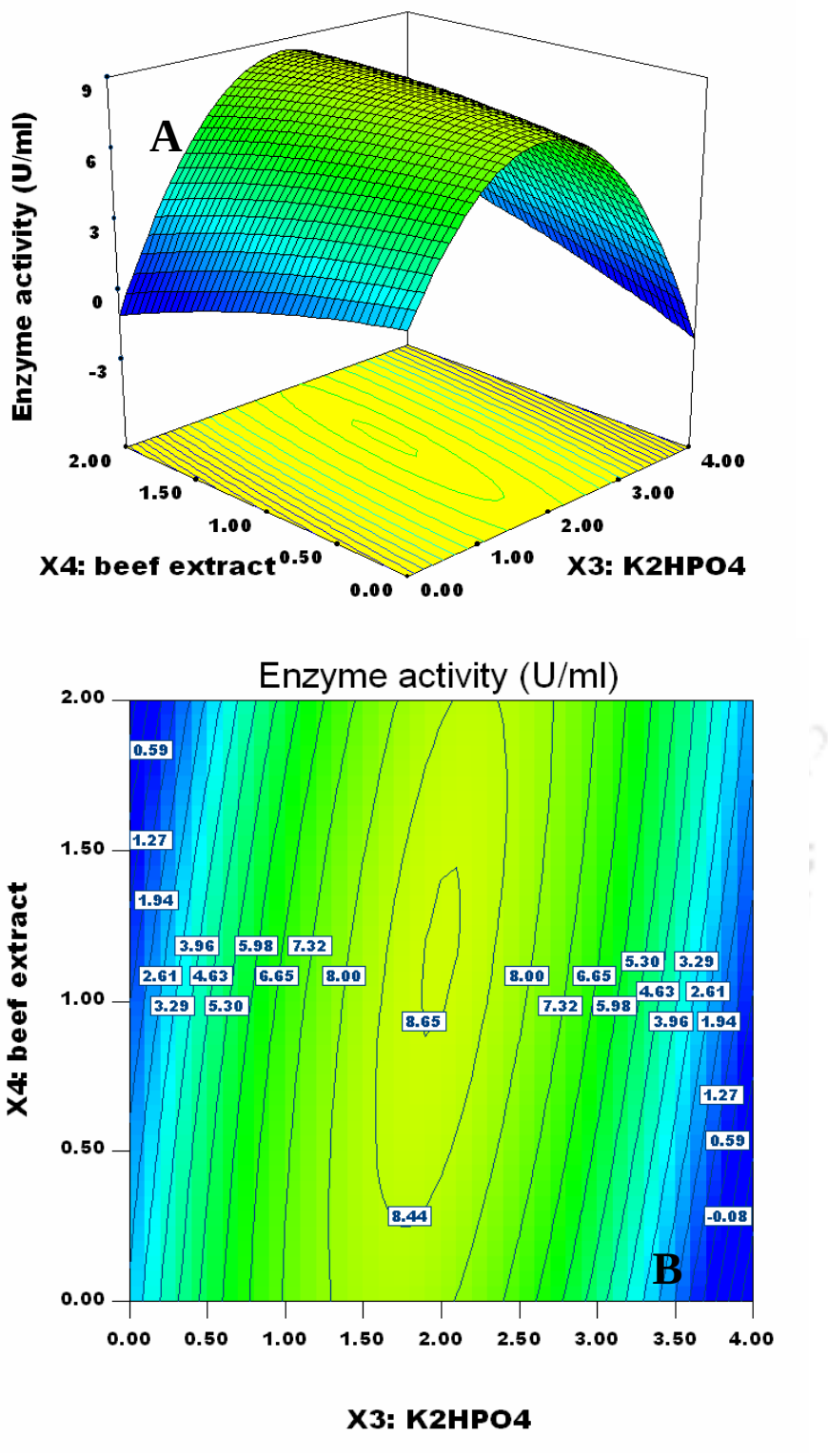

Fig. 5.6. Response surface plot (**A**) and Contour plot (**B**) of the combined effects of the combined effects of K_2HPO_4 and beef extract on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: sucrose = 2 and yeast extract = 2)

5.3.3 Optimization of medium composition for higher specific activity of dextransucrase by RSM

Model coefficients estimated by regression analysis for each variable representing for their effect on specific activity is shown in Table 5.8. The results revealed that sucrose (X_1) and yeast extract (X_2), with their positive coefficients and low *P*- values (P<0.0001), had a significant effect on dextransucrase specific activity. Although a positive coefficient was observed for K_2HPO_4 concentration (X_3) on dextransucrase specific activity, the higher *P* value (P = 0.058) indicated that it is least specific among all the four variables for dextransucrase specific activity. The negative coefficient observed for the beef extract indicated that decrease in its concentration can increase the dextransucrase production. Positive coefficients of X_1, X_2 and X_3 variables indicated a linear effect for the increase in dextransucrase specific activity. Negative coefficients were observed for quadratic terms of X_1, X_2 and X_3 variables, whereas a positive coefficient was observed for X_4. Low *P* values of X_1 (P < 0.001), X_2 (P < 0.0001) and X_3 (P < 0.0001) variables for quadratic terms indicated that they are also significant.

Among the interactions X_1X_2 (P < 0.847), X_1X_3 (P < 0.037) had negative coefficients, while X_1X_4 (P < 0.123), X_2X_3 (P < 0.386), X_2X_4 (P < 0.47) and X_3X_4 (P < 0.003) had positive coefficients. The interactions of $X_3X_4 > X_1X_3 > X_1X_4$ were found highly significant.

The Response surface plots can be used to visualize the effect of the concentration of variables on required response and can also measure the approximate required concentrations of variables for obtaining the maximum response (Tanyildizi *et al.* 2005).

Table 5.8. Model coefficient estimated by multiple linear regression for specific activity of dextransucrase.

Model Term	Parameter Estimate	Standard Error	Computed *t*-value	*P*-value
Intercept	-0.09631	0.020009	26.095	0.0001
X_1	0.32341	0.010806	12.566	0.0001
X_2	0.197668	0.010806	-6.506	0.0001
X_3	0.398364	0.010806	-2.039	0.058
X_4	-0.56326	0.010806	-5.814	0.0001
X_1^2	-0.0413	0.0099	-4.172	0.001
X_2^2	-0.07649	0.0099	-7.727	0.0001
X_3^2	-0.11865	0.0099	-11.985	0.0001
X_4^2	0.065301	0.0099	1.649	0.119
X_1X_2	-0.00259	0.013234	-0.196	0.847
X_1X_3	-0.03015	0.013234	-2.278	0.037
X_1X_4	0.043037	0.013234	1.626	0.123
X_2X_3	0.011796	0.013234	0.891	0.386
X_2X_4	0.019587	0.013234	0.740	0.470
X_3X_4	0.090882	0.013234	3.434	0.003

The well defined response surface plots in Fig (s). 5.7A, 5.8A and 5.10A depicted the amount of variable required for maximum dextransucrase specific activity at approximate concentration of 30 g/l sucrose in combination with 20 g/l of yeast extract and 20 g/l K_2HPO_4. Saddle shaped response surface plots in Fig (s). 5.9A, 5.11A and 5.12A indicated that low concentration of beef extract with higher concentration of sucrose, yeast extract and K_2HPO_4 favors the maximum dextransucrase specific activity. From the circular contour plots of Fig(s). 5.7B, 5.8B and 5.10B, it is evident that there is no significant interaction between sucrose with yeast extract and K_2HPO_4 and yeast extract with K_2HPO_4, respectively. The elliptical contours shown in Fig(s). 5.9B, 5.11B and 5.12B indicated a significant interaction occurred among sucrose with beef extract, yeast extract with beef extract and K_2HPO_4 with beef extract for dextransucrase specific activity.

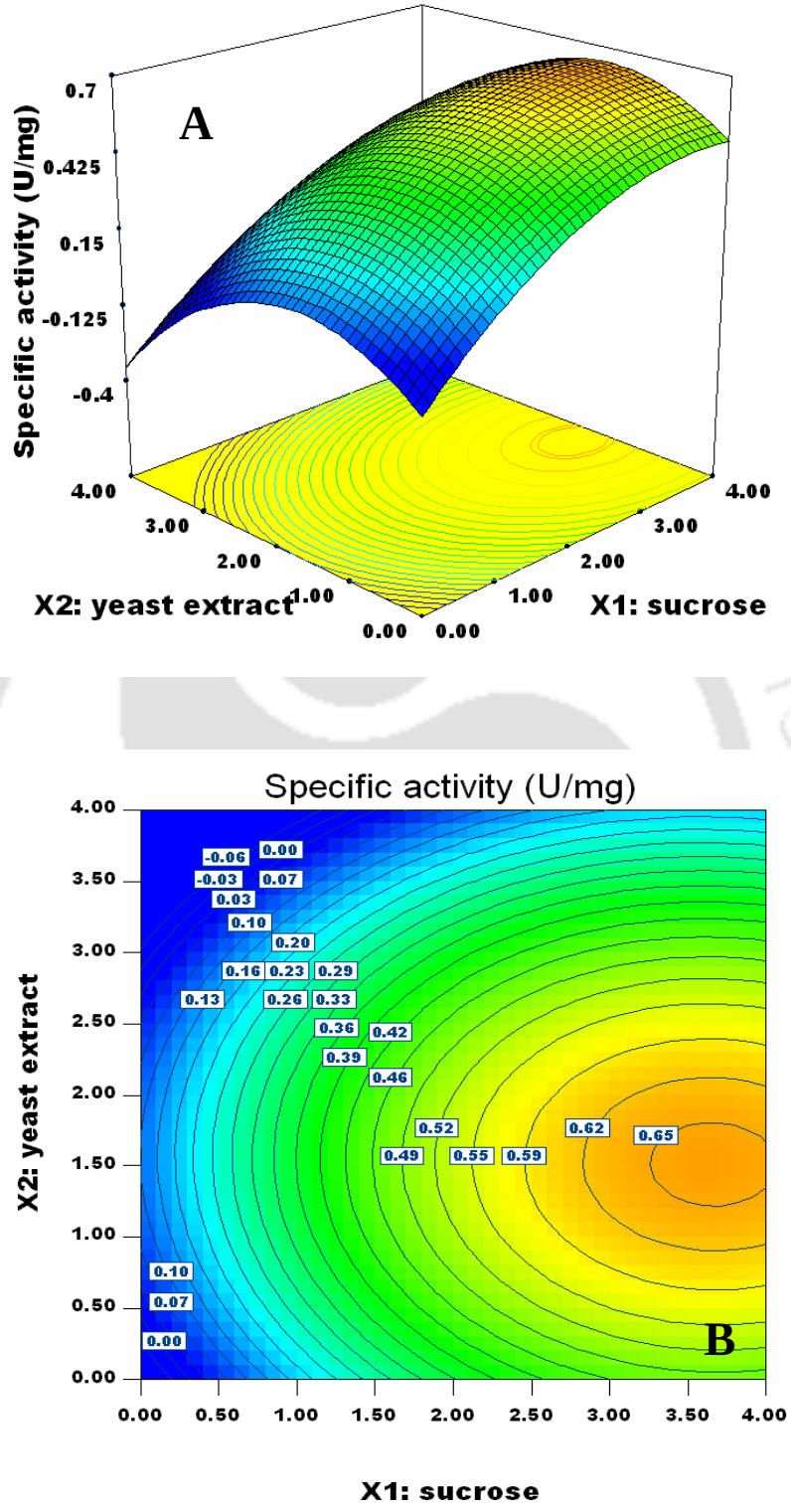

Fig. 5.7. Response surface plot (**A**) and Contour plot (**B**) of the combined effects of sucrose and yeast extract on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: K_2HPO_4= 2 and beef extract = 1)

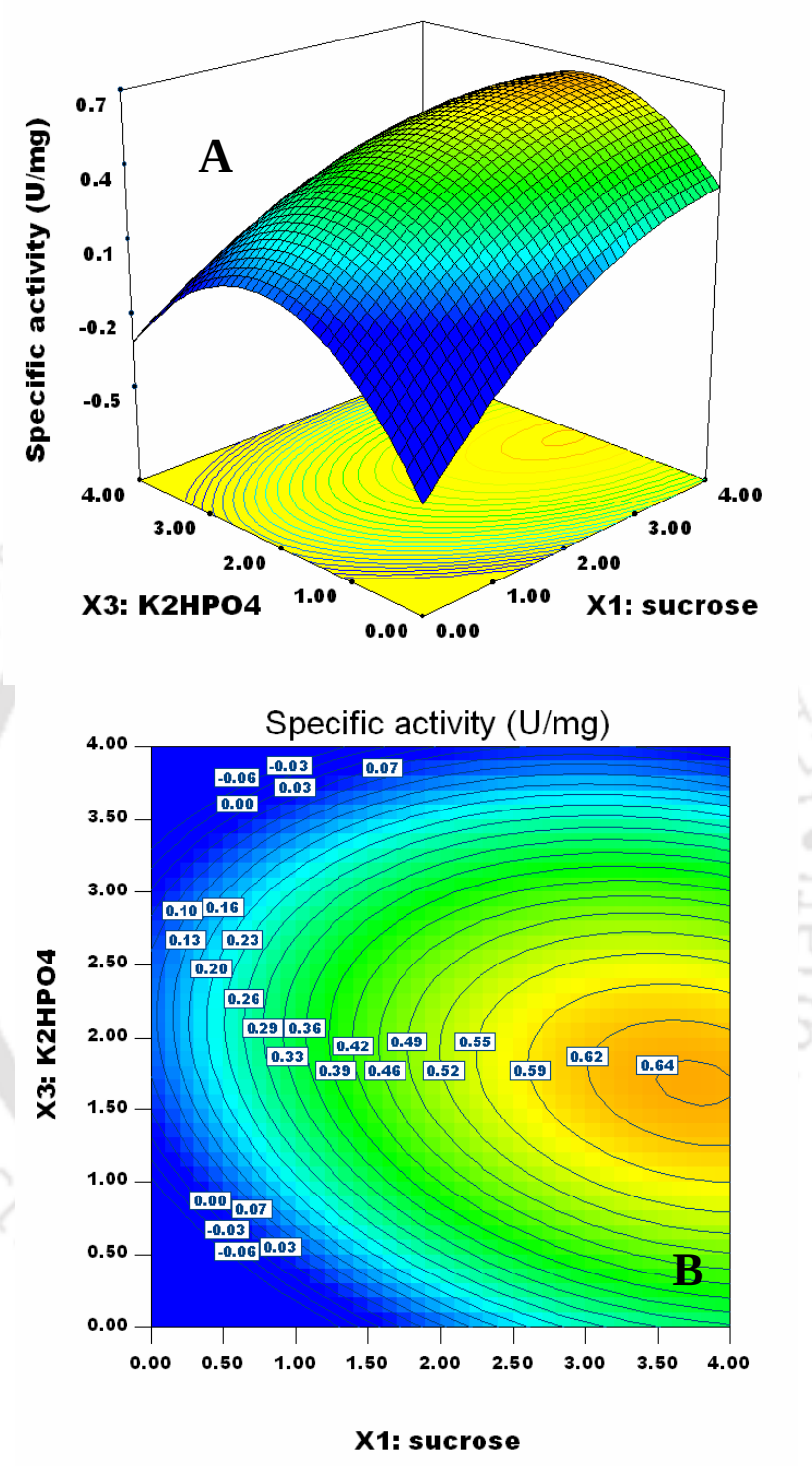

Fig. 5.8. Response surface plot (**A**) and Contour plot (**B**) of the combined effects of sucrose and K_2HPO_4 on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: yeast extract = 2 and beef extract = 1)

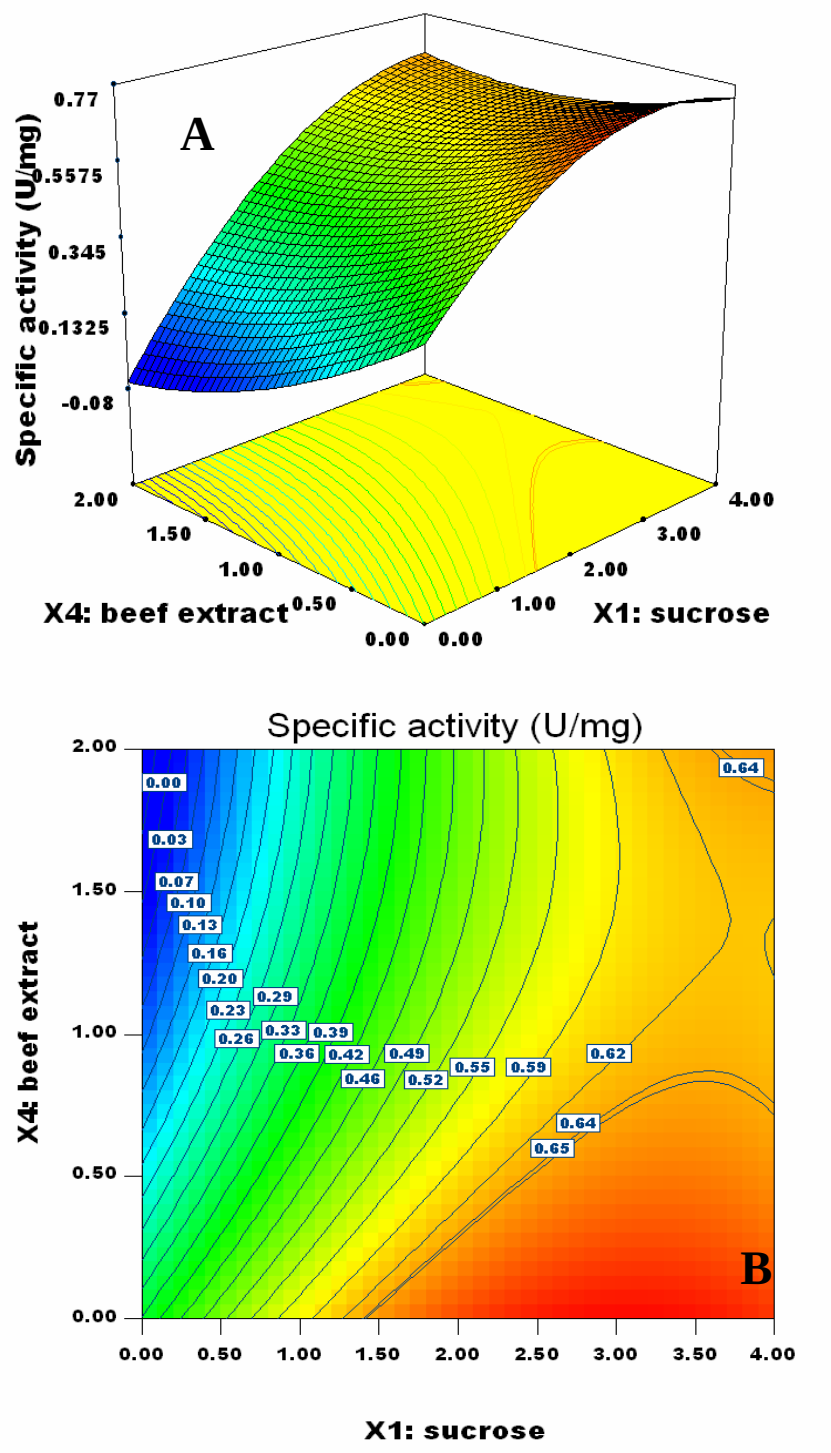

Fig. 5.9. Response surface plot (**A**) and Contour plot (**B**) of the combined effects of sucrose and beef extract on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: yeast extract = 2 and K_2HPO_4 = 2)

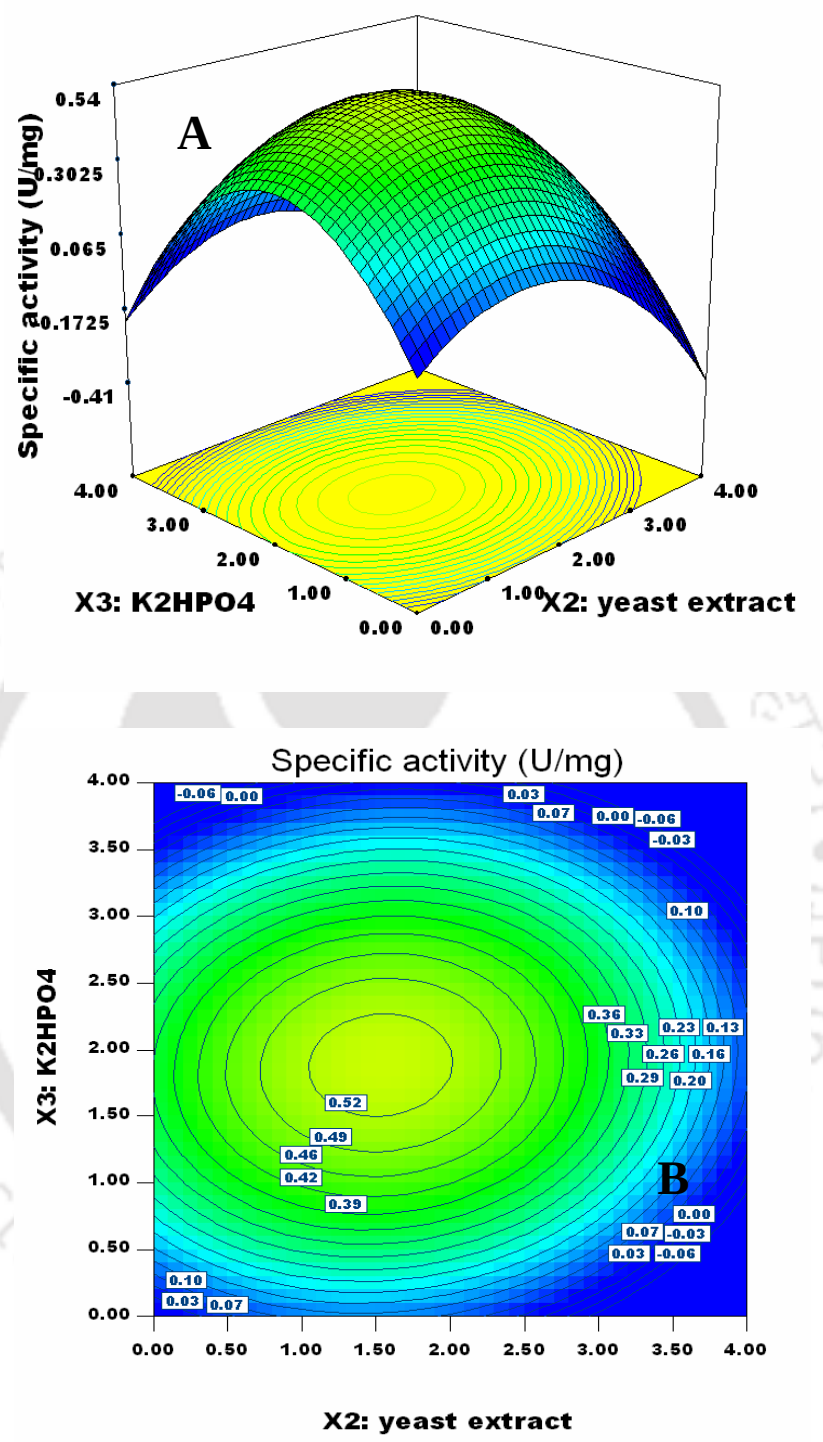

Fig. 5.10. Response surface plot (**A**) and Contour plot (**B**) of the combined effects of yeast extract and K_2HPO_4 on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: sucrose = 2 and beef extract = 1)

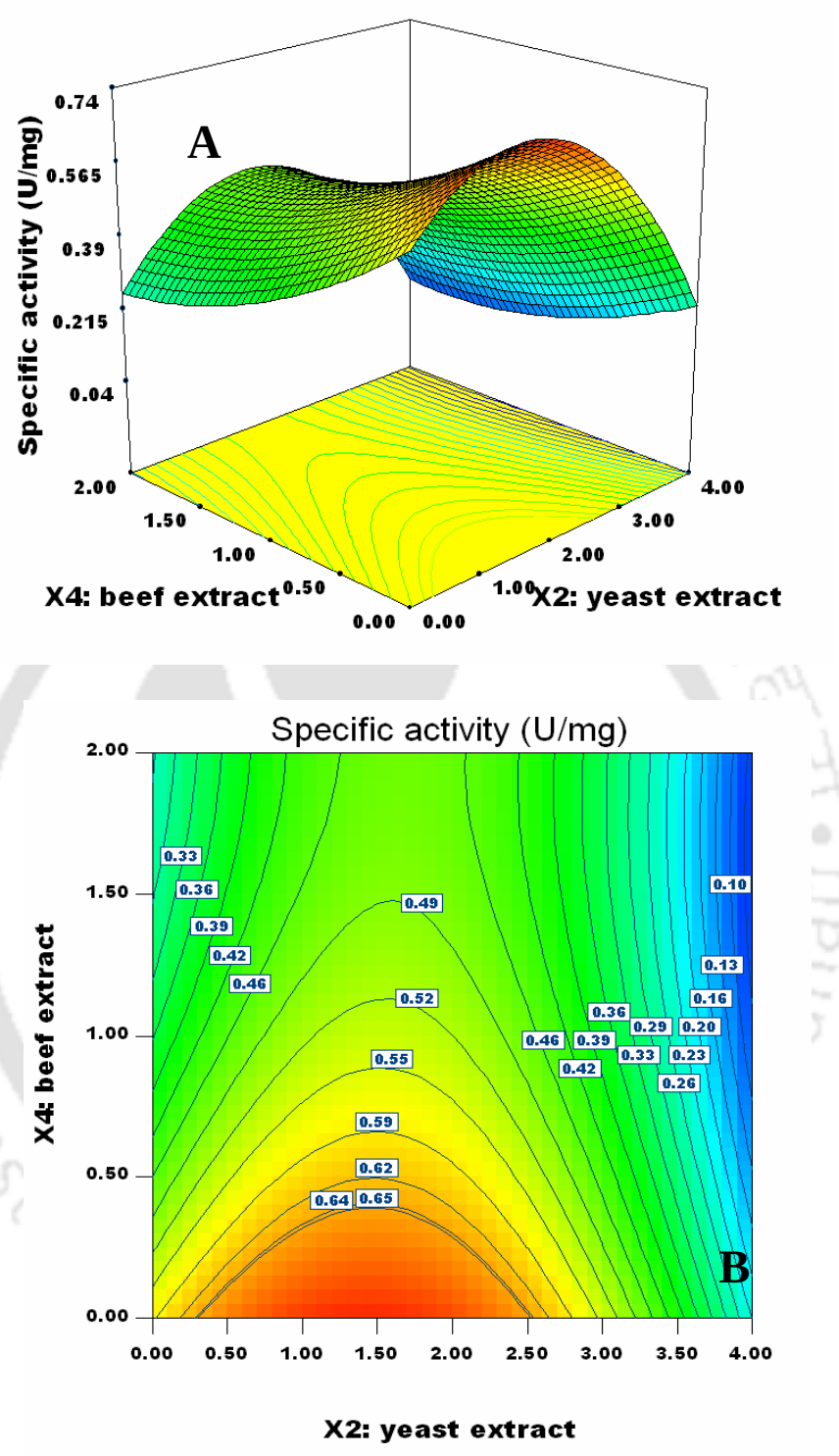

Fig. 5.11. Response surface plot (**A)** and Contour plot (**B)** of the combined effects of yeast extract and beef extract on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: sucrose = 2 and K_2HPO_4 = 2)

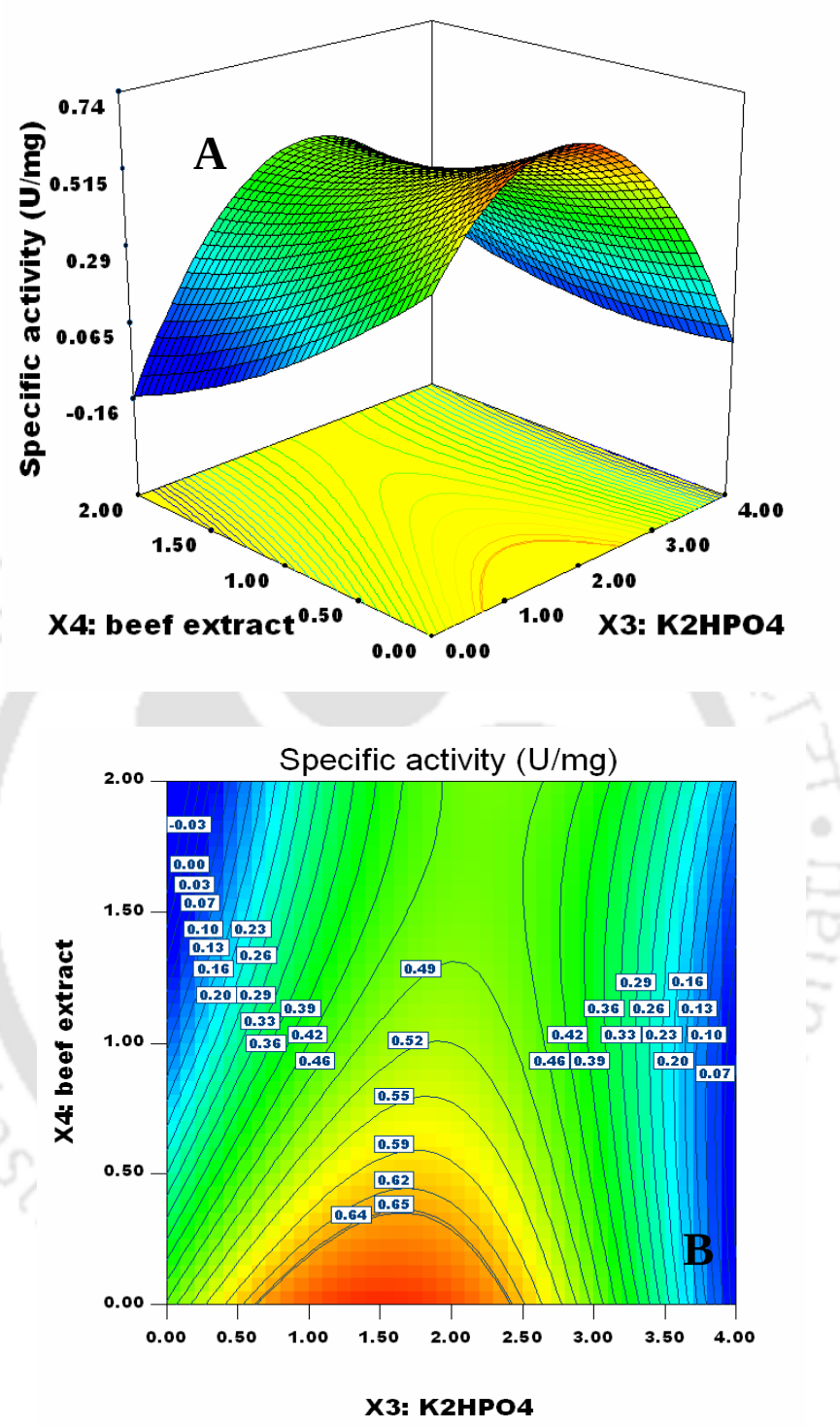

Fig. 5.12. Response surface plot (**A)** and Contour plot (**B)** of the combined effects of $K_2HPO_4$and beef extract on dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640. (Fixed level: Sucrose = 2 and yeast extract = 2)

5.3.4 Experimental validation of the optimized medium composition by flask culture and bioreactor

The optimum levels of the variables were obtained by solving the regression equation and also by analyzing the response surface contour plots using Design Expert software. The model predicted a maximum dextansucrase activity of 10.9 U/ml, with a specific activity of 0.64 U/mg appearing at: sucrose-30 g/l, yeast extract-18.9 g/l, K_2HPO_4-19.4 g/l and beef extract-15 g/l and by keeping the other components at their middle levels. To validate the predicted model three runs were conducted using this optimized medium composition at flask cultures with 100 ml medium. The dextransucrase activity of 10.7 U/ml obtained at this medium composition with a specific activity of 0.68 U/mg, perfectly matched with the model predicted value of 10.9 U/ml, 0.64 U/mg dextransucrase activity and specific activity, respectively (Table 5.9). This enzyme activity (10.7 U/ml) was higher (double) as compared to the control medium described by Tsuchiya *et al.,* 1952, which showed only 4.8 U/ml enzyme activity as described in Chapter 3, Section 3.3.2 and also reported earlier (Purama and Goyal 2007). The excellent correlation of enzyme activity between predicted and measured values of these experiments justifies the validity of the response model and the existence of an optimum point. Dextransucrase is associated with concomitant formation of dextran from sucrose present in the culture broth. The presence of higher sucrose concentration in the medium results higher viscosity of the broth, which makes the dextransucrase estimation and purification difficult, thus, a lower amount of sucrose is preferred for maximum dextransucrase production. Although, the dextransucrase activity of 12.3 U/ml and with a specific activity of 0.66 U/mg was predicted with medium composition containing 40 g/l sucrose, 20 g/l yeast extract, 20 g/l K_2HPO_4 and 10

g/l beef extract (Table 5.4), a dextransucrase activity and specific activities of 10.7 U/ml and 0.68 U/mg, respectively obtained by statistically optimized medium selected, could be justified, as it had lower sucrose concentration of 30 g/l, present in the medium. The statistical methods proved to be a powerful tool for maximizing dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640.

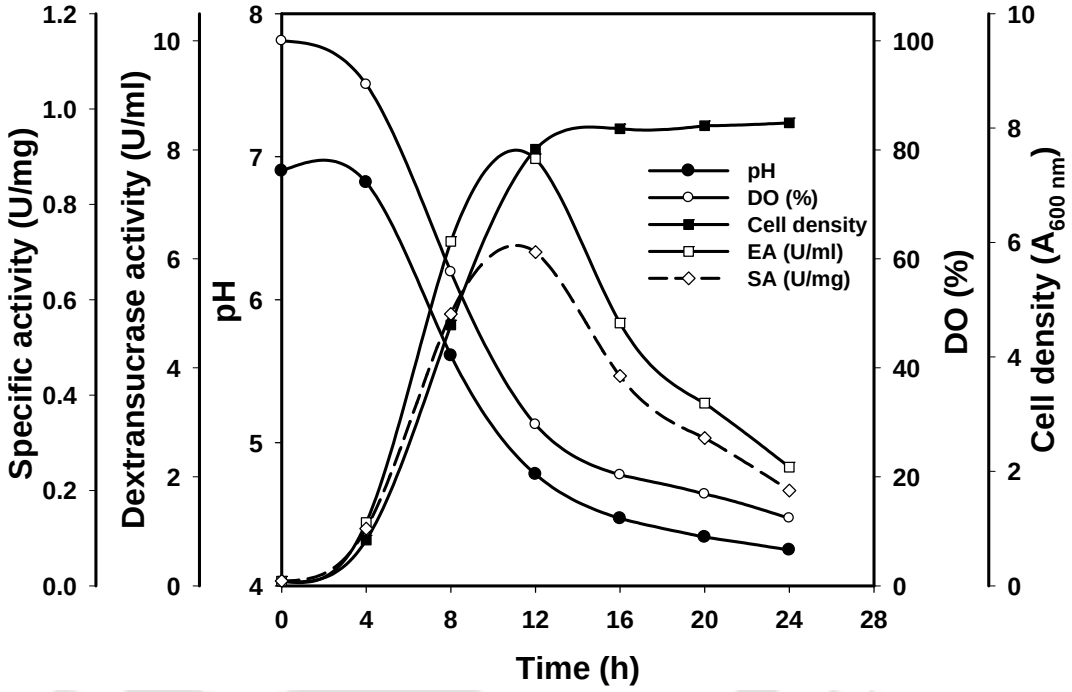

Fig. 5.13. Dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640 using the optimized medium in bioreactor at 1.5 vvm with no pH control

The scale-up of batch cultivation from the flask culture containing 100 ml medium to a bioreactor containing 1.4 litre of same optimized medium (sucrose-30 g/l, yeast extract-18.9 g/l, K_2HPO_4-19.4 g/l and beef extract-15 g/l and by keeping the other components at their middle levels) as used for flask culture for the dextransucrase production resulted in a dextransucrase activity of 9.9 U/ml (Fig. 5.13, Table 5.9). The same medium composition at a flask culture with 100 ml

medium gave a dextransucrase of 10.7 U/ml. Although, a loss of 8% in the enzyme activity was observed during the scale-up process using the optimized medium but it was two times higher than the enzyme activity (4.6 U/ml) obtained at fermenter level maintained under same culture conditions using the medium described by Tsuchiya *et al.* 1952 (Chapter 6, Section 6.3.1, Table 6.1, Fig. 6.1B). The enzyme activity (9.9 U/ml) obtained by bioreactor run was in good agreement with the model predicted a dextansucrase activity (10.9 U/ml) with the optimized medium composition (Table 5.9). The maximum enzyme activity in the bioreactor run was observed at 12h of the fermentation at a pH of 4.8 and at 38% DO and dry cell weight of 8.9 mg/ml (optical density of 8.0 at 600 nm) was observed.

Table 5.9. Comparison of dextransucrase activities of model prediction and experimental runs at flask culture and bioreactor

	Model Predicted Values	Flask Culture run	Bioreactor run
Enzyme activity (U/ml)	10.9	10.7	9.9
Specific activity (U/mg)	0.64	0.68	0.70

The model predicted a maximum specific activity of 0.64 U/mg for dextransucras (Table 5.9). The *Leuconostoc mesenteroides* NRRL B-640 grown under flask culture with 100 ml optimized medium at 25°C and shaking at 200 rpm gave dextransucrase with specific activity of 0.68 U/mg (Table 5.9), while the same medium composition under experimental conditions at 25°C, agitation speed 200 rpm and at a 1.5 vvm aeration rate under the batch cultivation using a bioreactor gave 0.7 U/mg (Fig. 5.13, Table 5.9). The results showed that the specific activities

for dextransucrase obtained with flask culture and bioreactor were in perfect agreement with the predicted values by the statistical model.

5.4 Conclusions

To improve dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 culture medium was screened and optimized using the statistical design techniques of Plackett-Burman and Response surface methodology. Plackett-Burman design with 6 variables viz. sucrose, yeast extract, K_2HPO_4, peptone, beef extract and Tween 80 was performed to screen the nutrients that were significantly affecting dextransucrase production. The proposed model equation illustrated the quantitative effect of variables and also of the interactions among the variables upon the dextransucrase production. The variables sucrose, K_2HPO_4, yeast extract and beef extract showed above 90% confidence levels for dextransucrase production and were considered as significant factors for optimization using Response surface methodology (RSM). 2^4-central composite design was used for RSM optimization. The experimental results were fitted to a second-order polynomial model which gave a coefficient of determination R^2 = 0.95 for dextransucrase activity and R^2 = 0.97 for dextransucrase specific activity. The model predicted a maximum dextansucrase activity of 10.9 U/ml, with a specific activity of 0.64 U/mg appearing at: sucrose-30 g/l, yeast extract-18.9 g/l, K_2HPO_4-19.4 g/l and beef extract-15 g/l and by keeping the other components at their middle levels. The dextransucrase activity of 10.7 U/ml obtained at above medium composition with a specific activity of 0.68 U/mg with flask culture, perfectly matched with predicted value of dextransucrase activity (10.9 U/ml) and specific activity (0.64 U/mg). The same optimized medium when used in bioreactor, gave an experimental value of

dextransucrase activity of 9.9 U/ml and a specific activity of 0.7 U/mg which again corresponded well with the predicted values by the statistical model.

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Chapter 6

Dextransucrase Production by Batch Fermentation in Bioreactor

6.1 Introduction

Leuconostoc mesenteroides NRRL B-640 elaborates a homopolysaccharide, dextran consisting essentially of α(1→6) linked glucose residues. Extracellular enzyme dextransucrase is responsible for the synthesis of dextran (Jeanes *et al.* 1954; Uzochukwu *et al.* 2001). The physical and chemical properties of dextran make it potentially attractive for several industrial and medical applications. They are used as stabilizing, emulsifying, prebiotic and gelling agents in food industry (Purama and Goyal, 2005). Dextran derivatives are used in industrial and biomedical applications such as gel filtration products and blood plasma substitutes (De Vuyst and Degeest, 1999; Purama and Goyal, 2005).

Isomalto-oligosaccharides obtained from dextransucrase acceptor reactions have many biological functions such as promotion of the growth of bifidobacteria in large intestine of humans and animals (Chung and Day 2002). Immobilization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F and *Leuconostoc mesenteroides* L strains in alginate was studied, in order to allow a continuous formation of defined oligosaccharides as prebiotic isomalto-oligosaccharides (Berensmeier *et al.* 2004; Kubik *et al.* 2004).

The *Leuconostoc mesenteroides* NRRL B-512F and its mutants B-512FMC and B-512FMCM (Kim and Robyt 1994; Hwa-Ja Ryu *et al.* 2000) have been extensively studied for the production of dextran. B-512F is the industrial strain used for the production of dextran which is linear in structure and the extensive use of B-512F was due to the yield of dextransucrase by this strain. The dextran from this strain was composed of 96% α (1→6) linkages (Jeanes *et al.* 1954). *Leuconostoc mesenteroides* NRRL B-640 is also another potential strain for the production of dextran and dextransucrase. The dextran from this strain is also highly homogenous and is known to synthesize the polysaccharide dextran with 95% α (1→6) linkages (Seymour *et al.* 1979, Uzochukwu *et al.* 2001). The strain of *Leuconostoc mesenteroides* NRRL B-640 produces 3.4 U/ml of dextransucrase in basal medium under non-aerated fermentation in a bioreactor without any strain improvement or optimization of medium constituents. This strain thus has a potential for the industrial production of dextran and is presently being investigated. *Leuconostoc mesenteroides* is a known micro-aerophile (Tsuchiya et al. 1952; Plihon *et al.* 1995) and several workers have studied aeration effects on the growth and enzyme production (Barker and Ajongwen 1991; Lazic *et al.* 1991, 1993, Veljkovic *et al.* 1992). The pH of the fermentation broth drops to 4.2 or 4.3 during the process (Tsuchiya et al. 1952; Goyal and Katiyar 1998). When the pH drops to a value of 5.0 to 5.5 the enzyme production is greater (Tsuchiya *et al.* 1952). Fermentations carried out at controlled pHs between 7.0 and 5.5 showed similar results (Lazic *et al.* 1991; Santos *et al.* 2000). It was found that transmembrane proton gradient is responsible for elaboration of the enzyme into the medium (Otts and Day 1988). This confirms that the pH has a role to play in dextransucrase production.

A mathematical model allows an easy data analysis and provides a strategy for solving problems encountered in the design. Kinetic studies play an important role in the overall investigation of fermentation. Mathematical models for dextransucrase and dextran production from *Leuconostoc mesenteroides* have been proposed (Landon *et al.* 1994; Santos *et al.* 2000). The aim of the present work was to determine the effect of aeration and pH on dextransucrase yield and activity from *Leuconostoc mesenteroides* NRRL B640 and to develop a model for its production in batch fermentation.

6.2 Materials and Methods

6.2.1 Microorganism and cultivation conditions

Leuconostoc mesenteroides NRRL B-640 was procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA. Ingredients required for maintenance and enzyme production media were from Hi-Media Pvt. Ltd., India. The culture was maintained in modified MRS (DeMan *et al.* 1961) with sucrose replaced by glucose as stab at 4°C and sub-cultured every 2 weeks. A loop full of culture from stab was transferred to 5 ml of medium as described by Tsuchiya *et al.* (1952). The cultures were grown at 25°C with shaking at 200 rpm. 1% of the culture inoculum was used to inoculate 100 ml enzyme production medium (Tsuchiya *et al.* 1952).

6.2.2 Production of dextransurase in bioreactor

Actively growing culture after 12h from the culture flask was used to inoculate the medium with (Tsuchiya *et al.* 1952).working volume of 1.4 litre in a 3 litre bioreactor (Applikon, model Bio Console ADI 1025). The volume of the inoculum

was 1% of the total medium in the fermenter. The temperature of bioreactor was maintained at 25°C with an agitation speed of 200 rpm. The samples (5 ml) were collected at regular time intervals and centrifuged at 9,200***g*** for 10 min at 4°C to separate the cells. The cell free extract was analyzed for enzyme activity and protein concentration. The growth rate, pH, and Dissolved Oxygen (DO) were monitored in the fermentor.

6.2.2 Enzyme activity assay

The assay of dextransucrase was carried out in 1 ml of a reaction mixture in 20 mM sodium acetate buffer, pH 5.4, containing 146 mM (5%) sucrose and using the cell free extract (10-20 µl) as the enzyme source (Purama and Goyal 2007). The reaction mixture was incubated at 30°C for 15 min. Aliquots (0.2 ml), from the reaction mixture were analyzed for reducing sugar concentration. The assay procedure is described in detail in Chapter 3, Section 3.2.7.

6.2.3 Cell growth measurement

Cells were separated from 1ml culture broth by centrifugation at 9,200***g*** for 5 min after regular intervals of 2h. The optical density at 600 nm of the culture broth was also recorded. The cells were washed with 0.9% NaCl and dried in the Eppendorf's tubes for 18-24h, to a constant weight. The cells were weighed and a graph was plotted for dry weight against the optical density at 600 nm.

$$\text{Cell concentration (mg/ml)} = 1.0985 + 0.9708 \cdot A_{600nm}$$

6.2.4 Effect of aeration on dextransucrase production in bioreactor

The production of dextransucrase in a bioreactor was studied at different aeration rates of 0.0, 1.5, 2.5 and 3.0 vvm. All the fermentations were carried out at an agitation speed of impeller at 200 rpm The samples were collected at regular time intervals and assayed for dextransucrase activity. The growth rate, pH, specific enzyme activity and Dissolved Oxygen (DO) were monitored.

6.2.5 Effect of controlled pH on dextransucrase production in bioreactor

The experiments on production of dextransucrase under batch fermentation in a bioreactor were carried out at an optimized aeration rate of 1.5 vvm and at controlled pHs of 6.0, 6.5 and 7.0 and were maintained by the addition of acid/base. The effect of high concentration of K_2HPO_4 (0.3 M) was also studied at uncontrolled pH. The samples were collected at regular time intervals and assayed for dextransucrase activity. The growth rate, pH, specific enzyme activity and Dissolved Oxygen (DO) were monitored.

6.3 Results and Discussion

6.3.1 Effect of aeration on dextransucrase production in bioreactor

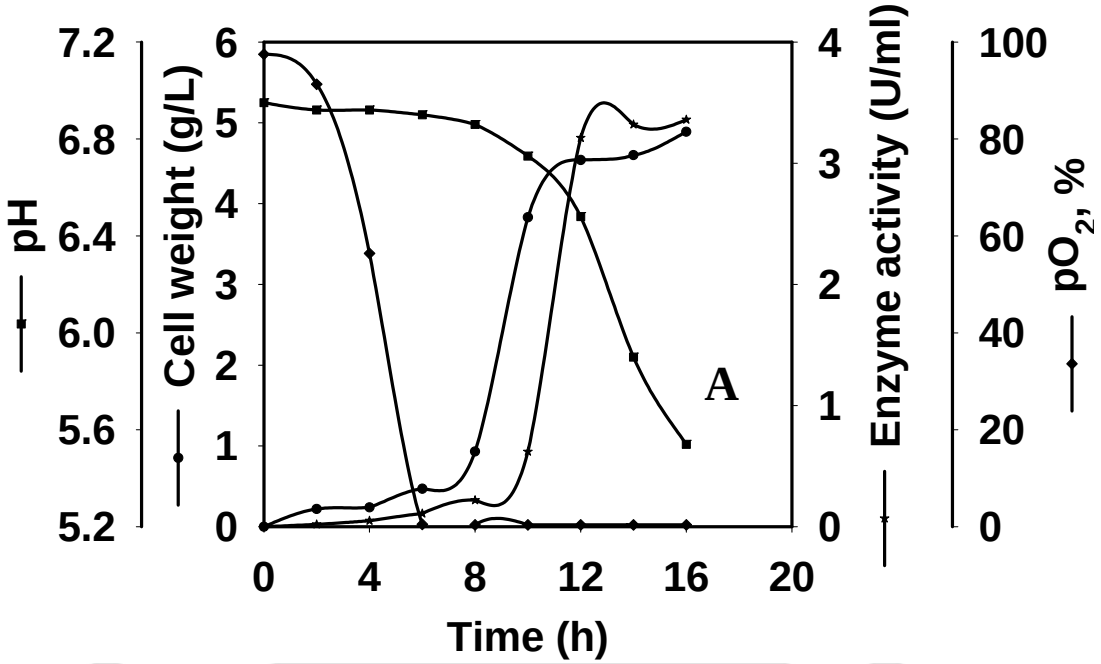
Leuconostoc mesenteroides NRRL B-640 was grown under different aeration conditions in batch fermentation in a bioreactor to study the effect of aeration on dextransucrase production. Dissolved oxygen, pH, enzyme activity and cell density profiles were plotted with time at different aeration rates. The results of different aeration rates are summarized in Table 6.1. The Fig. 1A-1D shows the profiles for dextransucrase production at aeration rates of 0.0, 1.5, 2.5 and 3 vvm. Fig. 6.1A depicts dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640 at 0 vvm aeration rate. The enzyme activity reached maximum at 3.4 U/ml after 16h and the dry cell weight was also highest at this point (4.9 mg/ml) (Table 6.1). The pH of the broth dropped to 5.5 after 10h and %DO dropped to zero after 6h of fermentation, a consequence of the micro-aerophilic nature of the bacterium. The specific growth rate (μ_m) was 0.5955 h^{-1} and enzyme yield ($Y_{E/X}$) was found to be 0.7 U/mg cell.

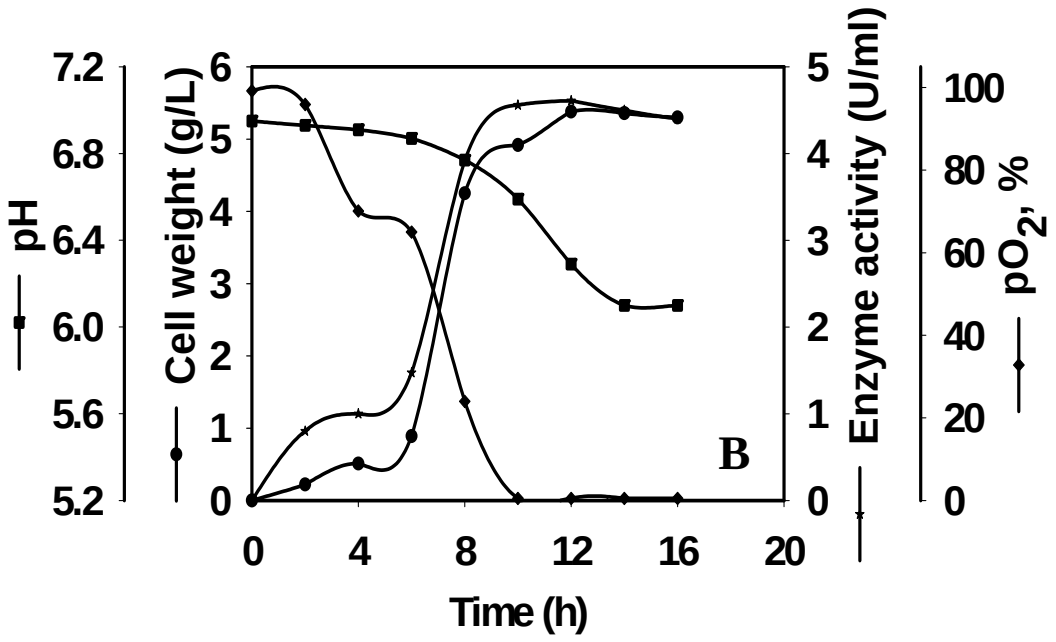
With the aeration rate at 1.5 vvm, the enzyme activity was 35% higher than that with 0 vvm aeration rate and reached a maximum of 4.6 U/ml after 12h and the cell growth attained stationary phase at 12h were also higher (Fig. 6.1B). Specific growth rate increased to 0.7295 h^{-1} and also the dry cell weight to 5.4 mg/ml (Table 6.1). The enzyme yield ($Y_{E/X}$) of 0.86 U/mg cells was higher than 0.7 U/mg obtained at 0 vvm aeration rate. The pH at the end of fermentation was 6.1 (16h) and %DO dropped to zero after 10h (Fig. 6.1B).

The specific growth rate (μ_m) further increased to 0.8745 h^{-1} and the enzyme activity marginally to 4.7 U/ml when aeration rate was increased from 1.5 to 2.5 vvm (Fig. 6.1C, Table 6.1). The maximum biomass produced was 5.7 mg/ml. The enzyme yield ($Y_{E/X}$) was also marginally higher 0.88 U/mg cells as compared to 1.5 vvm

aeration rate (Table 6.1). The pH dropped to 6.4 at 16h and %DO dropped to zero at 10h (Table 6.1). At 3 vvm aeration rate the specific growth rate dropped to 0.68 h^{-1} and the corresponding enzyme activity dropped to 4.4 U/ml (Fig. 6.1D, Table 6.1). The maximum biomass obtained was also less 5.3 mg/ml as compared to 5.7 mg/ml at 2.5 vvm aeration rate and also the enzyme yield reduced to 0.84 U/mg cells. The pH of the fermentation broth at the end of 12h was 6.7 at 3 vvm run. The %DO took longer (12h) to drop to zero at 3 vvm run as compared to 10h at 2.5 vvm run.

It is known that oxygen positively affects the growth of certain strains of *Leuconostoc mesenteroides* (Veljkovic *et al.* 1992) and that dextransucrase yields are consistently higher in shake flask cultures than in still ones. Dextransucrase biosynthesis was reported to be growth associated (Santos *et al.* 2000; Rodrigues *et al.* 2003). Thus it is expected that dextransucrase biosynthesis will be affected by the oxygen mass transfer rates. In *Leuconostoc mesenteroides* NRRL B-640 the maximum enzyme activity obtained increased as the rate of aeration increased. Although maximum activity of 4.7 U/ml was obtained at 2.5 vvm which was 39% higher than the activity at 0.0 vvm, it was only marginally better (2%) than at 1.5 vvm. Further increase to 3 vvm adversely affected the production of cell biomass and dextransucrase. Since there was no significant increase in enzyme activity at 2.5 vvm, aeration rate of 1.5 vvm was selected for further optimization experiments. In the present study, there was an increase in the specific growth rate (μ_m) and enzyme yield ($Y_{E/X}$) with an increase in aeration rate till 2.5 vvm and these were found to drop at 3 vvm (Table 6.1). The dextransucrase production increases up to its maximum value of 4.7 U/ml and then decreased, this might be the result of competition between the organism and dextransucrase induction for and by sucrose, the former using sucrose using as an energy source and the later being induced in its presence.





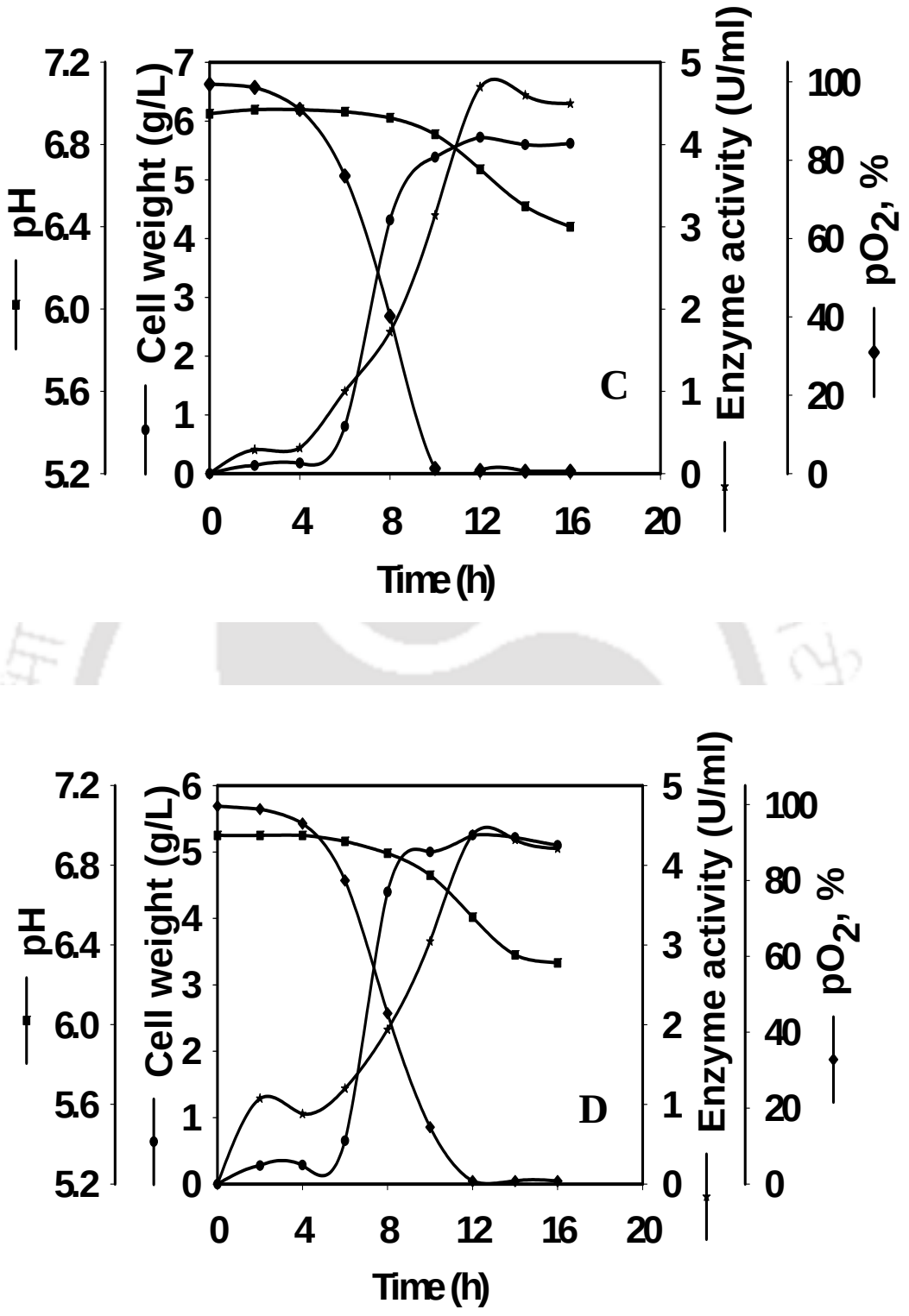


Fig. 6.1. Variation of cell density, pH, dissolved oxygen (%DO) and enzyme activity of dextransucrase during batch fermentation of *Leuconostoc mesenteroides* NRRL B-640 at aeration rates: (A) 0 vvm (B) 1.5 vvm (C) 2.5 vvm and (D) 3.0 vvm.

Table 6.1. Fermentation profile of dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 at different aeration rates.

Aeration rate (vvm)	Enzyme Activity (U/ml)	% DO reaching Zero (h)	X_{max}* (mg/ml)	μ_m* (h^{-1})	$Y_{E/X}$* (U/mg cell)
0.0	3.4	6	4.9	0.5955	0.70
1.5	4.6	10	5.4	0.7295	0.86
2.5	4.7	10	5.7	0.8745	0.88
3.0	4.4	12	5.3	0.6800	0.84

*Maximum biomass (X_{max}), specific growth rate (μ_m) and enzyme yield ($Y_{E/X}$) were calculated from the model equations using experimental data.

6.3.2 Mathematical model for cell growth and dextransucrase production

A sigmoidal shape was observed in the cell growth curve (Fig. 6.1). The most used approximation for this cell growth is the logistic equation that characterizes cell growth in terms of the maximum value obtained (Rodrigues *et al.* 2003). The specific growth rate μ (h^{-1}) is related to cell mass concentration X (g/l) as follows

$$\mu = \mu_m \left(1 - \frac{X}{X_{max}}\right) \quad (1)$$

where, μ_m and X_{max} are the maximum specific growth rate and maximum attainable biomass concentration, respectively. The cell growth rate r_X (g/l/h) is then:

$$r_X = \frac{dX}{dt} = \mu X = \mu_m \left(1 - \frac{X}{X_{max}}\right) X \quad (2)$$

Integrating the above equation with initial condition $X(0)=X_0$ (initial cell concentration) we have the logistic equation:

$$X(t) = \frac{X_0 \exp(\mu_m t)}{1 - (X_0 / X_{max})(1 - \exp(\mu_m t))} \quad (3)$$

or

$$\ln\left(\frac{\bar{X}}{1-\bar{X}}\right) = \mu_m t - \ln\left(\frac{X_{max}}{X_0} - 1\right) \quad \text{with} \quad \bar{X} = \frac{X(t)}{X_{max}} \quad (4)$$

The rate of enzyme production can be calculated from Eq. (5). The enzyme production was cell growth associated, so following model can be proposed for the yield of the enzyme activity.

$$r_E = dE/dt = Y_{E/X}\, dX/dt \quad (5)$$

where, r_E is enzyme production rate (U/ml/h), E is enzyme activity of dextransucrase produced (U/ml) and $Y_{E/X}$ is the yield of enzyme production per unit mass of cells produced (U/mg of cells).

The enzyme concentration as a function of time can be obtained from equations (2) and (5). After integration we get:

$$E(t) = Y_{E/X} X_0 \left(\frac{\exp(\mu_m t)}{1-(X_0/X_{max})(1-\exp(\mu_m t))} - 1 \right) \quad (6)$$

The yield of enzyme activity $Y_{E/X}$ was calculated using the formula:

$$Y_{E/X} = \frac{E_{max} - E_0}{X_{max} - X_0} \quad (7)$$

The results from the proposed model were compared with experimental results of the 1.5 vvm run, for enzyme activity and cell growth, were found in good agreement (Fig. 6.2A and B).

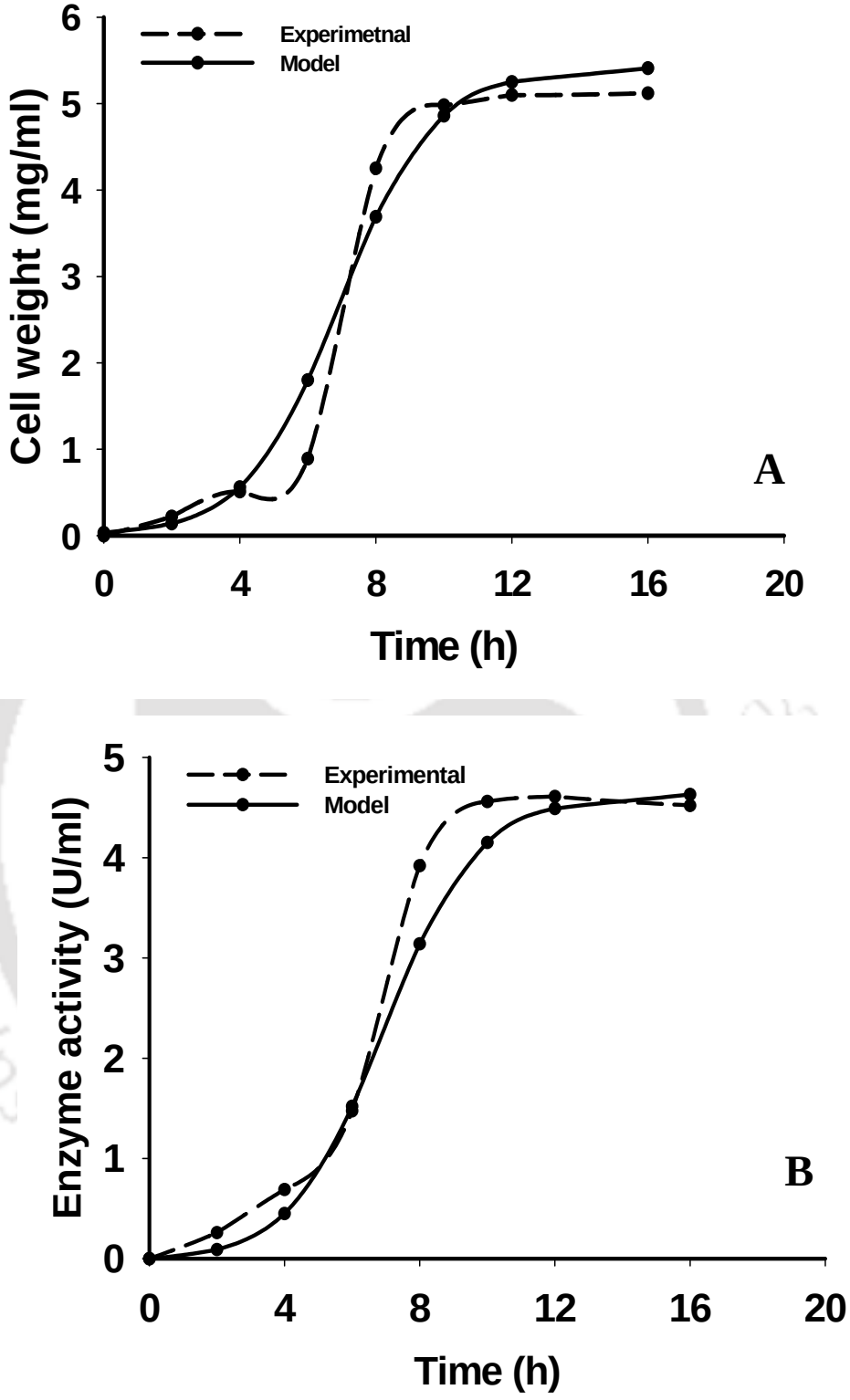


Fig. 6.2. Comparison of results (A) Cell dry weight and (B) Dextransucrase activity from predicted model with experimental fermentation results at aeration rate of 1.5 vvm at uncontrolled pH.

6.3.3 Effect of controlled pH on dextransucrase production in bioreactor

Leuconostoc mesenteroides NRRL B-640 was grown under different controlled pHs of 7.0, 6.5, 6.0 and at higher phosphate concentration of 0.3 M. The batch fermentations were carried out under optimized aeration rate of 1.5 vvm and at an agitation speed of 200 rpm. The parameters such as dissolved oxygen, enzyme activity and cell density were monitored or determined and were plotted against time. The results of fermentation run with uncontrolled pH were compared with the results of fermentation run at controlled pHs, and all runs were carried out at an aeration rate of 1.5 vvm (Table 6.2). With the fermentation at 1.5 vvm and uncontrolled pH, the maximum enzyme activity achieved was 4.6 U/ml and % DO dropped to zero in 10h (Table 6.2).

The Fig. 6.3A-D, show the fermentation profiles for pHs 7.0, 6.5, 6.0 and 0.3 M phosphate, respectively. At constant pH 7, the maximum enzyme activity of 4.7 U/ml was obtained which was only slightly higher than 4.6 U/ml with no pH control (Fig. 6.3A). However, the time taken by the cells to reach stationary phase (maximum value of 6 mg/ml) was significantly higher (24h) than the run with no pH control (10h), (Table 6.2). In contrast, the enzyme activity showed a steep decline after 16h, falling to almost zero at 24h. This can be ascribed to the instability of released dextransucrase at pH 7 of the medium.

The enzyme activity decreased slightly to 4.5 U/ml when pH was controlled at 6.5 (Fig. 6.3B). The dry cell weight showed maximum value of 3.9 mg/ml and the %DO dropped to 0 at 12h (Fig. 6.3B, Table 6.2). The enzyme activity further decreased to 4.1 U/ml at controlled pH 6.0, as compared to pH 6.5 and 7.0 (Fig. 6.3C). The cell density also decreased to 3.7 mg/ml and % DO dropped to the value of zero at the end of 12h (Fig. 6.3C, Table 6.2).

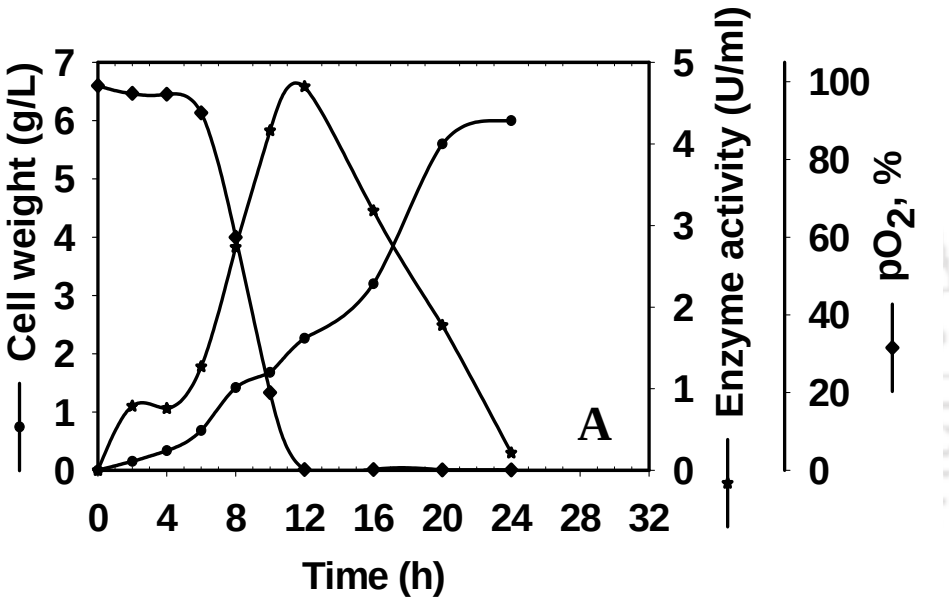
When the run was carried out at 0.3M concentration of K_2HPO_4, a maximum enzyme activity of 5 U/ml was achieved at 20h of fermentation (Fig. 6.3D) which was 9% higher than that obtained with no pH control fermentation. Also a higher cell density (7.3 mg/ml) was observed as compared to the 0.11 M run but the time taken for cell growth to reach at stationary phase was higher, 28h (Table 6.2). The pH remained stable and did not drop below 6.1. The %DO dropped to zero at 16h. From the maximum enzyme activities observed under controlled and uncontrolled pH and in presence of 0.3 M phosphate (Fig. 6.3D), it was observed that maintenance of pH does not have any significant effect on the production of dextransucrase and addition of phosphate had only a marginal increase of enzyme production.

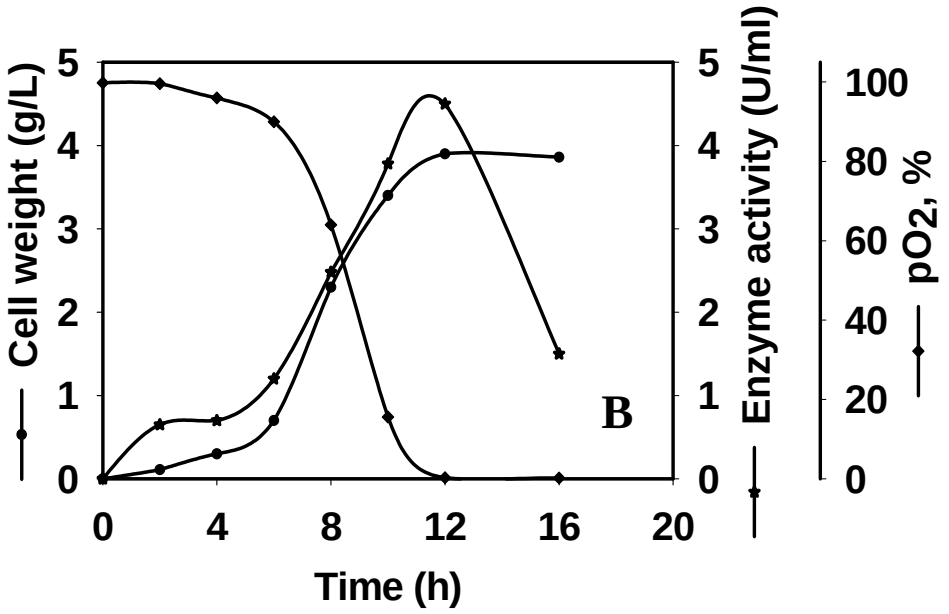
The mode of pH regulation, either by the continuous addition of alkali or by use of phosphate buffers in the medium, might be a critical factor affecting dextransucrase production. When the culture medium of *Leuconostoc mesenteroides* NRRL B-512F was adjusted by adding alkali (5M NaOH) in the range of 6.0 to 7.0, the maximum dextransucrase yield was obtained at pH 6.7, though maximum enzyme yield was achieved sooner in cultures held at the lower pH values (Tsuchiya *et al.* 1952). On the other hand, when the phosphate buffer (total phosphate concentration of 3.0%) was used to hold the pH steady during fermentation using the same strain NRRL B-512F, the maximum enzyme yield was obtained at pH 5.5 (Rodrigues *et al.* 2003). In *Leuconostoc mesenteroides* NRRL B-640 under controlled and uncontrolled pH and in presence of 0.3 M K_2HPO_4, it was observed that it had a significant effect on cell growth but did not have any significant effect on the production of dextransucrase and addition of 0.3 M K_2HPO_4 showed only a marginal increase of enzyme activity. The reduced enzyme activity at constant pH condition may be due to the instability of the enzyme at these conditions or this could be due to the absence of

sucrose as an inducer which was used up as an energy source to support the growth which was promoted at constant pH conditions.

Table 6.2. Experimental data for the effect of pH on dextransucrase production at 1.5 vvm aeration rate.

Parameters	No pH Control	pH 7.0	pH 6.5	pH 6.0	K_2HPO_4 (0.3 M)
Enzyme Activity (U/ml)	4.6	4.7	4.5	4.1	5.0
Dry cell weight (mg/ml)	5.4	6.0	3.9	3.7	7.3
Time for max. cell dry wt. (h)	12	24	12	12	28
%DO reaching zero value (h)	10	12	12	12	16





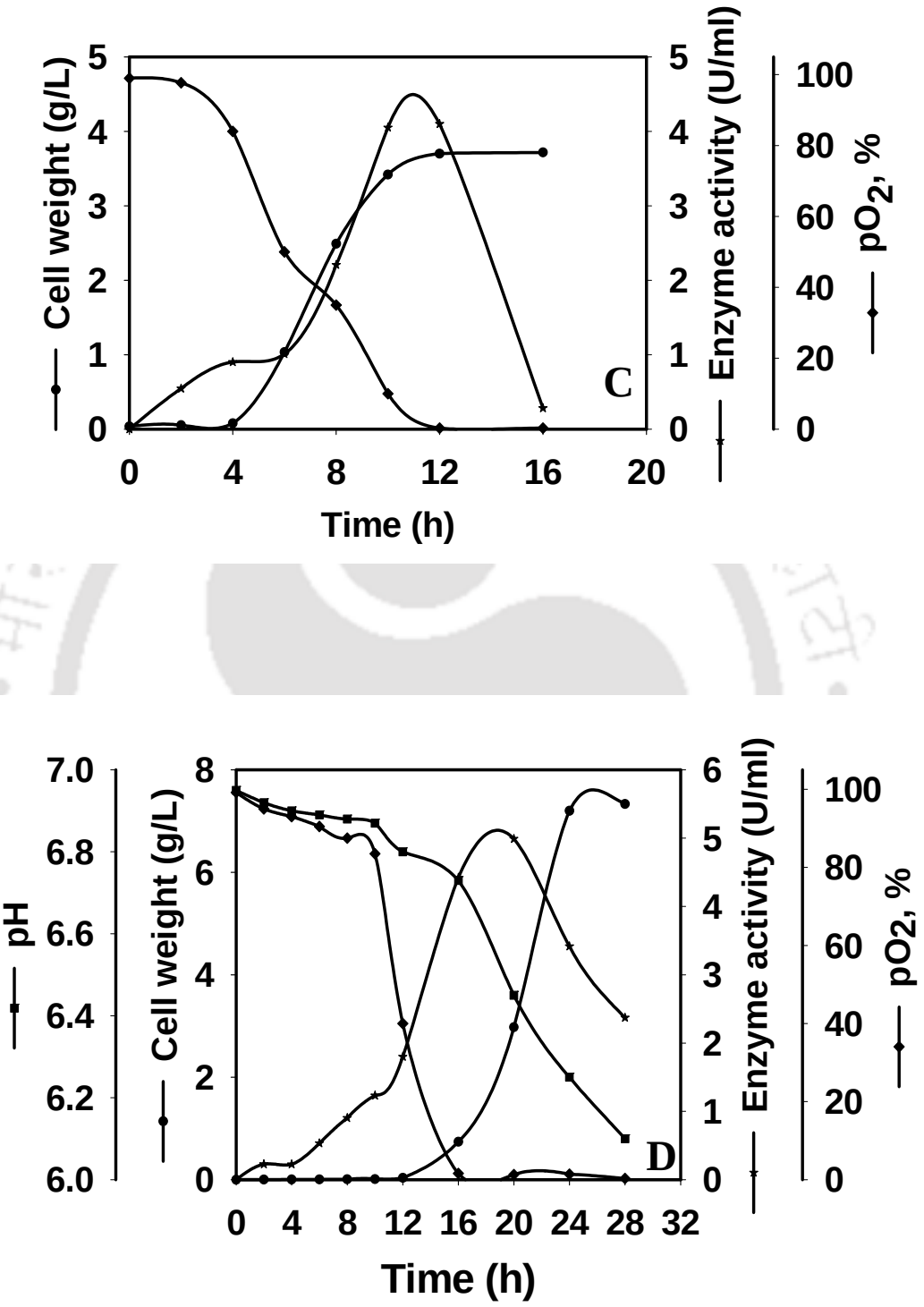


Fig. 6.3. Variation of cell density, dissolved oxygen and enzyme activity of dextransucrase during batch fermentation at controlled pHs; (A) pH 7.0 (B) pH 6.5 (C) pH 6.0 and (D) 0.3 M K_2HPO_4.

6.4 Conclusions

Experimental results of the batch fermentation of *Leuconostoc mesenteroides* NRRL B-640 with different aeration rates are presented in this report. Aeration significantly affected the production of dextransucrase. The maximum attainable enzyme yield was 0.86 U/mg cell at 1.5 vvm. The enzyme activity of 4.6 U/ml at 1.5 vvm aeration was 36% higher than with non-aerated conditions at 0 vvm. Although, a higher specific growth rate was observed at 2.5 vvm aeration rate only a marginal increase in the enzyme activity was observed resulting in the lower enzyme yields than the cultures grown under 1.5 vvm aeration rates. Further increase of aeration rates to 3 vvm from 2.5 vvm did not result in the higher specific growth rates and enzyme activity. The extracellular dextransucrase production was dependent on the bacterial cell growth. A mathematical model was developed for enzyme production and cell growth. The experimental values perfectly correlated with the predicted values by the model for both the cell biomass and the enzyme production rates under 1.5 vvm aeration rates. The results of the pH maintenance experiments showed that there was a marginal increase in dextransucrase production when the fermentation was carried out at controlled pH at 7.0 when compared to the fermentation at uncontrolled pH. The pHs 6.0 and 6.5 did not enhance the enzyme activity. At uncontrolled pH run higher K_2HPO_4 concentration (0.3 M) in the medium showed a 10% increase of the enzyme activity as compared to control medium containing 0.11 M K_2HPO_4. These results showed that aeration of 1.5 vvm and the maintenance of enzyme production medium at pH 7.0 in batch fermentation could obtain a higher dextransucrase production. The dextransucrase in high yields is of great importance for dextran biosynthesis.

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Chapter 7

Purification, Identifcation and Confirmation of Dextransucrase

7.1 Introduction

Leuconostoc mesenteroides produces an extracellular dextransucrase. Dextansucrase synthesizes high molecular weight glucose polymer called dextran from sucrose. Dextran has numerous applications in pharmaceutical, food and fine chemical industries (Robyt 1986; Naessens *et al.* 2005; Purama and Goyal 2005). Oligosaccharides synthesized using dextransucrase are used as neutraceuticals, stabilizers and prebiotics (Goulas *et al.* 2004; Naessens *et al.* 2005). Various methods have been reported for purification of dextransucrase. The purification methods such as fractionation by polyethylene glycol, ultra-filtration, salt, glycerol and alcohol precipitation, chromatography and phase-partitioning have been standardized and successfully used for purification of dextranucrase from different strains (Tsuchiya *et al.* 1955; Fukui *et al.* 1974; Itaya and Yamamoto 1975; Robyt and Taniguchi 1976; Russell 1979; Monsan and Lopez 1981; Paul *et al.* 1984; Lopez-Munguia 1993; Goyal and Katiyar 1994; Dols *et al.* 1998; Kitakota and Robyt 1998; Nigam *et al.* 2006; Purama and Goyal 2007a; Majumder *et al.* 2007).

Among all the reported purification methods, fractionation by polyethylene glycol (PEG) is a simple, effective and single step method for dextransucrase

purification (Russell 1979; Goyal and Katiyar 1994). Polyethylene glycols are nonionic hydrophilic polymers and are known to selectively precipitate proteins, which have high molecular weights or exist in aggregated forms (Honig and Kula 1976; Miekka and Ingham 1978). They also have an advantage of being readily removed by dialysis. The dextransucrase is associated with dextran polymer and forms high molecular weight aggregates in solution, leading to the formation of high molecular weight aggregates (Russell 1979; Funane *et al.* 1995). The dextransucrase from *Streptococcus mutans* was purified by precipitation using PEG-400 and PEG-6000, and it was reported that the higher molecular weight PEG-6000 precipitated other non-dextransucrase proteins, while PEG-400 gave higher specificity of precipitation (Russell 1979). The crude dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F was subjected to PEGs of different molecular weights (Goyal and Katiyar 1994). It was reported that the PEG-400 gave dextransucrase with highest specific activity of 8.7 U/mg with a 80% yield in a single step. The purification by two phase partitioning using different PEGs have been reported for dextransucrase from various strains of *Leuconostoc mesenteroides* (Fukui 1974; Paul *et al.* 1984; Otts and Day 1988; Lopez-Munguia *et al.* 1993; Remaud-Simeon 1991; Dols *et al.* 1998; Quirasco *et al.* 1999; Nigam *et al.* 2006).

Leuconostoc mesenteroides NRRL B-640 is shown to produce dextransucrase that gives highly linear and soluble dextran (Uzochukwu *et al.* 2002) and the strain is least studied for its dextransucrase elaboration. An attempt was made to purify the dextransucrase produced by this strain in order to explore the characteristics of dextran it can produce that can be exploited commercially. No report is available on the purification of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. In the present study an efficient method of purification of dextransucrase from

Leuconostoc mesenteroides NRRL B-640 using polyethylene glycol was developed. This is to our knowledge is the first study describing the purification of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. The crude dextransucrase in cell free extract and purified dextransucrase were identified and confirmed by Periodic Acid Schiff's (PAS) staining procedure using sucrose and raffinose as substrates.

7.2 Materials and Methods

7.2.1 Microorganism and reagents

Leuconostoc mesenteroides NRRL B-640 was procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA. Ingredients required for the maintenance and enzyme production media were from Hi-Media Pvt. Ltd., India. All the chemicals required for reducing sugar estimation, protein estimation and buffer preparation were of highest purity grade commercially available. PEG-200 (Hi-Media Pvt. Ltd., India), PEG-400 (Merck India Ltd.), PEG-1500 (BDH India Ltd.) were used for fractionation of dextransucrase.

7.2.2 Production of dextransucrase

Leuconostoc mesenteroides NRRL B-640 was grown in the medium as described by Tsuchiya *et al.* 1952 containing 2% sucrose as carbon source and details are described in Chapter 3, Section 3.2.6.

7.2.3 Enzyme activity assay

The assay of dextransucrase was carried out in 1 ml of a reaction mixture in 20 mM sodium acetate buffer, pH 5.4, containing 146 mM (5%) sucrose and using the cell free extract (10-20 µl) as the enzyme source (Purama and Goyal 2007b). The reaction mixture was incubated at 30°C for 15 min. Aliquots (0.2 ml), from the reaction mixture were analyzed for reducing sugar concentration. The assay procedure is described in detail in Chapter 3, Section 3.2.7.

7.2.4 Protein determination

The total protein content of the cell free extract was estimated by the method of Lowry *et al.* (1951). Bovine serum albumin ranging from, 25 µg/ml to 500 µg/ml concentration was used as a reference to plot a standard curve. The details are given in Chapter 3, Section 3.2.9.

7. 2.5 Purification of dextransucrase by PEG fractionation

Different molecular weights ice cold polyethylene glycols (PEG-200, PEG-400) were added to the 200 ml cell free extract to obtain the final concentrations (%, v/v) of 20, 25, 33, 40 and 50. A 60% PEG-1500 solution was prepared with distilled water and added to the cell free extract to get the final concentrations 10, 15, 20, 25 and 30 (%, w/v). They were incubated for 12h at 4°C to allow the dextransucrase fraction to precipitate. The mixture was centrifuged at 13,200g for 30 min at 4°C to separate the dextransucrase fraction. The pellet was dissolved in 20 mM sodium acetate buffer (pH 5.4). These fractions were subjected to dialysis using 5 kDa cut off membrane. The dextransucrase fractions were analyzed for enzyme activity and protein estimation.

7.2.6 Purification of dextransucrase by repeated steps of fractionation

To the purified dextransucrase by 33% PEG-200, 25% PEG-400 and 10% PEG-1500 fractions obtained after dialysis from the first step of fractionation. The PEG-200, PEG-400 and PEG-1500 were added to get the final concentrations of 33% PEG-200, 25% PEG-400 and 10% PEG-1500, respectively. The steps of precipitation, separation and dialysis of purified dextransucrase were performed as mentioned in earlier section. The same purification protocol was repeated 4 times. After each step of fractionation the fraction was assayed for protein concentration and enzyme activity. All the fractions were analyzed by on SDS-PAGE for protein and *in-situ* dextransucrase activity detection.

7.2.7 SDS-PAGE analysis of purified enzyme

SDS-polyacrylamide gel electrophoresis was performed with a vertical slab mini gel unit (BioRad) using 1.5 mm thick gels, following the method of Laemmli (1970). 7.5% (w/v) acrylamide for resolving gel and 4% (w/v) for stacking gel were used. The protein samples were prepared in 0.0625 M Tris-HCl buffer (pH 6.8) containing 2.3% (w/v) sodium dodecyl sulfate, 10% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol and 0.05% (w/v) bromophenol blue. The samples from all the purified fractions with different molecular weights were loaded on 7.5% acrylamide gel under denaturing conditions. Electrophoresis was carried out with a current of 2 mA per lane. The protein bands were fixed with solution containing acetic acid (5%, v/v) for 5 min, then stained for 30 min with 0.25% (w/v) Coomassie brilliant blue, and destained by repeated washing using a solution containing 20% methanol and 10% (v/v) acetic acid. Molecular mass marker proteins (Myosin from Rabbit Muscle, 205000; Phosphorylase b 97400; Bovine serum albumin, 66000; Ovalbumin, 43000;

Carbonic anhydrase, 29000 Da) purchased from Genei, India, were used as standard for SDS-PAGE.

7.2.8 Identification of dextransucrase by activity staining using Periodic Acid Schiff's (PAS) protocol

In-situ activity of dextransucrase was detected on a 7.5% acrylamide gels run under SDS non denaturing conditions using the protocol described by Holt *et al.* (2001) with certain modifications. The 7.5% acrylamide resolving gel, running buffer and the sample buffer contained SDS (the cationic detergent for movement of samples under charged conditions). For running the enzyme samples under non denaturing conditions the sample buffer (5x) used did not contain β-mercaptoethanol and the enzyme sample mixed with sample buffer was not subjected to heat denaturation. The samples for loading on to the gel were prepared by mixing 1 part of 5x sample buffer to 4 parts of the enzyme sample and allowing the mixture for 2h at 37°C for allowing the samples to get the charge by SDS. The samples from all fractions were loaded on two identical, 7.5% acrylamide gels (without the stacking gel) and were under SDS-non denaturing condition. After the run, SDS was removed by incubating the gel in sodium acetate buffer (20 mM sodium acetate, pH 5·4, 0·3 mM $CaCl_2$ and 0·1% Tween 80) at 4°C 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 5% sucrose for 48h at 30°C. 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) for 45 min at room temperature. After the periodic acid treatment, the gel was washed with water for 2h with several changes. The gel was then stained with 15 ml Schiff reagent (0.5% w/v Fuchsin basic, 1% sodium bisulphite and 0·1N HCl) until the discrete magenta

bands within the gel matrix appeared, which confirmed dextransucrase activity. The other gel was incubated in 5% raffinose, which is a specific substrate for fructosyltransferases (inulansucrase and levansucrase), in order to confirm dextransucrase and exclude the presence of fructosyltransferases.

7.2.9 Identificaction, location and confirmation of dextransucrase by CBB and PAS staining

Three 7.5% SDS-polyacrylamide gels were prepared only with only the resolving gel (without stacking). One gel was loaded with the samples prepared under denaturing conditions (with β-mercaptoethanol and heat treatment) and stained with Coomassie Brilliant Blue (CBB) R-250 (Fig. 7.11A). The other two gels were loaded with samples prepared under SDS-non denaturing conditions (without β-mercaptoethanol and heat treatment), and one gel was stained with Coomassie Brilliant Blue R-250 (Fig. 7.11B) and the other gel was used for *in-situ* activity detection by Periodic acid Schiff (PAS) staining protocol (Fig. 7.11C) as described in Section 7.2.8.

7.2.10 Purification of dextransucrase by gel-filtration chromatography

The purified dextransucrase after the first step of fractionation by 10% PEG-1500 was dialyzed extensively against 20 mM sodium acetate buffer (pH 5.4). 2.2 ml of the dialyzed enzyme (0.58 mg/ml, with specific activity of 23 U/mg) was applied to a glass column (1.5 cm x 30 cm) containing Sephacryl S-200HR with a bed volume of 40 ml previously equilibrated with 20 mM sodium acetate buffer (pH 5.4) containing 0.02% sodium azide. The column was run on chromatography system with UV detector and fraction collector (Biologic LP, BioRad Labs. India). The enzyme was eluted at a flow rate of 0.2 ml/min using the same buffer and 2 ml fractions were

collected and analyzed for enzyme activity and protein concentration. Fractions containing the highest specific activity were pooled. The pooled enzyme fraction was used for the estimation of enzyme activity and protein concentration and lyophilized and analyzed by SDS-PAGE as described earlier.

7.3 Results and Discussion

Dextransucrase have been reported to fractionate from the cell free extracts by polyethylene glycols of different molecular weights. In the present study the PEGs of different molecular weights were screened to purify dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. The dextransucrase purified by different PEGs was also analyzed for purity and confirmed by staining the dextran produced by it.

7.3.1 Fractionation by PEG-200

Dextransucrase purification by fractionation with PEG-200 was carried out using the cell free extract with a specific activity of 0.6 U/mg. Various concentrations of PEG-200 was added to obtain the final concentrations ranging from 20-50% (v/v). The fractionation resulted in a dextransucrase with specific activity of 5.1 U/mg at 33% (v/v) PEG-200 final concentration (Fig. 7.1). The purification by other fractions on either side of 33% final concentration resulted in a purified dextransucrase with lower specific activities (Fig. 7.1). A maximum of 9 fold purification and 3.2 % over all yield was obtained with 33% PEG-200 final concentration (Table 7.1). SDS-Polyacryalmaide gel analysis of all the purified fractions showed a prominent band at molecular weight equivalent to 180 kDa size with all PEG-200 fractions (Fig. 7.2). The presence of dextransucrase of 180 kDa molecular size band was further confirmed by detection of *in-situ* dextransucrase activity in the gels as described in Section 7.3.6.

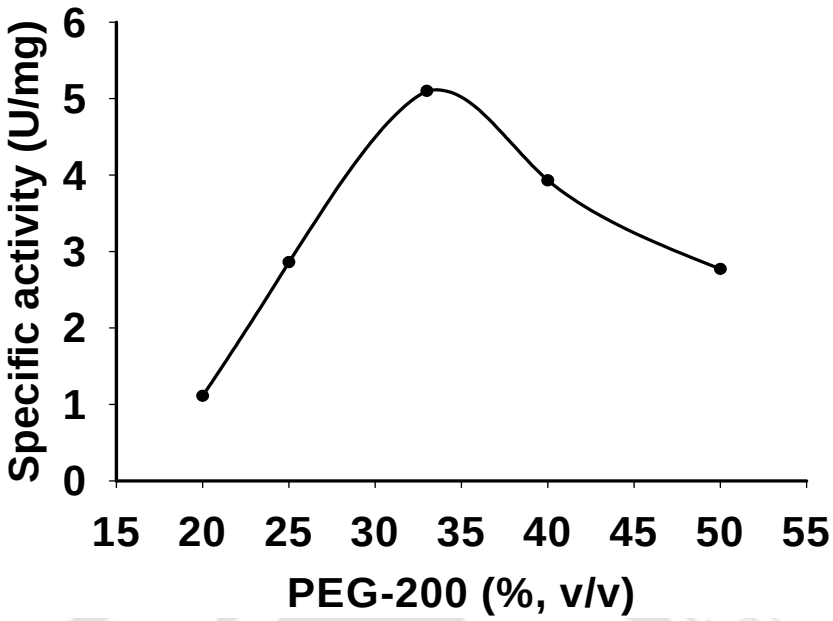

Fig. 7.1. Fractionation of dextransucrase with PEG-200. Specific activity of dextransucrase obtained were plotted with various fractions (%, v/v).

Table 7. 1. Purification of dextransucrase by fractionation with PEG-200

PEG-200	Volume	Dextransucrase			Protein	Total	Specific activity	Fold Purification
(%)	(ml)	Activity (U/ml)	Total Units	Overall % Yield	(mg/ml)	(mg)	(U/mg)	
Crude	200	4.2	848	–	7.4	1474	0.6	–
20	1.8	0.1	0.3	0.03	0.127	0.2	1.1	1.9
25	2.0	1.6	3.2	0.38	0.56	1.1	2.9	5.0
33	8.6	3.1	26.7	3.14	0.61	5.2	5.1	8.9
40	9.8	2.5	24.5	2.89	0.64	6.2	3.9	6.8
50	10.4	2.4	25.0	2.94	0.87	9.0	2.8	4.8

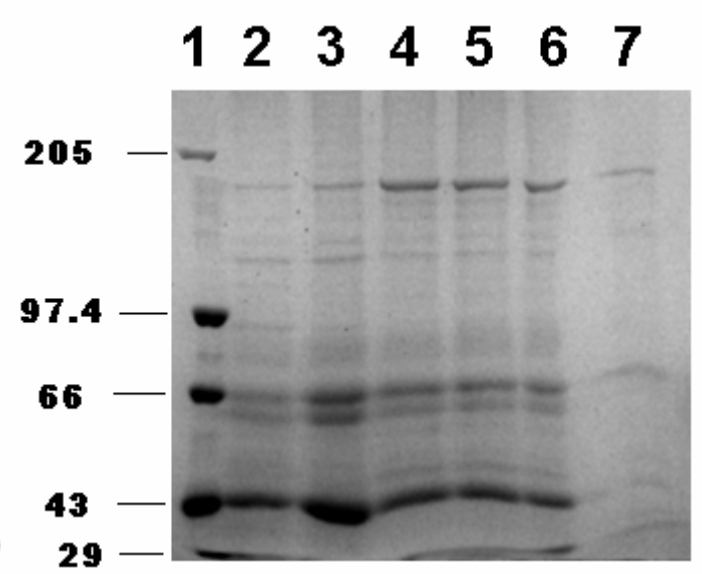

Fig.7.2. SDS-polyacrylamide gel electrophoresis of dextransucrase fractions obtained after PEG-200 fractionation. Lanes: 1-Protein molecular weight marker: 29-205 kDa; 2-20%; 3-25%; 4-33%; 5-40% and 6-50% (v/v) PEG-200 fractions; 7-Cell free extract.

7.3.2 Fractionation by PEG-400

Dextransucrase purification by fractionation using PEG-400 ranging from 20 to 50% (v/v) was carried out from the cell free extract with a specific activity of 0.6 U/mg. The fractionation by 25% (v/v) PEG-400 gave the maximum specific activity of 9.2 U/mg (Fig. 7.3) with 16 fold purification and 3.8% over all yield (Table 7.2). The purification by higher concentrations of PEG-400 did not favor the enzyme activity (Table 7. 2). The increase in the PEG-400 concentration beyond 25% resulted in decrease of specific activity of dextransucrase (Table 7. 2).

SDS-polyacrylamide gel electrophoretic analysis of all the purified dextransucrase fractions showed multiple bands. There was a prominent protein band of approximately 180 kDa molecular size obtained from 25% PEG-400 fraction (Fig. 7.4, lane 4). This was also confirmed by activity staining as described in the Section 7.3.6 for identification, confirmation and location of dextransucrase.

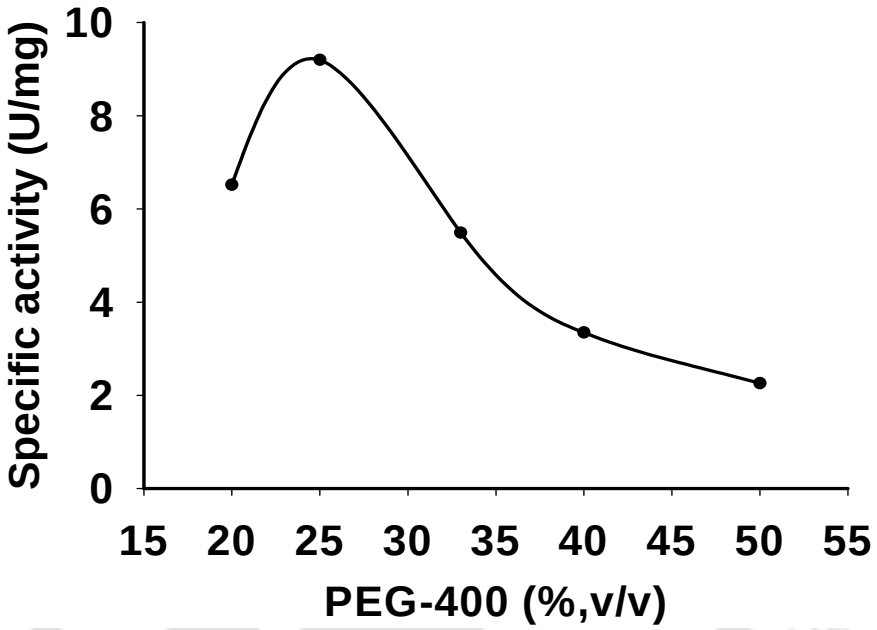

Fig.7.3. Fractionation of dextransucrase by PEG-400. The specific activity of dextransucrase obtained were plotted with various PEG-400 concentrations (%, v/v) dextransucrase fractions.

Table 7. 2. Purification of dextransucrase by PEG-400 fractionation

PEG-400 (%)	Volume (ml)	Dextransucrase			Protein (mg/ml)	Total (mg)	Specific activity (U/mg)	Fold Purification
		Activity (U/ml)	Total Units	Overall % Yield				
Crude	200	4.2	848	–	7.4	1474	0.6	–
20	5.3	3.8	20.0	2.4	0.58	3.1	6.5	11.3
25	5.8	5.5	32.1	3.8	0.60	3.5	9.2	16.0
33	7.8	3.4	26.1	3.1	0.61	4.8	5.5	9.5
40	10.8	2.2	23.5	2.8	0.65	7.0	3.4	5.8
50	15.9	1.7	27.3	3.2	0.76	12.1	2.3	3.9

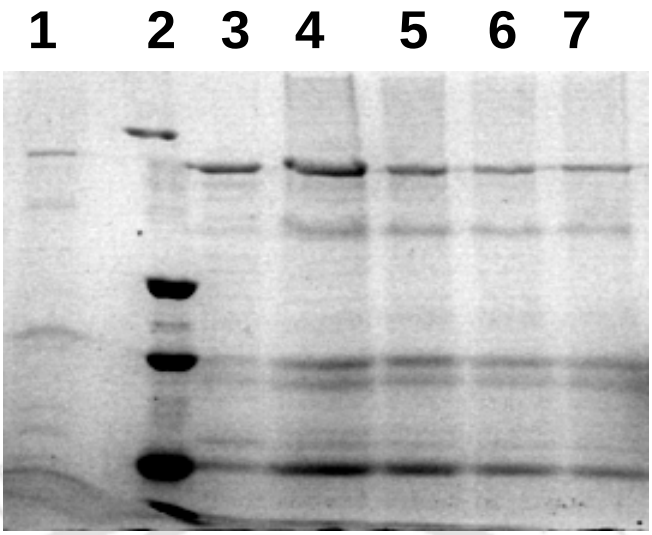

Fig.7.4. SDS-polyacrlamide gel of various dextransucrase fractions obtained after PEG-400 fractions. Lanes: 1-Cell free extrcat; 2-Protein molecular weight marker: 29-205 kDa; 3-20%; 4-25%; 5-33%; 6-40%; 7-50% (v/v) PEG-400 fractions.

7.3.3 Fractionation by PEG-1500

The dextransucrase purification was carried out by PEG-1500 fractionation with varying its concentrations between 10 to 30% (w/v) using cell free extract with a specific activity of 0.6 U/mg. A 10% (w/v) PEG-1500 fraction gave the dextransucrase with maximum specific activity of 23 U/mg (Fig. 7.5) with 40 fold purification (Table 7.3). With an increase in the concentration of PEG-1500 beyond 10%, a decrease in specific activity of enzyme (Fig. 7.5) was observed (Table 7.3). A prominent band of 180 kDa molecular size on SDS-PAGE gel was observed in all PEG-1500 fractions corresponding to dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 (Fig. 7.6).

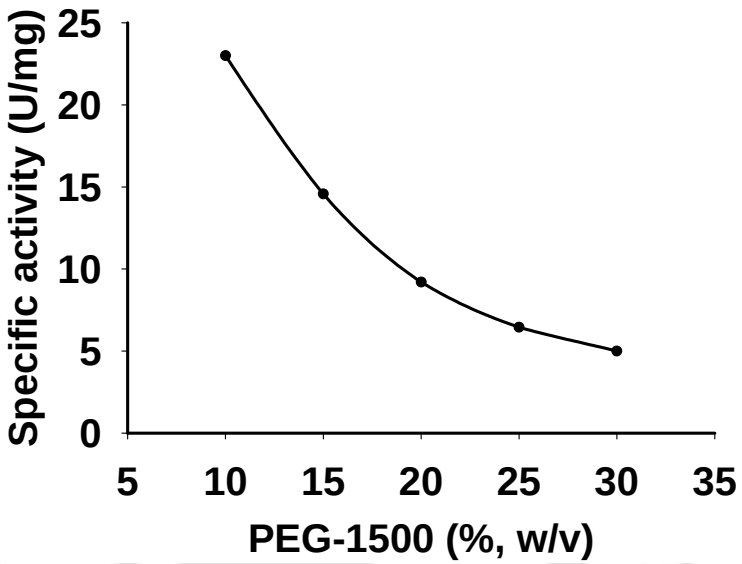

Fig. 7.5. Fractionation of dextransucrase by PEG-1500. Specific activity profiles of various fractions by PEG-1500 were plotted.

Table 7. 3. Purification of dextransucrase by PEG-1500 fractionation

PEG-1500 (%,w/v)	Volume (ml)	Dextransucrase			Protein (mg/ml)	Total (mg)	Specific activity (U/mg)	Fold Purification
		Activity (U/ml)	Total Units	Overall % Yield				
Crude	200	4.2	848	–	7.4	1474	0.6	–
10	2.2	13.3	28.8	3.4	0.58	1.3	23.0	40.0
15	3.5	10.2	35.7	4.2	0.70	2.5	14.6	25.3
20	4.7	8.3	38.9	4.6	0.90	4.2	9.2	16.0
25	3.5	7.1	24.6	2.9	1.10	3.8	6.5	11.2
30	4.8	6.5	30.9	3.6	1.30	6.2	5.0	8.7

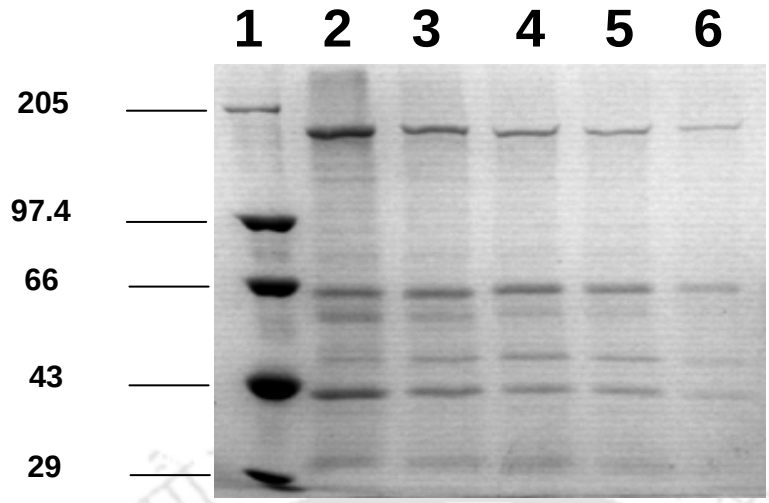

Fig. 7.6. SDS-polyacrylamide gel of purified dextransucrase fractions by PEG-1500. Lanes: 1-Protein molecular weight marker: 29-205 kDa; 2-10%; 3-15%; 4-20%; 5-25%; 6-30% (w/v) PEG-1500 fractions.

7.3.4 Comparison of dextransucrase fractionation by different PEGs

The culture broth obtained after 12h growth of *Leuconostoc mesenteroides* NRRL B-640 was centrifuged and the cells were discarded. The cell free extract containing exocellular dextransucrase was subjected to fractionation by PEG-200, 400 and 1500. Varied concentrations of three PEGs were used as described earlier. Fig. 7.7 shows the specific activity profiles of dextransucrase with corresponding concentrations of PEG-200, PEG-400 and PEG-1500. Fractionation with PEG-1500 gave dextransucrase with significantly higher specific activity than PEGs of low molecular weights (PEG-200 and PEG-400). The specific activity of dextransucrase was maximal at 10% and it decreased with increasing the concentration of PEG-1500 from 10% to 30% (Fig. 7.7), whereas, in the case of PEG-400 and PEG-200 the specific activity increased with increase in concentration to from initial 20% to 25% and 20% to 33%, respectively (Fig. 7.7). The maximum specific activity and the extent of purification achieved with each PEG are listed in Table 7.4. The maximum specific activity with a final 10% (w/v) PEG-1500 was 23 U/mg resulting in 40 fold

purification in a single step (Table 7.4). A concentration of 25% (v/v) PEG-400 gave the enzyme with 9.2 U/mg of protein with 16 fold purification and a concentration of 33% (v/v) PEG-200 gave dextransucrase with 5.1 U/mg of protein with 9 fold purification (Table 7.4). A higher percent of concentration of PEG was required for fractionating the dextransucrase of maximum specific activity with a molecular weight PEG. For example, a 33% concentration of PEG-200, 25% of PEG-400 and only a 10% PEG-1500 were required for maximum specific activity. Consequently, the specific activity also increased from 5.1 to 23 U/mg from 33% PEG-200 to 10% PEG-1500, respectively (Table 7.4).

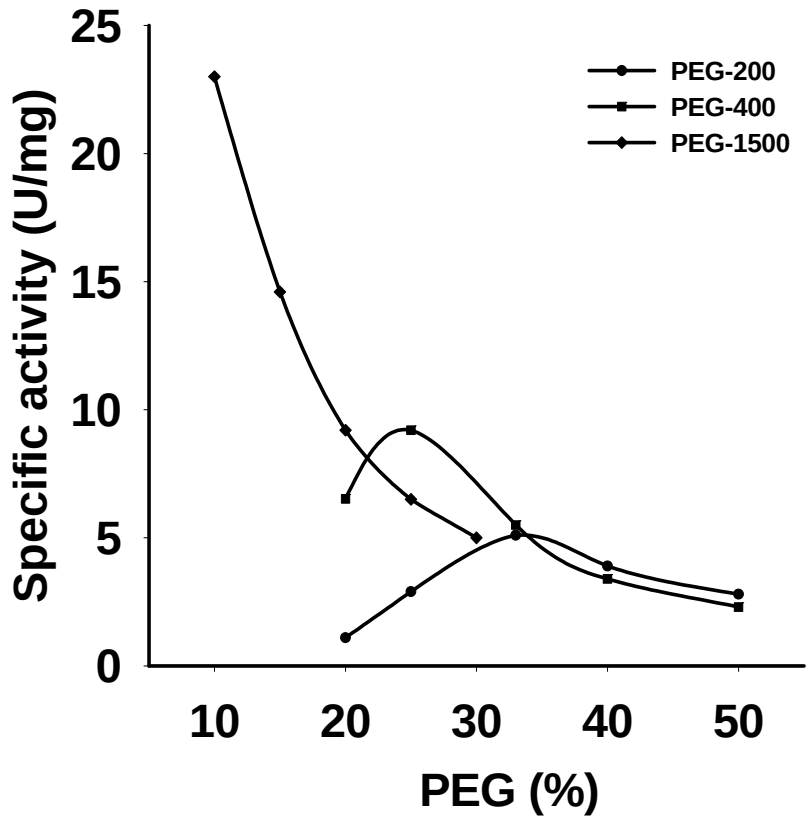

Fig. 7.7. Purification of dextransucrase from *Leuconostoc* me*senteroides* NRRL B-640 by PEG-200, 400 and 1500. The specific activity of purified dextransucrase by different final concentrations of PEGs were plotted.

Table 7. 4. Comparison of the dextransucrase purification by fractionation with different molecular weight polyethylene glycols.

PEG (%)	Vol. (ml)	Enzyme activity (U/ml)	Total Units	Overall % Yield	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Fold Purifi-cation
Crude	200	4.2	848	–	7.4	1474	0.6	–
33% PEG-200	8.6	3.1	26.7	3.1	0.61	5.2	5.1	8.9
25% PEG-400	5.8	5.5	32.1	3.8	0.60	3.5	9.2	16.0
10% PEG-1500	2.2	13.3	28.8	3.4	0.58	1.3	23.0	40.0

7.3.5 SDS-PAGE analysis of dextransucrase fractionation by PEGs

The dextransucrase fractions obtained with all the three PEG's (200, 400 and 1500) were also analysed by SDS-PAGE to check the purity of dextransucrase. The SDS-PAGE results showed the presence of multiple protein bands (Fig(s). 7.2, 7.4 and 7.6). Dextransucrase appears to exist in aggregated multiple forms of 45 kDa monomers. These results are similar to the results of Kobayashi and Mastuda (1986). However, a prominent band of 180 kDa size was observed in all the fractions of all the three PEGs used. The active form of the enzyme was confirmed by activity staining as described in the next section (Section 7.3.6).

7.3.6 Identification, confirmation and location of dextransucrase by activity staining

The dextransucrase fractions obtained from PEG-200, 400 and 1500 were run on SDS-PAGE gels under non denaturing conditions for *in-situ* activity detection (Fig. 7.8A, B and C). The white bands were observed on the gels incubated in sucrose after 48h. These white bands turned in to magenta color after PAS staining, which confirmed the presence of polysaccharide formed on polyacrylamide gels (Fig. 7.8A, B and C). The PAS staining of the sucrose incubated gels showed that the magenta colour activity bands are the native and active form of the purified dextransucrase

which corresponded to approximately 180 kDa molecular size that appeared on the denaturing gels stained with Coomassie Brilliant Blue (Fig(s). 7.2, 7.4 and 7.6). This comparison of activity staining and Coomassie Brilliant Blue staining gels identified the presence of enzyme dextransucrase.

Further confirmation of dextransucrase was done by using raffinose which is the substrate for fructosyltransferases (inulansucrase and levansucrase). When the SDS-PAGE gels run under non denaturing conditions were incubated with raffinose before PAS staining (i.e. the activity staining) no white band(s) appeared. However, the lanes showed only at the top, the native dextran associated with the fractionated dextransucrase samples from PEG-200 (Fig. 7.9A) and PEG-400 (Fig. 7.9B). This confirmed that the purified fractions contain only dextransucrase and not levansucrase or inulansucrase because raffinose is not a substrate for dextransucrase. The optimized concentrations of all the three PEGs giving highest dextransucrase specific activity as described in Section 7.3.4 were corroborated by the highest intensity bands obtained after activity staining (Fig. 7.8A, B and C lanes 4, 3, 2).

The dextransucrase was located and its molecular size was determined by comparing the three identical polyacrylamide gels (with out the stacking gel). One gel was loaded with the denatured (β-mercaptoethanol and heat treated) PEG-200 fractionated enzyme samples (Fig. 7.10A). The other two gels were loaded with PEG-200 fractionated enzymes samples prepared under non denaturing (without β-mercaptoethanol and without heat treatment) conditions (Fig. 7.10B and C). The sample preparation is given in detail in Section 7.2.9. All the PEG-200 purified dextransucrase fractions showed multiple bands on the SDS-PAGE gel ran under denaturing conditions (Fig. 7.10A). However, a prominent band of 180 kDa molecular size was observed with all the PEG-200 fractions.

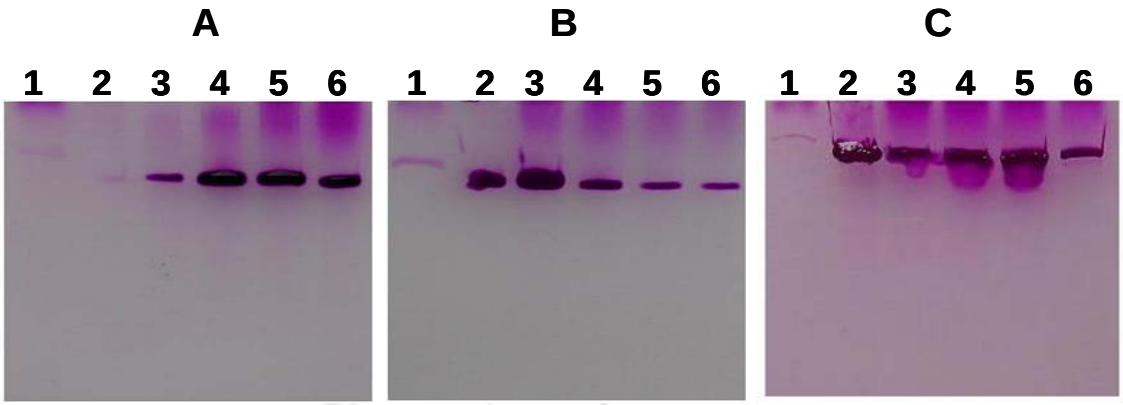

Fig. 7.8. Identification of purified dextransucrase by staining of dextran formed using sucrose. (**A)** PEG-200 (Lanes: 1-CFE, 2-20%, 3-25%, 4-33%, 5-40% and 6-50% PEG-200 fractions); (**B)** PEG-400 (Lanes: 1-CFE, 2-20%, 3-25%, 4-33%, 5-40% and 6-50% PEG-400 fractions); (**C)** PEG-1500 (Lanes: 1-CFE, 2-10%, 3-15%, 4-20%, 5-25% and 30% PEG-1500 fractions).

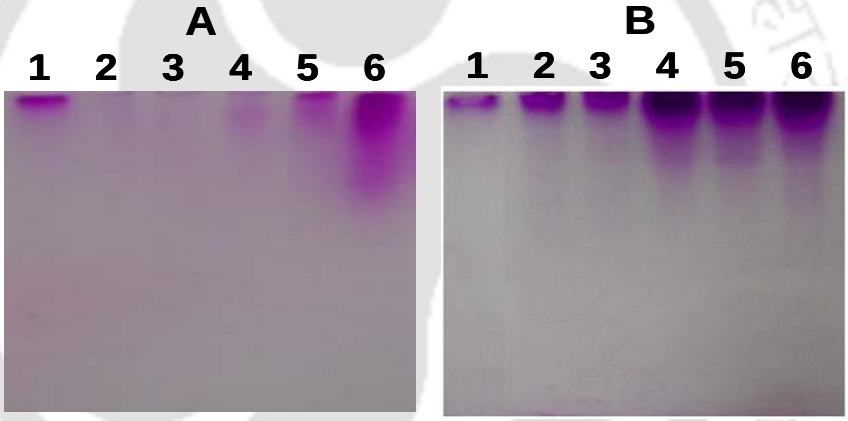

Fig.7.9. Confirmation of dextransucrase by staining method using raffinose as substrate. (**A)** PEG-200 (Lanes: 1-CFE, 2-20%, 3-25%, 4-33%, 5-40% and 6-50% PEG-200 fractions); (**B)** PEG-400 (Lanes: 1-CFE, 2-20%, 3-25%, 4-33%, 5-40% and 6-50% PEG-400 fractions).

The dextransucrase fractions obtained from lower concentrations of PEG-200 (20, 25 and 33%) gave a single band of approximate size 180 kDa on SDS-PAGE run under non denaturing conditions (Fig. 7.10B) corresponding to the activity bands which appeared on the activity stained gel (Fig. 7.10C). Multiple bands appeared with the dextransucrase fractions obtained from higher concentrations (40% and 50%) of PEG-200, indicating that the higher PEG-200 concentrations resulted in precipitating

other non-dextransucrase unwanted bulk proteins from the culture supernatant (Fig. 7.10B) which might be causing the decrease in recovered specific activities as described in Section 7.3.1.

The two gels, one with denatured samples (Fig. 7.10A) and the other with non denatured samples both stained with Coomassie Brilliant Blue R-250 (Fig. 7.10B) were compared with the third gel with nondenatured samples stained by Periodic acid Schiff's staining protocol (Fig. 7.10C). One of the prominent bands of the multiple molecular forms of enzyme that appeared on SDS-denaturing gel (Fig. 7.10A) corresponded to the single band of SDS-nondenaturing gel (Fig. 7.10B) and both these bands corresponded to the activity bands of native and active form of the purified dextransucrase (Fig. 10C), all having an approximate molecular size of 180 kDa.

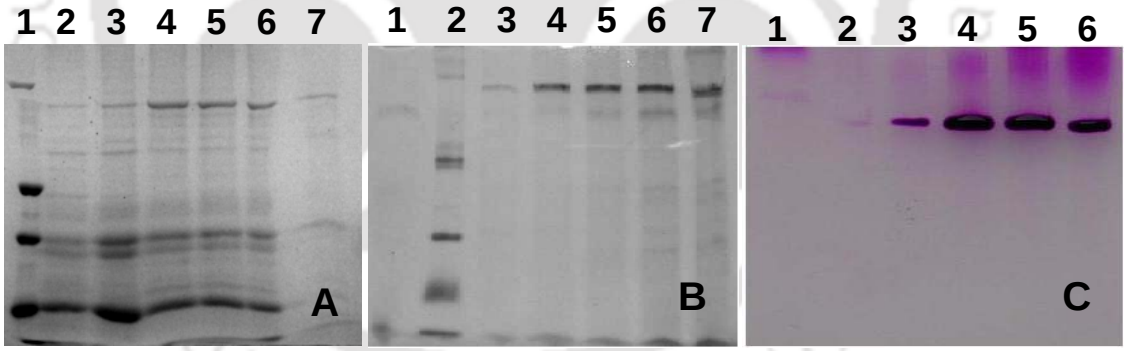

Fig. 7.10. Location of active and native form of dextransucrase and its molecular size. **(A)** CBB R-250 staining of dextransucrase fractions from PEG-200 obtained after denaturing SDS-polyacrylamide gel (Lanes: 1-Protein molecular weight marker: 29-205 kDa; 2-20%; 3-25%; 4-33%; 5-40%; 6-50%, v/v; 7-Cell free extract) and **(B)** CBB R-250 staining of various dextransucrase fractions from PEG-200 obtained after non-denaturing SDS-polyacrylamide gel (Lanes: 1-Cell free extract, 2-1-Protein molecular weight marker: 29-205 kDa; 3-20%, 4-25%, 5-33%, 6-40% and 7-50%, v/v). **(C)** PAS staining of dextransucrase fractions from PEG-200 obtained after non-denaturing SDS-polyacrylamide gel (Lanes:1-Cell free extract, 2-20%; 3-25%; 4-33%, 5-40% and 6-50%, v/v).

7.3.7 Dextransucrase purification by repeated steps of fractionation by PEGs

Four repeated steps of fractionation were carried out using the optimized concentrations of three PEG's of different molecular weights (200, 400 and 1500). The fractionation of the same crude dextransucrase (4.2 U/ml, 0.60 U/mg) by 33% PEG-200, gave specific activity of 12.7 U/mg protein with a 22 fold purification after the third repetitive step (Fig. 7.11), which was 2.5 times higher than the specific activity obtained after first step (Table 7.5). The fractionation using 25% PEG-400 resulted with maximum of dextransucrase activity of 17 U/mg after the third step which was twice higher than that of the first step (Fig. 7.12) and a maximum of 30 fold purification was observed (Table 7.6). Both polyethylene glycols 200 and 400 resulted in a sharp decrease in the specific activity after the third step which could be due to removal of dextan by PEG, dextran being the stabilizer for dextransucrase as reported earlier (Goyal and Katiyar 1994).

In the case of PEG-1500 the four steps of fractions were performed using three concentrations *viz.* 10%, 15% and 20% (w/v) (Fig. 7.13). Dextransucrase purification by fractionation in four successive steps using three concentrations of PEG-1500 gave a maximum enzyme activity after the second step and decreased after the second step (Fig. 7.13). A maximum of 47, 30 and 25 fold purification was obtained from 10%, 15% and 20% PEG-1500 fractions at their maximum specific activity steps (Table 7.7). 15% PEG-1500 and 20% PEG-1500 did not result higher specific activity of dextransucrase after the first step, rather lower specific activities of 17 and 14 U/mg were obtained as compared to 27 U/mg obtained after the first step of purification by 10% PEG-1500 (Table 7.7). The fractionation by 10% PEG-1500 showed an increase in the specific activity of dextransucrase from 23 U/mg to 27 U/mg giving 47 fold purification after the second step although the increase was not significant and

showed only a 17% increase (Table 7.7, Fig. 7.13). The results suggested that the fractionation by 10% PEG-1500 (w/v) can be the most appropriate method for purification of dextransucrase that yields a specific activity of 23 U/mg in a single step.

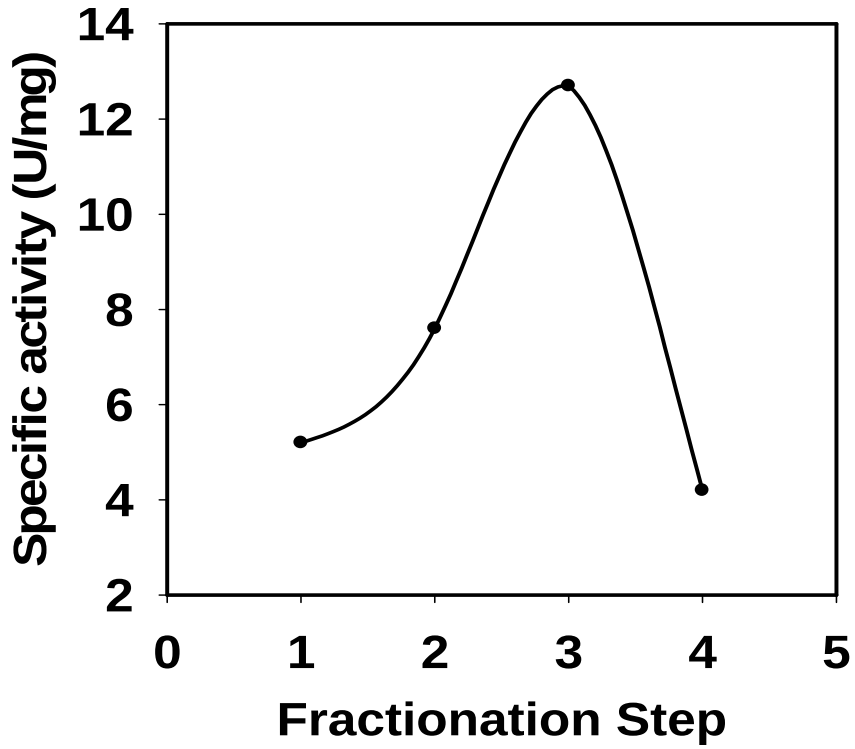

Fig. 7.11. Fractionation of dextransucrase by PEG-200 in four successive steps. Specific activity profile of dextransucrase fractions after each step were plotted.

Table 7. 5. Fractionation of dextransucrase in successive steps by 33% PEG-200

Steps PEG-200	Volume (ml)	Dextransucrase			Protein (mg/ml)	Total mg	Specific activity (U/mg)	Fold Purification
33%		Activity (U/ml)	Total Units	Overall % Yield				
Crude	200	4.2	848	–	7.4	1474	0.6	–
1	11	2.8	31.1	3.7	0.55	6.0	5.2	9.0
2	11	2.0	22.4	2.6	0.27	3.0	7.6	13.1
3	11	2.4	26.7	3.2	0.19	2.1	12.7	22.0
4	11	0.5	5.6	0.7	0.12	1.3	4.2	7.4

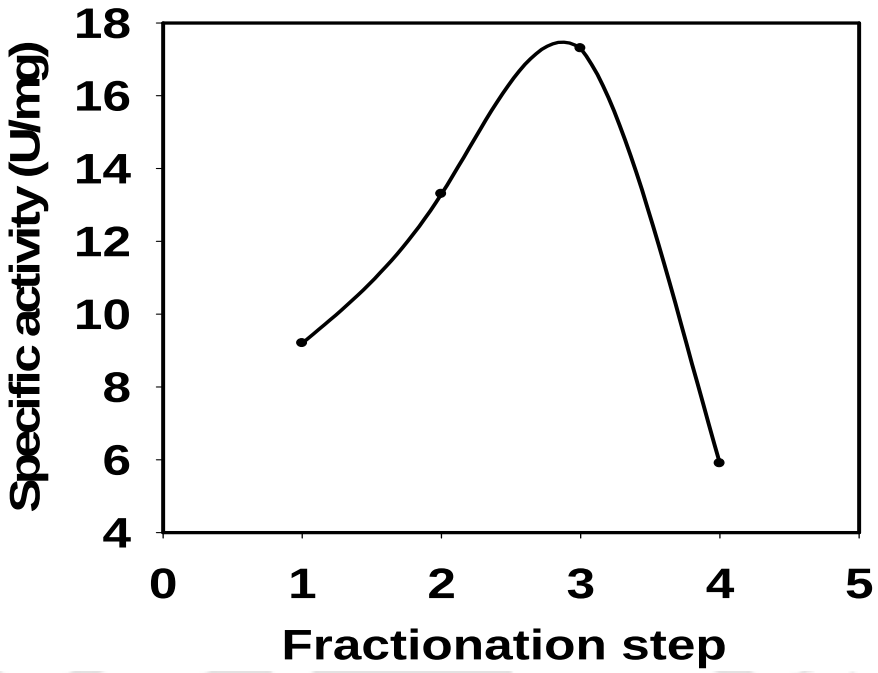

Fig. 7.12. Fractionation of dextransucrase by PEG-400 in four successive steps. Specific activity of dextransucrase fraction after each step were plotted.

Table 7. 6. Fractionation of dextransucrase in successive steps by 25% PEG-400

Steps PEG-400	Volume (ml)	Dextransucrase			Protein (mg/ml)	Total mg	Specific activity (U/mg)	Fold Purification
25%		Activity (U/ml)	Total Units	Overall % Yield				
Crude	200	4.2	848.0	–	7.4	1474.0	0.6	–
1	11	4.9	53.8	6.3	0.53	5.8	9.2	16.0
2	11	3.8	42.0	5.0	0.29	3.2	13.3	23.1
3	11	3.6	39.8	4.7	0.21	2.3	17.3	30.1
4	11	0.7	7.4	0.9	0.11	1.2	5.9	10.3

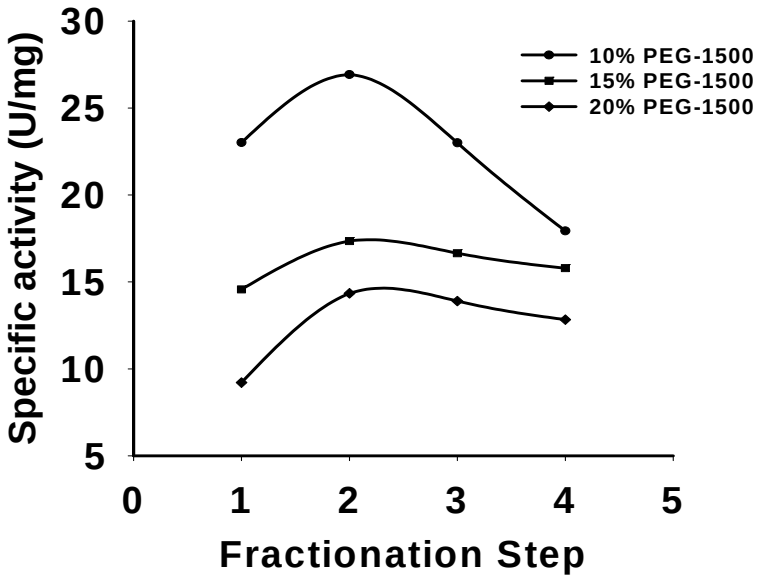

Fig. 7.13. Fractination of dextransucrase in four successive steps using 10%, 15% and 20% (w/v) PEG-1500 fractions. Specific activities obtained after each successive step of all concentrations were plotted.

Table 7. 7. Fractionation of dextransucrase in successive steps by PEG-1500

Steps PEG-1500 (%)	Vol. (ml)	Enzyme activity (U/ml)	Total Units	Overall % Yield	Protein (mg/ml)	Total mg	Specific activity (U/mg)	Fold Purification
Crude	200	4.2	848	-	7.4	1474	0.6	-
10% PEG-1500								
1	11	4.4	48.4	5.7	0.191	2.10	23.0	40.0
2	11	3.8	41.5	4.9	0.140	1.54	26.9	46.8
3	11	3.2	34.8	4.1	0.137	1.51	23.0	40.0
4	11	1.8	19.8	2.3	0.100	1.10	17.9	31.2
15% PEG-1500								
1	11	6.3	69.0	8.1	0.430	4.73	14.6	25.3
2	11	5.8	64.1	7.6	0.336	3.70	17.3	30.2
3	11	4.4	48.4	5.7	0.264	2.91	16.6	28.9
4	11	3.9	42.9	5.1	0.247	2.72	15.8	27.5
20% PEG-1500								
1	11	6.3	69.1	8.1	0.682	7.50	9.2	16.0
2	11	6.0	66.0	7.8	0.418	4.60	14.3	24.9
3	11	4.6	50.6	6.0	0.331	3.64	13.9	24.2
4	11	4.0	44.0	5.2	0.312	3.43	12.8	22.3

In order to check if in all the successive steps using three PEGs (200, 400 and 1500) are giving only dextransucrase and not any other protein, the fractions obtained after each step of purification were run on SDS-PAGE for CBB staining for checking purity as well as on non denaturing-PAGE for activity staining using PAS. The purified fractions from all steps of PEG-200 and PEG-400 (Fig. 7.14A) and PEG-1500 (Fig. 7.15A) showed multiple bands of aggregated molecular forms of enzyme on CBB staining. However, the fractions of all steps of purified fractions from PEG-200 (Fig. 7.14B), 400 (Fig. 7.14B) and PEG-1500 (Fig. 7.15B) showed single band upon activity staining. These results once again confirmed the presence of purified and active form of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 and its molecular size of 180 kDa.

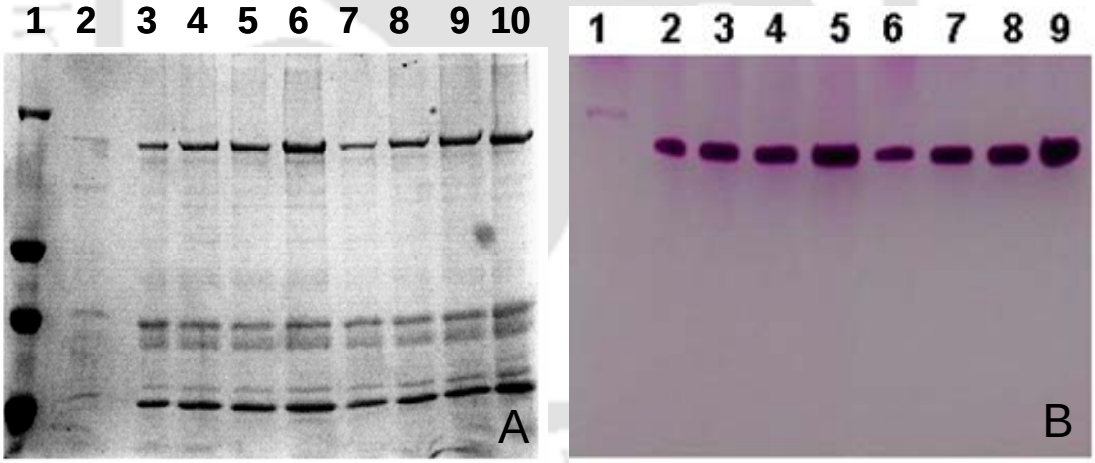

Fig. 7.14. SDS-PAGE and PAS analysis of purified dextransucase in successive steps. (**A)** CBB staining of SDS-PAGE of PEG-200 and PEG 400 fractions (Lanes: 1-Protein molecular weight marker (29-205 kDa), 2,-CFE, 3-Step1; 4-Step2; 5-Step3; 6-Step 4 from 33% PEG 200 and lanes: 7-Step1; 8-Step2; 9-Step3, 10-Step4 from 25% PEG 400 using 4 successive steps of purification); (**B)** PAS staining of non-denaturing PAGE of PEG-200 and PEG 400 fractions (Lanes: 1-CFE, 2-Step1; 3-Step2; 4-Step3; 5-Step 4 from 33% PEG 200 and lanes: 6-Step1; 7-Step2; 8-Step3, 9-Step4 from 25% PEG 400 using 4 successive steps of purification).

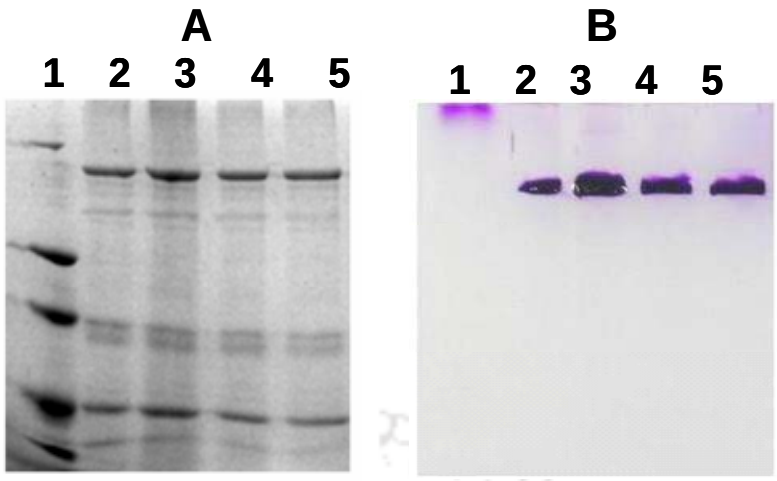

Fig. 7.15. Identification and confirmation of purified dextransucrase in successive steps by staining the identical gels by **(A)** CBB (SDS-denaturing gel) and **(B)** PAS (dextran formed on non-denaturing gel incubated in sucrose). (**A)** PEG-1500 (Lanes: 1-Protein molecular weight marker (29-205 kDa); 2-Step1; 3-Step2; 4-Step3; 5-Step 4 from 10% PEG-1500 using 4 successive steps of purification); (**B)** PEG-1500 (Lanes: 1-CFE, 2-Step1; 3-Step2; 4-Step3; 5-Step 4 from 10% PEG-1500 using 4 successive steps of purification).

7.3.8 Purification of dextransucrase by gel-filtration chromatography

The dextransucrase purified by 10% PEG-1500 in single step with a specific activity of 23 U/mg was subjected to Sephacryl S-200HR gel-filtration chromatography. The Fig. 7.16A shows the enzyme activity and protein (absorbance, A_{280}) profiles of dextransucrase from the eluted 2 ml fractions. The fractions were analysed for specific activity and purity by SDS-PAGE (Fig. 7.16B). The three enzyme fractions obtained by elution from gel-filtration chromatography of 10% PEG-1500 fractionated enzyme showed multiple bands of different molecular forms of dextransucrase enzyme on SDS-PAGE analysis as shown earlier dextransucrase purification by fractionation (Fig. 7.16B). The fractions containing the highest

specific activity were pooled. The pooled enzyme fraction was used for the estimation of enzyme activity and protein concentration. A specific activity of 35 U/mg with 61 fold purification was obtained by gel-filtration chromatography of 10% PEG-1500 purified enzyme (Table 7.8). From the results of the purification of enzyme by successive steps of 10% PEG-1500 and the gel-filtration chromatography, it is clear that the single step fractionation of dextransucrase by 10% PEG-1500 followed by gel-filtration chromatography resulted in much higher specific activity of enzyme and a higher fold of purification.

Table 7.8. Purification of dextransucrase by PEG-fractionation and gel-filtration chromatography

PEG (%)	Vol. (ml)	Enzyme activity (U/ml)	Total Units	Over-all % Yield	Protein (mg/ml)	Total protein (mg)	Specific activity	Fold Purifi Cation
Crude	200	4.24	848	–	7.37	1474	0.58	–
Fractionation by PEG of different molecular weights								
33% PEG-200	8.6	3.1	26.7	3.1	0.61	5.2	5.1	8.9
25% PEG-400	5.82	5.5	32.1	3.8	0.60	3.5	9.2	16.0
10% PEG-1500	2.16	13.3	28.8	3.4	0.58	1.3	23.0	40.0
Fractionation by PEG-1500 in repeated steps								
Step 1	11	4.40	48.4	5.7	0.191	2.10	23.0	40.0
Step 2	11	3.77	41.5	4.9	0.140	1.54	26.9	46.8
Step 3	11	3.16	34.8	4.1	0.137	1.51	23.0	40.0
Step 4	11	1.80	19.8	2.3	0.100	1.10	17.9	31.2
Gel-Filtration Chromatography of 10% PEG-1500 enzyme fraction								
Sephacryl S-200HR	12	0.88	10.6	1.25	0.025	0.3	35.3	60.9

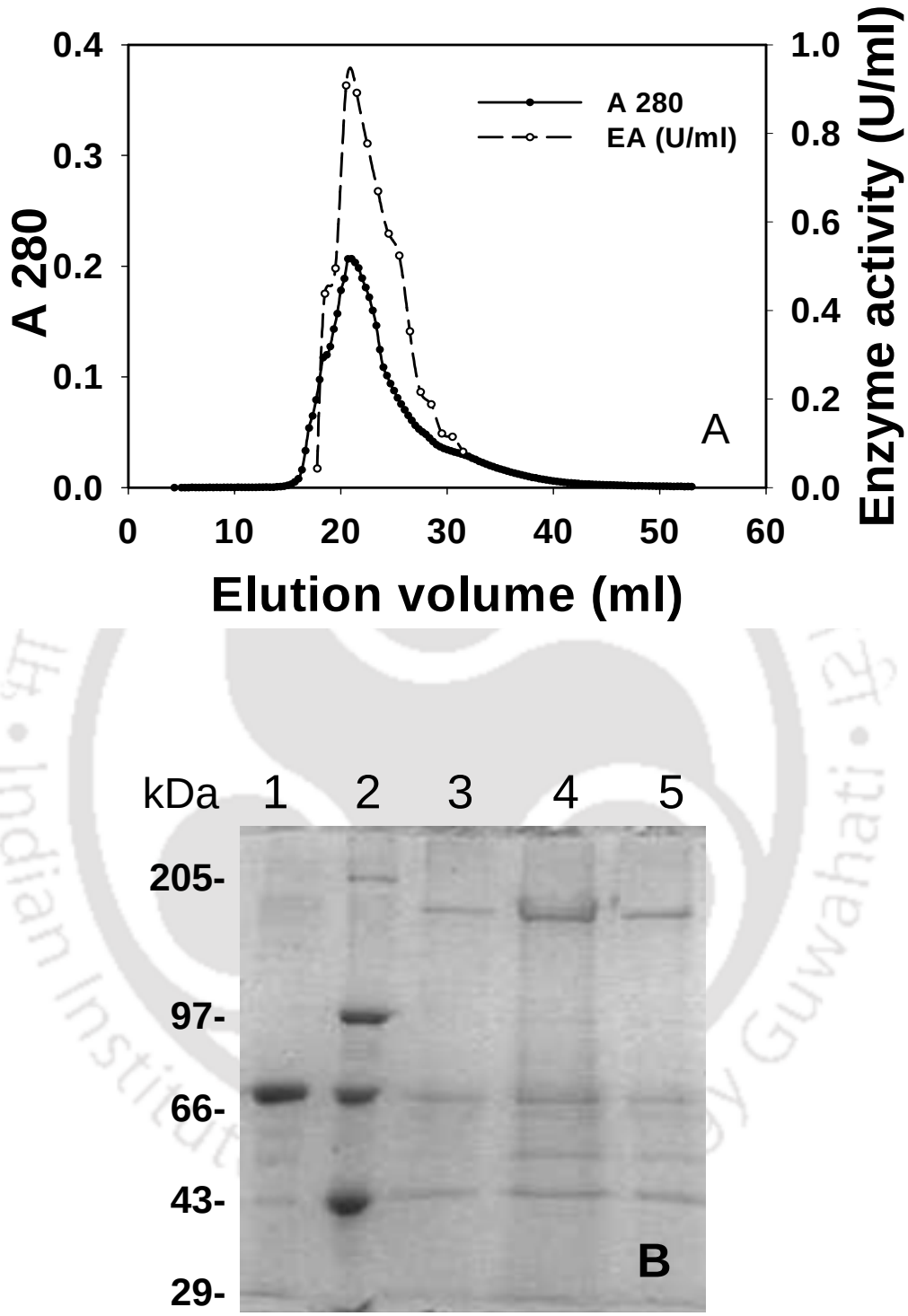

Fig. 7.16. (A) Elution pattern of dextransucrase from Sephacryl S-200 HR column (•) A_{280}; (◦) enzyme activity (U/ml). **(B)** SDS-PAGE analysis of fractions from the column (Lanes: 1, BSA; 2, Protein molecular weight marker (29-205 kDa); 3, 4 & 5 fractions) showing highest specific activity of dextransucrase.

7.4 Conclusions

The higher molecular weight polyethylene glycols favored the effective purification of dextransucrase produced by *Leuconostoc mesenteroides* NRRL B-640 from the cell free extract. Of the three PEGs (PEG-200, 400 and 1500), PEG-1500 gave the best results for purification of dextransucrase. A final concentration of 10% PEG-1500 was the optimum concentration for the purification of dextransucrase resulting in maximum specific activity of 23 U/mg in a single step. The results showed that as the molecular weight of PEG increased the specific activity of the fractionated dextransucrase also increased, and moreover a lower concentration of higher molecular weight PEG was required to achieve the maximum specific activity. The purified dextransucrase existed in multiple molecular forms as shown by SDS-PAGE, which is similar to those from other strains as reported earlier by several workers.

The enzyme was confirmed for dextransucrase by activity staining bands of the dextran produced, on the non denaturing PAGE by the purified fractions when incubated in sucrose. To rule out the presence of inulansucrase or levansucrase the purified enzyme on the gels was incubated with raffinose which is a substrate for these enzymes. The gels did not show any activity bands upon staining. This confirmed the presence of only dextransucrase (Fig. 7.9).

One of the prominent bands of the multiple molecular forms of enzyme that appeared on SDS-denaturing gel (Fig. 7.10A) corresponded to the single band of SDS-nondenaturing gel (Fig. 7.10B) and both these bands corresponded to the activity bands of native and active form of the purified dextransucrase (Fig. 10C), all having an approximate molecular size of 180 kDa.

The repeated steps of dextransucrase fractionation using 25% PEG-400 and 33% PEG-200 resulted with maximum of dextransucrase activity of 17 U/mg and 12.7 U/mg, respectively after the third step. The repeated steps of dextransucrase fractionation by 10% PEG-1500 showed an increase in the specific activity of dextransucrase from 23 U/mg to 27 U/mg after the second step, which was only 17% higher than the single step fractionation. The purification of dextransucrase by gel-filtration chromatography on Sephacryl S-200HR column using the single step purified enzyme by 10% PEG-1500 resulted in specific activity of 35 U/mg with 61 fold purification.

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Chapter 8

Characterization of Purified Dextransucrase and Dextran

8.1 Introduction

The stability of enzymes is a critical issue in the biotech industry. Enzymes are highly sensitive to the change of the environmental conditions such as temperature, pH and ionic strength. Both operational and storage stabilities of enzyme affect the enzyme based processes. Stabilizing an enzyme means suppressing the unfolding of the protein and retaining the catalytic activity (Kuhlmeyer and Klein 2003). Stabilization of the enzymes could be achieved by several ways; modification of the protein structure by protein engineering, chemical modification and immobilization (Costa *et al.* 2002). The stabilization can be achieved by the addition of additives like substrates, products, inhibitors, cofactors, metal ions, proteins, sugars and natural or synthetic polymers (Bryjak and Noworyta 1994; Costa *et al.* 2002; Yoon and Robyt 2003; Fagain 2003; Joo *et al.* 2005). The enzymes that can withstand extreme conditions can be isolated from extremophiles. The use of enzymes for industrial purposes usually depends on their stability during isolation, purification and storage (Joo *et al.* 2005). Stabilization by additives is the widely used procedure for various industrial enzymes (Kobayashi and Matsuda 1976; Robyt and Walseth 1979; Kobayashi and Matsuda 1980; Goyal *et al.* 1995; Fagain 2003; Yang *et al.* 2007).

Dextrasucrase is glucosyltransferase which synthesizes dextran, a glucopolysaccharide from sucrose. Dextransucrase is generally associated with the dextran. The removal of associated dextran from dextransucrase resulted in the loss of enzyme activity (Robyt and Walseth 1979). The dilution of the purified dextransucrase also resulted in the loss of activity (Miller and Robyt 1984). In general, dextransucrases are highly sensitive to pH, temperature and the dilution (Miller and Robyt 1984; Goyal *et al.* 1995). Certain metal ions and additives have been reported to stabilize dextransucrase from some strains *Leuconostoc mesenteroides* (Kobayashi and Matsuda 1980; Goyal *et al.* 1995). Among the metals ions; Ca^{2+}, Mg^{2+}, Co^{2+} and among stabilizers, the most widely used are Triton X-100, glycerol, Tween-80, polyethylene glycols, dextran, glutaraldehyde and polyvinyl alcohols on various enzyme systems (Kobayashi and Matsuda 1980, Miller and Robyt 1984; Goyal *et al.* 1995). The dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 has been purified to homogeneity using PEG fractionation method (Purama and Goyal 2007a) and its assay conditions have been optimized (Purama and Goyal 2007b). However, there is no report on biochemical characterization and stabilization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. In the present study the effects of certain metal ions (Ca^{2+}, Mg^{2+} and Co^{2+}), stabilizers (glycerol, PEG-8000, dextran, Tween-80 and glutaraldehyde) and different storage temperatures were investigated on dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. In the present chapter an effective method of stabilization of dextransucrase using Tween 80 has been described.

Leuconostoc mesenteroides NRRL B-640 is shown to produce dextransucrase that gives highly linear and soluble dextran (Seymour 1979a; Uzochukwu 2001). Dextran, is a class of homopolysaccharides composed exclusively of monomeric α-D-

glucose units linked mainly by α(1→6) bonds and a variable amount of branched α(1→2), α(1→3) or α(1→4) linkages. The dextran synthesized by *Leuconostoc mesenteroides* NRRL B-512F strain is a homopolysaccaharide containing 95% α(1→6) glucosidic linkages in the main linear chain and remaining 5% of α(1→4), α(1→3) and a very few α(1→2) branched linkages. The branches are mostly 1-2 glucose units long. The solubility of dextran occurs as a function of linkage pattern. The importance and applications of soluble dextran synthesized by *Leuconostoc mesenteroides* has been described in Chapter 1, Section 1.7.2. Sidebotham (1974) reported that various dextrans have ^{1}H-NMR spectral resonances (C-2, C-3, C-4, C-5 and C-6) in the 3-4 ppm region and the hemiacetal C-1 resonance in 4-6 ppm region. Seymour (1979b) showed that the ^{1}H-NMR spectral region for anomeric carbon of dextran from *Leuconostoc mesenteroides* NRRL B-1355 contained a resonance at 4.95 ppm and the branched linkages contained the resonance peak at 5.3 ppm.

Seymour *et al.* (1979a) employed ^{13}C-NMR spectroscopy to examine the structure of a series of dextrans and established that linear dextran has six prominent resonances. The ^{13}C-NMR spectra displayed two major regions; (a) the 95-105 ppm region, which is the anomeric region and (b) the 75-85 ppm region for dextrans branched at C-2, C-3, or C-4. ^{13}C-NMR resonances with in the 70-75 ppm region are associated with free positions at C-2, C-3 and C-4 residues. The FT-IR spectral data was useful in determining the nature of dextrans in terms of their monomeric units and their linkages (Shingel 2002). Several other repots also supported the use of FT-IR spectral data for the chatacterization of glucans showing anti-cancer properties (Cao *et al.* 2006; Liu *et al.* 2007). In the present study the dextran was synthesized using dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 and the structure was determined by FT-IR, ^{1}H-NMR and ^{13}C-NMR techniques.

8.2 Materials and Methods

8.2.1 Enzyme preparation

The enzyme was purified by a single step fractionation method by 10% PEG-1500 as described earlier (Purama and Goyal 2007a). The purification protocol is described in Chapter 7, Section 7.3.3.

8.2.2 Enzyme assay

The assay of dextransucrase was carried out in 1.0 ml of a reaction mixture in 20 mM sodium acetate buffer, pH 5.4, containing 146 mM (5%) sucrose and dextransucrase (0.55 mg/ml, 23 U/mg) (Purama and Goyal 2007b). The reaction mixture was incubated at 30°C for 15 min. Aliquots (0.2 ml), from the reaction mixture were analyzed for reducing sugar concentration. The assay procedure is described in detail in Chapter 3, Section 3.2.7.

8.2.3 Effect of salts and denaturing agents on dextransucrase activity

The effects of $CaCl_2$, $MgCl_2$, $CoCl_2$ and $MnSO_4$ between 0-20 mM and EDTA between 0-6 mM concentrations were studied on dextransucrase activity. The assays were carried out in 1.0 ml reaction mixture containing the salt, enzyme and the substrate and the activity was determined as described above (Section 8.2.2). The effect of urea was studied by incubating enzyme with urea (0-5 M final concentration) at 30°C for 30 min. Aliquots (10-20 µl) were taken and assayed for residual enzyme activity.

8.2.4 Thermal stability of dextransucrase

For determining the thermostability, the enzyme was incubated at different temperatures (10-50°C) for 10 min and the aliquots (10-20 µl) were assayed for residual enzyme activity in 1 ml reaction mixture containing 5% sucrose in 20 mM sodium acetate buffer, pH 5.4 by incubating at 30°C following the method as described above (Section 8.2.2).

8.2.5 Effect of storage temperature and additives on stability of dextansucrase

Storage stability was studied by incubating the dextransucrase at different temperatures (30°C, 4°C and –20°C). The samples at different time intervals were analysed for dextransucrase activity. Aqueous solutions of dextran (500 kDa) and PEG-8000 and solutions of glutaraldehyde, glycerol, Tween-80 were added to dextansucrase solution (0.55 mg/ml, 23 U/mg specific activity) in sodium acetate buffer, pH 5.4 to obtain the final concentrations of 2 µg/ml dextran (500 kDa), 10 µg/ml PEG-8000, 0.1% glutaraldehyde, 0.5% glycerol and 10 µg/ml Tween 80, respectively, in a final volume of 0.6 ml. The enzyme with or without Tween 80 was incubated at three different temperatures –20°C, 4°C and 30°C for 14 days. The aliquots (10-20 µl) were taken periodically for activity assay as described above (Section 8.2.2). All the experiments in triplicates and assays in duplicate were carried out and the results represent mean values with less than 2% of error.

8.2.6 Preparation and characterization of dextran

The dextran was produced by incubating 0.2 ml dextransucrase (0.58 mg/ml protein of specific activity 23 U/mg, purified by 10% PEG-1500 fractionation) in 100 ml of 5% sucrose in 20 mM sodium acetate pH 5.4 containing 0.3 mM $CaCl_2$ and 15

mM sodium azide at 28°C for 48h. After the completion of incubation, the dextran was precipitated using a final concentration of 65% ethanol and resuspended in water, this step was repeated. The precipitate was heat dissolved in water and dried by lyophilization. The optical rotation for dextran solution in water in a 10 ml quartz cuvette was recorded using a polarimeter (Perkin Elmer Instruments, Model 343 Polarimeter) using a sodium D-line (589 nm). The FT-IR spectrum using spectrometer (Perkin Elmer Instruments, Spectrum One FT-IR Spectrometer) was recorded for purified dextran in a KBr pellet. The ^{1}H-NMR and ^{13}C-NMR spectra of dextran in D_2O were recorded for analyzing the linkage composition using NMR spectrometer (Varian, Model AS400). Tetramethyl silane (TMS) was used as an internal reference.

8.3 Results and Discussion

8.3.1 Effect of salts and denaturing agents on dextransucrase activity

The effect of metal ions on enzyme activity is an established fact with several enzymes (Robyt and Walseth 1979; Goyal *et al.* 1995). These solutes stabilize the catalytic activity of enzymes by stabilizing the three-dimensional protein structure. The metal ions Ca^{2+} and Mg^{2+} at lower concentrations exhibited a marginal increase in the enzyme activity of dextransucrase (Fig. 8.1A and B), whereas the increase in activity by Co^{2+} was highly significant (Fig. 8.1C). The addition of 1 mM $CaCl_2$ and $MgCl_2$ to dextransucrase caused an enhancement of enzyme activity by 8% and 6%, respectively (Table 8.1). The addition of 4 mM $CoCl_2$ to dextransucrase resulted in an increase in the enzyme activity by 22% (Table 8.1 and Fig. 8.1C). Further increase in the salt concentrations did not favour the increase in the enzyme activity. Similar results were reported for purified *Leuconostoc mesenteroides* B-512F dextransucrase where, the activity of enzyme was enhanced by the addition of Ca^{2+} and Co^{2+} and inhibited by EDTA (Robyt and Walseth 1979; Kobayashi and Matsuda 1980). $MnSO_4$ had an inactivating effect on the dextransucrase activity (Fig. 8.1D). The addition of 1 mM $MnSO_4$ resulted in a 50% decrease of enzyme activity (Table 8.1). The addition of EDTA inhibited the enzyme activity and 50% inhibition was obtained with 1 mM EDTA (Fig. 8. 2). The greater extent of inhibition was observed with further increase in the concentration and saturation reached at 5 mM with to a 30% residual activity (Table 8.1). Urea displayed a deactivating effect on dextransucrase at all concentrations. The increase in time period of incubation of urea with enzyme had no further significant deactivating effect. However, with increase in the concentration of urea drastically affected the enzyme activity. The enzyme lost 45%, 70%, 90%, 95% and 98% of activity in 30 min when treated with 1, 2, 3, 4 and 5M urea, respectively

(Fig. 8.2, Table 8.1). These results were different from those observed with urea effect on dextransucrase of *Leuconostoc mesenteroides* NRRL B-1299 where the complete loss of enzyme activity was achieved with 1M urea (Kobayashi and Matsuda 1975).

Table 8.1. The effect of divalent metal ions, chelate compound and denaturant on dextransucrase activity.

	Relative activity (%)
$CaCl_2$ (1 mM)	108
$MgCl_2$ (1 mM)	106
$CoCl_2$ (4 mM)	122
$MnSO_4$ (1 mM)	50
EDTA (5 mM)	30
Urea (5 M)	02

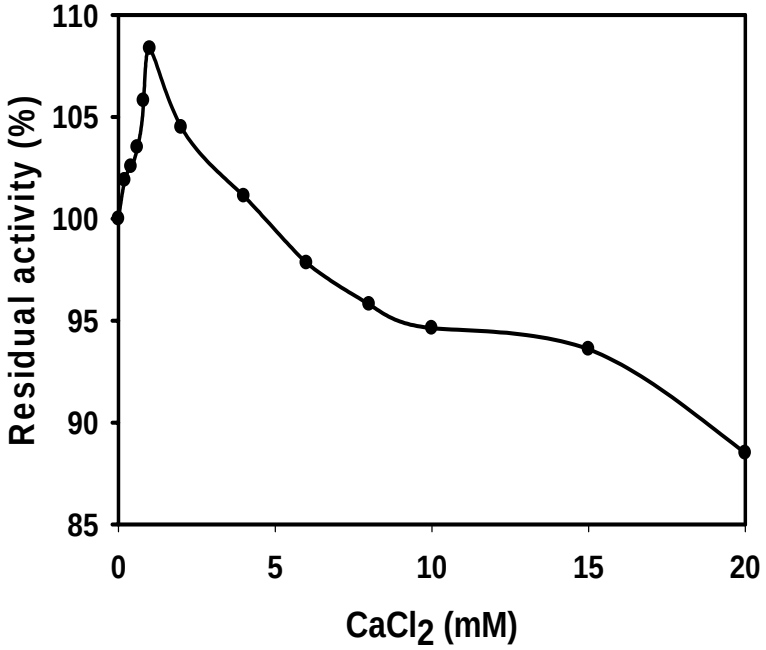

Fig. 8.1A. Effect of $CaCl_2$ on *in-vitro* activity of purified dextransucrase.

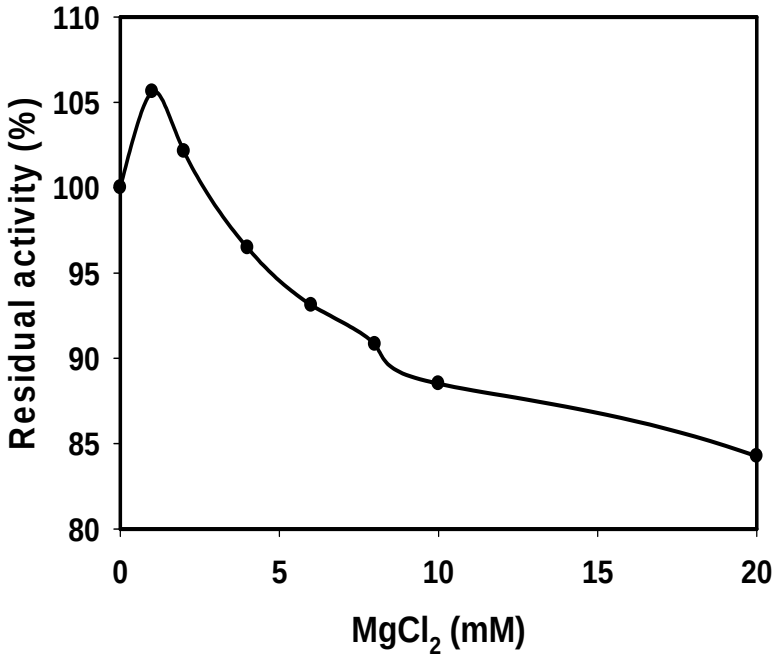

Fig. 8.1B. Effect of $MgCl_2$ *in-vitro* activity of purified dextransucrase.

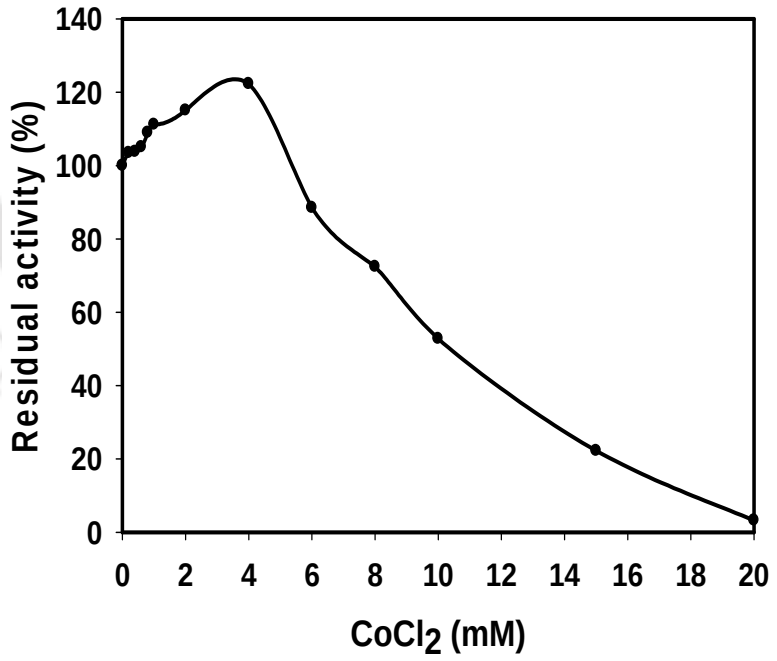

Fig. 8.1C. Effect of $CoCl_2$ on *in-vitro* activity of purified dextransucrase.

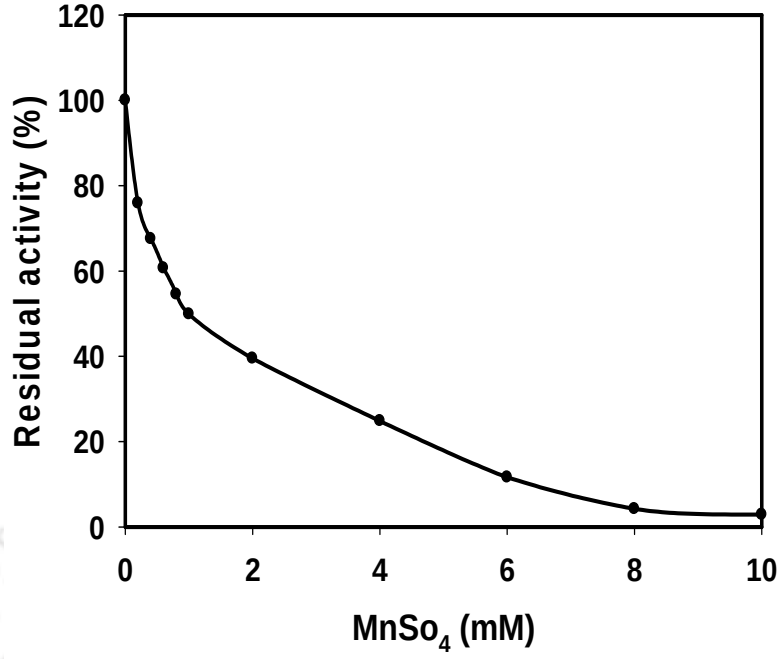

Fig. 8.1D. Effect of $MnSO_4$ on *in-vitro* activity of purified dextransucrase.

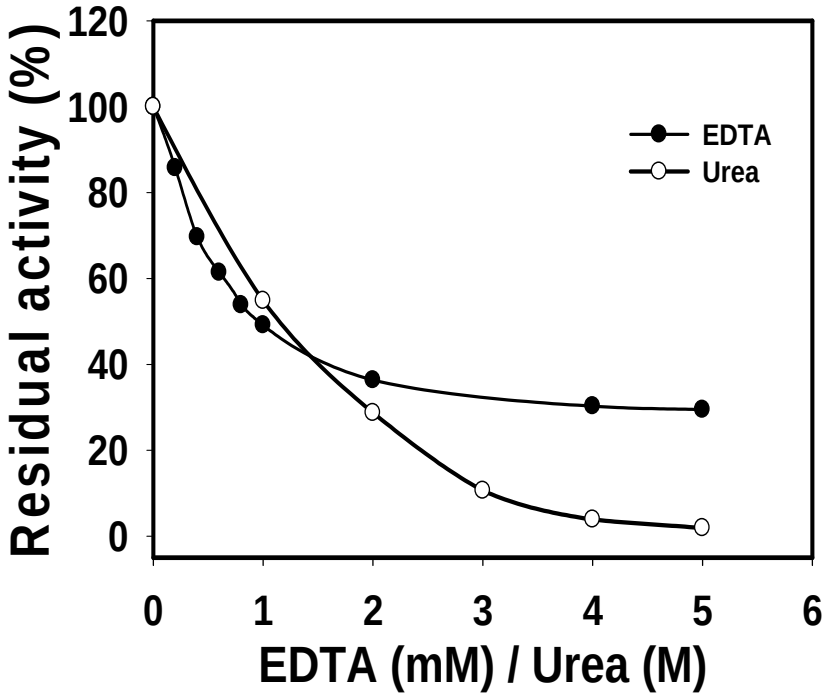

Fig. 8.2. Effect of urea and EDTA on dextransucrase: 0.55 mg/ml enzyme in 20 mM sodium acetate buffer (pH 5.4) was incubated with various concentrations of urea at 30°C for 10 min and the enzyme activity was estimated as described in methods. For EDTA effect on enzyme activity no pre-incubation was done.

8.3.2 Thermal stability of dextransucrase activity

The dextransucrase exhibited a mesophilic nature, and stable up to 30°C, above which it rapidly lost the enzyme activity upon incubation for 10 min (Fig. 8.3). Although, the dextransucrase showed an optimum temperature range for its assay between 30°C to 35°C, but enzyme was unstable in absence of its substrate sucrose at and above 35°C (Purama and Goyal 2007b). Similar results have been reported for dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299 (Kobayashi and Matsuda 1976).

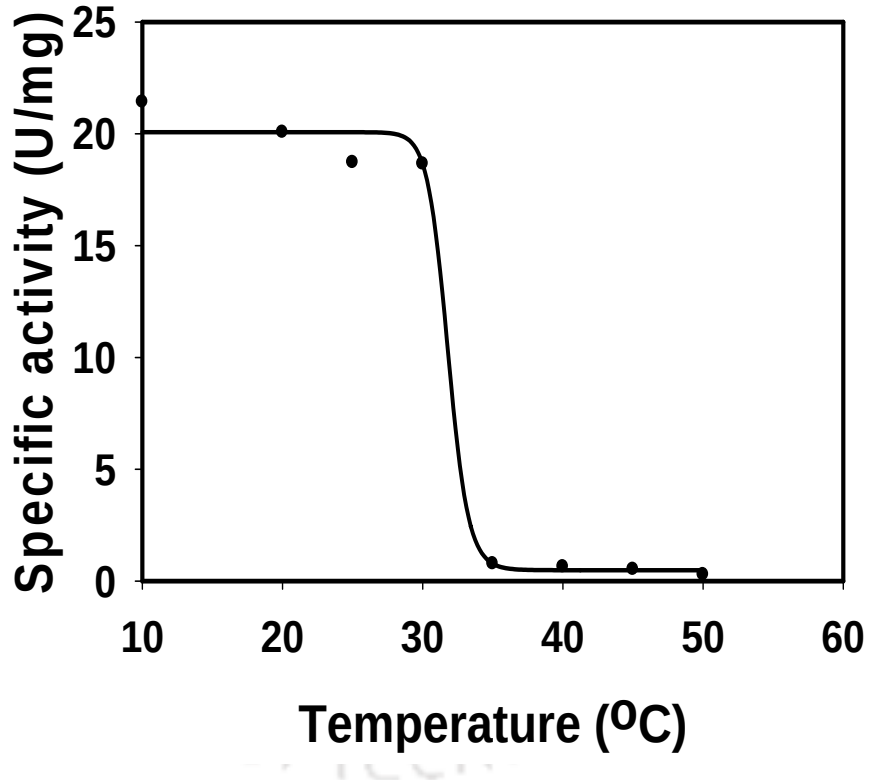

Fig. 8.3. Thermostability of dextransucrase at different temperatures pre-incubated for 10 min and assayed at 30°C in a 1 ml reaction mixture as described in Section 8.2.4.

8.3.3 Effect of additives and storage temperature on stability of dextansucrase

Five different additives were studied for their stability effect on dextransucrase. The additive used did not have any effect on the enzyme activity when included in the assay except glutaradehyde which showed inactivating effect on dextransucrase. The residual activity of dextransucrase at the end of 20h was 92%, 44%, 38%, 36%, 32% and 6% with Tween 80, glycerol, PEG 8000, dextran (500 kDa), control and glutaraldehyde, respectively at 30°C (Fig. 8.4). The Tween 80 was the best stabilizer for dextransucrase at 30°C. Glutaraldehyde did not support the stability, rather it inhibited the enzyme activity (Fig. 8.4). Surprisingly, dextran (500 kDa) did not provide stability to the dextransucrase from *Leuconostoc mesenteroides* B-640, though it has been reported with other strains that dextran imparts stabilization to dextransucrase (Goyal *et al.* 1995; Willemot *et al.* 1988). The other stabilizers glycerol, PEG-8000 displayed only a marginal or no effect at 30°C (Fig. 8.4). The glycerol (Goyal *et al.* 1995) and Tween 80 (Miller and Robyt 1984) have been reported earlier to impart stabilization to dextransucrase from *Leuconostoc mesenteroides* B-512F (Goyal *et al.* 1995).

The residual enzyme activity profile of storage stability at –20°C, 4°C and 30°C of purified enzyme are shown in Fig. 8.5. The enzyme lost its activity rapidly at 30°C after 1 day incubation retaining only 32% enzyme activity. Storage of dextransucrase at 4°C showed a loss of 10% enzyme activity after 6 days of incubation, whereas the enzyme lost 10% of activity in 10 days when stored at –20°C (Fig. 8.5). Similar results with more pronounced deactivation of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F were reported earlier (Ajongwen *et al.* 1993; Goyal *et al.* 1995). Dextransucrase lost 95% activity with in 4 days when stored at 30°C (Fig. 8.5). The addition of Tween 80 to dextransucrase provided stabilizing

effect at all the three temperatures –20°C, 4°C and 30°C (Fig. 8.5). The loss of activity in presence of Tween 80 was 8% and 30% at –20°C and 4°C in 14 days as against the losses of 25% and 65% with corresponding controls, which contained no Tween 80. Tween 80 provided significant stability to dextransucrase at 30°C up to 4 days, as the loss of the enzyme activity was only 32% in presence of Tween 80 as against 92% loss without Tween 80 in 4 days.

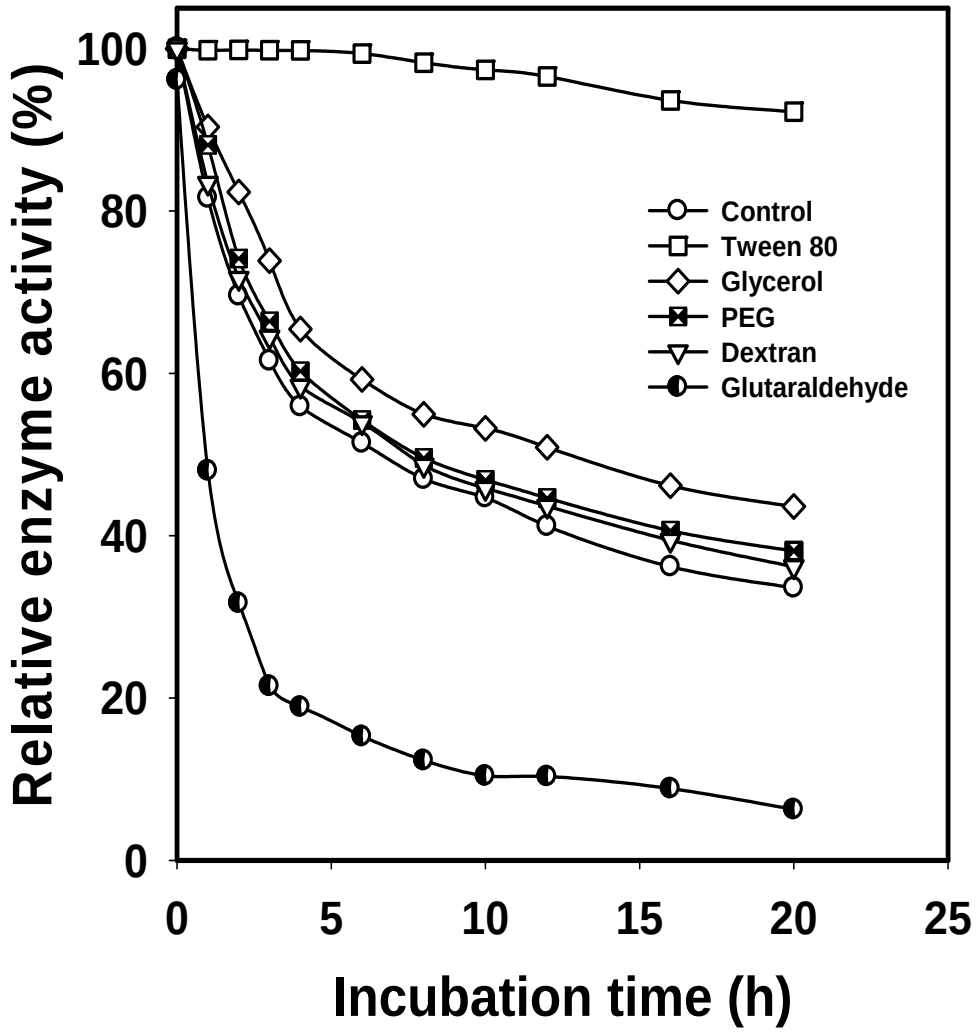

Fig. 8.4. Effect of various stabilizers on dextransucrase activity at 30°C. 0.55mg/ml dextransucrase in 20 mM sodium acetate buffer (pH 5.4) was pre-incubated with additives (Tween 80, glycerol, dextran (500 kDa), PEG-8000 and glutaraldehyde), at 30°C and the assay was carried out by taking 10-20 µl aliquots from enzyme-additive mixture in 1 ml reaction mixture containing 5% sucrose at 30°C as described in section 8.2.2.

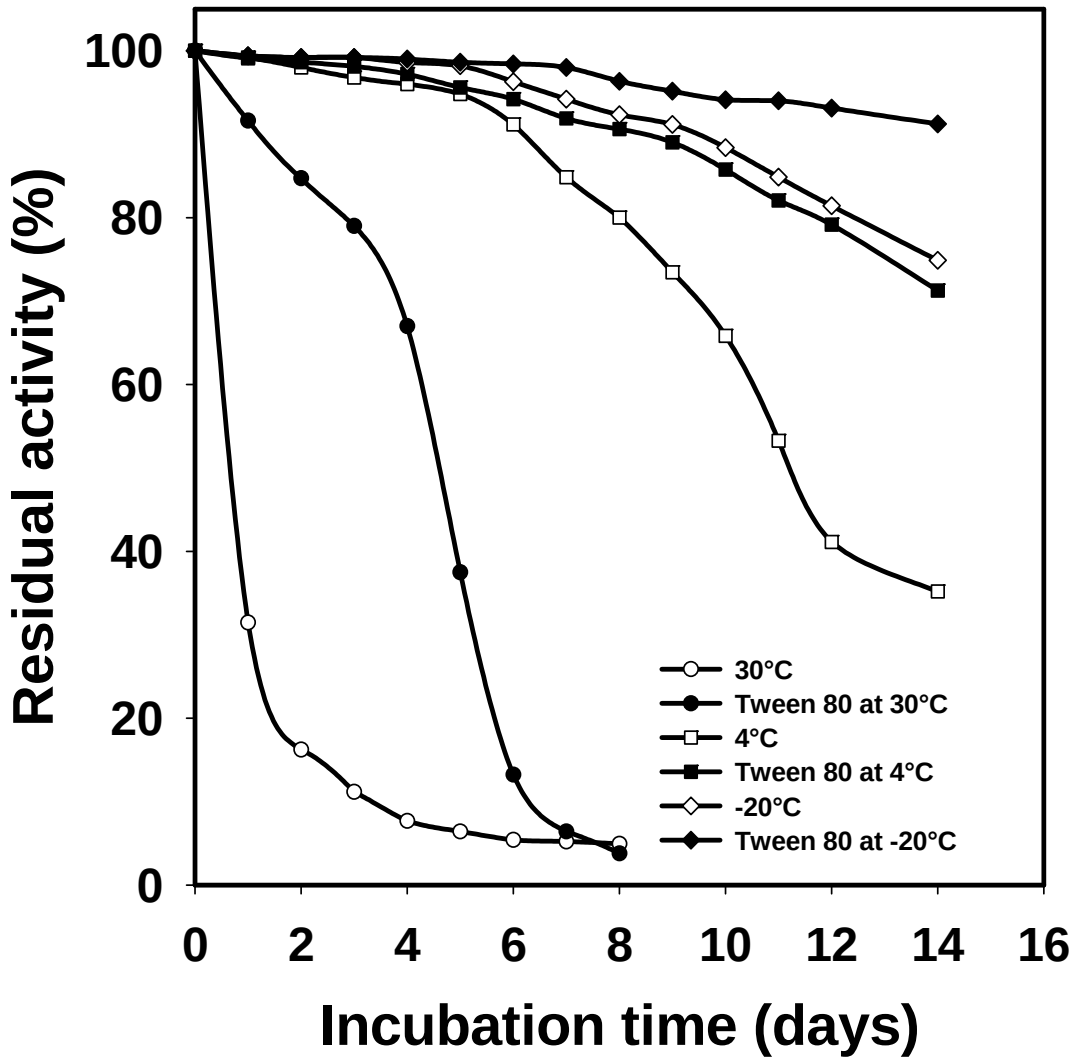

Fig.8.5. Effect of storage stability of dextransucrase and Tween 80 treated dextransucrase at temperatures 30°C, 4°C and –20°C. 0.55mg/ml dextransucrase in 20 mM sodium acetate buffer (pH 5.4) was pre-incubated with Tween 80 (10 µg/ml, final concentration) and aliquots were assayed for dextransucrase activity at 1 day intervals.

8.3.4 Half-life time of dextransucrase and additive-treated dextransucrase

Half-life time ($t_{1/2}$) of dextransucrase and additives treated dextransucrase are listed in the Table 8.2. The $t_{1/2}$ of dextransucrase increases from 0.34d to 40d with decreasing temperature from 30°C to –20°C. This showed that the storage temperature used for dextransucrase can be –20°C. Amongst all the stabilizers used Tween 80 gave the maximum stabilization of dextransucrase with $t_{1/2}$ of 90h at 30°C. A marginal increase in the $t_{1/2}$ (12.1h) was observed with glycerol addition to dextransucrase at 30°C as compared to control (8.2h). No significant increase in the $t_{1/2}$ of dextransucrase was observed with PEG-8000 (9.6h) and dextran, 500 kDa (9.1h) at 30°C. Surprisingly, $t_{1/2}$ of dextransucrase drastically decreased with the addition of glutaraldehyde ($t_{1/2}$ = 1.3h) when compared to dextransucrase with no additive ($t_{1/2}$ = 8.2h) at 30°C. The addition of Tween 80 to dextransucrase, incubated at three different temperatures (30°C, 4°C and –20°C) resulted in higher $t_{1/2}$ than with no Tween 80. Taken together all these results it can be summarized that a temperature of –20°C and addition of Tween 80 ($t_{1/2}$ of 109d) can best serve for the stabilization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 against activity losses.

Table 8.2. Half-life of dextransucrase and additive treated dextransucrase at various temperatures.

Dextransucrase + additive	Half-life (h/d)		
	30°C	4°C	–20°C
Dextransucrase	8.2h (0.34d)	14.3d	40d
Dextransucrase + Tween 80	90h (3.75d)	35d	109d
Dextransucrase + glycerol	12.1h	nd	nd
Dextransucrase + PEG-8000	9.6h	nd	nd
Dextransucrase + dextran (500 kDa)	9.1h	nd	nd
Dextransucrase + glutaraldehyde	1.3h	nd	nd

nd - not determined; h - hour; d - day

8.3.5 Characterization of dextran

8.3.5.1 Optical rotation and Fourier-Transform Infrared Spectrometry (FT-IR) analysis

The structure of *Leuconostoc mesenteroides* NRRL B-640 dextan was characterized using FT-IR, ^{1}H-NMR and ^{13}C-NMR. Dextran was synthesized using purified dextransucrase. The dextran synthesized from dextransucrase of *Leuconostoc* mesenteroides NRRL B-640 showed an optical rotation $[\alpha]_D^{20}$ of + 159° at $C^{0.25}$. The FT-IR spectra were used to investigate the functional groups of commercial dextrans and pullulans (Shingel 2002). FT-IR spectrum of the purified dextran is presented in Fig. 8.6. The band in the region of 3400 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide (Liu *et al.* 2007). The band in the region of 2930 cm^{-1} was due to C-H stretching vibration and the band in the region of 1639 cm^{-1} was due to carboxyl group (Cao *et al.*2006; Liu *et al.* 2007). The absorption peak at 906 cm^{-1} indicates the existence of α-glycosidic bond. The main characteristic bands found in the spectra of dextran at 1154, 1103 and 1020 cm^{-1} are due to valent vibrations of C-O and C-C bonds and deformational vibrations of the CCH, COH and HCO bonds (Shingel 2002). The band at 1154 cm^{-1} is assigned to valent vibrations of C-O-C bond and glycosidic bridge. The peak at 1103 cm^{-1} is due to the vibration of the C-O bond at the C-4 position of glucose residue (Shingel 2002). The presence of peak at 1020 cm^{-1} is due to the great chain flexibility present in dextran around the α(1→6) glycosidic bonds as shown earlier (Shingel 2002). FT-IR spectral analysis of *Leuconostoc mesenteroides* NRRL B-640 dextan showed that it contains α(1→6) linkages. This was further confirmed by ^{1}H-NMR and ^{13}C-NMR analysis.

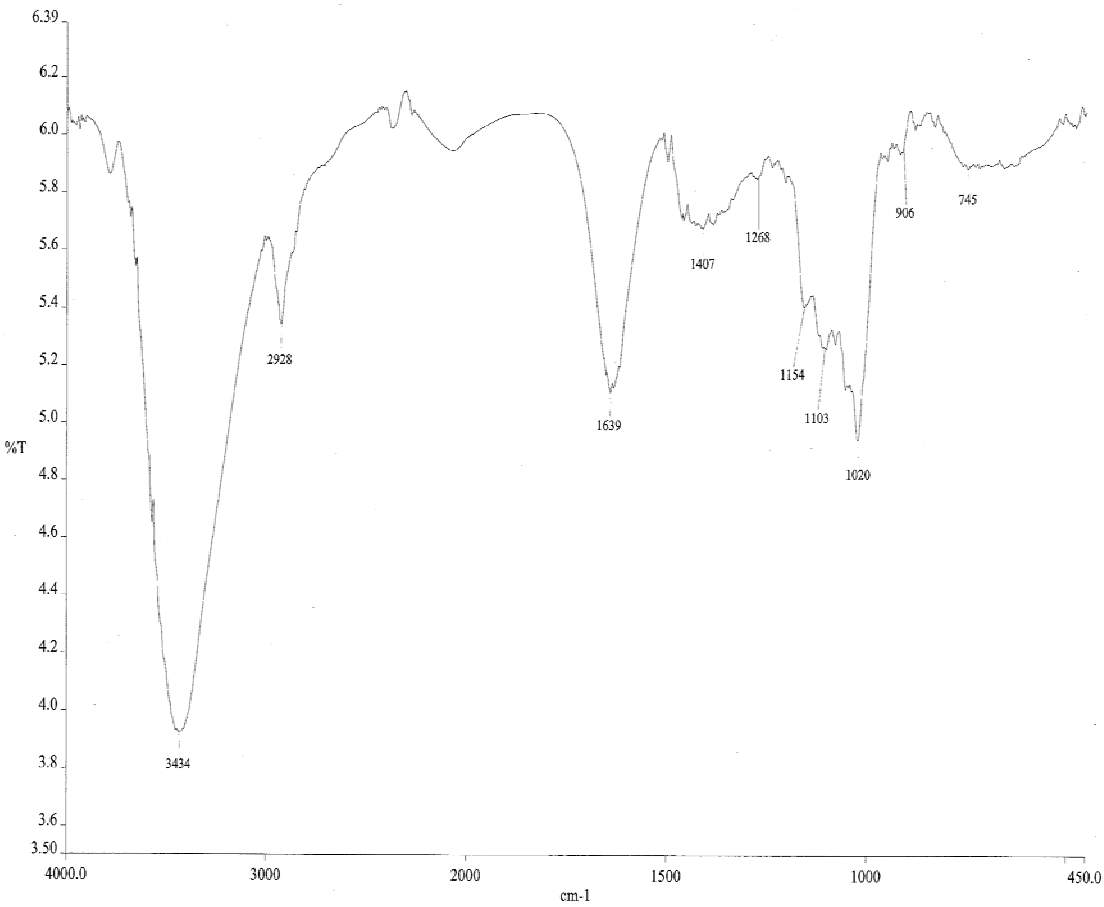

Fig. 8.6. FT-IR (KBr) spectrum of dextran produced from the purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-640.

8.3.5.2 ^{1}H-NMR and ^{13}C-NMR analysis of dextran

The anomeric proton resonances for the 400 MHz ^{1}H-NMR spectrum of α(1→6) dextran are shown in Fig. 8.7. Seymour 1979b observed the distribution of ^{1}H-NMR spectral resonances between 3-6 ppm for different dextrans. The resonance at 4.96 ppm is due to the H-1 of the α(1→6) glucosyl residues of main chain (Seymour 1979b). The dextan showed six prominent ^{13}C-NMR resonances at 100 MHz: 97.867, 71.563, 73.563, 69.660, 70.335 and 65.706 ppm (Fig. 8.8), which is characteristic of linear dextran (Seymour *et al.* 1979a; Uzochukwu *et al.* 2001). No additional peaks were observed in the region of 75-85 ppm indicating that the absence of branched linkages (Seymour 1979a). The major resonance in the anomeric region occurs at 98.7 (97.46) ppm showing that the C-1 is linked. An equally intense signal at 66.5 (65.30 ppm), indicates that most of the C-6 are also linked (Uzochukwu *et al.* 2001). The equal peak intensities at 97.86 and 65.7 confirmed that the glucose residues in dextran are linked by α(1→6) glycosidic bond and no additional peaks 75-85 ppm confirmed that the dextran synthesized by the purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 is a highly linear dextran with α(1→6) glycosidic bonds. The assignments for different resonances of ^{13}C and ^{1}H NMR are presented in Table 8.3.

Table 8.3. ^{1}H and ^{13}C-NMR chemical shifts of dextran from *Leuconostoc mesenteroides* NRRL B-640.

Sugar residue	Chemical shifts (δ) of ^{13}C/^{1}H (ppm)					
	C1	C2	C3	C4	C5	C6
α(1→6)	98.86	71.56	73.1	69.5	70.2	65.5
Glucose	H1	H2	H3	H4	H5	H6
polymer	4.96	3.48	3.62	3.44	3.83	3.90

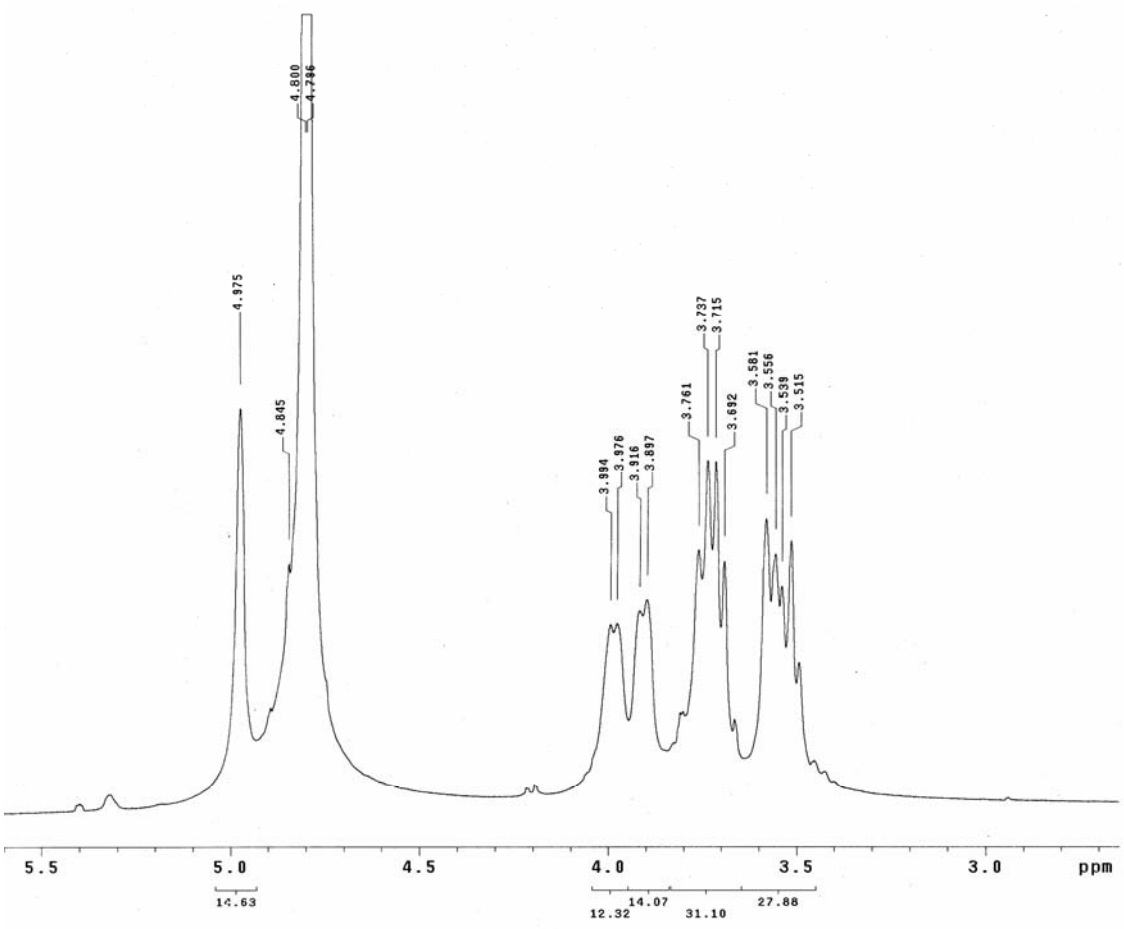

Fig. 8.7. ^{1}H-NMR (400 MHz, D_2O) spectrum of dextran produced from the purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-640.

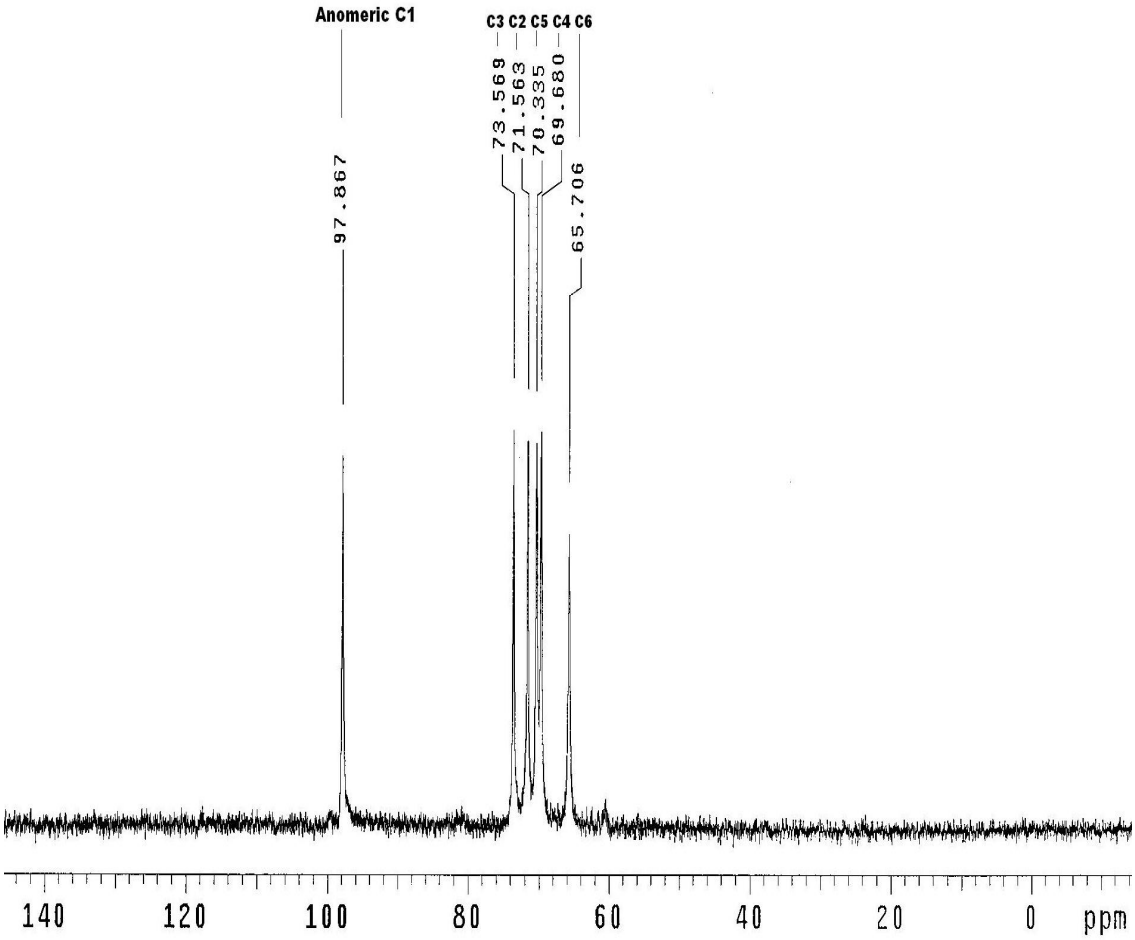

Fig. 8.8. ^{13}C-NMR (100 MHz, D_2O) spectrum of dextran produced from the purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-640.

8.4 Conclusions

It is important to obtain enzymes with high stability for their applications in industrial purposes. In the present study the effect of salts, storage temperature and stabilization efficiency of various additives on dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 were evaluated. Dextransucrase activity was enhanced by divalent cations (Ca^{2+}, Mg^{2+} and Co^{2+}) and 4 mM concentration of Co^{2+} ions showed an activation of the enzyme activity by 22%. EDTA showed an inhibitory effect on enzyme and 50% of the dextransucrase activity was lost by at 1 mM concentration. The enzyme activity loss was 98% with urea at 5M concentration when incubated for 30 min at 30°C. The thermo-stability results showed that dextransucrase is stable at lower temperatures (10-30°C) and loses rapidly the enzyme activity at temperatures higher than 30°C.

Amongst glycerol, dextran (500 kDa), Tween 80, PEG-8000 and glutaraldehyde, Tween 80 provided the maximum stabilization to the dextransucrase against activity loss at 30°C. Dextransucrase lost 92% activity with in 4 days when stored at 30°C, whereas in the presence of Tween 80 this loss was only 33% in 4 days.

The storage of dextransucrase at different temperatures showed that the enzyme retains 75% activity at -20°C in 14 days and retained only 35% activity at 4°C with in the same time period. Dextransucrase in presence of Tween 80 suffered only 8% loss of activity when stored at -20°C as against 28% loss at 4°C in 14 days. The results suggested that storage of dextransucrase at -20°C is better and Tween 80 can be used as a stabilizer for the enzyme which is an efficient method for preventing dextransucrase against the loss of enzyme activity.

The structure and type of linkage analysis of purified dextran produced from purified dextransucrase of *Leuconostoc mesenteroides* NRRL B-640 was explored

using FT-IR, ^{1}H-NMR and ^{13}C-NMR techniques. The peak at 1020 cm^{-1} in the FT-IR spectrum is characteristic of carbohydrate polymers with α(1→6) linkages. These results are in agreement with preliminary results of Uzochukwu *et al.* 2001. Preliminary results of ^{13}C-NMR showed that dextran produced by this strain contains α(1→6) linkage (Uzochukwu *et al.* 2001). The occurrences of six characteristic peaks in the ^{13}C-NMR spectra confirmed that the dextran produced by dexransucrase of *Leuconostoc mesenteroides* NRRL B-640 is a linear dextran. The anomeric resonance at 4.96 in the ^{1}H-NMR spectra indicated the presence of α-anomeric carbon, and equal intensity resonances at 97.86 and 65.70 in ^{13}C-NMR spectra indicated the presence of α-anomeric carbon of glucose residue is linked to C-6 residue of the neighboring glucose residue in the dextran and no additional peaks in the region of 75-85 ppm showed that the dextran is highly linear with α(1→6) linkages. The results of FT-IR, ^{1}H and ^{13}C-NMR spectral analysis confirmed that the polysaccharide produced from *Leuconostoc mesenteroides* NRRL B-640 is a highly linear dextran with α(1→6) linkages.

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2. **Ravi Kiran Purama** and Arun Goyal **(2007)** Identification, effective purification and functional characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. Bioresource Technology. DOI:10.1016/j.biortech. 2007.07.044 (in press). (Elsevier)
3. Avishek Majumder, **Ravi Kiran Purama** and Arun Goyal **(2007)** An overview of purification methods of glycoside hydrolase family 70 dextransucrase. Indian Journal of Microbiology, 47, 252-263. (Springer)
4. **Ravi Kiran Purama** and Arun Goyal **(2007)** Optimization of conditions of *Leuconostoc mesenteroides* NRRL B-640 for production of dextransucrase and its assay. Journal of Food Biochemistry. (in press) (Blackwell)
5. **Ravi Kiran Purama**, Gurtej Singh, Avishek Majumder, V.V. Dasu and Arun Goyal **(2007)** Dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 in batch fermentation. International Journal of Chemical Science (accepted).

Submitted

6. **Ravi Kiran Purama** and Arun Goyal **(2007)** Screening and optimization of nutritional factors for higher dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640 using statistical approach. Bioresource Technology (submitted).
7. **Ravi Kiran Purama** and Arun Goyal **(2007)** Application of response surface methodology for maximizing dextransucrase production from *Leuconotoc mesenteroides* NRRL-B-640 in a bioreactor. Applied Biochemistry and Biotechnology (submitted).
8. **Ravi Kiran Purama**, Mayur Aggarwal, Shadab Ahmed and Arun Goyal **(2007)** Stabilization and biochemical characterization of purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. Journal of Chemical Technology and Biotechnology (submitted).
9. **Ravi Kiran Purama**, Mayur Aggarwal, Avishek Majumder, Shadab Ahmed and Arun Goyal **(2007)** Antibiotic resistance and carbohydrate fermentation characteristics of glucansucrase producing *Leuconostoc* strains. Journal of Basic Microbiology. (submitted)
10. **Ravi Kiran Purama**, Gurtej Singh, Avishek Majumder, V.V. Dasu and Arun Goyal **(2007)** Effect of aeration on dextransucrase production from *leuconostoc mesenteroides* NRRL B-640 in bioreactor. Biochemical Engineering Journal (submitted).

List of conference papers

National

1. **Ravi Kiran Purama**, Mayur Agrawal and Arun Goyal **(2007)** Functional properties and stabilization of purified *Leuconotoc mesenteroides* NRRL-B-640 dextransucrase. 48th Annual Conference of Association of Microbiologists of India (AMI), December 18-21, 2007, Indian Institute of Technology Madras, Chennai, India.
2. **Ravi Kiran Purama**, Mayur Aggarwal, Avishek Majumder, Shadab Ahmed, Seema Patel and Arun Goyal **(2007)** Antibiotic susceptibility, carbohydrate fermentation and sucrose hydrolysing characteristics of glucansucrase producing *Leuconostoc* strains. 48th Annual Conference of Association of Microbiologists of India (AMI), December 18-21, 2007, Indian Institute of Technology Madras, Chennai, India.
3. **Ravi Kiran Purama**, Mayur Agrawal and Arun Goyal **(2007)** Application of statistical method for dextransucrase production from *Leuconostoc dextranicum* NRRL B-640 in batch fermentation. National Conference on Frontiers in Chemical Engineering, December 12-14, 2007, Indian Institute of Technology Guwahati
4. **Ravi Kiran Purama**, P. Goswami, L.H. Choudhury, A.T. Khan and Arun Goyal **(2007)** Structure of dextran from *Leuconostoc mesenteroides* NRRL B-640. 2nd Mid Year Symposium of Chemical Research Society of India, July 21, 2007, Indian Institute of Technology Guwahati, Guwahati, Assam, India.
5. Gurtej Singh, **Ravi Kiran Purama**, Avishek Majumder, V. Venkata Dasu and Arun Goyal **(2006)** Effect of pH and aeration on production of dextransucrase from *Leuconsotoc mesenteroides* NRRL B-640 in batch and fed-batch fermentations. 75th Annual Meeting of Society of Biological Chemists of India (SBCI), December 8-11, 2006, Jawahar Lal Nehru University, New Delhi, India, p179.
6. **Ravi Kiran Purama** and Arun Goyal **(2006)** Purification and characterization of a sucrose hydrolyzing enzyme from *Leuconostoc mesenteroides* NRRL B-640. 75th Annual Meeting of Society of Biological Chemists of India (SBCI), December 8-11, 2006, Jawahar Lal Nehru University, New Delhi, India, pp139-140.
7. **Ravi Kiran Purama** and Arun Goyal **(2006)** Production and purification of dextransucrase, a family 70 glycoside hydrolase from *Leuconostoc mesenteroides* NRRL B-640. 47th Annual Conference of Association of Microbiologists of India (AMI), December 6-8, 2006, University of Barkatullah, Bhopal (MP) India, pp191-192.
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International

9. **Ravi Kiran Purama** and Arun Goyal **(2007)** Application of response surface methodology for maximizing dextransucrase production from *Leuconotoc mesenteroides* NRRL-B-640 in a bioreactor. International Conference on New Horizons in Biotechnology, Nov. 26-29, 2007, National Institute of Interdisciplinary Science and Technology (NIST), Trivandrum, India
10. Gurtej Singh, **Ravi Kiran Purama**, Avishek Majumder, V. Venkata Dasu and Arun Goyal **(2007)** Effect of aeration, pH and nutrients on dextransucrase production from *Leuconsotoc mesenteroides* NRRL B-640 in batch fermentation. 7th Carbohydrate Bioengineering Meeting, April, 22-25, 2007, Technical University of Braunschweig, Germany, p199.
11. **Ravi Kiran Purama** and Arun Goyal **(2007)** Production, purification and identification of dextransucrase, a family 70 glycoside hydrolase from *Leuconostoc mesenteroides* NRRL B-640. 7th Carbohydrate Bioengineering Meeting, April, 22-25, 2007, Technical University of Braunschweig, Germany, p178.
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