Production, structure analysis and biotechnological applications of dextran from a novel strain of *Pediococcus pentosaceus* isolated from microbial diversity hot spot Assam

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

DOCTOR OF PHILOSOPHY

in

Biotechnology

by

Seema Patel

to the

Department of Biotechnology

Indian Institute of Technology Guwahati

February 2010

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

STATEMENT

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

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

February, 2010 *Seema Patel*

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

CERTIFICATE

It is certified that the work described in this thesis entitled "**Production, structure analysis and biotechnological applications of dextran from a novel strain of *Pediococcus pentosaceus* isolated from microbial diversity hot spot Assam**" by Ms. Seema Patel 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 at the Department of Biotechnology, Indian Institute of Technology Guwahati, India and this work has not been submitted elsewhere for a degree.

February, 2010

Arun Goyal
Professor and Head
(Supervisor)

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

CERTIFICATE OF COURSE WORK

This is to certify that Ms. Seema Patel has satisfactorily completed all the courses required for the Ph.D. degree program. These courses include:

BT 601 Analytical Biotechnology
BT 607 Plant Biotechnology
BT 615 Seminar Course
BT 616 Seminar Course
BT 617 Proteomics
BT 618 Biomaterials

Ms. Seema Patel was admitted to candidacy of PhD degree program after she successfully completed her Ph.D. qualifying comprehensive examination in August 2008.

Dr. Utpal Bora
Secretary
Departmental Post graduate Programme Committee
Department of Biotechnology
IIT Guwahati

Prof. Arun Goyal
Head
Department of Biotechnology
IIT Guwahati

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

Department of Biotechnology

Ph.D. GRADE CARD

Name: Seema Patel **Roll No.:** 07610607

Course No.	Course Name	Credit	Grade
BT 601	Analytical Biotechnology	6	AB
BT 607	Plant Biotechnology	6	AB
BT 615	Seminar Course	2	AA
BT 616	Seminar Course	2	AB
BT 617	Proteomics	6	AB
BT 618	Biomaterials	6	AA

Cumulative Performance Index: (CPI): 9.07

February, 2010

Assistant Registrar
(Academic Affairs)

ACKNOWLEDGEMENTS

The results embodied in this thesis are the outcome of cooperation of many helping hands and it could not have seen the light of the day without their voluntary involvement. Here I take the pleasant opportunity to express my gratitude to all of them.

First and foremost, with a deepest sense of respect, I would like to express my sincere gratitude to my supervisor Professor Arun Goyal, for his valuable suggestions, encouragement and inspiration. I am indebted to him for introducing me into the realm of this interesting work, training me with the techniques, giving me the freedom in designing and conducting the experiments. I earnestly thank him for imbibing scientific temperament and appreciable work ethics in me which enhanced my grasp of knowledge and made my learning pursuit exciting. I acknowledge his crucial role in sprucing up of my overall personality.

I would like to acknowledge my sincere gratitude to all my doctoral committee members, Dr. G. Saini, Dr. V.V. Dasu and Prof. T. Punniamurthy for their valuable suggestions and precious time.

My sincere thanks go to faculty members of the department, Dr. Utpal Bora and Dr. K. Pakshirajan, for their great help in materialization of some of my experimental endeavors. I thank Dr. Dubey, Dr. Bora, Dr. Ramesh and Dr. Sahoo for their instruction during course work.

I remain thankful to my seniors Dr. Ravi Kiran Purama and Dr. Avishek Majumder for their guidance and training. I am immensely thankful to my research group members Shadab, Damini, Deepmoni, Rishikesh and Shraddha. Hearty thanks to Naresh Kasoju and Subash Sahoo for their adept hands in fruition of some of my crucial experiments. I profusely thank Naresh Sahoo, Shampa, Mithilesh and Sanjay for clarifying my statistical doubts. Thanks are due to Achlesh and Vipin.

I am thankful to the summer trainees Nadeem and Rajesh, who not only learnt from me, but also taught me some techniques. I am thankful

to my Undergraduate trainees Mayur, Atul, Anurag, Debraj and Ankur for their wonderful ideas and help.

I am thankful to my labmates, Urmila, Nandini, Rachna, Vijay, Asim, Vinod, Pojul and Seraj for the humour we shared and the love they showered on me.

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

I am thankful to the Indian Institute of Technology Guwahati for providing me with the state of the art infrastructure for advance level of research. The financial support for scholarship from Ministry of Human Resource Development (MHRD), India is duly acknowledged.

No words would suffice to express my gratitude to Dr. Biswaranjan Meher for making my stay at IIT Guwahati cherishable with his constant affection and concern. Sanjukta madam's love and delicacies are never to be forgotten. Hostel friends Jasmini, Maya, Aditi, Rosy and Anu deserve to be thanked. Thanks to friends Smeeta Prusty, Sudipta Mazumder and Rajesh Sah for motivating me in different phases of my academic career.

Finally, my PhD. endeavor could not have been successful without the unfathomable love, trust, support and blessings of my family. I owe my achievements to my parents, elder sister Susmita and brother-in-law Dr. Birendra for their motivation. Cheering of my lovely niece Shreya and nephews Ankit and Swastik can not be underestimated. I fondly remember my brother Deepak, whose untimely demise made me realize the fragility of human life and immortality of work. Special thanks to Er. Suryakanta Panda for being a pillar of support and for inculcating in me, the dedication for work.

Seema Patel

February, 2010

Synopsis

Introduction

Dextrans are a class of homopolysaccharides composed of α-(1→6) glycosidic linkages in the main chains and α-(1→2), α-(1→3) and α-(1→4) branched glycosidic linkages. Dextrans may be linear or branched, with variable degree of branching. Dextran are used as food syrup stabilizers, matrix of chromatography columns, plasma substitute, anti-thrombogenic agent, biomaterials, paper and metal-plating processes, oil recovery. Porous dextran scaffolds have tissue engineering applications as drug delivery vehicles. Dextran derivatives as oligosaccharides have profound applications as neutraceuticals, prebiotics, immune stimulatory and anti cancer agents. Functionality of dextran depends on the strain of lactic acid bacteria synthesizing it. Dextran producing lactic acid bacteria have recently attracted much attention due to immense industrial potential of dextrans. The commonly known dextran producing lactic acid bacteria are *Lactobacillus*, *Leuconostoc* and *Streptococcus*. Biodiversity hotspots are promising niches for isolating commercially important strains of dextran producing lactic acid bacteria. North-East region, falling under the Indo-Burma biodiversity hotspot is expected to harbour such novel strains. With this objective, a high dextransucrase yielding strain of lactic acid bacterium was isolated from the soil collected from a place near Guwahati. 16s rRNA sequencing identified the isolate to be *Pediococcus pentosaceus*, belonging to lactic acid bacteria family. Certain strains of Pediococcus genus are industrially important as potent food preservatives for their

pediocin production ability. But, the dextran production aspect of this genus has never been explored or reported before. This work, reports for the first time, the dextran producing ability of the newly isolated novel strain of *Pediococcus pentosaceus*. The molecular, morphological and physiological characterization of the isolate *Pediococcus pentosaceus* (SPA) was conducted. Production, purification and a battery of characterization of both dextransucrase and dextran was carried out. Biotechnological utility of dextran from this isolate was studied and found suitable for food formulation and biomaterial fabrication. The wild-type isolate SPA was subjected to UV-mutagenesis and a high dextransucrase and dextran yielding SPAm1 mutant was developed. The dextransucrase activity and dextran production by the mutant SPAm1 was significantly enhanced by medium optimization using the statistical tool Response Surface Methodology.

Present work

The present investigations are carried out on the "Production, structure analysis and biotechnological applications of dextran from a novel strain of *Pediococcus pentosaceus* isolated from microbial diversity hot spot Assam". The thesis work comprises 7 Chapters.

Chapter 1 is the General Introduction which embodies the brief review of literature dedicated to the importance of lactic acid bacteria, their classification, characterization and the importance of isolation of new strains from the biodiversity hot spot North-East region of India. It describes the production, purification and characterization methods of the enzyme dextransucrase and the exopolysaccharide

dextran. The chapter also elaborates the potential applications of the dextran. Application of dextran as food additives and biomaterial are extensively reviewed. Mutagenesis of wild-type lactic acid bacteria to generate novel mutants yielding high dextransucrase activity and dextran concentration as a promising strategy is described. Statistical optimization of medium composition by applying the Response Surface Methods (RSM) for maximizing dextransucrase activity and dextran concentration is also described.

Chapter 2 describes the detailed protocol of screening of the natural isolate of lactic acid bacterium, SPA from the soil sample. The morphological and biochemical characterization of the isolate based on Gram nature test, catalase test, carbohydrate fermentation profile and antibiotic susceptibility test was carried out. The isolate was found Gram positive and catalase negative. Antibiotic susceptibility test showed its resistance to the antibiotics Norflaxacin, Ampicillin, Amikacin, Vancomycin, Tobramycin, Cephaloridine and Kanamycin. The isolate could ferment Cellobiose, Dextrose, Fructose, Galactose, Maltose, Mellibiose, Sucrose and Trehalose very efficiently whereas, Rhamnose and Xylose partially. The isolate could not metabolize Mannitol, Raffinose and Glycerol. The 16s rRNA sequencing based identification of the isolate and its phylogenetic tree was described. The isolate SPA was reported to be *Pediococcus pentosaceus* belonging to lactic acid bacteria family. For the first time, the novel dextran production capacity of this genus is reported. The culture conditions such as temperature and shaking or static were optimized to maximize the dextransucrase and dextran production from *Pediococcus pentosaceus*. The mesophilic and micro-aerophilic nature of the isolate was inferred as the

dextransucrase activity was maximum at 25°C under shaking flask condition. The shaking flask culture gave 17% higher dextransucrase activity than the static flask culture. Various growth parameters such as change in pH, cell optical density and enzyme activity were studied. The cell density and dextransucrase activity of the isolate was highest at 16h after incubation, which confirmed growth associated nature of enzyme activity.

Chapter 3 describes the purification, identification and characterization of dextransucrase from the isolate *Pediococcus pentosaceus* (SPA). The dextransucrase was purified by polyethylene glycol (PEG) fractionation using differenet percentages of polyethylene glycol. The cell free extract was subjected to fractionation by PEG-200, 400 and 1500. The 33% (v/v) PEG-200 gave dextransucrase with specific activity of 8 U/mg with 14 fold purification and 2.9% overall yield in a single step. The 25% (v/v) PEG-400 gave dextransucrase with specific activity of 18 U/mg with 31 fold purification and 8.5% overall yield. The 10% (w/v) PEG-1500 gave dextransucrase with maximum specific activity of 26 U/mg with 45 fold purification and 4.5% overall yield. The purified dextransucrase confirmed the *in-situ* activity of dextransucrase formation of dextran, when run on SDS-PAGE under non-denaturing condition and subjected to Periodic Acid Schiff staining. The single magenta colour activity band on the gel corresponded to approximately, 180 kDa band on the Coomassie Brilliant Blue stained SDS-PAGE gel. The conditions for maximum dextransucrase activity were optimized using PEG-400 purified enzyme (18 U/mg). The reaction conditions as temperature, pH, ionic strength, sucrose concentration were optimized for maximum dextransucrase activity. 5% sucrose, 30°C temperature, 10-20

mM sodium acetate buffer of pH 5.4 was found optimum for the dextransucrase activity of the isolate *Pediococcus pentosaceus* (SPA). The effects of different salts ($MgCl_2$, $CaCl_2$), and denaturants (Urea, EDTA) on the dextransucrase activity were studied. The stability of dextransucrase in organic solvents (Ethanol, Di Methyl Sulfoxide, Acetone and Acetonitrile) was studied.

Chapter 4 describes production, purification, structural characterization and application studies of dextran produced by the isolate *Pediococcus pentosaceus* (SPA). The dextran concentration in the cell free supernatant was determined by phenol-sulphuric method and was found to be 10.2 mg/ml. The crude dextran in the cell free supernatant was purified by ethanol precipitation and the structure of the lyophilized dextran was analyzed by optical rotation, surface morphology study by SEM, FT-IR, ^{1}H-NMR, ^{13}C-NMR and 2D NMR spectroscopic techniques. The rheological behaviour of dextran was determined. The purified dextran had an optical rotation $[\alpha]^{D}$ of + 125° at $C^{0.2}$ indicating the D-configuration of the glucosyl residues in its backbone. The Scanning Electron Microscopy revealed the reticular, porous structure and huge water holding capacity of the dextran. The FT-IR spectrum of the dextran showed bands at 3409, 2929, 1647, 1417, 1273, 1154, 1050 and 1017 cm^{-1}. The band in the region of 3400 cm^{-1} represents -OH group, 2930 cm^{-1} represents the C–H group and 1639 cm^{-1} represents -COOH group. The absorption peak at 906 cm^{-1} indicates the α-glycosidic bond. The characteristic bands at 1154, 1103 and 1020 cm^{-1} found in the spectra of dextran are due to valent vibrations of C–O and C–C bonds and deformational vibrations of the CCH, COH and HCO bonds. As shown by the

^{1}H-NMR spectrum, the resonance at 4.96 ppm was assigned to the C-1 of the α-(1→6) glucosyl residues of main chain of dextran. The absence of peak near 5.3 ppm confirms the linearity of the dextran of isolate SPA. ^{13}C-NMR spectrum elucidates the major resonance in the anomeric regions occurs at 97.9 ppm indicating that the C-1 is linked. The signal at 65.7 ppm indicates that most of the C-6s are also linked. The absence of peaks at 102.2 ppm and 78.5 ppm suggested the absence of α-(1→4) linkages in the dextran. The FT-IR, ^{13}C-NMR and ^{1}H-NMR data showed that the dextran produced by the isolate SPA is soluble, linear and main chain has α-(1→6) linkages. With increase of shear rate, shear stress increased and viscosity decreased confirming the typical polymeric non-Newtonian pseudoplastic behavior. The above illustrated structural features make dextran a very valued candidate for food, pharmaceutical and tissue engineering scaffold applications. To evaluate the safety and potential use of dextran from this isolate as neutraceuticals and biomaterials, its cytotoxicity tests on human cervical cancer (HeLa) cell line was conducted. The dextran was found non-toxic and biocompatible. The dextran produced by isolate *Pediococcus pentosaceus* (SPA) has potential use in food industry as gelling or stabilising agents. The dextran is also suitable as drug delivery agent.

Chapter 5 describes the UV-induced mutagenesis of the wild-type isolate *Pediococcus pentosaceus* (SPA) for strain improvement. The isolate SPA was subjected to UV radiation for different time intervals. After 30s and 60s UV irradiation, two mutants SPAm1 and SPAm2 were developed from the wild-type isolate SPA. The dextransucrase activity of SPAm1 was 4.9 U/ml and that of SPAm2

was 4.7 U/ml. The mutants SPAm1 and SPAm2, possessed about 44% and 38% enhanced dextransucrase activity over the wild type strain, respectively. The dextran concentration of SPAm1 was 12.2 mg/ml and that of SPAm2 was 11.3 mg/ml. The mutants SPAm1 and SPAm2, possessed about 20% and 11% enhanced dextran concentration over the wild type strain, respectively. The carbohydrate utilization of the mutant SPAm1 was carried out. No significant difference between the carbohydrate metabolism of SPA and SPAm1 was found except that the lactose metabolism in SPAm1 was not as strong as in SPA. Dextransucrase from the mutant SPAm1 was purified with PEG-400. The specific activity of this purified dextransucrase was 22.4 U/mg and the fold purification was 37. The specific activity of purified dextransucrase of SPAm1 was 24% higher than that of the wild-type isolate SPA. The surface morphology of the dextran produced by SPAm1 was also studied using Scanning Electron Microscopy. The pore size was larger in the dextran of mutant SPAm1 as compared to that of wild-type *Pediococcus pentosaceus* (SPA).

Chapter 6 deals with the statistical optimization of medium composition for enhancement of dextransucrase from the mutant SPAm1 developed from the wild-type isolate *Pediococcus pentosaceus* (SPA). Statistically-based experimental tools, Plackett-Burman factorial design and central composite design were applied for the optimization experiments. Six medium components *viz.* sucrose, yeast extract, beef extract, peptone, K_2HPO_4 and Tween 80 were examined for their significance on dextransucrase production. After regression analysis of the experimental data, sucrose, beef extract and Tween 80 were found the significant ingredients influencing the production of dextransucrase. The interactive effect of these three crucial variables on

dextransucrase production was studied using a 2^3 full-factorial central composite design. After ANOVA, a second-order polynomial equation was established to determine the relationship between the dextransucrase production and the three medium components. The optimal concentration of variables for maximum dextransucrase production were sucrose, (5.52% w/v), beef extract (0.23% w/v) and Tween 80 (0.83%, v/v). The maximum dextransucrase activity by the predicted model was 15.9 U/ml that was in perfect agreement with the actual experimental value (15.6 U/ml), when optimized medium was used. The dextransucrase activity of the mutant SPAm1 in unoptimised medium was 44% higher than the wild-type SPA; whereas, the increase in dextransucrase activity of the mutant in optimised medium was 218% more than that given by the unoptimised medium. There was 358% increase in dextransucrase activity of the mutant SPAm1 in optimised medium when compared to that of wild-type isolate SPA. Thus, the statistically-based experimental designs were found very promising in enhancing the dextransucrase activity by the *Pediococcus pentosaceus* mutant SPAm1.

Chapter 7 deals with the optimization of medium composition by the statistical tool Response Surface Methodology for maximization of dextran production from the mutant SPAm1. A 2-level Plackett-Burman factorial design and 5-level central composite design were applied for the optimization experiments. Five medium components *viz.* sucrose, yeast extract, K_2HPO_4, Tween 80 and $CaCl_2$ were examined for their significance on dextran production from the mutant *Pediococcus pentosaceus* (SPAm1). Regression analysis of the experimental data showed that sucrose and yeast extract were the significant ingredients influencing the production

of dextran. The interactive effect of these two crucial variables on dextran production was studied using a 2^2 full-factorial central composite design. After regression analysis, a second-order polynomial equation was established to determine the relationship between the dextran production and these two significant medium components. The optimal concentrations of variables for maximum dextran production were sucrose, (5.115% w/v) and yeast extract (0.635% w/v). The maximum dextran production by the predicted model was 40.8 mg/ml that was in perfect agreement with the actual experimental value (40.2 mg/ml) when optimized medium was used. The dextran concentration of the mutant SPAm1 in unoptimised medium was 20% higher than the wild-type SPA. Whereas, the increase in dextran concentration of the mutant in optimised medium was 294% more than that given by the unoptimised medium. There was 229% increase in dextran concentration by mutant SPAm1 in optimised medium when compared to that of wild-type isolate *Pediococcus pentosaceus* (SPA). Thus, the statistically-based experimental designs were found very promising a second step strategy to enhance the dextran production by the *Pediococcus pentosaceus* mutant SPAm1.

CONTENTS

Chapter 1

General Introduction

1.1 Introduction

A variety of food texturing microbial polysaccharides or hydrocolloid gums are produced by microorganisms. In recent years, significant progress has been made in discovery and development of new bacterial polysaccharides that possess novel biofunctional properties and are used as additives in foods such as ice creams, frozen deserts, yoghurt, salad dressings etc. (Sutherland, 1994). During the World War II, the usefulness of microbial polysaccharides was recognized with the discovery of dextran, which found clinical application (de Belder, 1996). It was after two decades in the year 1960 yet another useful microbial polysaccharide, xanthan gum produced by aerobic fermentation of *Xanthmonas campestris*, was developed by Kelco Company in USA, and it was one of the most widely used industrial polysaccharides (Garcia-Ochoa *et al*., 2000). The discovery and industrial acceptance of xanthan in food, industrial and oil field applications gave boost to further research and development in the field of microbial polysaccharides. Xanthan gum has practically all the functional properties like thickening, suspending, emulsion stabilising, gelling,

shear thinning rheology, thermo and acid stability required of a hydrocolloid for food and non-food applications (Garcia-Ochoa *et al*., 2000). Gellan gum, the extracellular polysaccharide produced by *Pseudomonas elodea* has potential to be employed in microbiological media and in gelled food formulations (Baird *et al*., 1983; Baird and Smith, 1989). Dextran produced by *Leuconostoc spp.*, curdlan produced by *Agrobacterium spp*., pullulan produced by *Aureobasidium pullana*, scleroglucan produced by *Sclerotium rolfsii*, welan produced by *Alcaligenes* ATCC31555 are certain other microbial polysaccharides having food applications. Till now, as many as a dozen microbial polysaccharides are already under commercial production or are being investigated as possible future industrial commodities. In most of the cases they act as water behaviour modifier, thickener, gelling agent, emulsifier, suspending agent and stabiliser (Lopes *et al*., 1995). These can serve as scaffold materials owing to their structural similarity to the extracellular matrix of many tissues (Drury and Mooney, 2003).

1.2 Microbial polysaccharides

Biosynthesis of polysaccharide is a normal metabolic feature of many anaerobic microorganisms. Microbial polysaccharides are generally divided into three groups, namely (1) The cell wall polysaccharides (2) The intercellular polysaccharides and (3) The exopolysaccharides. The first two group of polysaccharides are integral parts of cell wall or capsule, thus unseparable from cell biomass and hence, commercially unimportant. The exopolysaccharides, which constantly diffuse into the cell culture medium, making it slimy and viscous, are easy to isolate from the culture medium and are free of protein and cell organelles.

Exopolysaccharides (EPS) are long-chain polysaccharides produced extracellularly mainly by bacteria and microalgae (Sutherland, 1994). EPS consist of sugars or sugar derivatives, mainly glucose, galactose, mannose, N-acetylglucosamine, N-acetyl galactosamine and rhamnose, in variable ratios. EPS are not permanently attached to the surface of the microbial cell and are secreted into their surroundings during growth as loose slime. This distinguishes them from the structurally similar capsular polysaccharides, which remain permanently attached to the microbial cell surface. EPS protects them from adverse conditions as desiccation, nutrient shortage and antagonism (Looijestejn *et al.*, 2001). Microbes have a propensity for cation sequestration by virtue of the sticky nature of EPS liberated by them. EPS play key role in initial adhesion and firm anchorage of the bacteria to solid surfaces (Marshall, 1992). EPS facilitate aggregation of microbes by means of biofilm formation. EPS are instrumental in cellular recognition and pathogenecity (Whitfield and Vlvano, 1999). They also act as a barrier against bacteriophages. In general exopolysaccharides are not the reserve energy source, but can be catabolised to yield energy, when the nutrients in the culture media are exhausted (Cerning, 1990), but, *Streptococcus mutans* and *Streptococcus sobrinus* are capable of degrading the self synthesized dextran and *Streptococcus mutans* also utilizes the oligosachharide produced by it (Colby and Russel, 1997).

1.2.1 Classification of exopolysaccharides from lactic acid bacteria

Exopolysaccharides (EPS) of microbial origin are gaining importance because of their applications in food and other industries. EPS from microbial sources can be

classified into homopolysaccharides (HoPS) and heteropolysaccharides (HePS) Homopolysaccharides consist of identical monomers. HoPS are synthesized by transglycosylases which derive energy from the osidic bond of sucrose to catalyse the transfer of a corresponding glycosyl moiety (Monsan *et al.*, 2001). HePS are composed of variable monomers. The monomers of HePS are formed in the cytoplasm. These are assembled at the membrane by specific glycosyltransferases (GTF) through the sequential addition of sugar nucleotides and are delivered as building blocks for attachment with the growing repeating unit anchored on a lipid carrier. After completion of the monomer assembly, the repeating unit is externalized and polymerized (De Vuyst *et al.,* 2001).

From time immemorial lactic acid bacteria have been valued for their use in food fermentation and preservation. Recently, these bacteria have attracted attention for their exopolysaccharides production ability. The ability of lactic acid bacteria to produce EPS of variable composition, structure and size is widening their industrial applications. The strain, composition of growth medium and culture conditions as temperature, pH, oxygen tension and turbidity influence the EPS production.

1.2.2 Homopolysaccharides

Most of the homopolysaccharides secreted by lactic acid bacteria (LAB) are synthesized by extracellular glycansucrases, using sucrose as the glycosyl donor. Glycansucrases namely glucansucrase and fructansucrase, cleave the osidic bond of sucrose to catalyze the transfer of a corresponding glycosyl moiety which leads to the EPS polymer synthesis (Patel *et al.*, 2010). Glucansucrase produces glucans as dextran, mutan, alternan and reuteran. Similarly, fructansucrase produces levan and

inulin-type of fructans. Dextrans having α-(1→6) glycosidic bond in the main chain have versatile industrial use *viz*. food additives, therapeutic agents, biomaterials etc. (Patel *et al.*, 2010) Alternan having alternating α-(1→6) and α-(1→3) glucosidic linkages is commercially exploited as low viscosity bulking agent and extender in foods and cosmetics. Reuteran, a water soluble glucan having α-(1→6) and α-(1→4) glucosidic linkage has effective bakery applications (Brandt *et al.*, 2003; Tieking *et al.*, 2003). Levan is a fructan having β-(2→6) osidic bonds and is known for its antitumor (Yoo *et al.*, 2004) and cholesterol-lowering properties (Yamamoto *et al.*, 1999). Levan also holds promise as biothickener in food industry. Inulin-type glucans are fructooligosachharides containing β-(1→2) osidic bonds and function as prebiotics in humans (Bouhnik *et al.*, 2007).

1.2.3 Heteropolysaccharides (HePS)

Biosynthesis and secretion of the heteropolysaccharides from the lactic acid bacteria (LAB) occur at different phases of growth. The amount and type of the polymer is regulated by growth conditions (De Vuyst and Degeest, 1999). Structurally, HePS may be ropy or mucoid. A chemically defined medium containing carbohydrates, amino acids, vitamins, nucleic acid bases and mineral salts is suitable for studying the effects of nutrients on growth, metabolic pathways and biosynthesis of HePS by LAB. The media containing complex constituents like beef extract, peptone and yeast extract interfere with the monomer and thus the structure of the HePS (Degeest *et al.*, 2001). The total yield of EPS produced by the LAB depends on the composition of the medium, LAB strain and growth conditions like temperature, pH, oxygen tension and incubation period. Under optimal culture condition, HePS

yield ranges between 0.150 to 0.600 g/L (Cerning and Marshall, 1999). Kefiran is a water soluble heteropolysachharide consisting of glucose and galactose produced by *Lactobacillus kefiranofaciens, L. kefirgranum, L. parakefir, L. kefir* and *L. delbrueckii* subsp. *bulgaricus* (Arihara *et al.*, 1990). Kefiran is reported to have antimicrobial, wound healing properties, ability to lower cholesterol in blood, capacity to retard tumour growth and influence the systemic immunity (Vinderola *et al.*, 2006).

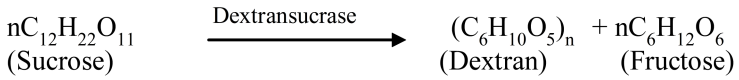
1.3 Dextran producing lactic acid bacteria

The chemical structure of the dextran is highly specific to the dextransucrase producing strain (Dols *et al.*, 1997). *L. mesenteroides* NRRL B-512F is the commercial dextran producing strain (Goyal *et al.*, 1995). *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2008a), *L. dextranicum* NRRL B-1146 (Majumder and Goyal, 2009), *L. mesenteroides* NRRL B-1299 (Edward *et al.*, 1974), NRRL B-523 (Padmanabhan and Kim, 2002), *Leuconostoc mesenteroides* NRRL B-742 (Kim and Robyt, 1995a) are some other well known dextran producing lactic acid bacteria. The cariogenic *Streptococcus* Ingbritt, *Streptococcus* OMZ 51 and the non-cariogenic *Streptococcus* ATCC 10558 produce dextrans (Sidebotham *et al.*, 1971). A human oral strain of *Lactobacillus casei* is reported to produce dextran-like, extracellular polysaccharide (Hammond, 1969). Smitinont *et al.* (1999) reported the probability of dextran production by *Pediococcus* genus.

1.3.1 Dextran biosynthesis and reaction mechanism

Chapter 1

Dextransucrases are extracellular enzymes produced by *Leuconostoc*, oral *Streptococcus*, *Lactococcus*, *Lactobacillus* (Sidebotham, 1974; Barker and Ajongwen, 1991). Owing to the classification based on the sequence similarities, dextransucrases belong to the family 70 of glycoside hydrolases (Moulis *et al.*, 2006). Sucrose is the inducer of dextransucrase (Neely and Nott, 1962). Dextransucrases belong to the family of glucansucrases, a group of enzymes that use the free energy of cleavage of sucrose to form new glycosidic bonds (Plou *et al.*, 2002). In the presence of other compounds, generally mono-, di- and short oligosaccharides, the synthesis of acceptor products may occur (de Segura *et al.*, 2006). The Leuconostoc species require sucrose in the culture medium as an inducer for the elaboration of glucansucrases, whereas in the Streptococcus species, glucansucrases are produced constitutively by growing on a glucose or fructose medium. The constitutive mutants *Leuconostoc mesenteroides* NRRL B-512FM, B-742, B-1355 and B-1299 produce glucansucrases in a glucose or fructose medium as a carbon source instead of sucrose (Kang *et al.*, 2003).



It is generally recognized that dextransucrase catalyzes three reactions, using sucrose as the substrate: (1) the synthesis of dextran, (2) the synthesis of dextran branch linkages, and (3) acceptor reaction in the presence of other carbohydrates, such as maltose, galactose etc. (Robyt *et al.*, 2008). The mechanism of dextran synthesis has been shown to involve a two-site insertion mechanism in which D-glucose and the growing dextran chain are covalently attached to the active site (Robyt *et al.*, 2008). The D-glucose is transferred to the reducing end of the growing dextran chain. The

branch linkages by the action of the synthetic enzyme (dextransucrase) form when a dextran chain acts as an acceptor and displaces either the D-glucose or the dextran chain from the active site, forming branch linkages (Robyt, 1995, Kitaoka and Robyt, 1999). They catalyse the synthesis of high molecular weight D-glucose polymers, dextrans, from sucrose. In the presence of efficient acceptors, they catalyse the synthesis of low molecular weight oligosaccharides (Koepsell *et al.*, 1953). The regioselectivity displayed by dextransucrases is highly strain dependent. With dextransucrase from *L. mesenteroides* NRRL B-512F different acceptors have been assayed, resulting in the synthesis of compounds with α-(1→6) linked glucose moieties. However, dextransucrase from the strain B-1299 is also able to form α-(1→2) linkages (Dols-Lafargue *et al.*, 2001). Elucidation of the factors determining the regiospecificity and the regioselectivity of dextransucrases is necessary. The cloning of dextransucrases encoding genes in addition to structure-function relationship studies have allowed the identification of important amino acid residues and have shown that dextransucrases are composed of two functional domains: a N-terminal core region (1000 amino acids) involved in sucrose binding and splitting and a C-terminal domain (500 amino acids) composed of a series of tandem repeats involved in dextran binding (Funane *et al.*, 2005). In the highly-conserved core region, about 700 amino acids make up a circularly permuted $(\beta/\alpha)_8$ barrel (MacGregor *et al.*, 1996), catalytic Asp (Tsumori *et al.*, 1997) and essential substrate-binding Gln (Monchois *et al.*, 2000) exist in this region.

Enzymology studies based on secondary structure prediction has led to a clearer knowledge of structure-function relationships of dextransucrases. Kobayashi and Matsuda (1978) reported that biosynthesis of dextran occurs by the addition of

glucosyl groups at the non-reducing end of the growing dextran. Dextransucrases, in addition to catalyzing the synthesis of dextran from sucrose, also catalyze the transfer of D-glucopyranosyl group from sucrose to acceptors such as D-glucose, maltose, isomaltose or compounds having free hydroxyl groups. A series of oligosaccharides and glycosides with isomaltodextrins of varying number of D-glucose units are attached by an α-(1→6) linkage to the nonreducing-end of the acceptor (Seo *et al.*, 2005). These acceptor reactions present a challenge for industrial realisation, because they offer new perspectives for the synthesis of defined oligosaccharides, which are up to now difficult to produce by chemical synthesis. A wide range of acceptors has been described by Demuth *et al.*, (2002). Maltose and isomaltose are efficient acceptors. Under eligible reaction conditions, mostly with high concentrated solution of the acceptor, it is possible to shift the reaction pathway towards a specific oligosaccharide formation resulting in a wide spectrum of products (Su and Robyt, 1993).

1.3.2 Structure of dextran

Dextrans $(C_6H_{10}O_5)n$ are a class of homopolysaccharide consisting of D-glucopyranosyl units polymerized predominantly in α-(1→6) linkage (Padmanabhan and Kim, 2002). The α-(1→2), α-(1→3) and α-(1→4) glycosidic linkages form branching (Kim *et al.*, 2003; Kang *et al.*, 2003). The degree of branching involving α-(1→2), α-(1→3) and α-(1→4) linkages in dextrans vary according to the origin of dextransucrase (Seymour and Knapp, 1980). Dextran from *Leuconostoc mesenteroides* NRRL B-512F consists of 95% of α-(1→6) osidic linkages and 5% of α-(1→3) branched linkages (Bertrand *et al.*, 2006). *L. mesenteroides* NRRL B-1299 produces a highly branched dextran containing between

27-35% of α-(1→2) branch linkages (Dols *et al.*, 1997). The molecular weights and degrees of branching of dextrans depend on the sucrose concentration, temperature and pH conditions (Kim *et al.*, 2003). The bio-funtionality of dextrans depend on their physico-chemical attributes, so, analytical characterization of the dextrans are of utmost importance. Differences in dextran structure are determined by Fourier-transform infrared spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance spectrometry (Holt and Cote, 1998). The FT-IR, NMR, scanning electron microscopy and rheological studies on dextrans of *Leuconostoc mesenteroides* NRRL B-640 and *Leuconostoc dextranicum* NRRL B-1146 have been carried out. *Leuconostoc mesenteroides* NRRL B-640 synthesizes a α-(1→6) linked linear dextran (Purama *et al.*, 2009) and *Leuconostoc dextranicum* NRRL B-1146 synthesizes a α-(1→6) linked dextran with α-(1→4) branching (Majumder and Goyal, 2009). Misaki *et al.* (1980) studied the structure of dextran from *Leuconostoc mesenteroides* B-1355, which revealed the branched structure containing α-(1→6) and α-(1→3) glucosidic linkages.

1.3.3 Applications of dextran

Dextrans have many industrial applications due to their non-ionic character and good stability under normal operating conditions. These are used for the matrix preparation of chromatography columns such as Sephadex (Shamala and Prasad, 1995). They are also used for preparing blood plasma substitutes, plasminogen activators and antithrombogenic agents (Soetaert *et al.*, 1995, Purama and Goyal, 2005). As the larger molecular weight dextrans can act as osmotic agents, they are used to treat hypovolemia (Alpar and Killampalli, 2004). Iron dextran is used to treat iron deficiency anaemia (Thayu and Mamula, 2005). Dextrans come handy in

microsurgery to reduce the risk of free tissue transfer loss. They are used as lubricant in eye drops and to increase blood sugar levels. By coupling with dextran T40, plasma half-life of Trichosanthin, a low molecular weight plant protein, capable of suppressing the replication of human immunodeficiency virus (HIV-1) can be prolonged as it escapes glomerular filtration (Ko *et al.*, 1991). Sodium salt of dextran sulfate is also reported to inhibit AIDS virus (Baba *et al.*, 1990). Dextran sulfates are highly active inhibitors of encephalomyocarditis hemagglutination (Kunin, 1967). 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). Use of dextrans have ramified into paper, metal-plating processes and enhanced oil recovery (Patel *et al.*, 2010). They are used as food syrup stabilizers and dough improvers. Dextrans provide a stabilising coating for protecting metal nanoparticles against oxidation (Bautista *et al.*, 2005). Dextran coating on biomaterials to prevent undesirable protein absorption is being explored to improve their biocompatibility (Sengupta *et al.*, 2006). Dextrans are expected to hinder protein adhesion by exerting repulsive steric effects and hydration pressure (Chen *et al.*, 2008). Dextrans have been frequently employed as drug carriers (Ulbrich and Subr, 2004). These are used in nanotechnology as tool for antigen delivery in vaccination (Sahoo *et al.*, 2007). Dextran conjugated dendritic nanoconstructs as potential vectors for anti-cancer agent are being evaluated (Agarwal *et al.*, 2009). Colloidal iron oxide formulated with dextran is clinically used as MRI contrast agents (Koo *et al.*, 2005). Encapsulated dextran acts as conjugate of cancer drug doxorubicin for tumour targeted delivery (Oh *et al.*, 2009). Low molecular weight dextran sulfate acts as endothelial and cytoprotectant in solid organ and islet

transplantation preventing complement-mediated damage of the donor graft endothelium (Spirig *et al.*, 2008). Dextran nanobubbles were prepared with a dextran shell and a perfluoropentan core to store oxygen to release in different hypoxic solutions (Cavalli *et al.*, 2009). The novel properties of dextran make it a versatile tool in regenative medicine. The recent studies have shown that not only water-soluble dextrans, but also water-insoluble dextrans are of high industrial values (Stewart and Fogler, 2001; Padmanabhan *et al.*, 2003). Dextran derivatives such as glucooligosaccharides containing α-(1→2) linkages are capable of promoting the development of the beneficial cutaneous flora detrimental to the undesirable microorganisms, either pathogenic or those associated with infections (de Segura, 2006).

1.4 Production of dextransucrase

Dextransucrase production is affected by several factors like temperature, aeration and medium components (Cortezi *et al.*, 2005). Tsuchiya *et al.* (1952) intensively studied the culture medium composition for dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F and found the conditions 27°C and pH 6.7 best for efficient growth without enzyme denaturation. Goyal *et al.* (1995) reported the maximum production of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F at 23°C under static condition. Santos *et al.* (2000) studied the same strain and observed maximum dextransucrase activity at 20°C and decrease in enzyme activity with increase in temperature. Purama and Goyal (2009) reported 25°C temperature and shaking condition was optimum for dextransucrase production from the *Leuconostoc mesenteroides* NRRL B-640. For glucansucrase production from

Leuconostoc dextranicum NRRL B-1146, 28°C temperature and static condition was optimum (Majumder and Goyal, 2008). However, the regulation of pH and aeration conditions have little effect on the enzyme production by strain *Leuconostoc mesenteroides* NRRL B-1299 (Dols *et al.*, 1997). Tsuchiya *et al.* (1952) assessed the effect of sucrose concentration on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F and concluded that 2% (w/v) sucrose was the optimum level. Tsuchiya *et al*. (1952), Barker *et al*. (1993) and Lazic *et al.* (1993) showed that when medium pH drops to 5.0-5.5, dextransucrase was more active in transforming sucrose to dextran. Majumder and Goyal (2007) studied the glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146 in a bioreactor. The increase in enzyme activity obtained in the bioreactor was 90% of the flask culture. Purama and Goyal (2008c) used statistically optimized medium for maximizing dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 in a bioreactor that gave specific activity of 0.70 U/mg. Purama *et al.* (2008) studied dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 in a bioreactor at different aeration rates and reported that the enzyme activity was 36% higher at 1.5 vvm aeration than that at 0 vvm. It has been reported that reported that the extracellular dextransucrase production is cell growth associated (Santos *et al.*, 2000; Purama *et al.*, 2008)

1.4.1 Purification of dextransucrase

Various purification methods such as precipitation by ammonium sulphate, ethanol or polyethylene glycol, phase partitioning, ultrafiltration and chromatography are used to purify the enzyme (Goyal and Katiyar 1994; Majumder *et al.*, 2007; Pijning *et al.*, 2008). Polyethylene glycol (PEG) fractionation is a reasonably

successful and cost-effective method for purification of dextransucrase. PEG is a non-ionic hydrophilic detergent known to selectively precipitate the proteins of high molecular weights or in aggregated forms. PEG fractionation method is inadequate for removing the associated polysaccharides from the dextransucrase. For characterization of the enzyme, it should be essentially free of the carbohydrate content and the yields of purification should be high. This is achieved by a combination of dextranase treatment, ion-exchange and affinity chromatography after PEG precipitation (Majumder *et al*., 2007). Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F was purified by concentration and dialysis of the culture supernatant followed by treatment with dextranase and chromatography on Bio-Gel A-5m. A 240-fold purification with a specific activity of 53 U/mg was obtained (Robyt and Walseth, 1979). Kobayashi *et al*. (1986) reported the efficacy of DEAE-cellulose and Sephadex G-100 column chromatography of dextransucrase from the same strain. A 679-fold purification with a specific activity 26 U/mg was obtained.

1.4.2 Biochemical characterization of dextransucrase

The properties of dextransucrase have been extensively studied and reviewed (Kim and Robyt, 1995b). Molecular mass of dextransucrase ranging from 64 to 185 kDa have been reported (Robyt, 1995). This variation has been associated with the presence of dextran in the purified dextransucrase preparations, disassociation of high molecular mass multimeric complex (Kim and Robyt, 1995b) or action of proteases (Miller and Robyt, 1986). Various dextransucrase molecular mass forms found in enzyme preparations as products of proteolytic activity were observed in *Leuconostoc mesenteroides* strains NRRL B-512F and B-512FMC (Sanchez-Gonzalez *et al.*,

1999). On hydrolysis by protease, the *L. mesenteroides* dextransucrase (173 kDa) resulted a product with molecular mass 120 kDa, retaining the dextransucrase activity (Sanchez-Gonzalez *et al.*, 1999). Multiple active lower molecular weight forms from *Leuconostoc mesenteroides* B512F dextransucrase have been reported, suggested as the proteolytic processing products of a 170 kDa precursor (Arguello-Morales *et al.*, 2005).

Dextrasucrase activity depends on 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- 35°C and at pH of 5.4 (Purama and Goyal, 2008a).

Divalent cations are associated with glucansucrases, hence they stabilize the activity of enzymes (Goyal *et al.*, 1995). The activity of enzyme was enhanced by the addition of alkaline earth metals and inhibited by chelating agent such as EDTA, indicating that glucansucrases are associated with alkaline earth metals (Kobayashi and Matsuda, 1980). Miller and Robyt (1986) reported the association of Ca^{2+} ions with the catalytic sites of glucansucrases. 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).

1.5 Microbial biodiversity of North East India

Chapter 1

At the confluence of the Indo-Malayan, Indo-Chinese and Indian biogeographical realms, the North East India region is unique in providing a profusion of habitats, which features diverse biota with a high level of endemism. The north eastern Indian region has been in focus for its high biodiversity and has been a priority for leading conservation agencies of the world. While World Wildlife Fund (WWF) has identified the entire Eastern Himalayas as a priority Global 200 ecoregion, Conservation International US has upscaled the Eastern Himalaya Hotspot to all the eight states of North-East India, along with the neighbouring countries of Bhutan, southern China and Myanmar. The Eastern Himalayas biodiversity 'hotspot' was modified to the 'Indo-Burma hotspot' covering central Nepal to whole of North East India, Andaman and Nicobar Islands, Hainan island in southern China, Myanmar, Thailand and southern Malaysian peninsula (Myers, 2000). This hotspot is, the second largest and next only to the Mediterranean basin with an area 2,20,60,000 sq km among the 25 identified globally. The Northeast region of India comprising the states of Arunachal Pradesh, Assam, Meghalaya, Manipur, Tripura, Mizoram, Nagaland and Sikkim can be physiographically categorized into the Eastern Himalayas, Northeast hills, the Brahmaputra and Barak Valley plains. North East India has still been able to retain significant proportion of its biodiversity, possibly due to long years of isolation and difficult terrain. So, North East India, a biodiversity hotspot needs special attention (Thakur *et al.*, 2007). This region harbours useful microbial population which needs to be exploited (Myers, 2000). In the present study, we focussed on isolation of dextran producing lactic acid bacteria strain that can be commercially exploited.

1.6 The genus *Pediococcus*

Pediococci are a heterogeneous group of homofermentative lactic acid bacteria (Collins *et al.*, 1990, Benachour *et al.*, 1995). Currently ten species are recognized, comprising *Pediococcus damnosus*, *P. parvulus*, *P. inopinatus*, *P. cellicola*, *P. ethanolidurans*, *P. claussenii*, *P. stilesii*, *P. acidilactici*, *P. pentosaceus* and *P. dextrinicus* (Dobson *et al.*, 2002, Albano *et al.*, 2007). *Pediococcus argentinicus* sp. nov. (De Bruyne *et al.*, 2008) and *Pediococcus lolii* sp. nov. (Doi *et al.*, 2009) are recently identified. The species of this genus are cosmopolitan in distribution as they have been isolated from sugarcane field soil (Patel and Goyal, 2010), plants (Di Cagno *et al.*, 2009); Todorov *et al.*, 2009), pickles (Jonganurakkun *et al.*, 2008; Huang *et al.*, 2009), wines (Edwards and Jensen, 1992; Beneduce *et al.*, 2004), beverages (de Nadra. *et al.*, 1998; Todorov and Dicks, 2005), cheese (Gurira and Buys, 2005), cereal kernels (Corsetti *et al.*, 2007; Laitila *et al.*, 2006), meat (Anastasiadou *et al.*, 2008), sausages (Nieto-Lozano *et al.*, 2010; Albano *et al.*, 2007), silage (Doi *et al.*, 2009) and flours (De Bruyne *et al.*, 2008). Different species of Pediococcus, their source of isolation and applications are presented in Table 1.1. Members of the Pediococcus genus are of economic importance in the fermented food industry (Dellaglio and Torriani, 1986). Pediococcus genus has found use as starter cultures in fermentation processes of milk (Caldwell *et al.*, 1996), meat (Pfannebecker and Frohlich, 2008; Rivera-Espinoza and Gallardo-Navarro, 2010), vegetable products (Huang *et al.*, 2009) and sausages (Nieto-Lozano *et al.*, 2010). *Pediococcus acidilactici* has GRAS (Generally Recognized As Space) status and its used in animal feeds as probiotic cultures and as nutritional enhancers in silage (O'Connor *et al.*, 2007; Jonganurakkun, 2008). *Pediococcus pentosaceus* has been predominant in

wines with high pH (Sato *et al.*, 2000). *Pediococcus pentosaceus* in association with *Lactobacillus plantarum* proved a promising starter culture, significantly enhancing malt processability (Laitila *et al.*, 2006). In addition to their contribution to fermented foods, several *Pediococcus* strains produce bacteriocins that inhibit the growth of major Gram-positive foodborne pathogens, as well as other food spoilage bacteria (Kantor 1997). Bacteriocinogenic strains of *Pediococcus* genus has been found active against lactic acid bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Listeria innocua*, *Listeria ivanovii* and *Listeria monocytogenes* (Todorov *et al.*, 2009). Bacteriocin production has been shown to be plasmid associated in strains of *P. pentosaceus* (pediocin A). Till date, there have been several reports on pediocin production from *Pediococcus* genus (Motlagh *et al.*, 1994; Bennik *et al.*, 1997; Todorov and Dicks, 2005; Albano *et al.*, 2007; Nieto-Lozano *et al.*, 2010). However, only sparse work on exopolysaccharide production by *Pediococci* strains has been reported. The widespread industrial potentials of exopolysaccharide warrants studies in *Pediococcus* genus like other exopolysaccharide producing lactic acid bacteria *viz. Lactobacillus*, *Leuconostoc* and *Streptococcus*. A plasmid associated glucan production by *P. damnosus* has been reported in wine (Gindreau *et al.*, 2001). Ropy *Pediococcus damnosus* (strain 2.6) was used for production of exopolysaccharide (EPS) in a semi-defined medium (Duenas-Chasco *et al.*, 1997; Duenas *et al.*, 2003). The exopolysaccharide was found to be a β-linked homopolymer of D-glucose. On the basis of methylation, ^{1}H, ^{13}C, 1D and 2D NMR analyses, the EPS was shown to consist of trisaccharide repeating units (Duenas-Chasco *et al.*, 1997). Lambo-Fodje *et al.* (2007) studied a ropy exopolysaccharide from *Pediococcus damnosus 2.6 which promised usage as a thickener in food systems. In*

some Argentinian wines, the ropiness inducing lactic acid bacteria include *Pediococcus pentosaceus* (de Nadra and de Saad, 1995). *Pediococcus pentosaceus* strains AP-1 and AP-3 isolated from traditional thai fermented foods produced exopolysaccharide in high yield (Smitinont *et al.*, 1999).

Pediococcus sp.	Source	Applications	Reference
Pediococcus acidilactici *Pediococcus acidilactici*	Sausage 'Alheira' sausage	inhibition of *L. monocytogenes* by pediocin	Nieto-Lozano *et al.*, 2010 Albano *et al.*, 2007
Pediococcus pentosaceus	Pork meat	Inhibit food spoilage and food-born pathogens	Anastasiadou *et al.*, 2008
Pediococcus pentosaceus	marula (*Scerocarya birrea*)	bacteriocin, active against lactic acid bacteria, *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Listeria*	Todorov *et al.*, 2009
Pediococcus pentosaceus NB-17	Japanese traditional vegetable pickles	functional foods	Jonganurakkun *et al.*, 2008
Pediococcus pentosaceus 05-10	a traditionally fermented vegetable product from China	anti-*Listeria* bacteriocin having potential application in food preservation, especially in the meat products industry.	Huang *et al.*, 2009
Pediococcus pentosaceus	raw tomatoes	Confer health-promoting and sensory properties to tomato juices	Di Cagno *et al.*, 2009
Pediococcus pentosaceus *Pediococcus pentosaceus*	Argentinian wines	Water soluble exopolysaccharides production andPediocin N5p	de Nadra. and de Saad, 1995 de Nadra. *et al.*, 1998
Pediococcus pentosaceus ST18	a traditional cereal beverage from Bulgaria	anti-listerial Pediocin ST18	Todorov and Dicks, 2005
Pediococcus pentosaceus *Pediococcus acidilactici*	meat, vegetable and dairy fermentation	Change the characteristic flavour, improve hygienic quality and extend shelf-life of food	Mora *et al.*, 1997
Pediococcusacidilactici *Pediococcuspentosaceus*	Cheddar cheese	Cause cheese ripening and confer cheese flavour inhibit *Bacillus cereus* and *Listeria monocytogenes*	Gurira and Buys, 2005
Pediococcus pentosaceus VTT E-90390	split barley kernels	balance the microbial community and enhance brewing of malt	Laitila *et al.*, 2006
Pediococcus spp.	commercial wines	-	Edwards and Jensen, 1992
Pediococcus parvulus	minimally processed vegetables	production of pediocin PA-1	Bennik *et al.*, 1997
Pediococcus damnosus	beer	antibacterial compounds against *Salmonella infantis*	Skytta *et al.*, 1993
Pediococcus ethanolidurans sp. nov.	walls of a distilled-spirit-fermenting cellar	Beverage fermentation	Liu *et al.*, 2006
Pediococcus lolli sp. nov.	Rye grass silage	Silage fermentation	Doi *et al.*, 2009

Table 1.1 Different species of Pediococcus, their source of isolation and applications

1.7 Dextransucrase and dextran production by *Pediococcus spp.*

Whittenbury (1965) reported that *Pediococcus* genus is incapable of synthesizing dextran. However, Pediococci possess the capabilities for biosynthesis of glucan-type EPS (Llauberes *et al.*, 1990, Martensson *et al.*, 2005). Dextran production ability of *Pediococcus* genus is not explored enough. It would be interesting to investigate the possible elaboration of dextransucrase by *Pediococcus pentosaceus* as it produces EPS (Smitinont *et al.*, 1999). *Pediococcus parvulus* 2.6 is reported to produce β-glucan (Velasco *et al.*, 2009). The sugar composition, methylation analysis and nuclear magnetic resonance spectroscopy of EPS from *P. pentosaceus* AP-1 and AP-3, revealed that the EPS were dextrans (Smitinont *et al.*, 1999). Both the EPSs differed from linear dextran by branching through 3,6-di-*O*-substituted α-D-glucopyranosyl residues. Similar dextrans from several strains of *Leuconostoc* and related genera (Seymour and Knapp, 1980) and *Lactobacillus brevis* (Pidoux *et al.*, 1988) have been described in literature. The ^{13}C-NMR spectral pattern, the ^{13}C chemical shifts and the degree of branching of both AP-1 and AP-3 exopolysaccharides are similar to those reported for dextran from *Streptococcus viridans* B-1351 fraction S (Seymour and Knapp, 1980). These results, however were deemed preliminary than conclusive in view of the crude methods used to purify the exopolysaccharides and determination of their sugar composition. The taxonomic

status of *Pediococcus dextrinicus* is questioned and transfer of this species to the genus *Lactobacillus*, with the name *Lactobacillus dextrinicus* comb. nov. is proposed (Haakensen *et al.*, 2009). This reclassification is supported by multilocus sequence analysis of the 16S rRNA gene and Cpn60, PheS, RecA and RpoA proteins. The mode of cell division and existing phenotypic information also corroborated that that *P. dextrinicus* does not belong to the genus *Pediococcus*, but rather to the genus *Lactobacillus* (Haakensen *et al.*, 2009).

1.8 UV-mutagenesis of lactic acid bacteria for product enhancement

The mutagenic effects of UV irradiation on three strains of *Streptococcus lactis* were investigated by Lautier *et al.* (1988). *Leuconostoc mesenteroides* NRRL B-1355 culture was mutagenized with UV radiation and the mutants exhibited changes from parent cultures in the production of glucansucrases and glucans (Smith *et al.*, 1994). UV mutagenseis of lactic acid bacteria as a tool for novel mutants generation has been tried by Kadam *et al.* (2006). *Lactobacillus delbrueckii* (NCIM 2365) was subjected to UV-irradiation and four mutants with enhanced lactic acid productivity than the wild type were developed successfully. UV mutagenesis and selection of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* for potential use as starter culture was carried out by Sudi *et al.* (2008). The constitutive mutants of *Leuconostoc dextranicum* NRRL B-1146 producing higher concentration of glucan were generated by UV mutagenesis technique (Singh *et al.*, 2009). UV-induced mutagenesis of the dextran producing lactic acid bacteria was carried out and two mutants exhibiting higher dextransucrase and dextran production were selected by Patel and Goyal (2010b).

1.9 Response Surface Methodology for optimization of dextransucrase and dextran production

It is important to study the interactive effects of the medium components for optimization. The conventional methods for multifactor experimental design are time-consuming and incapable of detecting the true optimum, due to the interactions among the factors (Liu and Wang, 2007). Response surface methodology is an empirical modelling technique used to evaluate the relationship between a set of experimental variables and observed results (Majumder and Goyal, 2008). This optimization involves three major steps comprising statistically designed experiments, regression analysis of a mathematical model and prediction of the response with subsequent validation of the model (Box and Behnken, 1960). Statistical methods as Plackett-Burman Design, Box-Behnken Design and Central Composite Design (CCD) are often used to optimize culture medium, enzyme synthesis, aqueous two phase separation of proteins and glucan production (Liu and Wang 2007; Majumder and Goyal, 2008). Majumder and Goyal (2008) optimized the medium for glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146. Purama and Goyal (2008b) screened the nutritional factors and optimized the dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 using Response surface methodology. Majumder *et al.* (2009a) optimized the medium for glucan production from *Leuconostoc dextranicum* NRRL B-1146 and Majumder *et al.* (2009b) optimized the medium for maximum dextran production from *Leuconostoc mesenteroides* NRRL B-640.

1.10 Objectives of the present study

Microbial exopolysaccharides have grasped immense attention for their multifold utility. A homopolysaccharide dextran has enormous applications in pharmaceutical, food, agriculture and fine chemical industries. Dextrans also hold prospect as anti-cancer, biosensor, tissue engineering scaffolds and drug delivery agents. Prebiotic oligosaccharides that are used as neutraceuticals, can be synthesized by acceptor reactions of dextransucrase.

The aim of the current study is to explore the natural biodiversity to isolate novel dextransucrase and dextran producing strains of lactic acid bacteria. On the basis of higher enzyme activity, the colonies will be selected. The Gram staining, catalase test, carbohydrate utilization pattern and antibiotic susceptibility experiments will be carried out. 16S rRNA sequencing based molecular identification of the isolate will be done and its taxonomic position will be traced by constructing a phylogenetic tree. The culture conditions as temperature and shaking will be optimized for obtaining maximum yields of dextransucrase and dextran. The growth parameters *viz.* the cell optical density, pH, enzyme activity and protein concentration of the selected isolate will be studied. The purification of dextransucrase from the isolate will be tried by the

polyethylene glycol fractionation protocol. Denaturing SDS-PAGE and non-denaturing SDS-PAGE will be performed as a part of biochemical characterization of the purified enzyme. The reaction conditions for enzyme such as sucrose concentration, temperature, pH and ionic strength of the buffer will be optimized. The effects of different organic solvents on the dextransucrase activity of the isolate will be studied. The purified dextransucrase will be used to synthesize dextran. The crude dextran will be purified by alcohol precipitation and subjected to lyophilisation. The dextran structure will be analysed by optical rotation, FT-IR, NMR and SEM studies. The dextran will be studied rheological properties. Cytotoxicity test of the dextran will be performed to evaluate its biomedical potential. UV mutagenesis of the isolate will be carried out to produce mutants having high dextransucrase and dextran yields. Statistical methods will be employed to further enhance the dextransucrase and dextran production from the mutant of the natural isolate of lactic acid bacterium.

Specific objectives of present study

1. Screening, identification and characterization of a new isolate of lactic acid bacterium producing dextransucrase and dextran.
2. Production, purification and functional characterization of dextransucrase.
3. Production, purification, structural and application studies of dextran.
4. Mutagenesis of lactic acid bacterium isolate for enhancement of dextransucrase and dextran production.
5. Medium optimization by Response Surface Methodology to enhance dextransucrase production from the mutant of lactic acid bacterium isolate.
6. Statistical optimization of medium for maximization of dextran production from the lactic acid bacterium mutant.

References

Agarwal, A., Gupta, U., Asthana, A. and Jain, N. K. (2009) Dextran conjugated dendritic nanoconstructs as potential vectors for anti-cancer agent. Biomaterials 30, 3588-3596.

Albano, H., Todorov, S. D., van Reenen, C. A., How, T., Dicks, L. M. T. and Teixeira, P. (2007) Characterization of a bacteriocin produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. Int. J. Food Microbiol. 116, 239-247.

Anastasiadou, S., Papagianni, M., Filiousis, G., Ambrosiadis, I. and Koidis, P. (2008) Growth and metabolism of a meat isolated strain of Pediococcus *pentosaceus* in submerged fermentation: Purification, characterization and properties of the produced pediocin SM-1. Enz. Microb. Technol. 43, 448-454.

Alpar, E. K. and Killampalli, V. V. (2004) Effects of hypertonic dextran in hypovolaemic shock: a prospective clinical trial. Injury. 35, 500-506.

Arguello-Morales, M., Sanchez-Gonzalez, M., Canedo, M., Quirasco, M., Farres, A. and Lopez-Munguia, A. (2005) Proteolytic modification of *Leuconostoc mesenteroides* B-512F dextransucrase. Antonie van Leeuwenhoek. 87, 131-141.

Arihara, K., Toba, T. and Adachi, S. (1990) Immunofluorescence microscopic studies on distribution of *Lactobacillus kefiranofaciens* and *Lactobacillus kefir* in kefir grains. Int. J. Food Microbiol. 11, 127-134.

Aumelas, A., Serrero, A., Durand, A., Dellacherie, E., Leonard, M. (2007) Nanoparticles of hydrophobically modified dextrans as potential drug carrier systems. Colloids and Surfaces B: Biointerfaces. 59, 74-80.

Baba, M., Schols, D., De Clercq, E., Pauwels, R., Nagy, M., Gyorgyi-Edelitnyi, J., Low, M. and Gorog, S. (1990) Novel sulfated polymers as highly potent and selective inhibitors of human immunodeficiency virus replication and giant cell formation. Antimicrob. Agents Chemother. 34, 134-138.

Baird, J. K., Sandford, P. A. and Cottrell, I. W. (1983) Industrial Applications of Some New Microbial Polysaccharides. Nature Biotechnol. 1, 778-783.

Baird, J. K. and Smith, W. W. (1989) An analytical procedure for gellan gum in food gels. Food Hydrocolloids. 3, 407-411.

Barker, P. E. and Ajongwen, N. J. (1991) The production of the enzyme dextransucrase using nonaerated fermentation techniques. Biotechnol. Bioeng. 37, 703-707.

Barker, P. E., Ganetsos, G. and Ajongwen, N. J. (1993) A novel approach to the production of clinical grade dextran. J. Chem. Technol. Biotechnol. 57, 21-26.

Bautista, M. C., Bomati-Miguel, O., Morales, M. P., Serna, C. J. and Veintemillas-Verdaguer, S. (2005) Surface characterisation of dextran-coated iron oxide nanoparticles prepared by laser pyrolysis and coprecipitation. J Magn Magn Mater 293, 20-27.

Beneduce, L., Spano, G., Vernile, A., Tarantino, D. and Massa, S. (2004) Molecular characterization of lactic acid populations associated with wine spoilage. J. Basic Microbiol. 44, 10-16.

Benachour, A., Frere, J. and Novel, G. (1995) pUCL287 plasmid from *Tetragenococcus halophila* (*Pediococcus halophilus*) ATCC 33315 represents a new theta-type replicon family of lactic acid bacteria. FEMS Microbiol. Lett. 128, 167-176.

Bennik, M. H. J., Smid, J. E. and Gorris, L. G. M (1997) Vegetable-associated *Pediococcus parvulus* produces pediocin PA-1. Appl. Environ. Microbiol. 63, 2074-2076.

Bertrand, A., Morel, S., Lefoulon, A. F., Rolland Y., Monsan, P. and Remaud-Simeon, M. (2006) *Leuconostoc mesenteroides* glucansucrase synthesis of flavonoid glucosides by acceptor reactions in aqueous-organic solvents. Carbohydr. Res. 341, 855-863.

Bouhnik, Y., Raskine, L., Champion, K., Andrieux, C., Penven, S., Jacobs, H. and Simoneau, G. (2007) Prolonged administration of low-dose inulin stimulates the growth of bifidobacteria in humans. Nutr. Res. 27, 187-193.

Brandt, M. J., Roth, K. and Hammes, W. P. (2003). Effect of an exopolysaccharide produced by *Lactobacillus sanfranciscensis* LTH1729 on dough and bread

quality. In: L. de Vuyst, Editor, Sourdough from fundamentals to applications, Vrije Universiteit Brussel (VUB), IMDO, Brussels. pp. 80.

Caldwell, S. L., McMahon, D. J., Oberg, C. J. and Broadbent, J. R. (1996) Development and characterization of lactose-positive. *Pediococcus* species for milk fermentation. Appl. Environ. Microbiol. 62, 936-941.

Cavalli, R., Bisazza, A., Giustetto, P., Civra, A., Lembo, D., Trotta, G., Guiot, C. and Trotta, M. (2009) Preparation and characterization of dextran nanobubbles for oxygen delivery. Int. J. Pharmaceutics 381, 160-165.

Cerning, J. (1990) Exocellular polysaccharides produced by lactic caid bacteria. FEMS Microbiol. Rev. 7, 113-130.

Chen, H., Yuan, L., Song, W., Wu, Z. and Li, D. (2008) Biocompatible polymer materials: Role of protein-surface interactions. Prog. Polym. Sci. 33, 1059-1087.

Colby, S. M. and Russell, R. R. B. (1997) Sugar metabolism by mutans streptococci. J. Appl. Microbiol. 83, 80-88.

Collins, M. D., Williams, A. M. and Wallbanks, S. (1990) The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16s rRNA sequence analysis: description of *Tetragenococcus* gen. nov. FEMS Microbiol. Lett. 70, 255-262.

Corsetti., A., Settanni, L., Lopez, C. C., Felis, G. E., Mastrangelo, M. and Suzzi, G. (2007) A taxonomic survey of lactic acid bacteria isolated from wheat (*Triticum durum*) kernels and non-conventional flours. Syst. Appl. Microbiol. 30, 561-571.

Cortezi, M., Monti, R. and Contiero, J. (2005) Temperature effect on dextransucrase production by *Leuconostoc mesenteroides* FT 045 B isolated from alcohol and sugar mill plant. Afr. J. Biotechnol. 4, 279-285.

De Belder, A. N. (1996) Medical application of dextran and its derivatives. In: Dumitriu, S. (Ed.) Polysaccharides in medicinal applications. University of Sherbrooke, Canada Marcel Dekker, Inc., Monticello, NY.

De Bruyne, K., Franz, C. M. A. P., Vancanneyt, M., Schillinger, U., Mozzi, F., de Valdez, G. F., De Vuyst, L. and Vandamme, P. (2008) *Pediococcus argentinicus* sp. nov. from Argentinean fermented wheat flour and identification of *Pediococcus* species by *pheS*, *rpoA* and *atpA* sequence analysis. Int. J. Syst. Evol. Microbiol. 58, 2909-2916.

Degeest, B., Vaningelgem, F. and De Vuyst, L. (2001) Microbial physiology, fermentation kinetics, and process engineering of heteropolysaccharide production by lactic acid bacteria. Int. Dairy J. 11,747-757.

Dellaglio, F. and Torriani, S. (1986) DNA-DNA homology, physiological characteristics and distribution of lactic acid bacteria isolated from maize silage. J. Appl. Bacteriol. 60, 83-92.

Demuth, K., Jordening, H.-J and Buchholz, K. (2002). Oligosaccharide synthesis by dextransucrase: new unconventional acceptors. Carbohydr. Res. 337, 1811-1820.

de Nadra, M. C. M. and de Saad, A. M. S. (1995) Polysaccharide production by *Pediococcus pentosaceus* from wine. Int. J. Food Microbiol. 27, 101-106.

de Nadra, M. C. M., de Lamelas, D. S. and de Saad, A. M. S. (1998) Pediocin N5p from *Pediococcus pentosaceus: adsorption on bacterial strains.* Int. J. Food Microbiol. 39, 79-85.

de Segura, A. G., Alcalde, M., Manuel Bernabe, M., Ballesteros, A. and Plou, F. J. (2006) Synthesis of methyl-d-glucooligosaccharides by entrapped

dextransucrase from *Leuconostoc mesenteroides* B-1299. J. Biotechnol. 124, 439-445.

De Vuyst, L. and Degeest, B. (1999) Heteropolysaccharides from lactic acid bacteria. FEMS Microbiol. Rev. 23, 153-177.

De Vuyst, L., De Vin, F., Vaningelgem, F. and Degeest, B. (2001) Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. Int. Dairy J. 11, 687-707.

Di Cagno, R. Surico, R. F., Paradiso, A., De Angelis, M., Salmon, J., Solange, B., , De Gara, L and Gobbetti, M. (2009) Effect of autochthonous lactic acid bacteria starters on health-promoting and sensory properties of tomato juices. Int. J. Food Microbiol. 128, 473-483.

Dobson, C. M., Deneer, H., Lee, S., Hemmingsen, S., Glaze, S. and Ziola, B. (2002) Phylogenetic analysis of the genus *Pediococcus*, including *Pediococcus claussenii* sp. nov., a novel lactic acid bacterium isolated from beer. Int. J. Syst. Evol. Microbiol. 52, 2003-2010.

Doi, K., Nishizaki, Y., Fujino, Y., Ohshima, T., Ohmomo, S. and Ogata, S. (2009) *Pediococcus lolii* sp. nov., isolated from ryegrass silage. Int. J. Syst. Evol. Microbiol. 59, 1007-1010.

Dols, M., Simeon-Remaud, M. and Monsan, P. F. (1997) Dextransucrase production by *Leuconostoc mesenteroides* NRRL B-1299. Comparsion with *Leuconostoc mesenteroides* NRRL B-512F. Enzyme Microb. Technol. 20, 523-530.

Dols-Lafargue, M., Willemot, R. M., Monsan, P. F. and Remaud-Simeon, M. (2001) factors affecting alpha-1,2 glucooligosaccharide synthesis by *Leuconostoc mesenteroides* NRRL B-1299 dextransucrase. Biotechnol. Bioeng. 74, 498-504.

Drury, J. L. and Mooney, D. J. (2003) Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials. 24, 4337-4351.

Duenas-Chasco, M. T., Rodríguez-Carvajal, M. A., Tejero Mateo, P. T., Franco-Rodriguez, G., Espartero, J., Irastorza-Iribas, A. and Gil-Serrano, A. M. (1997) Structural analysis of the exopolysaccharide produced by *Pediococcus damnosus* 2.6. Carbohydr. Res. 303, 453-458.

Duenas, M., Munduate, A., Perea, A. Irastorza, A. (2003) Exopolysaccharide production by *Pediococcus damnosus* 2.6 in a semidefined medium under different growth conditions. Int. J. Food. Microbiol. 87, 113-120.

Edwards, C. G. and Jensen, K. A. (1992) Occurrence and characterization of lactic acid bacteria from Washington State wines: *Pediococcus* spp. Am. J. Enol. Vitic. 43, 233-238.

Funane, K., Ishii, T., Ono, H. and Kobayashi, M. (2005) Changes in linkage pattern of glucan products induced by substitution of Lys residues in the dextransucrase. FEBS Lett. 579, 4739-4745.

Garcia-Ochoa, F., Santos, V. E., Casas, J. A., Gomez, E. (2000) Xanthan gum: production, recovery, and properties. Biotechnol. Adv. 18, 549-579.

Goyal, A., Nigam, M. and Katiyar, S. S. (1995) Optimal conditions for production of dextransucrase from *Leuconostoc mesenteroides* B-512F and its properties. J. Basic Microbiol. 35, 375-384.

Gindreau, E., Walling, E. and Lonvaud-Funel, A. (2001) Direct polymerase chain reaction detection of ropy *Pediococcus damnosus* strains in wine. J. Appl. Microbiol. 90, 535-542.

Gurira, O. Z. and Buys, E. M. (2005) Characterization and antimicrobial activity of *Pediococcus species isolated from South African farm-style cheese. Food* Microbiol. 22, 159-168.

Haakensen, M., Dobson, C. M., Hill, J. E and Ziola, B. (2009) Reclassification of *Pediococcus dextrinicus* (Coster and White 1964) back 1978 (Approved Lists 1980) as *Lactobacillus dextrinicus* comb. nov., and emended description of the genus Lactobacillus. Int. J .Syst. Evol. Microbiol. 59, 615-621.

Hammond, B. F. (1969) Dextran production by a human oral strain of *Lactobacillus casei.* Arch. Oral Biol. 14, 879-890.

Holt, S. M. and Cote, G. L. (1998) Differentiation of dextran-producing *Leuconostoc* strains by a modified randomly amplified polymorphic DNA protocol. Appl. Enviro. Microbiol. 64, 3096-3098.

Huang, Y., Luo, Y., Zhai, Z., Zhang, H., Yang, C., Tian, H., Li, Z., Feng, J. and Hao, Y. (2009) Characterization and application of an anti-*Listeria* bacteriocin produced by *Pediococcus pentosaceus 05-10 isolated from Sichuan Pickle, a* traditionally fermented vegetable product from China. Food Control. 20, 1030-1035.

Jonganurakkun, B., Wang, Q., Xu, S. H., Tada, Y., Minamida, K., Yasokawa, D., Sugi, M., Hara, H. and Asano, K. (2008) *Pediococcus* pentosaceus NB-17 for probiotic use. J. Biosci. Bioeng. 106, 69-73.

Kadam, S. R., Patil, S. S., Bastawde, K. B., Khire, J. M. and Gokhale, D.V. (2006) Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. Proc. Biochem. 41, 120-126.

Kang, H-K, Seo, E-S, Robyt, J. F. and Kim, D. (2003) Directed evolution of a dextransucrase for increased constitutive activity and the synthesis of a highly branched dextran. J. Mol. Catalysis B: Enzymatic 26, 167-176.

Kantor, A., Montville, T. J., Mett, A. and Shapira, R. (1997) Molecular characterization of the replicon of the *Pediococcus pentosaceus* 43200 pediocin A plasmid pMD136. FEMS Microbiol. Lett. 151, 237-244.

Kim, D. and Robyt, J. F. (1995a) Production, selection, and characteristics of mutants of *Leuconostoc mesenteroides B-* 742 constitutive for dextransucrases. Enz. Microbial Technol. 17, 689-695.

Kim, D. and Robyt, J. F. (1995b) Dextransucrase constitutive mutants of *Leuconostoc mesenteroides* B-1299. Enz. Microbial Technol. 17, 1050-1056.

Kim, D., Robyt, J. F., Lee, S-Y, Lee, J-H and Kim, Y-M (2003) Dextran molecular size and degree of branching as a function of sucrose concentration, pH, and temperature of reaction of *Leuconostoc mesenteroides* B-512FMCM dextransucrase. Carbohydr. Res. 338, 1183-1189.

Kitaoka, M. and Robyt, J. F. (1999) Mechanism of the action of *Leuconostoc mesenteroides* B-512FMC dextransucrase: kinetics of the transfer of D-glucose to maltose and the effects of enzyme and substrate concentrations. Carbohydr. Res. 320, 183-191.

Ko, W. H., Wong, C. C., Yeung, H. W., Yung, M. H., Shaw, P. C. and Tam, S. C. (1991) Increasing the plasma half-life of trichosanthin by coupling to dextran. Biochem. Pharmacol. 42, 1721-1728.

Kobayashi, M. and Matsuda, K. (1976) Purification and properties of the extracellular dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299. J. Biochem. 79, 1301-1308.

Kobayashi, M. and Matsuda, K. (1978) Inhibition of dextran synthesis by glucoamylase and endodextranase. Carbohydr. Res., 66, 277-288.

Kobayashi, M. and Matsuda, K. (1980) Characterization of multiple forms and main component of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Biochim. Biophys. Acta, 614, 46-62.

Kobayashi, M., Mihara, K. and Mastuda, K. (1986) Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Characterization of the enzyme bound to sephadex gel. Agric. Biol. Chem. 50, 551-556.

Koepsell, H. J., Tsuchiya, H. M., Hellman, N. N., Kasenko, A., Ho¡man, C. A., Sharpe, E.S. and Jackson, R.W. (1953) Enzymatic synthesis of dextran. acceptor specificity and chain initiation. J. Biol. Chem. 200, 793-801.

Koo, O. M., Rubinstein, I. and Onyuksel, H. (2005) Role of nanotechnology in targeted drug delivery and imaging: a concise review. Nanomedicine: Nanotechnol. Biol. Med. 1, 193-212.

Kunin, C. M. (1967) Distribution of cell receptors and simple sugar inhibitors during encephalomyocarditis virus-cell union. J. Virol. 1, 274-282.

Laitila, A., Sweins, H., Vilpola, A., Kotaviita, E., Olkku, J., Home, S. and Haikara, A. (2006) *Lactobacillus plantarum* and *Pediococcus pentosaceus* starter cultures as a tool for microflora management in malting and for enhancement of malt processability. J. Agric. Food Chem. 54, 3840-3851.

Lambo-Fodje, A. M., Leeman, M., Wahlund, K. G., Nyman, M., Oste, R. and Larsson, H. (2007) Molar mass and rheological characterisation of an exopolysaccharide from *Pediococcus damnosus 2.6. Carbohydr. Polym*. *68, 577-586.*

Lautier, M., Gillot, B., Auffray, Y., Castillo-Sanchez, X. and Boutibonnes, P. (1988) Mutagenesis in *Streptococcus lactis* exposed to UV irradiation and alkylating agents. Mutagenesis. 3, 245-247.

Lazic, M. L., Velzkovic, V. B., Vucetic, J. I. and Vrvic, M. M. (1993) Effect of pH and aeration on dextran production by *Leuconostoc mesenteroides*. Enz. Microb. Technol. 15, 334-338.

Liu, L., Zhang, B., Tong, H. and Dong, X. (2006) *Pediococcus ethanolidurans* sp. nov., isolated from the walls of a distilled-spirit-fermenting cellar. Int. J. Syst. Evol. Microbiol. 56, 2405-240.

Liu, G-C and Wang, X-L, (2007) Optimization of critical medium components using response surface methodology for biomass and extracellular polysaccharide production by *Agaricus blazei*. Appl. Microbiol. Biotechnol. 74, 78–83.

Llauberes, R. M., Richard, B., Lonvaud, A., Dubourdieu, D. and Fournet, B. (1990) Structure of an exocellular β-D-glucan from *Pediococcus* sp., a wine lactic bacteria. Carbohydr. Res. 203, 03-107.

Looijesteijn, P. J., Trapet, L., De vries, E., Abee, T. and Hugenholtz, J. (2001) Physiological function of exopolysaccharides produced by *Lactococcus lactis*. Int J Food Microbiol. 64, 71-80.

Lonvaud-Funel, A., Guilloux, Y. and Joyeux, A. (1993) Isolation of a DNA probe for identification of glucan-producing *Pediococcus damnosus* in wines. J. Appl. Bacteriol. 74, 41- 47.

Lopes, L., Andrade, C. T., Milas, M. and Rinaudo, M. (1995) Influence of aggregates on the sedimentation properties of welan gum. Polym. Bull. 34, 655-662.

MacGregor, E. A., Jesperson, H. M. and Svensson, B. (1996) A circularly permutated a-amylase-type α/β-barrel structure in glucan-synthesizing glucosyltransferases. FEBS Lett. 378, 263-266.

Majumder, A. and Goyal, A. (2007) Use of statistically designed medium for improved glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146 in a bioreactor. Int. J. Chem. Sci. 5, 1525-1531.

Majumder, A., Purama, R. K. and Goyal, A. (2007) An overview of purification methods of glycoside hydrolase family 70 dextransucrase. Ind. J. Microbiol. 47, 253-262.

Majumder, A. and Goyal, A. (2008) Enhanced production of exocellular glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 using response surface method. Bioresour. Technol. 99, 3685-369.

Majumder, A. and Goyal, A. (2009) Rheological and gelling properties of a novel glucan from *Leuconostoc dextranicum* NRRL B-1146. Food Res. Int. 42, 525-528.

Majumder, A., Singh, A. and Goyal, A. (2009a) Application of response surface methodology for glucan production from *Leuconostoc dextranicum* NRRL B-1146 and its structural characterization. Carbohydr. Polym. 75, 150-156.

Majumder, A., Bhandari, S., Purama, R. K., Patel, S. and Goyal, A. (2009b) Enhanced production of a novel dextran from *Leuconostoc mesenteroides* NRRL B-640 by Response Surface Methodology. Annals Microbiol. 59, 1-7.

Marshall, K. C. (1992) Biofilms: an overview of bacterial adhesion, activity and control at surfaces. Am. Soc. Microbiol. News. 58, 202-207.

Martensson, O., Biorklund, M., Lambo, A., Duenas-Chasco, M., Iras-torza, A., Holst, O., Norin, E., Welling, G., Oste, R. and Onning, G. (2005) Fermented, ropy, oat-based products reduce cholesterol levels and stimulate the bifidobacteria flora in humans. Nutr. Res. 25, 429-442.

Miller, A. W. and Robyt, J. F. (1984) Stabilization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F by nonionic detergents, poly(ethylene glycol) and high molecular weight dextran. Biochim. Biophys. Acta. 785, 89-96.

Miller A. W. and Robyt J. F. (1986) Detection of dextransucrase and levansucrase on polyacrylamide gels by the Periodic acid-Schiff stain: staining artifacts and their prevention. Anal. Biochem. 156, 357-363.

Misaki, A., Torii, M., Sawai, T. and Goldstein, I. J. (1980) Structure of the dextran of *Leuconostoc mesenteroides* B-1355. Carbohydr. Res. 84, 273-28.

Monchois, V., Vignon, M., Escalier, P. C., Svensson, B. and Russell, R. R. B. (2000) Involvement of Gln937 of *Streptococcus downei* GTF-I glucansucrase in transition-state stabilization. Eur. J. Biochem. 267, 4127-4136.

Monsan, P., Bozonnet, S., Albenne, C., Joucla, G., Willemot, R. and Remaud-Simeon, M. (2001) Homopolysaccharides from lactic acid bacteria. Int. Dairy J. 11, 675-685.

Mora, D., Fortina, M. G., Parini, C. and Manachini, P. L. (1997) Identification of *Pediococcus acidilactici and* Pediococcus *pentosaceus based on 16S rRNA* and ldhD gene-targeted multiplex PCR analysis. FEMS Microbiol. Lett. 151, 231-236.

Motlagh, A., Bukhtiyarova, M. and Ray, B. (1994) Complete nucleotide sequence of pSMB74, a plasmid encoding the production of pediocin AcH in *Pediococcus acidilactici*. Lett. Appl. Microbiol. 18, 305-312.

Moulis, C., Joucla, G., Harrison, D., Fabre, E. Potocki-Veronese, G., Monsan P. and Remaud-Simeon. M. (2006) Understanding the polymerization mechanism of Glycoside-Hydrolase family 70 glucansucrases. J. Biol. Chem. 281, 31254-31267.

Myers, N., Mittermeier, R. A., Mittermeier, C. G., Fonseca, G. A. B. and Kent, J. (2000) Biodiversity hotspots for conservation priorities. Nature. 403, 853-858.

Neely, W. B. and Nott, J. (1962) Dextransucrase, an induced enzyme from *Leuconostoc mesenteroides*. Biochem. 1, 1136-1140.

Nieto-Lozano, J. C., Reguera-Useros, J. I., Pelaez-Martinez, M. C., Sacristán-Pérez-Minayo, G., Gutiérrez-Fernández, A. J. and de la Torre, A. H. (2010) The effect of the pediocin PA-1 produced by *Pediococcus acidilactici* against *Listeria monocytogenes* and *Clostridium perfringens* in Spanish dry-fermented sausages and frankfurters. Food Control. 21, 679-685.

O'Connor, E. B., O'Sullivan, O., Stanton, C., Danielsen, M., Simpson, P. J., Callanan, M. J., Ross, R. P. and Hill, C. (2007) pEOC01: A plasmid from *Pediococcus acidilactici* which encodes an identical streptomycin resistance (aadE) gene to that found in *Campylobacter jejuni*. Plasmid. 58, 115-126.

Oh, J. K., Lee, D. I. and Park, J. M (2009) Biopolymer-based microgels/nanogels for drug delivery applications. Prog. Polymer Sci. 34, 1261-1282.

Padmanabhan, P. A. and Kim, D-S (2002) Production of insoluble dextran using cell-bound dextransucrase of *Leuconostoc mesenteroides* NRRL B-523. Carbohydr. Res. 337, 1529-1533.

Padmanabhan, P. A., Kim, D-S, Pak, D. and Sim, S-J (2003) Rheology and gelation of water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523. Carbohydr. Polym. 53, 459-468.

Patel, S. and Goyal, A. (2010a) Isolation of a new strain of exopolysaccharide producing lactic acid bacterium (SPO) from soil of sugarcane field of Orissa. J. Microb. World. (in press).

Patel, S. and Goyal, A. (2010b) Isolation, characterization and mutagenesis of exopolysaccharide synthesizing new strains of lactic acid bacteria. Int. J. Microbiol. (in press).

Patel, S., Majumder, A. and Goyal, A. (2010) Potentials of exopolysaccharides from lactic acid bacteria. Ind. J. Microbiol. (in press).

Pfannebecker, J. and Frohlich, J. (2008). Use of a species-specific multiplex PCR for the identification of *Pediococci.* Int. J. Food Microbiol. 128, 288-296.

Pidoux, M., Brillouet, J. M. and Quemener, B. (1988) Characterization of the polysaccharides from a *Lactobacillus brevis* and from sugary kefir grains. Biotechnol. Lett. 10, 415-420.

Pijning, T., Vujicic-Zagar, A., Kralj, S., Eeuwema, W., Dijkhuizen, L. and Bauke W. Dijkstra, B. W. (2008) Biochemical and crystallographic characterization of a glucansucrase from *Lactobacillus reuteri* 180. Biocat. Biotransform. 26, 12-17.

Plou, F. J., Martin, M. T., de Segura, A. G., Alcalde, M. and Ballesteros, A. (2002) Glucosyltransferases acting on starch or sucrose for the synthesis of oligosaccharides. Can. J. Chem. 80, 743-752.

Purama, R. K. and Goyal, A. (2005) Dextransucrase production by *Leuconostoc mesenteroides*. Ind. J. Microbiol. 2, 89-101.

Purama, R. K. and Goyal, A. (2008a) Identification, purification and functional characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. Bioresour. Technol. 99, 3635-3642.

Purama, R. K. and Goyal, A. (2008b) Screening and optimization of nutritional factors for higher dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640 using statistical approach. Bioresour. Technol. 99, 7108-7114.

Purama, R. K. and Goyal, A. (2008c) Application of Response Surface Methodology for maximizing dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 in a bioreactor. Appl. Biochem. Biotechnol. 151,182-192.

Purama, R. K., Singh, G., Majumder, A., Dasu, V. V. and Goyal, A. (2008) Dextransucrase production in bioreactor: effect of aeration and mathematical modelling. J. Appl. Biosci. Biotechnol. 4, 9-14.

Purama, R. K. and Goyal, A. (2009) Optimization of conditions of *Leuconostoc mesenteroides* NRRL B-640 for production of a dextransucrase and its assay. J. Food Biochem. 33, 218-231.

Purama, R. K., Goswami, P., Khan, A. T. and Goyal, A. (2009) Structural analysis and properties of dextran produced by *Leuconostoc mesenteroides* NRRL B-640. Carbohydr. Polym. 76, 30-35.

Rivera-Espinoza, Y. and Gallardo-Navarro, Y. (2010) Non-dairy probiotic products. Food Microbiol. 27, 1-11.

Robyt, J. F. and Walseth, T. F. (1979) Production, purification and properties of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Carbohydr. Res. 68, 95-111.

Robyt, J. F. (1995) Mechanisms in the glucansucrase synthesis of polysaccharides and oligosaccharides from sucrose. Adv. Carbohydr. Chem. Biochem., 51, 133-168.

Robyt, J. F., Yoon, S-H and Mukerjea, R. (2008) Dextransucrase and the mechanism for dextran biosynthesis. Carbohydr. Res. 343, 3039-3048.

Sahoo, S. K., Parveen, S. and Panda, J. J. (2007) The present and future of nanotechnology in human health care. Nanomed.: Nanotechnol., Biol. and Med. 3, 20-31.

Sanchez-Gonzalez, M., Alagon, A., Rodriguez-Sotres, R. and Lopez-Munguia, A. (1999) Proteolytic processing of dextransucrase of *Leuconostoc mesenteroides*. FEMS Microbiol. Lett. 181, 25-30.

Sato, H., Yanagida, F., Shinohara, T. and Yokotsuka, K. (2000) Restriction fragment length polymorphism analysis of 16S rRNA genes in lactic acid bacteria isolated from red wine. J. Biosci. Bioeng. 90, 335-337.

Santos, M., Teixeira, J. and Rodrigues, A. (2000) Production of dextransucrase, dextran and fructose from sucrose using *Leuconostoc mesenteroides* NRRL B512F. Biochem. Eng. J. 4, 177-188.

Sengupta, A., Wang, S., Link, E., Anderson, E. H., Hofmann, C., Lewandowski, J., Kottke-Marchant, K. and Marchant, R. E. (2006) Glycocalyx-mimetic dextran

modified poly (vinyl amine) surfactant coating reduces platelet adhesion on medical-grade polycarbonate surface. Biomaterials. 27, 3084-3095.

Seo, E. S., Lee, J. H. Park, J. Y. Kim, D., Han H. J. and Robyt, J. F. (2005) Enzymatic synthesis and anti-coagulant effect of salicin analogs by using the *Leuconostoc mesenteroides* glucansucrase acceptor reaction. J. Biotechnol. 117, 31-38.

Seymour, F. R. and Knapp, R. D. (1980) Structural analysis of dextrans, from strains of Leuconostoc and related genera, that contain 3-*O*-α-D -glucosylated α-D -glucopyranosyl residues at the branch points or in consecutive, linear positions. Carbohydr. Res. 81**,** 105-129.

Shamala, T. R. and Prasad, M. S. (1995) Preliminary studies on the production of high and low viscosity dextran by *Leuconostoc spp*. Proc. Biochem. 30, 237-241.

Sidebotham, R. L., Weigel, H. and Bowen, W. H. (1971) Dextrans elaborated by cariogenic organisms. Carbohydr. Res., 19, 151-159.

Sidebotham, R. L. (1974) Dextrans. Adv. Carbohydr. Chem. Biochem. 30, 371-44.

Singh, A., Majumder, A. and Goyal, A. (2009) Mutagenesis of *Leuconostoc dextranicum* NRRL B-1146 for higher glucan production. Int. J. Microbiol. 7(1).

Skytta, E., Haikara, A. and Mattlla-Sandholm, T. (1993) Production and characterization of antibacterial compounds produced by *Pediococcus damnosus* and *Pediococcus pentosaceus*. J. Appl. Bacteriol. 74, 134-142.

Smith, M. R., Zahnley, J. and Goodman, N. (1994) Glucosyltransferase mutants of *Leuconostoc mesenteroides* NRRL B-1355. Appl. Environ. Microbiol. 60, 2723-2731.

Smitinont, T., Tansakul, C., Tanasupawat, S., Keeratipibul, S.,Navarini, L., Bosco, M. and Cescutti, P. (1999) Exopolysaccharide-producing lactic acid bacteria strains from traditional thai fermented foods: isolation, identification and exopolysaccharide characterization. Int. J. Food Microbiol. 51, 105-111.

Soetaert, W., Schwengers, D., Bucholz, K. and Vandamme, E. J. (1995) A wide range of carbohydrate modifications by a single microorganism: Leuconostoc mesenteroides. In: Carbohydr. Bioeng. (Petersen, S.B., Svensson, B. and Pederson, S., Eds.), Elsevier, Amsterdam. Vol. 10, pp. 351-358.

Spirig, R., Gajanayake, T., Korsgren, O., Nilsson, B. and Rieben, R. (2008) Low molecular weight dextran sulfate as complement inhibitor and cytoprotectant in solid organ and islet transplantation. Mol. Immunol. 45 4084-4094.

Stewart, T. L. and Fogler, H. S. (2001) Biomass plug development and propagation in porous media. Biotechnol. Bioeng. 72, 353-363.

Su, D. and Robyt, J. F. (1993) Control of the synthesis of dextran and acceptor-products by *Leuconostoc mesenteroides* B-512FM dextransucrase. Carbohydr. Res. 248, 339-348.

Sudi, I. Y., De, N. and Ali-Dunkrah, U. (2008) Mutagenesis and selection of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* for potential use as starter culture. J. Am. Sci. 4, 80-87.

Sutherland, I. W. (1994) Structure-function relationships in microbial exopolysaccharides. Biotechnol. Adv. 12, 393-448.

Thakur, D, Yadav, A., Gogoi, B. K. and Bora, T. C (2007) Isolation and screening of *Streptomyces* in soil of protected forest areas from the states of Assam and

Tripura, India, for antimicrobial metabolites. J. Mycologie Medicale. 17, 242-249.

Thayu, M. and Mamula, P. (2005) Treatment of iron deficiency anemia in pediatric inflammatory bowel disease. Curr. Treat. Options Gastroenterol. 8, 411-417.

Tieking, M., Korakli, M., Ehrmann, M. A., Ganzle, M. G. and Vogel, R. F. (2003) In situ production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. Appl. Environ. Microbiol. 69, 945-952.

Todorov, S. D. and Dicks, L. M. T. (2005) Pediocin ST18, an anti-listerial bacteriocin produced by *Pediococcus pentosaceus ST18 isolated from boza, a traditional* cereal beverage from Bulgaria. Proc. Biochem. 40, 365-370.

Todorov, S. D., Leon, M. T. and Dicks, L. M. T. (2009) Bacteriocin production by *Pediococcus pentosaceus* isolated from marula (*Scerocarya birrea*). Int. J. Food Microbiol. 132, 117-126.

Tsuchiya, H. M., Koepsell, H. J., Corman, J., Bryant, M. O., Feger, V. H. and Jackson R. W. (1952) The effect of certain cultural factors on production of dextransucrase by *Leuconostoc mesenteroides.* J. Bact. 64, 521-527.

Tsumori, H., Minami, T. and Kuramitsu, H. K. (1997) Identification of essential amino acids in the *Streptococcus mutans* glucosyltransferases. Infect. Immun. 179, 3391-3396.

Ulbrich, K. and Subr, V. (2004) Polymeric anticancer drugs with pH-controlled activation. Adv. Drug Del. Rev. 56, 1023-1050.

Velasco, S. E., Areizaga, J., Irastorza, A., Duenas, M. T., Santamaria, A. and Munoz, M. E. (2009) Chemical and rheological properties of the β-glucan produced by *Pediococcus parvulus* 2.6. J. Agric. Food Chem. 57, 1827-1834.

Vinderola, G., Perdigon, G., Duarte, J., Farnworth, E. and Matar, C. (2006) Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity. Cytokine. 36, 254-260.

Whitfield, C. and Valvano, M. A. (1999) Biosynthesis and expression of cell-surface polysaccharides in gram-negative bacteria. Adv. Microbiol. Physiol. 35, 135-246.

Whittenbury, R. (1965) A Study of some *Pediococci* and their relationship to *Aerococcus viridans* and the *Enterococci.* J. Gen. Microbiol. 40, 97-106.

Yamamoto, Y., Takahashi, Y., Kawano, M., Iizuka, M., Matsumoto, T., Saeki, S. and Yamaguchi, H. (1999) In vitro digestibility and fermentability of levan and its hypocholesterolemic effects in rats. J. Nutr. Biochem. 10, 13-18.

Yoo, S. H., Yoon, E. J., Cha, E. and Lee, H. G. (2004) Antitumor activity of levan polysaccharides from selected microorganisms. Int. J. Biol. Macromol. 34, 37-41.

Chapter 2

Screening, identification and characterization of a new isolate of dextransucrase producing *Pediococcus pentosaceus* (SPA)

2.1 Introduction

Natural and in particular microbial biodiversity is our planet's greatest but least-developed resource for biotechnological innovation (Gupta *et al.*, 2002). Biodiversity includes the diversity of plants, animals and microbes and their habitats. Genetic diversity, species diversity and ecosystem diversity together give rise to biodiversity, which sustains life on earth through energy flow and food chain. Research interest in microbial biodiversity over the past 25 years has increased markedly as microbiologists have become interested in the significance of biodiversity as its industrial, medical, and agricultural applications have evolved (Morris *et al.*, 2002). Bioprospecting the rich, soil micro-organisms may lead to the discovery of novel industrial products. In this perspective, exploring the natural biodiversity for screening novel strains of dextran producing lactic acid bacteria is a crucial strategy towards discovery of bioactive dextran of industrial utility.

Chapter 2

This biosphere is dominated by microorganisms, yet to date less than 1% have been identified (Amann *et al.*, 1995). Conventionally, bacterial species were named and recognized based on phenotypic clustering (Rossello-Mora and Amann, 2001). However, the scope of traditional identification methods is limited as it fails to represent the enormous microbial diversity in nature (Rondon *et al.*, 2000). In recent decades, bacterial systematists have adopted molecular approaches that have allowed standardized species demarcation (Cohan and Perry, 2007). The advent of powerful analytical technique like Polymerase Chain Reaction (PCR) and the recognition of molecular marker like the 16S ribosomal DNA as species-specific identification-tag have emerged as reliable tools for identification of organisms. The 16S rRNA amplicons have been used for the sequencing and analysis to establish sequence identity and taxonomic relationships by comparing the results *in silico*, to existing information in diverse databases (Escalante *et al.*, 2001). The recent surge of research in molecular microbial ecology provides compelling evidence for the existence of many novel types of microorganisms in high numbers and varieties and it provides an entirely different approach for tapping the limitless resource of uncultured bacteria.

Despite the increased knowledge of dextran production of lactic acid bacteria, dextran producing *Pediococcus strains have hitherto not been reported. Here, we* report the isolation and characterization of a Pediococcus *with potential for dextran* synthesis. A novel isolate of *Pediococcus pentosaceus* belonging to lactic acid bacteria family was isolated. The isolate was identified based on a battery of analyses such as light and scanning microscopy, Gram staining, catalase test, carbohydrate fermentation profile, antibiotic sensitivity tests and 16S rRNA sequencing data analysis.

2.2 Material and Methods

2.2.1 Origin, culture and storage conditions of the isolate

The soil sample was collected from the sugarcane field of Assam (near Guwahati). 1 g soil from the sample was suspended in 10 ml 0.85% sterile saline solution and the soil suspension was subjected to serial dilution. 100 µl of the sample from 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions of soil sample was spread plated on sugarcane medium composed of filtered sugarcane juice fortified with sucrose (2%, w/v), peptone (1%, w/v), NaCl (0.1%, w/v), pH 7 (Bhatnagar *et al.*, 1985). The culture plates were prepared in duplicates. The plates were then incubated for 48h at 28^{o}C. Appearance of colonies were checked and counted on a colony counter. The large size colonies were selected and picked by inoculation loop and streaked on modified MRS agar plates (Goyal and Katiyar, 1996) which contained (%, w/v): glucose, 2; yeast extract powder, 0.5; beef extract and peptone, 1; dipotassium phosphate, 0.2; tri-ammonium citrate, 0.2; sodium acetate, 0.5; Tween 80, 0.1 (v/v); $MgSO_4.7H_2O$, 0.02; $MnSO_4.4H_2O$, 0.02 and agar, 1.5. The streaked plates were incubated for 12h at 28^{o}C and stored at 4°C. Loopful of cultures were inoculated in 5 ml enzyme production medium described by Tsuchiya *et al.* (1952) which contained (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; pH of the medium was adjusted to 6.9 with 0.1 M HCl solution. For enzyme production, the cultures were grown in

liquid enzyme production medium for 12h at 25°C and 180 rpm. 1% of these 12h grown culture was again inoculated in 5 ml sterile medium of Tsuchiya *et al.* (1952) in test tubes and grown for 16h at 25°C and 180 rpm for further inoculations and enzyme assays. All media were sterilized by autoclaving at a steam pressure of 10.3 kPa (15 lb/in^2) and a temperature of 121°C for 20 min. All inoculations and culture transfers were carried out under aseptic conditions in a laminar hood.

2.2.2 Selection of the isolate based on enzyme activity

The enzyme activities of all the natural isolates were determined and the higher enzyme activity isolates were selected. The enzyme assay was carried out in 1 ml reaction mixture containing 5% sucrose, 20 mM sodium acetate buffer (pH 5.4) and 20 µl cell free supernatant. The enzymatic reaction was performed at 30°C for 15 min. 100 µl aliquot from the reaction mixture was taken for reducing sugar estimation by the method of Nelson (1944) and Somogyi (1945). Fructose was used to plot the standard graph. One unit (U) of dextransucrase 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.

The isolate showing the highest enzyme activity was selected for further study and named as SPA after the author's and place's name. The isolate SPA was grown in modified MRS-agar stabs at 28°C for 20h and maintained at 4°C. The isolate was propagated by sub-culturing every 2 weeks. The culture was also preserved as glycerol stock frozen at -80°C in 22% (v/v) final sterile glycerol concentration for long term storage.

2.2.3 Morphological and biochemical characterization of isolate SPA

The phenotypic characterization of the isolate SPA was carried out following existing literature (Johnson and Case, 1995; Mathara *et al.*, 2004; Milliere *et al.*, 1989). The characteristics of this isolate, such as Gram staining, morphology, catalase activity test were studied. The Gram staining of the isolates was performed. The bacterial smear was air dried and flooded with crystal violet solution for 30s, the stain was decanted by rinsing with running water, the smear was treated with alcohol, then the slide was flooded with iodine solution for 30s, excess of this mordant was washed off with water and flooded with counter stain safranin for 30s, washed with water and the smear was observed under compound microscope.

The cell shape, size and their arrangement was studied by Scanning Electron Microscopy. The sample was prepared by centrifuging 1 ml of 12h grown culture at 5,000 rpm for 10 min. The cell pellet was dissolved in 1 ml of saline solution (0.85%, w/v). The sample was fixed with equal volume of glutaraldehyde (2.5%, v/v) for 2-4h. One drop of this bacterial smear was dehydrated using different percent of alcohol and dried in a vacuum desiccator. This dried sample was attached to the SEM stub with double-sided tape and coated with 10 nm Au in a sputter coater (SCH 620, Leo). The surface of the sample was viewed at various magnifications in Scanning Electron Microscope (Leo1330 VP) operated at 10.0 kV (Patel and Goyal 2010).

The catalase activity of the isolate was deduced by adding few drops of 3% H_2O_2 on 5 ml of 12h grown culture (Kannan, 2002). The *E. coli* culture was treated with H_2O_2 under identical conditions was taken as positive control for catalase activity.

The morphology of colony of isolate SPA was examined on modified MRS agar plate after 24h of growth (Goyal and Katiyar, 1996). Growth of the isolate in enzyme production medium of Tsuchiya *et al.* (1952) at initial pH of 3.3 and 9.6 was tested to determine its pH tolerance (Manes-Laazaro, 2008).

2.2.4 Optimization of growth condition of the isolate SPA

To determine the optimum incubation temperature for growth of the isolate, loopful of the isolate culture was grown in 5 ml enzyme production medium (Tsuchiya *et al.*, 1952) and incubated at five different temperatures 20°C, 22°C, 25°C, 28°C and 30°C in incubator shaker (Scigenics, model Orbitek LEX). After 16h, 1 ml from all the four samples were withdrawn, centrifuged and the cell free supernatants were analysed for enzyme activity as described in Section 2.2.2. To determine the effect of shaking on the growth of the isolate, loopful of the isolate culture from the modified MRS stab was grown in 5 ml enzyme production medium (Tsuchiya *et al.*, 1952) and kept in static and orbital shaking incubator at 180 rpm, 25°C± 0.2°C. After 16h, 1 ml from both the samples were withdrawn, centrifuged and were analysed for enzyme activity as described in Section 2.2.2.

2.2.5 Growth profile study

Various growth parameters such as change in pH, cell density and enzyme activity were studied at every 4h interval. The growth of culture was determined by measuring the optical density of cells at 600 nm on UV-Vis spectrophotometer

(Varian, model Cary 100) using the sterile enzyme production medium (Tsuchiya *et al.*, 1952) as blank.

2.2.6 Antibiotic sensitivity

The isolate SPA was tested for its susceptibility to thirty antibiotics using agar disc diffusion test (Barry and Thornsberry, 1980). The antibiotic test was performed using commercially available antibiotic octodiscs impregnated with Amoxyclav, Cephalexin, Ciproflaxacin, Clindamycin, Claxacillin, Erythromycin, Tetracyclin, Ampicillin, Carbenicilllin, Cephatoxamine, Chloramphenicol, Co-Trimazine, Gentamicin, Norflaxacin, Oxacillin, Amikacin, Amoxycillin, Bacitracin, Cephalothin, Novobiocin, Oxytetracyclin, Vancomycin, Penicillin-G, Tobramycin, Cephaloridine, Kanamycin, Linomycin, Methicillin, Norfloxacin and Oleandomycin purchased from Hi-media Pvt. Ltd., India. The petri-plates were first laid with MRS medium containing 1.8%, (w/v) agar. The isolate SPA was grown in MRS liquid medium, mixed in MRS-soft agar (0.8%, w/v agar) and poured over the MRS medium containing 1.8%, (w/v) agar. After 2 min, the antibiotic octodiscs were gently placed at the centre over the surface of the agar plates. The Petri plates were incubated in inverted position overnight at 28°C and were observed next day for zone of inhibition around the discs (Purama *et al.*, 2008).

2.2.7 Carbohydrate fermentation

The isolate SPA was tested for its carbohydrate fermentation ability (Kandler and Weiss, 1986). From the overnight grown MRS broth containing 2% glucose as carbohydrate source, 50 µl was inoculated in 5 ml liquid MRS medium lacking

glucose but containing Phenol red (0.04 g/L) as pH indicator and other test carbohydrates (Purama *et al.*, 2008). The test media were incubated at static condition, for 48h at 28°C. The acid production was observed between 24-48h. The acid production as a result of carbohydrate fermentation was indicated by a change in colour of phenol red to yellow.

2.2.8 16S rRNA based identification

2.2.8.1 Extraction of genomic DNA

The genomic DNA of the isolate SPA was extracted by GeNei^{TM} Genomic DNA Extraction kit (Bangalore Genei Pvt. Ltd.) The bacterial cell pellet was lysed using a solution containing guanidium thiocyanate (a chaotropic agent) and SDS (a detergent), to extract DNA. This lysis solution was used to disrupt the cell, remove proteins and polysaccharides and partial hydrolysis of RNA. DNA was then precipitated using alcohol and washed with 70% alcohol to remove contaminants. Pelleted DNA was solubilized in an appropriate buffer (solution B) at higher temperature (50-55°C). The extracted DNA was purified using Ultra pure prep kit (Bangalore Genei, India).

2.2.8.2 Amplification of 16S rRNA gene by Polymerase Chain Reaction

Genomic DNA of the isolate SPA was used for amplification of 16S rRNA gene using the universal 16S rDNA primers, forward primer BG395F (Sequence

hidden) and reverse primer BG396R (Sequence hidden). PCR amplifications were conducted in a solution containing ~10ng genomic DNA, 1µl dNTP mix (2.5 mM each), 100 ng each of forward and reverse primer, 1X Taq DNA polymerase assay buffer (10X), 3U Taq DNA polymerase enzyme (Bangalore Genei Ltd., Bangalore, India) and distilled water enough to make up the volume 50 µl. Amplification conditions were: 5 min initial denaturation at 94°C, 35 cycles of 30s denaturation at 94°C, 30s of primer annealing at 54°C, 1 min elongation at 72°C and a final extension of 10 min at 72°C to finalize the reaction. The reactions were carried out in a Thermal Cycler (Applied Biosystems, model ABI 2720).

2.2.8.3 Electrophoresis

Fifteen µl of PCR amplification product was electrophoresed on a 1% (w/v) agarose gel (low electro-endosmosis (EEO) in 1X TBE buffer (45 mM Tris-borate, pH 8.3 and 1 mM disodium EDTA) at 100 V for 2h. The gel was stained with Ethidium bromide in a final concentration of 0.5 µg/ml, visualized and photographed under UV illumination. A low range DNA ladder (1 Kb plus) (Bangalore genei Pvt Ltd, India) was used as a molecular weight marker.

2.2.8.4 Sequencing of the 16S rRNA gene

16S rRNA gene from the isolate SPA was amplified as mentioned above. A single 1,491bp band as an amplification product was eluted and purified from the gel slice using using the GeneiPure^{TM} Gel Extraction Kit (QIAGEN) and sequenced using an automated genetic analyser (Applied Biosystems, model ABI 3100) which uses the sequencing analysis software v.5.1 with the kb basecaller (Bafana *et al.*,

2009). The 16S rRNA sequencing was done using ABI's AmpliTaq Fluoroscent dye terminator cycle based on Sanger's method.

2.2.8.5 Sequence alignment of the 16S rRNA gene sequence

The 16S rRNA sequence of the isolate SPA was compared with about 1000 homologous sequences taken from the National Centre for Biotechnological Information (NCBI) genbank and Ribosomal Database Project (RDP) (Larsen *et al.*, 1993). Multiple sequence alignment of the sequence was conducted by ClustalW algorithm. Phylogenetic analysis was performed by applying distance matrix method.

2.3 Results and Discussion

2.3.1 Selection of the isolate SPA

After 48h incubation of the soil isolates on modified MRS agar medium (Goyal and Katiyar, 1996) and observation under the colony counter, a total of 46 colonies assumed to be lactic acid bacteria were picked up for their enzyme assay. Among them, six colonies showing higher enzyme activity were selected for further study. They were designated as S2, S6, S10, S12, S14 and S29 and their enzyme activity profile was presented in Table 2.3.1. Among the 6 isolates, S14 showed comparatively higher enzyme activity. S14 was renamed as SPA after the the author's and place's name. This isolate SPA was used for further characterization and study.

Table 2.3.1 Enzyme activities of the 6 selected isolates

Isolates	Enzyme activity (U/ml)
S2	2.7
S6	2.9
S10	2.4

S12	2.9
S14*	3.1
S29	2.2

*SP14 was designated as SPA for convenience of reporting.

2.3.2 Morphological and biochemical characterization

The appearance of purple colour of cells after Gram staining, confirmed the Gram positive nature of the isolate SPA, a characteristic of lactic acid bacteria (Fig. 2.3.1). The morphology of cells observed by scanning electron microscopy revealed coccus shape and random arrangement in groups or chains (Fig. 2.3.2).

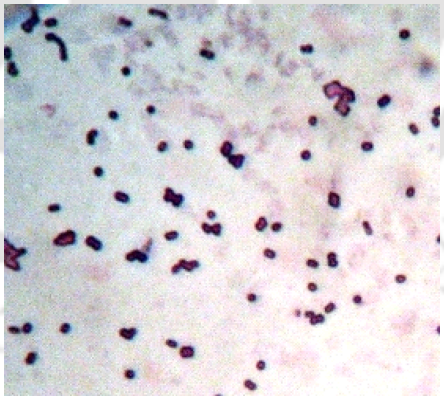


Fig. 2.3.1 Gram staining of the isolate SPA showing purple colour, indicating its Gram positive nature

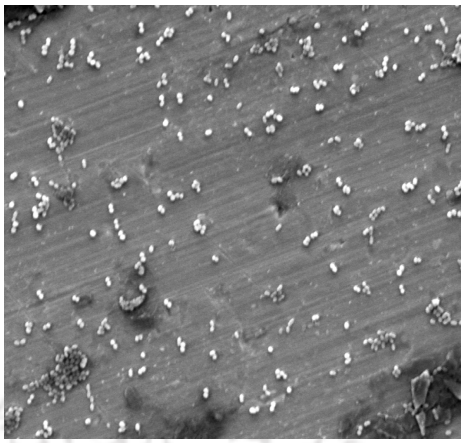


Fig. 2.3.2 Scanning Electron Microscopy of the isolate SPA showing its coccus shape and random arrangement

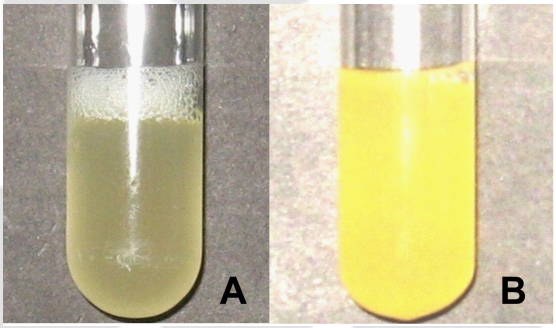


Fig. 2.3.3 Catalase activity test of the isolate SPA showing its catalase negative nature. *E.coli* (A) and SPA (B)

The catalase activity test proved the catalase negative nature of the isolate as it could not hydrolyse H_2O_2, whereas, *E. coli* hydrolysed it, as vigorous bubbling was observed (Fig. 2.3.3, A and B). The initial pH of 3.3 and 9.6 of the enzyme production medium of Tsuchiya *et al.* (1952) did not support the growth of the isolate. The colonies measured were 1-2 mm in diameter, white, smooth, circular and slimy when grown on modified MRS agar at 28°C for 24h. If the strains are catalase negative, gram-positive and cocci shaped they can be identified as lactic acid bacteria (Lin *et al.*, 2006).

2.3.3 Optimization of the growth conditions

The enzyme activity exhibited by the isolate at 20°C, 22°C, 25°C, 28°C and 30°C were 2.5 U/ml, 2.9 U/ml, 3.4 U/ml, 3.1 U/ml and 2.6 U/ml, respectively (Fig 2.3.4). The enzyme activity of the isolate SPA was maximum at 25°C incubation temperature, it was 17% higher than that at 22°C and 10% higher than that at 28°C. This result established the mesophilic nature of the isolate SPA (Fig. 2.3.4.1).

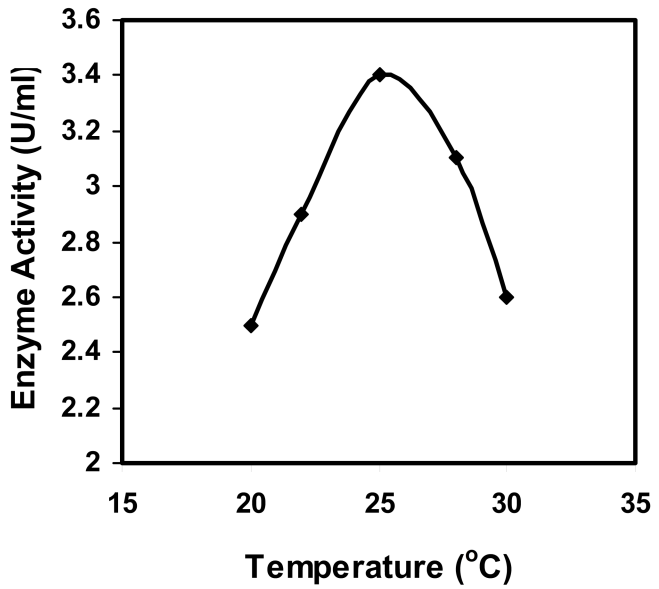


Fig. 2.3.4.1 Effect of temperature on enzyme activity during growth of isolate SPA

The enzyme activity exhibited by the isolate SPA in static condition was 2.9 U/ml, whereas it was 3.4 U/ml in shaking condition, showing a 17% enhancement in activity (Fig. 2.3.4.2). This observation explains the favourable effect of shaking condition on enzyme activity and establishes the microaerophilic nature of the isolate SPA.

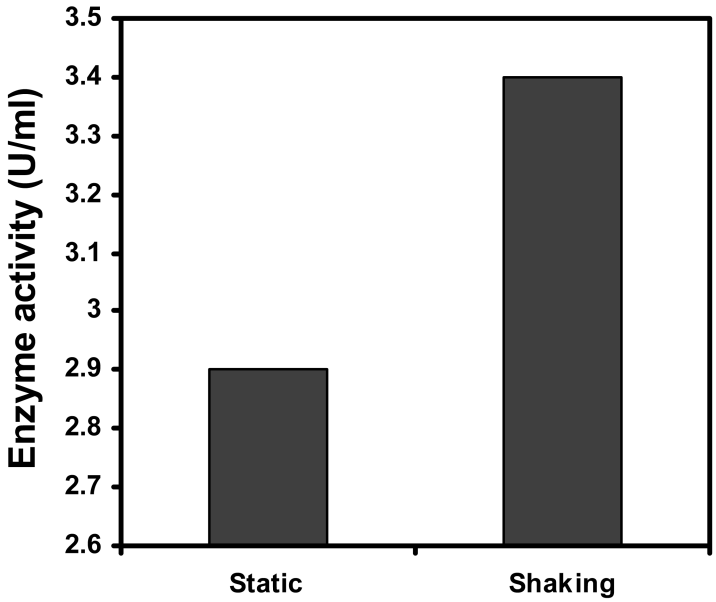


Fig. 2.3.4.2 Effect of shaking flask culture on enzyme activity during growth of isolate SPA

2.3.4 Growth profile study

The cell density of the isolate SPA measured at 600 nm by a spectrophotometer showed the peak at 16h of incubation (Fig. 2.3.5). The pH of the growth medium fell from 6.9 to 4.2, after 32h of incubation. Dextransucrase activity of the isolate SPA was highest at 16h. From these results, it was inferred that the enzyme activity is growth associated (Santos *et al.*, 2000). The lactic acid production led to pH fall, which inactivated the enzyme after 16h (Fig. 2.3.5).

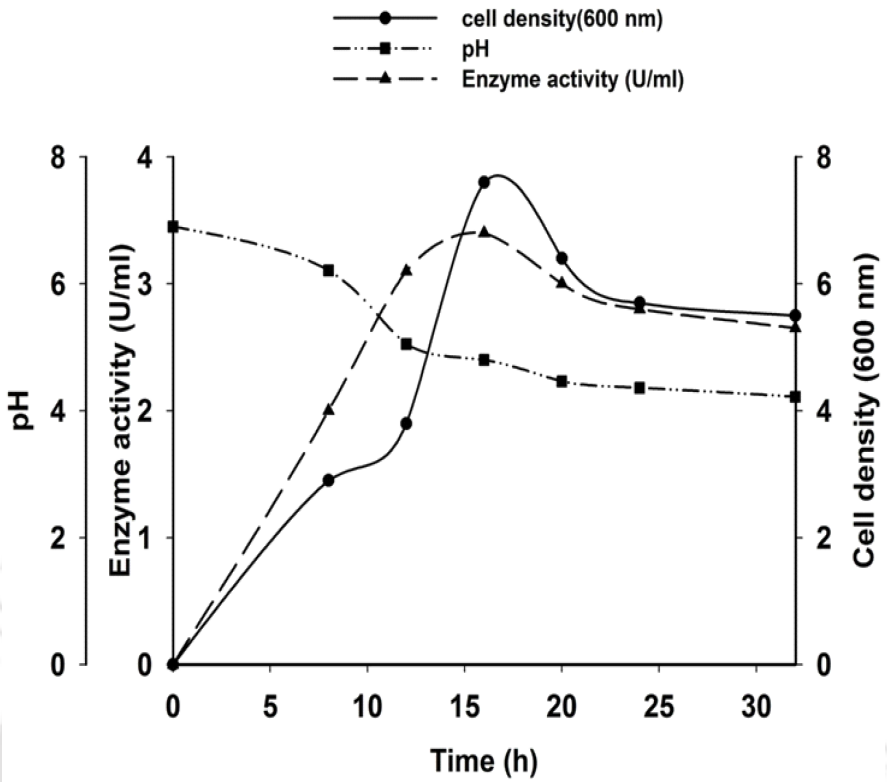


Fig. 2.3.5 The cell growth, pH and enzyme activity profile of the isolate SPA at 25°C under shaking condition.

2.3.5 Antibiotic susceptibility

In order to elucidate the antibiotic susceptibility of the isolate SPA, a standardized filter-paper disc-agar diffusion assay was carried out. This method determines the efficacy of the drug by measuring the diameter of the zone of inhibition which results from diffusion of the antibiotic from the disc into the medium. In this procedure, the filter-paper disc impregnated with specified concentrations of different antibiotics was placed centrally, on the surface of an agar plate seeded with the isolate SPA. After the overnight incubation at 28°C, the plates were examined for the zone of inhibition, surrounding the discs. The susceptibility of microorganism to a drug is determined by the size of this zone. A measurement of the

length of the zone of inhibition was made. Based on this comparison, the test organism is generally classified to be resistant, moderate or susceptible to the antibiotic. The isolate SPA was tested for susceptibility to thirty antibiotics belonging to tetracycline, glycopeptide, aminoglycoside and quinolone class. The isolate SPA was resistant to norflaxacin, ampicillin, amikcin, vancomycin, kanamycin, tobramycin, ciproflaxacin, claxacillin, co-trimize, gentamicin, cephaloridine and was sensitive to amoxycillin, carbenicillin, cephalothin, cephatoxamine, chlorampenicol, clindamycin, linomycin, oxytetracyclin, methicillin, oleandomycin, tetracycline (Fig. 2.3.6). The *Lactobacillus*, *Leuconostoc* and *Pediococcus* genera of lactic acid bacteria are intrinsically resistant to glycopeptide antibiotic vancomycin (Barton *et al.*, 2001).

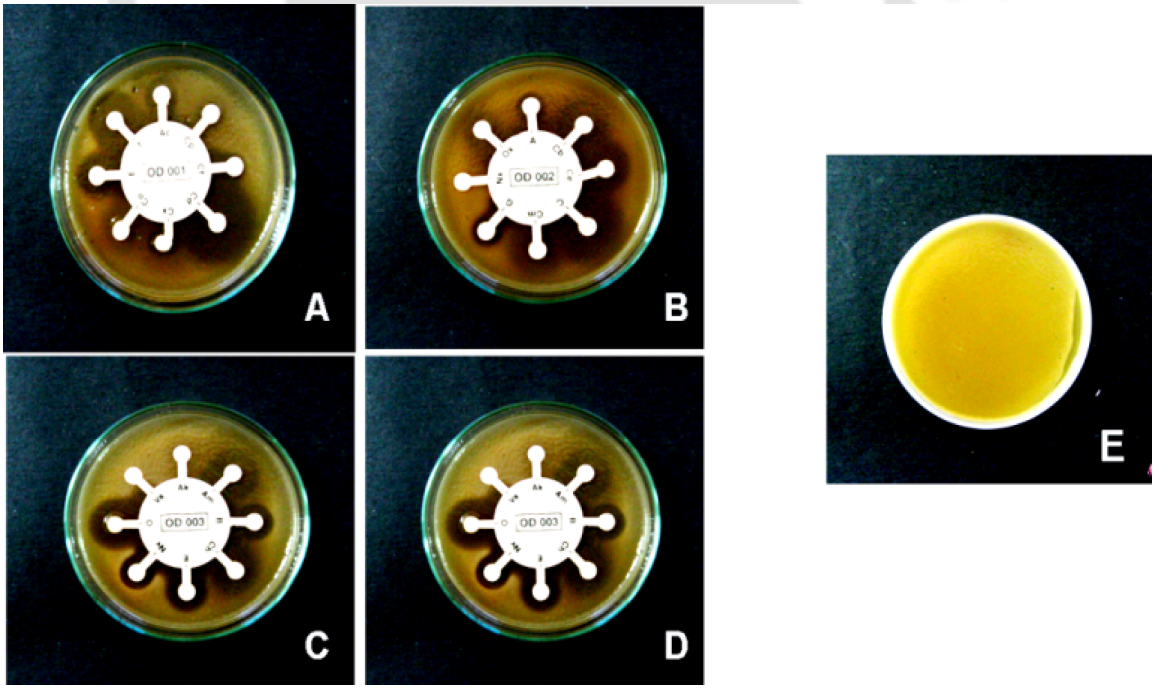


Fig. 2.3.6 Antibiogram of the isolate SPA using antibiotic octodiscs on MRS agar. Antibiotic treated culture of the isolate SPA (A-D); Control culture of the isolate SPA (E)

Table 2.3.1 Antibiogram of the isolate SPA using antibiotic octodiscs on MRS agar

S. No	Antibiotic	Concentration (mg)	Isolate SPA
1	Amoxyclav	10	M
2	Cephalexin	10	M
3	Ciproflaxacin	10	R
4	Claxaciln	1	R
5	Clindamycin	2	M
6	Erythromycin	15	S
7	Tetracyclin	30	S
8	Chloramphenicol	30	S
9	Co-Trimazine	25	R
10	Gentamicin	10	R
11	Norfloxacin	10	R
12	Oxacillin	5	M
13	Ampicilin	10	R
14	Carbenicillin	100	S
15	Cephatoxamine	30	M
16	Amikacin	10	R
17	Amoxycillin	10	S
18	Bacitracin	10 Units	M
19	Cephalothin	30	M
20	Novobiocin	30	M
21	Oxytetracyclin	30	S
22	Vancomycin	30	R
23	Penicillin-G	10 Units	S
24	Tobramycin	10	R
25	Cephaloridine	30	R
26	Kanamycin	30	R
27	Linomycin	2	S
28	Methicillin	5	S
29	Oleandomycin	15	S
30	Norflaxacin	10	R

Resistant (0-2 mm[a]); M- Moderate (3-6 mm[a]); S- Sensitive (7-13 mm[a])
[a]Values in millimetres are the distance of zone of inhibition of growth of microorganisms.

2.3.6 Carbohydrate fermentation pattern

The ability of the isolate SPA to degrade and ferment carbohydrates with the production of acid was tested. The isolate SPA utilized cellobiose, dextrose, fructose, galactose, maltose, melibiose, sucrose, trehalose as colour changed to yellow (Fig. 2.7). The isolate showed weak fermentation towards lactose, rhamnose, xylose and was unable to ferment mannitol, raffinose and glycerol (Fig. 2.3.7). The extent of fermentation of carbohydrates was categorized as shown in Table 2.3.2.

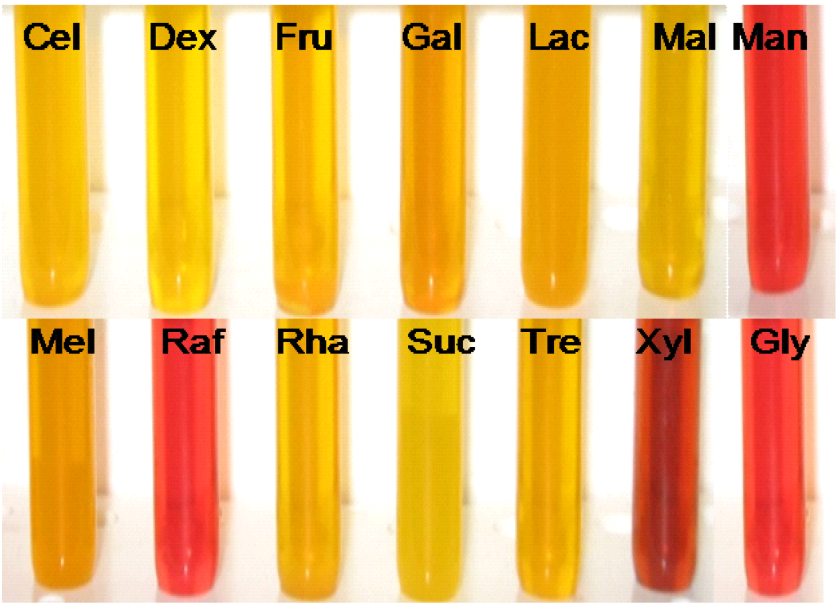


Fig. 2.3.7 The Carbohydrate fermentation pattern of the isolate SPA using 14 sugars. The change of colour from red to yellow indicates sugar fermentation

Table 2.3.2 Carbohydrate fermentation of the isolate SPA after 24h incubation

S. No.	Carbohydrates	SPA
1.	Cellobiose	+++
2.	Dextrose	+++
3.	Fructose	+++
4.	Galactose	+++
5.	Lactose	++
6.	Maltose	+++
7.	Mannitol	-
8.	Melibiose	+++
9.	Raffinose	-
10.	Rhamnose	++
11.	Sucrose	+++
12.	Trehalose	+++
13.	Xylose	+
14.	Glycrol	-

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

2.3.7 16S rRNA gene sequence data analysis

16S rRNA gene sequence analysis after PCR amplification was performed for identifying the isolate SPA. Profiles obtained by PCR amplification allowed identification of the isolate at both genus and species level. The 1,491bp 16S rRNA amplicon obtained by Polymerase Chain Reaction was electrophoretically resolved on a 1% agarose gel (Fig 2.3.8).

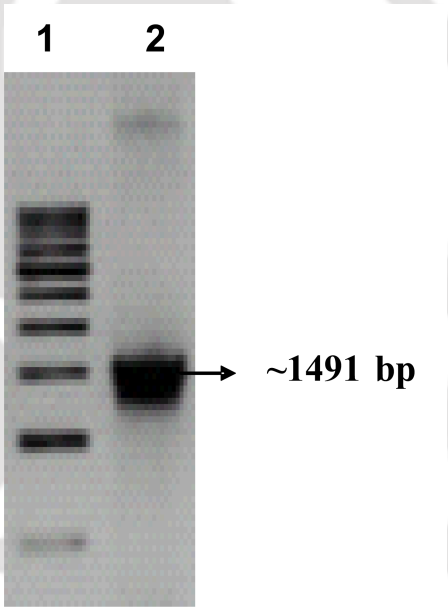


Fig. 2.3.8 Full length 16s rRNA gene (1491 bp) of the isolate SPA amplified with universal primers. The amplicon was electrophoretically resolved on a 1% agarose gel in 1X TBE buffer (A) Lane1: 1 Kb plus. DNA ladder (B) Lane 2: Amplified product of full length 16s rRNA gene

The full length sequencing of 1491bp 16S rRNA gene was conducted by Sanger's method and the sequence data used for alignment was presented (Fig. 2.3.9).

TGATGACGCTGGCGGCGTGCCTATTACATGCAAGTCGAACGAACTTCCGTTAATTGATTATGACGTACTTGTACTGATTGAGATTT
TAACACGAAGTGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGATAACACCTGGAAACAGATGCTAAT
ACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAG
TTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCG
GCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTTAGCGAGCGCAGGCGGTCTTTTAA
GTCTAATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCAT
GTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGGCTCGAAA
GCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAG
TGCTGCAGCTAACGCATTAAGTAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATTGACGGGGGCCCGCACAAT
CGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGACAGTCTAAGAGATTATAGGTT
CCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATTACTAGTTGCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAATGTGGGGACGACGTCAAA
TCATCATGCCCCTTATGACTTGGGCTATCACGTGCTACGATGGATGGTACAACGAGTCGCGAGACGCGAGATTAGCTAATCTCTTA
AAAACATTCTCAGTTCGGACTGCAGGTGCACTCCCTAAACGAAGTCGGATCGCGTAGTAACGGGGATCACATGCCCCGTTGATACC
TTCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAACCTTTTAGGAGCTAGCCGT
CTAAGGTGGGACAGAGATAGTGTTTCTGG

Fig. 2.3.9 16S rRNA gene sequence (1491 bp full length) of the isolate SPA

The 16S rRNA sequence of the isolate SPA was compared with 16S rRNA sequences from other reference bacteria obtained from National Centre for Biotechnological Information (NCBI) genbank (http://www.ncbi.nlm.nih.gov) and Ribosomal Database Project (RDP). Similarity searches were carried out using the BLAST algorithms available at (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments comparing the sequences were performed using ClustalW (http://www.ebi.ac.uk/clustalw/) (Parveen *et al.*, 2006). The alignment was checked visually and corrected manually using the sequence editor. Sequence similarity values were calculated by comparing nucleotides at the corresponding positions (Table 2.3.3). The homology in sequences helps to find common ancestry.

Table 2.3.3 Alignment results using combination of NCBI GenBank and RDP database

Identity	Alignment Results	Sequence Description
Isolate SPA	0.98	Studied sample
EU157914	0.94	*Pediococcus sp.*strain:MMZ60A
DQ267152	0.94	*Pediococcus pentosaceus*
EU569832	0.96	*Pediococcus pentosaceus* strain:KC007
FJ844982	0.97	*Pediococcus acidilactici* strain:IMAU20070
EU147312	0.96	*Pediococcus acidilactici* strain:BFE 8384
FJ405228	0.83	*Pediococcus claussenii strain* ZJ5
EU331259	0.98	*Pediococcus parvulus* strain Bpe301
FJ867642	0.91	*Pediococcus ethanolidurans* strain P2
AY956788	0.89	*Pediococcus cellicola* strain Bpe260
EU331313	0.95	*Pediococcus damnosus* strain Bpe260

After the alignment the sequences were subjected to Distance matrix based on nucleotide sequence homology using the Kimura 2 parameter (Casanellas and

Fernandez-Sanchez, 2008). Pairwise distance between and within the genotypes at the nucleotide levels were calculated with Kimura 2 parameters. Kimura 2 parameter is used to correct the rates of transition and transversion, to remove the base bias. Substitutions in base occurs randomly and the transitions (C to T, or A to G) are more frequent than transversions (A to T and C to G). This is the method of distance corrections in a phylogenetic analysis. The last step followed for identification was the phylogenetic tree based on Nearest Neighbour joining method, which actually identified the homology of the organism. Trees were drawn, the distance matrix for which was generated by MEGA 3.1 software (Kumar *et al.*, 2004) (Fig. 2.3.10). The stability of branching pattern and the statistical significance of the tree topology was confirmed by bootstrapping (Bafana *et al.*, 2009). Bootstrap values indicate the number of times a node was supported in 1000 sampling replications (Zhang and Zhirong, 2008). A cut-off of 97–98% similarity in 16S rRNA sequence was recommended as a criterion for demarcating species (Ward *et al.*, 2008). Based on the sequence similarity, the isolate was assigned accession number.

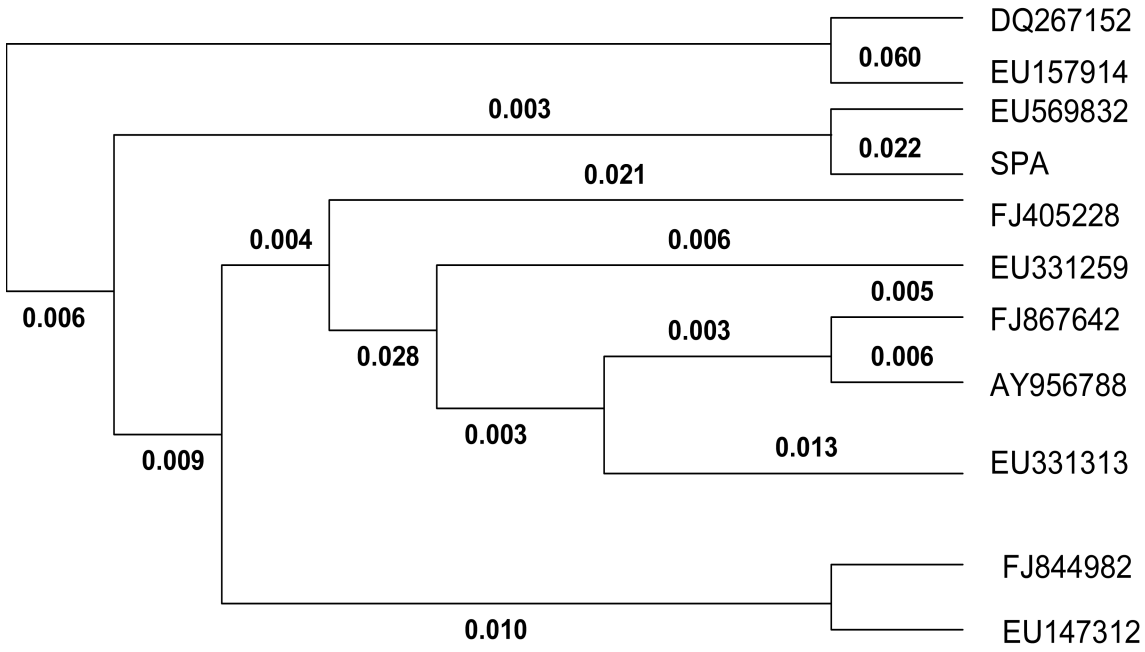


Fig. 2.3.10 Phylogenetic Tree made in MEGA 3.1 software using Neighbour joining method. Reference strains from NCBI database found as nearest homologs are indicated on the right side of the figure.

The results from phylogenetic analysis consistently resulted in the isolate SPA to be *Pediococcus pentosaceus* (Genbank Accession Number EU569832) (Fig 2.3.10). Distance matrix based on Nucleotide sequence homology (using Kimura-2 parameter) corroborated the above finding (Table 2.3.4).

Table 2.3.4 Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 parameter)

		1	2	3	4	5	6	7	8	9	10	11
EU569832	1	-	0.959	0.995	0.953	0.976	0.951	0.995	0.949	0.936	0.975	0.978
FJ405228	2	0.041	-	0.963	0.942	0.969	0.932	0.963	0.938	0.941	0.968	0.934
DQ267152	3	0.005	0.037	-	0.948	0.978	0.945	0.999	0.945	0.941	0.977	0.972
EU331259	4	0.047	0.058	0.052	-	0.941	0.985	0.948	0.982	0.979	0.941	0.930
FJ844982	5	0.025	0.031	0.022	0.059	-	0.940	0.979	0.940	0.942	0.999	0.952
FJ867642	6	0.049	0.068	0.055	0.015	0.060	-	0.945	0.988	0.979	0.941	0.928
EU157914	7	0.005	0.037	0.002	0.052	0.021	0.055	-	0.944	0.940	0.979	0.972
AY956788	8	0.051	0.062	0.055	0.018	0.060	0.013	0.056	-	0.975	0.941	0.926
EU331313	9	0.064	0.059	0.059	0.021	0.059	0.021	0.060	0.025	-	0.942	0.912
EU147312	10	0.025	0.032	0.023	0.059	0.001	0.059	0.021	0.059	0.058	-	0.951
Isolate SPA	11	0.022	0.066	0.028	0.070	0.048	0.072	0.028	0.074	0.087	0.049	-

The isolate SPA and the strain *Pediococcus pentosaceus* KC007 (Genbank Accession Number EU569832) clustered together showing 96% similarity. Hence the isolate was assigned the Genbank Accession Number EU569832 (Table 2.3.4). The closest homolog of the isolate was found to be *Pediococcus sp.* MMZ60A (EU157914). Other close homologs of the isolate SPA clustering separately are *Pediococcus pentosaceus* (DQ267152), *Pediococcus calssenii* ZJ5 (FJ405228), *Pediococcus parvulus* Bpe301 (EU331259), *Pediococcus ethnolidurans* P2 (FJ867642), *Pediococcus cellicola* Z-8 (AY956788), *Pediococcus damnosus* Bpe260 (EU331313). This isolate is farthest from the strain *Pediococcus acidilactici* IMAU20070 (FJ844982) and *Pediococcus acidilactici* BFE 8384 (EU147312) (Fig 2.3.10). Phylogenetic analysis of the complete 16S rRNA dataset resulted in trees with much greater resolution and well supported with high bootstrap pseudoreplicate score (1000 pseudoreplicates).

2.4 Conclusions

In light of the biochemical and physiological properties of the isolate SPA, its suitability for exploitation can be evaluated. The morphological studies, antibiotic resistance and carbohydrate fermentation profiles are important for understanding the characteristics of this industrially potential isolate. The isolate SPA was Gram positive, coccus shaped and did not possess catalase activity. The isolate was resistant to the antibiotics norfloxacin, ampicillin, amikcin, vancomycin, kanamycin, tobramycin, cephaloridine and norflaxacin. Resistance to vancomycin is shown by *Lactobacillus*, *Leuconostoc* and *Pediococcus* genera of lactic acid bacteria. The isolate was found sensitive to amoxycillin, carbenicillin, cephalothin, cephatoxamine, chlorampenicol, clindamycin, linomycin, oxytetracyclin, methicillin, oleandomycin

and tetracyclin. The isolate utilized cellobiose, dextrose, fructose, galactose, maltose, mellibiose, sucrose and trehalose. Rhamnose, lactose and xylose were partially fermented by the isolate. The isolate could not ferment mannitol, raffinose and glycerol.

The 16S rRNA gene sequence analysis revealed the identity of the isolate SPA which was found to be *Pediococcus pentosaceus* (Genbank Accession Number EU569832). The genetic relationships of this isolate with its neighbours were traced and a phylogenetic tree was constructed. These 16S rDNA based methodology is robust and superior over conventional identification methods based on phenotypic approaches, which are often ambiguous and unreliable. 16S rRNA sequence based PCR amplification method offers a viable option for the rapid and reliable identification of lactic acid bacteria. This study, revealed the dextransucrase production attribute of *Pediococcus* genus of lactic acid bacteria for the first time ever, heralding further rigorous investigation on this aspect. However, it is too early to claim the enzyme secreted by the isolate SPA as dextransuacrase. Further characterization of this enzyme is required to support this finding. This discovery is expected to be a major break-through in the history of dextran production. This investigation also unraveled the abundance of industrially valuable microbial flora in soil. Screening of the microbial biodiversity enriched North Eastern region of India needs proper attention.

The pursuit of novel discovery strategies targeting new dimensions of molecular diversity will certainly be the major field of development in next few years. The meteoric rise in the usage of dextrans in food, pharmaceutical and cosmetics

industries emphasizes the importance of exploration of the new strains and characterization of their traits.

References

Amann, R. I., Ludwig, W. and Schleifer, K. H. (1995) Phylogenetic identification and in situ detection of individual microbial cells with-out cultivation. Microbiol. Rev. 59, 143-169.

Bafana, A., Chakrabarti, T., Krishnamurthi, K. and Devi, S. S. (2009) Biodiversity and dye decolourization ability of an acclimatized textile sludge. Bioresour. Technol. 99, 5094-5098.

Barry, A. L. and Thornsberry, C. (1980) Susceptibility testing: diffusion procedures. In: Manual of Clinical Microbiology (Lennette, E. H., Balows, A., Hausler, W. J. Jr., Truant, J. P., ed). Washington, D.C: American Society for Microbiology, pp 463-74.

Barton, L. L., Rider, E. D. and Coen, R. W. (2001) Bacteremic infection with *Pediococcus*: vancomycin-resistant opportunist. Pediatrics. 107, 775-776.

Bhatnagar, R., Bhatnagar, N. and Prabhu, K. A. (1985) Extracellular dextransucrase from a new strain of *Leuconostoc mesenteroides*. IRCS Med. Sci. 13, 129-130.

Casanellas, M. and Fernandez-Sanchez, J. (2008) Geometry of the Kimura 3-parameter model. Adv. Appl. Math. 41, 265-292.

Cohan, F. M. and Perry, E. B. (2007) A systematics for discovering the fundamental units of bacterial diversity. Curr. Biol. 17, 373-386.

Escalante, A., Wacher, C. and Farres, A. (2001) Lactic acid bacterial diversity in the traditional Mexican fermented dough pozol as determined by 16S rDNA sequence analysis. Int. J. Food Microbiol. 64, 21-31.

Goyal, A. and Katiyar S. S. (1996) Regulation of dextransucrase productivity of *Leuconostoc mesenteroides* B-512F by the maintenance media. J. Gen. Appl. Microbiol. 42, 81-85.

Gupta, R., Beg, Q. K and Lorenz, P. (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 59, 15-32.

Johnson, T. R. and Case, C. L. (1995) Laboratory Experiments in Microbiology, Fourth ed. The Benjamin Cummings Publishing Co. Inc., Mealopark, CA, USA.

Kandler, O. and Weiss, N. (1986) Regular, nonsporing gram-positive rods. In: *Bergey's Manual of Systematic Bacteriology* (Sneath, P. H. A, Mair, N. S., Sharpe, M. E., Holt, J. G., ed). Baltimore: Williams and Wilkins, pp 1208-1219.

Kannan, N. (2002) Laboratory Manuals in General Microbiology. Panima Publishing Corporation, New Delhi, India.

Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5,150-163.

Larsen, N., Olsen, G. J., Maidak, B. L., McCaughey, M. J., Overbeek, R., Macke, T. J., Marsh, T. L. and Woese, C. R. (1993) The ribosomal database project. Nucleic Acids Res. 21, 3021-3023.

Lin, W-H, Hwang, C-F, Chen, L-W and Tsen, H-Y (2006) Viable counts, characteristic evaluation for commercial lactic acid bacteria products. Food Control. 23, 74-81.

Manes-Laazaro, R., Ferrer, S., Rossello-Mora, R. and Pardo, I. (2008) *Lactobacillus uvarum* sp. nov.-A new lactic acid bacterium isolated from Spanish Bobal grape musts. Syst. Appl. Microbiol. 31, 425-433.

Mathara, J. M., Schillinger, U., Kutima, P. M., Mbugua, S. K. and Holzapfel, W. H. (2004) Isolation, identification and characterisation of the dominant microorganisms of kule naoto: the Maasai traditional fermented milk in Kenya. Int. J. Food Microbiol. 94, 269-278.

Milliere, J.B., Mathot A-G, Schmitt, P and Divies, C. (1989) Phenotypic characterization of *Leuconostoc* species. J. Appl. Bacteriol., 67, 529-542.

Morris, C. E, Bardin, M., Berge, O., Frey-Klett, P., Fromin N., Girardin, H., Guinebretiere, M-H, Lebaron, P. Thiery, J. M. and Troussellier M. (2002) Microbial Biodiversity: Approaches to Experimental Design and Hypothesis Testing in Primary Scientific Literature from 1975 to 1999. Microbiol. Mol. Biol.Rev. 17, 242-249.

Nelson, N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153, 375-380.

Parveen, S., Sullender, W. M., Fowler, K., Lefkowitz, E. J., Kapoor, S. K. and Broor, S. (2006) Genetic variability in the G Protein gene of group A and B respiratory syncytial viruses from India. J. Clin. Mcrobiol. 44, 3055-3064.

Patel, S. and Goyal A. (2010) Isolation, characterization and mutagenesis of exopolysaccharide synthesizing new strains of lactic acid bacteria. Int. J. Microbiol. (in press).

Purama, R. K, Agrawal, M., Majumder, A., Ahmed, S. and Goyal, A. (2008) Antibiotic sensitivity, carbohydrate fermentation characteristics and plasmid profiles of glucansucrase producing four *Leuconostoc* strains. J. Pure Appl. Microbiol. 2, 139-146.

Rondon, M. R., August, P. R., Bettermann, A. D., Brady, S. F., Grossman, T. H., Liles, M. R., Loiacono, K. A., Lynch, B. A., MacNeil, I. A., Minor, C., Tiong, C. L., Gilman, M., Osburne, M. S., Clardy, J., Handelsman, J. and Goodman, R. M. (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of un-cultured microorganisms. Appl. Environ. Microbiol. 66, 2541-2547.

Rossello-Mora, R. and Amann, R. (2001) The species concept for prokaryotes. FEMS Microbiol. Rev. 25, 39-67.

Santos, M., Teixeira, J. and Rodrigues, A. (2000) Production of dextransucrase, dextran and fructose from sucrose using *Leuconostoc mesenteroides* NRRL B512F. Biochem Eng. J. 4, 177-188.

Somogyi, M. (1945) A new reagent for the determination of sugars. J. Biol. Chem. 160, 61-68.

Tsuchiya, H. M., Koepsell, H. J., Corman, J., Bryant, G., Bogard, M. O., Feger, V. H. and Jackson, R. W. (1952). The effect of certain cultural factors on production of dextransucrase by *Leuconostoc mesenteroides*. J. Bacteriol. 64, 521-526.

Ward, D. M., Cohan, F. M., Bhaya, D., Heidelberg, J. F., Kuhl, M. and Grossman, A. (2008) Genomics, environmental genomics and the issue of microbial species. Heredity. 100, 207-219.

Zhang, W. and Zhirong, S. (2008) Random local neighbor joining: A new method for reconstructing phylogenetic trees. Mol. Phylogenet. Evol. 47, 117-128.

Chapter 3

Production, purification and functional characterization of dextransucrase from *Pediococcus pentosaceus* (SPA)

3.1 Introduction

Dextransucrase is an extracellular enzyme belonging to Glycoside hydrolase family 70 (Majumder *et al.*, 2007). This enzyme is generally elaborated by lactic acid bacteria *viz. Leuconostoc* and *Streptococcus* (Purama and Goyal, 2008). *Pediococcus* belonging to lactic acid bacteria family may also produce dextransucrase (Smitinont *et al.*, 1999). However, not much work has been done on the dextransucrase production aspect of *Pediococcus*. This enzyme is of high industrial importance due to its dextran biosynthesizing property. This enzyme, cleaves its substrate sucrose, to release free glucose and fructose. The free glucosyl moieties are polymerized to form the homopoysaccharide dextran. The dextran has numerous industrial and medical applications (Neubauer *et al.*, 2003). The purification methods such as salt, alcohol precipitation, fractionation by polyethylene glycol, ultra-filtration, chromatography and phase-partitioning have been successfully employed for purification of dextranucrase from different strains of lactic acid bacteria (Majumder *et al.*, 2007). Among all the reported purification methods, polyethylene glycol (PEG) is an

effective, rapid and single step purification method for dextransucrase from *Leuconostoc mesenteroides* (Purama and Goyal, 2008). Dextransucrase purification by this non-ionic hydrophilic polymer polyethylene glycol has been found reasonably effective. Literature studies reveal suitability of polyethylene glycol (PEG) for selective precipitation of high molecular weight or aggregated form of proteins (Goyal and Katiyar, 1994). Dextransucrases are all phylogenetically closely related and are large proteins with an average molecular mass of 160 kDa (Neubauer *et al.*, 2003). The dextransucrase from *L. mesenteroides* NRRL B-512F was purifed by fractionation with PEG of different molecular weights (Goyal and Katiyar, 1994). Remaud-Simeon *et al.* (1991) purified dextransucrase from *L. mesenteroides* B-742 with an activity of 83 U/mg protein using PEG-1500. The extracellular dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 was purified using polyethylene glycol (PEG-400) fractionation giving specific activity of 9.2 U/mg in a single step (Purama and Goyal, 2008). The dextransucrase from *Leuconostoc dextranicum* NRRL B-1146 was purified using the same protocol which gave specific activity of 4.5 U/mg (Majumder *et al.*, 2008).

It has been reported that dextransucrase exists in either single or multiple forms having molecular weights in the range of 64-245 kDa (Willemot *et al.*, 1988). The enzyme remains in an aggregated form in the presence of dextran resulting in a high molecular weight. SDS-PAGE has been used for molecular weight determination of dextransucrase of two major forms of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. The approximate molecular weights found on SDS-PAGE were 177 kDa and 158 kDa (Miller and Robyt, 1986a). However, multiple dextransucrase forms of different molecular masses were observed in

SDS-PAGE analysis of the same dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F (Kobayashi and Matsuda, 1986; Goyal and Katiyar, 1994; Funane *et al.*, 1995; Quirasco *et al.*, 1999). Goyal and Katiyar (1994) reported 188 kDa for dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Quirasco *et al.* (1999) characterized four bands of 170, 160, 116 and 97 kDa with polymerizing activity after SDS-PAGE. Whereas, the 170 kDa protein corresponded to the known dextransucrase of *Leuconostoc mesenteroides* NRRL B-512F, the 160 kDa band was suggested to be a proteolytic degradation derivative of the original 170 kDa protein (Sanchez-Gonzalez *et al.*, 1999). The purified dextransucrase of *Leuconostoc mesenteroides* B-512FMC had a molecular size of 184 kDa on SDS-PAGE (Kim and Robyt, 1994). On heat denaturation at 100°C, two protein bands of 63 and 59 kDa were obtained on SDS-PAGE, supposed to be from 184 kDa active enzyme (Kim and Robyt, 1994). The PEG-400 purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 showed multiple protein bands on SDS-PAGE with a prominent band corresponding to the size 180 kDa (Purama and Goyal, 2009a). However, the same enzyme sample showed a single band on non-denaturing native-PAGE analysis (Purama and Goyal, 2009). This result confirmed the multimeric entity of this dextransucrase which remains in single molecular form in the native state and separates into multiple bands under denaturing milieu of boiling, SDS and 2-mercaptoethanol.

The presence of glucansucrase was identified and its glucan synthesizing activity was confirmed by activity staining bands of the glucan produced, on the non-denaturing PAGE incubated in sucrose (Majumder *et al.*, 2008). The purified

dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 confirmed *in-situ* dextran synthesis activity, when run on non-denaturing SDS-PAGE and the gel was subjected to Periodic Acid Schiff staining. The single magenta colour activity band corresponded to the native form of the purified dextransucrase of approximately, 180 kDa (Purama and Goyal, 2008).

The dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F exhibited maximum activity at 30°C temperature (Goyal *et al.*, 1995). *Leuconostoc mesenteroides* NRRL B-640 exhibited maximum dextransucrase activity at 30°C temperature and buffer pH 5.4 (Purama and Goyal, 2009b). Glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 showed highest activity at 10% sucrose, 30°C and 10-50 mM buffer of pH 5.4 (Majumder *et al.*, 2008).

The metal ions, Ca^{2+}, Mg^{2+}, Fe^{2+}, and Co^{2+} stimulated the activity of extracellular dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299 (Kobayashi and Matsuda, 1976). The specific activity of the dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F was greatly enhanced by $CaCl_2$ addition to the purified enzyme (Lopez and Monsan, 1980; Goyal *et al.*, 1995). The glucansucrase activity of *Leuconostoc dextranicum* NRRL B-1146 was enhanced by divalent cations (Ca^{2+}, Mg^{2+}, and Co^{2+}) and the Ca^{2+} ion showed enhancement in glucansucrase activity by 3 fold, whereas Mg^{2+} exhibited only a marginal increase in the glucansucrase activity (Majumder *et al.*, 2008). The association of alkaline earth metals with glucansucrases can be deduced from these findings (Robyt and Walseth, 1979).

Urea displayed deactivating effect on glucansucrase of *Leuconostoc dextranicum* B-1146 at concentrations ranging from 1-5 M (Majumder *et al.*, 2007). EDTA being a chelating agent, caused potent inactivation of the dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299 (Kobayashi and Matsuda, 1976). The addition of 0.8 mM EDTA led to 50% loss of glucansucrase activity of *Leuconostoc dextranicum* NRRL B-1146 and 90% of the glucansucrase activity was lost at 2 mM EDTA.

Dextransucrase is capable of acceptor reactions, but only a few of these reactions involving non-conventional acceptors have been successful. The low water solubility of the potential acceptors (e.g alkylglycoside and arylglycoside) in water is the hurdle. The use of organic solvents could allow them to dissolve and facilitate interactions between the acceptors and enzymes (Girard and Legoy, 1999). Since the beginning of the 1980s, it has been clearly shown that enzymes can be used in organic solvents with great efficiency (Brink *et al.*, 1988). The ability of glucansucrases to catalyse the glucosylation in aqueous-organic solvents of biologically important non-water soluble compounds is a crucial challenge. Phenolic compounds flavonoids, despite their versatile potential, have limited practical applications due to poor water solubility. Thus, in order to improve the flavonoid hydrophilicity, enzymatic glucosylation has been reported on various (Sato *et al.*, 2000). The effect of organic solvents on the glucansucrase activity was investigated and observed that different organic solvents *viz.* dimethyl sulfoxide (DMSO) and Bis methoxyethyl ether (MEE inactivated dextransucrase to different extent (Bertrand *et al.*, 2006).

In the present study, purification of dextransucrase from the isolate *Pediococcus pentosaceus* was carried out by polyethylene glycol fractionation. The enzyme was identified and confirmed by Periodic Acid Schiff's (PAS) staining and

the approximate molecular weight was determined. The reaction conditions for the enzyme activity were optimized. The effects of various salts, chaotropic agents and organic solvents on the enzyme activity were studied.

3.2 Materials and methods

3.2.1 Screening and maintenance of the isolate *Pediococcus pentosaceus*

The isolate *Pediococcus pentosaceus* was screened from the soil sample collected from a sugarcane field of Assam (near Guwahati) as described in Section 2.2.2 of Chapter 2. This isolate was maintained as stab in modified MRS agar (containing 2% sucrose, w/v) (Goyal and Katiyar, 1996) at 4°C and sub-cultured every two weeks. For the development of inoculum, a loopful of culture from modified MRS agar stab was transferred to 5 ml of medium described by Tsuchiya *et al*. 1952. The isolate *Pediococcus pentosaceus* culture was incubated at 25°C at 180 rpm for 12h.

3.2.2 Production of dextransucrase from the isolate *Pediococcus pentosaceus*

1% of the broth was again inoculated in 100 ml enzyme production medium in a 250 ml Erlenmeyer flask and incubated for 16h at 25°C under shaking conditions at 180 rpm. 1 ml sample was withdrawn and centrifuged at 10,000 rpm for 10 min at 4°C. The cell free supernatant was analyzed for enzyme activity and protein

concentration. All the experiments were performed in duplicates for accuracy of results.

3.2.3 Enzyme activity assay

The assay of dextransucrase was conducted in 1 ml of a reaction mixture containing 20 mM sodium acetate buffer (pH 5.4), 5% sucrose and 20 µl cell free supernatant as the enzyme source. The reaction mixture was incubated at 30°C for 15 min. The enzyme activity was analysed by estimating the reducing sugars by the Nelson-Somogyi protocol (Nelson, 1944; Somogyi, 1945). Aliquots (0.2 ml), from the reaction mixture were analyzed for reducing sugar. The absorbance was recorded at 500 nm in a UV-visible spectrometer (Varian, model Cary 100) against a blank. D-fructose was used as a standard.

The enzyme activity was calculated as follows:

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

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

3.2.4 Protein concentration estimation

The protein content of the cell free supernatant was estimated by the modified method of Lowry *et al.* (1951) using BSA as standard. To 200 µl of sample containing

10 µl cell free extract, 1 ml of reagent C was added. After 15 min incubation, 100 µl of Folin's reagent was added and the optical density was measured after 30 min at 750 nm in a UV-visible spectrometer (Varian, model Cary 100) against a blank. Bovine serum albumin (BSA) was used as standard. The specific activity (U/ mg) of the cell free supernatant was calculated.

The concentration of protein was calculated as follows:

$$\text{Protein Concentration (mg/ml)} = \frac{\Delta A_{750} \times C \times v}{V} = \text{(mg/ml)}$$

C = 1 OD equivalent of BSA from standard plot

ΔA_{750} = change in absorbance of the sample

V = volume of the sample

v = volume of the enzyme source (ml) for protein estimation

3.2.5 Purification of dextransucrase with polyethylene glycol

For purification of dextransucrase, the cell free supernatant was subjected to fractionation by different molecular weight of polyethylene glycol (Purama and Goyal, 2008). Different percentages of the ice cold polyethylene glycol of various molecular weights (PEG-200, PEG-400, PEG-1500) were added to 50 ml of cell free supernatant. PEG-200 and PEG-400 were used in the range of 25-50% and PEG-1500 was used in the range of 10-30%. They were incubated at 4°C for 12h to allow the dextransucrase to fractionate. The mixture was centrifuged at 13,200 rpm for 30 min at 4°C to separate the fractionated dextransucrase. The enzyme pellets obtained were dissolved in 1 ml of 20 mM sodium acetate buffer (pH 5.4). The enzyme samples were subjected to dialysis using 5 kDa cut off membrane. The partially purified enzyme samples were analyzed for enzyme activity and protein content. The purified enzyme was lyophilized and stored at -20^{o}C for further biochemical characterization.

3.2.6 SDS-denaturing PAGE of dextransucrase

SDS-denaturing polyacrylamide gel electrophoresis was performed following the method of Laemmli (1970). 7.5% (w/v) acrylamide for resolving gel and 4% (w/v) for stacking gel were used. The loading dye buffer contained 0.0625 M Tris-HCl buffer (pH 6.8), 2.3% (w/v) SDS, 10% (w/v) glycerol, 5% (w/v) β-mercaptothanol and 0.05% (w/v) bromophenol blue. The purified enzyme sample was mixed with 5X loading dye buffer in the ratio 4:1. The sample mixture was subjected to heat denaturation for 5 min and centrifuged for 1 min. The sample was loaded on 7.5% acrylamide gel and the electrophoresis was carried out using 1X running buffer (200 mM glycine, 0.1% SDS, 50 mM Tris-HCl pH 8.3) with a current of 2.5 mA per lane. The protein bands were fixed with Coomassie Brilliant Blue dye for 40 min and destained by repeated washing with a solution of methanol, acetic acid and water in ratio 40:10:50. Broad range protein marker (Myosin from Rabbit Muscle, 205000; Phosphorylase b 97400; Bovine serum albumin, 66000; Ovalbumin, 43000; Carbonic anhydrase, 29000 Da) purchased from Bangalore Genei, India, was used as standard.

3.2.7 Identification of dextransucrase by Periodic Acid Schiff Protocol

The dextran synthesizing activity of dextransucrase was detected by conducting SDS-nondenaturing PAGE on 7.5% gels (Holt *et al.*, 2001). Two gels were run simultaneously at identical conditions. The loading dye buffer contained 0.0625 M Tris-HCl buffer (pH 6.8), 2.3% (w/v) SDS, 10% (w/v) glycerol and 0.05% (w/v) bromophenol blue, but did not contain β-mercaptothanol. The enzyme sample was mixed with 5X sample buffer in the ratio 4:1. The heat denaturation step was

omitted. The sample was loaded on 7.5% acrylamide gel and the electrophoresis was carried out using 1X running buffer (200 mM glycine, 0.1% SDS, 50 mM Tris-HCl pH 8.3) with a current of 2.5 mA per lane.

After the run, one gel was stained with Coomassie Brilliant Blue dye for 40 min for fixing the protein bands and destained by repeated washing with a solution of methanol, acetic acid and water in ratio 40:10:50. Broad range protein marker (29-205 kDa) purchased from Bangalore Genei, India, were used as standard.

The other gel was subjected to SDS removal by incubating in sodium acetate buffer (20 mM sodium acetate, pH 5·4, 0·3 mM $CaCl_2$ and 0·1% Tween 80) at 4°C for 30 min. Then the gel was incubated in sodium acetate buffer (20 mM sodium acetate, pH 5·4, 0·3 mM $CaCl_2$) supplemented with 5% 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 composed of 0.5% (w/v) Fuchsin basic, 1% sodium bisulphite and 0·1N HCl, until the magenta colour band within the gel matrix appeared, for confirmation of dextransucrase activity (Majumder *et al.*, 2008).

3.2.8 Optimization of reaction conditions for maximum dextransucrase activity

3.2.8.1 Effect of sucrose concentration on dextransucrase activity

To study the effect of sucrose concentration on the enzyme activity of the purified dextransucrase (specific activity 18 U/mg), the enzyme assay was conducted

at different sucrose concentration ranging from 0.1-15%. The reaction was carried out in 1 ml mixture in 20 mM sodium acetate buffer pH 5.4 containing 20 µl of enzyme (0.24 mg protein/ml) and varying concentration of sucrose. The mixture was incubated at 30°C in water bath for 15 min and the activity was determined by estimating the released reducing sugar, as described earlier in Section 3.2.3.

3.2.8.2 Effect of temperature on dextransucrase activity

To study the effect of temperature on the enzyme activity of purified dextransucrase (specific activity 18 U/mg), the enzyme assay was conducted at 10 different temperatures ranging from 20-40°C ± 0.2°C. The enzyme reaction was carried out in 1 ml reaction mixture in 20 mM sodium acetate buffer (pH 5.4) containing 146 mM (5%) final concentration of sucrose, 20 µl of enzyme (0.24 mg protein/ml) for 15 min. The assay mixtures were 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 3.2.3.

3.2.8.3 Effect of ionic strength of buffer on dextransucrase activity

To study the effect of different ionic strength of buffer on the enzyme activity of purified dextransucrase (specific activity 18 U/mg), the enzyme assay was conducted at different molar concentrations of buffer ranging from 1-500 mM. The reaction was carried out in 1 ml mixture in sodium acetate buffer of pH 5.4 containing 146 mM (5%) final concentration of sucrose, 20 µl of enzyme (0.24 mg protein/ml) and varying molar concentrations of sodium acetate buffer (pH 5.4). The mixture was incubated at 30°C in water bath for 15 min and the enzyme activity was determined by estimating the released reducing sugar, as described earlier in Section 3.2.3.

3.2.8.4 Effect of pH of buffer on dextransucrase activity

To study the effect of different pH of buffer on the enzyme activity of purified dextransucrase (specific activity 18 U/mg), the enzyme assay was conducted at different buffer pH ranging from 4.2-6.0. The reaction was carried out in 1 ml mixture in 10mM sodium acetate buffer of varying pH containing 146 mM (5%) final concentration of sucrose and 20 µl of enzyme (0.24 mg protein/ml). The mixture was incubated at 30°C in water bath for 15 min and the enzyme activity was determined by estimating the released reducing sugar, as described earlier in Section 3.2.3.

3.2.9 Effect of salts and chaotropic agents on the activity of dextransucrase

The effect of the 0-6 mM $MgCl_2$ and 0-10 mM $CaCl_2$ on the dextransucrase activity of the isolate *Pediococcus pentosaceus* was studied. Also, the effect of 0-7 M urea and 0-2.5 mM EDTA on the dextransucrase activity of the isolate was studied. The purified dextransucrase with protein concentration of 0.24 mg/ml and 18 U/mg was used. The enzyme assays were carried out in 1ml reaction mixture containing salt or the chaotropic agents, sucrose (5%) in 10 mM sodium acetate buffer (pH 5.4) and 20 µl enzyme. The enzyme activity was determined as described in Section 3.2.3. The effect of urea was studied by incubating enzyme with urea at 30°C for 30 min. The aliquots (20 µl) were taken and assayed for residual enzyme activity as described earlier in Section 3.2.3.

3.2.10 Effect of organic solvents on solubility, activity and stability of dextransucrase

The effect of various concentrations (%, v/v) of dimethysulfoxide (DMSO), dimethylformamide (DMF), ethanol, acetone and acetonitrile on purified dextransucrase from *Pediococcus pentosaceus* was studied. 0.75 mg of lyophilised dextransucrase was dissolved in 50% ethanol, 90% DMSO, 50% acetone and 20% acetonitrile in 1 ml. Equal amount of enzyme dissolved in 1 ml of 20 mM sodium acetate buffer, pH 5.4 was taken as control. The residual enzyme activity was estimated as described earlier in section 3.2.3.

3.3 Results and Discussion

3.3.1 Purification with polyethylene glycol

The enzyme activity of the cell free supernatant of the isolate *Pediococcus pentosaceus* was 3.4 U/ml and protein concentration was 5.9 mg/ml. The specific activity was 0.6 U/mg. The purification of the crude enzyme by polyethylene glycols increased the dextransucrase activity significantly. The enzyme purification results are

presented in (Table 3.3.1). The enzyme activity profiles with PEG-200 400 and 1500 are shown in Fig. 3.3.1.

PEG	Vol. (ml)	E A (U/ml)	Total Units	Overall % Yield	Protein (mg/ml)	Total protein (mg)	S A (U/mg)	Fold Purifi-c ation
Crude	50	3.4	170	–	5.9	295	0.6	–
33% PEG-200	1.6	3.1	5	2.9	0.35	0.6	8.3	14
25% PEG-400	3.3	4.4	14.5	8.5	0.24	0.8	18	31
10% PEG-1500	1.5	5	7.5	4.5	0.2	0.3	25.9	45

Table 3.3.1 Purification of dextransucrase of isolate SPA by fractionation with different molecular weight polyethylene glycols.

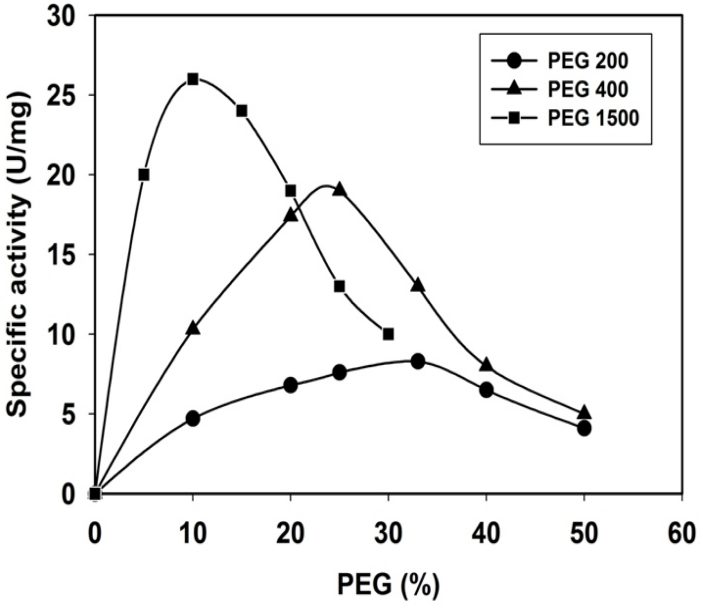


Fig. 3.3.1 Purification of dextransucrase from the isolate *Pediococcus pentosaceus* with different percentages (v/v) of PEG-200, 400 and 1500.

A concentration of 33% (v/v) PEG-200 gave the highest specific activity of 8.9 U/mg with 15 fold purification and 2.9% overall yield (Table 3.3.1) and 25% (v/v) PEG-400 gave the highest specific activity of 18 U/mg with 31 fold purification and 8.5% over all yield, whereas 10% (v/v) of PEG-1500 (60%, w/v) gave the maximum specific activity of 26 U/mg with 45 fold purification and 4.5% overall yield (Table 3.3.1).

3.3.2 Identification of the dextransucrase and molecular weight determination

The dextransucrase obtained after PEG purification was run on two identical SDS-PAGE gels under non denaturing conditions for *in-situ* activity detection. The gels were incubated in 5% sucrose for 48h and stained with Periodic Acid Schiff (PAS) reagent. The location of activity staining was determined with a Coomassie Brilliant Blue stained gel as control. A single magenta color band appeared on the

PAS stained gel. It confirmed the presence of dextran formed on polyacrylamide gels (Fig. 3.3.2). From the presence of magenta colour activity band, it was inferred that the purified dextransucrase was active. The comparison of activity staining and Coomassie Brilliant Blue stained gels identified the presence of dextransucrase and showed the approximate molecular weight of *Pediococcus pentosaceus* as 180 kDa. This band also corresponded to 180 kDa on the SDS-denaturing gel stained with Coomassie Brilliant Blue.

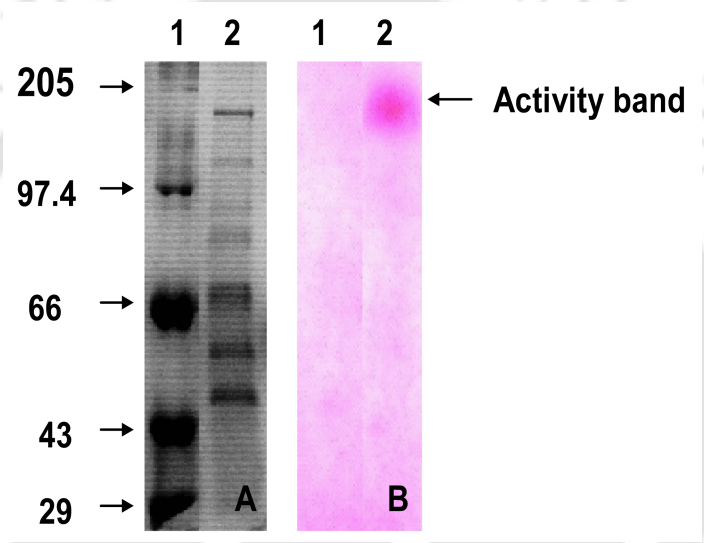


Fig. 3.3.2 Identification of purified dextransucrase from the isolate *Pediococcus pentosaceus* (SPA) (A) SDS-denaturing PAGE with Coomassie Brilliant Blue staining: Lane 1-Protein molecular weight marker, Lane 2-Purified dextransucrase from the isolate SPA (B) SDS-nondenaturing PAGE: Lane 1-Protein molecular weight marker 2. Periodic Acid Schiff staining of the dextran formed (The protein marker consists of Myosin from rabbit muscle, 205 kDa; Phosphorylase b 97.4 kDa; Bovine serum albumin, 66 kDa ; Ovalbumin, 43 kDa; Carbonic anhydrase, 29 kDa)

3.3.3 Optimization of reaction conditions for maximum dextransucrase activity

3.3.3.1 Effect of sucrose concentration on dextransucrase activity

The purified dextransucrase (specific activity 18 U/mg) was used to study the effect of sucrose concentration on dextransucrase activity by varying sucrose concentration between 0.1-15% final concentrations in the assay mixture. The results showed that saturation of enzyme activity reached at 5% (Fig. 3.3.3). It was observed that 5% sucrose concentration gave the maximum enzyme activity of 4.4 U/ml and specific activity of 18 U/mg. This finding of 5% sucrose as an optimum concentration for dextransucrase activity corroborates with findings of *Leuconostoc mesenteroides* NRRL B-640 (Purama and Goyal, 2009b). However, it deviates from the results of dextransucrases of *Leuconostoc mesenteroides* NRRL B-512F (Goyal and Katiyar, 1995) and *Leuconostoc dextranicum* NRRL B-1146 (Majumder *et al.*, 2008).

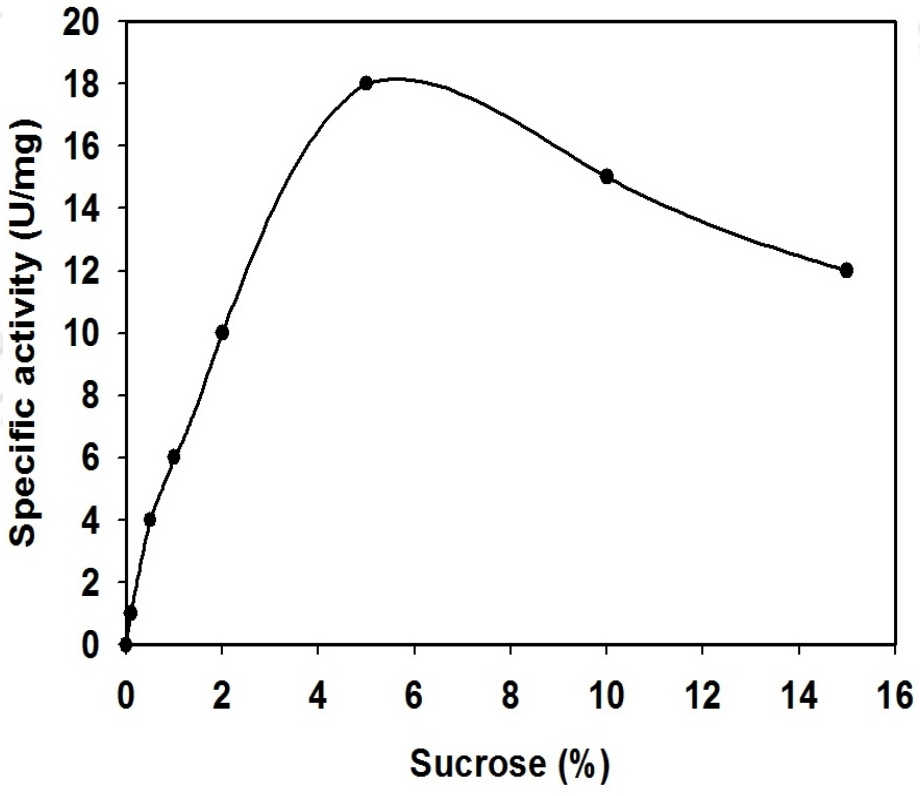


Fig. 3.3.3 Effect of sucrose concentration on dextransucrase activity *Pediococcus pentosaceus* assayed at 30°C in 20 mM sodium acetate buffer, pH 5.4.

3.3.3.2 Effect of temperature on dextransucrase activity

The purified dextransucrase (specific activity 18 U/mg) was used to study the effect of different temperature on dextransucrase activity by varying reaction temperature between 20-40°C. The results showed 30°C was the optimum temperature for dextransucrase activity (Fig. 3.3.4). The enzyme activity gradually decreased after 30°C. This result is supported by the findings that optimum temperature for dextransucrase activities of *Leuconostoc* strains such as B-512F and B-1355 falls in the range of 30 to 35°C (Purama and Goyal, 2009b).

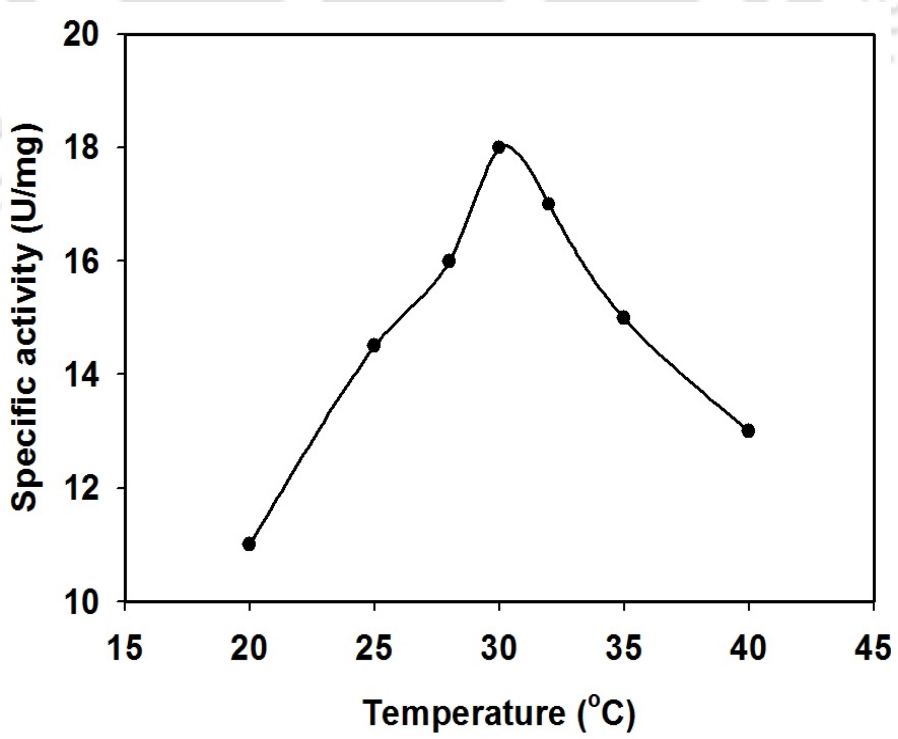


Fig. 3.3.4 Effect of temperature on dextransucrase activity of *Pediococcus pentosaceus*. The enzyme activity was determined by carrying out the assay in 20 mM sodium acetate buffer (pH 5.4) containing 5% sucrose.

3.3.3.3 Effect of ionic strength of buffer on dextransucrase activity

The purified dextransucrase (specific activity 18 U/mg) was used to study the effect of different ionic strength of buffer on dextransucrase activity by varying the molar concentrations between 1-500 mM. The results showed that 10-20 mM was appropriate concentration of buffer for dextransucrase activity (Fig. 3.3.5). Beyond 50 mM, the decrease in enzyme activity was rapid. The loss of enzyme activity at 200 mM was 50% and at 300 mM concentration it was about 33%.

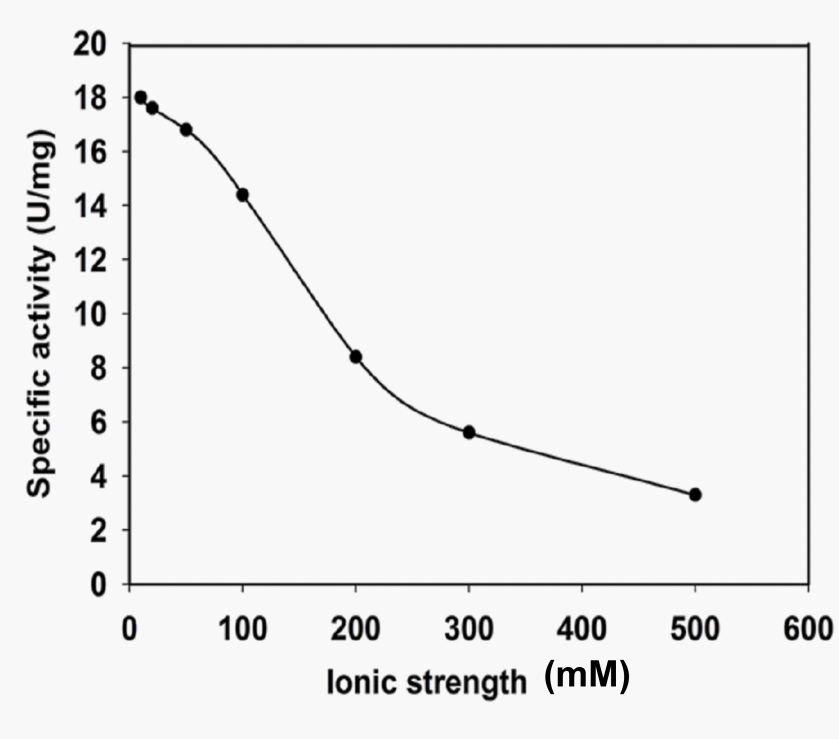


Fig. 3.3.5 Effect of ionic strength on dextransucrase activity of *Pediococcus pentosaceus*. The enzyme activity was determined by carrying out the assay at 30°C, in sodium acetate buffer of different ionic strengths (pH 5.4) containing 5% sucrose.

3.3.3.4 Effect of buffer pH on dextransucrase activity

The purified dextransucrase (specific activity 18 U/mg) was used to study the effect of different pH of buffer on dextransucrase activity by varying the pH between 4.2- 6.0. The maximum enzyme activity was observed at pH 5.4 (Fig. 3.3.6). The enzyme was quite stable below the pH 5.2, up to pH 4.2. 6% reduction in activity was observed at pH 5.2 and 11% reduction in activity was observed at pH 5.6. At pH 4.2, the activity was reduced to about 50% and at pH 4.8, 39% of the original activity was lost. The optimum pH 5.2-5.4 of dextransucrase was also observed in the strains, *Leuconostoc mesenteroides* NRRL B-512F (Goyal *et al.*, 1995), NRRL B-640 (Purama and Goyal, 2009b) and *Leuconostoc dextranicum* NRRL B-1146 (Majumder *et al.*, 2008).

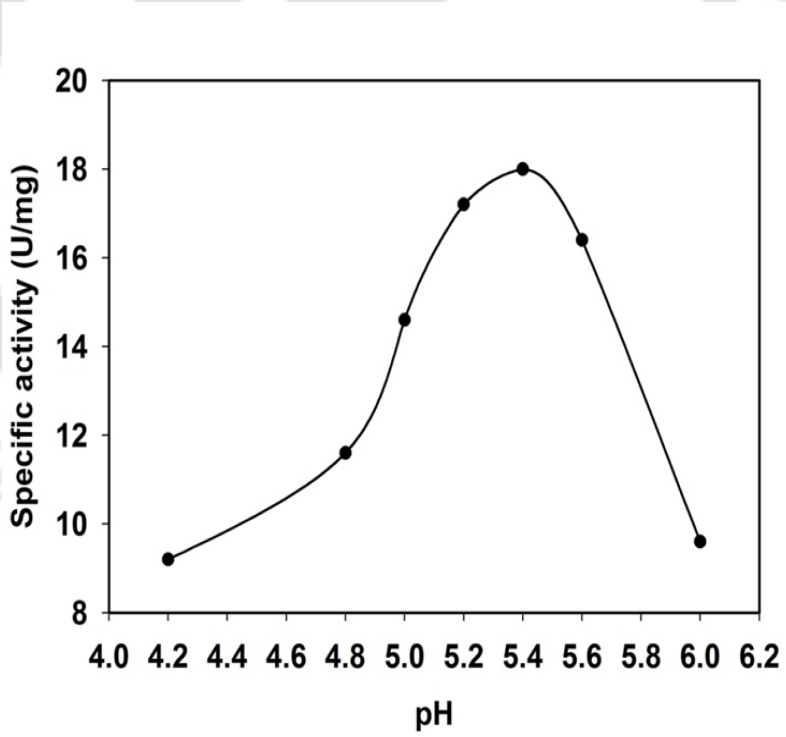


Fig. 3.3.6 Effect of pH on dextransucrase activity of*Pediococcus pentosaceus*. The enzyme activity was determined by carrying out the assay at 30°C in 10 mM sodium acetate buffer containing 5% sucrose.

3.3.4 Effect of salts on the activity of dextransucrase

The effect of different divalent metal ions, Mg^{2+} and Ca^{2+} on dextransucrase activity of *Pediococcus pentosaceus* was studied. Salts are believed to affect the water structure of enzymes thus affecting the solubility and activity (Forman and Kennedy, 1977). Mg^{2+} and Ca^{2+} salts stabilize the catalytic activity of enzymes by stabilizing the three-dimensional protein structure (Goyal *et al.*, 1995). The Mg^{2+} ions exhibited a marginal increase in the enzyme activity of dextransucrase. The enzyme activity increased by 5% at 1 mM $MgCl_2$ (Fig. 3.3.7). However, beyond 1 mM $MgCl_2$, the activity drastically decreased. At 6 mM $MgCl_2$, 25% of the initial activity was lost.

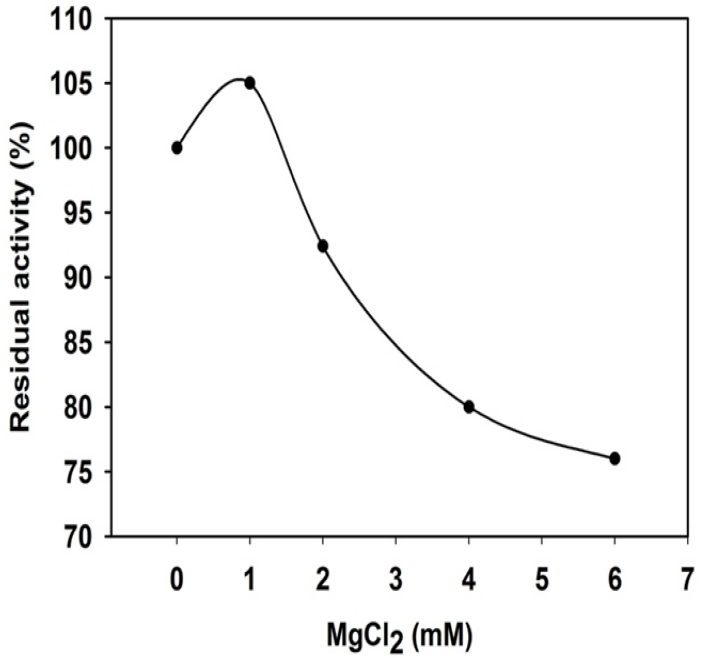


Fig. 3.3.7 Effect of $MgCl_2$ on the activity of purified dextransucrase of *Pediococcus pentosaceus*

The Ca^{2+} ions significantly enhanced the enzyme activity of dextransucrase. The enzyme activity increased by 150% at 6 mM $CaCl_2$ (Fig. 3.3.8). Similar results were reported for *Leuconostoc mesenteroides* B-512F dextransucrase (Goyal *et al.*, 1995). The Ca^{2+} ion has been reported to be associated with the catalytic sites of dextransucrases (Miller and Robyt, 1986b).

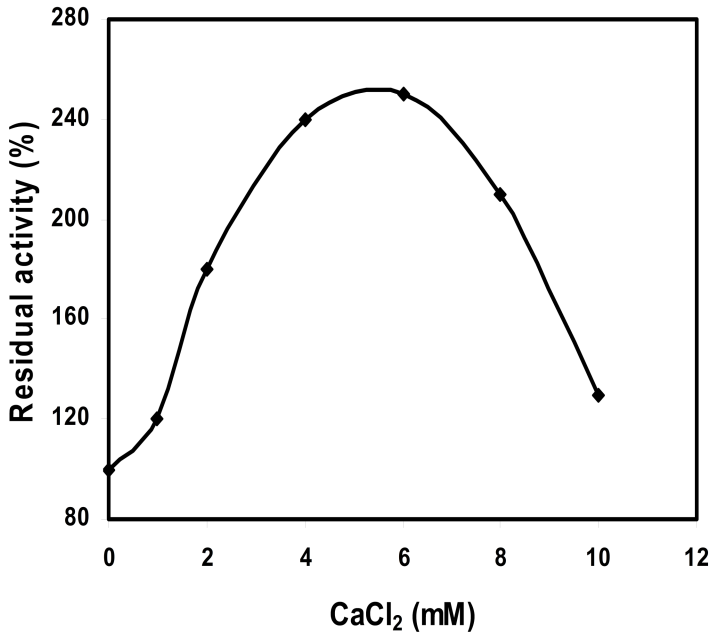


Fig. 3.3.8 Effect of $CaCl_2$ on the activity of purified dextransucrase of *Pediococcus pentosaceus*

3.3.5 Effect of chaotropic agents on the activity of dextransucrase

Urea inactivated dextransucrase at all concentrations ranging from 1 to 7M. With an increase in the concentration of urea there was a rapid decrease in enzyme activity. The enzyme lost 9%, 38%, 48%, 64%, 72%, 88% and 96% of activity on treatment with 1, 2, 3, 4, 5, 6 and 7M urea, respectively (Fig. 3.3.9). Similar pattern of activity loss was observed in glucansucrase of *Leuconostoc dextranicum* NRRL B-1146 (Majumder *et al.*, 2008). 5M urea completely inactivated the glucansucrase.

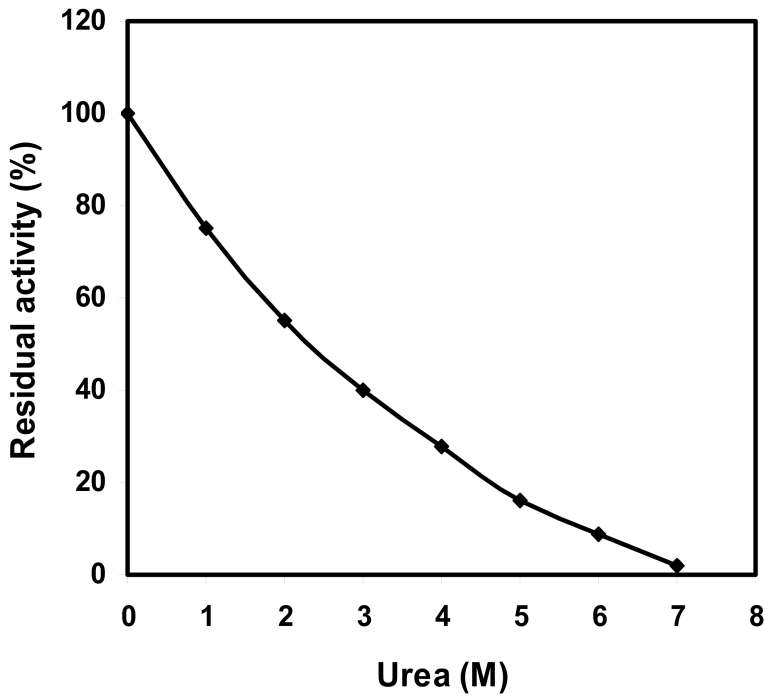


Fig. 3.3.9 Effect of urea on dextransucrase activity of *Pediococcus pentosaceus*. Dextransucrase (specific activity18 U/mg) in 20 mM sodium acetate buffer (pH 5.4) was incubated with various concentrations of urea at 30°C for 30 min and the aliquots (0.1 ml) were taken and enzyme activity was estimated as described in Section 3.2.3.

The addition of EDTA inhibited the dextransucrase activity of *Pediococcus pentosaceus*. 10% inactivation was obtained with 0.5 mM EDTA and 75% inactivation was obtained with 1 mM EDTA (Fig. 3.3.10). The saturation reached at 1.5 mM EDTA with about 20% residual activity.

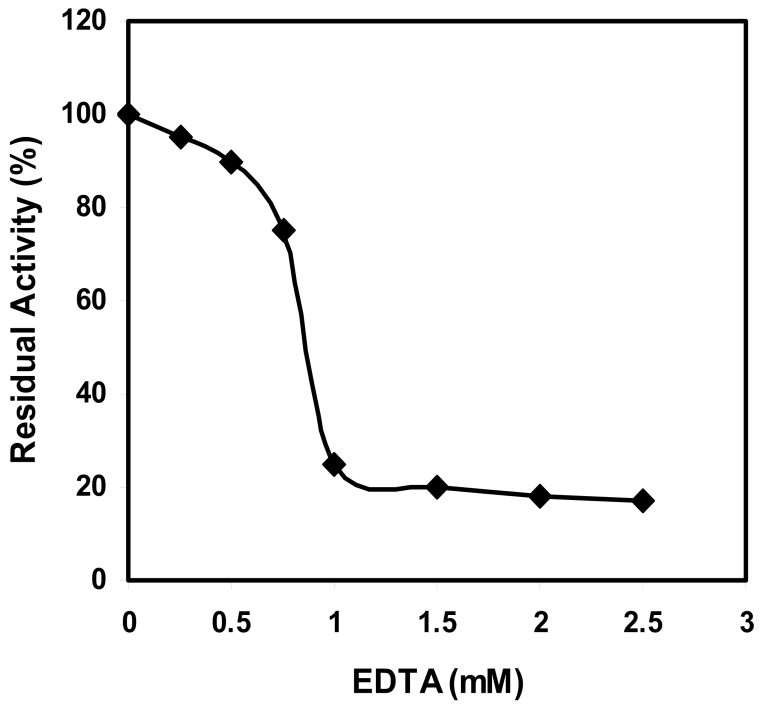


Fig. 3.3.10 Effect of EDTA on dextransucrase activity of *Pediococcus pentosaceus*. Dextransucrase (specific activity18 U/mg) in 20 mM sodium acetate buffer (pH 5.4) was incubated at 30°C along with different concentrations of EDTA. The enzyme activity was estimated as described in Section 3.2.3.

3.3.6 Effect of organic solvents on the activity of dextransucrase

The solubility of lipases, proteases or oxidases in organic solvents are well studied. But comparatively, fewer studies have been performed on solubility of glycosidases in organic solvents. The behavior of dextransucrase from *Leuconostoc mesenteroides* NRRL-B-512F in various organic solvents were studied by Girard and Legoy (1999). This study was conducted to gain some insight on the solubility, activity and stability of acceptors in organic solvents that may improve the interactions between the acceptors and enzymes facilitating the acceptor reactions.

The influence of various organic solvents on dextransucrase stability of *Pediococcus pentosaceus* was investigated. The enzyme activity loss by 50% ethanol, 90% DMSO, 50% acetone and 20% acetonitrile was 80%, 91%, 94% and 80%, respectively (Fig. 3.3.11). It was suggested that the enzyme structure modifications by the organic solvents, led to the activity losses (Girard and Legoy, 1999).

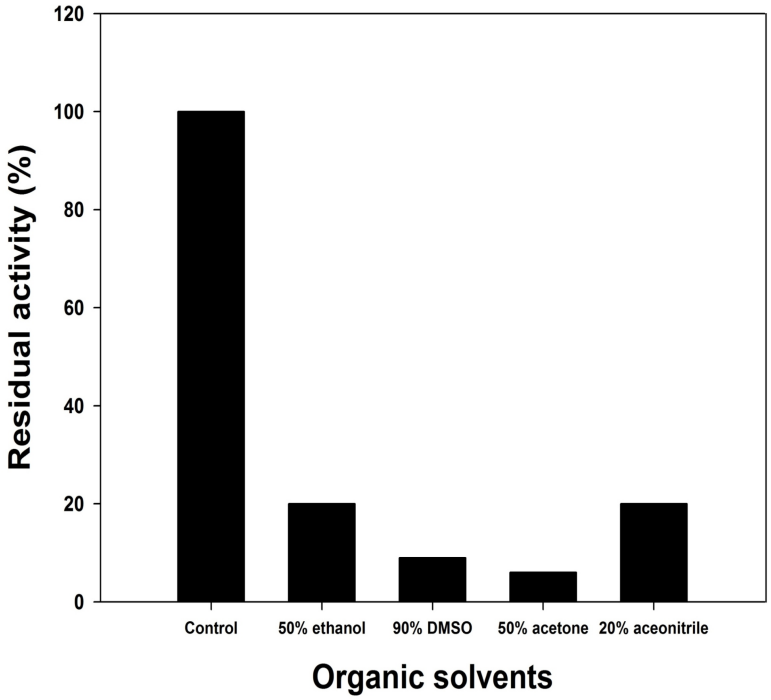


Fig. 3.3.11 Effect of different organic solvents on dextransucrase activity of *Pediococcus pentosaceus*. Dextransucrase (specific activity18 U/mg) in 20 mM sodium acetate buffer (pH 5.4) was incubated at 30°C along with the organic solvents. The enzyme activity was estimated as described in Section 3.2.3.

Conclusions

Polyethylene glycol (PEG) fractionation method was used to purify dextransucrase from *Pediococcus pentosaceus*. A 33% concentration of PEG-200, 25% of PEG-400 and 10% of PEG-1500 resulted in maximum specific activity of dextransucrase. A final concentration of 10% PEG-1500 resulted in dextransucrase of maximum specific activity 25.9 U/mg with 45 fold purification in a single step. PEG-400 showed better yield than PEG-1500. A final concentration of 25% PEG-400 was the optimum concentration for the purification of dextransucrase that resulted in maximum specific activity of 18 U/mg with 31 fold purification in a single step. The dextran synthesizing activity of the purified enzyme was identified and confirmed by activity staining. A single magenta colour band was observed on Periodic acid Schiff staining. The denaturing SDS-PAGE of the purified dextransucrase determined the approximate molecular size to be 180 kDa. The optimization of reaction conditions revealed that, 5% sucrose, 30°C reaction temperature, 10-20 mM ionic strength of buffer and 5.4 buffer pH were optimum for maximum activity of dextransucrase from the isolate *Pediococcus pentosaceus*. The dextransucrase activity was enhanced by Mg^{2+} and Ca^{2+} ions. 1 mM $MgCl_2$ enhanced activity of dextransucrase by 5%, however higher concentrations of $MgCl_2$ showed deactivating effect on the enzyme. 6 mM $CaCl_2$ enhanced activity of the enzyme by 150%. The enzyme activity loss with 1M and 7M concentrations of urea was 25% and 98%, respectively. EDTA being a chelating agent, removed the stabilising metal ions and caused 80% of the dextransucrase activity loss at 1.5 mM EDTA. Dextransucrase activity loss by 50% ethanol, 90% DMSO, 50% acetone and 20% acetonitrile were 80%, 91%, 94% and 80%, respectively.

References

Bertrand, A., Morel, S., Lefoulon, F., Rolland, Y., Monsan, P. and Remaud-Simeon, M. (2006) *Leuconostoc mesenteroides* glucansucrase synthesis of flavonoid glucosides by acceptor reactions in aqueous-organic solvents. Carbohydr. Res. 341, 855-863.

Brink, L. E. S., Tramper, J., Luyben, K. CH. A. M. and Van't Riet, K. (1988) Biocatalysis in organic media. Enz. Microbiol. Technol. 10, 736-743.

Forman, H. J. and Kennedy, J. (1977) Effects of chaotropic agents versus detergents on dihydroorotate dehydrogenase. J. Biol. Chem. 25, 3379-3381.

Funane, K., Yamada, M., Shiraiwa, M., Takahara, H., Yamamoto, N., Ichishima, E. and Kobayashi, M. (1995) Aggregated form of dextransucrases from *Leuconostoc mesenteroides* NRRL B-512F and its constitutive mutant. Biosci. Biotechnol. Biochem. 59, 776-780.

Girard, E. and Legoy, M-D (1999) Activity and stability of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F in the presence of organic solvents. Enz. Microb. Technol. 24, 425-432.

Goyal, A., and Katiyar, S. (1994) Fractionation of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase by polyethylene glycol: a simple and effective method of purification. J. Microbiol. Methods 20, 225-231.

Goyal, A., Nigam, M. and Katiyar, S. S. (1995) Optimal conditions for production of dextransucrase from *Leuconostoc mesenteroides* B-512F and its properties. J. Basic Microbiol. 35, 375-384.

Goyal, A. and Katiyar S. S. (1996) Regulation of dextransucrase productivity of *Leuconostoc mesenteroides* B-512F by the maintenance media. J. Gen. Appl. Microbiol. 42, 81-85.

Holt, S. M., Al-Sheikh, H. and Shin, K. J. (2001) Characterization of dextran-producing *Leuconostoc* strains. Lett. Appl. Microbiol. 32, 185-189.

Kim, D. and Robyt, J. F. (1994) Properties of Leuconostoc mesenteroides B-512FMC constitutive dextransucrase. Enz. Microb. Technol. 16, 1010-1015.

Kobayashi, M. and Matsuda, K. (1976) Purification and properties of the extracellular dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299. J. Biochem. 79, 1301-1308.

Kobayashi, M. and Matsuda, K. (1986) Electrophoretic analysis of the multiple forms of dextransucrase from *Leuconostoc mesenteroides*. J. Biochem. 100, 615-621.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227, 680-685.

Lopez, A. and Monsan, P. (1980) Dextran synthesis by immobilized dextran sucrase. Biochimie. 62, 323-329.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Majumder, A., Purama, R. K. and Goyal, A. (2007) An overview of purification methods of glycoside hydrolase family 70 dextransucrase. Ind. J. Microbiol. 47, 197-206.

Majumder, A., Mangtani, A. and Goyal, A. (2008) Purification, identification and functional characterization of glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. Curr. Trends Biotechnol. Pharm. 2, 493-505.

Miller A. W. and Robyt J. F. (1986a) Functional molecular size and structure of dextransucrase by radiation inactivation and gel electrophoresis. Biochim. Biophys. Acta. 870, 198-203.

Miller A. W. and Robyt J. F. (1986b) Activation and inhibition of dextransucrase by calcium. Biochim. Biophys. Acta. 880, 32-39.

Nelson, N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153, 375-380.

Neubauer, H., Bauche, A. and Mollet, B. (2003) Molecular characterization and expression analysis of the dextransucrase DsrD of *Leuconostoc mesenteroides* Lcc4 in homologous and heterologous *Lactococcus lactis* cultures. Microbiol. 149, 973-982.

Purama, R. K. and Goyal, A. (2008) Identification, effective purification and functional characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. Bioresour. Technol. 99, 3635-3542.

Purama, R. K. and Goyal, A. (2009a) Purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 exists as single homogeneous protein: analysis by non-denaturing Native-PAGE. Int. J. Microbiol. 6, 1-7.

Purama, R. K. and Goyal, A. (2009b) Optimization of conditions of *Leuconostoc mesenteroides* NRRL B-640 for production of dextransucrase and its assay. J. Food Biochem. 33, 218-231.

Quirasco, M., Lopez-Munguia, A., Remaud-Simeon, M., Monsan, P. and Farres, A. (1999) Induction and transcription studies of the dextransucrase gene in *Leuconostoc mesenteroides* NRRL B-512F. Appl. Environ. Microbiol. 65, 5504-5509.

Remaud-Simeon, M., Paul, F., Monsan, P., Heyraud, A. and Rinaudo, M. (1991) Molecular weight characterization and structural properties of controlled molecular weight dextrans synthesized by acceptor reaction using highly purified dextransucrase. J. Carbohydr. Chem. 10, 861-876.

Robyt, J. F. and Walseth, T. F. (1979) Production, purification and properties of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Carbohydr. Res. 68, 95-111.

Sanchez-Gonzalez, M., Alagon, A., Rodriguez-Sotres, R. and Lopez-Munguia, A. (1999) Proteolytic processing of dextransucrase of *Leuconostoc mesenteroides*. FEMS Microbiol. Lett. 181, 25-30.

Sato, T., Nakagawa, H., Kurosu, J., Yoshida, K., Tsugane, T., Shimura, S., Kirimura, K., Kino, K. and Usami, S. (2000) α-anomer-selective glucosylation of (+)-catechin by the crude enzyme, showing glucosyl-transfer activity of *Xanthomonas campestris* WU-9701. J. Biosci. Bioeng. 90, 625-630.

Smitinont, T., Tansakul, C., Tanasupawat, S., Keeratipibul, S.,Navarini, L., Bosco, M. and Cescutti, P. (1999) Exopolysaccharide-producing lactic acid bacteria strains from traditional thai fermented foods: isolation, identification and exopolysaccharide characterization. Int. J. Food Microbiol. 51, 105-111.

Somogyi, M. (1945) A new reagent for the determination of sugars. J. Biol. Chem. 160, 61-68.

Tsuchiya, H. M., Koepsell, H. J., Corman, J., Bryant, M. O., Feger, V. H. and Jackson R. W. (1952) The effect of certain cultural factors on production of dextransucrase by *Leuconostoc mesenteroides.* J. Bact. 64, 521-526.

Willemot, R., Monsan, P. and Durand, G. (1988) Effects of dextran on the activity and stability of dextransucrase from *Leuconostoc mesenteroides*. Annals NY Acad. Sci. 542, 169-172.

Chapter 4

Production, purification, structural and application studies of dextran from *Pediococcus pentosaceus* (SPA)

4.1 Introduction

Dextrans are homopolysaccharides of D-glucose, synthesized by dextransucrase, an extracellular enzyme elaborated by lactic acid bacteria *viz.*, *Leuconostoc, Lactobacillus* and *Streptococcus spp.* (Majumder and Goyal, 2008). Smitinont *et al.* (1999) reported the possible production of dextran from *Pediococcus pentosaceus* strain. Dextransucrases cleave the osidic bond of its substrate sucrose and catalyze the polymerization of the glucopyranosyl moieties to dextran (Purama and Goyal, 2008). 96 strains of dextran-producing lactic acid bacteria were studied to classify dextrans by their structural and functional properties (Jeanes *et al.*, 1954). Structural and functional attributes of dextran are dependent on the dextransucrase produced by the strain (Leathers, 2002).

Dextrans have prolific usage in food, clinical, fine chemicals, cosmetics and agricultural industries (Purama and Goyal, 2005; Patel *et al.*, 2010). Dextrans are used as viscosifying, texturizing, stabilizing, emulsifying or gelling agents in food

formulations (Majumder and Goyal, 2009). Dextran has been used in microsurgery to reduce the risk of free tissue transfer loss and improve micro circulation (Ridha *et al.*, 2006). Dextran has been investigated suitable for cell-resistant coatings on biomaterial surfaces (Massia *et al.*, 2000). Dextran hydrogels have biomedical applications in contact lenses, cell encapsulation for drug-delivery, tissue engineering sacffolds, burn dressing and spinal cord regeneration (Hoffman, 2002; Van Tomme and Hennink, 2007).

Dextrans differ in the type of glucosidic linkages, degree and type of branching, length of chains, molecular mass and their conformation (Majumder *et al.*, 2009a). The physical properties such as surface morphology, gelling nature, structure and rheology are crucial in determining the applications potential of dextrans (Stoodley *et al.*, 1999). The NMR spectroscopic techniques were used to analyze the dextran structures of *Leuconostoc citreum* E497 and *Weissella confusa* E392, which revealed α-(1→2) linkages in the dextran of *Leuconostoc citreum* E497 and linearity of dextran from *Weissella confusa* E392 (Maina *et al.*, 2008). The structural characteristics of dextran from *Leuconostoc dextranicum* NRRL B-1146 was investigated by FT-IR, ^{1}H NMR and ^{13}C NMR spectroscopic techniques, which showed the presence of α-(1→6) and α-(1→4) linkages (Majumder *et al.*, 2009a). The structure of dextran from *Leuconostoc mesenteroides* NRRL B-640 showed linear α-(1→6) linkages (Purama *et al.*, 2009). Rheometric study of the same dextran established the typical non-Newtonian pseudoplastic behaviour and Scanning electron microscopy revealed the porous surface morphology.

Considering their immense industrial and pharmaceutical applications, the production, purification, structural and functional study of dextrans with novel

properties are of great interest. In this regard, the physico-chemical attributes and application potentials of dextran from the new isolate of *Pediococcus pentosaceus* has been reported. The structure of this dextran was analysed by FT-IR, NMR, scanning electron microscopy and rheological studies. The cytotoxicity of the dextran was explored using human cervical cancer cell line (HeLa) cell line to evaluate its potential in biomedical applications.

4.2 Materials and methods

4.2.1 Microorganism and maintenance

Natural isolate of *Pediococcus pentosaceus* (Genbank Accession Number EU569832) isolated from the soil of Assam (near Guwahati) as described in Section 2.2.2 of Chapter 2 was used for the studies on its dextran. *Pediococcus pentosaceus* is a microaerohilic and mesophilic lactic acid bacterium growing at 25°C. Every 15 days, a loopful of the isolate culture was transferred to modified MRS agar medium as stabs (Goyal and Katiyar, 1996), grown for 16h, and stored at 4°C.

4.2.2 Production and estimation of dextran

Dextran from the isolate *Pediococcus pentosaceus* was produced by using the enzyme production medium (Tsuchiya *et al.*, 1952). The isolate was grown at 25°C under shaking condition at 180 rpm. After 24h of fermentation, the broth was centrifuged at 10,000 rpm for 10 min to obtain the cell free supernatant which contained dextran. The cell pellet obtained was discarded. The carbohydrate content in the cell free supernatant of the natural isolate *Pediococcus pentosaceus* was determined by phenol-sulphuric acid method (Dubois *et al.*, 1956) in a micro-titre plate (Fox and Robyt, 1991). To 25 µl of sample containing dextran in a microtitre plate, 25 µl of 5% (v/v) phenol was added and mixed by shaking the plate on a vortex mixer for 30s. Then the plate was placed on an ice bath and 125 µl of concentrated sulphuric acid was added to each well containing the mixture. The plate was again

shaken for 30s to ensure proper mixing of the contents of the wells. Then the plate was wrapped in cling film and incubated in water bath at 80°C for 30 min. After cooling to room temperature, the absorbance was determined at 490 nm on a ELISA plate reader (Bio-Rad, model 680). A standard graph was plotted using dextran (40 kDa) in the concentration range 0.1-1 mg/ml.

4.2.3 Purification of dextran of *Pediococcus pentosaceus*

The 25% PEG-400 purified dextransucrase produced by the natural isolate of *Pediococcus pentosaceus* was used for dextran synthesis as described in Section 3.2.4 of Chapter 3. For dextran synthesis, 0.8 ml of enzyme (0.24 mg protein/ml of specific activity 18 U/mg) was incubated in 10 ml of 5% sucrose as substrate in 20 mM sodium acetate (pH 5.4) containing 0.3 mM $CaCl_2$ and 15 mM sodium azide. The reaction mixture was incubated at 28°C for 48h. The polymeric mass produced was centrifuged at 13,000 rpm for 20 min and washed three times with 3 volumes of ethanol (90%, v/v), resuspended in deionized water to ensure removal of any remaining sucrose. The jelly-like mass was frozen at -20°C. The solidified sample was then freeze dried using a lyophilizer (Christ GmbH, model ALPHA 1-4 LD) at -51°C at a vacuum pressure of $35x10^{-3}$ mbar for 24h. The sample was stored at room temperature for physical, spectroscopic, microscopic and rheological characterization.

4.2.4 Physical properties of dextran

4.2.4.1 Gelling property of dextran

The concentration required for gelling is studied by taking different percentages of freeze dried dextran (0.1, 0.5 and 1%, w/v) in distilled water. All samples were

allowed to stand for 12h at room temperature. The themoreversible gelling nature was studied by heating and cooling the dextran solution.

4.2.4.2 Optical rotation

The optical rotation property of dextran from isolate *Pediococcus pentosaceus* was recorded using a polarimeter (Perkin-Elmer Instruments, model 343 Polarimeter) using a sodium D-line (589 nm) at 25°C.

4.2.5 Spectroscopic analyses of dextran

4.2.5.1 FT-IR spectrum

The FT-IR spectra of the purified dextran mixed with KBr was recorded using a spectrometer (Perkin-Elmer Instruments, model Spectrum One FT-IR Spectrometer).

4.2.5.2 NMR analyses

NMR spectroscopic analyses of the dextran were conducted using a spectrometer (Varian, model AS400) to find out the type of glycosidic linkage. The purified dextran was dissolved in 0.5 ml of D_2O (10 mg/ml, Sigma Aldrich). Tetramethyl silane (TMS) was used as an internal reference. 1-dimensional ^{1}H NMR was recorded at base frequency of 400 MHz and 1-dimensional ^{13}C NMR spectra was recorded at base frequency of 100 MHz. 2-dimensional Correlation spectroscopy (COSY) NMR analysis was conducted for further confirmation of data.

4.2.6 Scanning electron microscopic analysis

A little amount of lyophilised dextran was applied to the SEM stub by means of an adhesive tape and coated with 10 nm Au in a sputter coater (Leo, model SCH 620). The surface of the sample was viewed in Scanning Electron Microscope (Leo, model 1330 VP) at magnifications 2kx and 5 kx operated at 10.0 kV.

4.2.7 Rheological analysis of dextran

The change in steady shear viscosity and shear stress with change in shear rate were measured using the viscous colloid of dextrans (5 mg/ml) at 25°C by rheometer (Thermo Electron, model Haake rheostress RSI) interfaced with a HAAKE RheoWin 323 software. The applied shear rate was in the range of 0.05-500s^{-1}. The experiment was carried out in duplicate to ensure accuracy of results.

4.2.8 Cytotoxicity and biocompatibility test of dextran

4.2.8.1 Culture and maintenance of Human cervical cancer cell line

Cell culture vessels, media components and other chemicals were obtained from Sigma Aldrich, Bangalore, India. The Human cervical cancer (HeLa, derived from **He**nrietta **La**cks) cell lines were procured from the National Centre for Cell Science (NCCS) Pune, India. The cell lines were maintained in Eagle's minimum essential medium containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate, supplemented with 10% fetal bovine serum and 1% antibiotic antimycotic solution (1000 U/ml penicillin G, 10 mg/ml streptomycin sulfate, 5 mg/ml gentamycin and 25 µg/ml amphotericin B). The Cells were cultured in T-25 culture flask (Corning®) and were incubated at 37°C in a

humidified atmosphere supplied with 5% CO_2. The cells were cultured in T-25 culture flask (Corning®) and the monolayer was trypsinized when cells reached 80-90% confluence. The cell count was done using a haemocytometer before proceeding for further experiments.

4.2.8.2 Indirect contact based *in vitro* cytotoxicity assay

With an aim to establish the dextran derived from the natural isolate *Pediococcus pentosaceus*, as a safe food neutraceutical and potent biomaterial for various biomedical applications, we performed *in vitro* indirect contact based cytotoxicity assay. The lyophilized dextran powder was dissolved in sterile serum free DMEM medium (Kasoju *et al.*, 2009) to prepare a stock solution of 1 mg/ml. About $1x10^4$ cells/ well were seeded in a 96 well culture plate (Corning®) and cultured for 24h. Cells were then exposed to different concentrations of the dextran sample ranging from 10 µg/ml to 1000 µg/ml and incubated for 24, 48 and 72h. The cell viability was estimated using a 3-(4,5-Dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At the end of each incubation period, the contents of the each well were replaced with equal volume of MTT solution (0.5 mg/ml in serum free DMEM) and incubated for 4h at 37°C. The yellow coloured MTT is reduced to an insoluble purple formazan complex by mitochondrial reductases that are active only in the mitochondria of living cells. Thus, the formation of coloured complex can be directly correlated to the number of viable cells. After 4h of incubation with MTT solution, the contents of each well were replaced with equal volume of Dimethyl

sulfoxide. The absorbance of the purple coloured solution was quantitatively measured at 570 nm on a UV visible spectrophotometer (Varian Inc., Model Cary 100) and the viability (%) was calculated as follows:

$$\text{Viability (\%)} = (Nt/ Nc) \times 100$$

Where, Nt is the absorbance of the cells treated with dextran and Nc is the absorbance of the untreated cells.

4.2.8.3 Live dead staining assay

For live dead staining assay, cells were cultured in 35 mm cell culture dishes, treated with various concentrations ranging from 10 µg/ml to 1000 µg/ml and incubated in a CO_2 incubator. Cells were periodically observed for morphological changes under an inverted light microscope (Nikon, model TS 100-F) equipped with a digital camera (Nikon, model Coolpix 5400) after staining with Acridine Orange and Ethidium Bromide (AO/EB) solution. AO emits green fluorescence when bound to DNA of live cells and EB gives red fluorescence when bound to DNA of dead cells and hence AO/EB staining gives an estimate of live/dead cells.

4.2.8.4 Statistical analysis

All experiments were done in five independent runs and the data was expressed as Mean ± Standard deviation (n=5). To minimize the experimental errors, exponentially growing cells of the same passage were used for each of the experiment.

4.3 Result and Discussion

4.3.1 Dextran content in the cell free supernatant of lactic acid bacterium isolate *Pediococcus pentosaceus*

The dextran concentration in the cell free supernatant of the natural isolate *Pediococcus pentosaceus* determined by phenol-sulphuric acid method was 10.2 mg/ml. It is higher than the dextran concentrations of *Leuconostoc dextranicum* NRRL B-1146, which was reported to be 1.1 mg/ml (Majumder *et al.*, 2009a) as well as *Leuconostoc mesenteroides* NRRL B-640, which was reported to be 7.0 mg/ml (Majumder *et al.*, 2009b).

4.3.2 Purification of dextran

The dextran of *Pediococcus pentosaceus* was purified from cell supernatant by ethanol precipitation in 3 cycles as described in Methods. The purified dextran was lyophilised as white flakes and was used for structural and application studies.

4.3.3 Physical properties of dextran

4.3.3.1 Gelling property

The minimum concentration of dextran required to form the gel was 1% (w/v). The dextran showed thermoreversible nature when heated and cooled, similar to the bacterial polysaccharide gellan produced by *Sphingomonas paucimobilis* (Arockiasamy and Banik, 2008). This property has direct implications as stabilising, emulsifying, texturising and gelling agent in the food industry.

4.3.3.2 Optical rotation

The dextran synthesized by the natural isolate *Pediococcus pentosaceus* showed an optical rotation of $[\alpha]_D$ of +125°at $C^{0.2}$. It confirmed the *dextro* rotatory property of the biopolymer.

4.3.4 Structural analysis of dextran by spectroscopy

4.3.4.1 FT-IR analysis

The FT-IR spectrum provided information on the functional groups, monomeric units and linkages present in the dextran (Shingel, 2002). The FT-IR spectrum of this dextran is compared to spectra obtained for commercial xanthan gum, Guar gum and sodium alginate. The FT-IR spectrum of the purified dextran from the natural isolate *Pediococcus pentosaceus* is presented in Fig. 4.3.1. It shows bands around 3400 cm_{-}^{1}, 2939 $cm_{-}^{1}$and 990–1200 cm_{-}^{1}, common to all polysaccharides, representing O–H stretching, C–H stretching of the $–CH_2$ groups and saccharides, respectively

(Freitas *et al.*, 2009). A band around 1660 cm_^{1} in the Guar gum spectrum is attributed to ring stretching of galactose and mannose (Wang and Somasundaran, 2007). Similarly, band around 1647 cm^{-1} in the dextran can be assigned for the glucose. The band in the region of 3400 cm^{-1} represents the hydroxyl stretching vibration of the polysaccharide (Liu *et al.*, 2007). The band in the region of 2930 cm^{-1} is due to the C–H stretching vibration and the band in the region of 1639 cm^{-1} is due to carboxyl group (Liu *et al.*, 2007). The absorption peak at 906 cm^{-1} indicates the α-glycosidic bond. The characteristic bands at 1154, 1103 and 1020 cm^{-1} found in the spectra of dextran are due to valent vibrations of C–O and C–C bonds and deformational vibrations of the CCH, COH and HCO bonds. The band at 1154 cm^{-1} is assigned to valent vibrations of C– O–C bond and glycosidic bridge. The peak at 1103 cm^{-1} is due to the vibration of the C–O bond at the C-4 position of D-glucose (Shingel, 2002). The peak at 1020 cm^{-1} indicates the great chain flexibility around the α-(1→6) glycosidic bonds (Shingel, 2002). The band at 1732 cm^{-1} present in xanthan gum spectra is attributed to acetyl groups (Alvarez-Mancenido *et al.*, 2008). In the dextran under study, this band is lacking indicating the absence of acetyl group. The band at 1416 cm^{-1}, observed in most of the polymers, represents the symmetric stretching of –COO (Pongjanyakul and Puttipipatkhachorn, 2007). The dextran has this conspicuous band at 1417cm^{-1}.

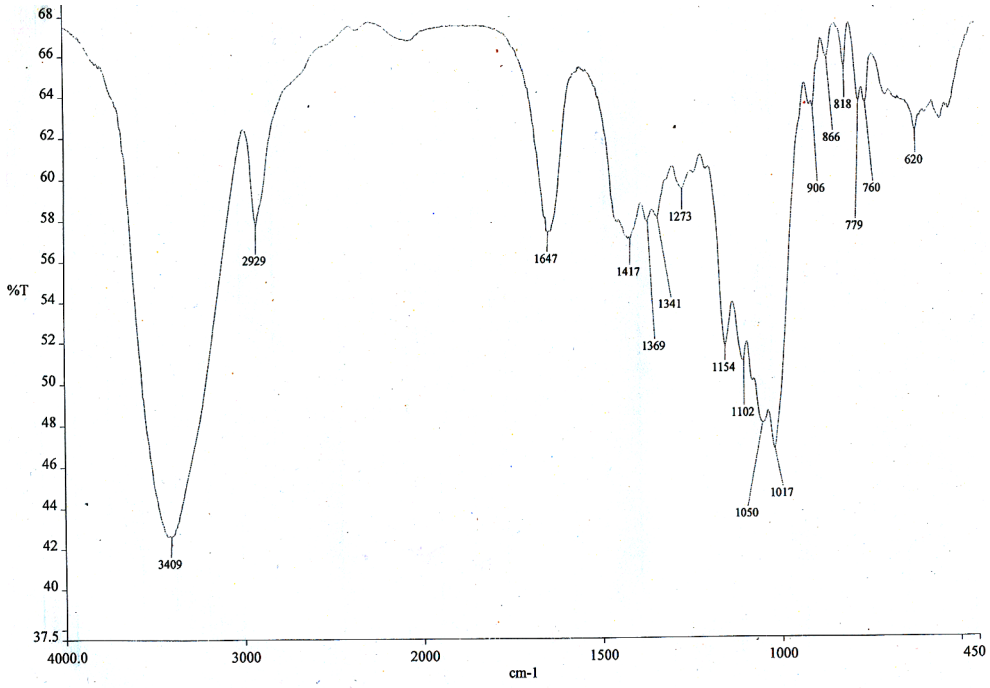


Fig. 4.3.1 FT-IR (KBr) spectrum of dextran produced from the purified dextransucrase of isolate *Pediococcus pentosaceus*.

4.3.4.2 ^{1}H NMR analysis

The anomeric proton resonances for the dextrans produced by the natural isolate *Pediococcus pentosaceus* are shown in Fig. 4.3.2. It has been observed that the distribution of ^{1}H NMR spectral resonances range between 3 and 6 ppm for different dextrans (Seymour, 1979b). It was reported that various dextrans have ^{1}H NMR spectral resonances (C-2, C-3, C-4, C-5 and C-6) in the 3- to 4-ppm region and the hemiacetal C-1 resonance in 4–6 ppm region (Sidebotham, 1974). The resonance at 4.96 ppm was assigned for the C-1 of the α-(1→6) glucosyl residues of main chain (Seymour, 1979b). The various resonances of ^{1}H NMR of the dextran of the natural isolate *Pediococcus pentosaceus* are identified in Table 4.3.1. The resonance at 4.91 ppm was assigned for the C-1 of the α-(1→6) linked backbone of dextran from the

natural isolate *Pediococcus pentosaceus* (Seymour, 1979b). *L. mesenteroides* NRRL B-640 produces highly linear and soluble dextran (Purama *et al.*, 2009). Studies on the ^{1}H NMR spectral region for anomeric carbon of dextran from *L. mesenteroides* NRRL B-1355 shows a resonance at 4.95 ppm and the branched linkages shows the resonance peak at 5.3 ppm (Seymour, 1979a). The absence of peak near 5.3 ppm showed that there is no branching in dextran of the natural isolate *Pediococcus pentosaceus*.

Table 4.3.1 ^{1}H and ^{13}C NMR resonances of dextran from the natural isolate *Pediococcus pentosaceus*.

Atoms	1	2	3	4	5	6
^{13}C	97.867	73.516	71.563	70.335	69.687	65.691
^{1}H	4.917	4.042	3.921	3.730	3.571	3.522

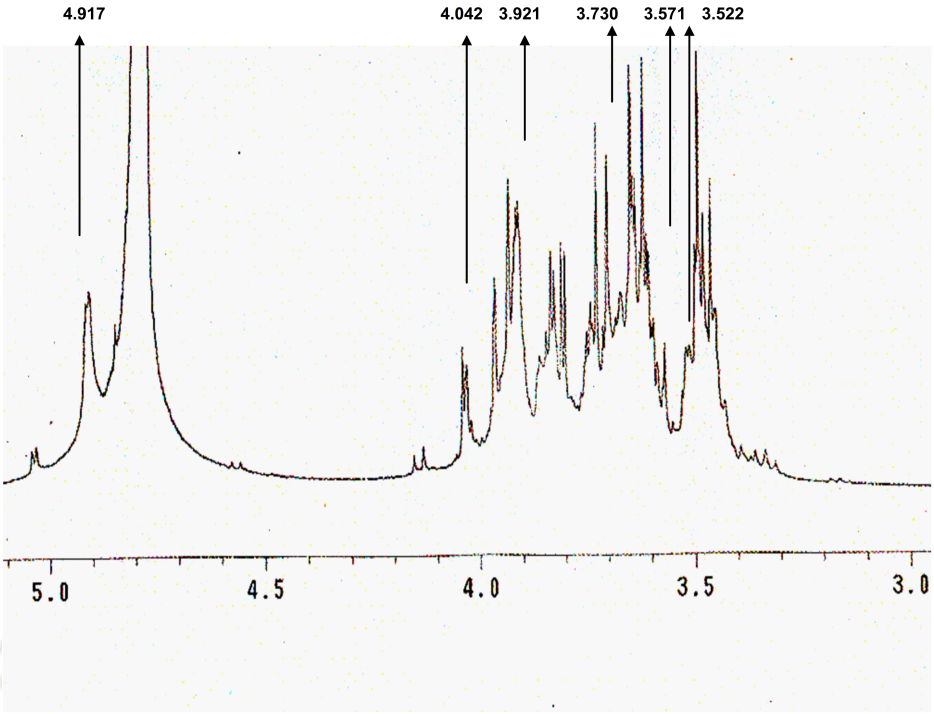


Fig. 4.3.2 ^{1}H NMR (400 MHz, D_2O) spectrum of dextran produced by the purified dextransucrase from the natural isolate of *Pediococcus pentosaceus*.

4.3.4.3 ^{13}C NMR analysis

Assignments to C-1 to C-6 carbons were made based on typical ^{13}C chemical shift regions reported in exopolysaccharide produced by a *Pseudomonas* strain (Freitas *et al.*, 2009). There were ^{13}C NMR resonances appearing above 92 ppm, typical of anomeric carbons C-1 involved in glycosidic bond linked carbohydrates. The dextran from the natural isolate *Pediococcus pentosaceus* showed six ^{13}C NMR resonances at 100 MHz: 97.867, 73.516, 71.563, 70.335, 69.687 and 65.691 ppm (Fig. 4.3.3.), which are characteristic of linear dextrans (Seymour, 1979a). ^{13}C NMR spectroscopy was used to examine the structure of a series of dextrans and established that linear dextran has six prominent resonances (Seymour, 1979a). No additional peaks were observed in the region of 75-85 ppm which indicates the absence of

branched linkages (Seymour, 1979a). The major resonance in the anomeric region at 98.7 (97.46) ppm proves that the C-1 is linked. An equally prominent peak at 66.5 (65.30 ppm), indicates that most of the C-6 are also linked. The ^{13}C NMR data presented in the current work shows that the dextran synthesized by the natural isolate *Pediococcus pentosaceus* is nearly identical to the ^{13}C NMR spectra of highly linear α-(1→6) glycosidic bond linkage containing dextran from the *L. mesenteroides* NRRL B-640 (Purama *et al.*, 2009).

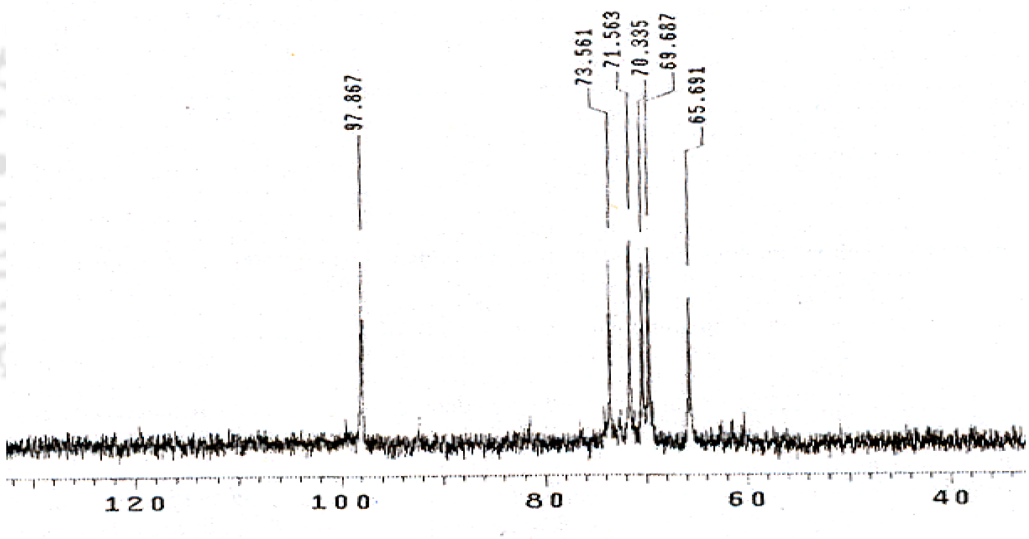


Fig. 4.3.3 ^{13}C NMR (100 MHz, D_2O) spectrum of dextran produced by the purified dextransucrase from the natural isolate of *Pediococcus pentosaceus*.

4.3.4.4 Two-dimensional NMR analysis

2D COSY (COrrelation SpectroscopY) NMR experiment is a simple and widely used technique for confirming the results. It is a homonuclear chemical shift correlation experiment based on the transfer polarization by a mixing pulse between

directly J-coupled spins. Thus, homonuclear chemical shifts through-bond interactions can be traced out by simple analysis of the 2D map providing a more general and more useful alternative to classical 1D homo decoupling experiments. This NMR was done to correlate the ^{1}H results for its improved sensitivity and resolution over the one-dimensional methods. 2D NMR of dextran from the natural isolate *Pediococcus pentosaceus* showed eight correlations (Fig. 4.3.4). This correlation corroborates the finding that the natural isolate *Pediococcus pentosaceus* synthesizes only linear dextran with α-(1→6) glycosidic bonds.

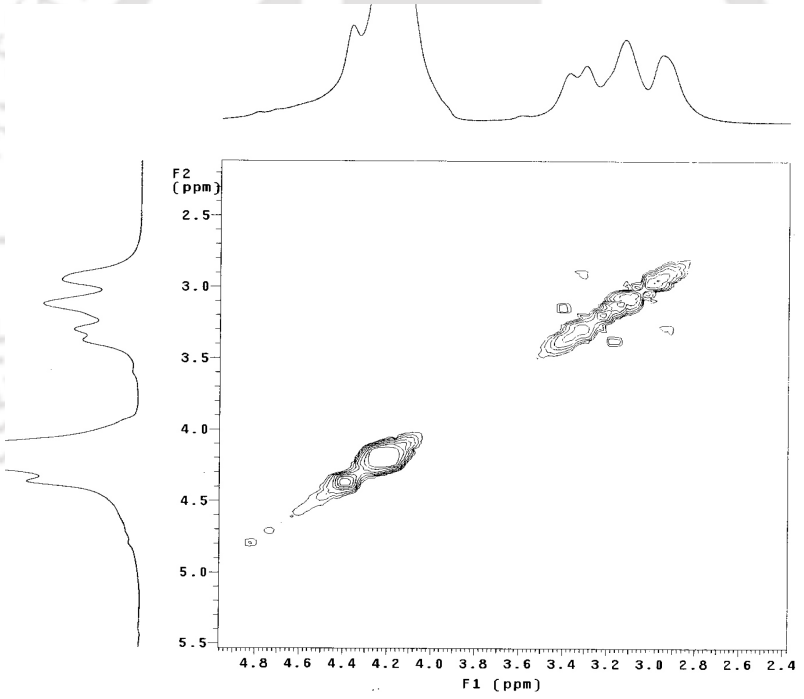


Fig. 4.3.4 2D NMR spectrum of dextran produced by the purified dextransucrase from the natural isolate *Pediococcus pentosaceus*.

4.3.5 Scanning electron microscopic analysis

The surface morphology dextran of the natural isolate *Pediococcus pentosaceus* was studied by scanning electron micrograph at various magnifications. Numerous

pores were observed in the reticular surface of dextran (Fig. 4.3.5). The porous structure promised high water holding capacity and consequent potential to be used in food industry as additives. From the porous dextrans, hydrogels can be created by either physical or chemical crosslinking, taking advantage of the abundant hydroxyl groups present on the α-(1→6) linked D-glucose residues (Levesque *et al.*, 2005). Hydrogels being composed of hydrophilic polymeric networks can absorb considerable amount of water and exhibit compatibility with proteins and living tissue (Hoffman, 2002). Hydrogels designed for tissue engineering scaffolds should also contain pores large enough to allow the migration, penetration and proliferation of living cells into the wound bed (Maire *et al.*, 2005). The key factors controlling the pore sizes, volume fraction and the interconnections are the composition of the network polymer chains and crosslink density (Hoffman, 2002).

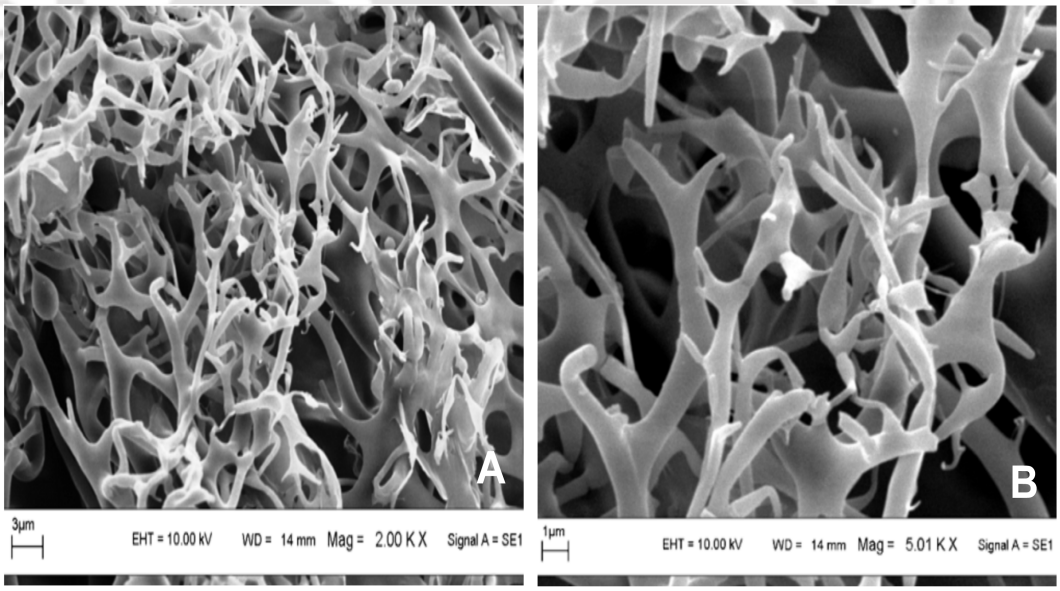


Fig. 4.3.5 Scanning Electron Micrograph of dextran from the natural isolate *Pediococcus pentosaceus* at magnification 2kX and 5kX showing the porous reticular surface morphology.

4.3.6 Rheological properties

On analysis of the experimental data obtained from the influence of shear rate on the apparent viscosity and shear stress, the results showed that the viscosity is inversely proportional to the shear rate and the semi liquid dextran exhibited a typical non-Newtonian behavior (Fig. 4.3.6). This is a typical characteristic of pseudoplastic fluid showing a shear thinning behavior, normally expected in polymer solutions, where the fluid molecular weight is high. In these systems, as the shear rate increases, the stress also increases, but its dependence on shear rate is less than linear; therefore viscosity decreases (Padmanabhan *et al.*, 2003).The non-Newtonian fluids formed by the polymers are employed in the food industry as gelling, stabilizers or thickening agents (Feddersen and Thorp, 1993). Alginates, carrageenans, agar, guar gum, arabic gum, methylcellulose and carboxymethylcellulose are hydrocolloids employed in numerous food formulations like sauces, ice creams etc. (Williams and Phillips, 2000). Therefore, rheology has emerged as a crucial quality control tool for the biopolymers (Zakaria and Rahman, 1996). The dextran from natural isolate *Pediococcus pentosaceus* conferring similar rheological property holds potential to be used in food industries.

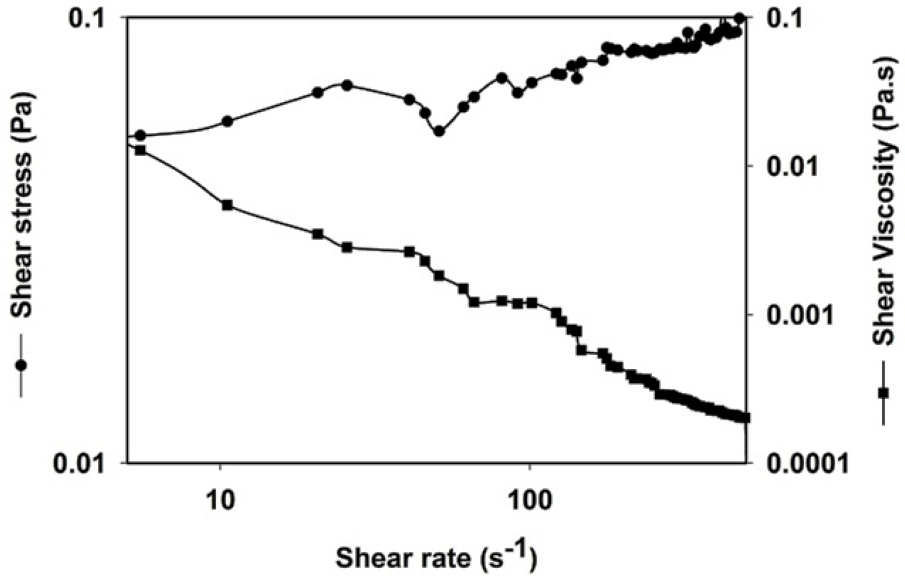


Fig. 4.3.6 Correlation of shear viscosity and shear stress of dextran of *Pediococcus pentosaceus* with respect to shear rate.

4.3.7 Cytotoxicity and cellular compatibility test of dextran

From the results of the MTT assay as shown in Fig. 4.3.7., it was clear that there is no effect of dextran from the natural isolate *Pediococcus pentosaceus* on the viability of the HeLa cells over a period of 72h even at high concentration i.e. 1000 µg/ml. In all the cases, the viability of treated cells was equivalent to the control cells.

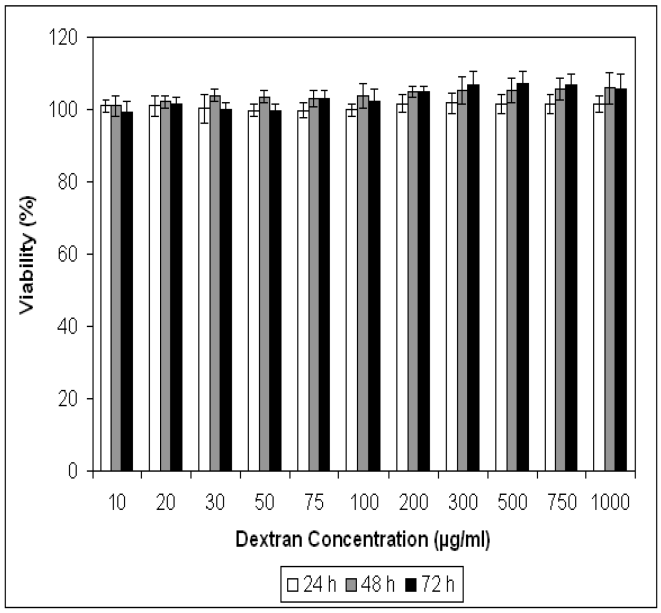


Fig. 4.3.7 The indirect contact based *in vitro* cytotoxicity assay showing the cell viability was unaltered after treatment with various concentrations of dextran (10 µg/ml to 1000 µg/ml) from the natural isolate *Pediococcus pentosaceus* over a period of 24 to 72h incubation.

Further, the live dead staining assay also revealed that the size, shape and viability of the cells remained unaffected even after 72h of treatment with high amount of dextran (1000 µg/ml) (Fig. 4.3.8). It was observed that the cells fluorescing bright green in abundant number both in treated and control experiments which indicated that the dextran has no effect on the morphology and metabolism of the cells.

The current study reveals that the dextran produced by *Pediococcus pentosaceus* is non-toxic and biocompatible and hence, is safe for consumption and can be used as a biomaterial for various biomedical applications. The results of the cytotoxicity assay were in good agreement with previous reports where the dextran from other resources

was found to be non-toxic and biocompatible (Draye *et al.*, 1998; De Groot *et al.*, 2001).

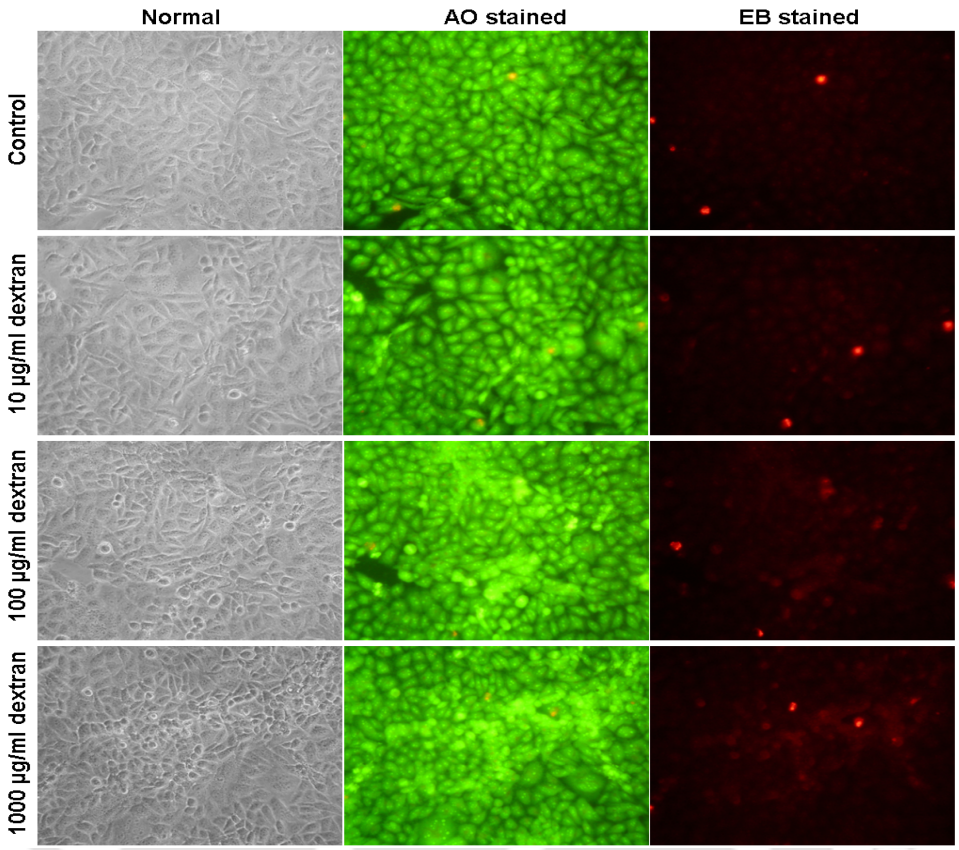


Fig. 4.3.8 The live dead staining assay showing the HeLa cell morphologies under fluorescence inverted light microscope after treatment with various concentrations of dextran (10 µg/ml, 100 µg/ml and 1000 µg/ml) from the natural isolate *Pediococcus pentosaceus*. After 48h incubation, the cells were stained with AO/EB to distinguish between live and dead cells. AO stains live cells and emits green fluorescence and EB stains dead cells and emits red fluorescence. No difference in the viability of dextran treated and control HeLa cells were observed.

4.4 Conclusions

Dextran yield of the natural isolate *Pediococcus pentosaceus* was more than the standard strains *viz. Leuconostoc mesenteroides* NRRL B640 and *Leuconostoc*

dextranicum NRRL B-1146. Thermoreversible nature of dextran of this isolate promises its food additive prospects. The results obtained from the FT-IR, ^{1}H, ^{13}C NMR and 2D NMR, SEM, optical rotaion and rheometic analyses threw light on the structural and functional aspects of the dextrans synthesised by the natural isolate *Pediococcus pentosaceus*. FT-IR report helped to identify the functional groups present in the dextrans and revealed the abundance of hydroxyl groups. NMR data confirmed the linear structure and abundance of α-(1→6) glycosidic bonds in the backbone of the dextrans. The surface morphology observed by Scanning Electron Micrograph revealed its network like highly porous structure. Porosity promises high water holding capacity enabling this dextran to be exploited in food industries as texturizing and viscosifying agent. The non-Newtonian pseudoplastic behaviour of the dextran analyzed by rheometric studies holds it as a potential gelling agent in food formulations. Ideally, a drug delivery vehicle should evoke no or minimal macrophage activation and it must have suitable permeability, chemical stability, physical durability, controllable biodegradability to withstand the new in vivo environment. The dextran from the natural isolate *Pediococcus pentosaceus* is non-toxic and biocompatible as evident from the *in vitro* contact based cytotoxicity assay and live dead staining assay. Hence, this biodegradable, natural biopolymer from the natural isolate *Pediococcus pentosaceus* can be explored for further applications in food formulations, drug delivery, tissue engineering and various other biomedical, pharmaceutical and biotechnological applications.

References

Alvarez-Mancenido, F., Landin, M., Lacik, I. and Martinez-Pacheco, R. (2008) Konjac glucomannan and konjac glucomannan/xanthan gum mixtures as excipients for controlled drug delivery systems. Diffusion of small drugs. Int. J. Pharmacol. 349, 11-18.

Arockiasamy, S. and Banik, R. M. (2008) Optimization of gellan gum production by *Sphingomonas paucimobilis* ATCC 31461 with non-ionic surfactants using central composite design. J. Biosci. Bioeng. 105, 204-210.

De Groot, C. J., Van Luyn, M. J., Van Dijk-Wolthuis, W. N., Cadee, J. A., Plantinga, J. A., Den Otter, W. and Hennink, W. E. (2001) In vitro biocompatibility of biodegradable dextran-based hydrogels tested with human fibroblasts. Biomaterials. 22, 1197-1203.

Draye, J. P., Delaey, B., Van de Voorde, A., Van Den Bulcke, A., De Reu, B. and Schacht, E. (1998) In vitro and in vivo biocompatibility of dextran dialdehyde cross-linked gelatin hydrogel films. Biomaterials. 19, 1677-87.

Dubois, M., Gilles, K. A., Hamilton, J. K. Rebers, P. A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356.

Feddersen, R. L. and Thorp, S. N. (1993) In R. L. Whistler and J. N. BeMiller (Eds.), Industrial gums (3rd ed.). San Diego: Academic Press.

Fox, J. D. and Robyt, J. F. (1991) Miniaturization of three carbohydrate analyses using microsample plate reader. Anal. Biochem. 19, 593-596.

Freitas, F., Alves,V.D., Pais, J., Costa, N., Oliveira, C., Mafra, L., Hilliou, L., Oliveira, R. and Reis, M. A. M. (2009) Characterization of an extracellular

polysaccharide produced by a *Pseudomonas* strain grown on glycerol. Biores. Technol. 100, 859-865.

Goyal, A. and Katiyar S. S. (1996) Regulation of dextransucrase productivity of *Leuconostoc mesenteroides* B-512F by the maintenance media. J. Gen. Appl. Microbiol. 42, 81-85.

Hoffman, A. S., (2002) Hydrogels for biomedical applications, Adv. Drug Del. Rev. 43, 3-12.

Jeanes, A., Haynes, W. C., Wilham, C. A., Rankin, J.C., Melvin E. H. and Austin, M. J. (1954) Characterization and classification of dextrans from ninety-six strains of bacteria. J. Am. Chem. Soc. 76, 5041-5052.

Kasoju, N., Bhonde, R. R. and Bora, U. (2009) Preparation and characterization of *Antheraea assama* silk fibroin based novel non-woven scaffold for tissue engineering applications. J. Tissue Eng. Reg Med. 3, 539-552.

Levesque, S. G., Lim, R. M. and Shoichet, M. S. (2005) Macroporous interconnected dextran scaffolds of controlled porosity for tissue-engineering applications. Biomaterials. 26, 7436-7446.

Leathers, T. D. (2002) Biopolymers. In E. J. Vandamme, S. DeBaets, & A. Steinbuchel (Eds.), Polysaccharides I: Polysaccharides from prokaryotes. Wiley-VCH: Weinheim. pp 229-321.

Liu, C., Lin, Q., Gao, Y., Ye, L., Xing Y. and Xi, T. (2007) Characterization and antitumor activity of polysaccharide from *Strongylocentrotus nudus* eggs. Carbohydr. Polym. 67, 313-318.

Maire, M., Logeart-Avramoglou, D., Degat, M-C and Chaubet, R. (2005) Retention of transforming growth factor β1 using functionalized dextran-based hydrogels. Biomaterials. 26, 1771-1780.

Majumder, A. and Goyal, A. (2008) Enhanced production of exocellular glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 using response surface method. Bioresour. Technol. 99, 3685-3691.

Majumder, A., Singh, A. and Goyal, A. (2009a) Application of response surface methodology for glucan production from *Leuconostoc dextranicum* NRRL B-1146 and its structural characterization. Carbohydr. Polym. 75, 150-156.

Majumder, A. Bhandari, S., Purama, R. K., Patel, S. and Goyal, A. (2009b) Enhanced production of a novel dextran from *Leuconostoc mesenteroides* NRRL B-640 by Response Surface Methodology. Annals Microbiol. 59, 1-7.

Majumder, A. and Goyal, A. (2009) Rheological and gelling properties of a novel glucan from *Leuconostoc dextranicum* NRRL B-1146. Food Res. Int. 42, 525-528.

Maina, N. H., Tenkanen, M., Maaheimo, H., Juvonen, R. and Virkki, L. (2008) NMR spectroscopic analysis of exopolysaccharides produced by *Leuconostoc citreum* and *Weisella confusa*. Carohydr. Res. 343, 1446-1455.

Massia, S. P., Stark J. and Letbetter, D. S. (2000) Surface-immobilized dextran limits cell adhesion and spreading. Biomaterials. 21, 2253-2261.

Padmanabhan, P. A., Kim, D-S, Pak, D. and Sim, S. J. (2003) Rheology and gelation of water-insoluble dextran from *Leuconostoc mesenteroides* NRRL B-523. Carbohydr. Polym. 53, 459-468.

Patel, S., Majumder, A. and Goyal, A. (2010) Potentials of exopolysaccharides from lactic acid bacteria. Ind. J. Microbiol. (in press).

Pongjanyakul, T. and Puttipipatkhachorn, S (2007) Xanthan–alginate composite gel beads: molecular interaction and in vitro characterization. Int. J. Pharmacol. 331, 61-71.

Purama, R. K. and Goyal, A. (2005) Dextransucrase production by *Leuconostoc mesenteroides*. Ind. J. Microbiol. 2, 89-101.

Purama, R. K. and Goyal, A. (2008) Identification, effective purification and functional characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. Bioresour. Technol. 99, 3635-3642.

Purama, R. K., Goswami, P., Khan, A. T. and Goyal, A. (2009) Structural analysis and properties of dextran produced by *Leuconostoc mesenteroides* NRRL B-640. Carbohydr. Polym. 76, 30-35.

Ridha, H., Jallali, N. and Butler, P. E. (2006) The use of dextran post free tissue transfer. J. Plast. Reconstr. Surg. 59, 951-954.

Seymour, F. R. (1979a) Structural analysis of dextrans containing 2-o-a- glucosylated a-D-Glucopyranosyl residues at the branch points, by use of ^{13}C nuclear magnetic resonance spectroscopy and gas–liquid chromatography- mass spectrometry. Carbohydr. Res. 7, 231-250.

Seymour, F. R. (1979b) Correlation of the structure of dextran to their ^{1}H NMR spectra. Carbohydr. Res. 74, 77-92.

Shingel, K. I. (2002) Determination of structural peculiarities of dextran, pullulan and c-irradiated pullulan by Fourier-transform IR spectroscopy. Carbohydr. Res. 337, 1445-1451.

Sidebotham, R. L. (1974) Dextrans. Adv. Carbohydr. Chem. Biochem. 30, 371-444.

Smitinont, T., Tansakul, C., Tanasupawat, S., Keeratipibul, S., Navarini, L., Bosco, M. and Cescutti, P. (1999) Exopolysaccharide-producing lactic acid bacteria strains from traditional thai fermented foods: isolation, identification and exopolysaccharide characterization. Int. J. Food Microbiol. 51, 105-111.

Stoodley, P., Lewandowski, Z., Boyle, J. D. and Lappin-Scott, H. M. (1999). Structural deformation of bacterial biofilms caused by short-term fluctuations in fluid shear: an in situ investigation of biofilm rheology. Biotechnol. Bioeng. 65, 83-92.

Tsuchiya, H. M., Koepsell, H. J., Corman, J., Bryant, G., Bogard, M. O., Feger, V. H. and Jackson, R. W. (1952) The effect of certain cultural factors on production of dextransucrase by *Leuconostoc mesenteroides*. J. Bacteriol. 64, 521-526.

Van Tomme, S. R. and Hennink, W. E. (2007) Biodegradable dextran hydrogels for protein delivery applications. Expert Rev. Med. Devices. 4, 147-164.

Wang, J. and Somasundaran, P. (2007) Study of galactomannose interaction with solids using AFM, IR and allied techniques. J. Colloid Interf. Sci. 309, 373–383.

Williams, P. A. and Phillips, G. O. (2000) Introduction to food hydrocolloids. In G. O. Phillips and P. A. Williams (Eds.), Handbook of hydrocolloids, Cambridge: Woodhead. pp1-19.

Zakaria, M. B. and Rahman, Z. Ab. (1996) Rheological properties of cashew gum. Carbohydr. Polym. 29, 25-27.

Chapter 5

UV-mutagenesis of isolate *Pediococcus pentosaceus* (SPA) for enhancement of dextransucrase and dextran production

5.1 Introduction

Modification of the genetic structure of organisms to increase the product formation is one of the main features in microbial processes (Doelle, 1994). Mutations can be induced using chemicals or radiation. The overall cost of enzyme production and downstream processing is the major concern for successful application in an enzyme industry. Several methods have been devised to increase the enzyme yields to meet industrial requirements. Mutagenesis, either site-directed or random have paved the way to a great variety new enzyme preparations with improved catalytic efficiency and better stability towards temperature and oxidizing agents (Gupta *et al.*, 2002). UV light is stated to be lethal and mutagenic in a variety of organisms including bacteria (Witkin, 1976). Irradiation of cells with ultraviolet light (UV) induces the formation of several types of mutagenic DNA lesions *viz.* cyclobutane pyrimidine dimers (CPDs), the pyrimidine-pyrimidine (6-4) photoproducts, purine dimers and pyrimidine mono-adducts (Pfeifer *et al.*, 2005). Drugs like quinoline, spirocyclic and

naphyhydrine derivatives gave synergistic effects with UV radiation enhancing its mutagenic effect (Sideropoulos *et al.*, 1980).

UV-irradiation is universally used to induce genetically improved strains (Solaiman *et al.*, 2005). There are many evidences supporting the efficacy of UV-irradiation as a tool for strain improvement of micro-organisms. UV induced mutations in *Acidianus brierleyi* growing in a continuous stirred tank reactor generated a strain with improved chalcopyrite bioleaching capabilities, which was four-fold higher than the parent strain (Meng *et al.*, 2007). UV-mutation has been employed successfully with *Aspergillus niger* for the enhancement of citric acid productivity from beeet molasses and other unusual carbohydrate substrates (Pelechova *et al.*, 1990; Hamissa *et al.*, 1992). UV-mutation was performed on four halotolerant bacterial strains for the treatment of tannery soak liquor, however, no significant effect in degrading the soak liquor by the mutants was noticed (Sekar *et al.*, 2009). Tylosin is a macrolide antibiotic, being used as veterinary drug and growth promoter. Tylosin producing wild-type *Streptomyces fradiae* NRRL-2702 was UV irradiated to generate hyper producing mutants and 2.7 fold increase in tylosin production was observed. However, UV irradiation associated changes were found unstable with loss of tylosin activity on subsequent culturing (Khaliq *et al.*, 2009).

There are several reports on attempts to develop mutant strains for improved enzyme and biopolymer yield (Maw *et al.*, 2002). Wild type *Gongronella butleri* strain was mutagenized with UV irradiation and high chitosan yielding mutant strains which were selected through ELISA (Maw *et al.*, 2002). From an extracellular protease producing natural isolate of *Pseudomonas sp.* RAJR 044, a mutant JNGR

242 with 2.5 fold higher protease productivity was obtained by ultraviolet irradiation (Dutta and Banerjee, 2006). The fungal strain *Penicillium janthinellum* NCIM 1171 was subjected to Ethyl Methyl Sulfonate (EMS) treatment for 24h followed by UV-irradiation for 3 min and mutants showing enhanced cellulase production were raised (Adsul *et al.*, 2007). *Acremonium cellulolyticus* strain C-1 was subjected to UV-irradiation followed by *N*-methyl-*N*′nitro-*N*-nitrosoguanidine (NTG) mutagenesis and a mutant CF-2612 exhibiting higher cellulolytic enzymes was isolated (Fang *et al.*, 2009). *Aspergillus oryzae* IPT-301 was successfully mutated using UV irradiation at 253.7 nm and seven mutants with fructofuranosidase activity significantly higher than the parent culture were selected (Maresma *et al.*, 2010). There is plenty of literature available concerning the isolation and screening of microorganisms for enzyme production with high transfructosylating activity, but only few of them involved mutagenesis techniques. Classical mutagenesis with physical and/or chemical agents followed by titre test of a large number of isolates has been used successfully to improve the productivity of several fungal metabolites and enzymes (Bai *et al.,* 2004; Pandey *et al.*, 2000; Rubinder *et al.*, 2002). A α-amylase overproducing mutant of *Aspergillus oryzae*, obtained by random mutagenesis showed 6.73 times more dextrinizing and 5.13 times more saccharogenic activities than the parent strain (Azin and Noroozi, 2001). UV light mutagenesis is being conducted on *Aureobasidium pullulans* NRRL Y-6220 to produce higher yield of unpigmented and reasonably higher molecular weight pullulan (Sena *et al.*, 2006).

However, reports on ultraviolet (UV) mutation of lactic acid bacteria are very few. UV mutagenesis as a tool for strain improvement of *Lactobacillus delbrueckii*

NCIM 2365 was carried out and mutants showing faster growth rates, shorter lag phases, higher productivity and greater lactic acid yield were developed (Kadam *et al.*, 2006). Singh *et al.* (2009) developed high glucan producing *Leuconostoc dextranicum* NRRL B-1146 constitutive mutants by UV mutagenesis technique. Random mutagenesis using UV irradiation of the lactic acid bacteria was successful in enhancement of dextran production (Patel *et al.*, 2010).

Considering the multifarious utility of dextran, in the current work, the isolate *Pediococcus pentosaceus* screened from the soil of Assam was subjected to UV mutagenesis for enhancing the production of dextransucrase and dextran. Two novel mutants exhibiting higher dextransucrase activity and dextran production than the wild-type *Pediococcus pentosaceus* were obtained. The dextran structure of the mutant and the wild-type isolate was compared by Scanning Electron Microscopy.

5.2 Materials and methods

5.2.1 Microorganism and maintenance

The microorganism *Pediococcus pentosaceus* (Genbank Accession Number EU569832) used in this UV mutagenesis study was isolated from the soil sample collected from a sugarcane field of Assam (near Guwahati) as described in Section 2.2.2 of Chapter 2. *Pediococcus pentosaceus* is a microaerohilic and mesophilic lactic acid bacterium growing at 25°C. For maintenance and propagation of the culture, every two weeks, a loop of the isolate was transferred to modified MRS agar medium (Goyal and Katiyar, 1996) as stabs, grown for 16h and stored at 4°C.

5.2.2 UV-mutagenesis of *Pediococcus pentosaceus* and selection of mutants

A loopful from the culture of the natural isolate *Pediococcus pentosaceus*, maintained in modified MRS agar as stabs were inoculated in 10 ml modified MRS medium (Goyal and Katiyar, 1996) and grown at 25°C and 180 rpm for 12h. 1 ml from this culture was serially diluted in 9 ml of 0.85% saline solution until the required dilutions were obtained. 100 µl of the culture from the dilution factors 10^{-4},

10^{-5}, 10^{-6} and 10^{-7} were spread-plated on modified MRS agar (1.8%, w/v) Petri-plates. Each dilution factor was taken in duplicates for accuracy of results. The Petri-plates were exposed to UV radiations for 15s, 30s, 60s and 90s (Kadam *et al.*, 2006). Immediately after the UV irradiation, the plates were kept in the dark to avoid photo-reactivation (Maw *et al.*, 2002). The control and UV treated Petri-plates were incubated at 28°C for 36h. A total 35 larger colonies were selected from the different culture plates for the isolate that showed around 1% survival rate.

Survival rate was calculated from the following equations:

$$\text{Survival (\%)} = \frac{\text{Mean number of colonies developed after UV treatment}}{\text{Mean number of colonies in the control}} \times 100$$

5.2.3 Dextransucrase assay and dextran estimation

These colonies were analyzed for their enzyme assay. One loopful from each colony was grown in enzyme production medium (Tsuchiya *et al.*, 1952) at 25°C under shaking condition at 180 rpm for 24h. The cell free supernatants obtained by centrifugation at 10,000 rpm and 4°C for 10 min were analysed for dextransucrase activity using reducing sugar estimation (Nelson, 1944; Somogyi, 1945) as described in Section 3.2.3 of Chapter 3. Protein concentrations of the cell free supernatants were estimated (Lowry *et al.*, 1951) as described in Section 3.2.3 of Chapter 3. Specific activities of enzyme and percent increase in activity as compared to wild-type *Pediococcus pentosaceus* were calculated.

Dextran concentrations of the selected colonies were calculated. The isolate *Pediococcus pentosaceus* was grown in enzyme production medium (Tsuchiya *et al.*, 1952) at 25°C under shaking condition at 180 rpm for 24h. The supernatant obtained by centrifugation of the broth at 10,000 rpm for 10 min was collected. The carbohydrate content in the cell free supernatant of the natural isolate *Pediococcus pentosaceus* was determined by phenol-sulphuric acid method (Dubois *et al.*, 1956) in a micro-titre plate (Fox and Robyt, 1991). To 25 µl of sample containing dextran in a microtitre plate, 25 µl of 5% (v/v) phenol was added. The mixture was mixed by shaking the plate on a vortex mixer for 30 s. Then the plate was placed on an ice bath and 125 µl of concentrated sulphuric acid was added to each well containing the mixture and shaken for 30 s for proper mixing. The plate wrapped in cling film was incubated in water bath at 80^{o}C for 30 min. After cooling to room temperature, the absorbance was determined at 490 nm on a ELISA plate reader (Bio-Rad, model 680). A Standard graph was plotted using dextran (40 kDa) in the concentration range 0.1–1 mg/ml.

5.2.4 Purification of dextran from mutant SPAm1 and comparison of surface morphology with dextran of wild-type *Pediococcus pentosaceus*

The dextransucrase produced by the mutant SPAm1 and purified by 25% PEG-400 was used for dextran synthesis. 0.8 ml of this enzyme (0.22 mg/ml, 22.3 U/mg) was added to a total 10 ml of reaction mixture containing 5% sucrose as substrate in 20 mM sodium acetate (pH 5.4), 0.3 mM $CaCl_2$ and 15 mM sodium azide. The reaction was incubated at 28°C for 48h. The polymeric mass produced was centrifuged at 13,000g for 20 min and washed with 3 volumes of ethanol (90%, v/v)

thrice and resuspended in deionized water. The gel like mass was frozen at -20ºC and subsequently freeze dried at -51°C at a vacuum pressure of $35X10^{-3}$ mbar for 24h using a lyophilizer (Christ GmbH, model ALPHA 1-4 LD). The sample was used for surface morphology elucidation.

A little amount of lyophilised dextran from the mutant SPAm1 was applied to the SEM stub by means of an adhesive tape and coated with 10 nm Au in a sputter coater (Leo, model SCH 620). The surface of the sample was viewed in Scanning Electron Microscope (Leo, model 1330 VP) at magnification 1kx operated at 10.0 kV. The micrograph was compared with that of wild-type isolate *Pediococcus pentosaceus*.

5.3 Results and Discussion

5.3.1 UV-mutagenesis of the isolate *Pediococcus pentosaceus*

The colonies generated after UV irradiation of the isolate *Pediococcus pentosaceus* were enumerated by colony counter. It was observed that 30s and 60s exposure to UV radiation on the culture plates of 10^{-4}, 10^{-5} and 10^{-6} dilution, generated mutants with approximately 1% survival rate. It was observed that, the number of surviving colonies decreased with prolonged exposure time. In the 90s UV irradiated culture plates, no colonies developed (Table 5.3.1).

Table 5.3.1 Number of colonies of isolate *Pediococcus pentosaceus* obtained at different dilutions after UV exposure

UV exposure duration (s)	10^{-4}		10^{-5}		10^{-6}		10^{-7}	
	No.	Survival	No.	Survival	No.	Survival	No.	Survival
Control	2263	-	721	-	194	-	21	-
15	620	27	92	12.5	22	11	5	24
30	76	3.4	**11**	**1.5**	**4**	**2**	2	9.5
60	**25**	**1.1**	2	0.33	**1**	**0.51**	0	0
90	3	0.14	0	0	0	0	0	0

*Figures in bold are the number of colonies with survival rate selected.

The colonies of isolate *Pediococcus pentosaceus* appeared at 10^{-4} dilution without exposure and reduced survival with UV exposure are shown (Fig. 5.3.1, A and B).

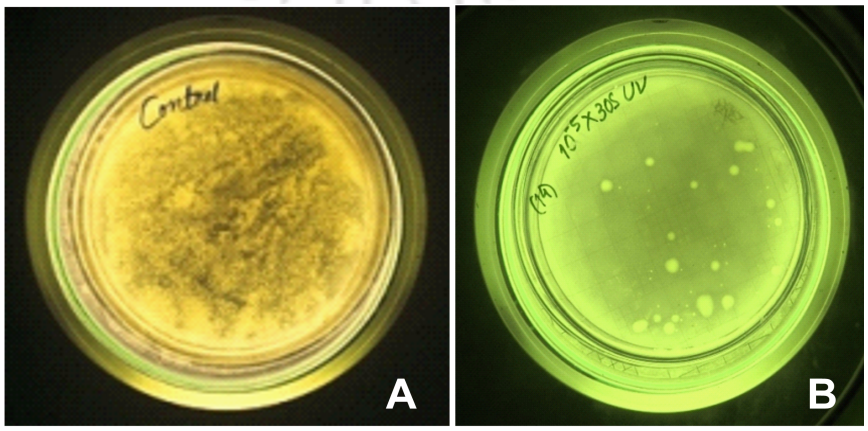


Fig. 5.3.1 (A). Colonies of wild-type isolate *Pediococcus pentosaceus* at dilution 10^{-4} without UV exposure as control (B). Mutant colonies of isolate *Pediococcus pentosaceus* at dilution 10^{-5} appeared after exposure to UV radiation for 30s.

From the selected plates of isolate *Pediococcus pentosaceus* having 41 colonies, 35 colonies were picked for enzyme assay (Table 5.3.2).

Strains	Enzyme activity (U/ml)	% increase	Strains	Enzyme activity (U/ml)	% increase
WT SPA	3.4	-	SPA18	2.9	-
SPA1	2.8	-	SPA19	3.1	-
SPA2	3.6	6	SPA20	3.9	15
SPA3	3.1	-	SPA21	3.5	3
SPA4	2.9	-	SPA22	3.2	-
SPA5	4.1	21	SPA23	3.7	9
SPA6	3.0	-	SPA24	3.0	-
SPA7	3.2	-	SPA25	2.8	-
SPA8	3.7	9	SPA26	3.1	-
SPA9*	4.9	**44**	SPA27	3.3	-
SPA10	2.8	-	SPA28	3.4	0
SPA11	2.7	-	SPA29	2.9	-
SPA12	3.1	-	SPA30	3.6	6
SPA13	4.0	18	SPA31	3.7	9
SPA14	2.7	-	SPA32	4.1	21
SPA15	3.0	-	SPA33	3.0	-
SPA16	3.8	12	SPA34	3.6	6
SPA17*	4.7	**38**	SPA35	3.2	-

Table 5.3.2 Enzyme activities of 35 selected mutants of isolate *Pediococcus pentosaceus*

*SPA9 and SPA17 were designated as SPAm1 and SPAm2 for convenience of reporting.

5.3.2 Selection of mutants

Out of the 35 mutants of isolate *Pediococcus pentosaceus*, 14 showed higher enzyme activity than the wild-type (Table 5.3.2). Some mutants showed decreased

enzyme activity in accordance with Gauss distribution (Pelechova *et al.*, 1990). The percent increase in dextransucrase activities of the 14 mutants were calculated (Table 5.3.2). Out of them, two mutants showed significant increase in enzyme activity and selected for propagation and further studies. The wild-type *Pediococcus pentosaceus* showed enzyme activity of 3.4 U/ml, whereas the mutant SPAm1 showed 4.9 U/ml and mutant SPAm2 showed 4.7 U/ml, exhibiting enhanced enzyme activity by 44% and 38%, respectively (Table 5.3.3). The specific activity of the mutant SPAm1 was 1 U/mg and that of SPAm2 was 0.94 U/mg, whereas the specific activity of the wild type SPA was 0.58 U/mg, exhibiting enhanced specific activity by 74% and 62%, respectively as compared to the wild-type *Pediococcus pentosaceus* (Table 5.3.3).

Table 5.3.3 Dextransucrase activity of mutants and wild-type isolate *Pediococcus pentosaceus*

Strains	E A* (U/ml)	% increase in E A	Protein (mg/ml)	S A * (U/mg)	% increase in S.A
WT SPA	3.4	-	5.9	0.58	-
SPAm1	4.9	44	4.9	1.0	74
SPAm2	4.7	38	5.0	0.94	62

*E A= Enzyme activity and S.A= Specific activity

The wild-type *Pediococcus pentosaceus* showed dextran concentration of 10.2 mg/ml, whereas the mutant SPAm1 showed 12.2 mg/ml and the mutant SPAm2 showed 11.3 mg/ml, exhibiting enhancement by 20% and 11%, respectively (Table 5.3.4).

Table 5.3.4 Dextran concentrations of mutants and wild-type *Pediococcus pentosaceus*

Strains	Dextran concentration (mg/ml)	% increase
WT SPA	10.2	-
SPAm1	12.2	20
SPAm2	11.3	11

5.3.3 Comparison of dextran surface morphology of the mutant and wild-type *Pediococcus pentosaceus*

The surface morphology of the dextran from the mutant SPAm1 and wild-type isolate *Pediococcus pentosaceus* was studied by scanning electron micrograph at magnification 1 kx. It was clearly observed that the pore sizes of the dextran of SPAm1 were larger than that of the wild-type isolate *Pediococcus pentosaceus* (Fig. 5.3.2).

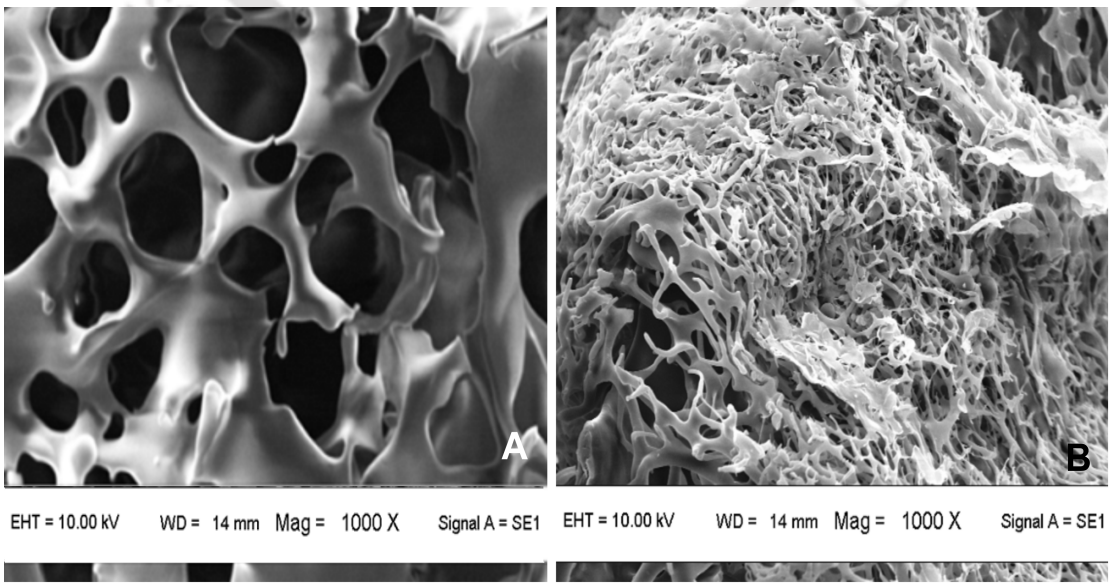


Fig. 5.3.2 Comparison of surface morphology of the (A) dextran produced by the mutant SPAm1 and (B) that of wild-type isolate *Pediococcus pentosaceus* as visualised under Scanning Electron Microscope at 1kx magnification.

5.4 Conclusions

Two mutants producing high dextransucrase and dextran were successfully obtained through UV mutagenesis of the wild-type *Pediococcus pentosaceus*. The wild-type *Pediococcus pentosaceus* exhibited dextransucrase activity of 3.4 U/ml, whereas the mutant SPAm1 exhibited 4.9 U/ml, accounting for a 44% increase. The mutant SPAm2 exhibited dextransucrase activity of 4.7 U/ml, accounting for a 38% increase over the wild-type strain. The wild-type *Pediococcus pentosaceus* exhibited dextran concentration of 10.2 mg/ml, whereas the mutant SPAm1 exhibited 12.2 mg/ml, accounting for a 20% increase. The mutant SPAm2 exhibited dextran concentration of 11.3 mg/ml, accounting for a 11% increase over the wild-type strain. The scanning electron micrographs revealed that the pore sizes of the purified dextran of the mutant SPAm1 were significanty larger than that of wild-type *Pediococcus pentosaceus*. These results justify the suitability of the *Pediococcus pentosaceus* mutant SPAm1 for commercial production of extracellular dextransucrase and dextran. However, the molecular basis of the enhanced yield from the mutant strains of *Pediococcus pentosaceus* needs to be investigated.

The meteoric rise in the usage of dextrans in food, pharmaceutical and cosmetics industries emphasises the importance strain improvement of the existing dextran

synthesizing lactic acid bacteria. UV-mutagenesis for scaling-up dextransucrase activity and dextran concentration can be considered as strategy towards achievement of this goal. The novel mutants can be potential candidates for commercial scale exploitation because of the increased enzyme activity and increased biopolymer synthesis attained.

References

Adsul, M. G., Bastawde, K. B. Varma, A. J. and Gokhale, D. V. (2007) Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulose production. Bioresour. Technol. 98, 1467-1473.

Azin, M. and Noroozi, E. (2001) Random mutagenesis and use of 2-deoxy-D-glucose as antimetabolite for selection of α-amylase-overproducing mutants of *Aspergillus oryzae*. World J. Microb. Biotech. 17, 747-750.

Bai, D. M.; Zhal, X. M.; Li, X. G. and Xu, S. M. (2004) Strain improvement of *Rhizopus oryzae* for over-production of L (+)-lactic acid and metabolic flux analysis of mutants. Biochem. Eng. J. 18, 41-48.

Doelle, H. W. (1994) Microbial Process Development, World Scientific Publishing Co. Pte. Ltd., Utopia Press, Singapore.

Dubois, M., Gilles, K. A., Hamilton, J. K. Rebers, P. A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356.

Dutta, J. R. and Banerjee, R. (2006) Isolation and characterization of a newly isolated *Pseudomonas* mutant for protease production. Braz. Arch. Biol. Technol. 49, 37-47.

Fang, X., Yano, S., Inoue, H. and Sawayama, S. (2009) Strain improvement of *Acremonium cellulolyticus* for cellulase production by mutation. J. Biosci. Bioeng. 107, 256-261.

Fox, J. D. and Robyt, J. F. (1991) Miniaturization of three carbohydrate analyses using microsample plate reader. Anal. Biochem. 19, 593-596.

Goyal, A. and Katiyar S. S. (1996) Regulation of dextransucrase productivity of *Leuconostoc mesenteroides* B-512F by the maintenance media. J. Gen. Appl. Microbiol. 42, 81-85.

Gupta, R., Beg, Q. K. and Lorenz, P. (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbiol. Biotechnol. 59, 15-32.

Hamissa, F. A., E1-Abyad, M. S., Abdu, A. and Gad, A. S. (1992) Raising potent UV mutants of *Aspergillus niger van*Tieghem for citric acid production from beet molasses. Bioresour. Technol. 39, 209-213.

Kadam, S. R., Patil, S. S., Bastawde, K. B., Khire, J. M. and Gokhale, D.V. (2006) Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. Proc. Biochem. 41, 120-126.

Khaliq, S., Akhtar, K., Ghauri, A. M., Iqbal, R., Khalid, A. M. and Muddassar, M. (2009) Change in colony morphology. Microbiol. Res. 164, 469-477.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Maw, T., Tan, T-K, Khor, E. and Wong, S-M (2002) Selection of *Gongronella butleri* strains for enhanced chitosan yield with UV mutagenesis. J. Biotech. 95, 189-193.

Maresma, B. G., Castillo, B. G.; Fernandez, R. C., da Silva, E. S., Maiorano, A. E. and de Andrade Rodrigues, M. F. (2010) Mutagenesis of *Aspergillus oryzae* IPT-301 to improve the production of β-fructofuranosidase. Brazilian J. Microbiol. 41, 186-195.

Meng, C., Shi, X., Lin, H., Chen, J. and Guo, Y. (2007) UV induced mutations in *Acidianus brierleyi* growing in a continuous stirred tank reactor generated a strain with improved bioleaching capabilities. Enz. Microb.Technol. 40, 1136-1140.

Nelson, N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153, 375-380.

Pandey, A., Nigam, P., Soccol, C.R., Soccol, V. T., Singh, D. and Mohan, R. (2000) Advances in microbial amylases. Biotechnol. Appl. Biochem. 31, 135-152.

Patel, S. and Goyal, A. (2010) Isolation, characterization and mutagenesis of exopolysaccharide synthesizing new strains of lactic acid bacteria. Int. J. Microbiol. (in press).

Pelechova, J., Petrova, L., Ujcova, E. and Martinkova, L. (1990) Selection of a hyperproducing strain of *Aspergillus niger* for biosynthesis of citric acid on unusual carbon substrates. Folia Microbiol. 35, 138-142.

Pfeifer, G. P., You, Y-H and Besaratinia, A. (2005) Mutations induced by ultraviolet light. Mut. Res. 571, 19-31.

Rubinder, K., Chadha, B. S., Singh, N., Saini, H. S. and Singh, S. (2002) Amylase hyperproduction by deregulated mutants of the thermophilic fungus *Thermomyces lanuginosus*. J. Ind. Microbiol. Biotechnol. 29, 70-74.

Sekar, S., Sivaprakasam, S. and Mahadevan, S. (2009) Investigations on ultraviolet light and nitrous acid induced mutations of halotolerant bacterial strains for the treatment of tannery soak liquor. Int. Biodet. Biodegr. 63, 176-181.

Sena, R. F., Costelli, M. C., Gibson, L. H. and Coughlin, R. W. (2006) Enhanced production of pullulan by two strains of *A. pullulans* with different concentrations of soybean oil in sucrose solution in batch fermentations. Braz. J. Chem. Eng. 23, 50-515.

Sideropoulos, A. S., Kacsuta, P. and Betz, M. K. (1980) Mutations induced by substituted quinolines in Ultraviolet-irradiated bacteria. Curr. Microbiol., 3, 207-212.

Singh, A., Majumder, A. and Goyal, A. (2009) Mutagenesis of *Leuconostoc dextranicum* NRRL B-1146 for higher glucan production. Int. J. Microbiol. 7(1).

Solaiman, E. A. M., Hegazy, W. K. and Moharam, M. E. (2005) Induction of overproducing alkaline protease *Bacillus* mutants through UV irradiation. Arab J. Biotech. 8, 49-60.

Somogyi, M. (1945) A new reagent for the determination of sugars. J. Biol. Chem. 160, 61-68.

Tsuchiya, H. M., Koepsell, H. J., Corman, J., Bryant, G., Bogard, M. O., Feger, V. H. and Jackson, R. W. (1952) The effect of certain cultural factors on production of dextransucrase by *Leuconostoc mesenteroides*. J. Bacteriol. 64, 521-526.

Witkin, E. M. (1976) Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. Bacteriol. Rev. 40, 869-907.

Chapter 6

Medium optimization for enhancement of dextransucrase production from the mutant *Pediococcus pentosaceus* (SPAm1) by Response Surface Methodology

6.1 Introduction

Dextransucrases are important class of enzymes with great industrial importance for their dextran biosynthesis capability. Dextrans are homopolysaccharides having various industrial applications, such as food additives, plasma substitute and tissue engineering (Patel *et al*., 2010). The biofunctionality of dextrans depend on the dextransucrases producing them. Considering the commercial importance of dextransucrases, there were several attempts to study and maximize their production (Purama and Goyal, 2008; Majumder *et al*., 2009a; Majumder *et al*., 2009b). Usually dextransucrases are produced by lactic acid bacteria *viz*. *Lactobacillus*, *Leuconostoc* and *Streptococcus*. Though, there is not any concrete evidence on dextran production ability of *Pediococcus* genus, Smitinont *et al*. (1999) emphasized on the possible production of dextransucrase by the *Pediococcus pentosaceus.* Media components were found to have great influence on extracellular enzyme production and are

specific for each microorganism (Oskouie *et al*., 2008). Therefore, the required constituents and their concentrations must be optimized accordingly.

The concentration of medium components and medium composition are crucial parameters that regulate enzyme activity (Tari *et al*., 2006). A number of optimization techniques could be used for this purpose (Oskouie *et al*., 2008). The Conventional "one factor at a time" measurement techniques are time consuming, labour intensive and often erroneous (Bandaru *et al*., 2006). Statistical approaches are most suitable for process optimization studies in biotechnology (Gupta *et al*., 2002). When many factors and interactions affect desired response, response surface methodology (RSM) is an effective statistical tool for optimizing the process by solving the multivariate equation (Puri *et al*., 2002). To enhance the production of dextransucrase, combinatorial interactions of medium components is a good strategy. Statistical methods such as Plackett-Burman Design, Central Composite and the Box-Behnken Design are commonly used methods (Liu and wang 2007; Majumder and Goyal 2008).

In the present study, the dextransucrase activity from the mutant of a new isolate of *Pediococcus pentosaceus* isolated from biodiversity hotspot Assam, India was optimized by modelling and analysis through Plackett-Burman and Central Composite Design. The effect of interaction between the three significant variables, sucrose, beef extract and Tween 80 on dextransucrase production was noted and their suitable concentration in the fermentation medium was optimized with response surface methodology.

6.2 Materials and Methods

6.2.1 Screening and maintenance of the isolate *Pediococcus pentosaceus* and generation of mutant SPAm1

The microorganism *Pediococcus pentosaceus* (Genbank Accession Number EU569832) used in this study was isolated from the soil sample collected from a sugarcane field of Assam (near Guwahati) as described in Section 2.2.2 of Chapter 2. The isolate was maintained as stab in modified MRS agar (containing 2% sucrose, w/v) (Goyal and Katiyar, 1996) at 4°C and sub-cultured every two weeks. Mutants of wild-type *Pediococcus pentosaceus* were generated by UV irradiation as described in Chapter 5. A mutant, SPAm1 showing significant increase in dextransucrase activity over the wild-type *Pediococcus pentosaceus* was studied for the medium optimization y statistical methods. From the mutant SPAm1 culture maintained as MRS agar stab at 4°C, 1 loopful was inoculated in the enzyme production medium described by Tsuchiya *et al.* (1952) and grown at 25°C at 180 rpm for 12h. This medium consisted of (%, w/v) sucrose, 2; yeast extract, 2; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.02; $MnSO_4.4H_2O$, 0.001; $FeSO4.7H_2O$, 0.001; $CaCl_2$, 0.001; NaCl, 0.001 and the pH was adjusted to 6.9. 1% of this broth was transferred to 250 ml Erlenmeyer flask containing 50 ml of statistically designed media and incubated at the above mentioned culture conditions for enzyme production.

6.2.2 Enzyme assay

1 ml of the sample from broth was withdrawn after 16h incubation and centrifuged at 10,000 rpm for 10 min at 4°C to remove the cells. The 1 ml assay mixture consisted of 5% sucrose in 20 mM sodium acetate buffer (pH 5.4) and 20 µl cell free supernatant containing enzyme. The reaction mixture was incubated at 30°C for 15 min and the reaction was terminated by adding 100 µl of Reagent D as described in Section 3.2.3 of Chapter 3, followed by boiling for 20 min. The reducing sugar content of the reaction mixture was estimated (Nelson, 1944; Somogyi, 1945). The dextransucrase activity was from the amount of released reducing sugar as described in Section 3.2.3 of Chapter 3. One unit of dextransucrase activity was defined as the amount of the enzyme resulting in the release of 1 µmole of reducing sugar per min at 30°C under the reaction conditions.

6.2.3 Experimental design and optimization

In order to characterize how the significant factors influence the responses, optimization of the medium composition was attempted by comparing different levels of crucial factors for dextransucrase production from the mutant SPAm1 of *Pediococcus pentosaceus*.

6.2.3.1 Plackett-Burman Design

Six medium components were selected for Plackett-Burman Design. Each of the 6 factors was examined in two levels: low level (-1) and high level (+1) (Plackett and Burman, 1946). The experiment designing was by Minitab statistical software (Minitab 15 English). The factors considered and their levels were shown in Table 6.3.1. The design matrix was presented in Table 6.3.2. 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 (dextransucrase 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 is useful for screening and evaluation of the important factors that influence the response, however, it does not describe the interaction among the factors. In this work, 6 variables were screened in 16 run orders. The experiments were carried out in duplicate and the averages of the dextransucrase activity were taken as response (Table 6.3.2). From the regression analysis the variables, which were significant at 90% level ($P<0.1$) were considered to have greater impact on dextransucrase production and were further optimized by a Central Composite Design. The experimental design and statistical analysis of the data were done by Minitab statistical software package.

6.2.3.2 Central Composite Design

The Central Composite Design (CCD) 2^3 full-factorial with three medium constituents, i.e. sucrose, beef extract and Tween 80 was generated by Minitab

statistical software (Minitab 15 English). In this study, the experimental plan consisted of 20 run orders. The experiments were carried out in duplicate and the averages of the dextransucrase activity were considered response. The relationships among the variables were determined by fitting the second-order polynomial equation to data obtained from the 20 experiments.

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

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, βij is the interaction coefficient. X_i is the factor variable in its coded form. Statistical analysis of data was performed to evaluate analysis of variance (ANOVA).

6.3 Results and Discussion

6.3.1 Screening of significant factors for dextransucrase production by Plackett-Burman Design

Among the nutrients, sucrose was selected as the carbon source as it is the substrate and inducer of dextransucrase production (Tsuchiya *et al*., 1952). The +1 level of sucrose taken was 5%. Different nitrogen sources, such as yeast extract, beef extract and peptone were chosen as they have been evidenced to significantly affect the dextransucrase production (Barker and Ajongwen, 1991). Lactic acid is known to

be a strong inhibitor for both cell growth and product formation in fermentation (Moldes *et al*., 2001). Maintenance of pH is important during lactic acid fermentation in order to provide the optimum pH to the organism to produce maximum product (Altaf *et al*., 2006). K_2HPO_4 was chosen as the buffering agent to arrest drastic fall in medium pH (Purama and Goyal, 2008). The surfactant Tween 80 was selected as it alters the membrane permeability and enhances the release of the enzyme (Goyal and Katiyar, 1997). On the basis of the calculated t and P-values (Table 6.3.3), sucrose, beef extract and Tween 80 was selected for further optimization of dextransucrase activity. All other variables used in all the trials were kept to the median level.

Interpretation of data in Table 6.3.2 revealed that there was an extreme variation in the dextransucrase activity in the 16 trials ranging from 0.30 U/ml to 10.3 U/ml. This variation can be attributed to the variable medium composition. Regression coefficients and t-value of the 6 ingredients were analysed (Table 6.3.3).

Variables with confidence levels greater than 90% were considered as significant. Sucrose and Tween 80 were significant and enhanced dextransucrase activity of the mutant SPAm1 of *Pediococcus pentosaceus*. Beef extract was found to be significant although with a negative coefficient.

Excluding the insignificant variables, the model equation for dextransucrase activity can be written as

$$Y = 3.6681 + 2.2319A - 0.9481C + 0.569F$$

Where, Y = Dextransucrase activity A = Sucrose, C = Beef extract and F = Tween 80

Table 6.3.1 Assigned concentrations of variables at different levels in Plackett-Burman Design for dextransucrase production

S.no.	Variable with designations	Lower level (-1) (%)	Higher level (+1) (%)
1	Sucrose (A)	1g	5g
2	Yeast extract (B)	1g	3g
3	Beef Extract(C)	1g	3g
4	Peptone (D)	1g	3g
5	K_2HPO_4 (E)	1g	3g
6	Tween 80 (F)	0.1ml	1ml

Table 6.3.2 Plackett-Burman Design for 6 variables with coded values along with the observed results for dextransucrase production.

Run Order	Sucrose	Yeast Extract	Beef Extract	Peptone	K_2HPO_4	Tween 80	Dextransucrase activity (U/ml)
1	-1	-1	-1	1	-1	1	2.7
2	-1	1	-1	1	1	-1	0.73
3	-1	-1	-1	-1	-1	-1	0.3
4	1	-1	1	1	-1	-1	3
5	-1	1	1	-1	-1	-1	0.61
6	1	-1	-1	-1	1	-1	7.4
7	1	1	-1	-1	-1	1	10.3
8	1	1	1	-1	1	-1	6.1
9	1	-1	1	-1	-1	1	3.8
10	-1	1	-1	-1	1	1	2.4
11	-1	-1	1	-1	1	1	1.9
12	-1	-1	1	1	1	-1	0.45
13	1	-1	-1	1	1	1	9.2
14	-1	1	1	1	-1	1	2.4
15	1	1	1	1	1	1	3.5
16	1	1	-1	1	-1	-1	3.9

Table 6.3.3 Statistical analysis of Plackett-Burman Design showing coefficient values, t and P-vale for each variable

Variable with designations	Levels (% w/v) (-1)	(+1)	Coefficient	t Stat	P-value
Sucrose (A)	1	5	2.2319	4.76	0.001
Yeast extract (B)	1	3	0.0744	0.16	0.877
Beef extract (C)	1	3	-0.9481	-2.02	0.074
Peptone (D)	1	3	-0.4331	-0.92	0.380
K_2HPO_4 (E)	1	3	0.2919	0.62	0.549
Tween 80 (F)	0.1	1	0.8569	1.83	0.101

6.3.2 Optimization of medium composition for dextransucrase production by Response Surface Methodology

At the end of screening experiments for different nutritional factors, a 2^3 full factorial Central Composite Design (CCD) was performed. The five levels of three crucial factors are given in Table 6.3.4. Twenty experiments were carried out from the design and responses are presented in Table 6.3.5.

Table 6.3.4 Experimental range and levels of independent variables

Variable	Symbol	Range and levels				
Sucrose (%, w/v)	X_1	0.48	1.5	3	4.5	5.523
Beef extract (%, w/v)	X_2	0.23	1.25	2.75	4.25	5.27
Tween 80 (%, v/v)	X_3	0.08	0.25	0.5	0.75	0.920

Table 6.3.5 Full factorial Central Composite Design matrix of 3 variables in uncoded units and the experimentally observed response

Run order	Sucrose (X_1)	Beef extract (X_2)	Tween 80 (X_3)	Dextransucrase Activity (U/ml)
1	3.0000	2.75000	0.50000	8.2
2	3.0000	2.75000	0.920448	9.3
3	3.0000	2.75000	0.079552	5.8
4	4.50000	1.25000	0.250000	12.3
5	4.50000	4.25000	0.750000	7.00
6	3.00000	2.75000	0.500000	8.1
7	3.0000	2.75000	0.50000	8.2
8	0.47731	2.75000	0.500000	2.10
9	3.00000	0.22731	0.500000	8.40
10	3.00000	2.75000	0.500000	8.2
11	1.50000	4.25000	0.250000	2.10
12	4.50000	1.25000	0.750000	13.5
13	3.00000	2.75000	0.500000	8.1
14	3.00000	5.27269	0.500000	4.90
15	5.52269	2.75000	0.500000	10.6
16	1.50000	4.25000	0.750000	3.00
17	1.50000	1.25000	0.750000	3.75
18	3.00000	2.75000	0.500000	8.2
19	4.50000	4.25000	0.250000	8.60
20	1.50000	1.25000	0.250000	2.30

The ANOVA is used to test the statistical significance of the ratio of mean square due to regression and mean square due to residual error (Kalavathy *et al*., 2009). The results of the second order response surface model fitting in the form of ANOVA are given below in Table 6.3.6.

Table 6.3.6 ANOVA for Dextransucrase activity

Source	DF	SS	MS	F-value	Prob. (P) > F
Model	9	194.956	21.6618	25.47	0.000
Residual (error)	10	8.505	0.8505	-	-
Lack of fit	5	8.492	16984	636.9	0.000
Pure error	5	0.013	0.0027	-	-
Total	19	203.461	-	-	-

R^2 = 95.8%; Adj R^2 =92.06%.
SS, sum of squares; DF, degrees of freedom; MS, mean square

To test the fit of the model the regression equation and determination coefficient R^2 were evaluated. The model presented a high R^2 value of 95.8% and adjusted R^2 value of 92.06 for dextarnsucrase activity. The high value of determination coefficient (0.958) indicates that only about 4.2% of the total variations were not satisfactorily explained by the model.

In general, the Fischer's F value with a low probability P-value indicates the high significance of the regression model. The significance of the regression coefficient of the parameter can be verified by the Student's t-test, while P values signify the pattern of interaction among the factors. The larger the value of *t* and smaller the P value, the more significant is the corresponding coefficient term. Generally P values lower than 0.001 indicates that the model is considered to be statistically significant at the 99% confidence level (Kalavathy *et al*., 2009). The ANOVA of quadratic regression models for dextransucrase activity demonstrated that the model is highly significant, and is evident from the Fisher's F-test with a very low

probability value [(Pmodel > F = 0.0000)]. The significance of each coefficient was determined by t and P-values which are listed below in Table 6.3.7.

Table 6.3.7 Model coefficient of dextransucrase activity estimated by multiple linear regression

Model Term	Coefficient	Standard Deviation	t-value	P-value
Intercept	8.1732	0.3761	21.729	0.000
X_1	3.2617	0.2496	13.070	0.000
X_2	-1.2475	0.2496	-4.070	0.001
X_3	0.5738	0.2496	2.299	0.044
X_1^2	-0.6848	0.2429	-2.819	0.018
X_2^2	-0.5787	0.2429	-2.382	0.038
X_3^2	-0.2605	0.2429	-1.072	0.309
X_1X_2	-1.1562	0.3261	-3.546	0.005
X_1X_3	-0.3437	0.3261	-1.054	0.317

X_2X_3	-0.4187	0.3261	-1.284	0.228

The coefficients of regression for the dextransucrase activity were calculated and the following regression equation was obtained.

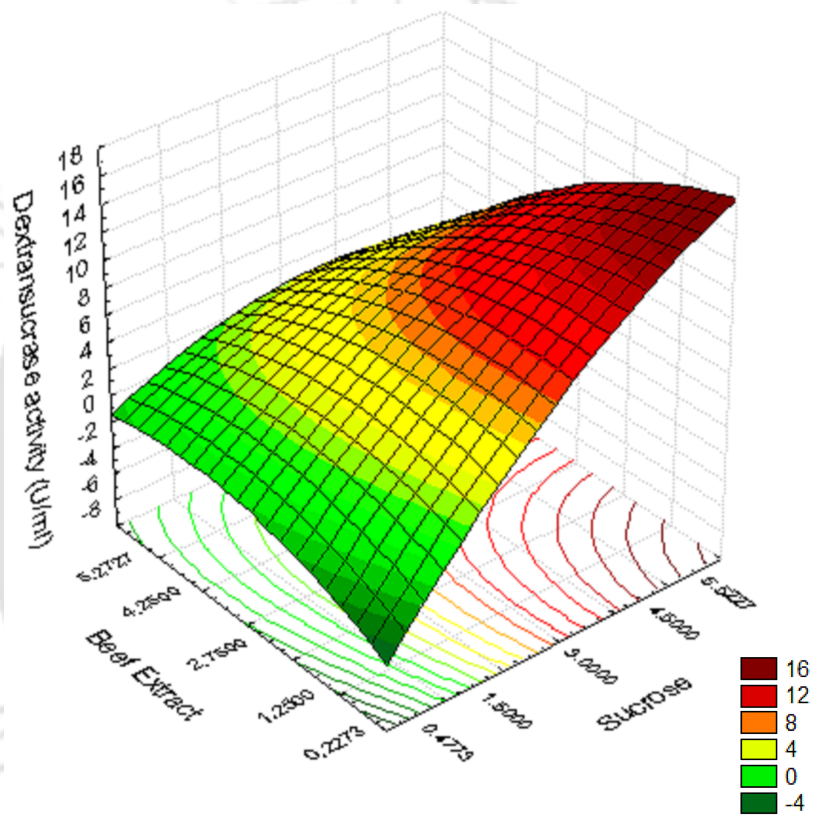
$$Y \text{ activity} = 8.1732 + 3.2617X_1 - 1.2475X_2 + 0.5738X_3 - 1.1562X_1X_2 - 0.3437X_1X_3 - 0.4187X_2X_3 - 0.6848X_1^2 - 0.5787X_2^2 - 0.2605X_3^2$$

Where, X_1 = Sucrose, X_2 =Beef extract and X_3 = Tween 80

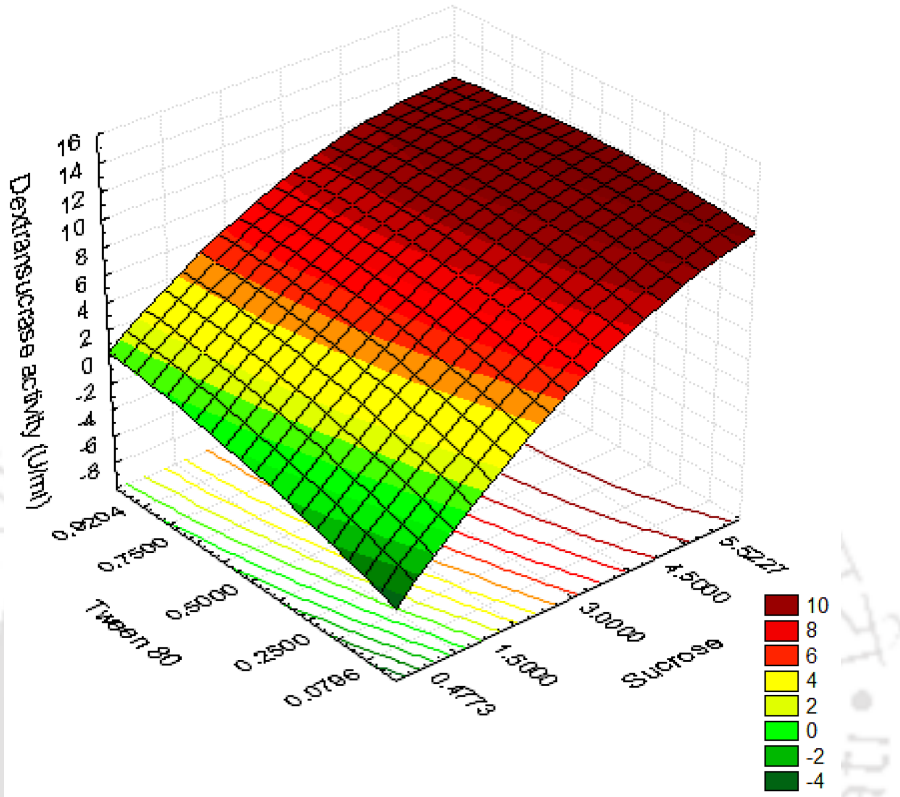
All three interactions, X_1X_2 (Sucrose. beef extract), X_1X_3 (Sucrose. Tween 80) and X_2X_3 (beef extract. Tween 80) have a negative coefficient. The small P-value (0.005) of the interaction between sucrose and beef extract (X_1X_2) shows that there is a very strong interaction between these two parameters. The possibility of maximum dextransucrase activity at high concentration of sucrose and low concentrations of

beef extract can be inferred. The surface plots and 2D contour plots are the graphical representations of the regression equation illustrating the function of two factors at a time, holding other factors at a fixed level (Kalavathy *et al*., 2009). The plots were generated by STATISTICA software and presented in Fig. 6.3.1 (A-C).

(A)



(B)



(C)

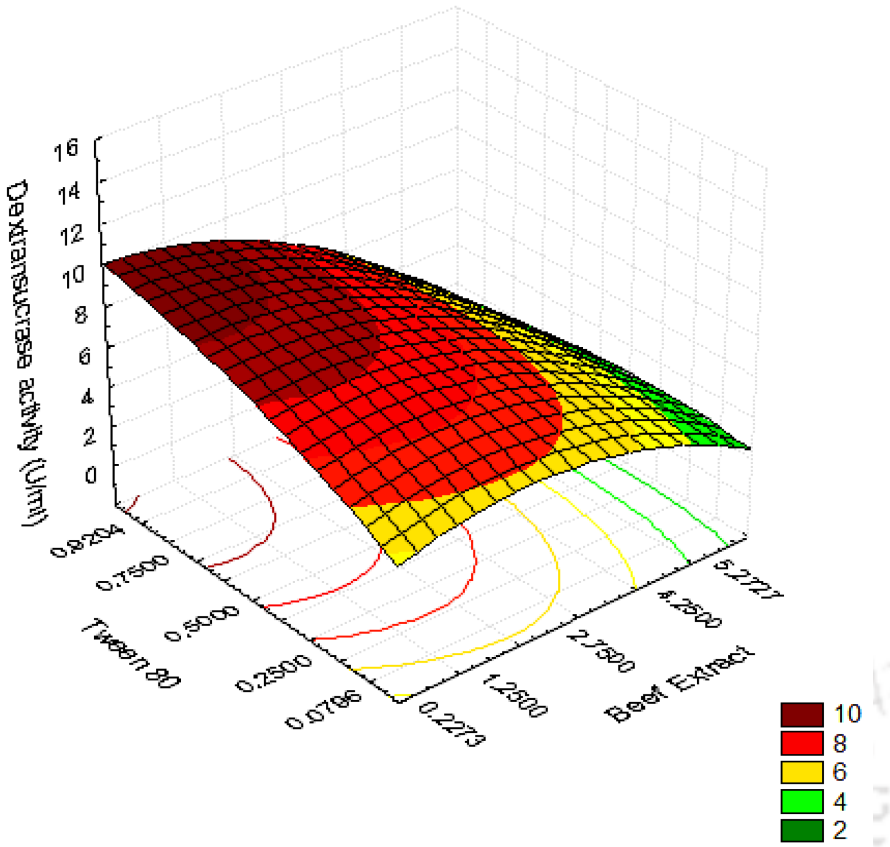


Fig. 6.3.1 Surface and Contour plot of the combined effects of the significant factors on dextransucrase activity of *Pediococcus pentosaceus*. (A) Sucrose and Beef extract (Fixed level: Tween 80 = 0.5) (B) Sucrose and Tween 80 (Fixed level: beef extract = 2.75) (C) Beef extract and Tween 80 (Fixed level: Sucrose = 3)

From the contour and surface plots, the interactions between two nutrients and also their optimum levels are conveniently illustrated (Majumder and Goyal, 2008). The surface plot depicting the interaction of sucrose and Tween 80 given in Fig. 6.3.1 (B) indicated that there is good interaction among the independent variables. The concentration of ingredients for the medium selected from Minitab statistical software were as follows: sucrose, (5.52% w/v), beef extract (0.23% w/v) and Tween 80

(0.83%, v/v). The maximum dextransucrase activity predicted using the above selected variables was 15.9 U/ml and that obtained experimentally was 15.6 U/ml showing a perfect agreement (Fig. 6.3.2).

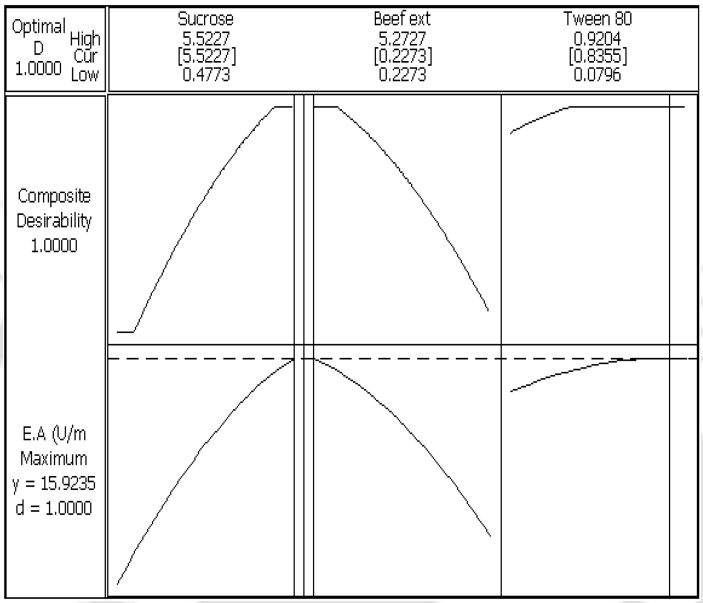


Fig. 6.3.2 Response optimization plot for dextransucrase activity of *Pediococcus pentosaceus* showing the optimum concentrations of the significant factors and the predicted dextransucrase activity.

The dextransucrase activity of wild-type isolate *Pediococcus pentosaceus* was 3.4 U/ml, whereas it was 4.9 U/ml for SPAm1 in unoptimized medium. Medium optimization of SPAm1,enhanced the enzyme activity to a significantly high 15.6 U/ml. There was 218% increase in dextransucrase yield by SPAm1 in optimized medium when compared to that in unoptimized medium. There was 358% increase in dextransucrase yield by SPAm1 in optimized medium when compared to that of wild-type isolate *Pediococcus pentosaceus* (Table 7.3.8).

Table 7.3.8 Comparison of dextransucrase activity from wild type *Pediococcus pentosaceus*, mutant SPAm1 in the unoptimized medium and in optimized medium.

Strains	Dextransucrase activity (U/ml)	Percent increase
Wild-type *Pediococcus pentosaceus*	3.4	-
Mutant SPAm1 in unoptimized medium	4.9	44
Mutant SPAm1 in optimized medium	15.6	358

6.4 Conclusions

The application of statistical design for screening and optimization of culture conditions allows quick identification of the crucial factors and mutual interactions existing among them. The essential step in the use of statistical experimental designing is to select the suitable ranges of the selected control factors for the initial experiments. The eventual objective of Response surface method is to determine the optimum operating conditions for the process. The dextransucrase activity predicted by the model at optimal conditions agreed fittingly with experimental data (R^2 = 95.8) thus confirming the model validity. The results indicated that this mutant of the natural isolate of *Pediococcus pentosaceus* can be used in industries owing to their high dextransucrase activity. By mere increase in sucrose concentration in the original enzyme production medium (55.2 g/L), and enrichment of the medium with small concentrations of beef extract (2.3 g/L) and Tween 80 (8.3 ml/L), the dextransucrase activity can be scaled up significantly. Response optimization projected a medium containing (g/L) sucrose, 55.2; yeast extract, 20; Beef extract, 2.3, 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 and Tween 80, 8.3 (ml/L) to be optimum for the production of dextransucrase by the mutant SPAm1.This medium predicted an enzyme activity of 15.9 U/ml which was experimentally validated and dextransucrase activity of 15.6 U/ml was obtained. Thus, the statistically-based experimental designs were found suitable tools to enhance the dextransucrase production by the mutant *Pediococcus pentosaceus* (SPAm1).

References

Altaf, Md., Naveena, B. J., Venkateshwar, M., Vijay Kumar, E. and Reddy, G. (2006) Single step fermentation of starch to 1(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using inexpensive nitrogen sources to replace peptone and yeast extract – Optimization by RSM. Proc. Biochem. 41, 465-472.

Barker, P. E. and Ajongwen N. J. (1991) The production of the enzyme dextransucrase using nonaerated fermentation techniques. Biotechnol. Bioeng. 37, 703-707.

Bandaru, V. V. R., Somalanka S. R., Menduc D. R., Madicherla N. R. and Chityala A. (2006) Optimization of fermentation conditions for the production of ethanol from sago starch by co-immobilized amyloglucosidase and cells of *Zymomonas*

mobilis using response surface methodology. Enz. Microb. Technol. 38, 209-214.

Goyal, A. and Katiyar, S. S. (1996) Regulation of dextransucrase productivity of *Leuconostoc mesenteroides* B-512F by the maintenance media. J. Gen. Appl. Microbiol. 42, 8185.

Goyal, A. and Katiyar, S. S. (1997) Effect of certain nutrients on the production of dextransucrase from *Leuconostoc mesenteroides* B-512F. J. Basic Microbiol. 37,197-204.

Gupta, R., Beg, Q. K. and Lorenz, P. (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microb. Biotechnol. 59, 1532.

Kalavathy, H. M., Regupathi, I., Pillai, M. G. and Miranda L. R. (2009) Modelling, analysis and optimization of adsorption parameters for H3PO4 activated rubber wood sawdust using response surface methodology (RSM). Colloids and Surfaces B: Biointerfaces 70, 35-45.

Liu, G-C and Wang, X-L (2007) Optimization of critical medium components using response surface methodology for biomass and extracellular polysaccharide production by *Agaricus blazei*. Appl. Microbiol. Biotechnol. 74, 78-83.

Nelson, N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153, 375-380.

Majumder, A. and Goyal, A. (2008) Enhanced production of exocellular glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 using response surface method. Bioresour. Technol. 99, 3685-3691.

Majumder, A., Singh, A. and Goyal, A. (2009a) Application of response surface methodology for glucan production from *Leuconostoc dextranicum* and its structural characterization. Carbohydr. Polym. 75, 150-156.

Majumder, A., Bhandari, S., Purama, R. K., Patel, S. and Goyal, A. (2009b) Enhanced production of a novel dextran from *Leuconostoc mesenteroides* NRRL B-640 by Response Surface Methodology. Annals Microbiol. 59, 1-7.

Moldes, A. B., Alonso, J. L., Parajo, J. C. (2001). Strategies to improve the bioconversion of processed wood into lactic acid by simultaneous saccharification and fermentation. J. Chem. Technol. Biotechnol. 76, 279-284.

Oskouie, S. F. G., Tabandeh, F., Yakhchali, B. and Eftekhar, F. (2008) Response surface optimization of medium composition for alkaline protease production by *Bacillus clausii*. Biochem. Eng. J. 39, 37-42.

Patel, S., Majumder, A. and Goyal, A. (2010) Potentials of exopolysaccharides from lactic acid bacteria. Ind. J. Microbiol. (in press).

Plackett, R. L. and Burman, J. P. (1946) The design of optimum multifactorial experiments Biometrika. 33, 305-325.

Purama, R. K. and Goyal, A. (2008) Screening and optimization of nutritional factors for higher dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640 using statistical approach. Bioresour. Technol. 99, 7108-7114.

Puri, S., Beg, Q. K. and Gupta, R. (2002) Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. Curr. Microbiol. 44, 286-290.

Smitinont, T., Tansakul, C., Tanasupawat, S., Keeratipibul, S.,Navarini, L., Bosco, M. and Cescutti, P. (1999) Exopolysaccharide-producing lactic acid bacteria strains from traditional thai fermented foods: isolation, identification and exopolysaccharide characterization. Int J. Food Microbiol. 51, 105-111.

Somogyi, M. (1945) A new reagent for determination of sugars. J. Biol. Chem. 160, 61-68.

Tari, C., Genckal, H and Tokatli, F. (2006) Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. Proc. Biochem. 41, 659-665.

Tsuchiya, H. M., Hellman, N. N., Koepsell, H. J., Corman, J., Stringer, C. S., Rogovin, S. P., Bogard, M. O., Bryant, G., Feger V. H., Hoffman C. A., Senti F. R. and Jackson, R.W. (1952) Factors affecting molecular weight of enzymatically synthesized dextran. J. Am. Chem. Soc. 77, 412-2419.

Chapter 7

Statistical optimization of medium for maximizing dextran production from mutant *Pediococcus pentosaceus* (SPAm1)

7.1 Introduction

The concentration of medium components and medium composition are crucial parameters that regulate enzyme activity and metabolite production. The direct and traditional measurement techniques as 'one factor at a time' used for optimization of multivariable system is time consuming, labour intensive and prone to erroneous data (Majumder *et al*., 2009a). Statistical approaches such as Plackett-Burman Design and Central Composite Design by Response surface methodology (RSM) are commonly used methods to optimize culture medium, enzyme synthesis, aqueous two phase separation of proteins and biopolymer production (Liu and Wang, 2007; Majumder and Goyal, 2008).

Response surface methodology has been employed for optimization of the fermentation conditions for pullulan production by the strain *Aureobasidium pullulans* SK1002 in shaking flask cultures and significant enhancement in pullulan production was achieved (Jiang, 2010). The cultural conditions for xanthan gum production by

Xanthomonas campestris were optimized by response surface methodology (Psomas *et al*., 2007; Salah *et al*., 2010). Gellan production by *Sphingomonas paucimobilis* ATCC-31461 was optimized applying response surface methodology (Banik *et al.*, 2007). Response Surface Methodology has been used as a strategy to optimize a novel glucan production from *Leuconostoc dextranicum* NRRL B-1146 (Majumder *et al.*, 2009a). The results of these experiments indicated that response surface methodology was a promising method for optimization of microbial biopolymers production.

Dextrans are used as food syrup stabilizers, matrix of chromatography columns, plasma substitute, antithrombogenic agent, biomaterials, paper and metal-plating processes, oil recovery (Majumder and Goyal, 2008; Purama *et al*., 2009; Patel *et al*., 2010). Porous dextran scaffolds have tissue engineering applications as drug delivery vehicles (Levesque *et al*., 2005). Majumder *et al.* (2009b) have enhanced production of a novel dextran from *Leuconostoc mesenteroides* NRRL B-640 using Response Surface Methodology.

In this work, efforts have been made to enhance dextran production from the mutant SPAm1 of a new isolate of *Pediococcus pentosaceus* (Genbank Accession Number EU569832) by statistical optimization of the medium. Plackett-Burman Design was used to screen the most significant factors and Central Composite Design was used to investigate their interactive effects, study the nature of the response surface and predict the optimum medium composition.

7.2 Materials and Methods

7.2.1 Screening, maintenance of isolate *Pediococcus pentosaceus* and generation of its mutant SPAm1

The isolate *Pediococcus pentosaceus* (SPA) (Genbank Accession Number EU569832) was screened from the soil sample collected from a sugarcane field of Assam (near Guwahati) as described in Section 2.2.2 of Chapter 2. The isolate was maintained as stab in modified MRS agar (Goyal and Katiyar, 1996) (containing 2% (w/v) sucrose) at 4°C and sub cultured every 2 weeks. The modified MRS medium for the bacterial stab contained in (w/v, %): Sucrose, 2; Yeast extract, 0.5; Beef extract, 1; Peptone, 1; K_2HPO_4, 0.2; Sodium acetate, 0.5; Tri-Ammonium citrate, 0.2; Tween 80, 0.1; $MgSO_4 \cdot 7H_2O$, 0.02; $MnSO_4 \cdot 4H_2O$, 0.02. The pH of the medium was adjusted to 6.4 using 1 N HCl before autoclaving.

Mutants of SPA were generated by UV exposure to different time intervals as described in Chapter 5. A mutant, SPAm1 showing significant increase in dextran concentration over the wild-type SPA, was further studied for higher dextran production by medium optimization using statistical methods.

7.2.2 Inoculum preparation

From the mutant culture maintained as MRS agar stab in 4°C, 1 loopful was inoculated in the 5 ml enzyme production medium described by Tsuchiya *et al.* (1952) and grown at 25°C at 180 rpm for 12h. This medium consists of (%, w/v) sucrose, 2; yeast extract, 2; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0.02; $MnSO_4.4H_2O$, 0.001; $FeSO4.7H_2O$, 0.001; $CaCl_2$, 0.001; NaCl, 0.001 and the pH of medium was adjusted to 6.9. 1% of this broth was transferred to 250 ml Erlenmeyer flask containing 50 ml of statistically designed medium with variable composition and incubated at the above mentioned culture conditions for dextran production.

7.2.3 Estimation of dextran concentration

The carbohydrate contents of the cell free supernatant of the *Pediococcus pentosaceus* mutant (SPAm1) grown in statistically optimized and unoptimized enzyme production medium (Tsuchiya *et al*., 1952) were determined by phenol-sulphuric acid method (Dubois *et al*., 1956) using a micro-titre plate (Fox and Robyt, 1991). To 25 µl of sample containing dextran in a microtitre plate, 25 µl of 5% (v/v) phenol was added and mixed by shaking the plate on a vortex mixer for 30 s. Then the plate was placed on an ice bath and 125 µl of concentrated sulphuric acid was added to each well containing the mixture. The plate was again shaken for 30 s to ensure proper mixing of the contents of the wells. Then the plate was wrapped in cling film and incubated in water bath at 80^{o}C for 30 min. After cooling to room temperature, the absorbance was determined at 490 nm on a ELISA plate reader (Bio-Rad, model 680). A Standard graph was plotted using dextran (40 kDa) in the concentration range 0.1-1 mg/ml.

7.2.4 Optimization procedure and experimental design

Statistical experimental designs were applied in two steps. The first step was to identify the significant nutrients for maximum production of dextran using Plackett-Burman Design (PBD) (Plackett and Burman, 1946) and the second was to optimize the significant nutrients resulted from PBD by using a Central Composite Design (CCD) in response surface methodology (RSM). The experimental design and statistical analysis of the data were done by using Minitab statistical software (Majumder *et al*., 2009a).

7.2.4.1 Plackett-Burman Design

Five medium components were selected for Plackett-Burman design *viz.* sucrose, yeast extract, K_2HPO_4, Tween 80 and $CaCl_2$. Among the nutrients, sucrose as the carbon source (Tsuchiya *et al*., 1952), yeast extract as the nitrogen source, (Majumder *et al*., 2009a), K_2HPO_4 as the buffering agent, the surfactant Tween 80 as the modulator of membrane permiability (Goyal and Katiyar, 1997) and $CaCl_2$ as the stimulator of dextransucrase (Lopez and Monsan, 1980). Each of the 5 factors was examined in two levels: low level (-1) and high level (+1) (Plackett and Burman, 1946). The factors considered and their levels were shown in Table 7.3.1. The design

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

matrix was presented in Table 2. Plackett-Burman experimental design is based on the first order polynomial model:

Where, Y is the response (Dextran concentration), 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 is useful for screening and evaluation of the key factors that influence the response. In this work, 5 variables were screened in 16 run orders. The experiments were carried out in duplicate and the averages of the dextran concentration were taken as response (Table 7.3.2.). From the regression analysis the variables, which were significant at 90% level ($P < 0.1$) were considered to have greater impact on dextran production and were further optimized by a Central Composite Design (Majumder *et al*., 2009a). The experimental design and statistical analysis of the data were done by Minitab statistical software package (Qader *et al*., 2008).

7.2.4.2 Central Composite Design

The 2^2 full-factorial Central Composite Design (CCD) with two medium constituents, i.e. sucrose and yeast extract was generated by Minitab statistical software. In this study, the experimental plan consisted of 13 run orders. The experiments were carried out in duplicate and the averages of the dextran concentration were taken as the response. The relationships among the variables were

determined by fitting the following second-order polynomial equation to the data obtained from 13 experiments.

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

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, βij is the interaction coefficient. Xi is the factor variable in its coded form.

The data were analyzed statistically by ANOVA method. P-values below 0.1 were regarded as statistically significant.

7.3 Results and Discussion

7.3.1 Screening of significant factors for dextran production by Plackett-Burman Design

Among the nutrients, sucrose was selected as the carbon source as it is the substrate and inducer of dextransucrase production (Tsuchiya *et al*., 1952). The +1 level of sucrose taken was 6%. Yeast extract was chosen as the organic nitrogen source, as it has been reported to enhance dextransucrase production significantly, due to other nutrients and growth enhancers present in it (Barker and Ajongwen, 1991; Zhang *et al*., 2009). K_2HPO_4 was chosen as the buffering agent to maintain medium pH for maximum product synthesis (Altaf *et al*., 2006; Purama and Goyal, 2008). The surfactant Tween 80 was selected as it was evidenced to stimulate the production of extracellular glucosyltransferase by acting either directly or indirectly at the level of enzyme synthesis (Wittenberger *et al*., 1978). Tween 80 is also reported to alter the membrane permeability and enhance the release of extracellular enzyme (Goyal and Katiyar, 1997). The specific activity of dextransucrase was greatly enhanced by addition of $CaCl_2$ to the purified enzyme, so it was chosen as a variable for Plackett-Burman Designing (Lopez and Monsan, 1980).

Table 7.3.1 Assigned concentrations of variables at different levels in Plackett-Burman Design for dextran production

S. No.	Variable with designations	Lower level (-1) (%)	Higher level (+1) (%)
1	Sucrose (A)	1	6
2	Yeast extract (B)	1	3
3	K_2HPO_4 (C)	1	3
4	Tween 80 (D)	0.1ml	1ml
5	$CaCl_2$ (E)	0.0002	0.002

Table 7.3.2 Plackett-Burman design for 5 variables with coded values along with the observed results for dextran concentrations

Run Order	Sucrose	Yeast Extract	K_2HPO_4	Tween 80	$CaCl_2$	Dextran (mg/ml)
1	1	-1	-1	1	1	15.6
2	-1	-1	1	-1	-1	5.2
3	1	-1	-1	-1	-1	16.5
4	-1	1	-1	-1	-1	2.9
5	-1	1	-1	1	1	3.0
6	-1	1	1	-1	1	4.2
7	1	1	-1	1	-1	11.5
8	-1	1	1	1	-1	3.0
9	1	-1	1	1	-1	16.8
10	1	1	1	-1	-1	13.7
11	-1	-1	1	1	1	5.4
12	-1	-1	-1	-1	1	5.6
13	1	-1	1	-1	1	21.7
14	1	1	1	1	1	12.2
15	1	1	-1	-1	1	11.8
16	-1	-1	-1	1	-1	7.2

The interpretation of data in Table 7.3.2 revealed that there was an extreme variation in the dextran concentration in the 16 trials ranging from 2.9 mg/ml to 21.7 mg/ml. This variation can be attributed to the variable medium composition. Regression coefficients of the 5 ingredients were analysed below in Table 7.3.3.

Table 7.3.3 Statistical analysis of Plackett-Burman Design showing coefficient, T and P values for each variable

Variable	Coefficient	T	P
Intercept	9.762	22.81	0.000
Sucrose (A)	5.212	12.16	0.000
Yeast extract(B)	-1.987	-4.63	0.001
K_2HPO_4 (C)	0.500	1.18	0.264
Tween 80(D)	-0.438	-1.01	0.338
$CaCl_2$ (E)	0.175	0.39	0.702

Lower the P-value, higher the significance of the variable (Majumder *et al*., 2009a). Sucrose and Yeast extract showing P value 0.000 and 0.001, respectively, are highly significant. Variable sucrose and Yeast extract have confidence levels greater than 95% as revealed by Pareto chart, so considered significant. Sucrose having coefficient 5.212 was positively significant. It's presence in the medium in higher concentration enhanced dextran production from the mutant SPAm1. Yeast extract having coefficient -1.987 was found negatively significant. It's presence in the medium in lower concentration is stimulating dextran production.

Excluding the insignificant variables, the model equation for dextran concentration after screening can be written as:

$$Y = 9.762 + 5.212A - 1.987B$$

Where Y= Dextran concentration, A = Sucrose and B = Yeast extract

Table 7.3.4 ANOVA of Plackett-Burman Design

Source	DF	SS	MS	F-value	P value
Main effects	5	505.48	101.095	34.36	0.000
Residual (error)	10	29.24	2.924	-	-
Total	15	534.72	-	-	-

On the basis of the significant coefficients (Table 7.3.3) and ANOVA result (Table 7.3.4), sucrose and Yeast extract were selected for further medium optimization to maximize dextran production. All other variables used in all the trials were kept to the median level.

7.3.2 Optimization of medium composition for dextran production by Response Surface Methodology

At the end of the screening experiments for different nutritional factors, the conditions were optimized by Central Composite Design (CCD). Taking five levels of both the significant factors thirteen experiments were carried out from the design (Table 7.3.5).

Table 7.3.5 Full factorial Central Composite Design matrix of two variables in uncoded units and the experimentally observed response

Run order	Sucrose (%, w/v) X_1	Yeast extract (%, w/v) X_2	Dextran concentration (mg/ml)
1	3.0	2.75	15.9
2	3.0	2.75	15.8
3	1.5	1.25	8.6
4	4.5	4.25	23.8
5	3.0	4.865	11.3
6	0.885	2.75	6.8
7	5.115	2.75	28.3
8	3.0	2.75	15.8
9	3.0	0.635	16.2
10	4.5	1.25	35.7
11	1.5	4.25	7.9
12	3.0	2.75	15.9
13	3.0	2.75	15.8

The results of the second order response surface model fitting in the form of ANOVA are given in Table 7.3.6.

Table 7.3.6 ANOVA of Central Composite Design for dextran concentration

Source	DF	SS	MS	F-value	Prob. (P) > F
Model	5	773.809	65.7429	23.40	0.000
Residual (error)	7	46.304	1.138	-	-
Lack of fit	3	46.292	2.2767	5143.50	0.000
Pure error	4	0.0120	0.0000	4.74	-
Total	12	-	-	-	-

R^2 = 94.35%; Adj R^2= 90.32%.

To test the fit of the model, the regression equation and determination coefficient R^2 were evaluated. The model presented a high R^2 value of 94.35% and adjusted R^2 value of 90.32%.

The ANOVA of quadratic regression models for dextran concentration demonstrated that the model is highly significant, and is evident from the Fisher's F-test with a very low probability value [(Pmodel > F = 0.0000)]. The significance of each coefficient was determined by t-values and P-values which are listed in Table 7.3.7.

Table 7.3.7 Model coefficient of dextran concentration estimated by multiple linear regression

Model Term	Coefficient	Standard error of Coefficient	T-value	P-value
Intercept	27.2171	15.936	1.708	0.131
X_1	24.5989	14.587	1.686	0.136
X_2	-25.3429	14.105	-1.797	0.115
X_1^2	7.5709	4.362	1.736	0.126
X_2^2	-0.9282	4.362	-0.213	0.838
X_1X_2	-12.5250	5.752	-2.177	0.066

The coefficients of regression for the dextran concentration were calculated and the following regression equation was obtained.

$$Y = 27.2171 + 24.5989X_1 - 25.3429X_2 - 12.5250X_1X_2 + 7.5709X_1^2 - 0.9282X_2^2$$

Where, Y = Response (Dextran concentration), X_1 = Sucrose and X_2 =Yeast extract

Larger magnitude of t-test and smaller P value, indicates the high significance of the corresponding coefficient (Tanyildizi *et al*., 2005). The result showed that the interaction, X_1X_2 (sucrose. Yeast extract) is highly significant with a P-value of 0.066 and a negative coefficient of -12.5250.

The contour and surface plot representing the regression equation graphically is presented in Fig. 7.3.1. The plot conveniently illustrates the interaction between the two variables sucrose and yeast extract and also locates their optimum levels (Majumder and Goyal, 2008). The plots indicated that the interaction between the independent variable sucrose and Yeast extract (X_1X_2) is negative but very strong. The dextran production increases at high sucrose and low yeast extract concentration (Fig. 7.3.1).

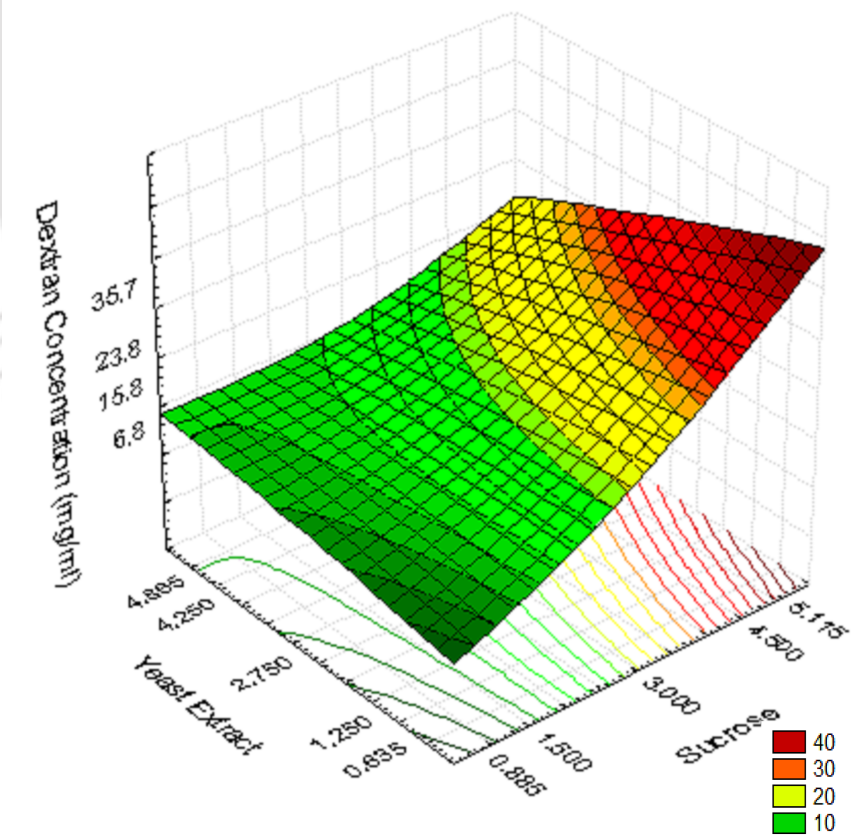


Fig. 7.3.1 Surface and Contour plot of the combined effects of sucrose and Yeast extract on dextran concentration of mutant of *Pediococcus pentosaceus*, (SPAm1).

Response optimization predicted the maximum dextran production of 40.8 mg/ml with desirability 1, at sucrose concentration 51.15 g/l and Yeast extract concentration 6.35 g/l (Fig. 7.3.2).

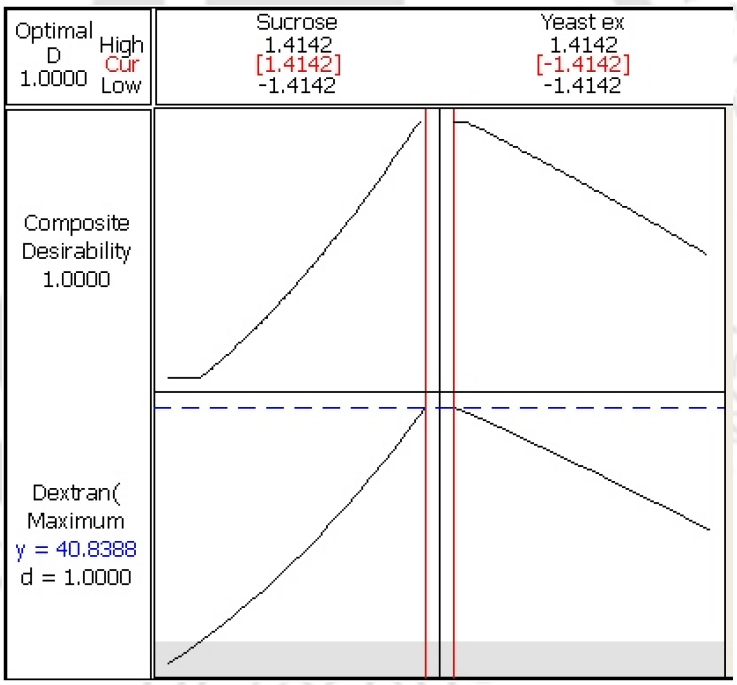


Fig. 7.3.2 Optimization graph for maximization of dextran production showing the optimum concentrations of sucrose and Yeast extract on dextran concentration of mutant of *Pediococcus pentosaceus* (SPAm1).

The maximum dextran concentration obtained experimentally by using the above composition of the variables was 40.2 mg/ml, which showed a perfect agreement with the predicted value of 40.8 mg/ml. Response surface methodology (RSM) showed that a medium containing (g/l) sucrose, 51.15; yeast extract, 6.35; 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 and Tween 80, 5 (ml/L) to be optimum for the production of dextran. This medium was projected to produce dextran concentration of 40.2 mg/ml. The comparison of the dextran production by wild-type isolate *Pediococcus pentosaceus* (SPA) in unoptimized medium, the mutant SPAm1 in unoptimized medium and SPAm1 in the optimized medium was presented in Table 7.3.8. The dextran concentration of the mutant SPAm1 in unoptimized medium was 20% higher than the wild-type *Pediococcus pentosaceus* (SPA). Whereas, the increase in dextran concentration of the mutant in optimized medium was 230% more than that given by the unoptimized medium. There was 294% increase in dextran concentration by mutants in optimized medium when compared to that of wild-type isolate *Pediococcus pentosaceus* (SPA) (Table 7.3.8).

Table 7.3.8 Comparison of dextran from wild type *Pediococcus pentosaceus* and its mutant SPAm1 in the unoptimized medium and in optimized medium

Strains	Dextran concentration (mg/ml)	Percent increase
Wild-type *Pediococcus pentosaceus* (SPA)	10.2	-
Mutant SPAm1 in unoptimized medium	12.2	20
Mutant SPAm1 in optimized medium	40.2	294

Thus, the statistically-based experimental designs were found very promising second step strategies to enhance the dextran production by the mutant *Pediococcus pentosaceus* (SPAm1).

7.4. Conclusions

To optimize fermentation medium for dextran production by the mutant SPAm1 of *Pediococcus pentosaceus*, 5 nutrients were tested using Placket -Burman Design and two nutrients *viz.* sucrose and yeast extract showing significant effect on dextran production were selected. The nutrients concentration was optimized by applying central composite design and the most suitable medium composition for dextran production was predicted as (g/l) sucrose, 51.15 and yeast extract, 6.35.

The present investigation, demonstrates the feasibility of enhancement of dextran production manifold from the *Pediococcus pentosaceus* mutant SPAm1 with mere shuffling of concentrations of crucial medium components. The dextran

concentration of the mutant SPAm1 in unoptimized medium was only 20% higher than the wild-type *Pediococcus pentosaceus* (SPA), which could be enhanced up to 294% in the optimized medium. Medium optimization resulted in 230% higher dextran production as compared to the mutant SPAm1. The results indicate that Response Surface Method comprising of Plackett-Burman Design and 2^2 full factorial Central Composite Design is very effective in attaining significant increase in dextran yield. This study shows that Response Surface Method can be applied for implementation of dextran production on a large scale.

References

Altaf, M., Naveena, B. J., Venkateshwar, M., Vijay Kumar, E. and Reddy, G. (2006) Single step fermentation of starch to 1(+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using inexpensive nitrogen sources to replace peptone and yeast extract – Optimization by RSM. Proc. Biochem. 41, 465-472.

Banik, R. M., Santhiagu, A. and Upadhyay S. N. (2007) Optimization of nutrients for gellan production by *Sphingomonas paucimobilis* ATCC-31461 in molasses

based medium using response surface methodology. Bioresour.Technol. 98, 792-797.

Barker, P. E. and Ajongwen N. J. (1991) The production of the enzyme dextransucrase using nonaerated fermentation techniques. Biotechnol. Bioeng. 37, 703-707.

Dubois, M., Gilles, K. A., Hamilton, J. K. Rebers, P. A. and Smith, F. (1956) Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356.

Fox, J. D. and Robyt, J. F. (1991) Miniaturization of three carbohydrate analyses using microsample plate reader. Anal. Biochem. 19, 593-596.

Goyal, A. and Katiyar, S. S. (1996) Regulation of dextransucrase productivity of *Leuconostoc mesenteroides* B-512F by the maintenance media. J. Gen. Appl. Microbiol. 42, 81-85.

Goyal, A. and Katiyar, S. S. (1997) Effect of certain nutrients on the production of dextransucrase from *Leuconostoc mesenteroides* B-512F. J. Basic Microbiol. 37, 197-204.

Jiang, L. (2010) Optimization of fermentation conditions for pullulan production by *Aureobasidium pullulan* using response surface methodology. Carbohydr. Polym. 79, 414-417.

Levesque, S. G., Lim, R. M. and Shoichet, M. S. (2005) Macroporous interconnected dextran scaffolds of controlled porosity for tissue-engineering applications. Biomaterials. 26, 7436-7446.

Liu, G-C and Wang, X-L (2007) Optimization of critical medium components using response surface methodology for biomass and extracellular polysaccharide production by *Agaricus blazei*. Appl. Microbiol. Biotechnol. 74, 78-83.

Lopez, A. and Monsan, P. (1980) Dextran synthesis by immobilized dextran sucrase. Biochimie. 62, 323-329.

Majumder, A. and Goyal, A. (2008) Enhanced production of exocellular glucansucrase from *Leuconostoc dextranicum* NRRL B-1146 using response surface method. Bioresour. Technol. 99, 3685-369.

Majumder, A. and Goyal, A. (2009) Rheological and gelling properties of a novel glucan from *Leuconostoc dextranicum* NRRL B-1146. Food Res. Int. 42, 525-528.

Majumder, A., Singh, A. and Goyal, A. (2009a) Application of response surface methodology for glucan production from *Leuconostoc dextranicum* NRRL B-1146 and its structural characterization. Carbohydr. Polym. 75, 150-156.

Majumder, A., Bhandari, S., Purama, R. K., Patel, S. and Goyal, A. (2009b) Enhanced production of a novel dextran from *Leuconostoc mesenteroides* NRRL B-640 by Response Surface Methodology. Annals Microbiol. 59, 1-7.

Patel, S., Majumder, A. and Goyal, A. (2010) Potentials of exopolysaccharides from lactic acid bacteria. Ind. J. Microbiol. (in press).

Plackett, R. L. and Burman, J. P. (1946) The design of optimum multifactorial experiments Biometrika. 33, 305-325.

Psomas, S. K., Liakopoulou-Kyriakides, M. and Kyriakidis D. A. (2007) Optimization study of xanthan gum production using response surface methodology. Biochem. Eng. J. 35, 273-280.

Purama, R. K. and Goyal, A. (2008) Screening and optimization of nutritional factors for higher dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640 using statistical approach. Bioresour. Technol. 99, 7108-7114.

Purama, R. K., Goswami, P., Khan, A. T. and Goyal, A., (2009) Structural analysis and properties of dextran produced by *Leuconostoc mesenteroides* NRRL B-640. Carbohydr. Polym. 76, 30-35.

Qader, S. A., Aman,. A., Saeeda, B., Syed, N. and Azhar, A. (2008) Role of calcium ions and temperature on dextransucrase production. Ind. J. Biotechnol. 7, 404-406.

Salah, R. B., Chaari, K., Besbes, S., Ktari, N., Blecker, C., Deroanne, C. and Attia, H. (2010) Optimisation of xanthan gum production by palm date (*Phoenix dactylifera* L.) juice by-products using response surface methodology. Food Chem. (in press).

Tanyildizi, M. S., Ozer, D. and Elibol, M. (2005) Optimization of α-amylase production by *Bacillus* sp. using response surface methodology. Proc. Biochem. 40, 2291-2296.

Tsuchiya, H. M., Hellman, N. N., Koepsell, H. J., Corman, J., Stringer, C. S., Rogovin, S. P., Bogard, M. O., Bryant, G., Feger V. H., Hoffman C. A., Senti F.

R. and Jackson, R. W. (1952) Factors affecting molecular weight of enzymatically synthesized dextran. J. Am. Chem. Soc. 77, 412-2419.

Wittenberger, C. L., Beaman, A. J. and Lee, L. N. (1978) Tween 80 effect on glucosyltransferase synthesis by *Streptococcus salivarius*. J. Bacteriol. 133, 231-239.

Zhang, H-B, Mao, X-Q, Wang, Y-J and Hu, X-Q (2009) Optimization of culture conditions for high-level expression of dextransucrase in *Escherichia coli*. J. Food Agric. Environ. 7, 75-78.

List of Publications

Published/in press/communicated

1. **Seema Patel** and Arun Goyal **(2010)** Isolation, characterization and mutagenesis of exopolysaccharide synthesizing new strains of lactic acid bacteria. ***Internet Journal of Microbiology*** (**in press**).
2. **Seema Patel**, Avishek Majumder and Arun Goyal **(2010)** Industrial potentials of Exopolysaccharides from Lactic acid bacteria. ***Indian Journal of Microbiology*** (**in press**).
3. **Seema Patel** and Arun Goyal **(2010)** Isolation of a new strain of exopolysaccharide producing lactic acid bacterium (SPO) from soil of sugarcane field of Orissa. ***Journal of Microbial World*** (**in press**).
4. Avishek Majumder, Anshuma Mangtani, **Seema Patel**, Rishikesh Shukla and Arun Goyal **(2009)** Gluco-oligosaccharides production from glucan of *Leuconostoc mesenteroides* NRRL B-742 by microwave assisted hydrolysis. ***Current Trends in Biotechnology and Pharmacy*** 3(4) 405-411.
5. Avishek Majumder, Sourabh Bhandari, Ravi Kiran Purama, **Seema Patel** and Arun Goyal **(2009)** Enhanced production of a novel dextran with gelling properties from *Leuconostoc mesenteroides* NRRL B-640 by statistical optimization. ***Annals of Microbiology*** 59 (2) (**in press**).
6. **Seema Patel**, Naresh Kasoju, Utpal Bora and Arun Goyal **(2010)** Structural analysis and biomedical applications of dextran produced by a new isolate (SPA) of lactic acid bacteria isolated from biodiversity hot spot Assam. Bioresource Technology (submitted)
7. **Seema Patel,** Damini Kothari, and Arun Goyal **(2010)** Enhancement of dextran production from the mutant of a new strain of *Pediococcus pentosaceus* by mutagenesis and Response surface method. Carbohydrate Polymers (submitted)
8. **Seema Patel**, Deeplina Das and Arun Goyal **(2010)** Structural characterization of the exopolysaccharide produced by a new strain of *Pediococcus pentosaceus* (SPO) isolated from Orissa. Proceedings of National Academy of Sciences (submitted).
9. **Seema Patel**, Damini Kothari and Arun Goyal **(2010**) Medium optimization for maximizing dextransucrase production from the mutant SPAm1 of new isolate of *Pediococcus pentosaceus* by Response Surface Methodology. Journal of Basic Microbiology (submitted).
10. **Seema Patel** and Arun Goyal (**2010**) 16S rRNA based phylogenetic analysis of an exopolysaccharide producing *Pediococcus pentosaceus* isolated from soil of Orissa. Journal of Applied Biology and Biotechnology (submitted).
11. **Seema Patel**, Damini Kothari and Arun Goyal (**2010**) Production, purification and characterization of dextransucrase from a new strain of Pediococcus pentosaceus isolated from Assam. World Journal of Microbiology and Biotechnology (submitted).

12. **Seema Patel** and Arun Goyal **(2010)** 16S rRNA based phylogenetic analysis of an dextran producing *Pediococcus pentosaceus* isolated from the Eastern Himalayan microbial biodiversity. Current Trends in Biotechnology and Pharmacy (submitted).

List of conference papers (14)

International (07)

1. **Seema Patel**, Arabinda Ghosh and Arun Goyal **(2010)** 16S rRNA based identification of a bioactive dextran producing *Pediococcus pentosaceus* isolated from soil of biodiversity hotspot Assam. International Conference on Environmental Health and Technology (EH&T 2010), March 15-17 Centre for Environmental Science and Engineering, Indian Institute of Technology Kanpur, Kanpur, India.
2. **Seema Patel** and Arun Goyal **(2009)** Production, purification and characterization of homopolysaccharides produced by two natural isolates of lactic acid bacteria, SPO and SPA. International Symposium on Emerging trends in Biomedical and Nanobiotechnology, Dec. 19-21 Acharya Nagarjuna University , Guntur, A.P., India. p32 (Oral presentation).
3. **Seema Patel**, Damini Kothari, Rajesh Singampalli and Arun Goyal **(2009)** UV induced mutagenesis of exopolysaccharide synthesizing natural isolate of lactic acid bacteria SPA for strain improvement. International Conference on Emerging Trends in Biotechnology, Dec 2-6 2009, Banaras Hindu University p178.
4. **Seema Patel**, Shraddha Shukla and Arun Goyal **(2009)** Production, purification and characterization of dextransucrases from two natural isolates of lactic acid bacteria, SPO and SPA. International Conference on Emerging Trends in Biotechnology, Dec 2-6 2009, Banaras Hindu University. p220
5. **Seema Patel** and Arun Goyal **(2009)** Characterization of two new isolates of Leuconostoc and their dextransucrases and dextrans. 8th Carbohydrate Bioengineering Meeting, May, 10-13, 2009, Ischia Island, Naples, Italy. p89
6. **Seema Patel** and Arun Goyal (**2008**) Structural analysis of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. International Symposium on Microbial Biotechnology: Diversity, Genomics and Metagenomics (49th Annual Conference of Association of Microbiologists of India), December 18-20, 2008, University of Delhi, Delhi, India. p7
7. **Seema Patel** and Arun Goyal (**2008**) Isolation of exopolysaccharide producing enzymes from natural bacterial isolates. International Congress on Bioprocesses in Food Industries (5th Convention of Biotech Research Society of India, November 6-8, 2008 Osmania University, Hyderabad, India. p99

National (07)

8. **Seema Patel** and Arun Goyal (**2010**) 16S rRNA based identification and phylogenetic analysis of an exopolysaccharide producing *Pediococcus pentosaceus* isolated from soil of Orissa. 55th annual Technical Session of Assam Science Society March 15-17, 2010, Gauhati University, Assam, India (Oral presentation).
9. **Seema Patel**, Damini Kothri and Arun Goyal **(2010)** Production, purification and characterization of dextransucrase from a novel natural isolate of *Pediococcus pentosaceus* isolated from Assam. National Conference on Biotechnology for Better Tomorrow (NCBBT 2010) Feb 6-7, 2010, Shri Shivaji College of Arts, Science and Commerce, Akola, Maharashtra, India.
10. **Seema Patel**, Damini Kothari, S. Krishna Bindu, Deeplina Das and Arun Goyal **(2009)** Statistical optimization of medium as a strategy to enhance the dextransucrase activity of mutant of a new isolate of lactic acid bacteria.50th Annual conference Association of Microbiologists of India (AMI- 2009) held on 15-18th December, 2009 at National Chemical Laboratory, Pune, Maharashtra, India. p260
11. **Seema Patel**, Damini Kothari, Arabinda Ghosh and Arun Goyal **(2009)** Optimization of critical medium components using Response Surface Methodology for enhancing the dextran production by the mutant of a new isolate of lactic acid bacteria. 50th Annual conference Association of Microbiologists of India (AMI- 2009) held on 15-18th December, 2009 at National Chemical Laboratory, Pune, Maharashtra, India. p253
12. **Seema Patel** and Arun Goyal **(2009)** Antibiotic sensitivity profile and characterization of two new bacterial isolates of *Leuconostoc sp*. North East Conference of Medical Microbiologists, April 25-26 2009. Downtown Hospital Complex, Guwahati, Assam.
13. Mayur Agrawal, **Seema Patel** and Arun Goyal **(2009)** Characterization of two high dextran-yielding mutants of *Leuconostoc mesenteroides* NRRL B-640. North East Conference of Medical Microbiologists, April 25-26 2009. Downtown Hospital Complex, Guwahati, Assam.
14. Ravi Kiran Purama, Mayur Agrawal, **Seema Patel** and Arun Goyal **(2007)** Functional properties and stabilization of purified *Leuconotoc mesenteroides* NRRL-B-640 dextransucrase. 48th Annual Conference of Association of Microbiologists of India (AMI), December 18-21, 2007, Indian Institute of Technology Madras, Chennai, India. p183

VITAE

The author was born on January 6, 1983 in Sundargarh, Orissa, India. She passed the Secondary School Examination conducted by Board of Secondary Education, Orissa, in 1998, and Council of Higher Secondary Education, Orissa, in 2000. She completed B.Sc. with Chemistry, Botany and Zoology as major subjects from Sambalpur University, Orissa, India, in 2003. She did her M.Sc. in Life Sciences from Sambalpur University, Orissa, India in 2005.

Ms. Seema Patel joined the Ph.D. programme in July 2007 at the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781 039, Assam, India after selection by the Department based on her Graduate Aptitude Test in Engineering (GATE) score of 98.6 percentile and performance at the written test and interview. She received Junior and Senior Research Fellowships through (GATE) under the scheme run by the Ministry of Human Resource and Development (MHRD), India. She successfully completed the course work with 9.1/10 Cumulative Point Index (CPI). She gave the Open (PhD Synopsis) Seminar on November 19, 2009 and presented her thesis work before the Doctoral Committee and her performance was satisfactory. She submitted the PhD thesis in February 2010.