Optimization of production and characterization of glucansucrase and glucan from *Leuconostoc dextranicum* NRRL B-1146

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

DOCTOR OF PHILOSOPHY
in
Biotechnology

by

Avishek Majumder

to the

Department of Biotechnology
Indian Institute of Technology Guwahati

January 2008

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Dedicated

to

Mom, Dad, Raja and Deepu

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.

January, 2008 *Avishek Majumder*

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

CERTIFICATE

It is certified that the work described in this thesis entitled "Optimization of production and characterization of glucansucrase and glucan from *Leuconostoc dextranicum* NRRL B-1146" by Mr. Avishek Majumder 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.

January, 2008

Arun Goyal
Associate Professor
(Supervisor)

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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CERTIFICATE OF COURSE WORK

This is to certify that Mr. Avishek Majumder 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 606	Food Biotechnology
BT 607	Plant Biotechnology
BT 710	Nanobiosciences

Mr. Avishek Majumder was admitted to candidacy of PhD degree program after he successfully completed his Ph.D. qualifying comprehensive examination in June 2006.

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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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BT 601	Analytical Biotechnology	6	AB
BT 602	Basic Biotechnology	6	AA
BT 604	Enzymology	6	AA
BT 606	Food Biotechnology	6	AB
BT 607	Plant Biotechnology	6	AA
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Avishek Majumder
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Synopsis

Introduction

Lactic acid bacteria produce a wide variety of exopolysaccharides, which are mainly involved in cell adhesion and protection. Until recently, industrial interest has resulted from their physico-chemical properties, but these polysaccharides have now raised new interest due to their potential for nutritional and health applications. In addition to heteropolysaccharides composed of glucose, galactose, fructose and rhamnose, lactic acid bacteria produce homopolysaccharides which contain only one type of monosaccharide, fructose or glucose, respectively, the fructans and the glucans. These homopolysaccharides share the feature of being synthesized by extracellular glucansucrases using sucrose as the glucosyl (fructose or glucose) donor. Homopolysaccharide synthesis in lactic acid bacteria has been mainly studied in oral *Streptococci, Leuconostoc* spp. and more recently from *Lactobacillus* spp. In general glucans and/or fructans can be used as viscosifying, stabilizing, emulsifying, sweetening, gelling, or water-binding agents, in the food as well as in the non-food industries. Glucooligosaccharides are potential prebiotics and they can be produced by glucansucrase in the presence of sucrose and suitable acceptors like maltose.

Glucans are synthesized by glucansucrases, which belong to glycoside hydrolases and have been grouped till date into 110 families (http://afmb.cnrs-mrs.fr/CAZY/). Depending on the main glucosidic linkages present in their glucan, three different types of α-glucans synthesized by *Leuconostoc* species are recognized: dextran, mutan, alternan. Dextrans have an abundance of α-(1→6) linkages with some branching points at position 2, 3 or 4. Mutan is a water-insoluble glucan having α-(1→3) glucosidic linkages. α-glucan composed of alternating α-(1→6) and α-(1→3)

glucosidic linkages was named alternan. Mutant strains of *Leuconostoc* have been reported to produce glucan with α-(1→6) and α-(1→2) linkages. There are sparse reports of the presence of α-(1→6) and α-(1→4) linkages from *Leuconostoc* species.

Polysaccharides from different sources are being screened and have found many potential applications recently, like polysaccharides showing anti-tumor capability, isolated from chinese herb *Angelica sinensis* (Oliv.) Diels, *Strongylocentrotus nudus* a sea urchin is composed of α-(1→6) and α-(1→4) linked glucan. *Leuconostoc dextranicum* NRRL B-1146 has been reported to produce a highly linear glucan with 96% α-(1→6) linkages. This data was based on a primitive structural characterization of polysaccharide using periodate oxidation. Our preliminary investigations showed that the polysaccharide from this strain is unique with linkages of α-(1→6) and α-(1→4) in its glucan. A neutral polysaccharide like this has applications in the food, cosmetic and pharmaceutical industries. The greater interest in this glucan arises due to the potential it holds, as an immunomodulatory agent. The structure of glucan is determined by the changes in the amino acid composition of the catalytic domain of glucansucrase. The purification and partial characterization of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 will add to the understanding of catalytic mechanism of glucan formation. Therefore, in the present study *Leuconostoc dextranicum* NRRL B-1146 was chosen for glucansucrase and glucan production. The microorganism was studied for nutrient, micronutrient and growth conditions requirement. The production of glucansucrase was enhanced using response surface method and artificial neural network. The glucansucrase was purified and biochemically characterized. Response surface method was used for increased production of glucan and structural characterization of glucan elaborated by *Leuconostoc dextranicum* NRRL B-1146 was carried out.

Present work

The present investigations are carried out on the "Optimization of production and purification of glucansucrase and glucan from *Leuconostoc dextranicum* NRRL B-1146". The thesis work comprises 9 Chapters. Chapter 1 is the General Introduction where the literature described is related to the characteristics of microorganism, the optimization of medium composition using response surface method and artificial neural networks for production of glucansucrase and glucan. It contains detailed reviews on production and purification methods and the properties of glucansucrase and glucan from *Leuconostoc* strains. The chapter also contains a brief review of the different types and structures of glucans synthesized by *Leuconostoc* spp. and their potential applications.

The chapter 2 describes the characteristics of the microorganism *Leuconostoc dextranicum* NRRL B-1146. The antibiotic resistance, carbohydrate fermentation profile, plasmid profile and sucrose hydrolyzing activity or polysaccharide synthesis activity of *Leuconostoc dextranicum* NRRL B-1146 were determined and compared with other strains. 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, vancomycin and kanamycin and was sensitive to amoxycillin, bacitracin, carbenicillin, cephalothin, cephatoxamine, chloramphenicol, clindamycin, linomycin, oxytetracyclin and tetracyclin. The ability of the *Leuconostoc* strains 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 dextranicum* B-1146 contained at least 3 plasmids that were estimated to be approximately, of 1.5 kb, 5.7 kb and 23.5 kb in size. Polyacrylamide gel electrophoresis was used for *in-situ* detection of enzyme activity to characterize glucansucrase production by glucan-producing *Leuconostoc* strain. This study was carried out to see if the 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-1146 showed a single activity band of glucansucrase corresponding to the approximate size, 205 kDa.

Chapter 3 is on optimization of culture conditions for glucansucrase production and its assay conditions. The aim of study was to optimize the culture conditions for obtaining the maximum yields of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. The optimum culture conditions for glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146 were investigated. The glucansucrase production was studied in the temperature range, 22 to 35°C under shaking and static flask conditions. The optimum temperature for glucansucrase production was 28°C. The static flask culture gave higher enzyme activity by 16% than the shaking flask culture. The conditions for maximum enzyme activity were optimized using purified glucansucrase with an activity of 4.5 U/mg. The effect of sucrose concentration on the enzyme activity was studied with varying sucrose concentration between 0.1-11% (2.92-321 mM) concentrations. The results showed that it follows the classical Michaelis-Menten kinetics and the saturation reached at 8% (234 mM) sucrose and K_m of 23.71 mM. The purified enzyme was maximally active at 30℃ and severe loss of activity was observed on either side of 30℃. The

maximum enzyme activity was observed at pH 5.4. The enzyme was quite stable below the pH 5.2, up to pH 4.2. The effect of ionic strength showed that the glucansucrase activity was stable with in the lower range of 10-50 mM concentration of sodium acetate buffer pH 5.4.

Chapter 4 describes the effect of nutrients on glucansucrase production using 'one-variable-at-a-time' approach from *Leuconostoc dextranicum* NRRL B-1146. The enzyme production medium used as control contained 2% sucrose and that gave an enzyme activity of 3 U/ml. With an increase in sucrose concentration to 4% there was a 25% increase in the activity. An increase in yeast extract concentration from 1.5% to 2.0% caused an increase in glucansucrase activity, further increase in yeast extract from 2% (control) to 3% the enzyme activity decreased. This showed that higher yeast extract did not support the enzyme production. The enzyme activity increased by 17% as the concentration of K_2HPO_4 was increased from 2% to 2.5%. Other nitrogenous sources like peptone and beef extract separately, enhanced the enzyme activity by 15%. Interestingly, Tween 80 significantly increased enzyme production. Increasing the concentration of Tween 80 up to 1.0% increased the activity by 65%, and saturation was also observed in the enzyme production after 1% Tween 80. Glucansucrase production decreased from 3 U/ml to 2 U/ml with the increase in $MgSO_4$ from 0.02 % to 0.1% showing a 33% decrease in the enzyme production. $FeSO_4$ at a concentration of 0.005% led to 20% increase in enzyme production from the control that contained 0.001% $FeSO_4$. A 37% increase was observed in the enzyme production at 0.01% $MnSO_4$ concentration. An increase in concentration of NaCl from 0.001% to 0.005% increased the enzyme production by 17%. The was 26% raise in the enzyme activity with an increase in $CaCl_2$ concentration to 0.002% as compared to the control medium that contained 0.001% $CaCl_2$.

Chapter 5 is devoted to the statistical approach to enhance the production of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. Statistically-based experimental designs were applied to optimize the fermentation for the production of glucansucrase by *Leuconostoc dextranicum* NRRL B-1146. Eleven medium components were examined for their significance on enzyme production using Plackett-Burman factorial design. Tween 80, sucrose and K_2HPO_4 significantly improved the enzyme production process. The combined effect of these nutrients on glucansucrase production were studied using a 2^3 full-factorial central composite design, a second-order polynomial was established to identify the relationship between the enzyme output and the three medium components. The optimal concentration of variables for maximum glucansucrase production were Tween 80, (0.55%, v/v); sucrose, (5.6%, w/v) and K_2HPO_4 (1%, w/v). The maximum enzyme activity by predicted model was 6.53 U/ml that was in perfect agreement with the actual experimental value (6.40 U/ml), when optimized medium was used. The process was scaled up to a bench top bioreactor with a working volume of 2 litres. The maximum activity obtained using the statistically designed medium in the bioreactor was 5.8 U/ml.

Chapter 6 reports the optimization of glucansucrase production using artificial intelligence. The development of accurate models for a biological reaction system on a chemical and physical basis is still a critical challenge, mainly due to the non-linear nature of the biochemical network interactions. It has been shown that machine learning techniques such as artificial neural network (ANN) and genetic algorithm (GA) mimic different aspects of biological information processing for data modeling and could prove to be useful in media optimization. Two different artificial intelligence techniques namely artificial neural network (ANN) and genetic algorithm

(GA) were integrated for optimizing fermentation medium for the production of glucansucrase. The experimental data reported for RSM were used to build the neural network. The ANN was trained using the back propagation algorithm. The ANN predicted values showed good agreement with the experimentally reported ones from a response surface based experiment. The concentrations of three medium components *viz* Tween 80, sucrose and K_2HPO_4 served as inputs to the neural network model and the enzyme activity as the output of the model. A model was generated with a coefficient of correlation (R^2) of 1.0 for the training set and 0.90 for the test data. A genetic algorithm was used to optimize the input space of the neural network model to find the optimum settings for maximum enzyme activity. This artificial neural network supported genetic algorithm predicted a maximum glucansucrase activity of 6.92 U/ml at medium composition of 0.54% (v/v) Tween 80, 5.98% (w/v) sucrose and 1.01% (w/v) K_2HPO_4. ANN-GA predicted model gave a 6.0% increase of enzyme activity over the regression (RSM) based prediction for optimized enzyme activity. The maximum enzyme activity experimentally obtained using the ANN-GA designed medium using flask culture was 6.75 ± 0.09 U/ml which was in good agreement with the predicted value.

Chapter 7 describes the purification, identification and confirmation of extracellular glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. The glucansucrase 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 30% (w/v) PEG-400 gave glucansucrase with maximum specific activity of 4.5 U/mg with 7.5 fold purification in a single step. The purified enzyme showed a single band on SDS-PAGE. The purified glucansucrase fractions obtained from PEG-400, confirmed the presence of glucan, 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 glucansucrase of approximately, 205 kDa molecular size, when compared to the bands that appeared on the SDS-PAGE gels stained with Coomassie Brilliant Blue. Further purification of 30% PEG 400 fractionated glucansucrase by gel-filtration using Sephacryl S200HR column gave enzyme with specific activity of 9.0 U/mg with 15 fold purification. The effects of different metal ions (Ca^{2+}, Mg^{2+} and Fe^{2+} etc.), stabilizers (Tween 80, dextran etc.) and EDTA on purified glucansucrase were studied.

The Chapter 8 is devoted to the sequential optimization strategy based on statistical experimental designs for the production of glucan by *Leuconostoc dextranicum* NRRL B-1146 in the flask culture. The maximum glucan yield in Tsuchiya medium was 234 mg/l at 28°C under static conditions. RSM was used to enhance the glucan yield. The first design employed was a two-level Plackett-Burman design, where eleven factors were studied for their influence on glucan production. Sucrose, peptone and yeast extract were the most significant factors improving glucan production. A three-level Box-Behnken factorial design was employed for maximum glucan production. A mathematical model was developed to show the effect of each medium composition and also their combinatorial interactions affect on the production of glucan. The optimal medium compositions stimulating the maximum glucan production were; sucrose 5.95%, peptone 0.52% and yeast extract 2.9%. These values predicted 1063 mg/l glucan production. Using these ingredient compositions of medium the glucan production experimentally found was 1015 ± 4.5 mg/l which was in good agreement with the predicted value of 1063 mg/l and was 5 times higher than unoptimized medium.

Chapter 9 describes the purification and structural characterization of glucan produced by *Leuconostoc dextranicum* NRRL B-1146. The microorganism *Leuconostoc dextranicum* NRRL B-1146 has been reported to produce a highly linear glucan with 96% α-(1→6) linkages based on periodate oxidation method. Our investigations have led to the finding that the polysaccharide produced by this strain is unique with of α-(1→6) and α-(1→4) linkages. The glucan was purified and the structure was analyzed by FTIR, ^{1}H-NMR and ^{13}C-NMR spectroscopic techniques. The crude glucan from the culture supernatant was precipitated by the addition of 3 volumes of 95% (v/v) pre-chilled ethanol and further purified by passing through a column of Sephadex G-100. The purified glucan had an optical rotation ($[\alpha]^{20}_{D}$ +52, *c* 0.2, H_2O) indicating the D-configuration of the glucosyl residues. The FTIR spectrum of the polysaccharide showed bands at 3422, 2928, 1645, 1155, 1080 and 1020 cm^{-1}. The bands at 1020 cm^{-1} and 1080 cm^{-1} are present in glucan with α-(1→6) and α-(1→4) linkages and can be considered as a characteristic for the type of inter-unit link assigned to the hydroxyl stretching vibration of the polysaccharide. ^{13}C-NMR spectra confirmed the homogeneity of the purified polysaccharide. The spectral features were typical of polysaccharide with α-(1→6) and α-(1→4) linkages. Based on ^{13}C-NMR data available in literature, it was possible to identify that the resonances in the region of 102.2 and 97.7 ppm corresponded to C-1 of the 1,4-D-Glc*p* and 1,6-D-Glc*p* residues, respectively. The major resonance in the anomeric regions occur at 97.7 ppm rather than at about 90 ppm indicating that the C-1 is linked. The signal at 66.9 ppm rather than at 60 ppm indicated that most of the C-6s are also linked. Similarly, the peaks at 102.2 ppm and 78.5 ppm suggested the presence of α-(1→4) linkage. The linkages in polysaccharide are α-glucosidic linkages as indicated by the absence of chemical shifts downfield of 102.2 ppm. The configurations for glucan were further

confirmed by the ^{1}H-NMR spectrum which displayed signals for anomeric protons at 5.38 and 4.91 which are assigned to α-(1→4) Glc*p* and α-(1→6) Glc*p*, respectively. The FTIR, ^{13}C-NMR and ^{1}H-NMR data showed that *Leuconostoc dextranicum* NRRL B-1146 a produced glucan that contains α-(1→6) and α-(1→4) linkages. The surface morphology studied using Scanning Electron Microscopy revealed that the polymer has a porous structure and can be used as a texturing agent. The steady shear measurements for the semi-dilute polymer solution indicated that the viscosity (η) of the polymer solution decreased with the increase in shear stress (τ) and exhibited typical non-Newtonian pseudoplastic behavior, indicating branched nature of the polymer.

CONTENTS

Chapter 1

General Introduction

Lactic acid bacteria produce a wide variety of exopolysaccharides, which are mainly involved in cell adhesion and protection. Exopolysaccharides (EPS) are long chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives secreted by bacteria and microalgae (Sutherland 1998). Xanthan, alginate, pullulan, dextran, alternan, levan and inulan are some of the examples of exopolysaccharides. The food industry uses polysaccharides from microbial sources and sea weeds for their thickening and gelling properties. One of the main applications of microbial polysaccharides is in food formulations as food additive. These polysaccharides comprise mainly glucose, fructose galactose and rhamnose in different ratios (De Vuyst and Degeest 1999). They are secreted into the surroundings during growth and are not attached permanently to the surface of the microbial cell (Laws *et al.* 2001).

Glucans are homopolysaccharides of glucose synthesized by lactic acid bacteria, which share the feature of being synthesized by extracellular glucansucrases using sucrose as the glucosyl (glucose) donor with the release of free fructose.

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

Glucansucrase is produced by microorganisms belonging to families Lactobacillaceae and Streptococcaceae, especially by the genera *Lactobacillus*, *Leuconostoc* and *Streptococcus. Leuconostoc mesenteroides* is the most extensively studied and used for glucan and glucansucrase production. Glucansucrases from *Leuconostoc* genus are inducible enzymes, which are induced in the presence of sucrose. *Leuconostoc* species are commercially exploited for the production of glucans. A survey of ninety-six strains of glucan producing bacteria was done to classify dextrans by their structure and properties (Jeanes *et al.* 1954). Depending on the main glucosidic linkages present in their glucan, three different types of α-glucans synthesized by *Leuconostoc* species are recognized: dextran, mutan, alternan. Dextrans have an abundance of α-(1→6) linkages with some branching points at position 2, 3 or 4. Mutan is a water-insoluble glucan having α-(1→3) glucosidic linkages. α-Glucan composed of alternating α-(1→6) and α-(1→3) glucosidic linkages was named alternan. Mutant strains of *Leuconostoc* have been reported to produce glucan with α-(1→6) and α-(1→2) linkages (Smith *et al.*1998).

1.1 The genus *Leuconostoc*

Members of the *Leuconostoc* genus (*leucos*, clear, light; *nostoc*, algal generic name; *Leuconostoc*, colorless nostoc) are facultatively anaerobic, catalase-negative, gram-positive cocci arranged in pairs or chains (Garvie 1986). *Leuconostoc* genus is "generally recognized as safe" (GRAS) and play an important role in several industrial and food fermentation processes (Buckenhuskes 1993). Taxonomic studies of *Leuconostoc* are traditionally based on carbohydrate metabolism or physiological tests, and more recently on cellular fatty acid composition (Tracey and Britz 1989), DNA base composition and DNA–DNA hybridization (Schillinger *et al.* 1989)

Hontebeyrie and Gasser (1977) used DNA/DNA and RNA/DNA hybridizations and proposed four species, *Leuconostoc dextranicum* and *Leuconostoc cremoris* being species of *Leuconostoc mesenteroides*, which formed a single DNA homology group. There have been several changes in the taxonomic classification of species within the genus *Leuconostoc* (Farrow *et al.* 1989; Kim *et al.* 2003). Three major genera, *Leuconostoc*, *Oenococcus* and *Weissella* have been distinguished (Collins *et al.* 1993). The *Leuconostoc* genus comprises *L. mesenteroides* (with the three subspecies, *mesenteroides*, *dextranicum* and *cremoris*) and 13 other species, *L. citreum*, *L. carnosum*, *L. durionis*, *L. fallax*, *L. ficulneum*, *L. pseudoficulneum*, *L. fructosum*, *L. gasicomitatum*, *L. gelidum*, *L. inhae*, *L. kimchii*, *L. lactis*, *L. pseudomesenteroides* (Euzeby 1997).

Various selective media have also been created for specifically isolating and enumerating *Leuconostocs* (Billie *et al.* 1985; Benkerroum *et al.* 1993). Selection for *Leuconostoc* is facilitated through the use of vancomycin in the growth medium, as all the species of *Leuconostoc* are intrinsically resistant to vancomycin (Horowitz *et al.* 1983; Dyas and Chauhan 1988). *Leuconostoc* appear to be morphologically similar to the heterofermentative vancomycin-resistant lactobacilli but tend to be the more coccoid like (Ogier *et al.* 2008). The carbohydrate fermentation pattern gives other means for *Leuconostoc* strain identification at the species level. For example, *L. citreum* is distinguishable from the other related species of *Leuconostoc* as it is the only species that ferments neither raffinose nor melibiose (Facklam and Elliott 1995). 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 Culturing and maintenance of *Leuconostoc* sp.

The *Leuconostoc* sp. are stored and maintained in sucrose rich media (Stacey 1942; Hehre 1946; Bhatnagar *et al.*1985; Goyal and Katiyar 1996), which are also used for isolation of *Leuconostoc* sp. *Lactobacillus* MRS is traditionally used medium for maintaining all the organisms belonging to family Lactobacillaceae (DeMan *et al.* 1960). This medium is also used for longterm preservation of *Leuconostoc* sp. For short duration, the culture can be stored in the enzyme production medium as described by Tsuchiya *et al*. (1952). Various media for the maintenance of *Leuconostoc* sp. cultures have been reported (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). Jeanes in 1965 described two different media for the maintenance of stock cultures. The maintenance medium described by Jeanes (1965) did not give higher enzyme production, possibly due to the lack of certain micronutrients. El-Sayed *et al*. (1990a) described a tomato-tryptone medium for culture maintenance. A chemically defined media was also developed for the maintainance and growth of *Leuconostoc mesenteroides* (Foucaud *et al.* 1997). Goyal and Katiyar reported that *Leuconostooc mesenteroides* NRRL B-512F maintained in modified MRS medium containing 2% sucrose in place of 2% glucose gives 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). Low sucrose maintenance media induced higher enzyme activity than the higher sucrose media. It was reported that on transferring the culture from modified MRS medium to other media gave lower activity and the reverse transfer to modified MRS medium resulted in an increase of enzyme activity (Goyal and Katiyar 1996).

1.3 Glucansucrase from *Leuconostoc* sp.

The enzyme glucansucrases have been placed in family 70 of glycoside hydrolases based on sequence homology and glycoside hydrolases have been grouped till date into 111 families (http://www.cazy.org/fam/GH70.html) (Henrissat 1991). Glucansucrase use the energy of the osidic bond sucrose to catalyze the transfer of D-glucopyranosyl units from sucrose to acceptor molecules. Glucansucrases from lactic acid bacteria share a common structure and are composed of four distinct domains. An N-terminal signal peptide followed by a variable stretch, a highly conserved catalytic domain and a C-terminal glucan binding domain (Monchois *et al*. 1999). The catalytic domain possesses $(\beta/\alpha)_8$ barrel structure and is characterized by the presence of 8 β–sheets located in the core of the protein alternated with 8 α–helices located on the surface of the protein (Monchois *et al.* 1999, van Hijum *et al.* 2006). This region has several important amino acids which play an important role in determining the structure of glucan product. Site-directed mutagenesis in this region of enzyme led to drastic changes in the structure of synthesized glucan (Kralj *et al*. 2004).

No crystallographic data is available for glucansucrases and few structure prediction studies have been carried out (Devulapalle *et al.* 1997). Chemical modification of glucosyltransferases with various agents has shown histidine and lysine residues are also important for enzyme activity (Fu and Robyt 1988; Funane *et al.* 1993; Goyal and Katiyar 1998a). Statistical and kinetic analyses of the inactivation of enzyme by DEP showed that two histidine residues are essential for the enzymatic activity (Fu and Robyt 1988). 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 (Goyal and Katiyar 1998b) inactivation studies. Aspartate residue in the catalytic domain of glucansucrase was found to be involved in the formation of glucosyl enzyme intermediate (Van Hijum *et al.* 2006).

1.4 Production of glucansucrase from *Leuconostoc* sp.

The growth and production of glucansucrase of *Leuconostoc* sp. is affected by factors like temperature, aeration and medium components (Tsuchiya *et al.* 1952; Lazic *et al.* 1993; Goyal *et al.* 1995; Rodrigues *et al.* 2003; Cortezi *et al.* 2005). The fermentation temperature ranging from 20 to 30°C have been found optimum for *Leuconostoc* growth and glucansucrase production (Tsuchiya *et al*. 1952; Kobayashi and Matsuda 1976; Robyt and Walseth 1979; Cortezi *et al*. 2005). The optimum temperature for glucansucrase production from *Leuconostoc mesenteroides* NRRL B-640 was 25°C (Purama and Goyal 2007a). Goyal *et al.* (1995) reported the maximum production of glucansucrase from *Leuconostoc mesenteroides* NRRL B-512F at 23°C. The effect of temperature on glucansucrase production from *Leuconostoc mesenteroides* FT-045 and *Leuconostoc mesenteroides* NRRL B-512F was studied using fermentation by Cortezi *et al.* (2005). They reported 23°C as the optimum temperature for both the strains.

Alsop (1983) reported that at higher temperature of fermentation, the inactivation of enzyme was rapid at pH 6.1-6.7 causing the lower enzyme yield. The decrease in enzyme activity in all cultures, at the end of fermentation was attributed to the extreme sensitivity of glucansucrase to unfavorable pH conditions of broth (Goyal *et al.* 1995). Organisms of the *Leuconostoc* genus are generally micro-aerophilic. There are reports on the production of glucansucrase using shaken and static flask

cultures (Tsuchiya *et al*. 1952; Goyal *et al.* 1995; Shamala and Prasad 1995) but static conditions favour better growth owing to its micro-aerophilic nature though there are few exceptions (Purama and Goyal 2007a). Static flask culture gave higher glucansucrase production from *Leuconostoc mesenteroides* NRRL B-512F as compared to shake flask culture (Goyal *et al.* 1995). Santos *et al.* (2000) observed the cell growth was positively affected by aeration, but the enzyme activity decreased. Michelena *et al.* (2003) studied the advantages of micro-aerophilic system over the non-aerated culture system.

Voluminous information is available on increasing the enzyme yield by the bacterium by studying the effect of nutrients (Koepsell and Tsuchiya 1952; Tsuchiya *et al.* 1952; Lawford *et al.* 1979; Robyt and Walseth 1979; Lopez and Monsan 1980; Kaboli and Reilly 1980; Monsan and Lopez 1981; Alsop 1983; Paul *et al.* 1984; Monsan *et al.*1987; Landon and Webb 1990; Brown and McAvoy 1990; El-Sayed *et al.*1990a; El-Sayed *et al.*1990b; Pennell and Barker 1992; Veljkovic *et al.*1992; Ajongwen and Barker 1993; Lazic *et al.*1993). Glucansucrases from *Leuconostoc* genus are inducible enzymes, which are induced in the presence of sucrose (Neely and Nott 1962). Smith and Zahnley (1999) reported the production of glucansucrase by different wild-type *Leuconostoc mesenteroides* in media containing sugars other than sucrose. They observed that *Leuconostoc mesenteroides* produced the inducible enzymes, glucosyltransferases and fructosyltransferases that synthesized glucan and levan from sucrose. The wild strains of *Leuconostoc mesenteroides* produced low, but detectable glucansucrase activity when grown on glucose, maltose or melibiose instead of sucrose. This was in agreement with the report of Dols *et al.* (1997) who showed that strain B-1299 produced glucansucrase when grown with glucose or fructose instead of sucrose as the sole carbon source. The glucosyltransferase activity

with sucrose medium was higher than with other sugars. The effects of nutrients and growth conditions of *Leuconostoc mesenteroides* NRRL B-512 were studied. The optimum concentration of sucrose was 2% for production of glucansucrase (Tsuchiya *et al.* 1952).

Different nitrogen sources, such as yeast extract, corn steep liquor, beef extract and peptone as nitrogen sources were found to significantly affect glucansucrase production (Tsuchiya *et al*. 1952; Goyal *et al.* 1995; Barker and Ajongwen 1991; Shamala and Prasad 1995). The yield of enzyme is also affected by the type of yeast extract used (Barker and Ajongwen 1991). Peptone and beef extract separately in addition to yeast extract resulted in enhanced enzyme activity (Goyal and Katiyar 1997). An optimum concentration of 4% yeast extract was reported for dextransucrase production form *Leuconostoc mesenteroides* NRRL B-1299 (Dols *et al*. 1997).

The influence of nitrogen/carbon ratio on glucansucrase production by *Leuconostoc mesenteroides* NRRL B-512F was studied (Lopretti *et al.* 1999). Using sucrose as substrate and also other complementary sugars, the enzyme concentration was measured 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.

K_2HPO_4 maintains the pH for longer duration and acts as a buffering agent for the lactic acid produced during fermentation (Tsuchiya *et al*. 1952; Rodrigues *et al.* 2003). Tsuchiya *et al.* (1952) observed that the high amount of phosphate was required for maintaining the pH in the production stage of the cultures. NaOH or

KOH could also serve the purpose. Rodrigues *et al*. (2003) studied the effect of phosphate concentration on the production of glucansucrase and cell propagation by *Leuconostoc mesenteroides* NRRL B-512F in a semi continuous shake flask culture. They used 0.3M K_2HPO_4 instead of 0.1M of the standard medium and reported increase in biomass and enzyme activity. Maintaining constant pH did not have any significant effect on the production of enzyme from *Leuconostoc mesenteroides* NRRL B-640 (Purama *et al.* 2007)

It has been reported that use of a surfactant Tween 80, increases the enzyme production (Sato *et al*. 1989; Goyal and Katiyar, 1997). The addition of Tween 80 to the medium resulted in an increase in the glucansucrase 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 glucansucrase.

Glucansucrases from the genera *Leuconostoc* show considerable response to the presence of micronutrients in the medium (Foucaud *et al*. 1997; Kim *et al.* 2000). 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). Mg^{2+} ions had no effect on the growth rate of the bacterium. Magnesium sulphate was not included in the enzyme production medium of Tsuchiya *et al.* (1952) during the study. Robyt and Walseth (1979) reported a two-fold increase in enzyme production by the addition of calcium chloride to the medium of *Leuconostoc mesenteroides* NRRL B-512F. *Leuconostoc* sp. are known to be micro-aerophilic microorganisms and $MnSO_4$ decreases the oxygen toxicity of the

Leuconostoc mesenteroides cells (Bellinger *et al.* 1997). Bellinger *et al.* (1997) reported that the addition of Mg^{2+}, Mn^{2+} and amino acids stimulate the growth of most of *Leuconostoc* strains.

1.5 Exopolysaccharides

Exopolysaccharides from microbial sources have been classified into two groups i.e. homopolysaccharides (e.g. cellulose, dextran, mutan, alternan, pullulan, reuteran, levan, curdlan etc.) and heteropolysaccharides (e.g. gellan and xanthan etc.) (Laws *et al.* 2001). In addition to heteropolysaccharides composed of glucose, galactose, fructose and rhamnose. Lactic acid bacteria also produce fructans and glucans, homopolysaccharides which contain only one type of monosaccharide, fructose or glucose, respectively. Homopolysaccharide synthesis by lactic acid bacteria has been mainly studied in oral *Streptococci, Leuconostoc* sp. and *Lactobacillus* sp. (Monsan *et al.* 2001). Glucans differ in the type of glucosidic linkages, degree and type of branching, length of glucan chains, molecular mass and conformation of polymers. All these properties strongly contribute to specific polysaccharide properties such as solubility, rheology and other physical characteristics (Monchois *et al.* 1999). Depending on the main chain glucosidic linkages in glucan, three different types of α-glucans synthesized by *Leuconostoc* species are known *viz.* dextran with α-(1→6), mutan with α-(1→3) and alternan with alternate α-(1→6) and α-(1→3) linkages.

Pastuer (1861) discovered the microbial origin of the jellification of cane sugar syrups. The product causing the jellification was named dextran due to its positive optical activity. The corresponding extracellular enzyme was named dextransucrase (Hehre 1946). A common feature of all dextrans is the abundance of

α-(1→6) linkages with some branching points at position 2, 3 or 4. Dextrans are produced by *Leuconostoc mesenteroides* strains (Cerning 1990). Guggenheim (1970) showed that water-insoluble glucan from *S. mutans* OMZ176 contained a high proportion (up to 90%) of α-(1→3) glucosidic linkages. He proposed the name mutan for this polymer. The corresponding enzyme was named mutansucrase. Mutan polymers are mainly produced by various *Streptococci* (Hamada and Slade 1980). Cote and Robyt (1982) isolated an α-glucan polymer from *L. mesenteroides* NRRL B-1335 composed of alternating α-(1→6) and α-(1→3) glucosidic linkages. The polymer was named alternan and the corresponding enzyme alternansucrase (Cote and Robyt 1982). The glucan containing α-(1→4), α-(1→6) glucosidic bonds was identified from *Lactobacillus reuteri* 121 (Kralj *et al.* 2004). The glucan was named reuteran and the corresponding enzyme reuteransucrase (Kralj *et al.* 2004). A glucan containing α-(1→6) linkages and a substantial amount of α-(1→2) linkages is produced by two strains of *L. mesenteroides* NRRL B-1299 and a mutant strain (R510) of NRRL B-1335 (Smith *et al.* 1998).

1.5.1 Production of glucan from *Leuconostoc* sp.

Conventional fermentation used for the production of dextran involves three phases: cell growth, enzyme production phase and glucan synthesis (Alsop 1983). Extensive work has been done on optimization and modification of the fermentation processes for improved production of glucan (Stacey 1942; Hehre 1946; Koepsell and Tsuchiya 1952; 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; Barker *et al.* 1993). 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, glucan synthesis takes place outside the cell in presence of glucansucrase, decoupling of the enzyme and glucan production was explored for optimizing the glucan synthesis (Landon and Webb 1990). Fed batch reactor maintained at constant pH of 6.7, was used for enhancing the production of glucan (Brown and McAvoy 1990; Ajongwen and Barker 1993). Other approaches included immobilization of *Leuconostoc mesenteroides* NRRL B-512F like encapsulation in calcium alginate beads (El-Sayed *et al.* 1990a; El-Sayed *et al.* 1990b), an amino-Spherosil support activated with glutaraldehyde, an alkylamine porous silica (Lopez and Monsan 1980; Kaboli 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 glucan by purified glucansucrase (El-Sayed *et al.* 1990b). Fructose, which is the by product of glucan synthesis was retained complexing with calcium ions on the resin used in the reactor, which facilitated the higher production of glucan having molecular weight in the range of 10,000 and 200,000 (El-Sayed *et al.* 1990b). The production of dextran and fructose from carob pod extract and cheese whey as carbon source by *Leuconostoc mesenteroides* NRRL B-512F resulted in 8.56 and 7.78 g/l, dextran and fructose (Santos *et al.* 2005).

1.5.2 Applications of glucans

The immunomodulatory and anti-cancer effects of many polysaccharides and polysaccharide-protein complexes isolated from bacteria, mushrooms, fungi, yeasts, algae, lichens and plants has attracted much attention recently in the medical and

biochemical areas (Ooi and Liu 2000). It is generally accepted that polysaccharides enhance various immune responses *in vivo* and *in vitro*. Anti-tumor polysaccharides from chinese herb, *Angelica sinensis* (Oliv.) Diels (Cao *et al.* 2006) and a sea urchin, *Strongylocentrotus nudus* are composed of α-(1→6) and α-(1→4) linked glucan (Liu *et al.* 2007). In recent years more and more polysaccharides have been reported to exhibit a variety of biological activities, including anti-tumor, and immunostimulation (Wasser, 2002).

In general glucans can be used as viscosifying, stabilizing, emulsifying, sweetening, gelling or water-binding agents in the food as well as in the non-food industries (Sutherland 1998; Welman and Maddox 2003). Certain oligosaccharides (e.g. isomaltooligosaccharides and lactulose) and polysaccharides (e.g. fructans) are used as prebiotic food additives. Additionally, oligosaccharides containing α-(1→2) glucosidic bonds in some cases are used as feed additives (Monsan and Paul 1995).

Neutral glucans like 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; Purama and Goyal 2005). 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). Dextran hydrogels are used in various pharmaceutical and biomedical applications

such as contact lenses, cell encapsulation for drug delivery, burn wound dressing and in spinal cord regeneration (Aumelas *et al*. 2007).

Native polyelectrolytes generated by sulphation of branched glucans are used as anticoagulants, cholesterol lowering agents, anti-ulceratives, as anti-metatastatic agents in the treatment of sepsis, AIDS and human prostatic carcinoma (Robyt 1986). Magnetic iron-glucan 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 L-Glucose (Zhang *et al*. 2006).

The glucansucrase 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.* 1991). 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* sp. and *Bacteroides* sp. (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). 1,5-Anhydro-D-fructose oligosaccharides generated by glucansucrase 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). Applications of glucans have been well documented in several reviews (Robyt 1986; Naessens *et al.* 2005; Purama and Goyal 2005).

1.5.3 Structure of glucans

Glucans derived from different *Leuconostoc* sp. show large variation in composition, charge, spatial arrangement and rigidity. No defining corelation between glucan concentrations and viscosities has been established yet (Duboc and Mollet 2001). The correlation between glucan concentration and viscosity is particularily important because it will provide a foundation for a strategy aimed at producing functionally valuable polysaccharides, which will behave in a predictable manner when incorporated into food products. The generalized trends observed in polysaccharides are, to have high viscosity, long chains of subunits (high molecular mass) and/or stiff chains are required (Laws *et al.* 2001). α-Linkages result in more flexible chains than β-linkages (Laws *et al.* 2001) and α-(1→4) linked polymers (amylose) show lesser flexible character in comparison with the polymers with α-(1→6) linked units (Shingel 2004).

A survey of ninety-six strains of glucan producing bacteria was done to classify dextrans by their structure and properties and identify a suitable strain, for pharmaceutical industry (Jeanes *et al.* 1954). The structural classification based on linkages was done using periodate oxidation. Important results concerning the mobility and confirmation of carbohydrate chains were obtained from the analysis of polysaccharides by the methods of IR spectroscopy (Bernazzani *et al.* 2001). This is in particular, because of the fact that steric factors and the spatial location of individual groups strongly contribute to the formation of vibrational spectra of carbohydrates. Fourier-transform infrared spectroscopy (FTIR) is now widely used to study the composition of complex carbohydrate systems, the molecular orientation, molecular interactions and conformational transitions of polysaccharides in solution or upon hydration (Kaourokova and Wilson 2001). The carbohydrates show high

absorbances in the region 1200-950 cm^{-1}, that is within the so called fingerprint region, where the position and intensity of the bands are specific for every polysaccharide allowing its possible identification (Filippov 1992).

Colson *et al.* 1974 assigned the ^{13}C-NMR spectra of several linear glucans by comparing with the spectra of glucose, some of specifically *O*-methylated glucose derivatives and a number of differently linked glucobioses and glucotrioses such as maltose α-(1→4); isomaltose α-(1→6); maltotriose α-(1→4) and α-(1→6); nigerose α-(1→3) and laminaribiose β-(1→3). They were able to assign unambiguously, the chemical shifts of C1–C6 of linear α-(1→6) linked glucans and establish the effect of branched linkages on the chemical shifts of the various carbon atoms. The elucidation of the composition, sequence and conformation of glucans with mixed linkages was achieved by the full assignment of these disaccharide and homoglucan resonances.

The structures of dextrans established by methylation and by periodate oxidation were shown to agree with that obtained by ^{13}C NMR. Seymour *et al.* 1976; 1979a; 1979b; 1979c) employed ^{13}C NMR spectroscopy to examine the structure of a series of dextrans and established that, the linear dextran has six prominent ^{13}C-NMR resonances which they termed A, B, C, D, E and F. Branching contributes additional resonances to the ^{13}C-NMR spectra of dextrans. The positions of these additional resonances in the anomeric region (95–105 ppm) and the 80–85ppm region are diagnostic for the type of branch linkage present. The relative peak heights of these diagnostic resonances resulting from branching are in general, proportional to the degree of branching.

Sidebotham (1974) reported that various glucans have specific ^{1}H-NMR spectral resonances. A linear polymer like dextran has ^{1}H-NMR 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 branched glucan 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 i.e. branching contributes additional resonances. The assignments in present study are based mainly on the work of these two groups of workers and literature available of structural characterization of glucans from various other sources.

1.6 Optimization strategies for glucan and glucansucrase production

1.6.1 One Variable At a Time (OVAT) approach

Optimization is an important step that helps determine the most suitable reaction conditions and the concentration of the medium components which maximize or minimize important process variables (Weuster-Botz 2000). The efficiency of already established bioprocesses can be increased either by introducing more productive strains or by optimizing the cultivation medium itself (Kovarova-Kovar *et al.* 2000). The composition of a fermentation medium consisting of carbon sources, nitrogen sources, mineral salts and trace elements determines the chemical and nutritional environment of cells in a reactor (Weuster-Botz 2000). This is essential for the effective manufacturing of products accumulated or secreted into the medium. The aim of optimisation is to determine suitable reaction conditions (pH, temperature and medium composition) for the respective biological system in order to maximise process variable of interest. Tsuchiya medium has been traditionally used as production media for glucansucrase production (Purama and Goyal 2005). The literature discussed in this section is based on OVAT approach. The nutritional requirements of *Leconostoc* vary considerably with species. Tsuchiya *et al.* (1952) reported the requirement of higher nitrogen sources and other nutrients for the

maximal enzyme production from *Leuconostoc mesenteroides* NRRL B-512. Dols *et al.* (1997) reported only a marginal increase in glucansucrase production from *Leuconostoc mesenteroides* NRRL B-1299 with an increase in yeast extract concentration from 2% to 4% and its further increase did not cause any increase in the glucansucrase production (Dols *et al*. 1997).

One Variable At a Time (OVAT) approach for optimization of enzyme production helps in the identification of basic requirements of the microorganism for its suitable growth and enzyme production (Weuster-Botz 2000). It also helps in identifying any additional requirements in addition to the carbon source, nitrogen sources and trace elements. Like in the case of *Leuconostoc* sp. the addition of surfactants like Tween 80, which alters the membrane structure, helps in secretion of extracellular glucansucrase (Goyal and Katiyar 1997). The micronutrient requirements of the microbes also vary significantly among species. Optimization of medium by classical method involves changing one independent variable keeping the other factors constant. The one dimensional search with successive variation of variables is the method most frequently used (Goyal and Katiyar 1997; Foucad *et al.* 1997; Zhinan and Peilin 1999).

Goyal and Katiyar (1997) studied the effect of certain nutrients on the production of dextransucrase from *Leuoconostoc mesenteroides* NRRL B-512F. They observed that increase in the concentration of sucrose 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 (Goyal and Katiyar 1997). The enzyme production decreased with an increase in the

concentration of yeast extract above 1.5%. 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). For the production of glucan Behravan *et al.* (2003) used different concentrations of molasses and wheat-bran extract for growing *Leuconostoc mesenteroides* NRRL B-512F for the production of dextran (glucan with α-(1→6) linkages). They found that with 20% sugar-beet molasses containing 9.4 g of sucrose, the dextran yield was high. 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 (Behravan *et al.* 2003).

1.6.2 Statistical designs for optimization

It is important to study the interactive effects of medium components for optimization. The conventional methods for multifactor experimental design are time-consuming and incapable of detecting the true optimum, due especially to the interactions among the factors (Wang and Lu 2005). In fermentation process, the operational variables interact and influence each other and affect the response. It is important that the optimization method accounts for its interactions so that a set of optimal experimental condition can be determined (Naveena *et al.* 2005; Liu and Wang 2007). The limitation of a single factor optimization process can be eliminated by different techniques. One of the techniques extensively used is response surface methodology (RSM). It is a collection of experimental strategies, mathematical methods and statistical inference. RSM explains the combined effects of all the factors in a fermentation process (Elibol 2004). The statistical methods give complete consideration of all possible component interactions in the experimental design.

Statistical methods such as Plackett-Burman design, the Box–Behnken design and central composite design (CCD) are some of the popular choices.

To reduce the number of experiments despite the large number of variables, methods of statistical experimental design are used. In general the procedure applied in statistical experimental design for the optimization of fermentation media can be subdivided into three steps (Weuster-Botz 2000). (i) identification of the most important medium components 'screening' (ii) identification of optimum 'optimum search' and (iii) experimental verification of the identified optimum. The identification of the most important medium components is done using Plackett-Burman design, where it is assumed that no interactions between medium components occur. Plackett-Burman experimental design is based on the first order polynomial model:

$$Y = \beta_o + \sum \beta_i x_i$$

where, Y is the response (enzyme activity), β_o is the model intercept and β_i is the linear coefficient, and x_i is the level of the independent variable.

To identify an optimum with the selected variables, the response surface method (RSM) is established, where it is assumed that the estimated response surface can be described using a second-order polynomial.

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

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. X_i is the factor variable in its coded form.

To determine the regression coefficients of the second-order polynomial using the fewest possible experimental approaches and with high accuracy, various

optimum experimental designs are proposed which differ in the number of minimum necessary experiments. The commonly used designs are the central composite design and Box-Behnken design.

Chellapandian *et al*. (1998) studied the production and properties of glucansucrase 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 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.

A 2^{5-1} fractional factorial central composite experimental design was used for optimizing the dextran production in shake flask fermentations from *Leuconostoc mesenteroides* NRRL B-512F (Karthikeyan *et al.* 1996). A dextran yield of 154 g/l was obtained with sucrose, 300 g/l; yeast extract, 10 g/l; K_2HPO_4, 30 g/l; temperature, 23°C and initial pH 8.3. Statistically-based experimental designs were applied to optimize the fermentation for the production of glucosyltransferase by *Leuconostoc dextranicum* NRRL B-1146 (Majumder and Goyal 2007). Eleven different medium components screened for their significance on enzyme production using Plackett-Burman factorial design. A 2^2 full-factorial central composite design, was used to establish the relationship between the enzyme output and the three medium components Tween 80, sucrose and K_2HPO_4 which significantly improved the enzyme production process. A maximum enzyme activity of 6.40 U/ml could be attained.

1.6.3 Artificial intelligence based optimization

Machine learning techniques such as artificial neural network (ANNs) and genetic algorithms (GAs) mimic different aspects of biological information processing for data modelling and could prove to be useful in medium optimization. The development of accurate models for a biological reaction system on a chemical and physical basis is still a critical challenge, mainly due to the non-linear nature of the biochemical network interactions (Franco-Lara *et al.* 2006). An artificial neural network is a superior and more accurate modelling technique as compared to RSM as it represents the non-linear ties in a much better way (Dutta *et al.* 2004). Its application has been reported by many in predictive microbiology (Lou and Nakai 2001). The use of advanced non-linear data analysis tools such as ANN in food science, environmental biotechnology and biochemical engineering is a well established tradition. The contribution of neural networks to biotechnology has been reviewed by Montague and Morris (1994) and Patnaik (1999).

Genetic algorithms are artificial intelligence based stochastic non–linear optimization formalisms (Goldberg 1989). They have been used with great success for optimizing complex expressions, which in this case is a neural network (Sumanwar *et al.* 2002). Since early nineties, a marked increase in the applications of genetic algorithms has been observed in many fileds of applied sciences. GAs are capable of searching a large parameter space in a highly directed and systematic manner (Freyer *et al.* 1992). ANN-GA based approach was used for simultaneous maximization of biomass and conversion of pentafluoroacetophenon with *Synechococcus* PCC 7942 (Franco-Lara *et al.* 2006) and optimization of fermentation medium for the production of exopolysaccharide from *Lactobacillus plantarum* (Desai *et al.* 2006).

1.7 Purification of glucansucrase

Bulk of the information on purification of extracellular glucansucrase 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 (Majumder *et al.* 2007). 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 (Moulis *et al.* 2006). Glucansucrase of *Leuconostoc* spp. is induced by sucrose, which leads to concomitant glucan synthesis during enzyme production. The presence of glucan results in aggregated forms of enzyme. In addition, glucans 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 are used for purification (Robyt and Walseth 1979; 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; Purama and Goyal 2007b)

1.7.1 Fractionation by Polyethylene glycol (PEG)

Polyethylene glycol (PEG) fractionation method is relatively easier and faster procedure to obtain purified form of glucasucrase. PEG is known to selectively precipitate proteins, which have high molecular weights or exist in aggregated forms. The large size of glucansucrase, together with its tendency to form aggregates in solution has led to the use of non-ionic hydrophilic polymer PEG for precipitation of

glucansucrase. Glucansucrase from *Streptococcus mutans* was purified by PEG precipitation using PEG-400 and 6000 (Russel 1979). A simple and effective method of purification of glucansucrase from *Leuconostoc mesenteroides* NRRL B-512F, using PEG precipitation was developed (Goyal and Katiyar 1994). The PEG-400 reproducibly gave glucansucrase with specific activity of 8.7 U/mg, with 80% yield. 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. The major disadvantage of this procedure is the contamination of enzyme by the glucan 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). Glucansucrase from *Leuconostoc mesenteroides* NRRL B-640 was fractionated using PEG-1500 to obtain specific activity of 23 U/mg (Purama and Goyal 2007b).

1.7.2 Phase partitioning

Phase partitioning occurs between glucan and PEG (Paul *et al.*1984) when sucrose is added to the culture supernatant, the enzyme synthesizes glucan in large amounts. The top phase is rich in PEG while the bottom one is rich in glucan, this phase-partitioning allows the purification of extracellular glucansucrase. Glucansucrase preferentially goes in to the lower glucan-rich phase because of the covalent association of glucansucrase and glucan.

The glucansucrase 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). Successive phase partitioning steps using PEG 1500 and glucan 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). The culture supernatant of *Leuconostoc mesenteroides* ATTC 10830 was added with 10% dextran T-500 solution followed by 20% PEG-3350 addition (Otts and Day 1988) to obtain glucansucrase 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 *et al.* 1991).

Phase partitioning using PEG-6000 and 400 was carried out for glucansucrase purification from *Leuconostoc mesenteroides* B-512F (Nigam *et al.* 2006). The specific activity of 42.1 U/mg and an overall yield of 84% obtained after the three steps of phase-partitioning by PEG-6000 (Nigam *et al.* 2006). 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). Commercially available glucan Dextran T70 was added to culture supernatant from *Leuconostoc mesenteroides* sp. followed by PEG fractionation (Dols *et al.* 1998; Quirasco *et al.* 1999). 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.

1.7.3 Chromatography

Glucansucrase has been purified using columns containing hydroxyapatite, DEAE-cellulose, DEAE-sephadex, sephadex, sephacryl, ultrogel AcA34. Glucansucrase 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. The affinity of Sephadex has been found to be good for glucansucrase from *Streptococcus*, but poor for *Leuconostoc* due to the endogenous dextran present in the *Leuconostoc* preparations (Suzuki and Kobayashi 1975; Kobayashi and Matsuda 1976). The enzyme precipitate obtained by ammonium sulfate precipitation of culture supernatant of *Leuconostoc mesenteroides* B-512F was subjected to chromatography on DEAE-cellulose column and the enzyme adsorbed on the column was eluted with NaCl gradient (Robyt and Taniguchi 1976). 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 (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 (Lawford *et al.* 1979). When the crude extract of enzyme was applied to DEAE-cellulose column, the glucansucrase activity could not be eluted from the column, even at very high ionic strengths (Robyt and Walseth 1979). This was because glucansucrase tends to aggregate in the presence of dextran. Most of the glucansucrase 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 glucansucrase 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. The precipitate was put on DEAE-cellulose column and 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). Gel-filtration chromatography of glucansucrase from *Leuconostoc mesenteroides* NRRL B-640 using Sephacryl S200HR resulted in purified glucansucrase with a specific activity of 35 U/mg with a 61-fold purification was obtained (Purama and Goyal 2007b).

1.8 Properties of glucansucrase

Several workers have reported that glucansucrase exists in single or multiple forms having molecular weight in the range 64,000–245,000 (Kobayashi and Matsuda 1980; Miller *et al*. 1986; Fu and Robyt 1990; Goyal and Katiyar 1994; Purama and Goyal 2007b). Glucansucrases are highly sensitive to pH, temperature and the dilution (Miller and Robyt 1984). A purified glucansucrase from *Leuconostoc mesenteroides* B512F enzyme exhibited maximum activity at 30°C and pH 5.2 (Goyal *et al.* 1995). The glucansucrase 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 glucansucrase from *Leuconostoc mesenteroides* IBT-PQ was 35°C with half-life of 137 min

(Chellapandian *et al*. 1998). Kinetic studies of the glucansucrases are affected by the presence of glucan. Different reports are available on the K_m by glucansucrases. Glucansucrase from *Leuconostoc mesenteroides* NRRL B-1299 exhibited a K_m of 10.7 mM (Kobayashi and Matsuda 1976). The K_m of 14.9 mM, 16 mM and 47 mM was reported for glucansucrase from *Leuconostoc mesenteroides* NRRL B-512F (Kobayashi *et al.* 1986; Miller *et al.* 1986; Goyal *et al.* 1995).

Divalent cations are associated with glucansucrases, they stabilize the catalytic activity of enzymes by stabilizing the three-dimensional protein structure (Robyt and Walseth 1979; Goyal *et al.* 1995). The activity of enzyme was enhanced by the addition of Ca^{2+} and Co^{2+} and inhibited by EDTA indicating that glucansucrases are associated with alkaline earth metals (Robyt and Walseth 1979; Kobayashi and Matsuda 1980). Ca^{2+} ions have been reported to be associated with the two catalytic sites of glucansucrases (Miller and Robyt 1986). One of the sites is activating and the other involved in sucrose binding is inhibitory. Ca^{2+} at the inhibitory site prevents binding of sucrose (Miller and Robyt 1986). Heavy metal ions such as zinc, cadmium, lead, mercury and copper ions showed varied inhibitory effect on the enzyme activity (Robyt and Walseth 1979). Glucansucrase 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).

The removal of associated glucan from glucansucrase resulted in the loss of enzyme activity (Robyt and Walseth 1979). The dilution of the purified glucansucrase 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-20000, methylcellulose and non-ionic

detergent Tween 80 (Miller and Robyt 1984). The glucansucrase stability was observed at different pH and temperature with time (Rodrigues *et al.* 2003). The activity loss at -15°C and 4°C was much less as compared to the loss at 25°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 glucansucrase 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).

1.9 Objectives of the present study

Newer polysaccharides from different microbial sources are being screened for potential industrial applications. More and more polysaccharides specifically glucans have been reported to exhibit a variety of biological activities, while traditionally they have found numerous applications in the pharmaceutical, food, agriculture and fine chemical industries. A glucan comprising α-(1→6) and α-(1→4) linkages is produced by *Leuconostoc dextranicum* NRRL B-1146. The greater interest in this glucan arises due to the potential it holds, as an anti-cancer and immunomodulatory agent. A neutral polysaccharide like this has applications in the food, cosmetic and pharmaceutical industries. Prebiotic oligosaccharides have a beneficial impact on human health. Oligosaccharides with α-(1→6) and α-(1→4) linkages can be obtained from glucansucrase in the presence of suitable acceptor like maltose along with sucrose. Glucooligosaccharides have been produced by immobilized glucansucrase.

The aim of this present study was to study the strain of *Leuconostoc dextranicum* NRRL B-1146 for the production of glucan and glucansucrase. The optimization of medium composition and culture conditions for obtaining the maximum yields of glucansucrase and glucan were carried out. Different optimization strategies like OVAT, RSM and ANN-GA were used for medium optimization. The purification of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 was carried out using polyethylene glycol fractionation and gel-filtration chromatography. The purified glucansucrase was biochemically characterized. The glucan formed by the glucansucrase was purified by alcohol precipitation and chromoatography. The structure of glucan synthesized by glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 was carried out using FT-IR, ^{1}H-NMR and ^{13}C-NMR techniques. The

surface morphology of the glucan was studied using scanning electron microscopy. The rheological properties of the glucan were stdudied with respect to viscosity.

Specific objectives of the present study

1. Determine the characteristics of *Leuconostoc dextranicum* NRRL B-1146.
2. Optimization of glucansucrase production and its assay.
3. Effect of nutrients on glucansucrase production.
4. Optimization of glucansucrase production using response surface methodology.
5. Optimization of glucansucrase production using artificial neural network and genetic algorithm techniques.
6. Purification, identification and properties of glucansucrase.
7. Optimization of glucan production by statistical approach.
8. Structural determination and surface characterization of glucan.

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Chapter 2

Characteristics of *Leuconostoc dextranicum* NRRL B-1146

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). The organisms of *Leuconostoc* genus are micro-aerophilic. The first classification of the genus separated three species, *Leuconostoc mesenteroides*, *Leuconostoc dextranicum*, *Leuconostoc citrovorus*, on the basis of sucrose and pentoses (arabinose, xylose) fermentation (Hucker and Pederson 1931). The genus was divided into six groups based on phenotypic characterization (Garvie 1960). Hontebeyrie and Gasser (1977) used DNA/DNA and RNA/DNA hybridizations and proposed that six species should be reduced to four, *Leuconostoc dextranicum* and *Leuconostoc cremoris* being species of *Leuconostoc mesenteroides*, which formed a single DNA homology group. Phenotypic features were used by Garvie (1984) to propose differentiation of the genus *Leuconostoc* from other lactic acid bacteria based on carbohydrate fermentation patterns between species. *Leuconostoc dextranicum* is commonly found in starter cultures used in dairy industry, where they are involved in texture and flavor production.

Glucansucrases from *Leuconostoc* genus are inducible enzymes, which are induced in the presence of sucrose (Neely and Nott 1962). 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 dextranicum* NRRL B-1146. In the present study, three glucansucrase producing *Leuconostoc* strains *viz. Leuconostoc mesenteroides* B-512F, *Leuconostoc dextranicum* NRRL B-1146 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

The *Leuconostoc* strains *viz. Leuconostoc mesenteroides* B-512F, *Leuconostoc dextranicum* B-1146 and *Leuconostoc citreum* 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 the strains were grown at 28°C for all experiments.

2.2.2 Antibiotic sensitivity

The *Leuconostoc* strains were tested for susceptibility to 30 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 was added to the lysis mixture, mixed by vortexing 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. The

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 in 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 analyzed by using agarose gel (0.8%) electrophoresis and 1xTAE (40 mM Tris, 20 mM acetic acid, 1 mM 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 glucansucrase 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 three *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 colour bands within the gel matrix appeared, which confirmed glucansucrase 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 resistance of Leuconostoc strains

A standardized filter-paper disc-agar diffusion assay was 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 organism to be tested. Following the incubation, the plates are examined for the presence of growth inhibition, which is indicated by a clear zone surrounding each disc. 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 (Fig. 2.3.1). A measurement of the diameter of the zone of inhibition is made and its size is compared to that contained in a standardized chart. Based on this comparison, the test organism is determined to be resistant or susceptible to the antibiotic (Fig. 2.3.1). The three *Leuconostoc* strains were tested for susceptibility to thirty antibiotics representing the major antibiotics. All the three strains were resistant to the antibiotics co-trimazine, norflaxacin, norfloxacin and vancomycin (Table 2.3.1). All the three strains were sensitive to amoxycillin, bacitracin, carbenicillin, cephalothin, cephatoxamine, chlorampenicol, clindamycin, linomycin, oxytetracyclin and tetracyclin. The data is 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 Table 2.3.1 it can be seen that the antibiotic sensitivity test of the strain B-1146 was 90% similar to B-512F and 83 % similar to B-742 and the strain B-512F was 77% similar to the strain B-742 in their antibiotic sensitivity patterns.

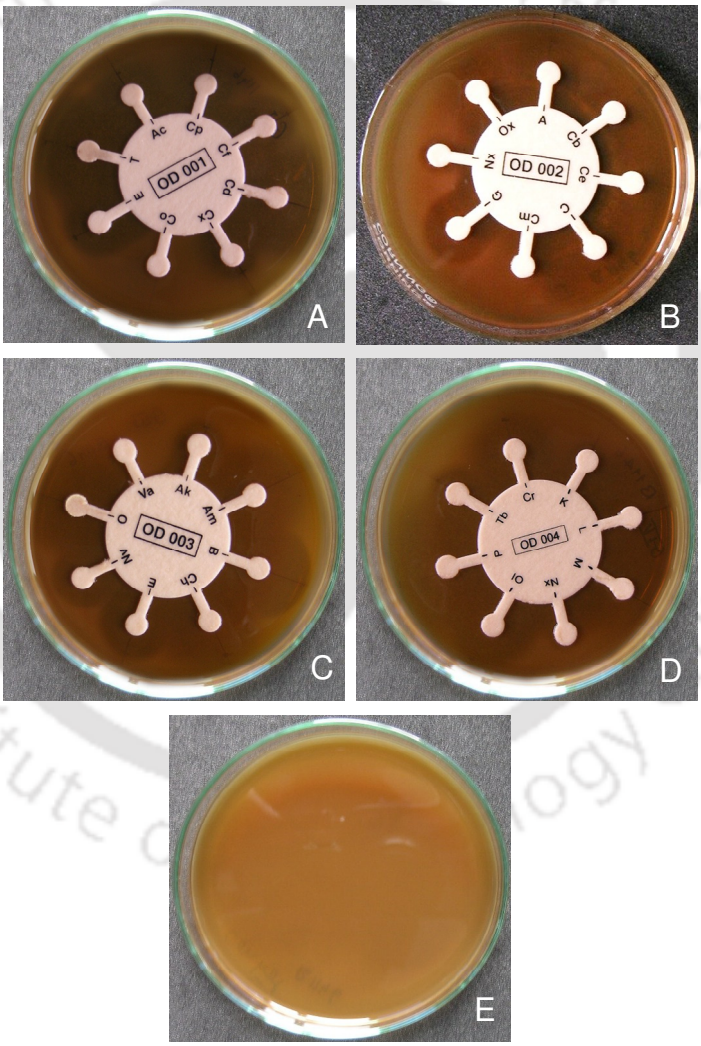

Fig. 2.3.1. Antibiotic susceptibility patterns of *Leuconostoc dextranicum* NRRL B-1146. Fig(s). A-D show susceptibility of *Leuconostoc dextranicum* NRRL B-1146 against different antibiotics abbreviated (as explain in Table 2.3.1) and Fig. 2.3.1E is the control showing the lawn of the grown microorganism without the antibiotic octodisc.

Table 2.3.1. Antibiogram of *Leuconostoc* strains using antibiotic discs on MRS medium

S. No.	Antibiotic	Concentration (µg)	B-512F	B-742	B-1146
1.	Amoxyclav (Ac)	10	M	S	M
2.	Cephalexin (Cp)	10	M	S	M
3.	Ciproflaxacin (Cf)	10	M	M	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	R	M
14.	Norflaxacin (Nx)	10	R	R	R
15.	Oxacillin (Ox)	5	M	S	M
16.	Amikacin (Ak)	10	R	R	M
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	R	M
25.	Cephaloridine (Cr)	30	M	M	M
26.	Kanamycin (K)	30	M	R	R
27.	Linomycin (L)	2	S	S	S
28.	Methicillin (M)	5	S	S	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.

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 made it imperative that all cultures should be observed after 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 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 B-1146. 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 2.3.2). *Leuconostoc* strains B-512F and B-1146 were able to weakly ferment galactose and xylose (Table 2.3.2). The *Leuconostoc* strain B-742 fermented cellobiose. And the raffinose was fermented by both B-512F and B-1146 (Table 2.3.2). *Leuconostoc* strains B-512F and B-1146 had nearly identical fermentation profiles, whereas, *Leuconostoc* strain B-742 showed quite different fermentation pattern. Most of these results of strains 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 after 7 days of incubation. As mentioned earlier that prolonged incubation might have caused production of alkali as a result of certain enzymatic action on substrates other than the carbohydrate (Holt *et al.* 2001).

Table 2.3.2. Carbohydrate fermentation of *Leuconostoc* strains after 48h.

S. No.	Carbohydrate	B-512F	B-1146	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

2.3.3 Plasmid profile of *Leuconostoc dextranicum* NRRL B-1146

The plasmid profiles of three glucansucrase producing *Leuconostoc* strains were determined (Fig. 2.3.2). The *Leuconostoc* strain B-742 did not contain any plasmid and B-512F contained a single plasmid and showed a single band (data not shown) as reported earlier (Holt *et al.* 2001). *Leuconostoc dextranicum* B-1146 contained at least 3 plasmids (Fig. 2.3.2 A,B, lane 2) that were estimated to be approximately, 1.5 kb (Fig. 2.3.2 A, lane 2), 5.7 kb (Fig. 2.3.2 A, lane 2 and also Fig. 2.3.2 B, lane 2 when compared with lane 3 marker) and 23.5 kb (Fig. 2.3.2 A,B, lane 2) in size. (The size of the plasmid is just an approximation as they are compared with linear DNA ladder, however the size 5.7 kb was compared with a recombinant

plasmid of the same size Fig. 2.3.2 B, lane 3). 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 Vaughan *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.

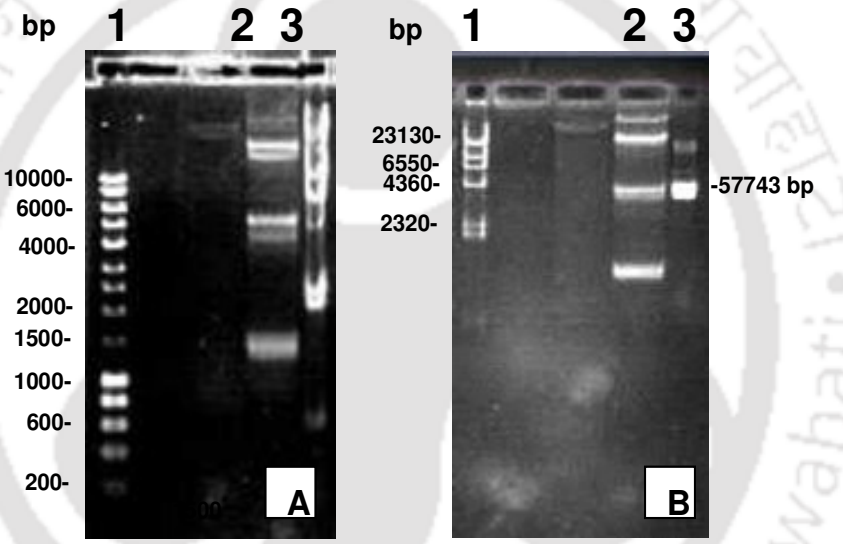

Fig. 2.3.2. Plasmid profile of *Leuconostoc* strains examined by 0.8% agarose gel electrophoresis. **A)** Lane 1: DNA ladder (10,000 bp - 200bp); lane 2: *Leuconostoc* strain B-1146 and lane 3; λDNA Hind III digest **B)** Lane 1: λDNA Hind III digest; lane 2: *Leuconostoc* strain B-1146 and Lane 3: a recombinant plasmid of 5.7 kbp size

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 glucan-producing *Leuconostoc* strains (Leathers *et al.* 1997). Polyacrylamide gel electrophoresis was used for *in situ* detection of enzyme activity to characterize sucrase production by dextran-producing *Leuconostoc* strains (Leathers *et al.* 1997). This study, however, 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. 2.3.3 A). The strain B-1146, showed extremely faint band that could be detected only by activity staining. All three *Leuconostoc* strains produced at least one detectable glucan-synthesizing activity bands (Fig. 2.3.3 B). *Leuconostoc* strain B-1146 was the only strain to produce single faint activity band for glucansucrase (Fig. 2.3.3 lane 3) of the molecular size 205 kDa. The *Leuconostoc* strain B-512F showed a band of 180 kDa, however, B-512F showed an extra faint band (Fig. 2.3.3 B). The *Leuconostoc* strain B-742 displayed the multiple activity bands compared with the other strains tested (Fig. 2.3.3 B, lane 2). *Leuconostoc* strain B-742 produced three close activity bands, and two were prominent and one band was very faint (Fig. 2.3.3). The results of B-742 are similar to those reported earlier (Holt *et al.* 2001) except that we found more intensity of the two bands. However, for the strain B-512F our results were different from earlier reported (Holt *et al.* 2001). We found that B-512F shows two bands corresponding to 188 kDa and 146 kDa sizes, whereas they reported two bands of 146 kDa and 118 kDa sizes. It has also been reported earlier that the purified glucansucrase from B-512F resulted molecular size of 188 kDa (Goyal and Katiyar

1994). The results of B-742 are similar to those reported earlier (Holt *et al.* 2001) except that we found more intensity of the two bands.

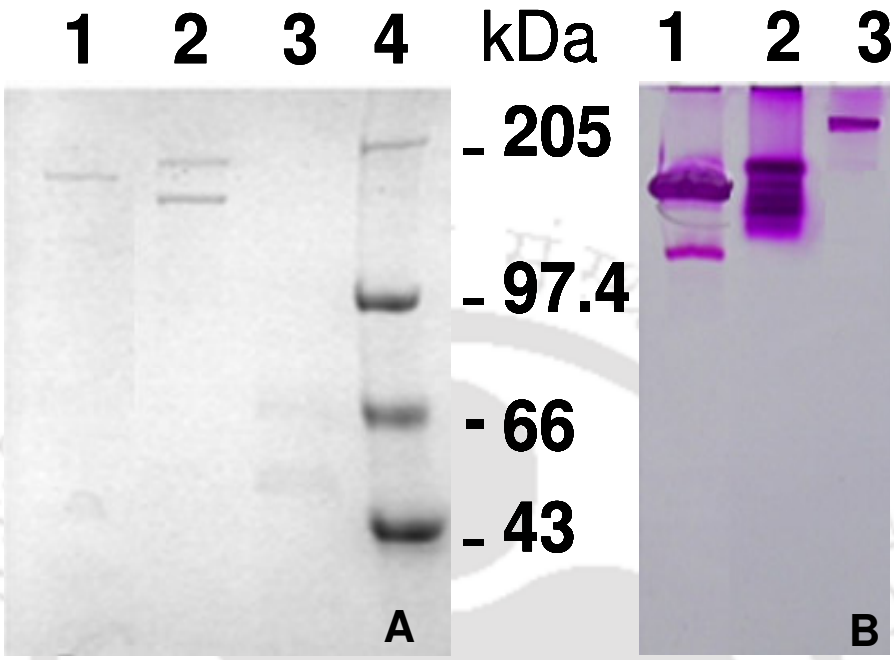

Fig. 2.3.3. Glucansucrase activity patterns by glucan producing *Leuconostoc* strains; **A)** Coomassie Brilliant Blue staining **B)** Activitiy staining using periodic acid Schiff protocol.
Lanes: (1) B-512F; (2) B-742; (3) B-1146 and (4) Mol wt marker.

2.4 Conclusions

Three glucansucrase producing *Leuconostoc* strains *viz. Leuconostoc dextranicum* NRRL B-1146, *Leuconostoc mesenteroides* B-512F, *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 glucansucrase producing *Leuconostoc* strain B-1146 have never been reported earlier. All the three strains were resistant to the antibiotics cotrimazine, norflaxacin, norfloxacin and vancomycin. All 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-1146 possessed 3 plasmids of sizes 1.5 kb, 5.7 kb and 23.5 kb. The strain B-1146 showed sucrose hydrolyzing activity by displaying the glucan formation pattern in the native polyacrylamide gel. In present study it was found that B-512F shows two bands corresponding to 188 kDa and 146 kDa molecular sizes whereas, Holt *et al.* 2001 reported two bands but of 146 kDa and 118 kDa molecular sizes. The glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 showed a single band of 205 kDa molecular size. 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 glucansucrase producing new *Leuconostoc* strains, which can prove to be better strains for industrial applications.

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Chapter 3

Optimization of Conditions for Glucansucrase Production and its Assay

3.1 Introduction

Glucansucrases are large extracellular enzymes capable of synthesizing various glucans from sucrose *viz*. dextran, mutan and alternan (Monchois *et al*. 1999). Four different genera of lactic acid bacteria: *Streptococcus*, *Leuconostoc*, *Weissella* and *Lactobacillus* are known to produce glucansucrases (Purama and Goyal 2005). Glucansucrases from *Leuconostoc* genus are inducible enzymes, which are induced in the presence of sucrose (Neely and Nott 1962). The production of glucansucrases is affected by factors like temperature, aeration, medium components (Tsuchiya *et al.* 1952; Lazic *et al.* 1993; Goyal *et al.* 1995; Rodrigues *et al.* 2003; Cortezi *et al.* 2005). The fermentation temperature ranging from 20 to 30°C have been used for glucansucrase production from *Leuconostoc* (Tsuchiya *et al*. 1952; Kobayashi and Matsuda 1976; Robyt and Walseth 1979; Cortezi *et al*. 2005). Organisms of the *Leuconostoc* genus are considered micro-aerophilic. There are reports on the production of glucansucrase using shaken and static flask cultures (Tsuchiya *et al*. 1952; Goyal *et al.* 1995; Shamala and Prasad 1995). The glucansucrase from

Leuconostoc mesenteroides NRRL B-512F (Miller and Robyt 1984, Goyal *et al.* 1995) and *Leuconostoc mesenteroides* NRRL B-640 (Purama and Goyal, 2007) exhibited an apparent optimum temperature of 30˚C (Miller and Robyt 1984, Goyal *et al.* 1995). The glucansucrase 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 2007). There is no report available on the optimization of culture conditions for *Leuconostoc dextranicum* NRRL B-1146. The present chapter describes the optimization of culture conditions for glucansucrase production and its assay from *Leuconostoc dextranicum* NRRL B-1146.

3.2 Materials and Methods

3.2.1 Microorganism

The bacterial culture strain *Leuconostoc dextranicum* NRRL B-1146 was obtained from the Agricultural Research Service Culture Collection, National Centre for Agricultural Utilization Research, Peoria, USA. The culture was maintained in modified MRS (sucrose in place of glucose, DeMan *et al*. 1960) as a stab at 4°C and sub cultured every 2 weeks. The medium for the bacterial stab contained in (g/l): sucrose, 20; yeast extract, 5; beef extract, 10; K_2HPO_4, 2; sodium acetate, 5; tri-sodium citrate, 2; Tween 80, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.2.

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 inoculations and culture transfers were carried out under aseptic conditions using laminar air flow chamber.

3.2.3 Maintenance and sub-culturing of *Leuconostoc dextranicum*

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. The modified MRS medium was prepared by substituting sucrose with glucose as a carbon source.

3.2.4 Inoculum preparation

A freshly grown 12h culture of *Leuconostoc dextranicum* NRRL B-1146 in Tsuchiya medium (Tsuchiya *et al.* 1952) containing (g/l) sucrose, 20; yeast extract, 20; K_2HPO_4, 20; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01; NaCl 0.01. The pH of the medium was adjusted to 6.9 with 0.1 M HCl solution. Inocula were prepared by transferring a loopful from stab culture to 5 ml Tsuchiya medium incubated at 28°C. Then 1 ml was transferred to 250 ml Erlenmeyer flask containing 100 ml of sterile Tsuchiya medium. 5 ml of this inoculum whose growth was judged by optical density (600 nm) was used for inoculation. The inoculum cell density was maintained constant for every experiment and appropriate dilutions were made when required.

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 Glucansucrase production

The standard production medium of Tsuchiya was used as a basal medium. The effect of physical parameters like temperature and agitation on glucansucrase production was tested in 250 ml Erlenmeyer flask containing 100 ml of sterile Tsuchiya medium. The fermentation was allowed to proceed for 12h at 28°C under static conditions. 5 ml of the sample from fermenting broth was withdrawn and centrifuged at 9200***g*** and 4°C for 15 min and the glucansucrase activity in the

supernatant was determined. All the experiments were performed in triplicates and the enzyme assay were performed in duplicates.

3.2.7 Enzyme activity assay

The assay of glucansucrase was carried out in 1 ml of a reaction mixture volume in 20 mM sodium acetate buffer (pH 5.4) containing 292 mM (10%) sucrose and using the cell free extract (20 µl) as the enzyme source. The reaction mixture was incubated at 30°C for 15 min. The enzyme activity was determined 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 glucansucrase activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar per min. The glucansucrase 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 glucansucrase and partially purified glucansucrase was estimated by the method of Lowry *et al.* 1951. Bovine serum albumin (BSA) 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 glucansucrase under different culture conditions

3.2.11.1 Effect of temperature

To determine the optimum incubation temperature for maximum enzyme production *Leuconostoc dextranicum* NRRL B-1146 was grown at five different temperatures 22°C, 25°C, 28°C, 30°C, 32°C and 35°C ± 0.2°C in the flasks containing 100 ml medium as described earlier (Section 3.2.6) in an incubator at static condition. The samples (5.0 ml) were withdrawn at different time intervals and centrifuged at 9,200***g*** for 10 min at 4°C to pellet out the cells. The cell free extract was analyzed for enzyme activity as described earlier in section 3.2.7.

3.2.11.2 Effect of static and shaken flask culture

The production of glucansucrase was compared under shaken flask condition with the static flask culture at 28°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 9,200***g*** for 10 min at 4°C to pellet out the cells. The cell free extract was analyzed for enzyme activity and as described earlier in section 3.2.7.

3.2.12. Partial purification and characterization of glucansucrase

3.2.12.1 Purification of glucansucrase

The crude glucansucrase with (0.5 U/ml) was purified by adding ice-cold polyethylene glycol 400 to obtain a final concentration of 30% (v/v). The mixture was kept at 4°C for 12h and the glucansucrase fraction was allowed to precipitate. The fractionated glucansucrase was separated by centrifugation at 13,200***g*** for 30 min at

4°C. The partially purified glucansucrase was subjected to dialysis using a 5 kDa cut off membrane using 20 mM sodium acetate buffer pH 5.4 with three changes. The partially purified glucansucrase having a specific activity of 4.5 U/mg and a concentration of 1.45 mg/ml was used for further biochemical characterization.

3.2.12.2 Effect of sucrose concentration on glucansucrase activity

The effect of sucrose concentration on enzyme activity was studied by varying its concentration between 0.1-11% in the enzyme reaction mixture. The purified glucansucrase with 4.5 U/mg specific activity obtained after 30% PEG-400 fractionation was used for the study of effect of sucrose concentration on glucansucrase activity. The reaction was carried out in 1 ml mixture in 20 mM sodium acetate buffer pH 5.4 containing 20 µl of enzyme (1.45 mg/ml) and varying concentration of sucrose. The mixture was incubated at 30°C for 15 min in water bath 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 glucansucrase activity

The enzyme activity of partially purified glucansucrase with specific activity 4.5 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 292 mM (10%) final concentration of sucrose and 20 µl of enzyme (1.45 mg/ml) for 15 min. The assay mixture was incubated at different temperatures for 15 min. Aliquots (0.1 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 glucansucrase activity

The glucansucrase activity assay was performed at 8 different pH points ranging from 4.2 to 5.6, with 0.2 unit variation. The assays were carried out in 1 ml reaction mixture containing 292 mM (10%) final sucrose concentration in 20 mM sodium acetate buffer of different pH. The reaction mixture was incubated at 30°C for 15 min. Aliquots (0.1 ml) of reaction mixture was taken for reducing sugar analysis and enzyme activity determination as described earlier in Section 3.2.7.

3.2.12.5 Effect of ionic strength on glucansucrase activity

The glucansucrase activity was determined in a broad range of ionic strength between 5 mM-500 mM sodium acetate buffer pH 5.4. The assays were performed in 1 ml reaction mixture containing 292 mM (10%) final concentration of sucrose at 30°C for 15 min. Aliquots (0.1 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 glucansucrase

Temperatures ranging from 22°C to 35°C were studied for production of enzyme under static condition. An incubation temperature of 28°C gave a maximum of 3.0 U/ml enzyme activity (Fig. 3.3.1). The activity achieved at 22°C and 25°C was 10% and 7% respectively, lower than the optimum temperature. At these temperatures the cell growth was slow which might be the cause for lower enzyme production and activity. The enzyme activity decreased as the temperature increased above 28°C, which might be due to the enzyme deactivation at higher temperatures. Even at 30°C a 16% decrease in enzyme activity was observed (2.5 U/ml) and the enzyme activity reduced by 27% at 35°C when compared with activity at 28°C. Most of the *Leuconostoc* strains were reported to produce glucansucrase 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.* 2005). The enzyme production from *Leuconostoc mesenteroides* NRRL B-640 was favoured, when the culture was grown at 25°C (Purama and Goyal 2007), while Goyal *et al.* (1995) reported the maximum production of glucansucrase from *Leuconostoc mesenteroides* NRRL B-512F at 23°C. The effect of temperature on glucansucrase production from *Leuconostoc mesenteroides* FT-045 in comparison to *Leuconostoc mesenteroides* NRRL B-512F was studied using fermentation by Cortezi *et al.* (2005). They reported 23°C as the optimum temperature for both the strains FT-045 and B-512F.

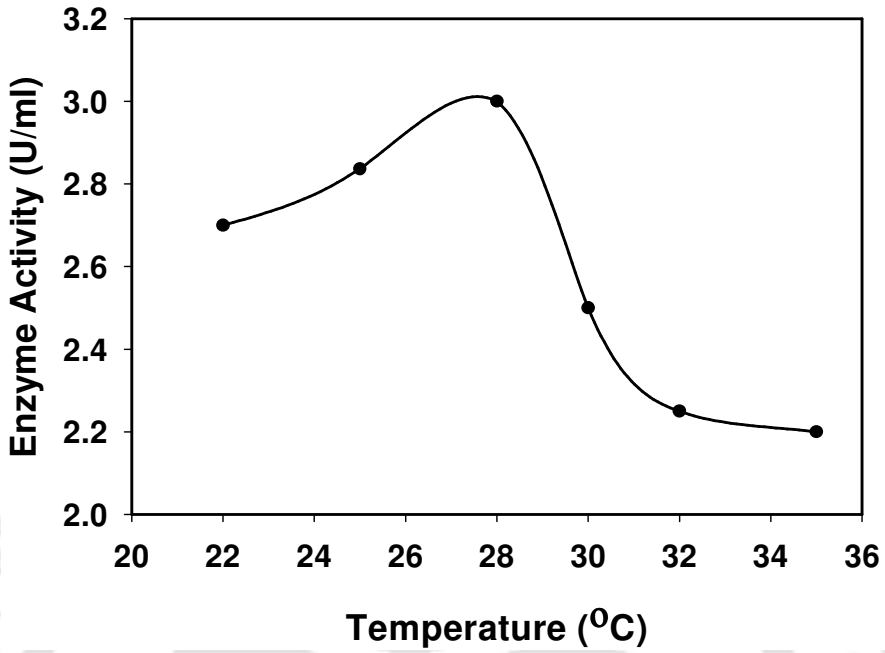

Fig. 3.3.1. Effect of temperature on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146. The maximum enzyme activity obtained at each temperature was plotted.

3.3.2 Effect of shaking on production of glucansucrase

The production of glucansucrase was observed under shaking and static conditions at 28°C. An activity of 3 U/ml was observed in static condition while it was 2.5 U/ml in shaking condition at 200 rpm. The static condition favoured the enzyme production by 16% over shaking condition, which is in accordance with microaerophilic nature of *Leuconostoc* sp. Similar results were reported for *Leuconostoc mesenteroides* NRRL B-512F where, the maximum enzyme production occurred under static conditions (Goyal *et al.* 1995).

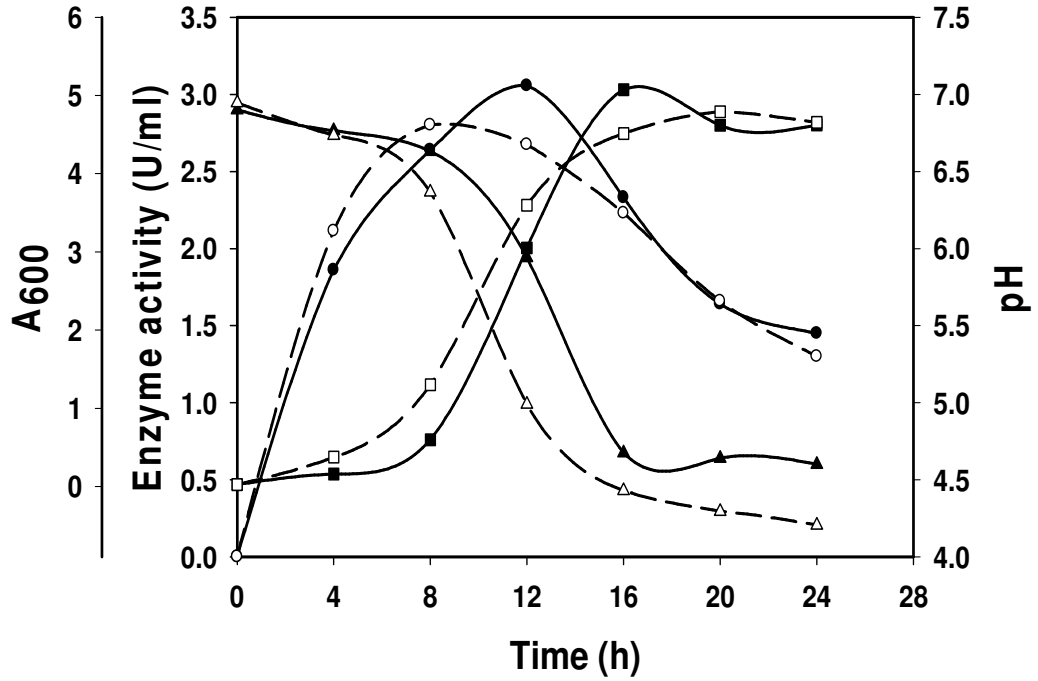

Fig. 3.3.2. Effect of shaken and static flask culture on glucansucrase production.The enzyme activity (○), cell growth (□) and pH profiles (Δ) of *Leuconostoc dextranicum* NRRL B-1146 under shaken (----) culture condition. The enzyme activity (●), cell growth (■) and pH profiles (▲) of *Leuconostoc dextranicum* under static flask (—) culture condition are shown.

3.3.3 Optimization of conditions for glucansucrase assay

3.3.3.1 Effect of sucrose concentration on glucansucrase activity

Partially purified glucansucrase with 4.5 U/mg was used to study the effect of sucrose concentration on glucansucrase activity by varying sucrose concentration between 0.1-11% final concentrations in the assay mixture. The results showed that it follows the classical Michaelis-Menten kinetics and the saturation reached at 10% (Fig. 3.3.4). The final sucrose concentration of 10% was taken as optimum concentration for further glucansucrase activity assay. Similar result of 10% sucrose as an optimum concentration has been reported for glucansucrase from *Leuconostoc mesenteroides* NRRL B-512F (Goyal and Katiyar 1995). However, 5% sucrose was the optimal concentration for the glucansucrase from *Leuconostoc mesenteroides* NRRL B-640 (Purama and Goyal 2007).

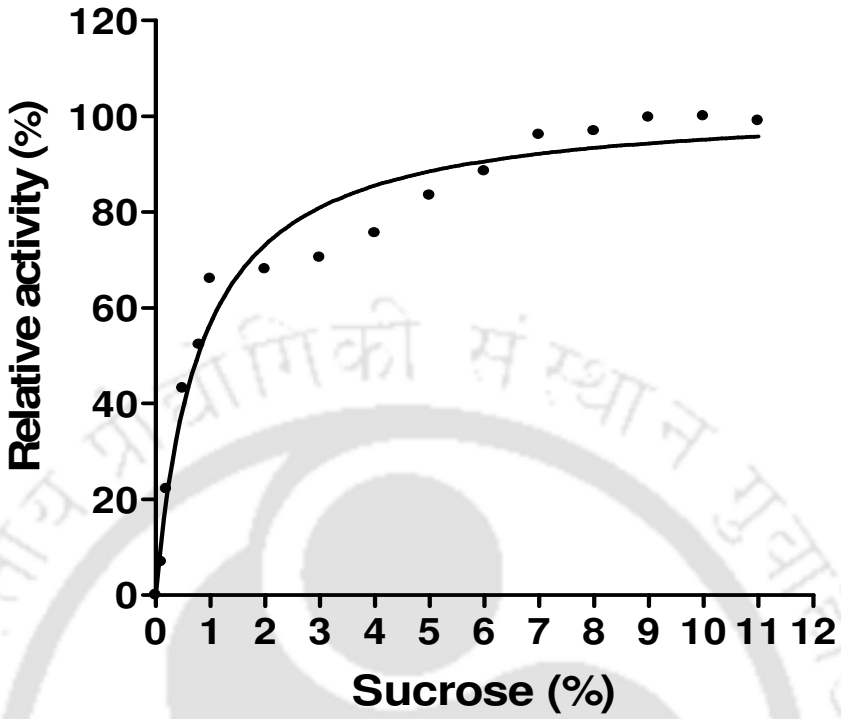

Fig. 3.3.4. Effect of sucrose concentration on glucansucrase activity assayed at 30°C in 20 mM sodium acetate buffer, pH 5.4.

3.3.3.2 Effect of temperature on glucansucrase activity

The partially purified glucansucrase was maximally active at 30℃ with a specific activity of 4.5 U/mg at pH 5.4 in 20 mM sodium acetate buffer (Fig. 3.3.5). Severe loss of activity was observed on either side of 30℃. The enzyme activity drastically decreased after 35℃ and it decreased by 60%. The enzyme activity was completely lost at 40℃. These results are in accordance with the earlier findings that glucansucrase from most of the *Leuconostoc* strains such as IBT-PQ, B-512F and B-1355 exhibit the optimum temperature for enzyme activity with in the range of 30 to 35°C (Lopez *et al.* 1980; Chellapandian *et al.* 1998; Purama and Goyal 2007).

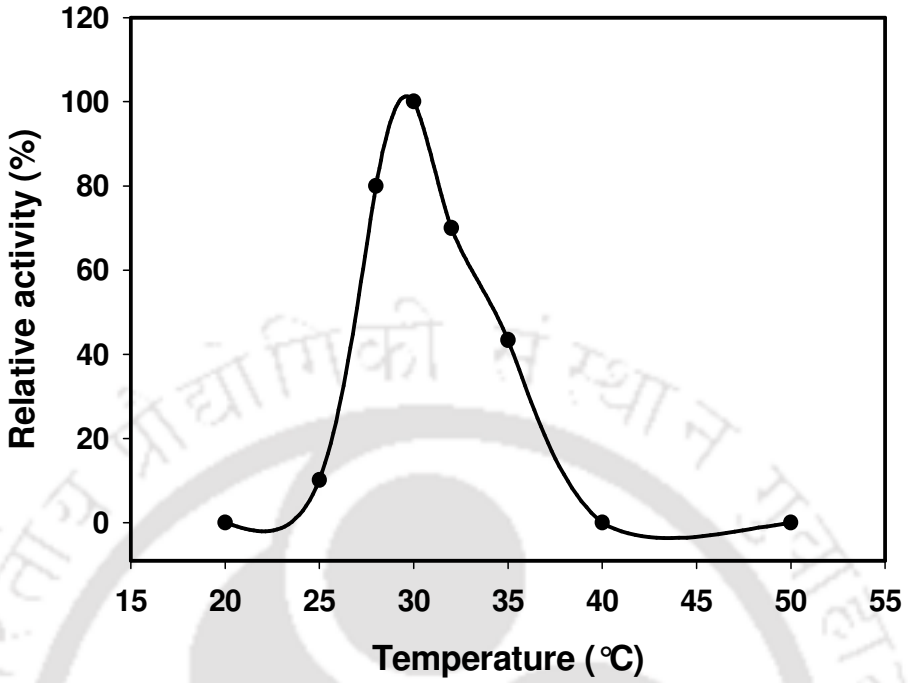

Fig. 3.3.5. Effect of temperature on glucansucrase activity. The enzyme activity was determined by carrying out the assay in 20 mM sodium acetate buffer (pH 5.4) containing 10% sucrose.

3.3.3.3 Effect of pH on activity of glucansucrase

The maximum glucansucrase activity was observed at pH 5.4 with specific activity of 4.5 U/mg (Fig. 3.3.6). The maximum enzyme activity was observed at pH 5.4. The enzyme was quite stable below the pH 5.2, up to pH 4.2. A 4% reduction in activity was observed at pH 5.2. The enzyme was stable below the pH 5.2, up to pH 4.2 and retained 60% of the initial enzyme activity. However, by increasing the pH by only 0.2 units to 5.6, the enzyme activity dropped to 10%. The optimum pH for maximum glucansucrase activity is 5.4. The optimum pH of 5.4 of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 is similar to the strains, *Leuconostoc mesenteroides* NRRL B-512F (Goyal *et al.* 1995) and NRRL B-640 (Purama and Goyal 2007).

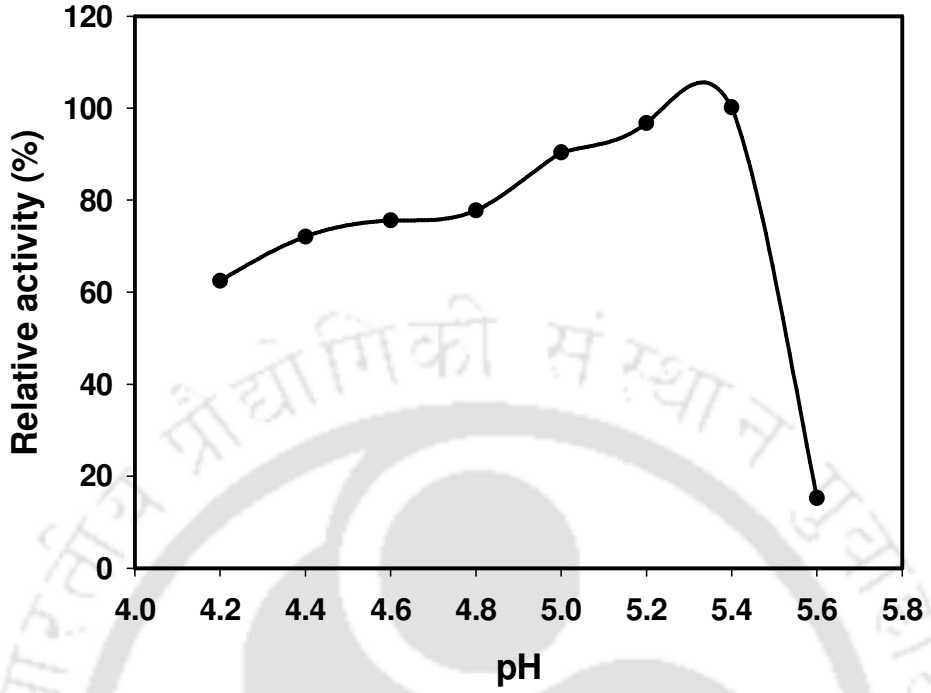

Fig. 3.3.6. Effect of pH on glucansucrase activity. The enzyme activity was determined by carrying out the assay in 20 mM sodium acetate buffer containing 10% sucrose.

3.3.3.4 Effect of ionic strength on glucansucrase activity

The glucansucrase activity was stable at lower ionic strength of 5-50 mM sodium acetate buffer with a variation in specific activity between 4.5-4.3 U/mg (Fig. 3.3.7). The decrease in enzyme activity was significant with increase of ionic strength beyond 50 mM. The loss of activity at 100 mM was 20% and at 500 mM concentration there was 50% loss of enzyme activity. Glucansucrases are generally fairly stable at all ionic strengths of acetate buffer. The glucansucrase of *Leuconostoc mesenteroides* NRRL B-640 was stable in sodium acetate buffer in range of 10 to 500 mM concentration.

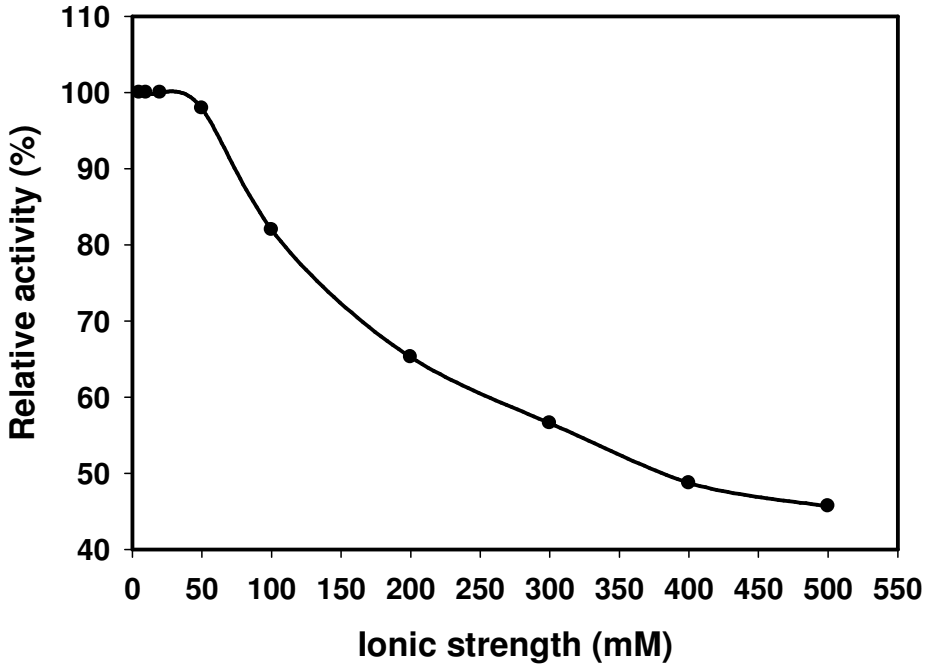

Fig. 3.3.7. Effect of ionic strength on glucansucrase activity. The enzyme activity was determined by carrying out the assay in sodium acetate buffer of different ionic strengths (pH 5.4) containing 10% sucrose.

3.4 Conclusions

The optimum culture conditions for enzyme production are species as well as strain specific. The culture conditions like incubation temperature and shaking or static flask conditions affect the growth of microorganism and also influence the enzyme production. In this study, optimization of the cultural conditions for glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146 was investigated. The enzyme production was favoured, and resulted in higher enzyme activity when the culture was grown at 28°C and under static flask culture. Most of the *Leuconostoc* strains were reported to produce glucansucrase with maximum activity in the temperature range of 20-30 °C (Goyal *et al.* 1995; Dols *et al.* 1998; Santos *et al.* 2000; Cortezi *et al.* 2005).

Glucansucrase was partially purified by PEG 400 fractionation. The partially purified enzyme gave an activity of 4.5 U/mg. The assay conditions for the glucansucrase were optimized using partially purified enzyme. The glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 was maximally active at 10% sucrose, 30°C and at pH 5.4 of sodium acetate buffer of lower ionic strength between 10-50 mM. Similar results were reported from the strains, *Leuconostoc mesenteroides* NRRL B-512F (Goyal *et al.* 1995) and NRRL B-640 (Purama and Goyal 2007) except that the strain NRRL B-640 is maximally active at 5% sucrose. Further studies on the optimization of medium composition by One Variable At a Time Approach (OVAT), Response Surface Methodology (RSM) and Artificial Intelligence for maximizing the enzyme production from *Leuconostoc dextranicum* NRRL B-1146 are described in Chapters 4, 5 and 6, respectively.

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Chapter 4

Effect of Nutrients on Glucansucrase Production

4.1 Introduction

Glucansucrases from *Leuconostoc* genus are inducible enzymes, which are induced in the presence of sucrose (Neely and Nott 1962). Some *Leuconostoc* strains are shown to produce glucansucrase by the media containing sugars other than sucrose, though the glucansucrase levels are minimal, but when sucrose was used as substrate in the medium, the enzyme activity observed was several fold higher (Dols *et al*. 1998). Various nitrogen sources such as yeast extract, corn steep liquor, beef extract and peptone has been used for glucansucrase production Tsuchiya *et al*. 1952; Goyal *et al.* 1995; Shamala and Prasad 1995). The yield of enzyme is also affected by the type of yeast extract used. It was shown that different commercial grades of yeast extracts had significantly different effects on the final cell concentration and the enzyme yield (Barker and Ajongwen 1991). Peptone and beef extract separately in addition to yeast extract resulted in enhanced enzyme activity (Goyal and Katiyar 1997). An optimum concentration of 4% yeast extract was reported for glucansucrase production form *Leuconostoc mesenteroides* NRRL B-1299 (Dols *et al*. 1997). K_2HPO_4 acts as a buffering agent maintains the pH for longer duration and counter balances the pH lowered by the lactic acid produced during fermentation (Tsuchiya *et*

al. 1952; Goyal and Katiyar 1997; Rodrigues *et al*. 2003). At higher concentration of K_2HPO_4 higher production of glucansucrase in *Leuconostoc mesenteroides* NRRL B-512F was reported (Rodrigues *et al*. 2003). A 20% increase in the enzyme activity was reported at 0.3 M K_2HPO_4. It has been reported that use of a surfactant Tween 80, increases the enzyme production (Sato *et al*. 1989; Goyal and Katiyar 1997). The presence of Tween 80 in the medium affects the homogeneity of the broth and facilitates the nutrient and oxygen transfer to the microorganism. Micronutrients play a vital role in the growth of microorganisms. They affect the metabolic process and in turn affect the production of enzyme. Glucansucrases from the genera *Leuconostoc* show considerable response to the presence of micronutrients in the medium (Jeanes *et al*. 1948; Goyal and Katiyar 1997; Foucaud *et al*. 1997). Bellinger *et al.* (1997) showed the addition of Mg^{2+}, Mn^{2+} and amino acids stimulated the growth of most *Leuconostoc* strains. *Leuconostoc dextranicum* NRRL B-1146 produces a novel polysaccharide with α-(1→6) linkage and with α-(1→4) branches (as confirmed by ^{13}C-NMR and FTIR, data shown Chapter 9). The glucansucrase from this strain can be used for production of oligosaccharides by both fermentation and enzymatic synthesis under controlled conditions. The chapter describes the effect of nutrients on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146 studied by one variable at a time approach. The effects of different medium components were tested by changing the concentration of one variable, while keeping other variables constant.

4.2 Materials and Methods

4.2.1 Microorganism

Leuconostoc dextranicum NRRL B-1146 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 of culture and inoculum preparation

The cultures of *Leuconostoc dextranicum* NRRL B-1146 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 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 28°C, static conditions for 12-16h. 1% of the culture inoculum was used for the enzyme production.

4.2.4 Enzyme activity assay

The assay of glucansucrase was carried out in 1 ml of a reaction mixture in 20 mM sodium acetate buffer, pH 5.4, containing 292 mM (10%) sucrose and using the cell free extract (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.

4.2.5 Effect of nutrients on glucansucrase 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 28°C in static condition. 5 ml broth samples were periodically withdrawn and analyzed for glucansucrase activity as described in Chapter 3, Section 3.2.7. All the experiments were performed in triplicate and the enzyme assays were performed in duplicate.

4.2.5.1 Effect of sucrose

The effect of sucrose on glucansucrase production was studied by varying its concentration from 1 to 6% 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 used as control. The culture conditions and assay conditions were used as described in Section 4.2.5.

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 glucansucrase 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 and enzyme assay conditions were used as described in, Section 4.2.5.

4.2.5.3 Effect of peptone and beef extract

The effects of peptone and beef extract on glucansucrase 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 0.5% to 2%, whereas the beef extract was varied from 0.5% to 2% taking the medium as described by Tsuchiya *et al.* (1952) as control that contained no peptone or beef extract. The culture conditions and enzyme assay conditions were used as described in, Section 4.2.5.

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 1% (v/v) in the medium. The medium as described by Tsuchiya *et al.* (1952) containing no Tween 80 served as the control.

4.2.5.5 Effect of micronutrients on glucansucrase production

The effect of $MgSO_4$ on glucansucrase production was studied by varying the concentrations from 0.02 to 0.1%. The medium described by Tsuchiya *et al.* (1952) containing 0.02% $MgSO_4$ was taken as control. The effects of $FeSO_4$, $MnSO_4$, NaCl and $CaCl_2$ on enzyme production were studied separately by varying the concentration of the salts from 0.001 to 0.005%, taking the medium described by Tsuchiya *et al.* (1952) as control that contained 0.001% concentration of all the four salts in the production medium.

4.3 Results and Discussion

4.3.1 Effect of Sucrose

Leuconostoc dextranicum NRRL B-1146 when grown in enzyme production medium containing 2% sucrose that was used as control gave glucansucrase activity of 3 U/ml. With an increase in sucrose concentration from 2% to 4% the glucansucrase activity increased by 25% to 3.75 U/ml. The glucansucrase production attained saturation at 5% sucrose (Fig. 4.3.1, Table 4.3.1). This is in agreement with other reports where it was reported that, the maximum enzyme production was achieved at lower sucrose levels and above 4% sucrose there was no increase in enzyme activity for other *Leuconostoc mesenteroides* strains (Goyal and Katiyar 1997; Lopretti *et al*. 1999). They also reported a decrease in glucansucrase production at higher than 4% concentration of sucrose. The viscosity of the broth increased with sucrose concentration due to the subsequent formation of exopolysaccharide from the available and residual sucrose by the released enzyme, in the medium. Some *Leuconostoc* strains are shown to produce glucansucrase by the media containing sugars other than sucrose (Dols *et al*. 1998; Smith and Zahnley 1999), though the glucansucrase levels are minimal, but when sucrose was used as substrate the enzyme activity observed was several fold higher in the medium (Dols *et al*. 1998).

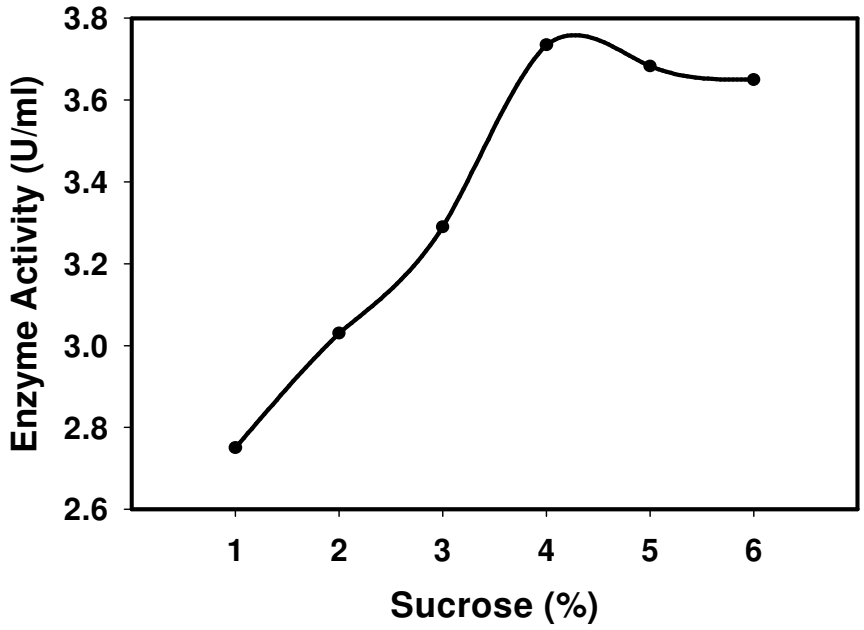

Fig. 4.3.1. Effect of sucrose on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146.

4.3.2 Effect of yeast extract and K_2HPO_4

An increase in yeast extract concentration from 1.5% to 2.0% caused an increase in glucansucrase activity at all concentrations of K_2HPO_4 (Fig. 4.3.2). However, with an increase in yeast extract from 2% (control) to 3% the enzyme activity decreased at all K_2HPO_4 concentration. This shows higher yeast extract does not support the enzyme production. Increase in the K_2HPO_4 concentration from 1.5% to 2.5% resulted in higher enzyme production at all yeast extract concentrations, whereas 3% K_2HPO_4 decreased the enzyme activity at all yeast extract concentrations. A maximum of 3.75 U/ml of enzyme activity was obtained with 2.5% yeast extract and 2.5% K_2HPO_4 (Fig. 4.3.2, Table 4.3.1), showing a 25% increase, in comparison

to the control. Different nitrogen sources, such as yeast extract, beef extract and peptone were chosen, as nitrogen sources were found to significantly affect glucansucrase production (Tsuchiya *et al*. 1952; Barker and Ajongwen 1991). Tsuchiya *et al.* (1952) reported requirement of higher nitrogen sources and other nutrients for the maximal enzyme formation. 2% yeast extract was found to be optimal for the production of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146, increasing the concentration of yeast extract inhibited the production of enzyme. These results are similar to those reported by Dols *et al.* (1997), where an increase in yeast extract concentration from 2% to 4% showed only a marginal increase in glucansucrase production from *Leuconostoc mesenteroides* NRRL B-1299 and its further increase did not cause any increase the glucansucrase production (Dols *et al*. 1998). Goyal and Katiyar (1997) also reported a decrease in enzyme production from *Leuconostoc mesenteroides* NRRL B-512F with increasing the yeast extract concentration.

K_2HPO_4 acts as a buffering agent to the lactic acid produced during the fermentation (Tsuchiya *et al*. 1952; Rodrigues *et al*. 2003). In *Leuconostoc dextranicum* NRRL B-1146 there was an increase in the enzyme production with increasing concentrations of K_2HPO_4. At a concentration of 2.5% and there was a 17% increase in the enzyme activity. The increase in concentration of K_2HPO_4 increased the production of glucansucrase in *Leuconostoc mesenteroides* NRRL B-512F and 20% increase of the enzyme activity was reported at 0.3 M K_2HPO_4 (Rodrigues *et al*. 2003).

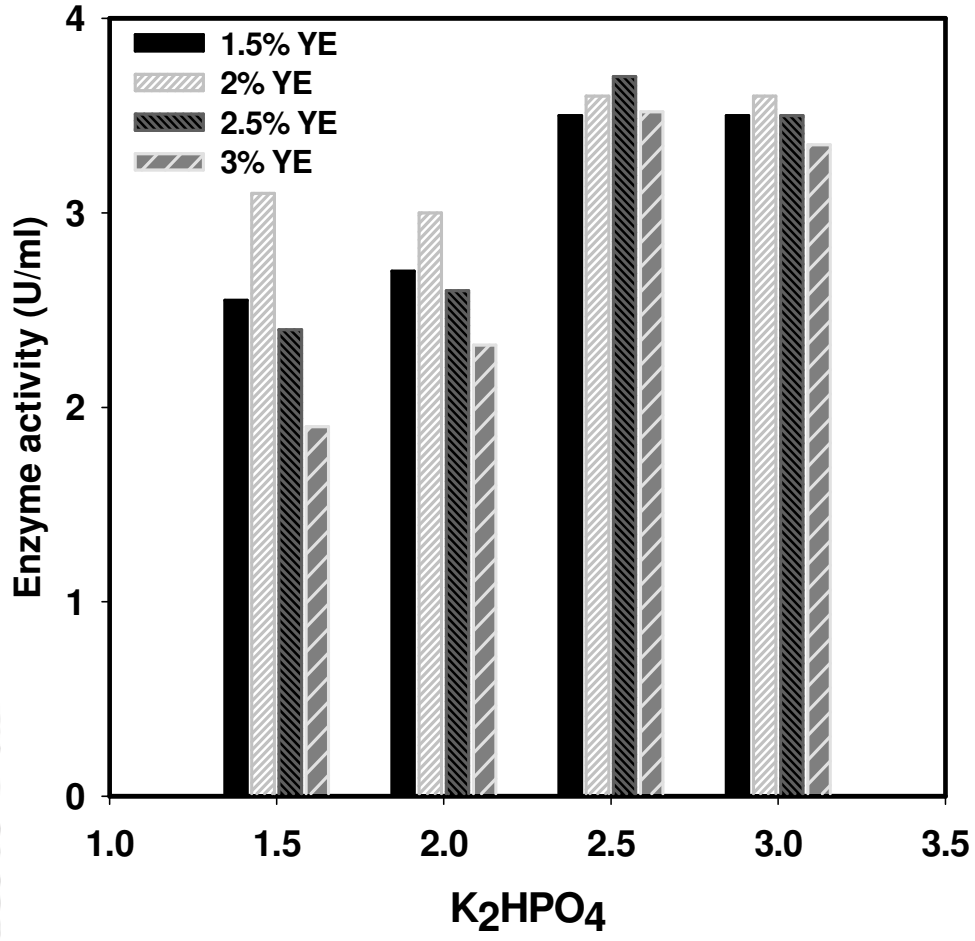

Fig. 4.3.2. Effect of yeast extract and K_2HPO_4 on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146.

4.3.3 Effect of beef extract and peptone

Effect of beef extract on glucansucrase production was studied by varying the concentration from 0.5% to 2%. The addition of 1% beef extract to control medium showed 10% increase in glucansucrase activity (Fig. 4.3.3, Table 4.3.1). Further increase in beef extract concentration beyond 1%, did not favor the enzyme production, rather a decrease in enzyme production was observed. This might be due to the effect of certain trace elements present in beef extract.

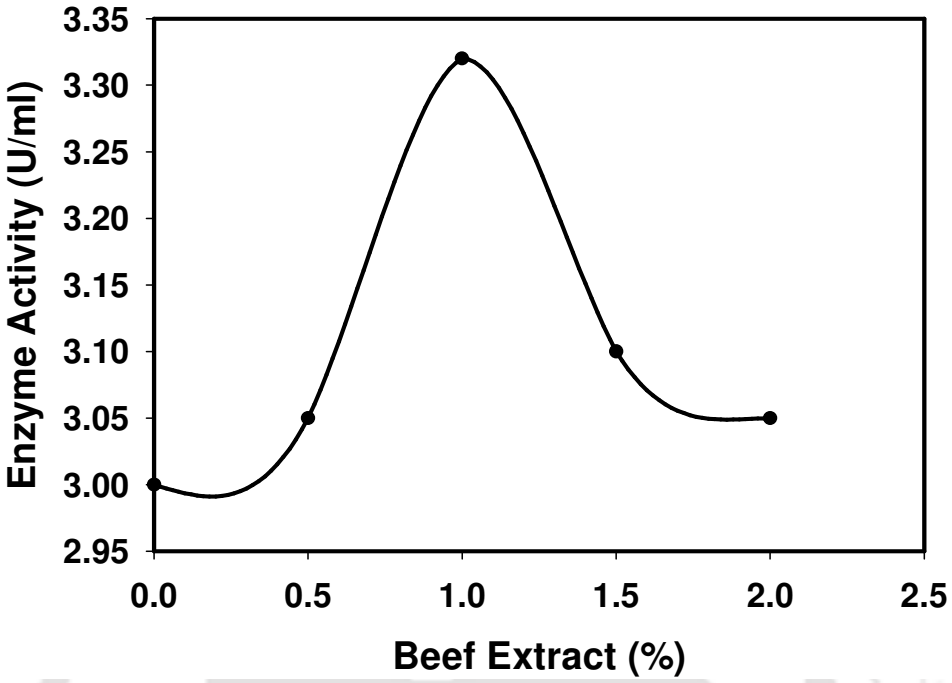

Fig. 4.3.3. Effect of beef extract on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146.

The effect of peptone on glucansucrase production was studied by varying its concentration from 0.5 to 2%. A sigmoidal shape of curve for enzyme activity was observed (Fig. 4.3.4). With an increase in the peptone to 1% an increase in enzyme production was observed (Fig. 4.3.4). The addition of 1% peptone gave 16% increase in enzyme production over control medium (Table 4.3.1). Further increase in peptone beyond 1% did not increase the enzyme production. Ul-Qader *et al.* (2003) also observed higher glucansucrase 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.

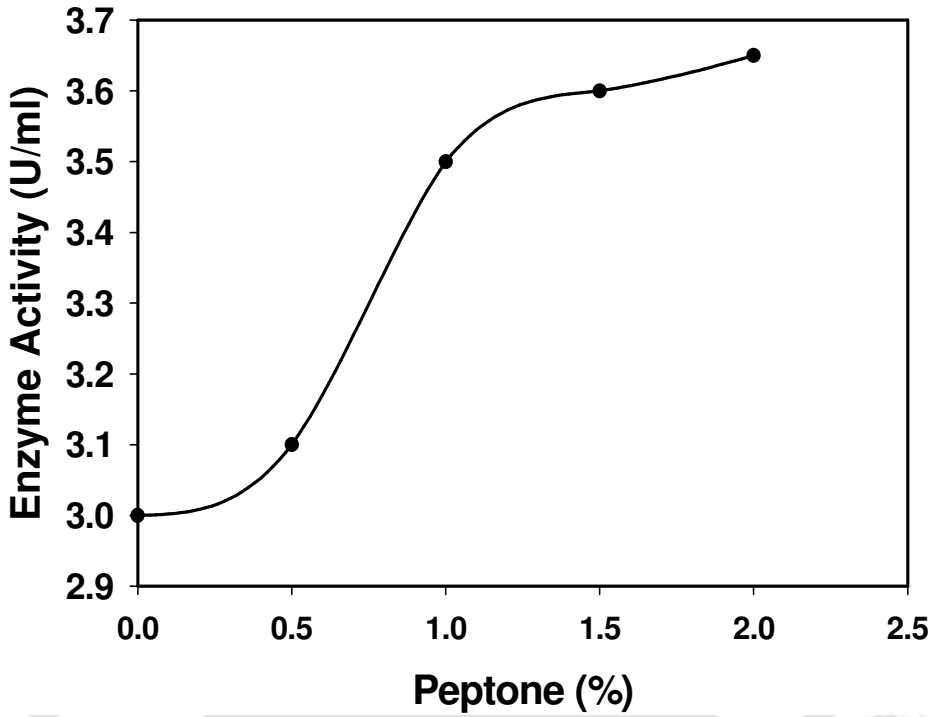

Fig. 4.3.4. Effect of peptone on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146.

4.3.4 Effect of Tween 80

The addition of Tween 80 to the medium stimulated the production of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. A sigmoidal shape pattern of enzyme activity was observed (Fig. 4.3.5). A maximum of 5 U/ml was observed with the addition of 1.0% Tween 80 (Fig. 4.3.5). Increasing the concentration of Tween 80 up to 1.0% increased the activity by 65%, and saturation was observed in the enzyme production after 1% Tween 80 (Fig. 4.3.5, Table 4.3.1). The enzyme activity increased by 50% to 4.4 U/ml at 0.5% Tween 80. It has been reported that the use of a surfactant Tween 80, increases the glucansucrase secretion from the cells (Sato *et al*. 1989; Goyal and Katiyar 1997). Tween 80 is a well known

industrial surfactant. Its presence in the medium affects the homogeneity of the broth and facilitates the nutrient and oxygen transfer to the microorganism. The addition of Tween 80 to the enzyme production medium alters the fatty acid composition of the membrane thus enhancing the secretion of the glucansucrase and its activity (Umesaki *et al.* 1977; Sato *et al.* 1987).

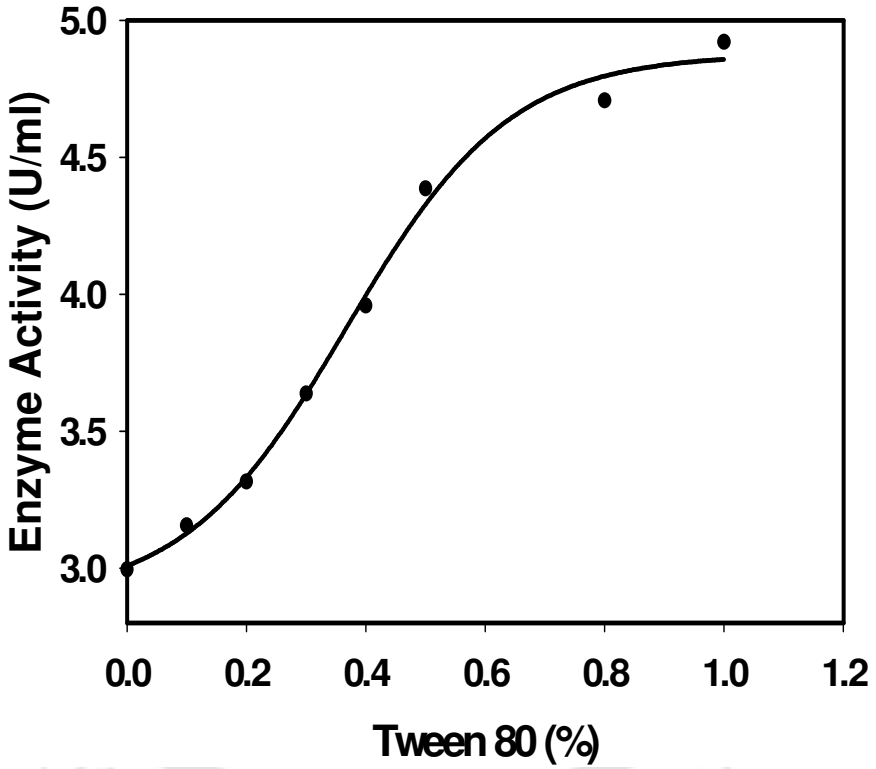

Fig. 4.3.5. Effect of Tween 80 on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146.

4.3.5 Effect of micronutrients on glucansucrase production

4.3.5.1 Effect of $MgSO_4$

The effect of $MgSO_4$ on glucansucrase production was studied by increasing the concentration in the production medium from 0.02% to 0.1%. The glucansucrase production decreased from 3 U/ml to 2 U/ml with the increase in $MgSO_4$ from 0.02 % to 0.1% showing a 33% decrease in the enzyme production (Fig. 4.3.6, Table 4.3.1). Mg^{2+} ion have also been shown to increase the production of dextransucrase in *Leuconostoc mesenteroides* NRRL B-512F (Goyal and Katiyar 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). Bellinger *et al.* (1997) reported that the addition of Mg^{2+}, Mn^{2+} and amino acids stimulate the growth of most of *Leuconostoc* strains.

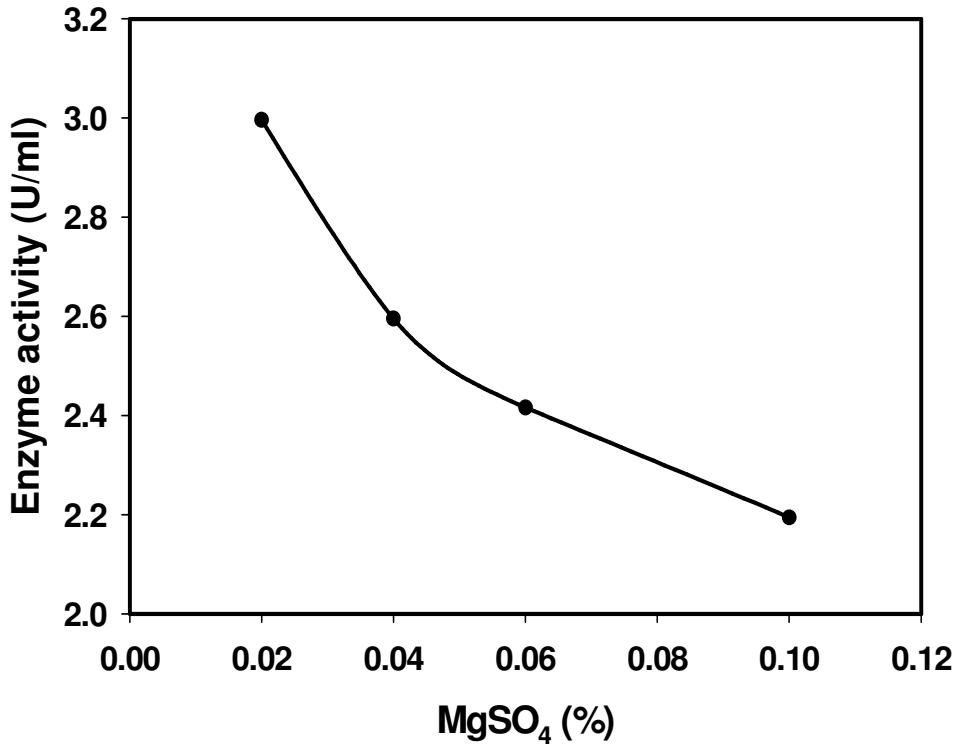

Fig. 4.3.6. Effect of $MgSO_4$ on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146.

4.3.5.2 Effect of $FeSO_4$ and $MnSO_4$

The production of glucansucrase increased with increase in $FeSO_4$ up to 0.005%. $FeSO_4$ at a concentration of 0.005% led to the maximum elaboration of the enzyme as compared to control where 0.001% $FeSO_4$ was present (Fig. 4.3.7). A maximum of 3.6 U/ml enzyme activity with 20% increase was obtained with 0.005% $FeSO_4$ (Fig. 4.3.7). Further increase in the concentration beyond 0.005% of $FeSO_4$ led to a decrease in the enzyme activity.

The effect of the $MnSO_4$ on the glucansucrase production was studied by varying the concentration from 0.001% to 0.01% and a 37% increase was observed in the enzyme production at 0.01% $MnSO_4$ concentration (Fig. 4.3.8, Table 4.3.1). *Leuconostoc* sp. are known to be micro-aerophilic microorganisms. $MnSO_4$ decreases the oxygen toxicity of the *Leuconostoc mesenteroides* cells (Bellinger *et al.* 1997). Micronutrients play a vital role in the growth of microorganisms. They affect the metabolic process and in turn affect the production of enzyme. The metal ion requirement of *Leuconostoc mesenteroides* NRRL B-1146 was determined, changing the concentration of each metal ion. Bellinger *et al.* (1997) showed the addition of Mg^{2+}, Mn^{2+} and amino acids stimulated the growth of most *Leuconostoc* strains. Similar results were reported for Mn^{2+}. Mn^{2+} was stimulatory for the growth and enzyme production and 0.01% $MnSO_4$ in the medium was optimal for the production of glucansucrase (Carlson and Rosano 1951; Foucad *et al*. 1996).

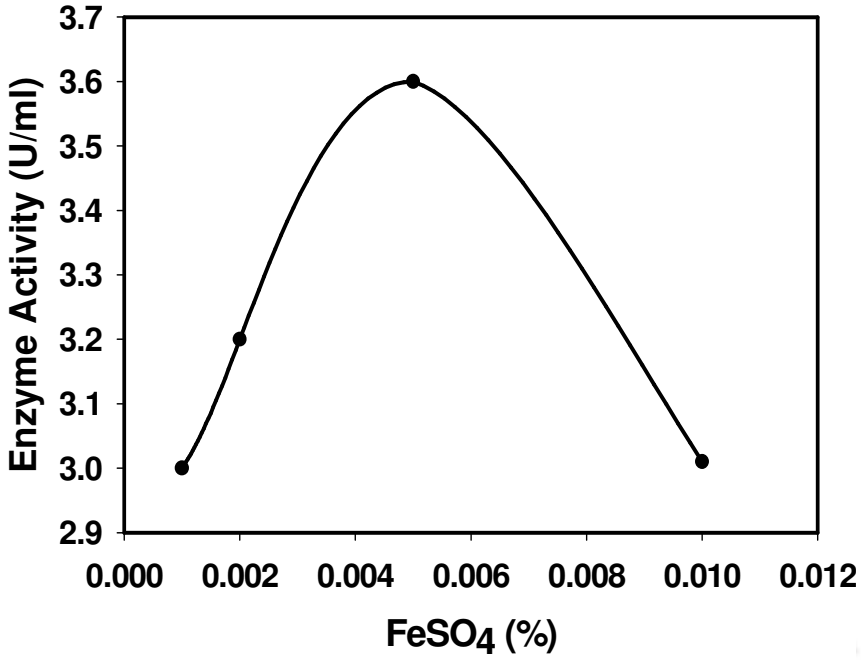

Fig. 4.3.7. Effect of $FeSO_4$ on glucansucrase production from *Leuconostocdextranicum* NRRL B-1146.

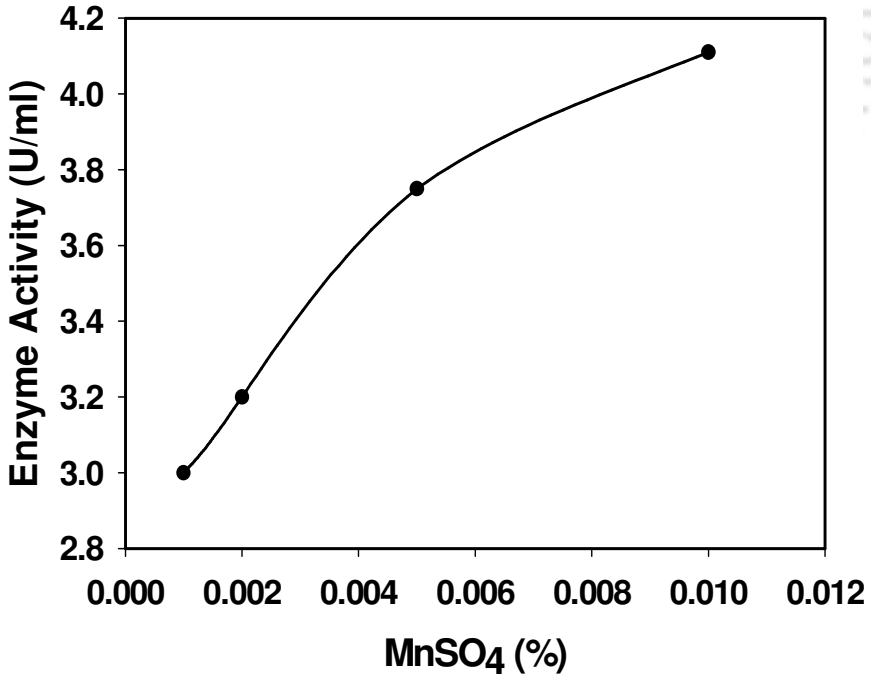

Fig. 4.3.8. Effect of $MnSO_4$ on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146.

4.3.5.3 Effect of NaCl and $CaCl_2$

The effect of sodium ions was studied by increasing the concentration of NaCl from 0.001% to 0.01% in the enzyme production medium. An increase in NaCl concentration from 0.001% to 0.05%, increased the enzyme production by 17% from 3 U/ml and further increase rather reduced the enzyme production (Fig. 4.3.9, Table 4.3.1).

The effect of $CaCl_2$ was studied by increasing the concentration from 0.001% to 0.01% in the enzyme production medium (Fig. 4.3.10). There was a 26% increase in enzyme activity with the increase in $CaCl_2$ concentration to 0.002% as compared to the control medium (Table 4.3.1). Further increase in $CaCl_2$ did not alter the enzyme production. A 2-fold increase in the enzyme production from *Leuconostoc mesenteroides* NRRL B-512F was reported by the addition of $CaCl_2$ to the medium (Robyt and Walseth 1979).

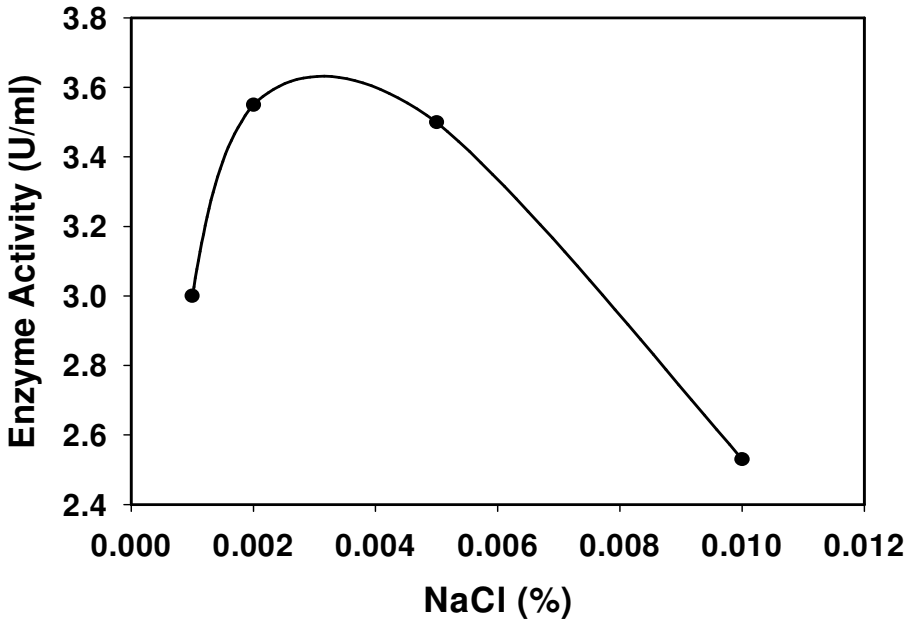

Fig. 4.3.9. Effect of NaCl on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146.

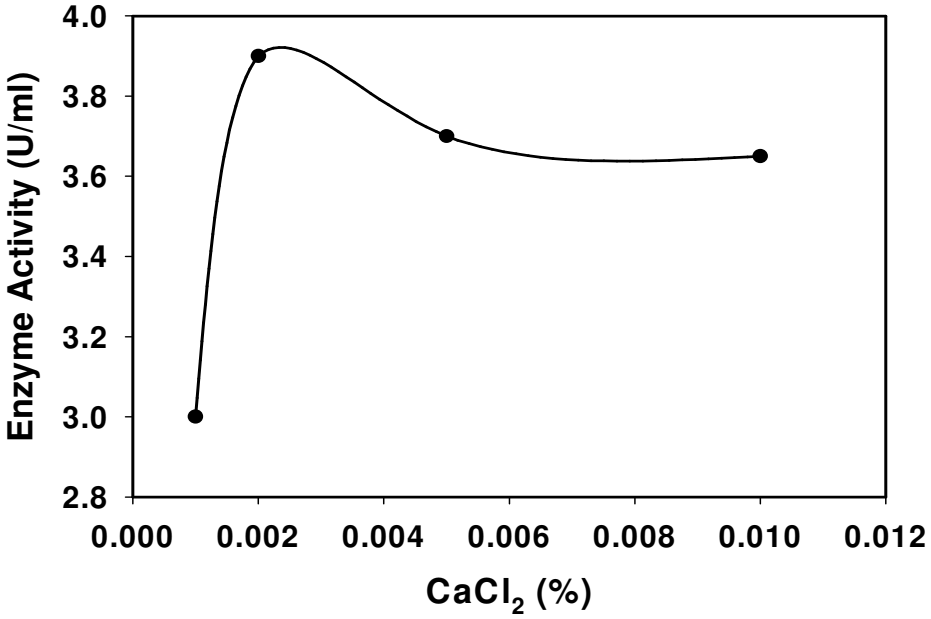

Fig. 4.3.10. Effect of $CaCl_2$ on glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146.

Table 4.3.1. Maximum activity of glucansucrase achieved with variation in concentration of nutrients. The effects of nutrients were compared with control medium except in the case of beef extract and peptone, where the control medium contained no beef extract or peptone.

S.No.	Nutrient (Concentration)	Enzyme activity (%)
1.	Control (3.0 U/ml)	100
2.	Sucrose (4%)	125
3.	Tween 80 (1% v,v)	165
4.	K_2HPO_4 (2.5%)	120
5.	Yeast extract (2.5%) + K_2HPO_4 (2.5%)	125
6.	Beef extract (1%)	115
7.	Peptone (1%)	116
8.	$MgSO_4$ (0.1%)	67
9.	$FeSO_4$ (0.005%)	120
10.	$MnSO_4$ (0.01%)	137
11.	NaCl (0.005%)	117
12.	$CaCl_2$ (0.002%)	126

4.4 Conclusions

Various strains of *Leuconostoc* have been screened earlier for glucan production with unique linkages. The nutrient requirements of those strains have been studied. All the reports state varied requirements for each strain. The effect of nutrients on the production of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 was studied using one variable at a time approach. The control medium gave an enzyme activity of 3 U/ml. Sucrose at a concentration of 4% led to 3.75 U/ml activity. The yeast extract at 2% was optimal for glucansucrase production. There was an increase in the enzyme production with increasing concentrations of K_2HPO_4 and a 17% increase in the enzyme activity was observed at 2.5% concentration. The addition of 1% beef extract and 1% peptone separately to the control medium showed 10% and 16% increase in glucansucrase activity. $MgSO_4$ did not favor enzyme production. Tween 80, $MnSO_4$, $CaCl_2$ significantly enhanced the glucansucrase production. 1% Tween 80 in the enzyme production medium led to 5 U/ml production of enzyme. Tween 80 leads to changes in the fatty acid composition of *Leuconostoc dextranicum* and is responsible for the release of extracellular glucansucrase. $MnSO_4$ at 0.01% in the production medium led to 4 U/ml, while $CaCl_2$ at 0.01% resulted in 3.8 U/ml. The studies have indicated that it is imperative to identify the nutrient requirements of *Leuconostoc dextranicum* for maximum glucansucrase elaboration. The limitation of this approach is that the interaction among different media components cannot be understood. There is still scope for improving glucansucrase production by statistically designed experiments based on response surface methodology (RSM) that shows the combinatorial interactions among different medium components and can be used to optimize fermentation medium. The present study provides a base for further studies and media optimization using RSM.

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Chapter 5

Optimization of Glucansucrase Production by Response Surface Methodology

5.1 Introduction

The production of economically important glucansucrase is essential for the synthesis of novel glucan and oligosaccharides (Monsan and Paul 1995). Overproduction of the enzyme can be achieved by both genetic manipulations and genetic engineering. As excretion of products of metabolism is a part of survival strategy of microbes in certain environments, overproduction of enzymes by medium manipulation can be considered a good strategy (Goyal *et al*. 1995; Goyal and Katiyar 1997; Tari *et al*. 2006). Optimization of medium by the classical method involves changing one independent variable keeping the others constant. The conventional methods for multifactor experiments are time consuming and incapable of detecting the true optimum, due especially to the interaction among the factors (Weuster-Botz 2000). In fermentation process, the operational variables interact and influence each other effect on the response, its important that the optimization method account for this interaction so that a set of optimal experimental condition can be determined (Weuster-Botz 2000). This limitation of single factor experiments can be overcome by using response surface methodology (RSM) which is used to explain the combined

effects of all factors in a fermentation process, is a collection of experimental strategies, mathematical methods and statistical interface. Statistical methods such as Plackett-Burman design, the Box–Behnken design and central composite design are some of the popular choices (Naveena *et al*. 2005; Liu and Wang 2007).

In the present work, the production of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 was optimized in order to obtain its high yield. First, Plackett-Burman screening design was applied to address the most significant factors affecting enzyme production. Secondly, a central composite design was used to describe the nature of the response surface in the experimental region. A sequential optimization strategy was applied for glucansucrase production by *Leuconostoc dextranicum* B-1146 through statistically designed experiments as an effective tool for medium engineering.

5.2 Materials and Methods

5.2.1 Microorganism

A glucan producing strain of *Leuconostoc dextranicum* NRRL B-1146 was obtained from the Agricultural Research Service Culture Collection, National Centre for Agricultural Utilization Research, Peoria, USA. The culture was maintained in modified MRS (sucrose in place of glucose, DeMan *et al*. 1961) as a stab at 4°C and subcultured every 2 weeks. The modified MRS medium for the bacterial stab contained in (g/l): Sucrose, 20; Yeast extract, 5; Beef extract, 10; K_2HPO_4, 2; Sodium acetate, 5; Tri-Sodium citrate, 2; Tween 80, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.2. The pH of the medium was adjusted to 6.4 using 1 N HCl before autoclaving.

5.2.2. Inoculum

Inocula were prepared by transferring a loopful from stab culture to 5 ml Tsuchiya medium (Tsuchiya *et al*. 1952) and incubated at 28°C, for 16 h. Then 1% (1 ml) was transferred to 250 ml Erlenmeyer flask containing 100 ml of sterile Tsuchiya medium containing (g/l) Sucrose, 20; Yeast extract, 20; K_2HPO_4, 20; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01; NaCl 0.01. The pH of the medium was adjusted to 6.9 using 1 N HCl before autoclaving. 5% (v/v) inoculum was used to inoculate 100 ml of enzyme production medium (Tsuchiya medium) for all the experiments. All the experiments were performed in duplicate. The inoculated flasks were incubated at 28°C, under static condition for 12 h.

5.2.3 Glucansucrase activity assay

The assay of glucansucrase was carried out in 1 ml of a reaction mixture in 0.2 M sodium acetate buffer, pH 5.4, containing 292 mM (10%) sucrose and using the

cell free extract (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. One unit (U) of glucansucrase activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar (fructose) in 1 min at 30°C and pH 5.4.

5.2.4 Growth kinetics of *Leuconostoc dextranicum*

The dry weight of cells was determined by centrifugation of the broth at 10,000***g*** for 10 min, and drying at 55°C until a constant weight was achieved. The widely used approximation for cell growth kinetics is the logistic equation that characterizes the cell growth with time. The rate of cell growth (r_X) expressed as (g/l/h), was calculated from Eq. 1 and Eq. 2. X is dry cell mass concentration (g/l), t is time (h) and μ is specific growth rate (h^{-1}). The specific growth rate (μ) was determined graphically and confirmed using Eq. 3 at the exponential growth phase.

$$r_X = \frac{dX}{dt} \quad (1)$$

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad (2)$$

$$\ln X = \mu t \quad (3)$$

The rate of enzyme production (r_E) was calculated from Eq. 4. The enzyme production was cell growth associated, so following model was proposed for the yield of the enzyme activity.

$$r_E = dE/dt = Y_{E/X}\, dX/dt \quad (4)$$

Where, r_E is enzyme production rate (U/ml/h), E is enzyme activity of glucansucrase produced (U/ml) and $Y_{E/X}$ is the yield of enzyme expressed as activity in Units per mg cells (U/mgcells). The enzyme yield ($Y_{E/X}$) was calculated by Eq. 5

using experimental results of the fermentation, where E_{max} and E_0 are maximum and initial enzyme activity, respectively.

$$Y_{E/X} = \frac{E_{max} - E_0}{X_{max} - X_0} \quad (5)$$

5.2.5 Optimization procedure and experimental design

5.2.5.1 Plackett-Burman design

For screening purpose, various medium components and culture parameters were evaluated. Based on Plackett-Burman factorial design, each factor was examined in two levels: -1 for low level and +1 for high level (Plackett and Burman 1946). Table 5.2.1 shows the factors under investigation as well as levels of each factor used in the experimental design, whereas Table 5.2.2 represents the design matrix. Plackett-Burman experimental design is based on the first order polynomial model:

$$Y = \beta_o + \sum \beta_i x_i \quad (6)$$

where, Y is the response (enzyme 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, eleven assigned variables were screened in twelve experimental designs. All experiments were carried out in duplicate and the averages of the glucansucrase activity were taken as response (Table 5.2.2). From the regression analysis the variables, which were significant at 90% level ($P < 0.1$) were considered to have greater impact on glucansucrase production and were further optimized by a central composite design. The experimental design and statistical analysis of the data were done by using Minitab statistical software package (Release 15).

Table 5.2.1. Assigned concentrations of variables at different levels in Plackett-Burman design for glucansucrase production.

S.No.	Variables with designation	Lower level (% w/v)	Higher level (% w/v)
1	Sucrose (X_1)	1.0	6.0
2	Peptone (X_2)	0.5	3.0
3	Yeast Extract (X_3)	0.5	3.0
4	Beef Extract (X_4)	0.5	3.0
5	K_2HPO_4 (X_5)	1.0	4.0
6	Tween 80 (X_6)	0.1	1.0
7	$MgSO_4$ (X_7)	0.01	0.1
8	$MnSO_4$ (X_8)	0.01	0.001
9	$FeSO_4$ (X_9)	0.01	0.001
10	$CaCl_2$ (X_{10})	0.01	0.001
11	NaCl (X_{11})	0.01	0.001

Table 5.2.2. Plackett-Burman design for 11 variables with coded values along with the observed results for glucansucrase production.

Run Order	Sucrose	Peptone	Yeast Extract	Beef Extract	K_2HPO_4	Tween 80	$MgSO_4$	$MnSO_4$	$FeSO_4$	$CaCl_2$	NaCl	Activity (U/ml)
1	-1	1	-1	-1	-1	1	1	1	-1	1	1	1.85±0.034
2	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.46±0.136
3	-1	1	1	1	-1	1	1	-1	1	-1	-1	2.72±0.142
4	-1	-1	1	1	1	-1	1	1	-1	1	-1	0.40±0.068
5	1	1	-1	1	-1	-1	-1	1	1	1	-1	1.64±0.023
6	1	-1	1	-1	-1	-1	1	1	1	-1	1	1.56±0.074
7	1	-1	1	1	-1	1	-1	-1	-1	1	1	3.65±0.056
8	1	-1	-1	-1	1	1	1	-1	1	1	-1	3.70±0.055
9	1	1	-1	1	1	-1	1	-1	-1	-1	1	1.75±0.091
10	1	1	1	-1	1	1	-1	1	-1	-1	-1	4.57±0.029
11	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.86±0.050
12	-1	-1	-1	1	1	1	-1	1	1	-1	1	2.83±0.010

5.2.5.2 Central composite design

The central composite design (CCD) with 2^2 full-factorial central composite experimental plan with three medium constituents, i.e. Tween 80, sucrose and K_2HPO_4 at five levels, was generated by Minitab statistical software (Release 15). In this study, the experimental plan consisted of 20 trials and the value of the dependent response was the mean of two replications. The relationships and interrelationships of the variables were determined by fitting the second order polynomial equation to data obtained from 20 experiments.

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \qquad (7)$$

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. X_i is the factor variable in its coded form. The following equation was used for coding the actual experimental values of the factors in the range of (-1 to +1):

$$X = \frac{\left[actual - \left(low - level + high - level\right)/2\right]}{\left(high - level - low - level\right)/2} \qquad (8)$$

Statistical analysis of data was performed to evaluate analysis of variance (ANOVA).

5.2.6 Experimental validation and fermentation profile of the optimized media in flask culture and bioreactor

The statistically optimized medium composition for glucansucrase production was confirmed and validated experimentally by triplicate fermentation runs in 250 ml culture flask containing 100 ml medium at 28°C static condition in triplicate sets (as described in Chapter 3, Section 3.2.6) and duplicate fermentation runs in a 5 l stirred tank fermentor. The cylindrical stirred tank bioreactor (260 mm i.d. x 390 mm height, Sartorius, model Biostat B plus) was used with a working volume of 2 l and at a

temperature of 28°C. The fermentor consisted of a glass vessel with stainless-steel endplates and three equally spaced vertical baffles. Agitation was provided by a six-flat-blade impeller (diameter 64 mm). The pH of broth was measured using a built in pH meter (Hamilton, USA) equipped with a glass electrode, and dissolved oxygen concentration (DO) in the fermentation broth was determined with a polographic electrode (Hamilton, USA). The values of the DO readings were expressed as percentage of the initial level of saturation. The total biomass dry weight was determined by centrifugation of the broth at 10,000***g*** for 10 min, and drying at 55°C until a constant weight was achieved. The fermentor along with the production medium was sterilized at 121°C for 20 min. After cooling, the medium was inoculated with 100 ml inoculum. In order to allow efficient mass transfer in the system agitation speed of 200 rpm was provided.

5.3 Results and Discussion

5.3.1 Evaluation of factors affecting glucansucrase activity by Plackett-Burman design

The data in Table 5.2.2 indicated that there was a wide variation of glucansucrase activity from 0.40 U/ml to 4.6 U/ml in the twelve trials. This variation reflected the importance of medium optimization to attain higher yields. The main effects of the examined factors on glucansucrase production are presented graphically in Fig. 5.3.1. On analysis of regression coefficients and *t*-value of 11 ingredients (Table 5.3.1), those which showed a positive effect for glucansucrase activity were sucrose, peptone, yeast extract, K_2HPO_4, Tween 80 and $FeSO_4{\cdot}7H_2O$. Beef extract, $MgSO_4{\cdot}7H_2O$, $MnSO_4{\cdot}4H_2O$, $CaCl_2{\cdot}2H_2O$, NaCl had a negative effect on enzyme production. Variables with confidence levels greater than 90% were considered as significant. Sucrose and Tween 80 were significant at 99.99% level and produced high glucansucrase activities. K_2HPO_4 was also significant at a confidence level of 92%. $MgSO_4{\cdot}7H_2O$ was found to be significant although with a negative coefficient. The addition of $MgSO_4{\cdot}7H_2O$ may be avoided in subsequent experiments as it does not enrich the glucansucrase production and the minor requirement of the microorganism can be fulfilled by the complex components of yeast extract. Neglecting the variables which were insignificant, the model equation for glucansucrase activity can be written as Eq. 9.

$$Y_{activity} = 2.1709 + 0.6454\, X_1 + 0.1853\, X_5 + 1.0532\, X_6 \qquad (9)$$

where, X_1 = sucrose, X_5 = K_2HPO_4 and X_6 = Tween 80.

Among the nutrients, carbon source sucrose was chosen as it induces the glucansucrase production and is also, a substrate for glucan production (Tsuchiya *et al.* 1952). The +1 level of sucrose was chosen at 6% as higher than 6% concentrations lead to increased viscosity, making the separation of cells difficult. Different nitrogen

sources, such as yeast extract, beef extract and peptone were chosen, as the nitrogen sources are reported to significantly affect the glucansucrase production (Barker and Ajongwen 1991). K_2HPO_4 was chosen as it acts as a buffering agent to the lactic acid produced during the fermentation and maintains the pH for a longer duration (Rodrigues *et al*. 2003; Tsuchiya *et al*. 1952). It has been reported that the use of a surfactant Tween 80, alters the surface property of the membrane and enhances the release of the glucansucrase (Goyal and Katiyar 1997; Sato *et al*. 1989). Its presence in the medium will affect the homogeneity of the broth and facilitate the nutrient and oxygen transfer to the microorganism. On the basis of the calculated *t*-values (Table 5.3.1), sucrose, K_2HPO_4 and Tween 80 were chosen for further optimization, since these factors had the most significant effects on the glucansucrase activity. All other variables used in all the trials were kept to the median level.

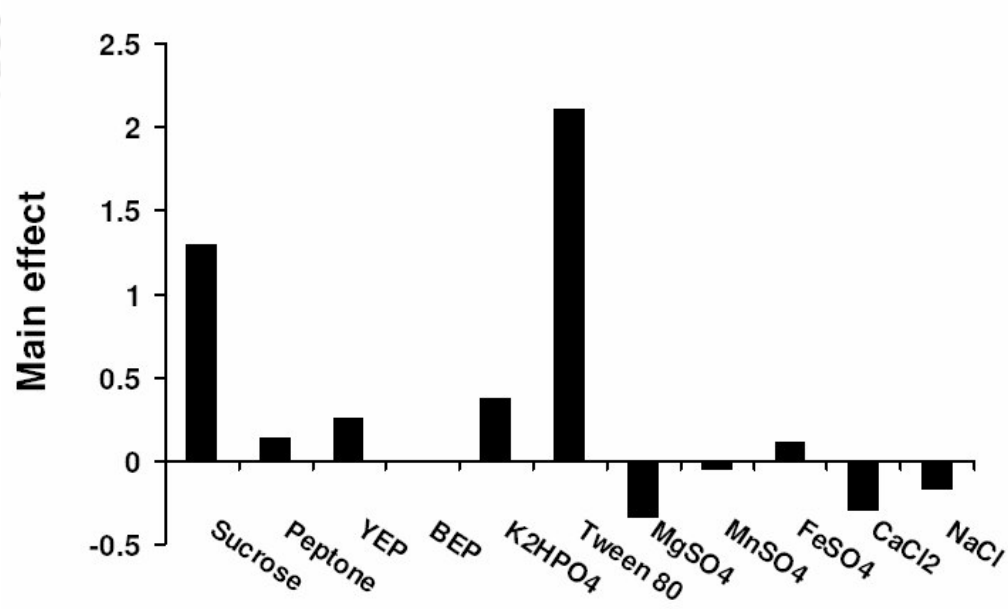

Fig. 5.3.1. Effect of nutrients on glucansucrase production by *Leuconostoc dextranicum*

Table 5.3.1. Statistical analysis of Plackett-Burman design showing coefficient values, *t* and *P*-value for each variable.

Variable	Glucansucrase activity			
	Coefficient	*t* Stat	*P*-value	Confidence level (%)
Intercept	2.17	22.6	0.0001	99.99
Sucrose	0.6454	6.72	0.0001	99.99
Peptone	0.0657	0.68	0.508	50
Yeast Extract	0.1277	1.33	0.211	79
Beef Extract	-0.007	-0.01	0.995	0.05
K_2HPO_4	0.1853	1.93	0.08	92
Tween 80	1.053	10.96	0.0001	99.99
$MgSO_4$	-0.1677	-1.75	0.109	89.1
$MnSO_4$	-0.0257	-0.27	0.794	21
$FeSO_4$	0.0524	0.55	0.596	41
$CaCl_2$	-0.1498	-1.56	0.147	86
NaCl	-0.0832	-0.87	0.405	60

5.3.2 Optimization of glucansucrase production by CCD

At the end of screening experiments for different nutritional factors, a CCD was performed. The levels of each factor are given in Table 5.3.2. Twenty experiments were carried out from the design (Table 5.3.3). The results of the second order response surface model fitting in the form of ANOVA are given in Table 5.3.4. To test the fit of the model the regression equation and determination coefficient R^2 were evaluated. The model presented a high determination coefficient (R^2 = 0.936) explaining 93% of the variability in the response (Table 5.3.4). The value of adjusted R^2 is also very high that indicated a high significance of the model (Khuri and Cornell 1987). The coefficients of regression were calculated and the following regression equation (Eq. 10) was obtained.

$$Y_{activity} = 5.0562 + 0.7662\ X_1 + 0.6648\ X_2 + -1.0170\ X_3 + 0.1012\ X_1X_2 + \ 0.5387\ X_1X_3$$

$$-0.1587\ X_2X_3 - 0.4824\ X_1^2 - 0.3233\ X_2^2 - 0.3198\ X_3^2 \qquad (10)$$

where, Y = response (glucansucrase activity), X_1 = Tween 80, X_2 = Sucrose,

$X_3 = K_2HPO_4$ in coded values

Table 5.3.2. Experimental range and levels of independent variables

Variable	Symbol	Range and Levels				
		-2	-1	0	1	2
Tween 80 (%, v/v)	X_1	0.1	0.28	0.55	0.82	1.0
Sucrose (%, w/v)	X_2	1.0	2.0	3.5	4.98	6.0
K_2HPO_4 (%, w/v)	X_3	1.0	1.6	2.5	3.4	4.0

Table 5.3.3. Full factorial central composite design matrix of three variables in coded units and the experimentally observed response.

Run No.	X_1	X_2	X_3	Activity (U/ml)
1	0	0	0	5.10 ± 0.081
2	1	-1	1	3.90 ± 0.087
3	-1	1	-1	5.00 ± 0.010
4	0	0	0	5.10 ± 0.110
5	0	-2	0	2.60 ± 0.060
6	0	0	0	5.10 ± 0.093
7	2	0	0	4.50 ± 0.018
8	1	1	1	4.92 ± 0.084
9	1	1	-1	5.90 ± 0.004
10	0	0	-2	6.02 ± 0.091
11	0	0	0	4.95 ± 0.067
12	0	2	0	5.90 ± 0.023
13	-1	-1	1	1.25 ± 0.004
14	0	0	0	5.10 ± 0.085
15	1	-1	-1	4.75 ± 0.127
16	-1	1	1	1.36 ± 0.021
17	0	0	2	2.50 ± 0.071
18	0	0	0	4.95 ± 0.192
19	-2	0	0	3.10 ± 0.012
20	-1	-1	-1	3.75 ± 0.005

The ANOVA of quadratic regression model demonstrated that the model is highly significant, and is evident from the Fisher's *F*-test with a very low probability value [($P_{model} > F = 0.0001$)] (Table 5.3.4). At the same time, relatively lower value of coefficient of variation (CV = 11.5%) indicates good precision and reliability of the experiments carried out. The significance of each coefficient was determined by *t*-values and *P*-values which are listed in Table 5.3.5. The larger the magnitude of *t*-test and value and smaller the *P*-value indicates the high significance of the corresponding coefficient (Karthikeyan *et al*. 1996; Tanyildizi *et al*. 2005). The result showed that among the independent variables, X_3 (K_2HPO_4) has significant effect as it has negative coefficient (Table 5.3.5, Eq. 10). The decrease in its concentration can increase the product yield. Among the interactions, X_1X_3 (Tween 80 x K_2HPO_4) has high positive coefficient, while X_1X_2 (Tween 80 x sucrose) has positive coefficient and X_2X_3 (sucrose x K_2HPO_4) has a negative coefficient.

Table 5.3.4. ANOVA for quadratic model.

Source	SS	DF	MS	*F*-value	Prob (*P*) > *F*
Model	36.1431	9	4.0159	16.508	< 0.0001
Residual (error)	2.4326	10	0.2432		
Lack of fit	2.4026	5	0.4805	80.088	< 0.0001
Pure error	0.0300	5	0.0060		
Total	38.5758	19			

$R^2 = 0.9369$; CV = 11.503; Adj $R^2 = 0.8801$.

SS, sum of squares; DF, degrees of freedom; MS, mean square

Table 5.3.5. Model coefficient estimated by multiple linear regression.

Model Term	Parameter Estimate	Standard Error	Computed *t*-value	*P*-value
Intercept	5.0562	0.2011	25.135	0.000
X_1	0.7662	0.1334	5.741	0.000
X_2	0.6648	0.1334	4.982	0.001
X_3	-1.0170	0.1334	-7.621	0.000
X_1^2	0.1012	0.1743	-3.714	0.004
X_2^2	0.5387	0.1743	-2.489	0.032
X_3^2	-0.1587	0.1743	-2.462	0.034
X_1X_2	-0.4824	0.1299	0.581	0.574
X_1X_3	-0.3233	0.1299	3.09	0.011
X_2X_3	-0.3198	0.1299	-0.91	0.384

The 2D contour plots are the graphical representations of the regression equation. The plots are presented in Fig(s). 5.3.2-5.3.4. From the contour plots it is easy and convenient to understand the interactions between two nutrients and also locate their optimum levels. There was an increase in glucansucrase production when high concentrations of sucrose (4-6% w/v) and Tween 80 (0.8% v/v) were used (Fig. 5.3.2). The plot depicting the interaction of Tween 80 and K_2HPO_4 is given in Fig. 5.3.3. The plot indicated that there is good interaction among the independent variable corresponding to the contour and also a smaller *P*-value (0.011) shows that there is relatively significant interaction between two parameters. The maximum activity can be obtained at low concentrations of K_2HPO_4 (1% w/v) and high concentration of Tween 80 (0.8% v/v). Maximal activity was obtained with low levels of K_2HPO_4 (1%) and high levels of sucrose (4-6%) (Fig. 5.3.4).

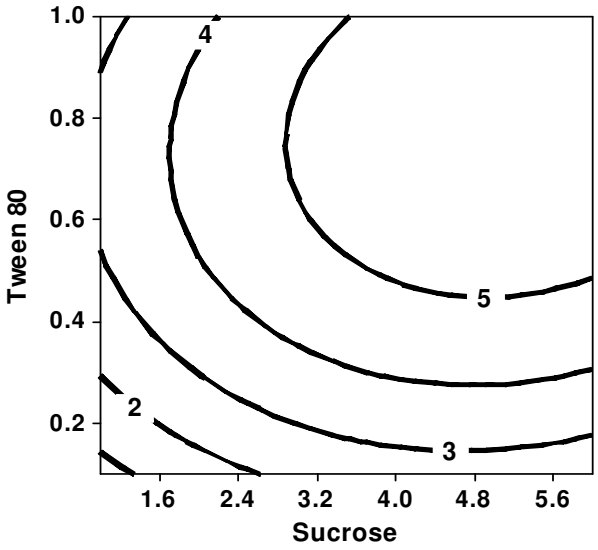

Fig. 5.3.2. Contour plot of the combined effects of Tween 80 and sucrose on glucansucrase production by *Leuconostoc dextranicum* NRRL B-1146. Fixed level: K_2HPO_4 = 2.5

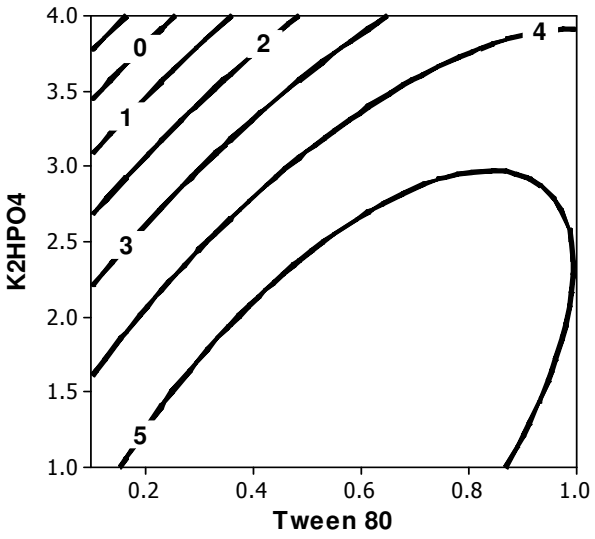

Fig. 5.3.3. Contour plot of the combined effects of Tween 80 and K_2HPO_4 on glucansucrase production by *Leuconostoc dextranicum* NRRL B-1146. Fixed level: sucrose = 3.5

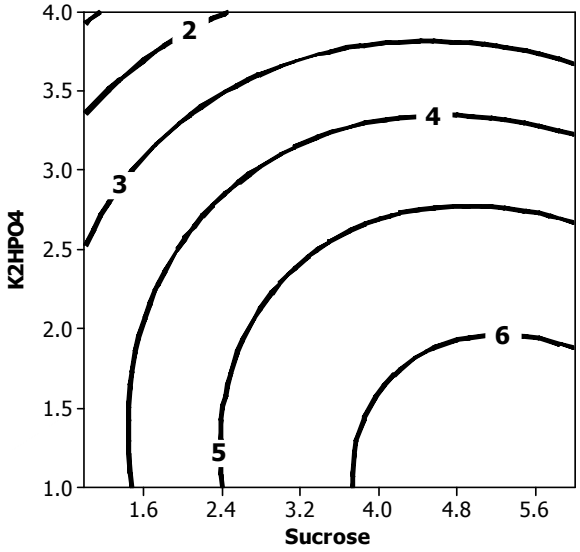

Fig. 5.3.4. Contour plot of the combined effects of sucrose and K_2HPO_4 on glucansucrase production by *Leuconostoc dextranicum* NRRL B-1146. Fixed level: Tween 80 = 0.55

5.3.3 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 Minitab statistical software package (Release 15). The concentration of ingredients for the medium predicted were as follows: Tween 80, (0.55% v/v); sucrose, (5.6% w/v) and K_2HPO_4 (1%, w/v). The maximum enzyme activity predicted by the model using above selected variables was 6.53 U/ml. And the maximum glucansucrase activity obtained experimentally in flask culture was 6.4 ± 0.02 U/ml and was 5.8 ± 0.4 U/ml in bioreactor. The experimentally obtained enzyme activity values showed a perfect agreement with the predicted model. The fermentation pattern of *Leuconostoc dextranicum* was studied with time, after inoculation of 100 ml broth into the 2.0 litre culture medium in the bioreactor. The dissolved oxygen (DO) level in the fermentor started dropping and the DO level dropped to zero after 8h (Fig. 5.3.5).

The pH of the medium dropped as the cell growth proceeded and stabilized at 4.7 after 8h (Fig. 5.3.5). The production of glucansucrase started, as soon as the cells entered the exponential phase (cell mass, *X* of 4.0 g/l) (Fig. 5.3.5). The enzyme activity increased constantly to 5.8 U/ml up to 28h of fermentation. The cell growth kinetics and enzyme yield of *Leuconostoc dextranicum* were compared with Tsuchiya medium and RSM optimized medium in flask culture and bioreactor and presented in Table 5.3.5. The results showed that RSM optimized medium gave more than double the enzyme activity and enzyme yield as compared to the un-optimised medium (Table 5.3.5).

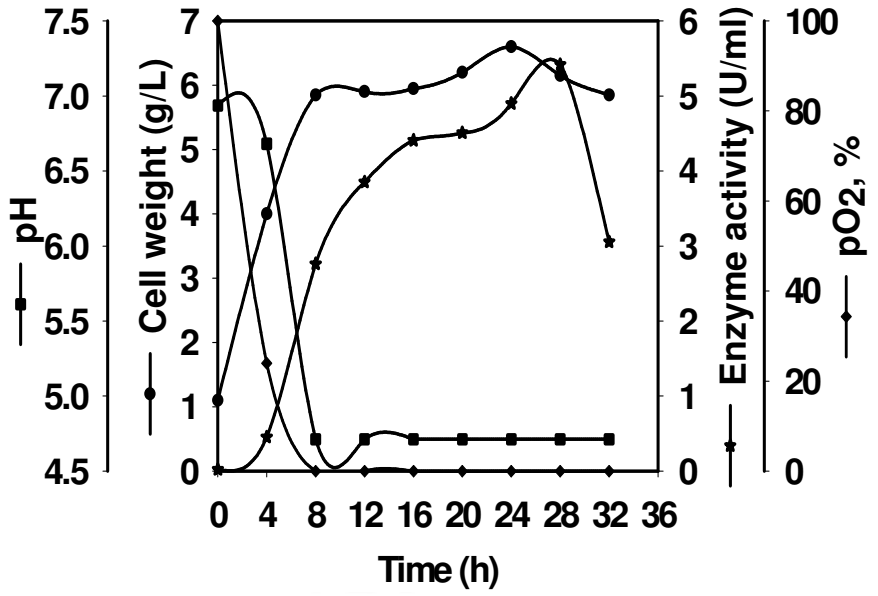

Fig. 5.3.5. Glucansucrase production by *Leuconostoc dextranicum* NRRL B-1146 using the optimized medium in bioreactor at 1.5 vvm with no pH control.

Table 5.3.5. Comparison of glucansucrase activity and growth of *Leuconostoc dextranicum* under different conditions.

	Cell mass (X_{max}) (g/l)	Specific growth rate (μ) (h^{-1})	Enzyme Activity (U/ml)	Enzyme Yield ($Y_{E/X}$) (U/mg cell)
Un-optimized Tsuchiya Medium	5.85	0.04	3.0	0.87
RSM optimized medium Using Flask Culture	5.95	0.04	6.4	1.85
RSM optimized medium using Bioreactor	6.6	0.03	5.8	1.7

5.4 Conclusions

The production of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 was maximized using response surface methodology. Various medium components were screened for their effect on glucansucrase production using Plackett-Burman design. Results showed that sucrose, Tween 80 and K_2HPO_4 significantly affected the enzyme production. These three ingredients were used as main variables in the central composite design (CCD) study at Tween 80, (0.55%, v/v); sucrose, (5.6%, w/v) and K_2HPO_4 (1%, w/v). The maximum enzyme activity predicted using above selected variables was 6.53 U/ml, and the maximum activity obtained experimentally was 6.4 ± 0.02 U/ml in flask culture and 5.8 ± 0.4 U/ml in bioreactor. The experimentally obtained enzyme activity showed a good agreement with predicted activity value. The fermentation in a bioreactor resulted glucansucrase activity of 5.8 U/ml which was 90% of that, achieved in a flask culture using the same medium composition. The maximum enzyme activity that could be obtained using un-optimized production medium of Tsuchiya was 3.0 U/ml, while 6.4 U/ml could be obtained using medium designed by RSM. The maximized activity (6.4 U/ml) is lower when compared to the glucansucrase activity of several strains of *Leuconostoc* sp. industrially used, though it should be taken into account that the glucansucrase from this strain has novel linkages in the glucan it produces. The result of RSM showed that medium optimization by statistical approach is a good option for maximizing enzyme production.

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Chapter 6

Artificial Neural Network and Genetic Algorithm based Optimization of Glucansucrase Production

6.1 Introduction

Glucansucrases (GS) are glycoside hydrolases, which belong to family 70 (GH70) of glycoside hydrolases (Majumder *et al.* 2007). They are large, extracellular proteins with average molecular masses of 160,000 Da which are involved in the synthesis of α-glucans, and are limited to lactic acid bacteria (http://www.cazy.org/fam/GH70.html) (Henrissat 1991). During the formulation of fermentation medium, optimization is an important step that helps determine the most suitable reaction conditions and the concentration of the medium components which maximize or minimize important process variables (Weuster-Botz 2000). The efficiency of already established bioprocesses can be increased either by introducing more productive strains or by optimizing the cultivation medium itself (Kovarova-Kovar *et al.* 2000). Since these factors have complex effects on the system, possible interactions among the various factors are often characterized through experiments. To account for these interactive influences, statistical techniques like response surface methodology (RSM) are increasingly being used (Gangadharan *et al.* 2007; Majumder and Goyal 2007).

The development of accurate models for a biological reaction system on a chemical and physical basis is still a critical challenge, mainly due to the non-linear nature of the biochemical network interactions (Franco-Lara *et al.* 2006). It has been shown that machine learning techniques such as artificial neural networks (ANNs) and genetic algorithms (GAs) mimic different aspects of biological information processing for data modelling and could prove to be useful in media optimization. An artificial neural network is a superior and more accurate modelling technique as compared to RSM as it represents the non-linear ties in a much better way (Dutta *et al.* 2004; Haider *et al.* 2007). Its application has been reported by many in predictive microbiology (Lou and Nakai 2001; Garcia-Gimeno *et al.* 2002). The use of advanced non-linear data analysis tools such as ANN in food science, environmental biotechnology and biochemical engineering is a well established tradition (Almeida 2002). The contribution of neural networks to biotechnology has been reviewed by Montague and Morris (1994) and Patnaik (1999).

Genetic algorithms are artificial intelligence based stochastic non–linear optimization formalisms (Goldberg 1989). They have been used with great success for optimizing complex expressions, which in this case is a neural network (Ramanathan *et al.* 2001; Sumanwar *et al.* 2002). GAs are capable of searching a large parameter space in a highly directed and systematic manner (Freyer *et al.* 1992). The features, advantages and working protocol described (Venkatasubramanian and Sundaram 1998), make GA an ideal technique to solve diverse optimization problems in bioprocess engineering (Baishan *et al.* 2003). ANN-GA based approach was used for simultaneous maximization of biomass and conversion of pentafluoroacetophenon with *Synechococcus* PCC 7942 (Franco-Lara *et al.* 2006) and optimization of fermentation medium for the production of exopolysaccharide from *Lactobacillus*

plantarum (Desai *et al.* 2006). RSM based data was further optimized using ANN-GA for the production of hydantoinase by *Agrobacterium radiobacter* (Nagata and Chu 2003) and production of lipase from a mixed culture (Haider *et al.* 2007).

Glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146 was optimized previously using RSM (Majumder and Goyal 2007) and in the present investigation, the evaluation of ANN supported GA for optimization of fermentation medium using the experimentally obtained data from a previously studied full-factorial central composite design of three variables, is addressed. A highly accurate artificial neural network was created. Subsequently, a genetic algorithm was used to optimize the medium composition using the neural network as its fitness function.

6.2 Materials and Methods

6.2.1 Microorganism and culture conditions for glucansucrase production

The strain *Leuconostoc dextranicum* NRRL B-1146 was procured from Agricultural Research Service Culture Collection, National Centre for Agricultural Utilization Research, Peoria, USA. The culture was maintained in modified MRS (sucrose in place of glucose (DeMan *et al.* 1961)), as a stab at 4°C and sub-cultured every 2 weeks. For inoculation of the culture medium two loops of culture was taken in 5 ml Tsuchiya medium (Tsuchiya *et al.* 1952) contained in a test tube and grown at 28°C for 12h. From the 5 ml broth, 1% of inoculum was transferred to 250 ml Erlenmeyer flasks containing 100 ml of Tsuchiya medium (pH 6.9). This 100 ml inoculum was used to inoculate 100 ml of sterile medium in Erlenmeyer flask containing ANN-GA designed medium and grown at 28°C for 12h. The experiment was performed in triplicate sets of flasks. The production medium consisted of (g/l) sucrose, 59.8; yeast extract, 17.5; beef extract, 17.5; peptone, 17.5; Tween 80, 5.4; K_2HPO_4, 10.1; $MgSO_4 \cdot 7H_2O$, 0.55; $MnSO_4 \cdot 4H_2O$, 0.055; $FeSO_4 \cdot 7H_2O$, 0.055; $CaCl_2 \cdot 2H_2O$, 0.055; NaCl 0.055. The pH of the medium was adjusted to 6.9 using 1N HCl before autoclaving.

6.2.2 Assay of enzyme activity

A sample (2 ml) from the fermented broth was taken after 12 h and centrifuged at 9,000***g*** for 5 min, at 4°C and the cell free supernatant was used for the estimation of enzyme activity. The assay of glucansucrase was carried out in 1 ml of a reaction mixture in 0.2 M sodium acetate buffer, pH 5.4, containing 292 mM (10%) sucrose and using the cell free extract (20 µl) as the enzyme source. The reaction mixture was incubated at 30°C for 15 min. The assay and reducing sugar estimation

procedures are described in Chapter 3, Section 3.2.7. One unit (U) of glucansucrase activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar (fructose) in 1 min at 30°C and pH 5.4.

6.2.3 Computational methods

6.2.3.1 Artificial Neural Network

A neural network is a computer program architecture for non linear computations and is a mathematical representation of the neurological functioning of a brain. It simulates the brain's learning process by mathematically modelling the network structure of inter connected node cells. The goal is to formalize human like decision making, behaviour and performance into a rigorous tool. It is composed of many simple elements operating in parallel. These elements, called processing elements are inspired by biological nervous systems and are highly connected. An individual processing element (neuron) can have any number of inputs, but only one output that is generally related to the inputs by a transfer function. The most frequently used transfer functions are sigmoid function, hyperbolic tangent function, sine function, linear and saturated linear transfer function. The argument of the transfer function is the sum of input elements of the corresponding neuron, each input being multiplied by the associated weight, which shows the strength of the connection between two neurons.

The typical neural network architecture has an input layer, one or more hidden layer and an output layer. The neurons in the hidden layer which are linked to the neurons in the input and output layers by adjusting weights, enable the network to compute complex associations between the input and output variables (Fig. 6.2.1). They are used as "black box models" of key variables whose relationship to other

process entities are neither formally described nor mathematically established but are assumed to occur. To train an ANN model, a set of data containing input nodes and output nodes are fed. The most commonly used algorithm is the back propagation algorithm.

$$o_j = \frac{1}{1+e^{-\sum_i w_{ji} o + w_j \Theta}} \qquad (1)$$

where, wji denote connection weights, oi denotes output of a previous layer neuron, wj denotes the adjustable threshold for the neuron, i denotes the number of inputs to the neuron from the previous layer and j denotes the current neuron.

In this training algorithm, the error between the results of the output neurons and the actual outputs is calculated and propagated backward through the network. The algorithm adjusts the weights in each successive layer. Validation is done by presenting the network the test, a data set not used for training and then evaluating the system performance under the situation. Once the training is over, ANN is capable of predicting the output when any input similar to the pattern that it has learnt is fed.

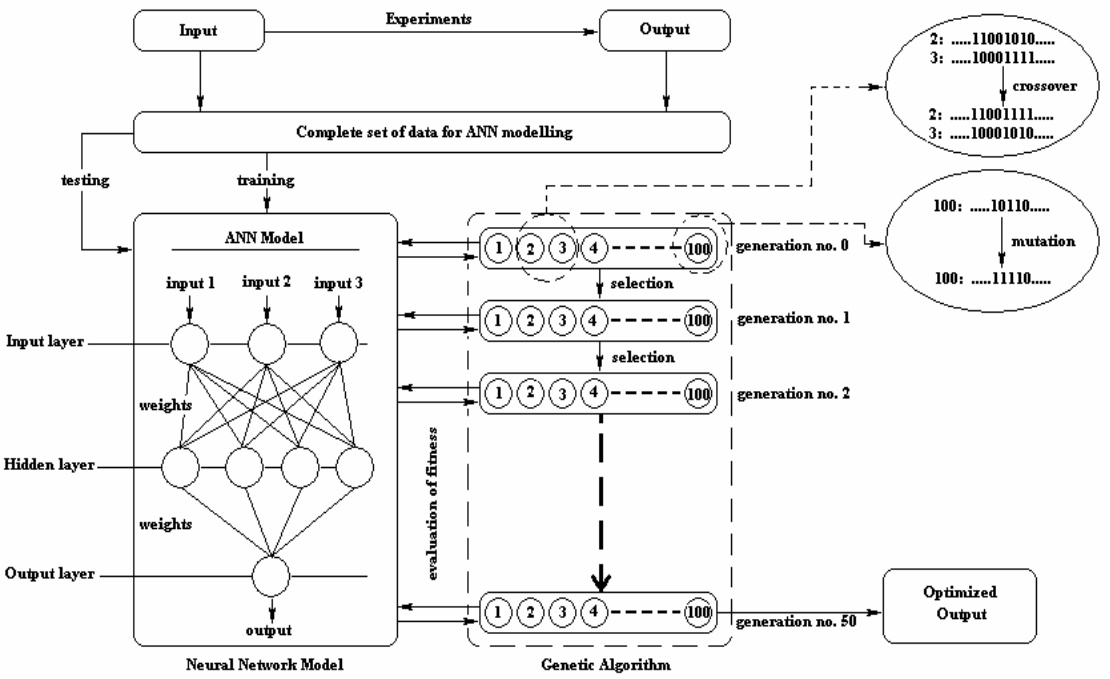

Fig. 6.2.1. Schematic representation of ANN-GA based optimization.

ANN was applied here to provide a non-linear mapping between input variables (Tween 80, sucrose and K_2HPO_4) and the output variable (enzyme activity). Regression based response surface models require the order of the model to be stated, while ANN tends to implicitly match the input vector (i.e. medium composition) to the output vector (enzyme activity). ANN has been applied for simulating the same experimental data used for RSM. The learning rate of the network was set to values that resulted in an optimal coefficient of correlation (R^2) for the neural network.

6.2.3.2 Genetic Algorithm

Genetic algorithms are general purpose search algorithms inspired by Charles Darwin's principle of "Survival of the fittest" to solve complex optimization problems (Goldberg 1989). A genetic algorithm starts off with an initial population of randomly generated chromosomes. Generally the chromosomes in the population are represented as strings of binary digits. Such an implementation of chromosomes is

usually called encoding scheme. During successive iterations, called generations, the initial chromosomes advance towards stronger chromosomes by reproduction among members of the previous generation.

New generations are created by three genetic operators: selection, crossover and mutation (Fig. 6.2.1). The offsprings generated at each step are evaluated for their fitness using the ANN model developed and the corresponding string is selected from the mating pool with a probability that is directly proportional to its fitness level. This selection ensures that only the best chromosomes cross over and mutate. The primary genetic operator is the crossover operator, which combines the features of two parent structures to form offsprings. In the crossover, two strings are selected randomly and then, beyond an arbitrary position along the two strings, the crossover takes place creating two offsprings. Mutations introduce diversity into the population. The most common form of mutation is the point mutation. The mode of introduction of a point mutation is to take a bit from the chromosome and then alter it with some predetermined probability. This helps avoid any convergence to a local minimum.

6.2.4 Software

The artificial neural network model was generated using NNMODEL version 1.4, USA. The genetic algorithm used for optimization was a modified form of the binary coded algorithm developed by researchers at Kanpur Genetic Algorithms Laboratory (http://www.iitk.ac.in/kangal/index.shtml), Indian Institute of Technology Kanpur, India.

6.3 Results and Discussion

6.3.1 Artificial Neural Network

The production of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 has been optimized previously using RSM (Majumder and Goyal 2007). Tween 80, sucrose and K_2HPO_4 were found to be the most important factors for glucansucrase production. The effect of these factors was studied at different levels with a 2^3 full factorial central composite design. Regression equation for glucansucrase activity was obtained using the experimental results of central composite design. The equation based on a second order polynomial with the optimized coefficients is given by

$$Y_{activity} = 0.9685 + 3.7421X_1 + 1.6310X_2 + 0.0476X_3 + 0.2546X_1X_2 + 2.2575X_1X_3 - 0.1197X_2X_3 - 6.7392X_1^2 - 0.1464X_2^2 - 0.4021X_3^2) \quad (2)$$

where, Y = response (glucansucrase activity), X_1 = Tween 80, X_2 = Sucrose, X_3 = K_2HPO_4 in uncoded values

The topology of the neural network designed for glucansucrase production consisted of three input variables and one output variable (Table 6.3.1). The model consisted of four hidden layers along with the input and the output layer. The corresponding weights of each of the above layers, which define the neural network, are given in Table 6.3.2. Each neuron has one additional weight as an input and this neuron is called Theta. This input allows an additional degree of freedom when minimizing the training error. While three quarters of the experimental data sets were used for adjusting the weights of the model during its training, the remaining 25% were used to test the network's performance after the each iteration (Franco-Lara *et al.* 2006). The model was found to be highly significant with an R^2 of 1.00 for training

data and 0.90 for the test data. The advantage of using ANN model for this fermentation is due to the corrective action arising from the training methodology.

Table 6.3.1. Network parameters of the artificial neural network.

Input variables	Tween 80 (%, v/v)	Sucrose (%, w/v)	K_2HPO_4 (%, w/v)
Lower limit of the variable	0.10	1.00	1.00
Upper limit of the variable	1.00	6.00	4.00
Output variables	Glucansucrase activity (U/ml)		
Data sets in training matrix	15		
Data sets in test matrix	05		
Number of hidden layers	04		
Error tolerance	01×10^{-3}		
Learning rate	0.8		

Table 6.3.2. The neural network generated for predicting glucansucrase activity.

Input to hidden layer weights				
	H1	H2	H3	H4
Tween 80	+0.013	-7.684	+8.152	-0.797
Sucrose	+0.456	+4.967	+2.774	+1.191
K_2HPO_4	+0.124	+5.644	-0.586	-1.541
Theta	-0.055	-3.688	-4.807	+1.792

Hidden to output layer weights	
Hidden 1	+0.664
Hidden 2	-3.546
Hidden 3	+7.933
Hidden 4	+2.363
Theta	+6.877

Input to output layer weights	
Tween 80	-12.599
Sucrose	-0.810
K_2HPO_4	+0.527

As seen in Table 6.3.3 the measured and calculated enzyme activity with ANN model were almost identical as compared to the predicted values by the regression equation. The difference between experimental and ANN predicted values is presented graphically as a residual plot (Fig. 6.3.2) which shows the accuracy of prediction by the neural network. Fig. 6.3.3 shows the plot of predicted glucansucrase activity by ANN model and regression equation against the experimentally determined values. The results showed that ANN based predictions are closer to the line of perfect prediction than those of quadratic polynomials. Similar results were obtained for the production of protease, where it was shown that ANN has better prediction accuracy than RSM (Dutta *et al.* 2004; Haider *et al.* 2007). ANN has been previously used for building predictive models with high accuracy. These studies involved the optimization of fed-batch process for the production of riboflavin (Kovarova-Kovar *et al.* 2000), studies on thermal inactivation of glucoamylase (Bryjak 2004), optimization of catalytic reaction of pancreas lipase (Manohar and Divakar 2005) and enhanced production of exopolysaccharide from *Lactobacillus plantarum* (Desai *et al.* 2006).

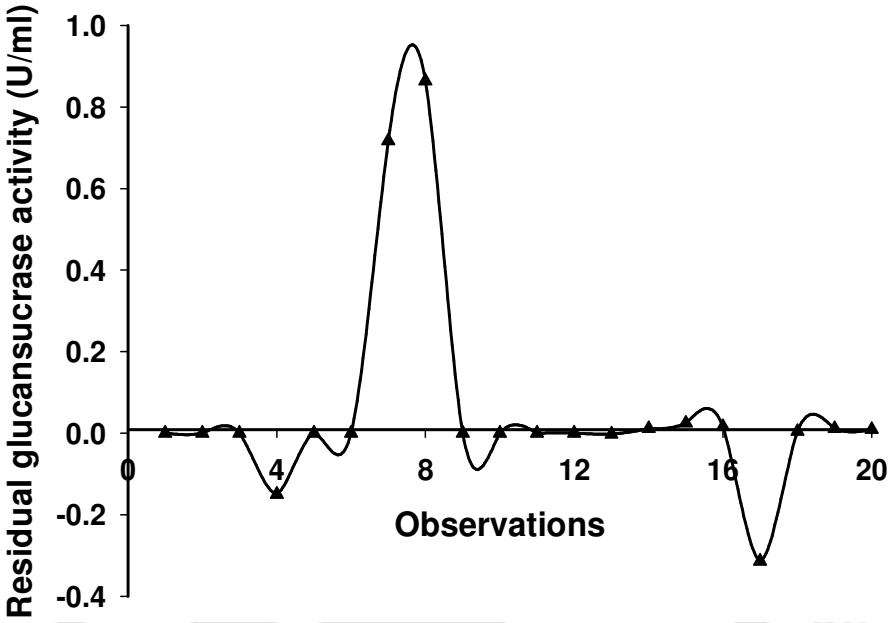

Fig. 6.3.2. Residual plot of measured and ANN predicted glucansucrase activity of *Leuconostoc dextranicum* NRRL B-1146.

Table 6.3.3. Central composite design matrix of three variables and the experimentally determined, regression model predicted and neural network predicted values of glucansucrase activity.

Tween 80 (%, v/v)	Sucrose (%, w/v)	K_2HPO_4 (%, w/v)	Enzyme activity (U/ml)	Predicted Enzyme activity (U/ml)	
			Experimental	Regression	ANN
0.55	3.50	2.50	5.10	5.06	5.10
0.55	3.50	2.50	5.10	5.06	5.10
0.55	3.50	2.50	5.10	5.06	5.10
0.55	3.50	2.50	4.95	5.06	5.10
0.55	3.50	2.50	5.10	5.06	5.10
0.55	3.50	2.50	5.10	5.06	5.10
0.10	3.50	2.50	3.10	2.40	2.38
1.00	3.50	2.50	4.50	4.98	3.64
0.55	1.00	2.50	2.60	3.02	2.60
0.55	6.00	2.50	5.90	5.26	5.90
0.55	3.50	1.00	6.02	5.86	6.02
0.55	3.50	4.00	2.50	2.44	2.50
0.28	2.01	1.61	3.75	3.98	3.75
0.82	2.01	1.61	4.75	4.24	4.74
0.28	4.99	1.61	5.00	5.43	4.98
0.82	4.99	1.61	5.90	6.09	5.88
0.28	2.01	3.39	1.25	1.19	1.56
0.82	2.01	3.39	3.90	3.61	3.90
0.28	4.99	3.39	1.36	2.00	1.35
0.82	4.99	3.39	4.92	4.83	4.91

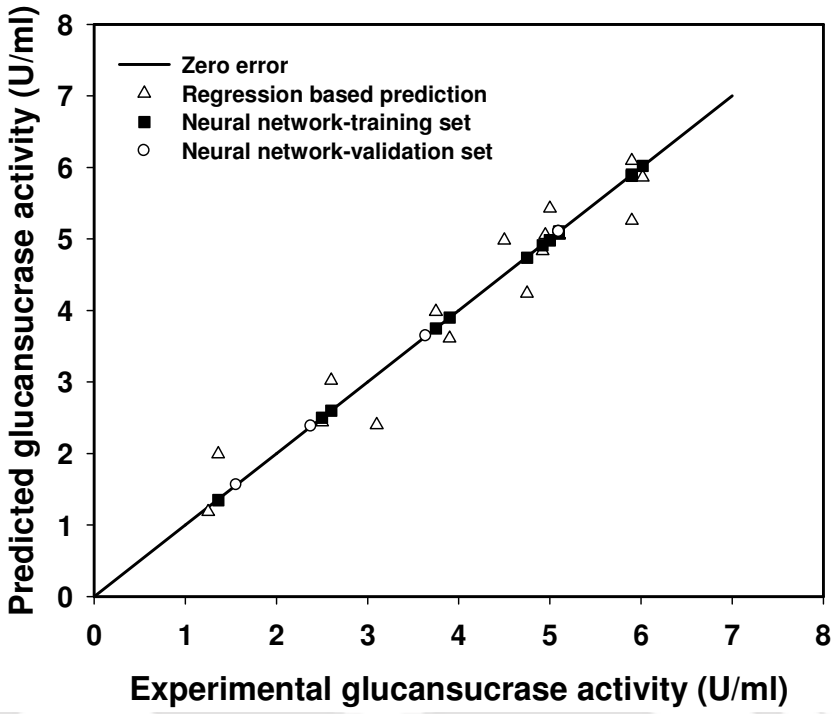

Fig. 6.3.3. Glucansucrase production predicted by neural network and regression equation (RSM) versus actual glucansucrase production.

6.3.2 Genetic Algorithm

Using this artificial neural network as the fitness function, the genetic algorithm was implemented to optimize the medium composition for maximum glucansucrase activity. The defining parameters of the algorithm are given in Table 6.3.4. The algorithm was run five times and the results at the end of 50 generations for each of the runs are reported in Table 6.3.4. The maximum enzyme activity exhibited by these runs was 6.92 U/ml. Starting from an average enzyme activity of lesser than 3.91 U/ml at generation 0, the average enzyme activity was 6.41 U/ml at the end of 50 generations. Fig. 6.3.4 shows the evolution of the algorithm, with successive generations. The average fitness of the algorithm increases as the number of healthy individuals increased till the 19^{th} generation. Stagnation was seen as the convergence to the maxima was achieved and was almost constant thereafter, all the way to the last

generation, the generation 50. The convergence of individuals to the same parameter can be directly correlated with its fit to the neural network in the preceding generations (Weuster-Botz and Wandrey 1995). For a simpler visualization of the convergence of the genetic algorithm, the response is divided into 7 identical subunits ranging from 0 U/ml to 7 U/ml with a gap of 1 U/ml. The population of 100 was then grouped into one of the above 7 groups. The population profile obtained at different generations is presented in Fig. 6.3.5. The population at generation 0 was selected at random to cover the entire parameter range; therefore the profile is spread across the seven groups. It was observed that majority of the population was in the group of 6-7 U/ml as the evolution progressed across the generations. After generation number 10, more than 75% of the population was consistently above 6 U/ml (Fig(s). 6.3.4 and 6.3.5). The interactions between the medium components cannot be exactly described using GA, but it has been described that convergence of different medium constituents to a single optimal value indicates no interactions between them (Weuster-Botz and Wandrey 1995; Haider *et al.* 2007).

Table 6.3.4. Working parameters of genetic algorithm.

Total number of generations	50
Population size	100
Total string length	45
Number of binary coded variables	03
Cross over probability	0.10
Mutation probability	0.05
Number of runs	5

Table 6.3.5. ANN-GA predicted concentration of medium components and the predicted glucansucrase activity.

	Predicted value of media components			Predicted Glucansucrase activity (U/ml)		
	Tween 80 (%, v/v)	Sucrose (%, w/v)	K_2HPO_4 (%, w/v)	Maximum	Minimum	Average
Run 1	0.53	5.98	1.01	6.91	4.38	6.36
Run 2	0.54	5.98	1.02	6.91	3.44	6.21
Run 3	0.54	5.95	1.01	6.92	4.12	6.37
Run 4	0.54	5.98	1.00	6.92	4.09	6.41
Run 5	0.54	6.00	1.00	6.92	3.16	6.28
Average	0.54	5.98	1.01	6.92	3.84	6.33

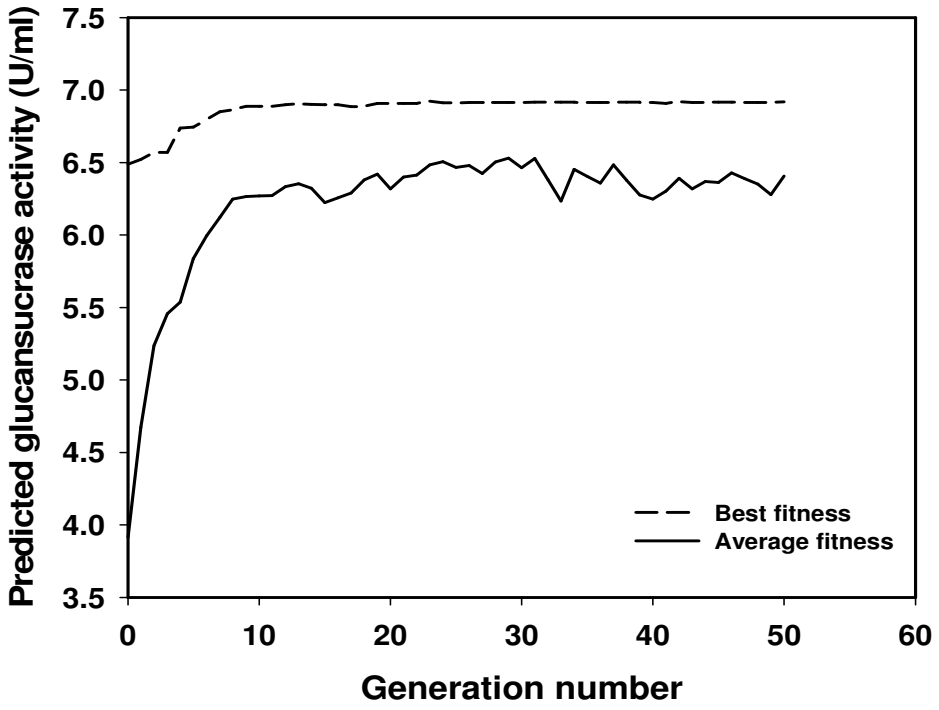

Fig. 6.3.4. Evolution of the best and average fitness (glucansucrase activity) over 50 generations in the genetic algorithm.

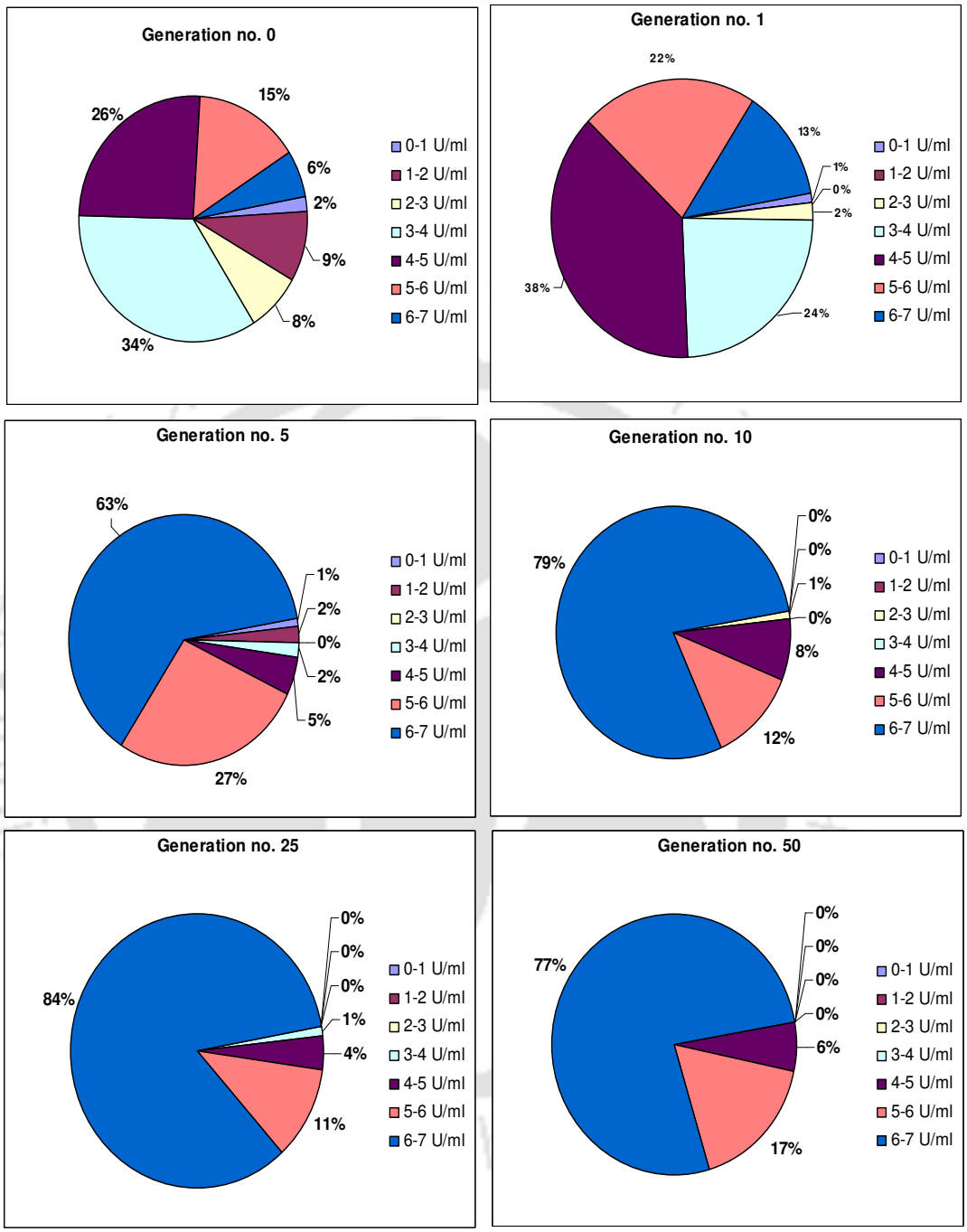

Fig. 6.3.5. Population profile at different generations in the genetic algorithm.

Using the second-order polynomial of RSM the optimal concentration of medium components for a predicted maximum glucansucrase production of 6.53 U/ml were Tween 80, (0.55%, v/v); sucrose, (5.6%, w/v) and K_2HPO_4 (1%, w/v) (Majumder and Goyal 2007). The ANN-GA predicted concentration of medium components and the predicted glucansucrase activity are presented in Table 6.3.5. The maximum glucansucrase activity predicted by ANN-GA was 6.92 U/ml at a medium composition of 0.54 % (v/v) Tween 80, 5.98 % (w/v) sucrose and 1.01 % (w/v) K_2HPO_4 which was 6% higher than the value of 6.53 U/ml predicted by that of regression based prediction. The result of the ANN-GA specified medium composition was verified by carrying out the fermentation run in triplicate sets. The glucansucrase activity obtained experimentally was 6.75 ± 0.1 U/ml. This showed a perfect agreement with the ANN-GA predicted model. The production of enzyme could be enhanced using ANN-GA (Table 6.3.6). ANN based prediction and GA based optimization led to an increase an enzyme activity from 6.4 to 6.75 U/ml.

Table 6.3.6. Comparison of different optimization procedures of RSM and ANN-GA.

	Predicted Enzyme Activity (U/ml)	**Experimental Enzyme Activity (U/ml)**	**Fold increase**
Unoptimzed Production Medium (Tsuchiya)	-	3.0	-
RSM optimized Flask Culture	6.53	6.4	2.1
ANN-GA optimized Flask culture	6.92	6.75	2.6

6.4 Conclusions

In the past few years, artificial intelligence based optimization techniques have gained great popularity. The reason for this is the fact, that they offer the promise of a general, cost-effective method for optimizing bioprocesses. ANN-GA differs from conventional optimization in their ability to learn about the system without the prior knowledge of the interactions of the process variables. The prediction of the ANN based model was found to be superior to that of the regression based model. The results showed that the training of an artificial neural network with the experimental data from *Leuconostoc dextranicum* NRRL B-1146 fermentation was quite successful. The input space of the neural network model was optimized using GA. The ANN-GA predicted optimized enzyme activity was 6.92 U/ml, which was higher than that of 6.53 U/ml predicted by regression based model. The ANN-GA based optimized solution when verified experimentally, resulted in glucansucrase activity of 6.75 ± 0.1 U/ml which was higher than that of RSM based 6.4 U/ml. The study demonstrates ANN-GA based optimization using the experimental data from a previous RSM data. It is a robust tool for optimizing the medium composition for maximal enzyme activity. The approach for ANN-GA can be varied with different data inputs and this method can be used as a viable alternative to the standard RSM approach and also can be employed for modelling and optimization of different bioprocess systems.

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Chapter 7

Purification, Identification and Characterization of Glucansucrase

7.1 Introduction

Majority of glucans are synthesized from sucrose by glucansucrase secreted mainly by bacteria belonging to genera *Leuconostoc, Streptococcus* and *Lactobacillus* (Purama and Goyal 2005). The enzyme glucansucrases have been placed in family 70 of glycoside hydrolases based on sequence homology and glycoside hydrolases have been grouped till date into 111 families (http://afmb.cnrs-mrs.fr/CAZY/). Three different types of α-glucans synthesized by *Leuconostoc* sp. are recognized *viz*. dextran, mutan and alternan depending on the main glucosidic linkages present in their glucan (Monsan *et al.* 2001).

Bulk of the information on purification of extracellular glucansucrase has been generated from *Leuconostoc species*. The presence of glucan in the enzyme preparations results in aggregated forms of enzyme. In addition, glucans 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 have been used for purification (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 *et al.* 1993; Goyal and Katiyar 1994; Dols *et al.* 1998; Kitaoka and Robyt 1998; Nigam *et al.* 2006; Purama and Goyal 2007; Majumder *et al.* 2007). Among all the reported purification methods, fractionation by polyethylene glycol (PEG) is a simple, effective and single step method for glucansucrase purification (Russell 1979; Goyal and Katiyar 1994; Purama and Goyal 2007). Polyethylene glycol (PEG) is known to selectively precipitate proteins, which have high molecular weights or exist in aggregated forms (Honig and Kula 1976; Miekka and Ingham 1978). The large size of glucansucrase, together with its tendency to form aggregates in solution has led to the use of non-ionic hydrophilic polymer PEG for precipitation of glucansucrase (Goyal and Katiyar 1994; Nigam *et al.* 2006; Purama and Goyal 2007). Several workers have reported that glucansucrase exists in single or multiple forms having molecular weight in the range 64,000–245,000 (Kobayashi and Matsuda 1980; Miller *et al*. 1986; Fu and Robyt 1990; Goyal and Katiyar 1994; Purama and Goyal 2007).

The enzyme remains in an aggregated form in the presence of glucan resulting in high molecular weight. Various metals ions; Ca^{2+}, Mg^{2+}, Co^{2+} and among stabilizers, Triton X-100, glycerol, Tween-80, polyethylene glycols, dextran, glutaraldehyde and polyvinyl alcohols have significant effect on glucansucrases (Kobayashi and Matsuda 1980, Miller and Robyt 1984; Goyal *et al.* 1995). The enzyme can be stabilized by high-molecular-weight dextran, polyethyleneglycol or nonionic detergents such as Tween 80 (Miller and Robyt 1984; Goyal and Katiyar 1994). Glucansucrases are inactivated by EDTA and can be reactivated with Ca^{2+} (Miller and Robyt 1986). Dextransucrase is strongly inhibited by metal ions such as Cu^{2+}, Fe^{3+} and Mn^{2+} (Kobayashi and Matsuda 1975; Robyt and Walseth 1979).

In the present study purification of glucansucrase from *Leuconostoc dextraniucm* NRRL B-1146 using polyethylene glycol and size-exclusion chromatography was carried out. The glucansucrase was identified and confirmed by Periodic Acid Schiff's (PAS) staining procedure using sucrose as substrate.

7.2 Materials and Methods

7.2.1 Microorganism and reagents

Leuconostoc dextranicum NRRL B-1146 was procured from ARS Culture collection, USDA, USA. Ingredients 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. PEGs-(200, 400, 4000, 6000) from (Merck India) were used for enzyme fractionation.

7.2.2 Production of glucansucrase

Leuconostoc dextranicum NRRL B-1146 was grown in 100 ml enzyme production medium (as described by Tsuchiya *et al.* 1952) at 28°C and static flask condition. The details are described in Chapter 3, Section 3.2.6.

7.2.3 Enzyme activity assay

The assay of glucansucrase was carried out in 1 ml of a reaction mixture in 20 mM sodium acetate buffer, pH 5.4, containing 292 mM (10%) sucrose and using 10-20 µl) of enzyme sample. The reaction mixture was incubated at 30°C for 15 min. Aliquots (0.1 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 protein concentration of the cell free extract and PEG fractions 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 glucansucrase by PEG fractionation

The ice cold polyethylene glycols of different molecular weights (PEG-200, PEG-400) were added to 200 ml cell free extract to obtain their final concentrations between 10-50% (v/v). A 60% PEG-4000 and PEG-6000 solution was prepared with distilled water and added to the cell free extract to get their final concentrations between 5-25% (w/v). They were incubated at 4°C for 12h to allow the glucansucrase to fractionate. The mixture was centrifuged at 13,200***g*** for 30 min at 4°C to separate the fractionated glucansucrase. The enzyme pellet was separated and dissolved in 20 mM sodium acetate buffer (pH 5.4). The resuspended enzyme samples were subjected to dialysis using 5 kDa cut off membrane. The glucansucrase samples were analyzed for enzyme activity and protein content.

7.2.6 Purification of glucansucrase by gel-filtration chromatography

The purified glucansucrase after fractionation by 30% PEG-400 was dialyzed extensively against 20 mM sodium acetate buffer (pH 5.4). 2 ml of the dialyzed enzyme (1.45 mg/ml, with specific activity of 4.5 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. The column was 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 using 20 mM sodium acetate buffer pH 5.4 at a flow rate of 0.2 ml/min. 4 ml fractions were collected and analyzed for enzyme activity and protein concentration. The 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 further analyzed by SDS-PAGE.

7.2.7 SDS-PAGE analysis of purified glucansucrase

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) and 0.05% (w/v) bromophenol blue. 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. Purified glucansucrase fraction was loaded on 7.5% acrylamide gel (without the stacking gel) and was run under SDS-non denaturing condition. Electrophoresis was carried out using running buffer (200 mM glycine, 0.1% SDS, 50 mM Tris-HCl pH 8.3) with a current of 2 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 glucansucrase by activity staining using Periodic Acid Schiff's (PAS) protocol

In-situ activity of glucansucrase 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, 4% (w/v) for stacking gel, running buffer and the sample buffer contained SDS (the cationic detergent for movement of samples under charged conditions). The samples for loading on to the gel were prepared and run as described in Section 7.2.7. 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 for 30 min. Then the gel was incubated in sodium acetate buffer (20 mM sodium acetate, pH 5·4, 0·3 mM $CaCl_2$) supplemented with 10% sucrose at 30°C for 48h. Following incubation, the gel was washed once with a solution of methanol: acetic acid (50:10) in water for 30 min, then with water for 30 min, and incubated in a periodic acid solution (1% periodic acid and 3% acetic acid) at 30°C for 45 min. After the periodic acid treatment, the gel was washed with water for 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 colour bands within the gel matrix appeared, which confirmed glucansucrase activity.

7.2.9 Determination of K_m and V_{max} of glucansucrase

The effect of sucrose concentration on enzyme activity was studied by varying its concentration between 0.1-15% in the enzyme reaction mixture to determine its K_m and V_{max}. The purified glucansucrase with 9 U/mg specific activity was used for the study of effect of sucrose concentration on glucansucrase activity. The reaction was carried out in 1 ml mixture in 20 mM sodium acetate buffer pH 5.4 containing 20 µl

of enzyme (0.45 mg/ml) and varying concentration of sucrose. The mixture was incubated at 30°C for 15 min in water bath and activity was determined by estimating the released reducing sugar, as described earlier in Section 7.2.3.

7.2.10 Effect of salts and denaturing agents on glucansucrase

The effects of $CaCl_2$, $MgCl_2$, and $CoCl_2$ between 0-10 mM and EDTA between 0-2 mM concentrations were studied on purified glucansucrase. The purified enzyme with protein concentration of 1.45 mg/ml and 4.5 U/mg was used. The assays were carried out in 1.0 ml reaction mixture containing the salt or additive, the substrate sucrose (10%) in 0.2 M sodium acetate buffer (pH 5.4) and 10-20 µl enzyme. The enzyme activity was determined as described earlier in Section 7.2.3. The effect of urea was studied by incubating enzyme with urea (0-5 M final concentration) at 30°C for 30 min. The aliquots (20 µl) were taken and assayed for residual enzyme activity as described earlier in Section 7.2.3.

7.2.11 Effect of stabilizers on glucansucrase

The stability was studied by incubating glucansucrase with different stabilizers at 30°C for 20h. The additives at final concentration of 2 µg/ml dextran (500 kDa) and 10 µg/ml Tween-80 were added to glucansucrase solution (0.45 mg/ml, 9.0 U/mg) in 20 mM sodium acetate buffer, pH 5.4. The aliquots of 0.1 ml were withdrawn at different time intervals and were analyzed for residual glucansucrase activity as described earlier in Section 7.2.3.

7.3 Results and Discussion

7.3.1 Fractionation by PEG-200

Glucansucrases 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 glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. The purified glucansucrase was analyzed for purity and confirmed by staining the glucan produced by it. Glucansucrase purification by fractionation with PEG-200 was carried out using the cell free extract having a specific activity of 0.6 U/mg. PEG-200 was added to obtain various final concentrations ranging from 10-50% (v/v). The maximum specific activity of 2.5 U/mg was obtained at 40% (v/v) concentration of PEG-200 (Fig. 7.3.1). The purification by other fractions on either side of 40% final concentration resulted in lower specific activities of glucansucrase (Fig. 7.3.1). A maximum, 4 fold purification with 5% overall yield of glucansucrase was obtained with 40% PEG-200 concentration (Table 7.3.1).

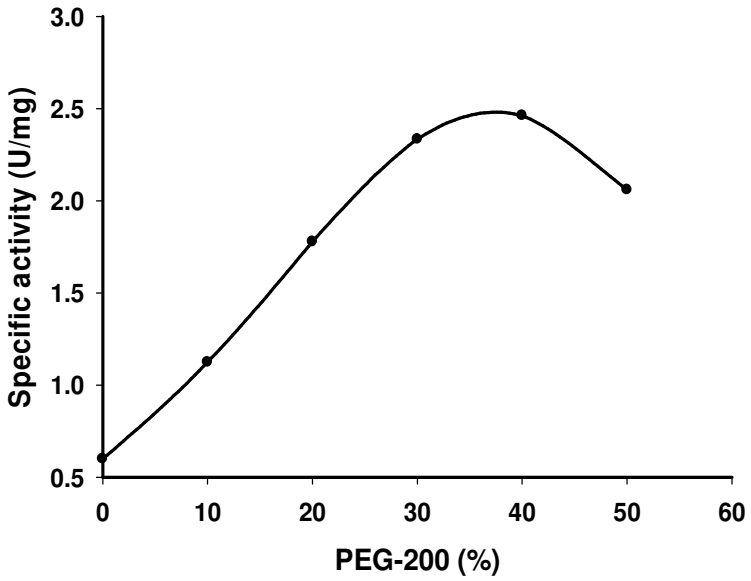

Fig. 7.3.1. Fractionation of glucansucrase with PEG-200. Specific activity of glucansucrase obtained were plotted with various fractions (%, v/v).

Table 7.3.1. Purification of glucansucrase by fractionation with PEG-200

PEG-200(%)	Volume (ml)	Glucansucrase			Protein (mg/ml)	Total (mg)	Specific activity (U/mg)	Fold Purification
		Activity (U/ml)	Total Units	Overall % Yield				
Crude	200	3.1	624	-	5.2	1040	0.6	-
10	10	0.9	9	1.4	0.8	8	1.1	1.9
20	10	1.6	16	2.6	0.9	9	1.8	3.0
30	10	2.8	28	4.5	1.2	12	2.3	3.9
40	10	3.2	32	5.1	1.3	13	2.5	4.1
50	10	2.8	28	4.5	1.36	13.6	2.1	3.4

7.3.2 Fractionation by PEG-400

Glucansucrase purification by fractionation using PEG-400 concentrations ranging from 10 to 50% (v/v) was carried out. The cell free extract having specific activity of 0.6 U/mg was used for PEG fractionation. The fractionation by 30% (v/v) concentration of PEG-400 gave the maximum specific activity of 4.5 U/mg (Fig. 7.3.2) with 7.5 fold purification and 10.5% over all yield (Table 7.3.2). The purification by higher concentrations of PEG-400 did not favor the enzyme activity and resulted in decrease of specific activity of glucansucrase (Table 7.3.2)

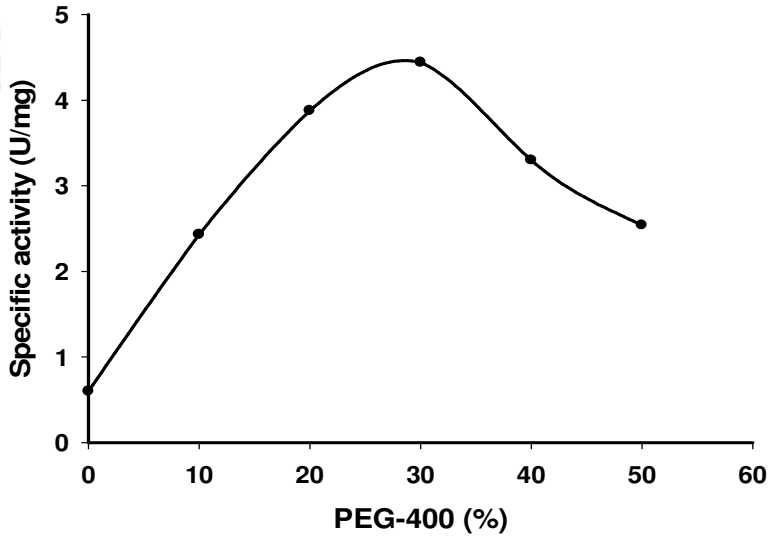

Fig.7.3.2. Fractionation of glucansucrase by PEG-400. The specific activity of glucansucrase obtained were plotted with various fractions (%, v/v).

Table 7.3.2. Purification of glucansucrase by PEG-400 fractionation

PEG-400 (%)	Volume (ml)	Glucansucrase			Protein (mg/ml)	Total (mg)	Specific activity (U/mg)	Fold Purification
		Activity (U/ml)	Total Units	Overall %Yield				
Crude	200	3.1	624	100	5.20	1040	0.6	-
10	10	2	20	3.2	0.82	8.2	2.4	4.1
20	10	3.8	38	6.1	0.98	9.8	3.9	6.5
30	10	6.4	64	10.3	1.45	14.5	4.5	7.4
40	10	5.6	56	9.0	1.70	17	3.3	5.5
50	10	4.6	46	7.4	1.82	18.2	2.5	4.2

7.3.3 Fractionation by PEG-4000

The glucansucrase purification was carried out by PEG-4000 fractionation by varying its concentrations between 5 to 25% (w/v) using cell free extract having a specific activity of 0.6 U/mg. A 10% (v/v) PEG-4000 fraction gave the glucansucrase with maximum specific activity of 2.55 U/mg (Fig. 7.3.3) with 4 fold purification (Table 7.3.3). With an increase in the concentration of PEG-4000 beyond 10%, a decrease in specific activity of enzyme was observed (Fig. 7.3.3, Table 7.3.3).

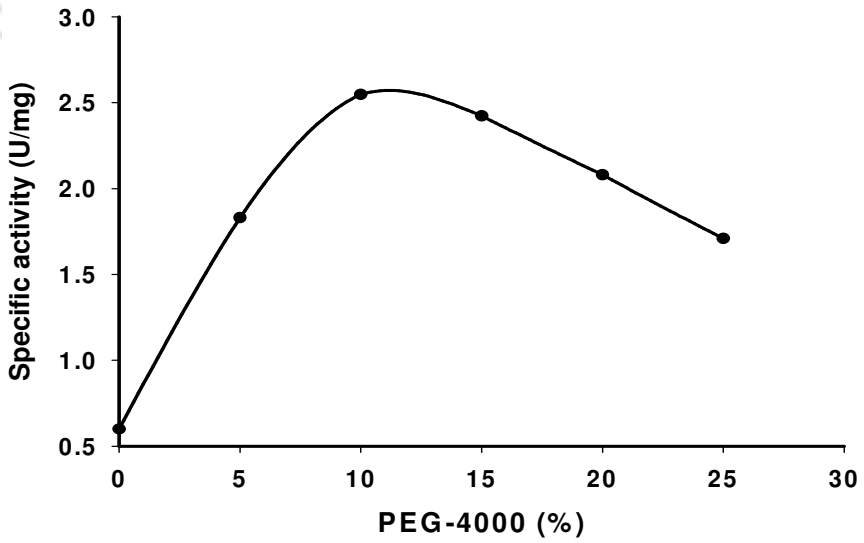

Fig. 7.3.3. Fractionation of glucansucrase by PEG-4000. Specific activity profiles of glucansucrase obtained were plotted with various fractions (%,w/v).

Table 7.3.3. Purification of glucansucrase by PEG-4000 fractionation

PEG-4000 (%,w/v)	Volume (ml)	Glucansucrase			Protein (mg/ml)	Total (mg)	Specific activity (U/mg)	Fold Purification
		Activity (U/ml)	Total Units	Overall %Yield				
Crude	200	3.1	624	–	5.2	1040	0.6	–
5	10	1.1	11	1.8	0.61	6.1	1.8	3.1
10	10	2.1	21	3.4	0.83	8.3	2.6	4.2
15	10	2.6	26	4.1	1.03	10.3	2.5	4.1
20	10	3.0	30	4.8	1.23	12.3	2.4	4.0
25	10	3.2	32	5.2	1.89	18.9	1.7	2.8

7.3.4 Fractionation by PEG-6000

Glucansucrase purification by fractionation using PEG-6000 ranging from 10 to 25% (v/v) was carried out from the cell free extract with a specific activity of 0.6 U/mg. The fractionation by 10% (v/v) PEG-6000 gave the maximum specific activity of 2.4 U/mg (Fig. 7.3.4) with 4 fold purification and 5% over all yield (Table 7.3.4). The purification by higher concentrations of PEG-6000 did not favour the enzyme activity (Table 7.3.4).

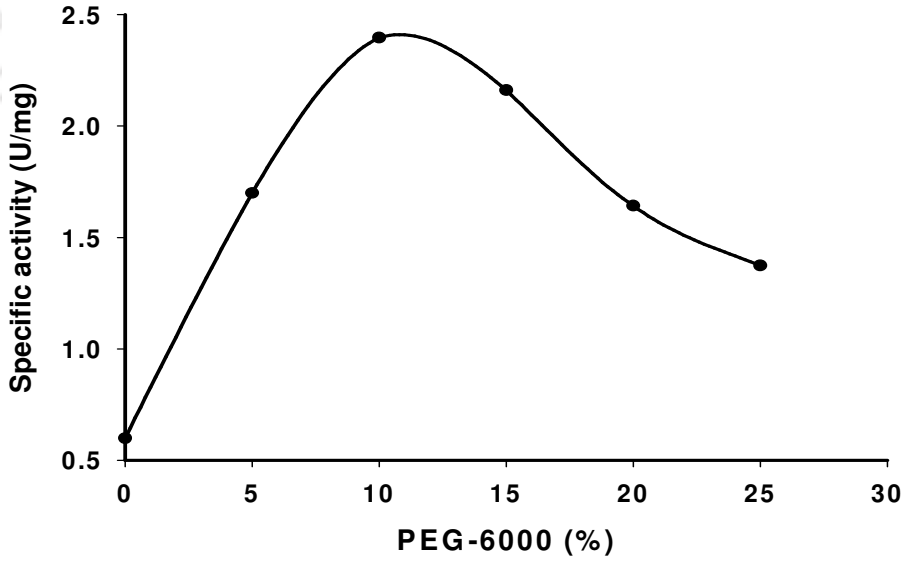

Fig. 7.3.4. Fractionation of glucansucrase by PEG-6000. Specific activity of glucansucrase obtained were plotted with various fractions (%, w/v).

Table 7.3.4. Purification of glucansucrase by PEG-6000 fractionation

PEG-6000 (%,w/v)	Volume (ml)	Glucansucrase			Protein (mg/ml)	Total (mg)	Specific activity (U/mg)	Fold Purification
		Activity (U/ml)	Total Units	Overall %Yield				
Crude	200	3.12	624	–	5.2	1040	0.6	–
5	10	1.45	14.5	2.3	0.9	9	1.7	2.8
10	10	2.6	26	4.1	1.1	11	2.4	4.0
15	10	2.65	26.5	4.2	1.4	14	2.0	3.4
20	10	2.7	27	4.3	1.6	16	1.6	2.7
25	10	2.8	28	4.4	2.0	20	1.4	2.3

7.3.5 Comparison of glucansucrase fractionation by different PEGs

The purification of glucansucrase by fractionation using different PEGs (PEG-200, 400, 4000 and 6000) were compared (Fig. 7.3.5). Table 7.3.5. shows the maximum specific activity, fold purification and % yield of glucansucrase with corresponding concentrations of PEG-200, PEG-400, PEG-4000 and PEG-6000. The fractionation with PEG-400 gave glucansucrase with significantly higher specific activity of 4.5 U/mg than other PEGs. The overall yield was 10.5% and fold purification was 7.5. Glucansucrase from *Streptococcus mutans* was purified by PEG precipitation using PEG 400 and 6000. PEG 6000 precipitated other non-glucansucrase proteins, while PEG 400 gave higher specificity of precipitation (Russell 1979).

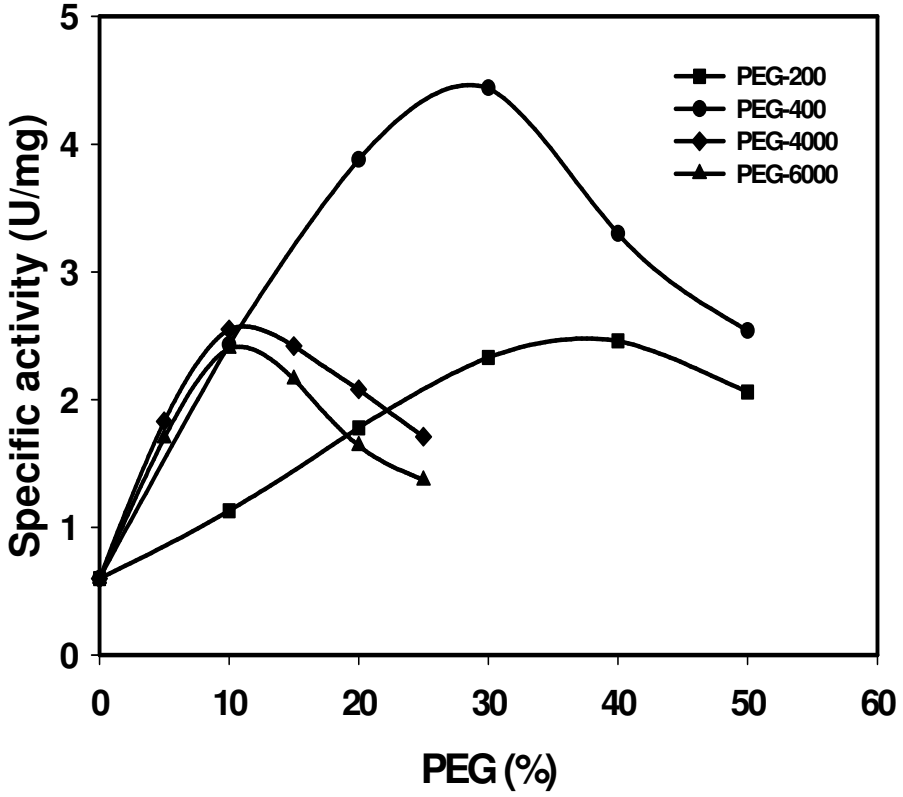

Fig. 7.3.5. Purification of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 by PEG-200, 400, 4000 and 6000. The specific activity of purified glucansucrase by different final concentrations of PEGs were plotted.

Table 7.3.5. Comparison of the glucansucrase 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	3.1	624	–	5.2	1040	0.6	–
40% PEG-200	10	3.2	32	5.1	1.3	13	2.5	4.1
30 % PEG-400	10	6.6	65.5	10.5	1.45	14.5	4.5	7.5
10% PEG-4000	10	2.1	21.2	3.4	0.83	8.32	2.6	4.2
10% PEG-6000	10	2.6	25.78	4.1	1.08	10.76	2.4	4.0
SephacrylHR200	12	4.1	48.6	7.8	0.45	5.4	9.0	15.0

7.3.6 Purification of glucansucrase by gel-filtration chromatography

The glucansucrase purified by 30% PEG-400 in single step with a specific activity of 4.5 U/mg was subjected to Sephacryl S-200HR gel-filtration chromatography. The Fig. 7.3.6 shows the enzyme activity and protein (absorbance, A_{280}) profiles of glucansucrase obtained from the eluted 4 ml fractions. The fractions were analysed for specific activity of glucansucrase. 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 9 U/mg with 15 fold purification was obtained by gel-filtration chromatography (Table 7.3.5). The PEG 400 reproducibly gave glucansucrase from *Leuconostoc mesenteroides* NRRL B-512F with specific activity of 8.7 U/mg and 15-fold purification (Goyal and Katiyar 1994). Similar method for purification was also reported for glucansucrase from *Leuconostoc mesenteroides* NRRL B-640 (Purama and Goyal 2007). The purification of glucansucrase with PEG 1500 resulted specific activity of 23 U/mg with 40 fold purification and further purification by gel-filtration gave a specific activity of 35 U/mg with 61 fold purification (Purama and Goyal 2007).

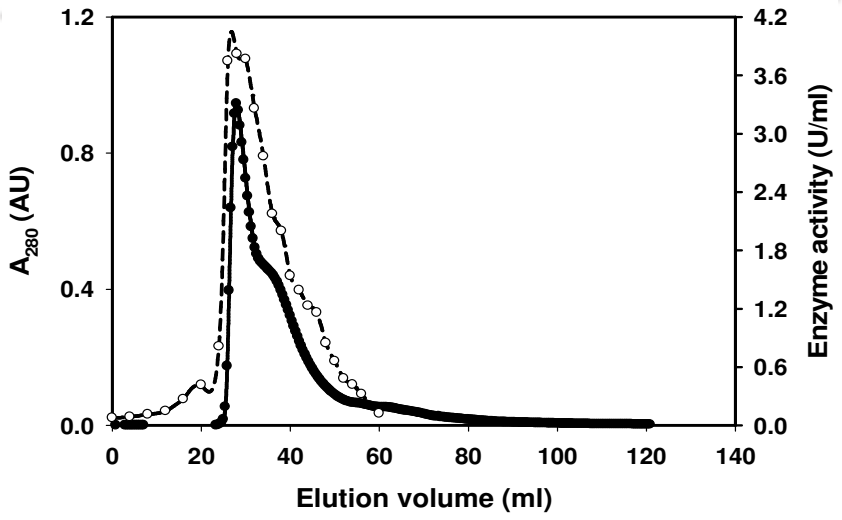

Fig. 7.3.6. Elution pattern of glucansucrase on Sephacryl S-200HR (•) A280; (◦) enzyme activity (U/ml).

7.3.7 Identification, confirmation and molecular size analysis of glucansucrase

The glucansucrase obtained after purification from Sephacryl S-200HR column was run on 2 identical SDS-PAGE gels under non denaturing conditions for *in-situ* activity detection. The native form of the purified glucansucrase corresponded to approximately 205 kDa molecular size that appeared on the denaturing gels stained with Coomassie Brilliant Blue (Fig. 7.3.7A). White bands appeared on the gels when incubated in 10% sucrose for 48h. These white bands turned in to magenta color after PAS staining, which confirmed the presence of glucan formed on polyacrylamide gels (Fig. 7.3.7B). The PAS staining of the sucrose incubated gels showed that the magenta colour activity bands are the native and active form of the purified glucansucrase. This comparison of activity staining and Coomassie Brilliant Blue staining of gels identified the presence of enzyme glucansucrase and also showed that molecular size of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 is 205 kDa.

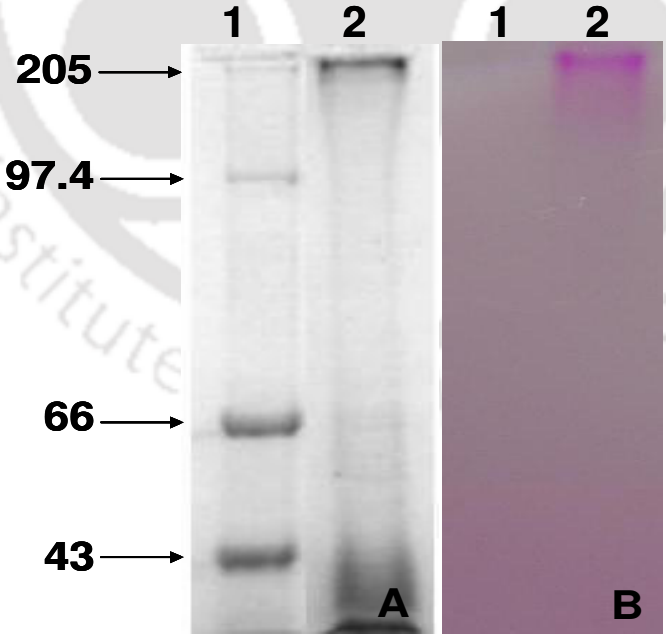

Fig. 7.3.7. Identification of purified glucansucrase by (A) CBB R-250 staining. (B) Activity staining of the glucan formed (Lanes: 1-Protein molecular weight marker: 29-205 kDa; 2-Sephacryl S-200HR pooled fraction).

7.3.8 Determination of Kinetic parameters of glucansucrase

The effect of sucrose concentration on the enzyme activity was studied by varying sucrose concentration between 1-500 mM final concentrations. The results showed that the enzyme glucansucrase follows the classical Michaelis-Menten kinetics and the saturation reached at 292 mM (10%) (Fig. 7.3.8). The data were analysed using GraphPad Prism software. The purified glucansucrase gave a K_m value of 18.7 mM and a V_m of 9.4 U/mg. Kinetic studies of the glucansucrases are affected by the presence of glucan. Different reports are available on the K_m by glucansucrases. Glucansucrase from *Leuconostoc mesenteroides* NRRL B-1299 exhibited a K_m of 10.7 mM (Kobayashi and Matsuda 1975). The K_m of 14.9 mM, 16 mM and 47 mM was reported for glucansucrase from *Leuconostoc mesenteroides* NRRL B-512F (Kobayashi *et al.* 1986; Miller *et al.* 1986; Goyal *et al.* 1995)

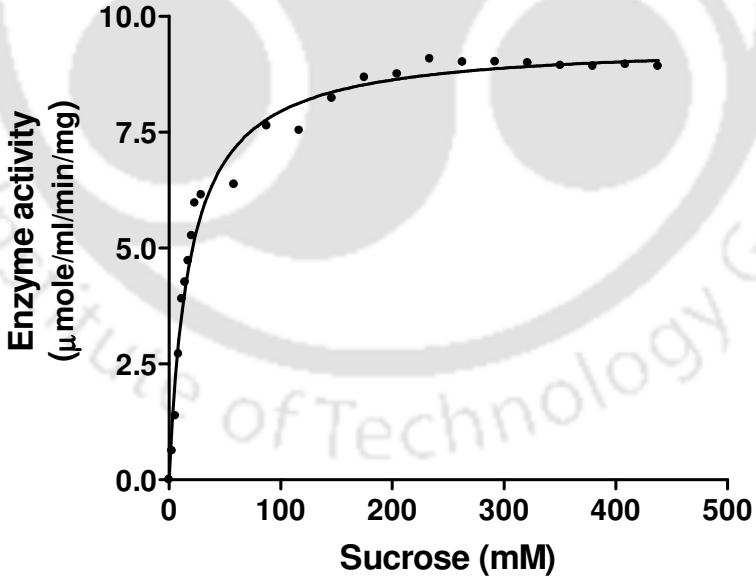

Fig. 7.3.8. Effect of sucrose concentration on glucansucrase activity assayed at 30°C in 20 mM sodium acetate, pH 5.4.

7.3.9 Effect of salts and denaturing agents on glucansucrase activity

The effect of different divalent metal ions Ca^{2+}, Mg^{2+} and Co^{2+} on activity of glucansucrase was studied. These solutes stabilize the catalytic activity of enzymes by stabilizing the three-dimensional protein structure (Robyt and Walseth 1979; Goyal *et al.* 1995). The Ca^{2+} ions had a significant activation effect on enzyme activity of glucansucrase. There enzyme activity increased by 2.8 fold at 6 mM $CaCl_2$ (Fig. 7.3.9A). Mg^{2+} and Co^{2+} exhibited a marginal increase in the enzyme activity of glucansucrase. (Fig. 7.3.9B and C). The addition of 6 mM $CaCl_2$, 4 mM $CoCl_2$ and 2 mM $MgCl_2$ to glucansucrase caused an enhancement of enzyme activity by 280%, 22% and 12%, respectively (Table 7.3.6). Further increase in the salt concentrations did not favour the increase in the enzyme activity. Similar results were reported for *Leuconostoc mesenteroides* B-512F glucansucrase where, the activity of enzyme was enhanced by the addition of Ca^{2+} and Co^{2+} and inhibited by EDTA indicating that glucansucrases are associated with alkaline earth metals (Robyt and Walseth 1979; Kobayashi and Matsuda 1980). Ca^{2+} has been reported to be associated with the two catalytic sites of glucansucrases (Miller and Robyt 1986). One of the sites is activating and the other involved in sucrose binding is inhibitory. Ca^{2+} at the inhibitory site prevents binding of sucrose (Miller and Robyt 1986).

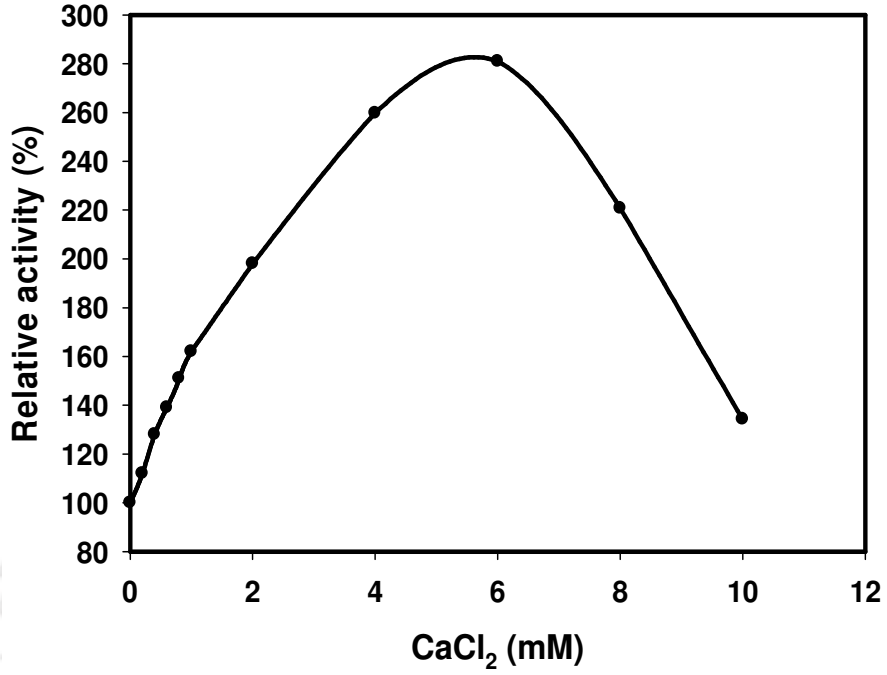

Fig. 7.3.9A. Effect of $CaCl_2$ on *in-vitro* activity of purified glucansucrase.

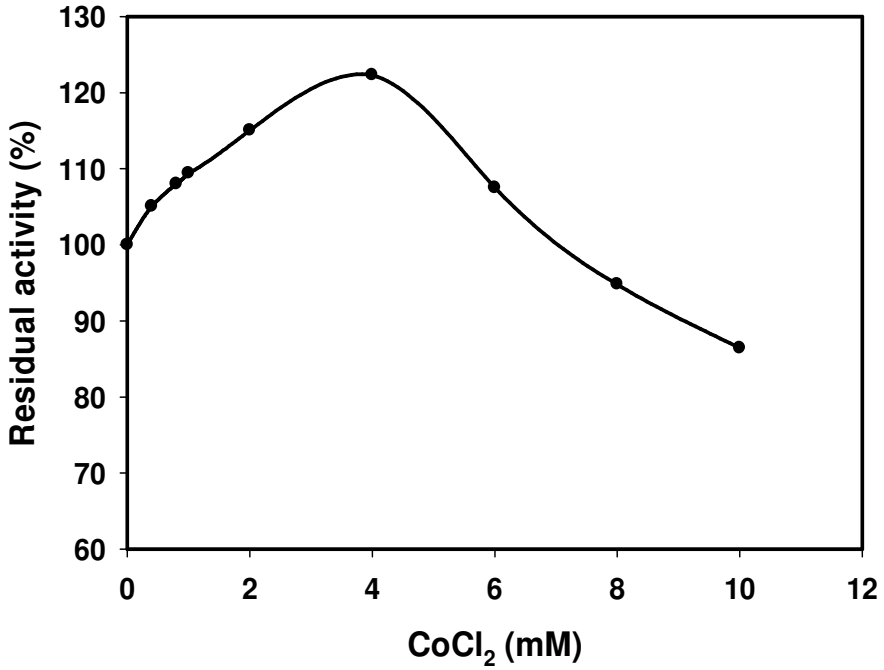

Fig. 7.3.9B. Effect of $CoCl_2$ on *in-vitro* activity of purified glucansucrase.

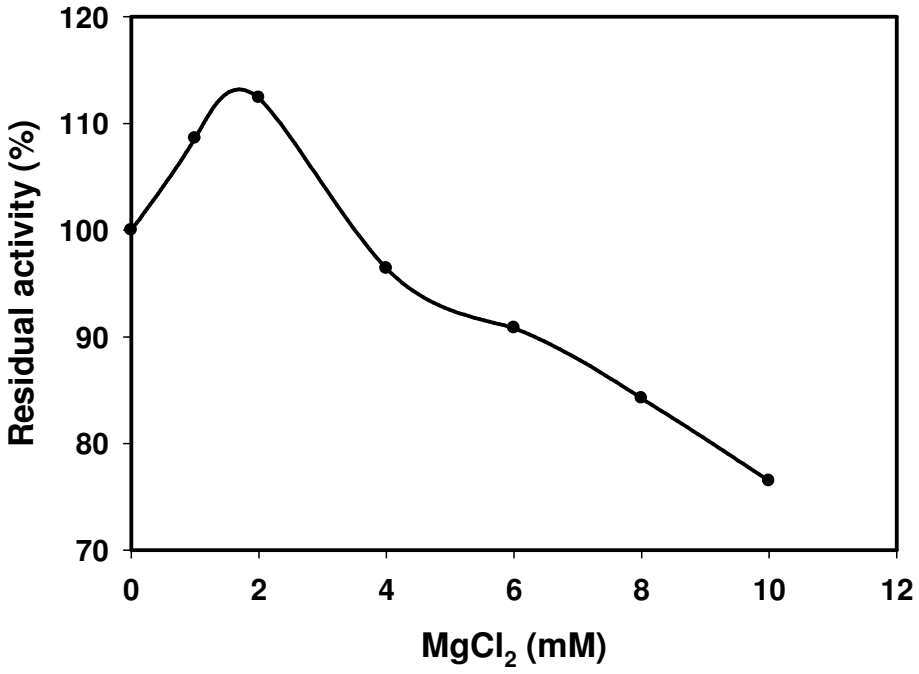

Fig. 7.3.9C. Effect of $MgCl_2$ on *in-vitro* activity of purified glucansucrase.

The addition of EDTA inhibited the enzyme activity of *Leuconostoc dextranicum* NRRL B-1146 glucansucrase and 50% inactivation was obtained with 0.8 mM EDTA (Fig. 7.3.10). The greater extent of inactivation was observed with further increase in the concentration and saturation reached at 2 mM EDTA with a 12% residual activity.

Urea displayed deactivating effect on glucansucrase at all concentrations. With an increase in the concentration of urea there was a drastic decrease in enzyme activity. The enzyme lost 49%, 72%, 88%, 93% and 99% of activity in 30 min when treated with 1, 2, 3, 4 and 5M urea, respectively (Fig. 7.3.11). These results were different from those observed with urea effect on glucansucrase of *Leuconostoc mesenteroides* NRRL B-1299 where the complete loss of enzyme activity was achieved with 1M urea (Kobayashi and Matsuda 1975).

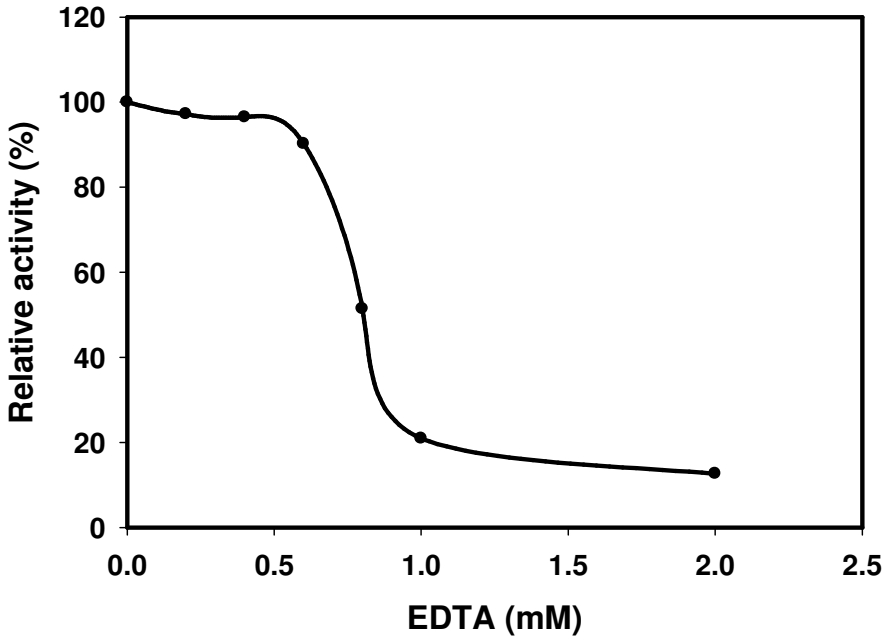

Fig. 7.3.10. Effect of EDTA on glucansucrase: 0.45 mg/ml enzyme in 20 mM sodium acetate buffer (pH 5.4) was incubated at 30°C along with EDTA. The enzyme activity was estimated as described in Section 7.2.3.

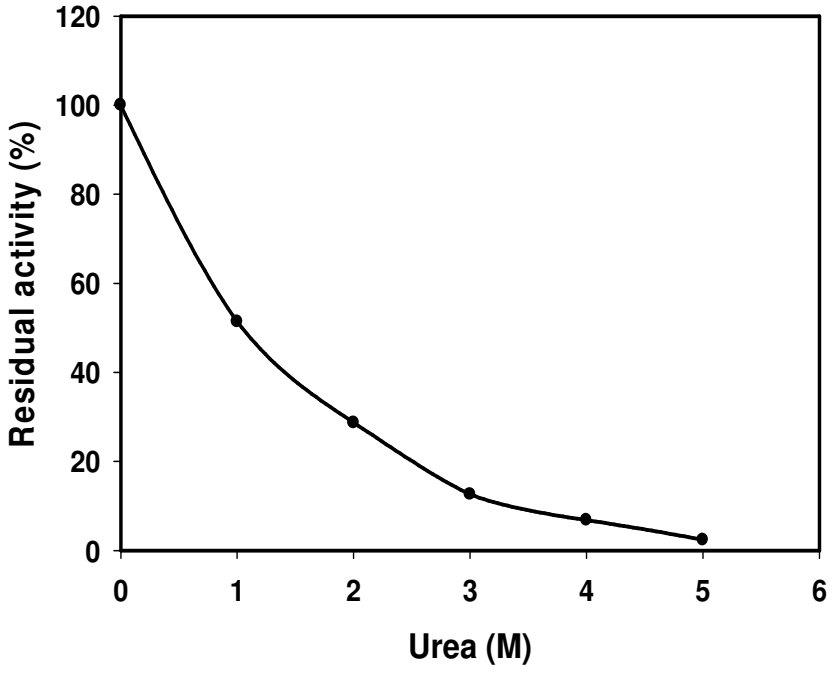

Fig. 7.3.11. Effect of urea on glucansucrase: 0.45 mg/ml enzyme in 20 mM sodium acetate buffer (pH 5.4) was incubated with various concentrations of urea at 30°C for 30 min and the aliquots (0.1 ml) were taken and enzyme activity was estimated as described in Section 7.2.3.

Table 7.3.6. The effect of divalent metal ions, chelate compound and denaturant on glucansucrase activity.

	Relative activity (%)
$CaCl_2$ (6 mM)	280
$MgCl_2$ (2 mM)	112
$CoCl_2$ (4 mM)	122
EDTA (2 mM)	12
Urea (5 M)	02

7.3.10 Effect of stabilizers on glucansucrase activity

The effect of 10 µg/ml Tween 80 and 10 µg/ml dextran (500 kDa) on glucansucrase activity was studied for their stabilising effect on glucansucrase. The residual activity of glucansucrase at the end of 20h was 86%, 45% and 33% with Tween 80, dextran (500 kDa) and control, respectively at 30°C (Fig. 7.3.12). Tween 80 was the best stabilizer for glucansucrase at 30°C. Tween 80 has also been reported to stabilize glucansucrase from other strains also (Goyal and Katiyar 1995). Surprisingly, dextran (500 kDa) did not provide stability to the glucansucrase from *Leuconostoc dextranicum* B-1146, though it has been reported with other strains that dextran imparts stabilization to glucansucrase (Willemot *et al.* 1988; Goyal *et al.* 1995).

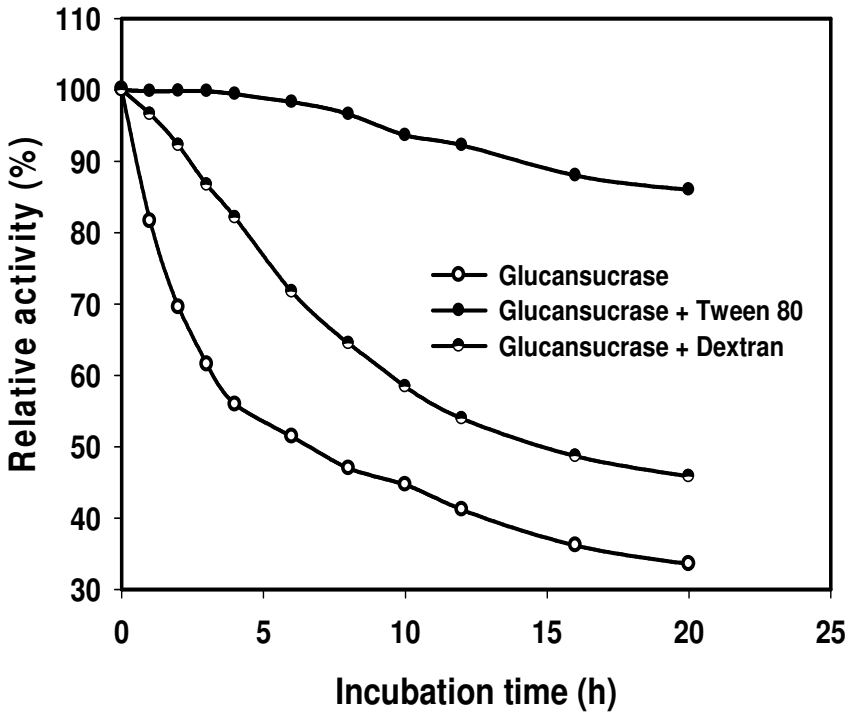

Fig. 7.3.12. Effect of Tween 80 and dextran (500 kDa) on glucansucrase activity at 30°C. 0.45 mg/ml glucansucrase in 20 mM sodium acetate buffer (pH 5.4) was pre-incubated with additives (Tween 80 and dextran (500 kDa)), at 30°C. The assay was carried out by taking 20 µl aliquots from enzyme-additive mixture in 1 ml reaction mixture containing 10% sucrose in 20 mM sodium acetate buffer pH 5.4 by incubating at 30°C as described in Section 7.2.3.

7.4 Conclusions

Polyethylene glycol (PEG) is known to selectively precipitate proteins, which have high molecular weights or exist in aggregated forms. Of the different molecular weight PEGs (PEG-200, 400, 4000 and 6000) used, PEG-400 gave the best results for purification of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. A final concentration of 30% PEG-400 was the optimum concentration for the purification of glucansucrase that resulted in maximum specific activity of 4.5 U/mg with 7.5 fold purification in a single step. The purification of glucansucrase by gel-filtration chromatography on Sephacryl S-200HR column using the single step purified enzyme by 30% PEG-400 resulted in specific activity of 9 U/mg with 15 fold purification. The presence of glucansucrase was identified and confirmed by its glucan synthesizing activity that was determined by activity staining bands of the glucan produced, on the non-denaturing PAGE by the purified glucansucrase when incubated in sucrose. The purified glucansucrase had a approximate molecular size of 205 kDa as determined by non denaturing SDS-PAGE. The purified glucansucrase gave V_m of 9.4 U/mg and K_m of 18.7 mM. The glucansucrase activity was enhanced by divalent cations (Ca^{2+}, Mg^{2+} and Co^{2+}) and 6 mM concentration of Ca^{2+} ion showed a significant activation of the enzyme by 280%. EDTA showed a chelating effect on enzyme associated with divalent cations and 90% of the glucansucrase activity was lost at 2 mM EDTA. The enzyme activity loss was 50% and 98% with urea at 1M and 5M concentrations, respectively when incubated for 30 min at 30°C. Of the two stabilisers dextran and Tween 80, Tween 80 provided higher stabilization to the glucansucrase against activity loss at 30°C. The residual activity with Tween 80 was 86% as compared to 33% with no additive after 20h incubation at 30°C.

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Chapter 8

Multi-response analysis of glucan production

8.1 Introduction

The immunomodulatory and anti-cancer effects of many polysaccharides and polysaccharide-protein complexes isolated from bacteria, mushrooms, fungi, yeasts, algae, lichens and plants, has attracted much attention in the medical and biochemical areas (Ooi and Liu 2000). It is generally known that polysaccharides enhance various immune responses *in vivo* and *in vitro*. Lactic acid bacteria produce a wide variety of exopolysaccharides. Homopolysaccharide synthesis in lactic acid bacteria has been mainly studied in oral *Streptococci*, *Leuconostoc* species and *Lactobacillus* species (Van Geel-Schutten *et al*. 1999). *Leuconostoc* species are commercially exploited for the production of glucans, homopolysaccharides which contain only one type of monosaccharide, glucose. They are potential therapeutic agents and are also used as viscosifying, stabilizing, emulsifying, sweetening, gelling, or water-binding agents, in the food as well as in the non-food industries (Korakli and Vogel 2006).

Glucans differ in the type of glucosidic linkages, degree and type of branching, length of glucan chains, molecular mass and conformation of polymers. All these properties strongly contribute to specific polysaccharide properties such as

solubility, rheology and other physical characteristics (Monchois *et al*. 1999). Depending on the main chain glucosidic linkages in glucan, three different types of α-glucans synthesized by *Leuconostoc* species are known *viz.* dextran with α-(1→6), mutan with α-(1→3) and alternan with α-(1→6) and α-(1→3) linkages. There are only few reports on the presence of α-(1→6) and α-(1→4) linkages from *Leuconostoc* species. *Leuconostoc dextranicum* NRRL B-1146 elaborates a glucan comprising α-(1→6) and α-(1→4) linkages (as described in Chapter 9).

The present interest in glucan with α-(1→6) and α-(1→4) linkages arises due to the potential it comprises as an anti-tumor and immunomodulatory agent. Response surface methodology is a collection of statistically based experimental designs, which have been established as a convenient method for optimizing various processes. Some of the popular choices include the Plackett-Burman design, the Box-Behnken design and the central composite design. In the present study a sequential optimization strategy for glucan production by *Leuconostoc dextranicum* B-1146 through statistically designed experiments was carried out. First, Plackett-Burman screening design was applied to address the most significant factors affecting enzyme production. Second, a Box-Behnken design was used to describe the interaction between different medium components and describe the nature of the response surface in the experimental region, to search optimal medium composition for maximizing the glucan yield.

8.2 Materials and methods

8.2.1 Microorganism

The strain *Leuconostoc dextranicum* NRRL B-1146 was procured from Agricultural Research Service Culture Collection, Peoria, USA. The culture was maintained in modified MRS (sucrose in place of glucose, DeMan *et al.* 1960), as stab at 4°C and sub-cultured every 15 days.

8.2.2 Inoculum preparation

Two loops of culture were taken from the stabs and grown in 5 ml medium described by Tsuchiya *et al.* 1952 incubated at 28°C for 12h. From the broth, 1% of inoculum was transferred to 100 ml of Tsuchiya medium contained in 250 ml Erlenmeyer flask and allowed to grow for 12h at 28°C. This inoculum culture was further used to inoculate various set of 100 ml medium in 250 ml Erlenmeyer flasks at 5% (v/v) level.

8.2.3 Flask culture using Tsuchiya medium and statistically designed medium

All the experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of Tsuchiya medium and 100 ml of medium as per the statistical experimental design (Tables 8.3.1 and 8.3.3). The pH of the medium was adjusted to 6.9 before autoclaving. The inoculated flasks were kept under static conditions at 28°C for 48h. All the runs were replicated and the glucan content was estimated. The culture supernatant was obtained by centrifugation the broth at 10,000***g*** for 10 min at 4°C. The crude glucan was precipitated by the addition of 3 volumes of 95% (v/v) pre-chilled ethanol at 0°C and centrifuged at 13,000***g***. The process was repeated and the polysaccharide content was determined by phenol-sulfuric acid method (Dubois *et al.*

1956) in a micro titer plate (Fox and Robyt 1991).

8.2.4 Estimation of glucan

To 0.025 ml of sample containing glucan in a microtitre plate, 0.025 ml of 5% (w/v) phenol was added. The plate was mixed at slow speed on a vortex mixer for 30 s. The plate was then placed onto ice bath and 0.125 ml of concentrated sulfuric acid was added to each well containing sample and phenol. The plate was again mixed for 30 s incubated in water bath at 80°C for 30 min and cooled and the absorbance was read at 490 nm. Standard curve was prepared using dextran (10 kDa) in the concentration range 0.1-1 mg/ml.

The concentration of glucan was calculated as follows:

$$\text{Glucan concentration (mg/ml)} = \frac{\Delta A_{490} \times C}{V} = \text{(mg/ml)}$$

C = 1 OD equivalent of dextran from standard plot

ΔA_{490} = change in absorbance of the sample

V = volume of the sample

8.2.5 Plackett-Burman design

The purpose of the first optimization step was to identify the ingredient(s) of the medium that has a significant effect on glucan production. Based on Plackett-Burman factorial design, each factor was examined in two levels: -1 for low level and +1 for high level (Plackett and Burman 1946). Table 8.3.1 shows the factors under investigation as well as levels of each factor used in the experimental design, and the Table 8.2 represents the design matrix. 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 (glucan yield mg/ml), 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 the interaction among factors and it is used to screen and evaluate the important factors that influence the response. In the present work, eleven assigned variables were screened in twelve experimental sets. All experiments were carried out in duplicate and the average of the glucan content was taken as response (Table 8.2). From the regression analysis, the factors which were significant at 90% level ($P < 0.1$) were considered to have greater impact on glucan production. The experimental design and statistical analysis of the data were done by Minitab statistical software package (Release 15).

8.2.6 Box-Behnken design

In order to describe the nature of the response surface in the experimental region, a Box-Behnken (Box and Behnken 1960) design was applied. The factors of highest confidence levels were prescribed into three levels, coded -1, 0, and +1 for low, middle and high concentrations, respectively. A total of 15 runs, performed in duplicate, were used to optimize the range and levels of chosen factors, viz., sucrose, peptone and yeast extract. The range and levels of the variables investigated in this study are given in Table 8.3.3. The relationships and interrelationships of the factors were determined by fitting the second order polynomial equation 2, to the data obtained.

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \qquad (2)$$

where, Y is the predicted response variable; β_o, β_i, β_{ii} and β_{ij} are constant regression coefficients of the model; and X_i and X_j (i=1,3; j=1,3, $i \neq j$) represent the independent factors (medium components in the form of coded values). The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. Minitab statistical software package (Release 15) was used for performing the regression and graphical analysis of the obtained data.

8.3 Results and Discussion

8.3.1 Production of glucan in Tsuchiya medium

The growth and production of glucansucrase from *Leuconostoc dextranicum* is favoured under static condition at 28°C owing to the microaerophilic nature of *Leuconostoc* sp. The glucansucrase production medium of Tsuchiya *et al.* (1952) was used as the basal medium for glucan production. The cell growth entered the log phase at the end of 6h (Fig. 8.3.1). The pH of the medium dropped to 4.7 and remained constant till the end of 48h of fermentation. The production of glucan did not increase after 24h and the maximum glucan yield that could be obtained was 0.240 mg/ml (Fig. 8.3.1).

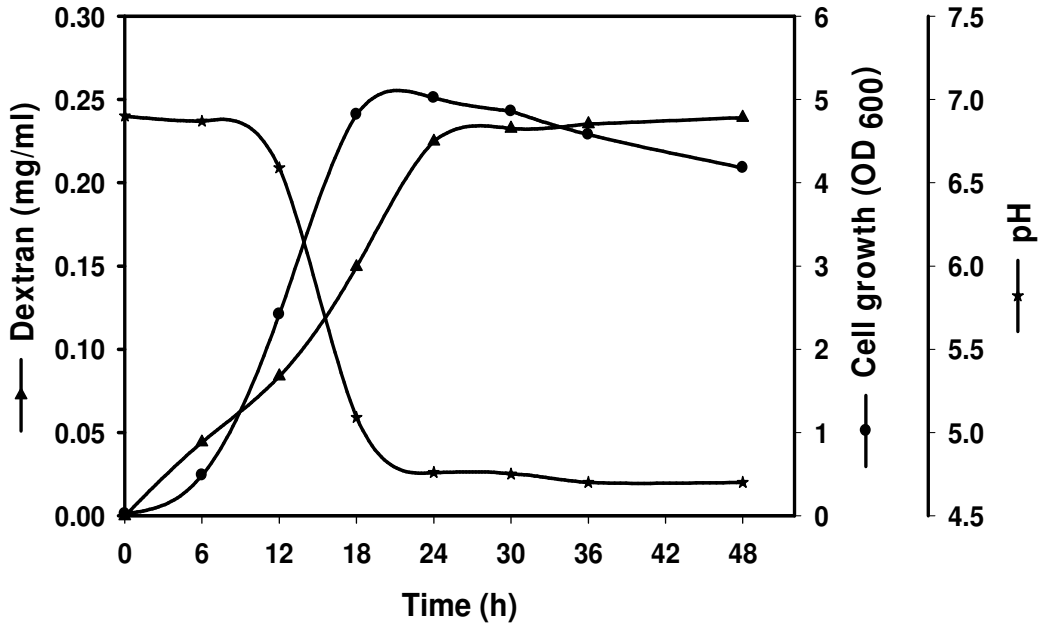

Fig. 8.3.1. Fermentation profile of *Leuconostoc dextranicum* in Tsuchiya medium and the production of glucan.

8.3.2 Evaluation of factors affecting glucan production

Plackett-Burman design was used as first approach to screen relatively important factors affecting the glucan production from *Leuconostoc dextranicum* NRRL B-1146. The averages of glucan production in (mg/ml) for 12 different sets of 11 medium components are presented in Table 8.3.2. The *t*-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance (Karthikeyan *et al.* 1996). Sucrose, peptone and yeast extract were found to be the most significant factors enhancing the glucan production (Table 8.3.1). The main effects of the examined factors on glucansucrase production are presented graphically in Fig. 8.3.2. $CaCl_2$ was found to be significant factor although with a negative coefficient. The addition of $CaCl_2$ was avoided in subsequent experiments as it did not aid in enhancing glucan production and the minor requirements of the microorganisms were fulfilled by the complex components of yeast extract and beef extract.

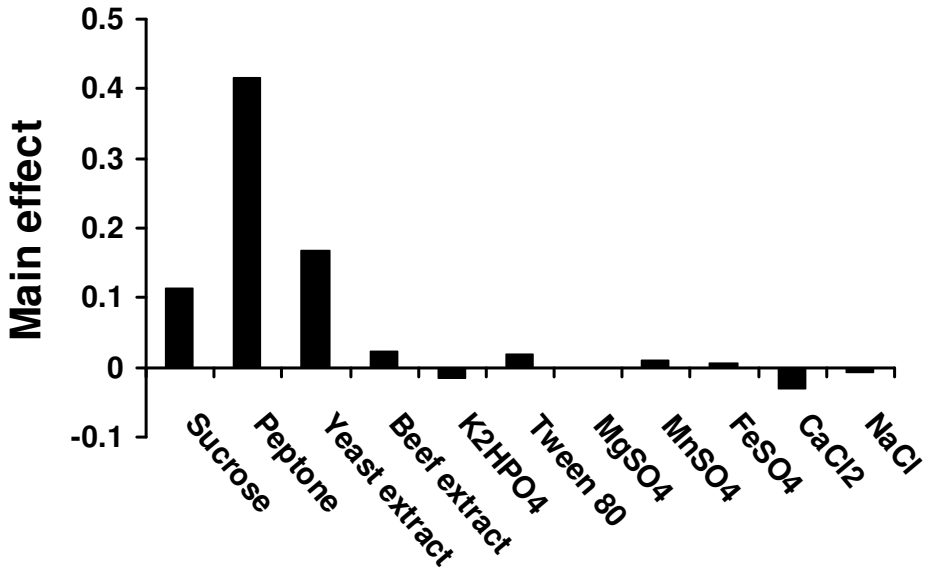

Fig. 8.3.2 Effect of nutrients on glucan production by *Leuconostoc dextranicum*.

The polynomial model describing the correlation between the 11 factors and the glucan yield could be presented as:

$$Y_{mg/ml} = 0.4145 + 0.1136X_1 + 0.4169X_2 + 0.1678X_3 + 0.0231X_4 - 0.0156X_5 + 0.0181X_6 - 0.0017X_7 + 0.011X_8 + 0.0057X_9 - 0.0305X_{10} - 0.0064X_{11} \quad (3)$$

Sucrose was chosen as the carbon source as it was the inducer for the production of glucansucrase and also the substrate for glucan production (Tsuchiya *et al.* 1952). The +1 level of sucrose was chosen at 6% (w/v) as higher concentrations than 6% lead to increased viscosity making the separation of cells difficult. Different nitrogen sources were chosen as they were found to significantly affect the production of glucan from *Leuconostoc* species (Barker and Ajongwen 1991; Karthikeyan *et al.* 1996).

Table 8.3.1. The factors, levels and the regression analysis of the Plackett-Burman design.

Factors (% w/v)	Levels		*t* Stat	*P*-value
	-1	+1		
Sucrose (X_1)	1.0	6.0	6.83	0.0001
Peptone (X_2)	0.5	3.0	2.51	0.029
Yeast Extract (X_3)	0.5	3.0	10.08	0.0001
Beef Extract (X_4)	0.5	3.0	1.39	0.193
K_2HPO_4 (X_5)	1.0	4.0	-0.93	0.37
Tween 80 (X_6)	0.1	1.0	1.09	0.299
$MgSO_4$ (X_7)	0.01	0.1	-0.07	0.945
$MnSO_4$ (X_8)	0.001	0.01	0.66	0.522
$FeSO_4$ (X_9)	0.001	0.01	0.34	0.739
$CaCl_2$ (X_{10})	0.001	0.01	-1.83	0.094
NaCl (X_{11})	0.001	0.01	-0.39	0.707

Table 8.3.2. Plackett-Burman design for 11 variables with coded values along with the observed results.

Run Order	Sucrose	Peptone	Yeast Extract	Beef Extract	K_2HPO_4	Tween80	$MgSO_4$	$MnSO_4$	$FeSO_4$	$CaCl_2$	NaCl	Glucan (mg/ml)
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	0.668 ± 0.12
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	0.416 ± 0.03
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	0.413 ± 0.07
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	0.659 ± 0.09
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	0.398 ± 0.05
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	0.761 ± 0.11
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	0.598 ± 0.00
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	0.396 ± 0.17
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	0.159 ± 0.00
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	0.268 ± 0.01
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	0.153 ± 0.04
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.087 ± 0.07

8.3.3 Interaction between medium components and their optimization for glucan production

At the end of screening experiment, sucrose, peptone and yeast extract were believed to play a significant role in glucan production. The Box-Behnken design for the three factors and the corresponding experimental data are shown in Table 8.3.3. The results of the second-order response surface model fitting in the form of ANOVA are given in Table 8.3.4. The goodness of the model was checked by the determination coefficient R^2 (0.9865) explaining 98.65% of the variability of the response. The value of adjusted R^2 (0.9692) was also very high, indicating high significance of the model. The coefficients of the regression analysis are shown in Table 8.3.5 and the polynomial model for glucan yield, Y can be expressed by Eq. 4.

$$Y = 0.4516 + 0.0933X_1 - 0.0440X_2 + 0.2395X_3 + 0.0682X_1^2 + 0.1050X_2^2 - 0.0003X_3^2 - 0.0278\,X_1X_2 + 0.0266\,X_1X_3 - 0.0324\,X_2X_3 \quad (4)$$

The ANOVA of quadratic regression model demonstrated that the model is highly significant, as is evident from the Fisher's *F*-test with a very low probability value [($P_{model} > F$) = 0.0001] (Table 8.3.4). The relatively lower value of coefficient of variation (CV = 6.58%) indicated a better precision and reliability of the experiments carried out.

The significance of each coefficient could be seen from the *t* and *P* values listed in Table 8.3.5. Yeast extract (X_3) had a significant effect (P<0.0001) on glucan yield *Y*, as it had the largest coefficient followed by sucrose (X_1). Positive coefficient was observed for the quadratic term of sucrose (X_1^2) and peptone (X_2^2) and interaction term X_1X_3. However, peptone (X_2), quadratic term of yeast extract (X_3^2) and interaction terms (X_1X_2 and X_2X_3) had negative effect on *Y*. The probability value of coefficient of quadratic effect of yeast extract (X_3^2) was very high (0.987) indicating that only 1.3% of the model was affected by this factor. Linear effect of yeast extract and sucrose were highly significant and explained that they can act as limiting nutrients and subtle variation in their concentration will alter the product formation.

Table 8.3.3. Box-Behnken design matrix of three variables and the experimentally observed response.

Run No.	Coded factor values			*Y* Glucan (mg/ml)
	Sucrose	Peptone	Yeast extract	
	X_1 (%,w/v)	X_2 (%,w/v)	X_3 (%,w/v)	
1	-1 (1.0)	-1 (0.5)	0 (1.75)	0.570 ± 0.0136
2	1 (6.0)	-1 (0.5)	0 (1.75)	0.796 ± 0.0277
3	-1 (1.0)	1 (3.0)	0 (1.75)	0.507 ± 0.0563
4	1 (6.0)	1 (3.0)	0 (1.75)	0.621 ± 0.0181
5	-1 (1.0)	0 (1.75)	-1 (0.5)	0.178 ± 0.0006
6	1 (6.0)	0 (1.75)	-1 (0.5)	0.327 ± 0.0085
7	-1 (1.0)	0 (1.75)	1 (3.0)	0.656 ± 0.0381
8	1 (6.0)	0 (1.75)	1 (3.0)	0.912 ± 0.0136
9	0 (3.5)	-1 (0.5)	-1 (0.5)	0.338 ± 0.0201
10	0 (3.5)	1 (3.0)	-1 (0.5)	0.345 ± 0.0228
11	0 (3.5)	-1 (0.5)	1 (3.0)	0.830 ± 0.0185
12	0 (3.5)	1 (3.0)	1 (3.0)	0.707 ± 0.0523
13	0 (3.5)	0 (1.75)	0 (1.75)	0.441 ± 0.0035
14	0 (3.5)	0 (1.75)	0 (1.75)	0.469 ± 0.0266
15	0 (3.5)	0 (1.75)	0 (1.75)	0.440 ± 0.0071

Table 8.3.4. ANOVA for quadratic model.

Source	SS	DF	MS	*F*-value	Prob (*P*) > *F*
Model	0.6246	9	0.0696	57.037	< 0.0001
Residual (error)	0.00854	7	0.0012	-	-
Lack of fit	0.00785	3	0.0026	15.268	0.0118
Pure error	0.00686	4	0.00017		
Total	0.6350	16			

R^2 = 0.9865; CV = 6.58; Adj R^2 = 0.9692 .

SS, sum of squares; DF, degrees of freedom; MS, mean square;

Table 8.3.5. Model coefficient estimated by multiple linear regression.

Model Term	Parameter Estimate	Standard Error	Computed *t*-value	*P*-value
Intercept	0.4516	0.02366	19.024	0.000
X_1	0.0933	0.01449	6.441	0.001
X_2	-0.0440	0.01449	-3.041	0.029
X_3	0.2395	0.01449	16.533	0.000
X_1^2	0.0682	0.02133	3.201	0.024
X_2^2	0.1050	0.02133	4.926	0.004
X_3^2	-0.0003	0.02133	-0.017	0.987
X_1X_2	-0.0278	0.02049	-1.358	0.232
X_1X_3	0.0266	0.02049	1.301	0.250
X_2X_3	-0.0324	0.02049	-1.584	0.174

2-Dimensional contour plots are the graphical representations of the regression equation that help in the identification of the type of interactions between test variables. The circular contour plots indicate that the interaction between the corresponding factors is negligible. An elliptical or saddle nature of the contour plots indicates significance of the interactions between the corresponding factors. The interaction between sucrose and peptone was negligible (Fig. 8.3.3) and there was an increase in glucan production when high concentrations of sucrose (4-6%) and lower concentrations of peptone (0.5-2%) were used. The interactions between yeast extract and peptone were not perfectly elliptical (Fig. 8.3.4) and optimum glucan production could be obtained at concentrations of yeast extract (2-3%) and peptone (1.5-2.5%). The plot for sucrose and yeast extract depicts interaction between them (Fig. 8.3.5) and maximum glucan production could be obtained at relatively high concentrations of sucrose (4-6%) and yeast extract (2-3%).

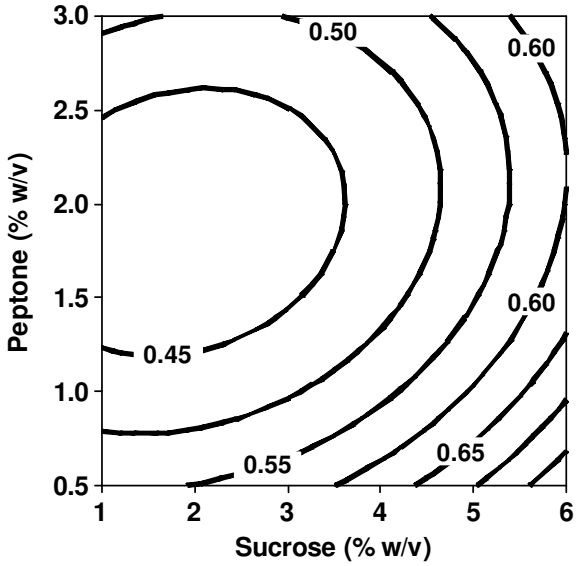

Fig. 8.3.3. Contour plot of the combined effects of peptone and sucrose on glucan production. Fixed level: yeast extract = 1.75

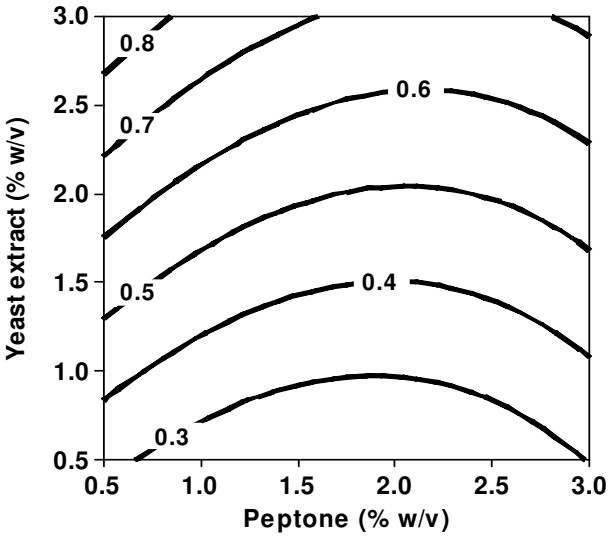

Fig. 8.3.4. Contour plot of the combined effects of yeast extract and peptone on glucan production. Fixed level: sucrose = 3.5

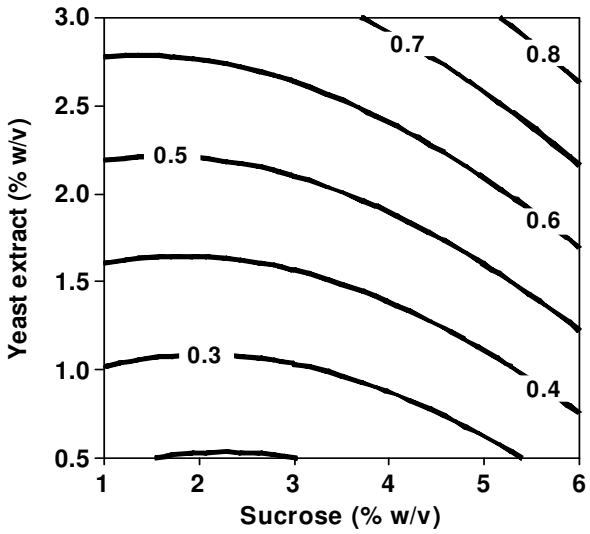

Fig. 8.3.5. Contour plot of the combined effects of yeast extract and sucrose on glucan production. Fixed level: peptone = 1.75

The concentration of medium components selected for maximum glucan production using the point prediction tool of Minitab software were as follows: sucrose 5.95%, peptone 0.52% and yeast extract 2.9%. These values predicted 1063 mg/l glucan production. These optimized values of nutrient parameters were validated by flask culture study in triplicate sets of experiments and the maximum glucan production obtained was 1015 ± 4.5 mg/l which was in good agreement with the predicted value.

8.4 Conclusions

In the present study, the effect of different nutrients on glucan production from *Leuconostoc dextranicum* NRRL B-1146 was studied. Using Plackett-Burman design different ingredients were screened for their effect on glucan production. The results showed that sucrose, peptone and yeast extract significantly affect glucan production. Using a Box-Behnken design for the three selected ingredients, the interaction between the components and their optimum levels for maximum glucan production were determined. The concentrations of medium components selected for maximum glucan production were sucrose 5.95%, peptone 0.52% and yeast extract 2.9%. These values predicted 1063 mg/l glucan production. The experimentally found value of glucan produced in the flask culture using statistically designed medium was 1015 ± 4.5 mg/l which was in agreement with the predicted value.

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Chapter 9

Structural Characterization of Glucan

9.1 Introduction

Lactic acid bacteria produce a wide variety of exopolysaccharides. Their synthesis has been mainly studied in oral *Streptococci*, *Leuconostoc* species and *Lactobacillus* species (Van Geel-Schutten *et al.* 1999; Monsan *et al*. 2001). *Leuconostoc* species are commercially exploited for the production of glucans, homopolysaccharides which contain only one type of monosaccharide, glucose. They are potential therapeutic agents (Korakli and Vogel 2006) and are also used as viscosifying, stabilizing, emulsifying, sweetening, gelling, or water-binding agents, in the food as well as in the non-food industries (Sutherland 1998; Welman and Maddox 2003). Glucans differ in the type of glucosidic linkages, degree and type of branching, length of glucan chains, molecular mass, and conformation of polymers. All these properties strongly contribute to specific polysaccharide properties such as solubility, rheology and other physical characteristics (Monchois *et al.* 1999). Depending on the main chain glucosidic linkages in glucan, three different types of α-glucans synthesized by *Leuconostoc* species are known *viz.* dextran with α-(1-6), mutan with α-(1-3) and alternan with α-(1→6) and α-(1→3) linkages.

Mutant strains of *Leuconostoc mesenteroides* have been reported to produce glucan with α-(1→6) and α-(1→2) linkages (Monsan *et al.* 2001). There are only few reports on the presence of α-(1→6) and α-(1→4) linkages from *Leuconostoc* species. Glucans comprising α-(1→6) and α-(1→4) linkages have been reported from other species. Pullulan has a regular alternation of α-(1→6) and α-(1→4) bonds in its linear α-D-glucan. It is produced as an exopolysaccharide by the polymorphic micromycete *Aureobasidium pullulans* (Leathers 1993). Pullulan is of economic importance having applications in food, pharmaceutical, agricultural, and chemical industries. Anti-tumor polysaccharides from chinese herb, *Angelica sinensis* (Oliv.) Diels (Cao *et al.* 2006) and a sea urchin, *Strongylocentrotus nudus* are composed of α-(1→6) and α-(1→4) linked glucan (Liu *et al.* 2007). Glucan with α-(1→6) and α-(1→4) bonds was also identified from *Lactobacillus reuteri* and the glucan product was named reuteran (Kralj *et al.* 2004). The interest in glucan with α-(1→6) and α-(1→4) linkages was due to the potential it holds as an anti-tumor and immunomodulatory agent.

Leuconostoc dextranicum NRRL B-1146 has been reported to produce a highly linear glucan with 96% α-(1→6) linkages (Jeanes *et al.* 1954). This data was based on a primitive structural characterization of polysaccharide using periodate oxidation. Our preliminary investigations showed that the polysaccharide from this strain is unique with linkages of α-(1→6) and α-(1→4) in its glucan. The glucan production from this strain was optimized as described in Chapter 8. The glucan was purified and the structure was analyzed by FTIR, ^{1}H- and ^{13}C-NMR spectroscopic techniques. The surface morphology of purified glucan was studied using Scanning Electron Microscopy. The shear measurement of semi-dilute polymer solution was carried out using Rheometer.

9.2 Materials and Methods

9.2.1 Microorganism

The strain *Leuconostoc dextranicum* NRRL B-1146 was procured from Agricultural Research Service Culture Collection, National Centre for Agricultural Utilization Research, Peoria, USA. The culture was maintained in modified MRS (sucrose in place of glucose, DeMan *et al.* 1960), as stab at 4°C and sub-cultured every 15 days.

9.2.2 Inoculum preparation

Two loops of culture were taken from the stabs and grown in 5 ml Tsuchiya medium (Tsuchiya *et al.* 1952) in a test tube at 28°C for 12h. The pH of the medium was adjusted to 6.9 before autoclaving. From the culture, 1% was transferred to 250 ml Erlenmeyer flasks containing 100 ml of medium and allowed to grow for 12h at 28°C under static conditions and used as inoculum.

9.2.3 Production of glucan from *Leuconostoc dextranicum*

The production of glucan was carried out in 250 ml Erlenmeyer flasks containing 100 ml optimized medium containing (g/l) sucrose, 59.5; yeast extract, 29; K_2HPO_4, 10; $MgSO_4 \cdot 7H_2O$, 0.1; $MnSO_4 \cdot 4H_2O$, 0.005; $FeSO_4 \cdot 7H_2O$, 0.005; $CaCl_2 \cdot 2H_2O$, 0.005; NaCl 0.005; as per the statistical experimental design. The pH of the medium was adjusted to 6.9 before autoclaving. The inoculated flasks were kept under static condition at 28°C for 48h.

9.2.4 Precipitation of glucan

The culture supernatant was obtained by centrifugation the broth at 10,000***g*** for 10 min at 4°C. The crude glucan was precipitated by the addition of 3 volumes of 95% (v/v) pre-chilled ethanol at 0°C and centrifuged at 13,000***g***. The process of precipitation was repeated to remove any trace impurities or free reducing sugars.

9.2.5 Determination of glucan concentration

The polysaccharide glucan content was determined by phenol-sulfuric acid method (Dubois *et al.* 1956) in a micro titer plate (Fox and Robyt 1991). The details are described in Chapter 8, Section 8.2.4.

9.2.6 Purification of glucan using Size Exclusion Chromatography

The ethanol precipitate of glucan obtained was dissolved in 50 mM sodium phosphate buffer, pH 7.0, to a concentration of 10 g/l, and loaded onto a column (1.5 cm x 30 cm) containing Sephadex G-100 (Sigma Chemical Co., St. Louis, USA). The glucan was eluted with the same buffer at a flow rate of 0.1 ml/min. The total sugar content of glucan was determined by phenol sulfuric acid method (Fox and Robyt 1991) using glucose as the standard. The fractions containing glucan were pooled. The pooled fractions were lyophilized for further analysis. The glucan could be associated with the protein so the protein concentration of the fractions was determined as described in next Section 9.2.7.

9.2.7 Determination of protein content

The protein content of the eluted fractions 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 of protein estimation are given in Chapter 3, Section 3.2.9.

9.2.8 Optical rotation and Fourier-Transform Infrared Spectrometric (FT-IR) analysis

The polysaccharide was characterized using a Fourier Transform Infra-Red Spectrophotometer (Perkin Elmer Instruments, Spectrum One FT-IR Spectrometer). The dried polymer was grinded with Potassium bromide (KBr) powder and pressed into pellets for FT-IR spectral measurement in the frequency range of 4000-400 cm^{-1}, with 20 scans. Optical rotation was measured at 27°C using a (Perkin Elmer Instruments, Model 343) polarimeter.

9.2.9 ^{1}H- and ^{13}C-Nuclear magnetic resonance (NMR) spectral analysis of glucan

^{1}H and ^{13}C-NMR spectra for glucan were recorded at 30°C with a NMR Spectrometer (Varian, Model AS400) 400 MHz equipped with VnmrX for Sun Microsystems Ver. 6.1 software (operating frequencies 400 MHz for ^{1}H and 100 MHz for ^{13}C-NMR). The samples were dissolved in D_2O (99.96%) at concentrations of 5 mg ml^{-1} (for ^{1}H-NMR) and 20 mg/ml (for ^{13}C-NMR). ^{1}H chemical shifts were referenced to internal D_2O (4.8 ppm at all temperatures).

9.2.10 Rheological analysis of the glucan

The steady shear viscosity (η) measurement were recorded for the semi-diute glucan polymer (0.5%) at 25°C using a rheometer (Make, Thermo Electron, model Haake rheostress RSI) interfaced with a, HAAKE RheoWin 323 software. The shear rate was in the range of 0.1-1000 s^{-1}.

9.2.11 Scanning Electron Microscopic analysis of glucan

A sample of the dried polymer glucan was attached to the S.E.M stub with double-sided tape, then coated with ~10 nm Au in a sputter coater (SCH 620, Leo). The surface of the sample was viewed in Scanning Electron Microscope (Leo1330 VP) operated at 10.0 kV.

9.3 Results and Discussion

9.3.1 Purification of glucan by Size Exclusion Chromatography

The purity of glucan produced from the optimized medium is an important factor for the potential use of the biopolymer in pharmaceutical and food-related applications. The crude glucan obtained by repeated alcohol precipitation was further purified by size-exclusion chromatography using Sephadex G-100 column. The glucan was eluted by 50 mM sodium phosphate buffer as a single peak as detected by the phenol-sulfuric acid assay (Fig. 9.3.1). The fractions of 1 ml were collected and the glucan content was estimated by phenol-sulfuric acid method (Fox and Robyt 1991). The protein content of the eluted fractions was determined by the method of Lowry (Lowry *et al.* 1951). Less than 1% protein was found and this was supposed to be protein associated with the glucan. The polysaccharide containing fractions were pooled and freeze-dried to give a white powder and used for subsequent analysis.

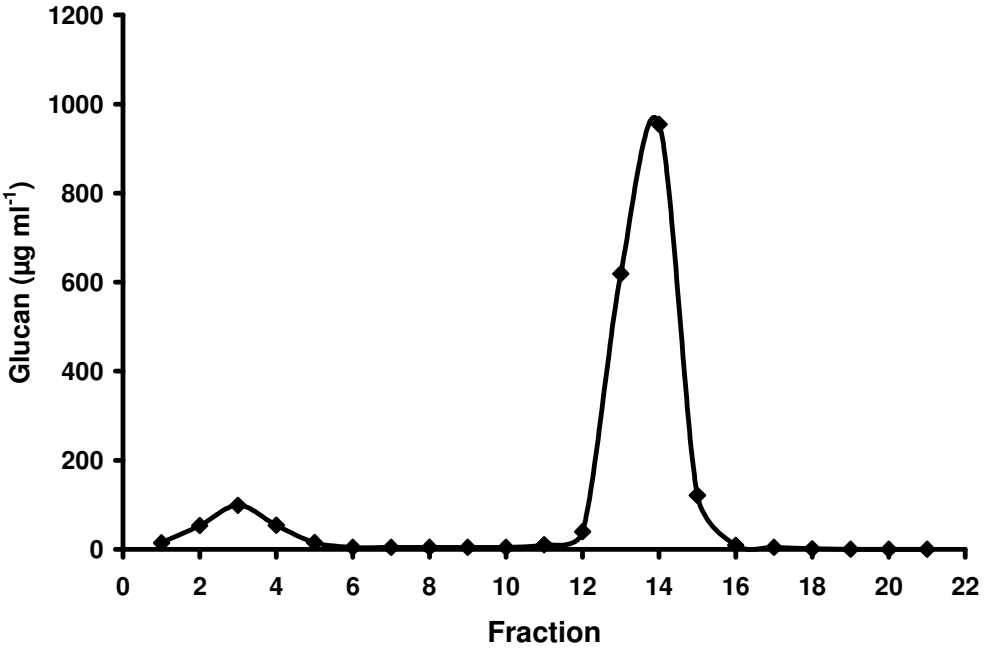

Fig. 9.3.1. Elution profile of glucan from Sephadex G-100 column using 50mM sodium phosphate buffer (pH 7.0), at flow rate 0.1 ml/min.

9.3.2 Optical rotation and structural analysis of glucan by FTIR spectroscopy

The glucan purified from Sephadex G-100 column and lyophilized was used for structural analysis. The glucan had an optical rotation ($[\alpha]^{20}_{D}$ +52 (*c* 0.2, H_2O)) indicating the D-configuration of the glucosyl residues (Liu et al., 2007).

The vibrational spectra have found important applications in the identification and analysis of sugars. The carbohydrates show high absorbance in the region of 1200-950 cm^{-1}, that is within the so-called fingerprint region, where the position and intensity of the bands are specific for every polysaccharide allowing its possible identification (Cerna *et al.* 2003). The FT-IR spectrum of the glucan from *Leuconostoc dextranicum* showed a strong band at 3422 cm^{-1} that was assigned to the hydroxyl stretching vibration of the polysaccharide (Fig. 9.3.2). The band in the region of 2928 cm^{-1} was due to C-H stretching vibration and the band in the region of 1645 cm^{-1} corresponded to associated water. A characteristic absorption at 850 cm^{-1} and 930 cm^{-1} were also observed indicating the α-configuration of the sugar units. There was no absorption at 890 cm^{-1} indicated the absence of β-configuration (Barker *et al.* 1954). The absorption at 1155 cm^{-1}, 1080 cm^{-1} and 1020 cm^{-1} indicated α-pyranose of the glucose residue. The band at about 1150 cm^{-1} was assigned to the valent vibrations of the C-O-C bond and glycosidic bridge. The bands at 1020 cm^{-1} and 1080 cm^{-1} are present in polysaccharide with α-(1→6) and α-(1→4) linkages and can be considered as a characteristic for the type of interunit link (Shingel 2002).

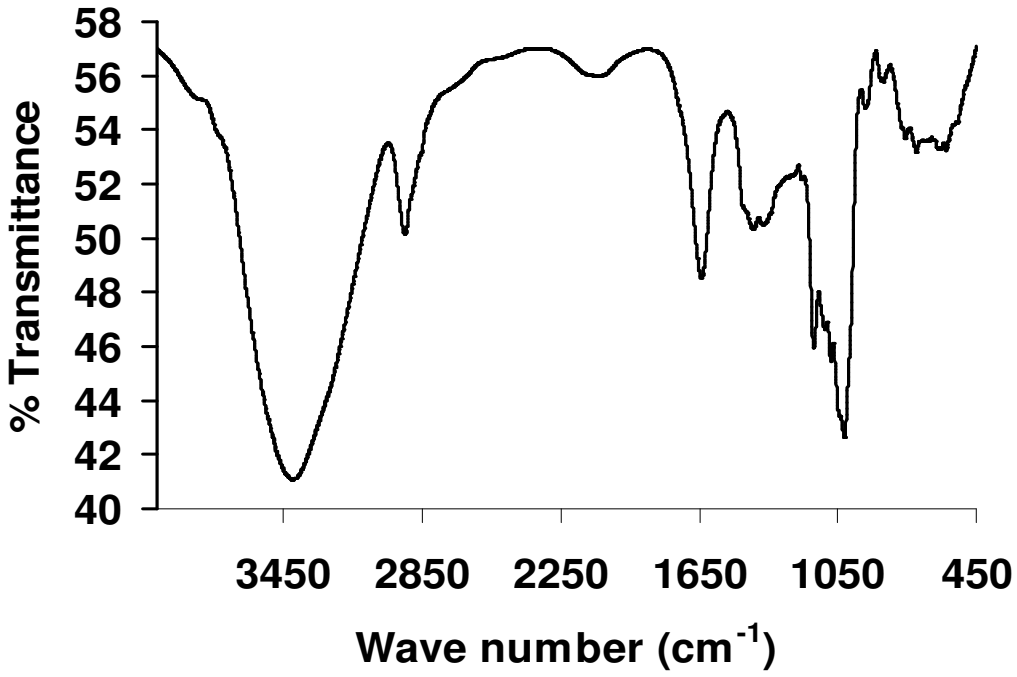

Fig. 9.3.2. FTIR spectrum of glucan produced from *Leuconostoc dextranicum* NRRL B-1146.

9.3.3 Structure determination of glucan by ^{1}H-NMR and ^{13}C-NMR spectroscopy

^{1}H-NMR and ^{13}C-NMR spectra further confirmed the homogeneity of the purified polysaccharide. The spectral features were typical of polysaccharide with α-(1→6) and α-(1→4) linkages based on the peak assignments from the literature (Uzochukwu *et al.* 2002; Cao *et al.* 2006; Liu *et al.* 2007). The ^{13}C-NMR spectrum of polysaccharide is shown in Fig. 9.3.3. Based on data available in literature, it was possible to identify that the resonances in the region of 102.2 and 97.7 ppm corresponded to C-1 of the 1,4-D-Glc*p* and 1,6-D-Glc*p* residues, respectively (Seymour *et al.* 1976; Uzochukwu *et al.* 2002; Cao *et al.* 2006). The major resonance in the anomeric regions occur at 97.7 ppm rather than at about 90 ppm indicating that the C-1 is linked. The signal at 66.9 ppm rather than at 60 ppm indicated that most of

the C-6 are also linked (Seymour *et al.* 1976). Similarly, the peaks at 102.2 ppm and 78.5 ppm suggested the presence of α-(1→4) linkage. The linkages in the polysaccharide are α-glucosidic linkages, as indicated by the absence of chemical shifts downfield of 102.2 ppm.

The configurations for glucan were further confirmed by the ^{1}H-NMR spectrum which displayed signals for anomeric protons at 5.38 and 4.91 which are assigned to α-(1→4) Glc*p* and α-(1→6) Glc*p*, respectively (Fig. 9.3.4). The strong water (HOD) signal at 4.8 ppm obscures the α-anomeric signal at 4.9 ppm. The chemical shifts from 3.4 to 4.0 ppm are assigned to protons of carbons C-2 to C-6 of glycosidic ring (Cheetam *et al.* 1991).

Peak intensities of the ^{13}C and ^{1}H- NMR have been previously used to determine the abundance of a particular linkage in the polysaccharide (Uzochukwu *et al.* 2002). The intensities of characteristic peaks of 97.7, 102.2 ppm and 5.38 and 4.9 ppm indicated the presence of equal abundance α-(1→4) linkages along with α-(1→6) linkages.

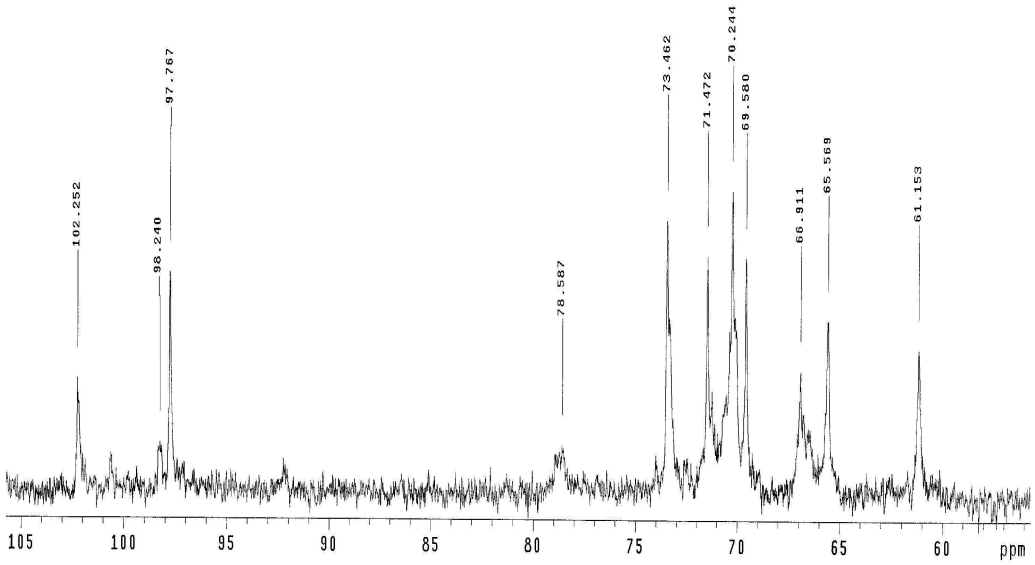

Fig. 9.3.3. ^{13}C-NMR spectrum of glucan from *Leuconostoc dextranicum.*

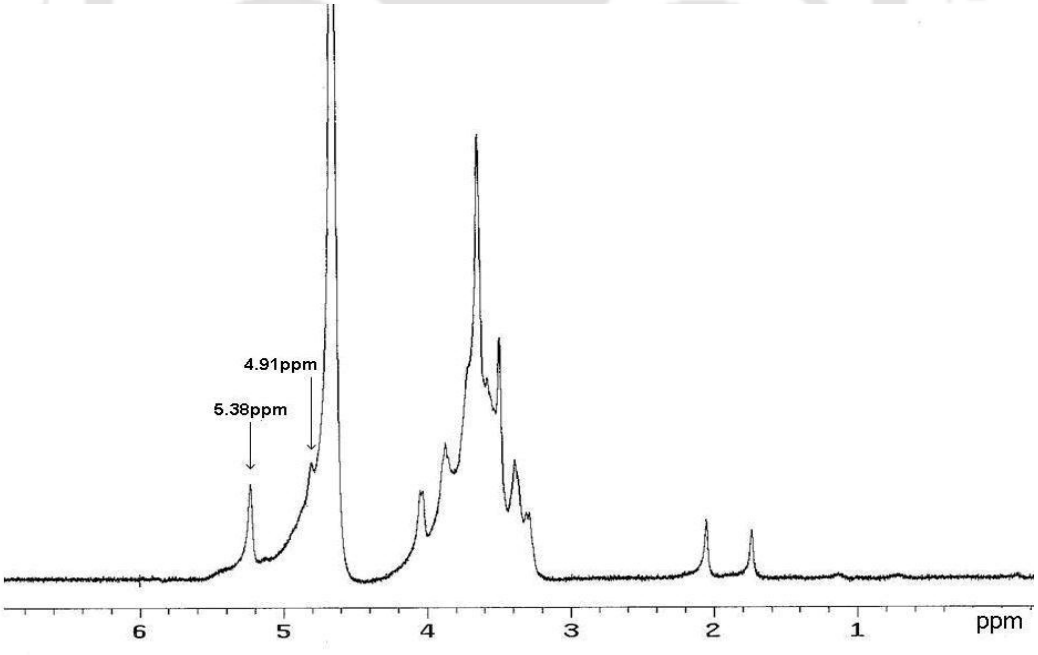

Fig. 9.3.4. ^{1}H-NMR spectrum of glucan from *Leuconostoc dextranicum*.

9.3.4 Rheological properties of glucan

The steady shear measurements for the semi-dilute glucan solution indicated that the viscosity (η) of the dilute polymer exponentially decreased with the increase in shear stress (τ) and exhibited a typical non-Newtonian psuedoplastic behavior (Fig. 9.3.5) indicating branched nature of the polymer. A similar pseudoplastic behaviour of viscosity was observed in case of exoploysaccharide, EPS-WN9 from *Paenibacillus* sp. WN9 (Seo *et al.* 1999) and EPS450 from *Bacillus* sp. I-450 (Kumar *et al.* 2003). The exponential decrease in viscosity with increase in shear rate (shear-thinning effect) was also noticed in case of rhamsam produced by *Alcaligenes* sp. which had the largest side chain, while gellan produced by *Psuedomonas elodea* exhibited least shear-thinning, since this polysaccharide had no side chain in the backbone structure (Kwon *et al.* 1987).

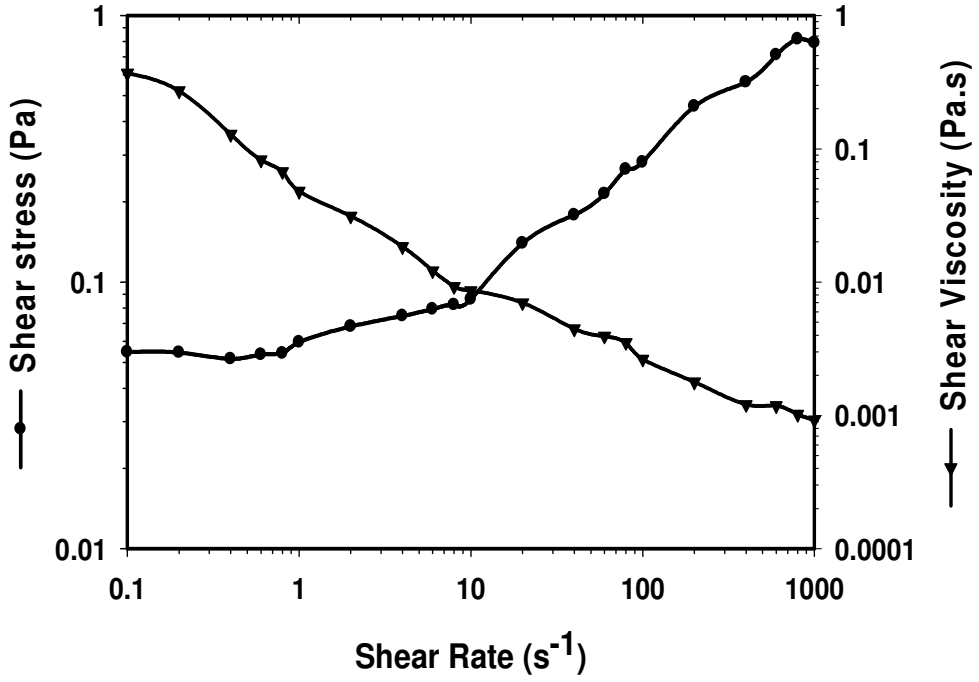

Fig. 9.3.5. Viscosity-shear rate profile of glucan at 0.5 g/l measured at 25°C.

9.3.5 Scanning electron microscopic analysis of glucan

Scanning Electron Micrograph of the glucan showed the surface morphology. The polymer showed a porous or web like structure (Fig. 9.3.6). Owing to its small pore size distribution the polymer can hold water and can be used as a texturing agent in food industry.

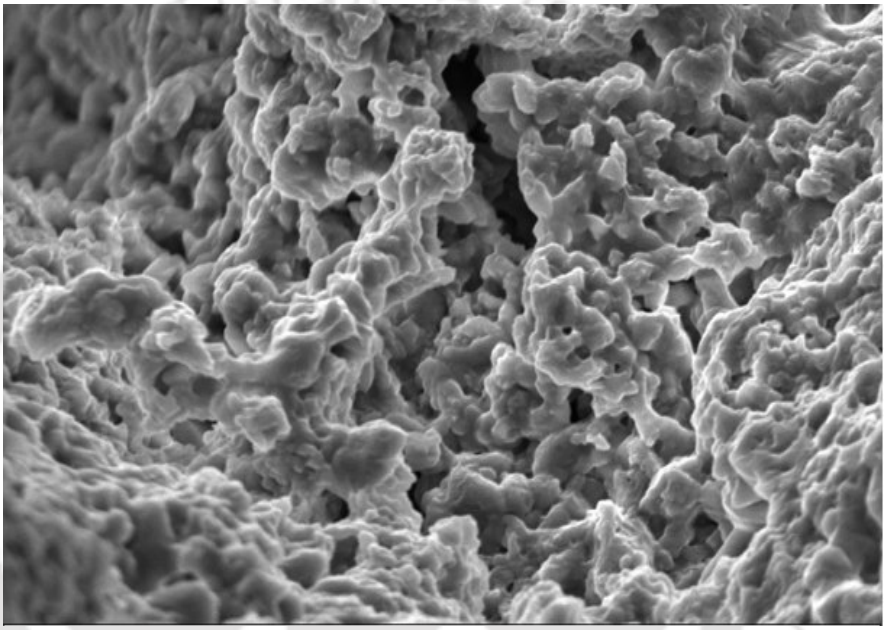

Fig. 9.3.6. Scanning Electron Micrograph (SEM) of glucan showing surface morphology.

9.4 Conclusions

It is important to screen exopolysaccharide producing microorganisms with novel properties that could be of commercial value. In the present study, glucan produced by *Leuconostoc dextranicum* NRRL B-1146 was purified and its structure was analyzed. The glucan produced from *Leuconostoc dextranicum* NRRL B-1146 with the statistically formulated medium was purified by size-exclusion chromatography and obtained as homogenous and free from contaminating polysaccharides. The optical rotation of the polysaccharide indicated the D-configuration of the glucosyl residues. Structural characterization of the glucan using FTIR, ^{1}H-NMR and ^{13}C-NMR techniques indicated the presence of high abundance of α-(1→4) linkages along with α-(1→6) linkages. Polysaccharides in general and α-D-glucans in particular with these linkages have been shown to be of therapeutic potential and very promising anti-tumor agents. The steady shear measurements for the semi-dilute glucan solution indicated that the viscosity (η) of the polymer solution decreased with the increase in shear stress (τ) and exhibited typical non-Newtonian pseudoplastic behavior, indicating branched nature of the polymer. The surface morphology studied using Scanning Electron Microscopy revealed that the polymer has a porous structure and can be used as a texturing agent in food industry.

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List of Publications

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1. **Avishek Majumder**, Anshuma Mangtani and Arun Goyal (**2008**) Purification, identification and functional characterization of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. Current Trends in Biotechnology and Pharmacy (in press).
2. **Avishek Majumder,** Angad Singh and Arun Goyal (**2008**) Artificial intelligence based optimization of exocellular glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146. Bioresource Technology (in press) doi:10.1016/ j.biortech.2008.03.038. (Elsevier)
3. **Avishek Majumder** and Arun Goyal (**2008**) Optimization of culture conditions of a novel glucan producing glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. Current Trends in Biotechnology and Pharmacy 3, 260-268. (a publication by Association of Biotechnology and Pharmacy, India)
4. **Avishek Majumder** and Arun Goyal (**2008**) Enhanced production of exocellular glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 using response surface method. Bioresource Technology. DOI:10.1016/j.biortech.2007.07.027 (in press) (Elsevier)
5. Ravi Kiran Purama, Gurtej Singh, **Avishek Majumder**, V.V. Dasu and Arun Goyal (**2008**) Dextransucrase production in bioreactor: effect of aeration and mathematical modelling. Journal of Applied Bioscience and Biotechnology (in press). (a publication of North East Biotechnology Association, Assam, India)
6. Ravi Kiran Purama, Mayur Agrawal, **Avishek Majumder**, Shadab Ahmed and Arun Goyal (**2008**) Antibiotic sensitivity, carbohydrate fermentation and plasmid profiles of glucansucrase producing four *Leuconostoc* strains. Journal of Pure and Applied Microbiology 2, 139-146. (Oriental Scientific Publishing Company)
7. **Avishek Majumder** and Arun Goyal (**2007**) Use of statistically designed medium for improved glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146 in a bioreactor. International Journal of Chemical Sciences 5(4), 1525-1531.
8. 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 Sciences 5(4), 1497-1504.
9. **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)

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10. **Avishek Majumder**, Angad Singh and Arun Goyal **(2008)** Multi-response analysis of α-D-glucan production from *Leuconostoc dextranicum* and its structural characterization. Bioresource Technology (communicated)

Paper Presentations

1. Anshuma Mangtani, **Avishek Majumder** and Arun Goyal (**2008**) Screening of purification methods for glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. National Conference on New Horizons in Biotechnology, February 8-9, 2008, Swami Vivekanand Mahavidyalaya, Udgir, Maharashtra, India.
2. **Avishek Majumder** and Arun Goyal (**2007**) Use of statistically designed medium for improved glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146 in a bioreactor. National Conference on Frontiers in Chemical Engineering, December 12-14, 2007, Indian Institute of Technology Guwahati, Guwahati, India

List of conference papers

International

1. **Avishek Majumder** and Arun Goyal (**2008**) Production purification and structure determination of a novel glucan from *Leuconostoc dextranicum*. 12th International Conference on "The Interface of Chemistry-Biology in Biomedical Research" February 22-24, 2008, Birla Institute of Technology and Science, Pilani, Rajasthan, India.
2. **Avishek Majumder** and Arun Goyal **(2007)** Statistical approach for optimized production of glucan, an exopolysaccharide from *Leuconostoc dextranicum* NRRL B-1146. International Conference on New Horizons in Biotechnology, Nov. 26-29, 2007, National Institute of Interdisciplinary Science and Technology (NIST), Trivandrum, India.
3. Angad Singh, **Avishek Majumder** and Arun Goyal **(2007)** Production of glucan and oligosaccharides using immobilized glucansucrase. International Seminar on Frontiers in Polymer Science and Technology, Nov 1-3, 2007, Guwahati, Assam India.
4. **Avishek Majumder** and Arun Goyal **(2007)** Analysis of an exocellular glucansucrase and the glucan from *Leuconostoc dextranicum* NRRL B-1146. 7th Carbohydrate Bioengineering Meeting, April, 22-25, 2007, Technical University of Braunschweig, Germany, p212.
5. 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.
6. Ravi Kiran Purama, **Avishek Majumder** and Arun Goyal **(2006)** Purification of family 70, glycoside hydrolase (GH70) from *Leuconostoc mesenteroides* NRRL B-742 by polyethylene glycol 200. International Symposium on Frontiers and Biotechnology-Retrospect and Prospect, January 8-10, 2006, Osmania University, Hyderabad, India.
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11. Ravi Kiran Purama, Mayur Agrawal, **Avishek Majumder**, Shadab Ahmed 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.
12. 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.
13. **Avishek Majumder** and Arun Goyal **(2006)** Optimization of conditions for production of dextransucrase, a glycoside hydrolase of family 70 from *Leuconostoc dextranicum* NRRL B-1146. 75th Annual Meeting of Society of Biological Chemists of India (SBCI), December 8-11, 2006, Jawahar Lal Nehru University, New Delhi, India, p177.
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