Dextransucrase and dextran with anticancer properties from probiotic *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber

A Thesis

Submitted in Partial Fulfillment of the
requirements for the Degree of

DOCTOR OF PHILOSOPHY

by

RISHIKESH SHUKLA

Under supervision of

Professor Arun Goyal

Jan 2014

Department of Biotechnology
Indian Institute of Technology Guwahati
Guwahati 781 039, Assam, India

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 described is based on the findings of other investigators.

Jan, 2014

Rishikesh Shukla

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

It is certified that the work described in this thesis entitled **"Dextransucrase and dextran with anticancer properties from probiotic *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber"** by Mr. Rishikesh Shukla 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 mainly in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

Dr. Arun Goyal
(FAMI, FBRS, FABAP, FNABS, FNAAS, FIFIB)
Professor of Biotechnology
(Thesis Supervisor)
Department of Biotechnology
Indian Institute of Technology Guwahati
Guwahati, 781 039, India

ACKNOWLEDGEMENTS

First of all I express my deep sincere gratitude to The Almighty God for the Blessings and strength bestowed upon me to complete this work. I have taken efforts in this project. However, it would not have been possible without the kind support and help of many individuals and organization who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study. I would like to extend my sincere thanks to all of them. At the end of my thesis, it is a pleasant task to express my thanks to all those who contributed in many ways and had profound impact in the success of this study deserves special acknowledgment.

At the inception, I am highly indebted to my thesis supervisor, Professor Arun Goyal, Department of Biotechnology, IIT Guwahati for his advice during my doctoral research endeavor as well as for providing necessary information regarding the project. This work would not have been possible without his guidance, support and encouragement. As my supervisor, he has constantly forced me and motivated me to remain focused on achieving my goal. His observations and comments helped me to establish the overall directions of the research and to move forward with the investigation in depth. I would also like to thank him for being an open person to ideas and for encouraging and helping me to shape my interest and ideas. I owe a *special thanks to him for reviewing my thesis progress and giving me valuable suggestions.*

I would also like to express my sincere gratitude to all my doctoral committee members Dr. K. Pakshirajan, Dr. V.K. Dubey and Prof. T. Punniyamurthy for their valuable suggestions and constructive criticism during my progress report presentations that has led to the successful completion of my thesis.

I would also like to express the deepest appreciation to Dr. Debasish Das and Dr. Nitin Chaudhary for their useful guidance in my thesis work.

I sincerely thank Dr. Ilia Iliev, Biochemistry Laboratory, Department of Biochemistry & Microbiology, University of Plovdiv, Plovdiv, Bulgaria who helped me during his visit to India to carry out a part of my doctoral research work.

I would also like to thank the present and previous heads of the Department of Biotechnology, IIT Guwahati, Prof. V. V. Dasu and Prof. Arun Goyal for providing me with the necessary facilities.

I take this opportunity to record my sincere thanks to my seniors Dr. Avishek Majumder, Dr. Ravi Kiran Purama, Dr. Seema Patel, Dr. Deepmoni Deka and Dr. Shadab Amhed for their help and suggestions. I am immensely thankful to all of my research group members. I am grateful to all my lab mates of Department of Biotechnology for their cooperation and support.

I am thankful to Mr. Sharan, Mr. Nurul, Mrs. Prarthana, Mrs. Anita and Mr. Niranjan for technical help and assistance. I wish to acknowledge the supports received from other teaching and non-teaching staffs of the Department of Biotechnology, IIT Guwahati.

I wish to acknowledge Central Instrumentation Facility, IIT Guwahati for providing instruments for my experiments. I wish to acknowledge IIT Guwahati for providing financial assistance and experimental facilities.

I would like to pay high regards to my Parents, loving brother Dr. Avdhesh, sister in law Dr. Sunita Shukla, niece Shubhi, my wife Shraddha, my friends Sanjay, Varun, Vishal, Rohitas, Santosh, Pankaj, Shuchi, Nidhi and Abhay for their sincere encouragement and inspiration throughout my research work and lifting me uphill this phase of life. I owe everything to them.

Besides this, several people have knowingly and unknowingly helped me in the successful completion of this project. I also place on record, my sense of gratitude to one and all who directly or indirectly, have lent their helping hand in this venture.

Rishikesh Shukla

Jan 2014

SYNOPSIS

Introduction

Dextransucrase (1,6-α-D-glucan-6-α-glucosyltransferase, EC 2.4.1.5) is an extracellular enzyme that catalyses the formation of dextran by transfer of D-glucosyl moieties of sucrose to the growing polymer chain. Dextrans are a class of homopolysaccharides containing a backbone of α-(1→6) glycosidic linkages and the branching of α-(1→2), α-(1→3) or α-(1→4). Dextrans may be linear or branched, with variable degree of branching. Dextran has been used as adjuvant, emulsifier, carrier and stabilizer in food and pharmaceutical industries. Dextran are also used as matrix of chromatography column, anticoagulant, as an anticancer agent, for enhanced oil recovery, metal-plating and biomaterials. Dextran has been found suitable for cell-resistant coatings on biomaterial surfaces. Functionality of dextran depends on its structure and the strain of lactic acid bacteria synthesizing it. Dextran producing lactic acid bacteria have attracted much attention due to immense industrial potential of dextrans. The commonly known dextran producing lactic acid bacteria are *Lactobacillus*, *Leuconostoc* and *Streptococcus.* However *Pediococcus* and *Weissella* spp. also produce dextran. Molecular weight and linkage pattern of dextran play key role in determining it's application. High molecular weight dextran with linear structure finds their application in food industries as hydrocolloids, whereas, low molecular weight dextrans are important for clinical use. The branched dextran can be used to produce branched oligosaccharides which have applications as prebiotics.

The lactic acid bacteria (LAB) have been isolated from different sources, but only few emphasized on isolation from fruits and vegetables. The LAB from lettuce, cucumber and cabbage are not much studied. Fermented cucumber serves as traditional food in some parts of the world. Therefore the isolation, screening and characterization of industrially important LAB from fermented cucumber of untapped Indo-Burma biodiversity hot spot, the Northeast India, deserve special attention. The hot and humid tropical climate of Assam is conducive for isolation of microbes from the soil producing rare and novel industrial enzymes and bioactive exopolysaccharides. With this objective, a high dextransucrase yielding strain of lactic acid bacterium "CRAG3" was isolated from the fermented cucumber. The isolate CRAG3 was physiologically and biochemically characterized using Gram staining, catalase test, carbohydrate utilization pattern and antibiotic susceptibility experiments to distinguish it from other closely related lactic acid bacteria. 16s rRNA sequencing identified the isolate to be *Pediococcus pentosaceus*, belonging to lactic acid bacteria family. The production, purification and characterization of dextransucrase and dextran from *Pediococcus pentosaceus* CRAG3 were carried out. *In vitro* cytotoxicity assay of dextran was performed using various mammalian cell lines to evaluate its biomedical applications. The production of both dextransucrase and dextran was enhanced using statistical methods. The structure and properties of dextran were studied. The isolate was also explored for its probiotic properties such as cell adhesion, resistance to biological barriers, utilization of prebiotics and antibacterial activity.

Present work

The present investigations are carried out on the **“Dextransucrase and dextran with anticancer properties from probiotic *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber”**. The thesis work comprises 6 Chapters.

Chapter 1 is the General Introduction which represents the brief review of literature dedicated to the importance of lactic acid bacteria, dextransucrase, dextran and their characterization. It describes the molecular architecture, three dimensional structure of dextransucrase and the mechanism of dextran production. The production, purification and characterization methods of the dextransucrase and the exopolysaccharide dextran have been described in this chapter. The chapter also elaborates the potential applications of the dextran. Applications of dextran as food additive, biomaterial and as anticancer agents are extensively reviewed. Statistical optimization of medium composition by applying the Taguchi’s orthogonal methodology for maximizing dextransucrase activity and dextran concentration is also described. The chapter 1 also describes the role of lactic acid bacteria as probiotics. Cucumber is a good source of lactic acid bacteria. The microorganism *Pediococcus pentosaceus* and their phylogenetic and metabolic traits, dextransucrase and dextran production capacity and their potential applications as probiotic are discussed.

Chapter 2 describes the detailed decorum of screening of the novel strain of lactic acid bacterium, CRAG3 from fermented cucumber on the basis of its ability to produce higher dextransucrase and dextran. The isolate CRAG3 displaying maximum enzyme activity (2.7 U/ml) and maximum total carbohydrate content (4.5 mg/ml) was selected for identification and further biochemical characterization. The morphological characteristics showed that it was Gram positive and coccus shaped bacterium, forming round colonies. The physiological and biochemical characterization were carried out to distinguish the isolate from other closely related lactic acid bacteria. The biochemical characterization of isolate CRAG3 displayed its ability to utilize carbohydrates such as cellobiose, dextrose, arabinose, sucrose, maltose, mannose and galactose. However, the isolate CRAG3 failed to utilize raffinose, inulin, lactose and melibiose. Triple sugar iron agar test also confirmed that the isolate CRAG3 was able to ferment sucrose and glucose. The isolate CRAG3 was catalase negative since no bubble formation was observed on addition of H_2O_2. The motility agar test proved CRAG3 as a non-motile bacterium since it did not spread in motility indole lyine agar slant. The antibiotic sensitivity test showed that the isolate was resistant to cefixime, ciprofloxacin, co-trimoxazole, kanamycin, nalidixic acid, sulphamethoxazole, tobramycin, trimethoprim, vancomycin and gentamicin. The resistance of *Pediococcus pentosaceus* CRAG3 to vancomycin, showed its common characteristics of lactic acid bacteria. Identification of isolate was carried out on the basis of 16S rRNA sequence analysis. Consensus sequence of 1,269 bp of 16S rDNA was generated from forward and reverse sequence data using aligner software. The isolate was clustered with *Pediococcus pentosaceus* strain ULAG75 (GenBank Accession Number: JN944736) and *Pediococcus pentosaceus* (GenBank Accession Number: DQ267152) which confirmed it as *Pediococcus pentosaceus*. The isolate CRAG3 was assigned the Genbank accession number JX679020. The members of *Pediococcus* genus have great economic importance in the fermented food industry, as starter cultures in fermentation processes of milk, meat, vegetable products and sausages and also as probiotics. Since the dextransucrase producing ability of *Pediococcus pentosaceus* has not been much explored, this isolate could serve as good candidate for potential applications in food industries.

Chapter 3 deals with purification, identification and characterization of dextransucrase from *Pediococcus pentosaceus* CRAG3 (Genbank accession number JX679020). The culture supernatant of the isolate showed maximum dextransucrase activity (4.35 U/ml) and total carbohydrate content (15.4 mg/ml) at optimum temperature and shaking condition of 25°C and 180 rpm, respectively. The crude dextransucrase (0.67 U/mg) was purified by PEG fractionation and gel filtration. The dextransucrase after fractionation with PEG-1500 (10%, w/v) gave 20.0 U/mg specific activity with 30 fold purification. The enzyme purified with PEG-1500 gave two bands of 224 and 188 kDa on SDS-PAGE under denaturing condition. However, the same enzyme sample gave single band of 224 kDa when run on native-PAGE and also on non-dentauring SDS-PAGE after PAS staining with sucrose, which confirmed a single molecular and pure form of dextransucrase. No band on gel incubated with raffinose was obtained which ruled out the presence of fructansucrase. The dextransucrase purified by PEG-1500 (10%, w/v) was further purified by gel filtration using Sephacryl S-300HR column which gave the specific activity of 46.0 U/mg with 69 fold purification. The enzyme purified by gel filtration also showed 224 kDa band of dextransucrase on denaturing SDS-PAGE. The presence of single magenta color band after PAS staining confirmed the presence of dextransucrase. The dextransucrase partially purified with 10% PEG-1500 was further biochemically characterized. The assay conditions for dextransucrase activity such as pH, temperature, sucrose concentration and ionic strength of buffer, were optimized. The enzyme showed maximum activity (20.0 U/mg) in 5% sucrose in 20 mM sodium acetate buffer (pH 5.4) at 30ºC. The effect of sucrose concentration on dextransucrase activity showed that the specific activity increased with increasing sucrose concentration to 5% but decreased thereafter, which suggested that it did not completely follow classical Michaelis Menton kinetics. The purified dextransucrase showed K_m of 15.42 mM and V_{max} of 20.9 U/mg. The effects of various metal ions on dextransucrase activity showed that Co^{2+} (4 mM), Ca^{2+} (2 mM), Mg^{2+} (2 mM) and Zn^{2+} (1 mM) enhanced the activity by 56%, 44%, 14% and 12%, respectively. This displayed the importance of these metal ions for the catalytic efficiency of dextransucrase from *Pediococcus pentosaceus* CRAG3. However, the dextransucrase activity was decreased by 89% and 90% in presence of Mn^{2+} (8 mM) and Ni^{2+} (8 mM), respectively. The dextransucrase was stable up to 40ºC when incubated for 1 h which showed its superior property over other previously reported dextransucrases which were stable up to 30ºC. The storage stability of dextransucrase was also studied at different temperatures and pH. The dextransucrase was most stable at -20ºC with half-life of 307 days, half-life ($t_{1/2}$) of 85.5 h and 15.53 h, respectively as compared to control (6.9 h). The effect of purified dextransucrase on sucrose supplemented milk showed the solidification of milk due to formation of dextran, signifying its importance in improving the texture of dairy and bakery products. The purified dextransucrase from *Pediococcus pentosaceus*

CRAG3 with higher activity and stability can serve as a potential candidate for industrial applications.

Chapter 4 describes the production, purification and structural characterization of dextran produced by the lactic acid bacterium isolate *Pediococcus pentosaceus* CRAG3. The dextran produced by dextransucrase from the isolate was purified and its structure and properties were studied. The purified dextran displayed number average molecular weight, 2.93×10^5. The high molecular weight dextran can be used as food ingredient in bakery products or in gel filtration material like Sephadex products. The SEM analysis of dextran revealed porous structure, which accounts for its importance as a carrier for drug delivery. The monosaccharide analysis of dextran confirmed the presence of only glucose monomeric units. The FTIR spectrum showed the presence of α-(1→6) and α-(1→3) linkages which were further confirmed by NMR spectroscopy. The ^{1}H-NMR spectrum confirmed the presence of α-(1→6) linear chain and α-(1→3) branching. The dextran possesses 75% α-(1→6) linear linkage and 25% α-(1→3) branched linkages. The ^{13}C-NMR spectrum of dextran also confirmed the presence of α-(1→6) and α-(1→3) linkages. The dextran with branching can be used for production of branched oligosaccharides which have superior prebiotic properties over linear oligosaccharides. The *in vitro* cytotoxicity effect of *Pediococcus pentosaceus* CRAG3 dextran on HeLa and HT29 cell lines showed its anti-cancer activity against both cell lines. Dextran at 1 µg/ml for HeLa cells and 500 µg/ml for HT29 cells, displayed per cent survival of 63.3±3.2% and 34.24±0.54%, respectively. The effect of dextran on murine macrophages (J774A.1) line showed increase in macrophage growth by 20%.

The anti-cancer activity of dextran from *Pediococcus pentosaceus* CRAG3 might be due to macrophage activation. This branched dextran from *Pediococcus pentosaceus* CRAG3 may potentially be used for clinical purposes. Till date not much attention has been paid to the dextran production capacity of *Pediococcus pentosaceus.* This study reveals a novel branched anticancer dextran producing bacterial isolate which could be a good candidate for dextran production in the pharmaceutical industry.

Chapter 5 deals with optimization of medium composition by the statistical tool, Taguchi methodology for maximization of dextransucrase and dextran production from *Pediococcus pentosaceus* CRAG3. The branched dextran producing *Pediococcus pentosaceus* CRAG3 was selected for optimization of medium components for enhanced dextransucrase and dextran production using One Variable at a Time (OVAT) approach and Taguchi orthogonal array design. The effects of sucrose, yeast extract, beef extract, peptone, K_2HPO_4 and Tween 80 on dextransucrase and dextran production were analysed. The dextransucrase activity and dextran concentration in control medium was 4.5 U/ml and 4.5 mg/ml, respectively. The sucrose at a concentration of 3.5% (w/v) resulted in 1.75 fold increase in dextransucrase activity (7.9 U/ml) with respect to control. 1% (w/v) yeast extract gave 6.5 U/ml dextransucrase which was 44% higher as compared to the control containing 2% (w/v) yeast extract. However, the addition of beef extract (1%, w/v) or peptone (1%, w/v) to the control medium increased the dextransucrase activity to 7.35 U/ml and 8.3 U/ml, respectively. There was an increase in dextransucrase activity with increase in concentrations of K_2HPO_4 and Tween 80. The increase in activity was 38% (6.2 U/ml) and 100% (9.0 U/ml) at 1%

(w/v) K_2HPO_4 and 1% (v/v) Tween 80, respectively. The effects of nutrients on dextran production showed that sucrose (3.5%, w/v), yeast extract (1%, w/v), peptone (1%, w/v) and K_2HPO_4 (1%, w/v) favoured its production with 180%, 27%, 6% and 178% increase in dextran concentration, respectively. However, beef extract (0.1%, w/v) and Tween 80 (0.05%, w/v) negatively affected dextran production with 17% and 20% decrease in dextran concentration. Based on the results of OVAT, the different levels of medium components were selected for medium optimization of dextransucrase and dextran production using Taguchi methodology. The effect of factors on response showed that Tween 80 had an ample effect and yeast extract had least effect on dextransucrase production by *P. pentosaceus* CRAG3. However, K_2HPO_4 showed most significant effect and Tween 80 showed least effect on dextran production by *P. pentosaceus* CRAG3. ANOVA showed significant effect of sucrose, Tween 80 and K_2HPO_4 on dextransucrase production. On the other hand, sucrose, peptone and K_2HPO_4 were found to be most significant for dextran production. The optimized medium for dextransucrase production contained (%, w/v): sucrose, 3.5%; yeast extract, 0.2%; beef extract, 0.5%; peptone, 3%; $MgSO_4.7H_2O$, 0.02%; $MnSO_4.4H_2O$, 0.001%; $FeSO_4.7H_2O$, 0.001%; $CaCl_2.2H_2O$, 0.001%; NaCl 0.001%; K_2HPO_4, 0.2% and Tween 80, 1% (v/v). However, the optimized medium for dextran production consisted (%, w/v): sucrose, 5%; yeast extract, 2%; beef extract, 0.5%; peptone, 1%; K_2HPO_4, 0.2%; $MgSO_4.7H_2O$, 0.02%; $MnSO_4.4H_2O$, 0.001%; $FeSO_4.7H_2O$, 0.001%; $CaCl_2.2H_2O$, 0.001%; NaCl 0.001% and Tween 80, 0.1%. Significant increase in dextransucrase activity and dextran concentration was observed in optimized medium (10 U/ml and 16 mg/ml, respectively) as compared to unoptimized medium (4.5 U/ml and 4.5 mg/ml, respectively). Validation of data was done at flask and bioreactor levels. The maximum dextransucrase activity achieved in shake flask and bioreactor level was 10.1 U/ml and 10.2 U/ml, respectively which was in good agreement with Taguchi predicted result (10.5 U/ml). The maximum dextran concentration at shake flask level was 16 mg/ml which was also in accordance with Taguchi predicted value (15.98 mg/ml). Interestingly, the enhancement in dextran concentration (24 mg/ml) was observed at bioreactor level which might be due to higher growth of cells. These results are encouraging for pilot scale or industrial scale production of dextran from hyper producing *Pediococcus pentosaceus* CRAG3.

Chapter 6 deals with the probiotic potential of *Pediococcus pentosaceus* CRAG3 (Genbank accession number JX679020). The probiotic potential of the isolate was determined by analyzing surface adhesive property, hydrophobicity and resistance to biological barriers. The isolate showed 33% hydrophobicity after 20 min which signifies the hydrophobic nature of its cell surface and significant factor in microbial adhesion. It displayed 43% autoaggregation and 10% coaggregation with *E. coli* after 5 h which are also related to cell adhesion properties of a probiotic microorganism. The coaggregation of *Pediococcus pentosaceus* CRAG3 with other microbes could increase its colonization potential if it was to be used in mixed culture as probiotics. It may also constitute an important host defence mechanism against infection, since it is a potent producer of lactic acid (an inhibitor to pathogenic bacteria). It displayed good adhesion property with mammalian cell lines (HeLa and J774A.1) in *in vitro* conditions which confirmed its ability to adhere with mammalian cells signifying in competing with other microorganisms in gastrointestinal tract. The isolate also showed ability to resist 100 μg/ml of lysozyme for 2 h with 75.0% survival displaying its ability to overcome the first biological barrier in mouth. *P. pentosaceus* CRAG3 displayed tolerance against simulated gastric juice (pH 3.0) with 58% survival showing its ability to transit through high acidic environment of stomach. It possessed tolerance (73%, 63% and 50% survival) in presence of 0.3%, 0.5% and 1.0% (w/v) bile salts, respectively, which are potent antimicrobial agents. *Pediococcus pentosaceus* CRAG3 also showed 1.04 U/ml bile salts hydrolase (BSH) activity with yellow halo (6 mm) around colonies. It exhibited antibacterial activity against *E. coli*, *S. aureus* and *B. subtilis* which might be due to the production of lactic acid signifying its capability to kill pathogenic microorganisms. *Pediococcus pentosaceus* CRAG3 displayed ability to utilize prebiotics such as inulin and raffinose with 37.5% and 6%, respectively, the relative cell growth which is also one of the important properties of a microorganism to behave as probiotic.

CONTENTS

Chapter 1. General Introduction

Chapter 2. Identification and biochemical characterization of *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber

Chapter 3. Production, purification and characterization of dextransucrase from *Pediococcus pentosaceus* CRAG3

Chapter 4. Production, purification and characterization of dextran from *Pediococcus pentosaceus* CRAG3

Chapter 5. Enhanced production of dextransucrase and dextran from *Pediococcus pentosaceus* CRAG3 in bioreactor using Taguchi methodology

Chapter 6. Probiotic potential of *Pediococcus pentosaceus* CRAG3

Chapter 1

General Introduction

1.1 Isolation and identification of lactic acid bacteria

The lactic acid bacteria (LAB) are a group of Gram-positive rod and coccus shaped, non-spore forming, non-motile microorganisms. The major product formed during the fermentation of carbohydrates is lactic acid. This group comprises the genera like *Carnobacterium, Enterococccus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella* (Stiles and Holzapfel, 1997; Holzapfel *et al.*, 2001). Although there are reports available on isolation of LAB from different sources, only few emphasized on isolation from fruits and vegetables (Padmaja *et al.*, 2011; Ravi *et al.*, 2011), but from lettuce, cucumber and cabbage are not much studied. Fermented cucumber serves as traditional food in many parts of the world (Chen *et al.*, 2012). It has been reported that the fermentation in cucumbers occur by lactic acid bacteria (Singh and Ramesh, 2008). Cai et al.

(1999) isolated and characterized *Pediococcus* species forage crops. The isolation and characterization of antimicrobial *Pediococcus* species from South African farm-style cheese was reported by Gurira and Buys (2005). The isolation and probiotic potential of *Pediococcus* spp. from Turkish-type fermented sausages (Sucuk) has been done by Yuksekdag and Aslim (2010).

Various methods have been used for identification of bacteria but the use of 16S rRNA sequences for phylogenetic analysis has been generally accepted (Fetsl *et al.*, 1986; Barry *et al.*, 1990). Woese and his co-workers compared a stable part of the genetic code to determine phylogenetic relationships of bacteria and other life forms (Woese *et al.*, 1985; Woese, 1987). This included the genes that code for the 5S, the 16S (also known as small subunit), and the 23S rRNA and the spaces between these genes. But now-a-days most commonly used part of DNA for bacterial taxonomy is the gene 16S rRNA gene (Bottger, 1989; Kolbert and Persing, 1999; Palys *et al.*, 1997; Garrity and Holt, 2001; Tortoli, 2003; Harmsen and Karch, 2004). The 16S rRNA gene is also designated as 16S rDNA, and the terms have been interchangeably used. The length of 16S rRNA is about 1,550 bp which is composed of both variable and conserved regions. Usually universal primers chosen are complementary to the conserved regions of the gene at either 540 bp region or at the end of the whole sequence which is about 1,550 bp region and the sequence of the variable region in between is used for comparative taxonomy (Chen *et al.*, 1989; Relman, 1999). The 16S rRNA gene sequence has been determined for a large number of strains and this gene is universal amongst bacteria which help in measuring relationships among all bacteria (Woese *et al.*, 1985; Woese, 1987). In other words the 16S rRNA gene sequence comparison allows differentiating organisms at genus level and also classifying strains at species and subspecies level. Now it is also important to consider that is it necessary to sequence the whole 1.5 kb length or whether the commonly reported shorter sequence can provide enough information. It has been reported that sometimes it is necessary to sequence the entire 1.5 kb region for identification (Sacchi *et al.*, 2002a; Sacchi *et al.*, 2002b). Usually the entire 1.5 kb sequence is required when describing a new species. However, the first 500 bp sequence of many clinical bacteria provides sufficient variation for identification. This also provides a large difference between strains due to the presence of more diversity per kilobase sequence in the region. Kattar *et al.* (2001) found in 16S rRNA of *Bordetella* spp. that there is 66% variability in initial 500 bp.

1.2 Glycosyltransferases from lactic acid bacteria

Glycosyltransferases are enzymes which catalyse the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds (Monsan *et al.*, 2010; van der Vlist *et al.*, 2011). The 'activated' sugar phosphates act as glycosyl donors that transfer glycosyl group to a nucleophilic group, usually an alcohol forming an O-, N-, S- or C- glycoside; the glycoside may be part of a monosaccharide, oligosaccharide or polysaccharide (Campbell *et al.*, 1997; Coutinho *et al.*, 2003; Lairson *et al.*, 2008; Robert and Spencer, 2009). Glycosyltransferases can be classified as either retaining or inverting enzymes according to the stereochemistry of the substrates and reaction products (Sinnott, 1990). Till date glycosyltransferases have been classified into 94 families of glycosyltransferase (GT) on the basis of sequence similarity as listed in CAZy database (http://www.cazy.org/GlycosylTransferases.html).

1.2.1 Dextransucrase

The dextransucrases are large size extracellular enzymes which catalyse the formation of various glucans (dextran, mutan, alternan and reuteran) using sucrose as a substrate (Purama and Goyal, 2005). Dextransucrases (also known as glucosyltransferases) have been classified in family 70 of glycoside hydrolases (GH70) on the basis of sequence homology and glycoside hydrolases have been grouped into 111 families (http://afmb.cnrs-mrs.fr/CAZY/). The first three-dimensional structure of GH70 GSs has become available in truncated forms of the enzyme (Vujicic-Zagar *et al.*, 2010; Ito *et al.*, 2011; Brison *et al.*, 2012). The nature of product form depends on the type of enzyme used, such as dextransucrase catalyses the formation of dextran, mutansucrase is used for the production of mutan, alternansucrase for alternan and reutransucrase for synthesis of reutran. Robyt (1995) reported that molecular mass of dextransucrase (sucrose: 1,6-α-D-glucan 6-α-glucosyltransferase; EC 2.4.1.5) vary from 64 to 185 kDa. However, dextransucrase has also been reported to exist in single or multiple forms having molecular weight in the range 64,000-245,000 (Kobayashi and Matsuda, 1980; Goyal and Katiyar, 1994; Purama and Goyal, 2007). Such variation is associated with the presence of dextran in the purified dextransucrase preparations, disassociation of high molecular mass multimeric complex (Kim and Robyt, 1994) or action of proteases (Miller and Robyt, 1986).

It has been reported that divalent cations have a significant role in maintaining the enzyme activity as most of them act as cofactors (Majumder *et al.*, 2008). The dextransucrase activity increased by 22, 14, and 13% by the addition of 2 mM $MgCl_2$, 2 mM $CoCl_2$, and 4 mM $CaCl_2$ to dextransucrase (Rao and Goyal, 2013). Shukla *et al.*

(2013b) also reported the positive effect of Co^{2+} and Ca^{2+} ions on WcCab3-DSR activity. There have been various reports for the use of stabilizers to enhance the stability of dextransucrase (Purama *et al.*, 2010; Kothari *et al.*, 2011; Rao and Goyal, 2013; Shukla *et al.*, 2013). For instance, glycerol and Tween 80 efficiently enhanced the Wc-Cab3 DSR (dextransucrase) stability and the half-life at 30° (Shukla *et al.*, 2013). Tween 80 also provided the maximum stabilization to dextransucrase from the mutant of *Pediococcus pentosaceuos* SPAm against activity loss at 30°C and 4°C (Kothari *et al.*, 2011). Rabelo *et al.* (2011) reported the effect of cashew apple juice as a stabilizer for the crude dextransucrase, from *Leuconostoc citreum* B-742. The crude enzyme presented good stability in cashew apple juice for 48 h at 25°C and pH 6.5.

1.2.2 Mechanism of action of dextransucrases

Dextransucrase catalyzes the transfer of glucosyl moiety from sucrose. The resulting product formed by the acceptor reaction of dextransucrases depends on the destination of these glucosyl units (Mooser, 1992) (Fig. 1.1). Depending upon the type of acceptor molecule dextransucrases catalyse two types of reactions

(A) Glucosyl transfer, when growing chain acts as acceptor. It can be

  i) Polymerisation, when growing dextran chain is an acceptor (Fig. 1.1A) or

  ii) Oligosaccharides synthesis, when acceptor molecules are oligosaccharides (such as maltose, isomaltose and galactose). This is called acceptor reaction and it leads to inhibition of dextran production (Fig. 1.1B) and

(B) Hydrolysis, when water is an acceptor molecule (Fig. 1.1C)

The acceptor reaction involves the transfer of glucosyl moiety to a growing dextran chain. However, the transfer of dextranyl group to a dextran chain leads to give dextranyl branched dextran chains (Robyt and Taniguchi, 1976). The transfer of the dextranyl chain can also occur on to water and/or to an acceptor saccharide such as D-glucose, D-fructose, sucrose, or maltose to release the dextran from the active site and terminate polymerization (Robyt and Walseth, 1978; Luzio and Mayer, 1983). With maltose as an acceptor molecule, dextransucrase synthesizes oligosaccharides with α-(1→2) glycosidic bonds (Paul *et al.*, 1992; Remaud-Simeon *et al.*, 1994). These oligosaccharides resist hydrolysis by the digestive enzymes in animals and humans because of the configuration of their glycosidic bonds and they selectively stimulate the growth of intestinal microflora such as *Bifidobacterium* spp., *Lactobacillus* spp. and *Bacteroides* spp. (Valette *et al.*, 1993). But whatever be the end product formed the transfer of glucosyl units occur through the formation of covalent glucosyl-enzyme complex (Ebert and Schenk, 1968; Robyt et al., 1974; Luzio and Mayer, 1983) (Fig. 1.1). The complex was first isolated for the glucansucrase produced by *S. sobrinus* by Mooser and Iwaoka (1989).

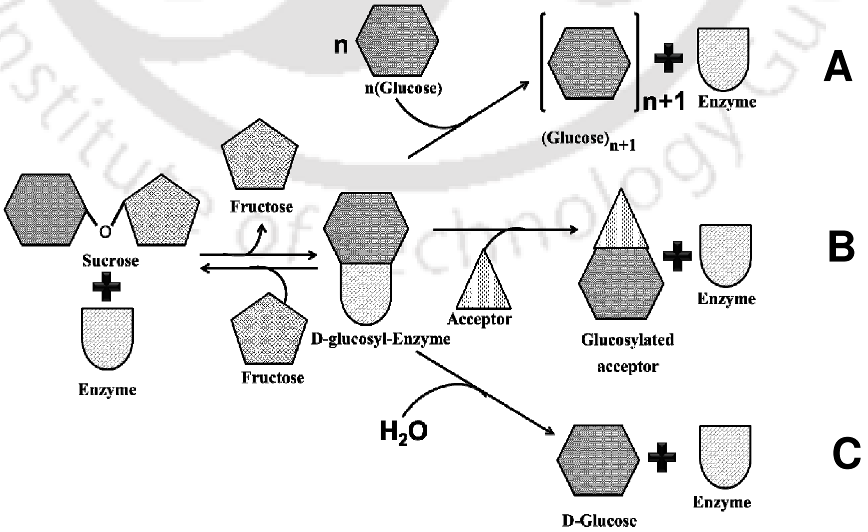

Fig. 1.1 Mechanism of reaction by glucosyltransferase showing transfer of glucosyl moiety to **(A)** Growing dextran chain, **(B)** Oligosaccharides and **(C)** Water (Mooser, 1992).

The growth of dextran chain occurs by two alternative mechanisms:

(A) Non-reducing end elongation in which there is participation of one site (an aspartate or glutamate) that acts as a nucleophilic group and another residue acts as a proton donor. The formation of covalent glucosyl-enzyme complex might takes place by nucleophilic attack of carboxyl group on C1 of the glucosyl unit of sucrose, while the other acidic group might release the fructose by giving a proton to the oxygen atom of glucosidic link. Mooser *et al.* (1991) gave the fact that only one site capable of making a covalent bond with the glucose moiety originating from the breakdown of sucrose which supports this hypothesis. The members of family GH70 elongate oligosaccharides at non reducing end (Monchois *et al.*, 2000; Arguello Morales *et al.*, 2001; Kralj *et al.*, 2005). But this hypothesis was considered inappropriate by Su and Robyt (1994). **(B)** The another mechanism is reducing end elongation which involves two step reaction with two sucrose binding sites (nucleophiles). Ebert and Schenk (1968) were the first to propose a mechanism where glucan elongation occurred at its reducing end. It was also supported by Robyt *et al.* (1974), Robyt (1996) and Monchois *et al.* (1999). According to this mechanism, the growth of dextran chain occurs by successive insertions of glucose moiety between catalytic site of enzyme and reducing end of polysaccharide. This mechanism consists of two distinct steps (Robyt et al., 1974)

i. Where the two nucleophilic sites attack two sucrose molecules resulting in the release of two fructose molecules and giving two glucosyl residues covalently linked to the enzyme and

ii. The –OH group of C6 of one of the two glucosyl residues may make a nucleophilic attack on the C1 of the other one forming an α(1→6) bond and release one of the two nucleophilic sites. The released nucleophilic sites can attack another sucrose molecule to create a new covalent glucosyl-enzyme complex.

.

Dextran elongation occurs by this mechanism where the two catalytic sites are alternately involved in covalent complexes with glucosyl residue or dextran and at each step, a glucosyl residue is inserted at the reducing end of the glucanosyl-enzyme complex. The same mechanism is expected to be used by amylosucrase (family 13 member) (Uitdehaag *et al.*, 1999, Albenne *et al.*, 2004, Jensen *et al.*, 2004), in which a covalent glucosyl-enzyme complex intermediate is formed. The similar set of experiments was conducted on *S. mutans* 6715 dextransucrase (Robyt and Martin, 1983) and with an immobilised dextransucrase free of glucan from *S. sanguis* ATCC 10558 (Ditson amd Mayor, 1984) in the support of fact that chain growth occurs at the reducing end. Recently, Robyt *et al.* (2008) have explained that the three conserved amino acids at one active-site can participate in the two catalytic-site, insertion mechanism. Robyt *et al.* (2008) also showed that the molecular size of dextran is inversely proportional to the concentration of the enzyme which indicated that the elongation of dextran is a highly processive reaction in which glucose is rapidly added to the reducing-end of the covalently linked, growing dextran chain.

However, a recent study on the crystal structure of a glucansucrase fragment (GTF180-ΔN) from *Lactobacillus reuteri* 180 have supported the non-reducing end growth mechanism (Vujicic-Zagar *et al.*, 2010). The crystal structure of the glucansucrase fragment (GTF180-ΔN) from *L. reuteri* 180 has confirmed that there was only one active site with no space for another covalently bound glucosyl residue or dextranyl chain (Vujicic-Zagar *et al.*, 2010).

1.2.3 Production of dextransucrases

Dextransucrases are commonly produced by four genera of lactic acid bacteria (LAB) namely *Streptococcus*, *Leuconostoc*, *Weissella* and *Lactobacillus* (Purama and Goyal, 2005). However, dextransucrase from *Pediococcus* spp. has also been reported (Patel *et al.*, 2011a). There are reports on the production of dextransucrase using shaken and static flask cultures (Tsuchiya *et al.*, 1952; Goyal *et al.*, 1995). The dextransucrase and dextran production from various lactic acid bacteria have been scaled up in the bioreactor (Patel *et al.*, 2011b; Shukla and Goyal, 2013a; Shukla and Goyal, 2013b). Purama and Goyal, 2008 scaled-up the batch cultivation from the flask culture to a bioreactor containing 1.4l of the optimized medium which resulted in a dextransucrase specific activity of 0.70 U/mg. The dextran concentration from *Pediococcus pentosaceus* SPAm was successfully enhanced in a 3l bioreactor using statistical optimized medium (Patel *et al.*, 2011b). The aeration conditions have also been optimized for production and scale up of dextransucrase from (Michelena *et al.*, 2003; Purama and Goyal 2008a).

Taguchi orthogonal design

The production of dextransucrases is affected by various factors like temperature, aeration, medium components (Tsuchiya *et al.*, 1952; Lazic *et al.*, 1993; Goyal *et al.*, 1995; Rodrigues *et al.*, 2003; Cortezi *et al.*, 2004). Hence there is a need of developing proper fermentation media to improve its efficient utilization in fermentation technology. The initial step in process of media optimization is to screen important factors affecting the production. However, alteration of one factor at a time, for conventional optimization of product formation, is a time and labour consuming process and also it does not effectively provide the combinatorial effect of studied factors (Beg *et al.*, 2003). Hence to overcome such situation, statistical approaches have been developed using a combination of mathematical and analytical tools (Box and Hunser, 1975). In this respect, recently developed orthogonal array (OA) optimization methodology provides offline quality control of system, parameter, and tolerance designs that help in the identification of key factors and their levels for best performance (Joseph and Piganatiells, 1988; Sreenivas *et al.*, 2004). For this, simultaneous study of various control factors and their optimization by statistical experimental designs have been done (Sreenivas *et al.*, 2004; Abdel-Fattah *et al.*, 2005). Various methods have been developed for optimization of nutrient's concentration such as neural networking (Singh *et al.*, 2008), Box Behnken method (Navaneeth *et al.*, 2009) and central composite design (CCD) (Patel *et al.*, 2011b). Among various statistical experimental designs studied, Taguchi experimental design was found to be better method by which the simultaneous examination of many factors can be done with the extraction of much quantitative information with a few experimental trials (Umesaki *et al.*, 1977; Stone and Veevers, 1994; Houng *et al.,* 2006). There are only few reports available on the application of Taguchi's method in the field of biotechnology (Cobb and Clarkson, 1994; Han *et al.,* 1998; Jeney *et al.*, 1999). However, the basic principle of this method serves as screening filters which examine the effects of many process variables and identify those factors which have major effects on process using a few experiments (Dasu *et al.*, 2003). Taguchi method of design of experiments (DOE) involves establishment of large number of experimental situation described as orthogonal arrays (OA) to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments (Krishna

Prasad *et al.,* 2005). Shukla and Goyal (2012) reported the use of Taguchi methodology for enhanced production of dextransucrase and dextran from *Pediococcus pentosaceus* CRAG3.

1.2.4 Purification of dextransucrases

Dextransucrase occurs in aggregated form resulting in its high molecular weight. The production of dextran increases viscosity of culture medium causing troubles in purification of enzyme. The purification of dextransucrases can be done by ultra-filtration, salt and PEG precipitation, phase-partitioning and chromatography, alone or in combination (Majumder *et al.*, 2007). The purification of dextransucrase from *L. mesenteroides* by ammonium sulphate precipitation was first done by Hehre (1946). Later, ammonium sulphate, alone or in combination with other solvents was used for the purification of dextransucrases from different strains. The dextransucrase from *L. mesenteroides* B-512 was purified by fractionation with ammonium sulphate and alcohol (Koepsell and Tsuchiya, 1952). But this method was found to be unsuccessful, as the reduction in about 90% activity of dextransucrase was observed at 80% (w/v) concentration of ammonium sulphate (Robyt and Walseth, 1978). The purification of dextransucrase from *L. mesenteroides* B-1299 using ammonium sulphate fractionation was also carried out by Gupta and Prabhu (1995). The purification of dextransucrase has also been done by fractionation with alcohol (Tsuchiya *et al.*, 1955) and 50% glycerol (Rodrigues *et al.*, 2003). The dextransucrase was successfully purified by precipitation with polyethylene glycol (PEG), which is one of the simplest, effective and one step purification method (Russell, 1979; Goyal and Katiyar, 1994; Purama and Goyal, 2007; Patel *et al.*, 2011a). Due to their large molecular weight and aggregate forming tendency in solution of dextransucrases, non-ionic hydrophilic polymer PEG is used for its purification (Goyal and Katiyar, 1994; Nigam *et al.*, 2006; Purama and Goyal, 2007). The PEGs of different molecular weights are used for precipitation of dextransucrase such as PEG 400 and PEG 6000 has been used for precipitation of dextransucrase from *Streptococcus mutans* (Russell, 1979), high molecular weight PEG 20000 has been used for purification of dextransucrase from *L. mesenteroides* B-512FM (Su and Robyt, 1994), while dextransucrase from *L. dextranicum* B-1146 was purified using PEG 200, PEG 400, PEG 4000 and PEG 6000 (Majumder *et al.*, 2008). Dextransucrase from *Pediococcus pentosaceus* isolated from soil of North East India was purified by polyethylene glycol (Patel *et al.*, 2011a). Although various PEGs are used for precipitation of dextransucrase, but high molecular weight PEGs such as PEG 6000 also allows other proteins to precipitate. Hence, PEG 400 is usually preferred for purification due to its ability to get removed after dialysis.

Another method for purification of dextransucrase is gel filtration. Various columns containing matrices such as Sephacryl S-200HR, Sephacryl S-300HR, hydroxyapatite, DEAE-cellulose, DEAE-sephadex, sephadex, ultragel AcA 34 have been used for the purification of enzyme. Sephadex has good affinity for dextran, hence can be used for purification of dextransucrase to get polysaccharide free enzyme. The purification of dextransucrase from *L. dextranicum* NRRL B-1146 (Majumder *et al.*, 2008) and *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2008) has been successfully done by gel filtration using Sephacryl S-200HR matrix.

1.3 Exopolysaccharides from lactic acid bacteria

Exopolysaccharides are high molecular weights biopolymers which attain sometimes several millions of Daltons. A number of lactic acid bacteria (LAB) including *Leuconostoc* and *Pediococcus* spp. produce long-chain polysaccharides and are mainly extracellular (Sutherland, 1994). Depending on the monomeric units present they can be heteropolymers (consisting of two or more different types of monomers) of neutral sugars (pentoses and hexoses) or anionic sugars (hexoses) or homopolymers (consisting of one type of monomers). Homopolymers are composed of a variety of sugar residues, chiefly glucose, galactose, fructose and rhamnose or sometimes charged groups such as acetate, phosphate or glycerolphosphate (de Vuyst & Degeest, 1999). The homopolymers consisting of glucose units are termed as glucans and those contains fructose monomers are known as fructans. These are synthesised by enzymatic reaction catalysed by glucosyltransferase (for glucans) or fructosyltransferase (for fructans) using sucrose (α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside) as a substrate. The applications of polysaccharides from various sources have been observed especially in pharmaceutical, food, agriculture and fine chemical industries (Sutherland, 1998; Majumder *et al.*, 2007; Patel *et al.*, 2012; Shukla and Goyal, 2013a). The exopolysaccharides are of two types; heteropolysaccharides and homopolysaccharides as described later in Section 1.3.1 and 1.3.2.

1.3.1 Heteropolysaccharides from lactic acid bacteria

Heteropolysaccharides (HePSs) are the exopolysaccharides that contain two or more different kind of monosaccharides. They are synthesized by intracellular sugar nucleotides, the intermediates of carbon metabolism and their monomers are formed in cytoplasm. They provide extracellular support to very different organisms, from prokaryotes to eukaryotes. The important heteropolysaccharides of extracellular matrix are hyaluronic acid, chondroitin sulfates and dermatan sulfates together with fibrous proteins, like collagen, elastin, fibronectin and laminin. These usually formed by the repetition of a disaccharide unit of an aminosugar and an acid sugar. Lactic acid bacteria synthesize HePSs at different stages of growth and the growth conditions regulate the amount and type of polymer (De Vuyst and Degeest, 1999). Structurally they are ropy or mucoid. The effects of nutrients on growth, metabolic pathways and biosynthesis of HePSs by lactic acid bacteria can be studied by chemically defined medium containing carbohydrates, amino acids, vitamins, nucleic acid bases and mineral salts. The production of heteropolysaccharides by LAB vary significantly amongst different strains such as 50-350 mg/l for *Streptococcus thermophilus*, 80-600 mg/l for *Lactococcus lactis* subsp. *cremoris*, 60-150 mg/l for *Lactobacillus delbrueckii* subsp. *bulgaricus* (Cerning, 1995), and approximately 140 mg/l for *Lactobacillus plantarum* (Tallon *et al.*, 2003 Tsuda and Miyamoto, 2010).

1.3.2 Homopolysaccharides from lactic acid bacteria

Homopolysaccharides are the polymers consist of single type of monomers such as cellulose (a linear polymer of D-glucose residues bonded by β-(1→4)-O-glycosidic linkages), dextran (a polymer consists of D-glucose residues bonded by α-(1→6)-O-glycosidic linkages) and fructan (a polymer of D-fructose residues bonded by β-(2→1) orβ-(2→6) linkages). The synthesis of homopolysaccharides (HoPSs) in lactic acid bacteria has been mainly studied in species of *Streptococci*, *Leuconostoc* and *Lactobacillus* (Monsan *et al.*, 2001). However the homopolysaccharides from *Pediococcus* spp. (Patel et al., 2010; Shukla and Goyal 2013a) and *Weisella* spp. (Shukla and Goyal, 2013b) has also been reported. These are synthesized by extracellular glycosytransferases using sucrose as the glucosyl donor. Glycosytransferases (glucosyltransferase and fructosyltransferases) catalyse the transfer of glycosyl moiety by cleaving the osidic bond of sucrose to synthesize corresponding EPS polymer (glucan or fructan) (Patel *et al.*, 2010). HoPSs are classified into two groups **i)** β-fructans and **ii)** Glucans (Fig. 1.2).

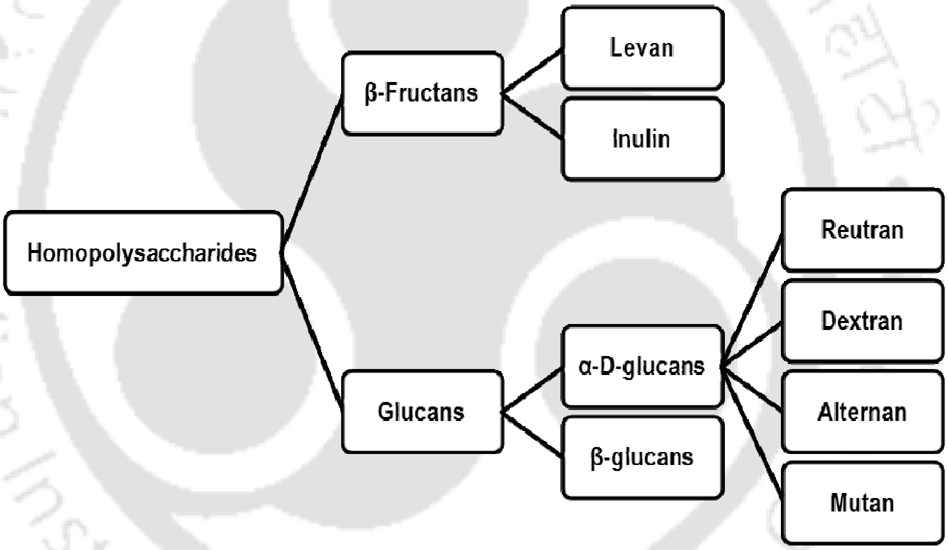

Fig. 1.2 Classification of homopolysaccharides into β-fructans and glucans.

i) β-fructans are mainly of two types **a)** Levans composed mainly of β-(2→6) linked fructose residues (Fig. 1.3A). The synthesis of levans has been observed in *Streptococci* (Simms *et al.*, 1990), *Leuconostoc mesenteroides* (Robyt and Walseth, 1978) and also in lactobacilli: *Lactobacillus reuteri* 121 (van Geel-Schutten *et al.*, 1999, van Hijum *et al.*, 2001). Some non-LAB also synthesize levans such as *Diazotrophicus* (Hernandez *et al.*, 1995), *Zymomonas mobilis* (Song *et al.*, 1993;

Kyono *et al.*, 1995) and *Bacillus* spp. (Li *et al.*, 1997, Perez *et al.*, 1996). **b)** inulin containing β-(2→1) linked fructose residues (Fig. 1.3B), are produced by (cariogenic) *Streptococcus* species, *Leunostoc citreum* CW28 and by inulosucrase of *Lactobacillus reuteri* 121 (van Hijum *et al.*, 2002, Olivares-Illana *et al.*, 2003).

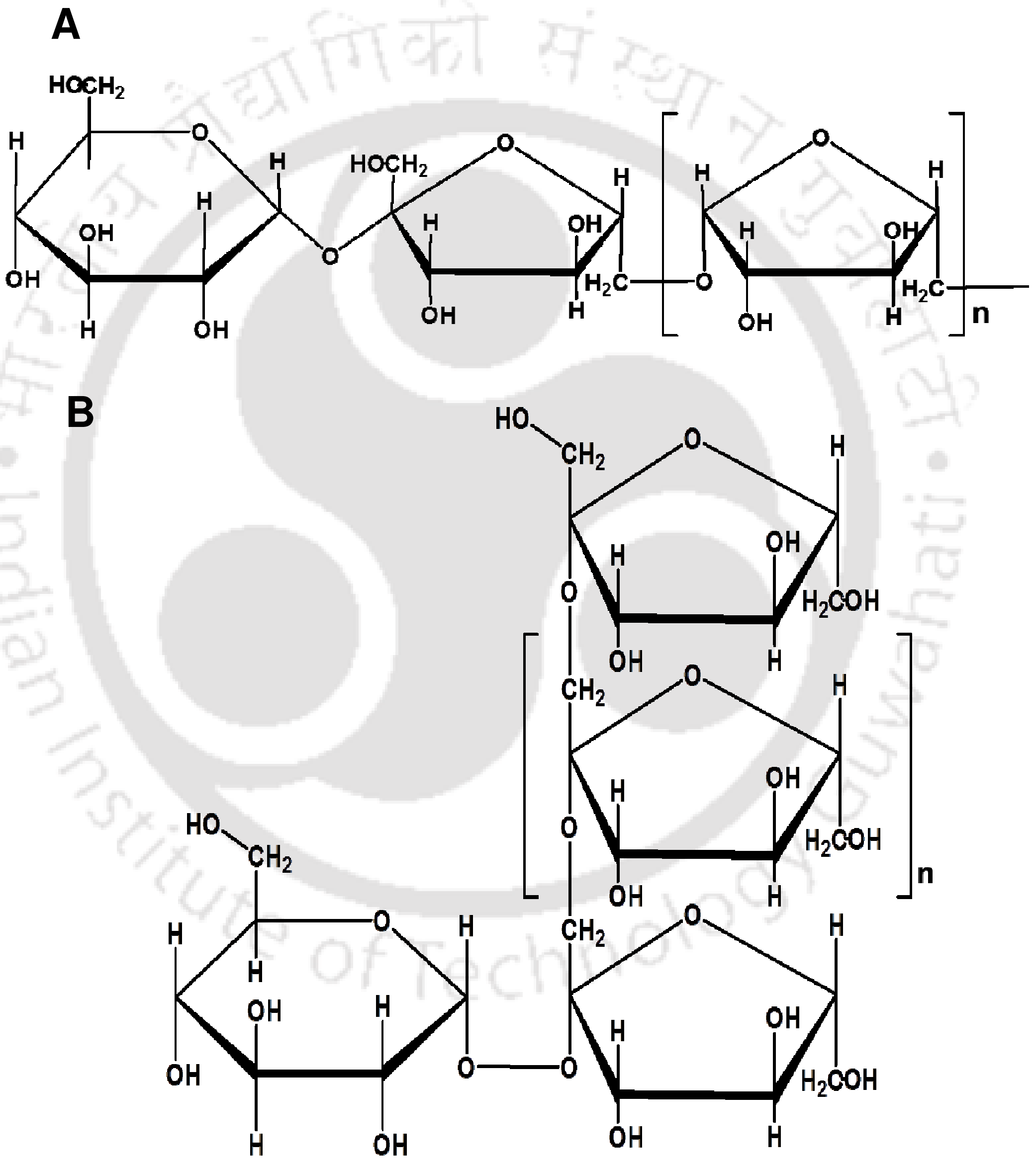

Fig. 1.3 Basic structure of β-fructans synthesised by fructosyl-transferases. Based on the dominant linkage type in main chain, the β-fructans are classified into two groups: **(A)** Levan containing β-(2→6) linkages in main chain and **(B)** Inulin having β-(2→1) linkages in main chain (Leemhuis *et al.*, 2013).

ii) Synthesis of α-glucan has been observed in four different genera of lactic acid bacteria: *Streptococci*, *Leuconostoc*, *Weissella* and *Lactobacilli* (van Geel-Schutten *et al.*, 1998; Monchois *et al.*, 1999; Kralj *et al.*, 2002; Tieking *et al.*, 2003; Kralj *et al.*, 2005; Kralj *et al.*, 2004). However, *Pediococcus* species also produce α-glucan (Smitinont *et al.*, 1999). The ability of *Pediococcus pentosaceus* to produce glucan was first reported by Patel *et al.* (2010). The α-D-glucan from LAB comprise five groups **a)** dextran, containing predominantly α-(1→6) linked glucopyranosyl units in the main chain (Fig. 1.4A) (Cerning, 1990); **b)** reuteran, containing large amounts of α-(1→4) glucosidic bonds (Fig. 1.4B) synthesized by *Lactobacillus reuteri* 121 and *Lactobacillus reuteri* BioGaia (Kralj *et al.*, 2002; Kralj *et al.*, 2005); **c)** mutan, a polyglucose with mainly α-(1→3) linkages (Fig. 1.4C) (various *Streptococci*) (Hamada and Slade, 1980) and *Lactobacillus reuteri* ML1 (Kralj *et al.*, 2004b); **d)** alternan with alternating α-(1→6) and α-(1→3) linked D-glucopyranosyl units (Fig. 1.4D) (*Leuconostoc mesenteroides* NRRL B-1355) (Arguello-Morales *et al.*, 2000), and **e)** glucan polymers containing large amounts of α-(1→2) linkages, produced by *Leuconostoc mesenteroides* strain NRRL-B1299 and a mutant strain R510 of *Leuconostoc mesenteroides* NRRL B-1355 (Bozonnet *et al.*, 2002, Smith *et al.*, 1998). The nature and amount of other glucosidic linkages present in glucans may differ in degree of branching, type of branching point, molecular weight, the length of the branching chains and their spatial arrangement (Monchois *et al.*, 1999). This results in large variations in solubility and other physical characteristics of glucans (Monchois *et al.*, 1999). It has been reported that the polysaccharides with α-(1→6) and α-(1→4) linkages acts like promising anti-cancer agents (Cao *et al.*, 2006; Liu *et* *al.*, 2007). The branched water soluble α-D-glucan had been isolated from various sources such as the mushrooms *Agaricus bisporus* and *Agaricus brasiliensis* (Smiderle *et al.*, 2011) and the fruit bodies of *Coprinus comatus* (Li *et al.*, 2013). Various species of *Leuconostoc* are known to produce glucans of varying molecular weights (Qader *et al.*, 2007; Farwa *et al.*, 2008; Aman *et al.*, 2012). *Leuconostoc mesenteroides* AA1 isolated from fermented cabbage produce high molecular weight α-(1→6) linked linear glucan (Aman *et al.*, 2012). The synthesis of galactan has been observed in *Lactococcus lactis* subspecies *cremoris* H414 (Gruter *et al.*, 1992). The synthesis of these polysaccharides takes place in the same way as heteropolysaccharides but without the involvement of sucrase enzymes.

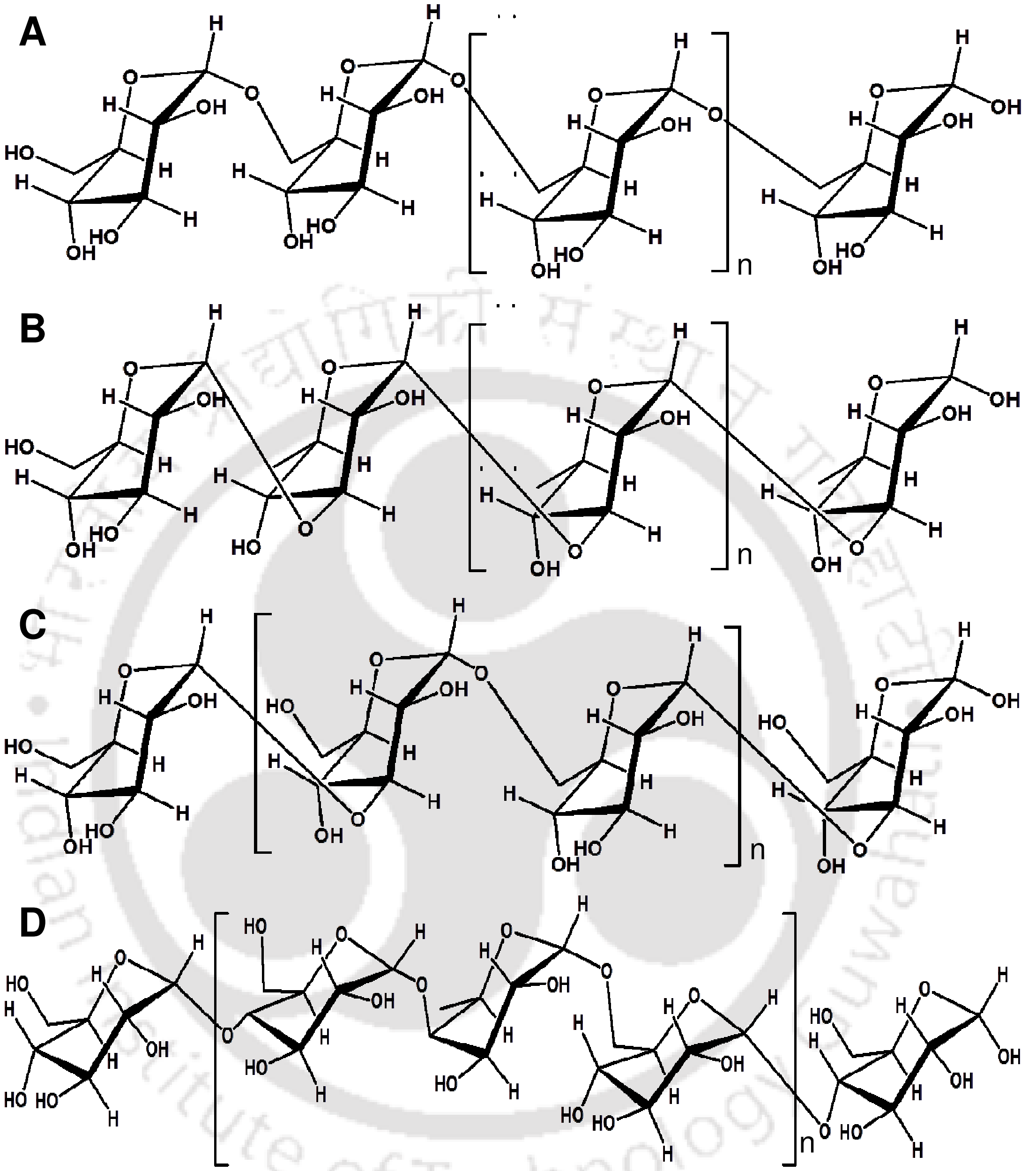

Fig. 1.4 Basic structures of α-glucans synthesised by the reactions catalysed by glucosyl transferases. **(A)** Dextran having predominant α-(1→6) linkages; **(B)** Reutran containing large number of α-(1→4) linkages; **(C)** Mutan possessing mainly α-(1→3) linkages and **(D)** Alternan with alternate α-(1→3) and α-(1→6) linkages (Leemhuis *et al.*, 2013).

1.4 Structure of dextran

Colson *et al.* (1974) studied the ^{13}C NMR of various linear dextrans and assigned the peaks by comparing with the spectrum of glucose. They clearly assigned chemical shifts of C-1 to C-6 of linear α-(1→6) dextrans and also showed the effects of branching on chemical shifts of various carbon atoms. Seymour *et al* (1979a, 1979b, 1979c, 1979d, 1979e) studied the ^{13}C NMR spectra of various linear dextrans and showed that

1. Linear dextran has 6 major resonances which were termed A, B, C, D, E and F.
2. Besides these 6 resonances, additional resonances in spectra indicate branching.
3. These additional resonances are present in anomeric region (95-105 ppm) and the diagnostic peaks for type of linkages are present in 80-85 ppm region.
4. The relative peak heights of these diagnostic resonances of branching are proportional to the degree of branching.

Structural characterization of glucans from various lactic acid bacteria such as *Leuconostoc dextranicum* NRRL B-1146 (Majumder *et al.*, 2009), *Leuconostoc mesenteroides* NRRL B-640 (Purama *et al.*, 2009), *Pediococcus pentosaceus* (Patel *et al.*, 2010), *Pediococcus pentosaceus* CRAG3 (Shukla and Goyal, 2013a) and *Weisella cibaria* JAG8 (Rao and Goyal, 2013) using NMR and FTIR spectroscopic techniques have been described. The dextran from *Leuconostoc dextranicum* NRRL B-1146 was highly linear with 97% α-(1→6) and 3% α-(1→3) linkages (Majumder *et al.*, 2009), while that from *Leuconostoc mesenteroides* NRRL B-640 was linear with with consecutive α-(1→6) linkages (Purama *et al.*, 2009). *Pediococcus pentosaceus* (Patel *et al.*, 2010) and *Weisella cibaria* JAG8 (Rao and Goyal, 2013) produce linear dextran having predominant α-(1→6) linkages as determined by NMR and FTIR spectroscopy techniques.

The structural analysis of dextrans from gum producing bacteria using NMR spectroscopy showed that *Leuconostoc dextranicum* contains mainly α-(1→3) branching along with some α-(1→4) linkages; *Leuconostoc mesenteroides* possessed generally α-(1→2) branching and *Lactobcillus* spp. appeared to be branched chiefly by α-(1→2) linkages in α-(1→6) backbone (Uzochukwo *et al.*, 2001). Wang *et al.*, (2007) studied the structure of water-insoluble (1→3)-α-D-glucan isolated from the *Penicillium chrysogenum.* FTIR spectral analysis of dextran from *Pediococcus pentosaceus* CRAG3 showed the presence of α-(1→6) and α-(1→3) linkages (Shukla and Goyal 2013a). The ^{1}H NMR spectrum of dextran from *Pediococcus pentosaceus* CRAG3 showed 75% α-(1→6) linked glucose and 25% α-(1→3) linked glucose (Shukla and Goyal 2013a). The percentage of branching in exopolysaccharide (EPS) from *Weissella confusa* E392 using integration of ^{1}H-NMR spectrum showed that it contained 97.3% of α-(1→6) linked linear chain with 2.7% of α-(1→3) linked branches (Maina *et al.*, 2008).

1.5 Applications of exopolysaccharides

Exopolysaccharides have many applications in industries due to their non-ionic character and good stability under normal operating conditions. Sutherland (1972) described viscosifying, stabilizing, emulsifying, prebiotic, gelling or water binding properties of exopolysaccharides for their use in food as well as in non-food industry. The polysaccharides from *Lactobacillus* are of great importance due to their contribution in human health as antitumoral (Oda *et al.*, 1983), antiulcer (De Roos and Katan, 2000), immunomodulating (Schiffrin *et al.*, 1995) or cholesterol-lowering activity (Roberfroid *et al.*, 1993).

1.5.1 Applications of dextran

1.5.1.1 Applications in pharmaceutical industry

Dextran displays wide range of applications in food industry, pharmaceutical and various other fields (Fig. 1.5). They are used as lubricant in eye drops and to increase blood sugar levels (Koepsell and Tsuchiya, 1952). Dextran sulphates are highly active inhibitors of encephalomyocarditis hemagglutination (Kunin, 1967). It has also been reported that sodium salt of dextran sulphate inhibits AIDS virus (Baba *et al.*, 1990). They can be used for matrix preparation of chromatography columns such as Sephadex (Shamala and Prasad, 1995). α-Glucans and their derivatives are used in industrial and biomedical applications such as gel filtration products, blood plasma substitutes, plasminogen activators and anti-thrombogenic agents (Soetaert *et al.*, 1995; De Vuyst and Degeest, 1999; Purama and Goyal, 2005). Due to their large size, dextran can act as osmotic agents, to treat hypovolemia (Alpar and Killampalli, 2004). Iron dextran is used to treat iron deficiency anaemia (Thayu and Mamula, 2005).

Dextran produced by oral *Streptococci* play major role in the cariogenesis process, by enhancing the attachment and colonization of cariogenic bacteria (Loesche, 1986). In addition, a carboxy-methylated derivative of (1→3)-α-D-glucan with the activity against sarcoma 180 was synthesized by substitution with chloroacetic acid (Kiho *et al.*, 1989). The relationship between the nature of functionalized groups and physicochemical properties of the chemically modified derivatives of linear (1→3)-α-D-glucan, obtained from the spores of *Ganoderma lucidum*, and their immunomodulating activity were reported (Perez *et al.*, 1996). The antitumor activity of sulphated linear (1→3)-α-D glucan from the fruiting body of *Agrocybe cylindracea* (DC. ex Fr.) Maire (Bolbitiaceae) was obtained and its antitumor activity against *Ehrlich ascites* carcinoma was investigated (Zhang *et al.*, 2000). Dextrans have been frequently employed as drug carriers (Ulbrich and Subr, 2004), for antigen delivery in vaccination (Sahoo *et al.*, 2007), as vectors for anti-cancer agent (Agrawal *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). The porous glucan particles from *Saccharomyces cerevisiae* are known to be used in macrophage targeted delivery of nanoparticles. Soto *et al.* (2010) reported the application of glucan particle encapsulated rifampicin in delivery of nanoparticles to macrophages. Dextran have been used in controlled drug-delivery systems (Dinu *et al.*, 2011). The pharmaceutical and biomedical applications of dextran also include their role in contact lenses, cell encapsulation for drug delivery, burn wound dressing and in spinal cord regeneration (Aumelas *et al.*, 2007; Chen *et al.*, 2013).

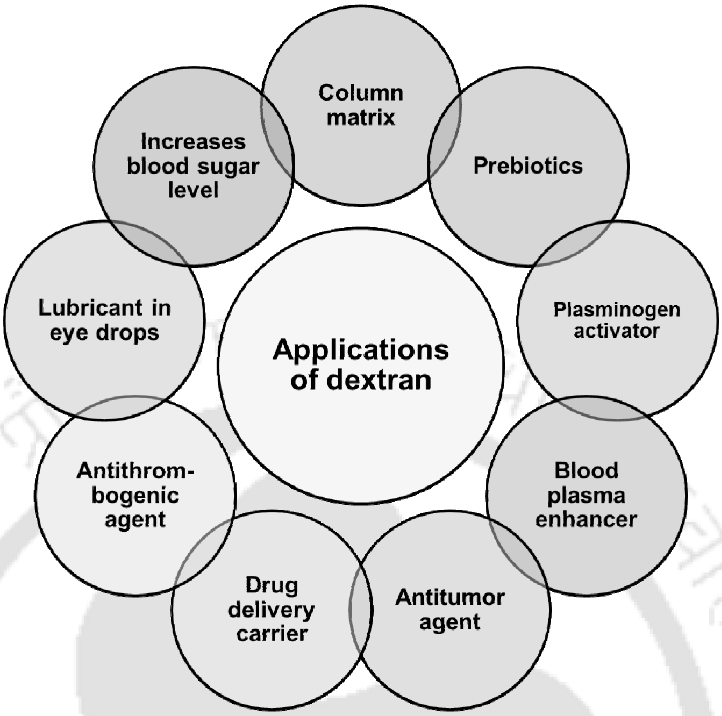

Fig. 1.5 Applications of dextran.

1.5.1.2 Applications in food industry

Dextrans provide a stabilizing coating for protecting metal nanoparticles against oxidation (Bautista *et al.*, 2005). They are expected to impede adhesion of protein by exerting repulsive steric effects and hydration pressure (Chen *et al.*, 2008). They can be used as food syrup stabilizers and dough improvers (Katina et al., 2009). The role of exopolysaccharides in improving rheology of dough and bread texture has been studied in dextran from *Weissella* spp. (Di Cagno *et al.*, 2006), *Leuconostoc mesenteroides* (Patel *et al.,* 2012) and levan from *Lactobacillus sanfranciscensis*; (Waldherr and Vogel, 2009). Dextran is known to improve functional properties such as solubility and surface properties of proteins through a Maillard reaction (Sun *et al.*, 2011; Zhang *et al.*, 2012). Pepe *et al.* (2013) demonstrated that the combined use of wheat flour and dextran-producing LAB strains in the sourdough process stimulated the LAB and yeast metabolism and led to shorter leavening times and higher EPS production in dough.

1.5.1.3 Role of dextran as anticancer agent

Glucans (both alpha and beta) from various sources such as lichens (Olafsdottir *et al.*, 1999), plants (Nair *et al.*, 2006), fungi (Lavi *et al.*, 2006) and bacteria (Shukla and Goyal, 2013a; Haroun *et al.*, 2013) have been known to possess immune-modulatory properties. However, the anticancer properties or immuno-stimulatory of α-glucans are not much studied as compared to β-glucans. There are few reports on anticancer α-glucans but only after their chemical modification such as carboxy-methylation (Bao *et al.*, 2001) and sulfation (Wiater *et al.*, 2010). The innate immunity is pleaded by neutrophils, natural killer cells and macrophages. During microbial invasion, the innate immunity acts as first host line defense and targets the structurally conserved pathogen-associated molecular patterns (PAMPs) through specific germ-line encoded receptors called pattern recognition receptors (PRRs) (Aderem and Ulevitch, 2000). There are receptors present on macrophages such as complement receptor 3 (CD11b/CD18 or CR3), Dectin-1 and Toll-like receptors (TLRs) 2 and 6 which recognize β-glucans. These receptors interact with glucans and stimulate the synthesis of cytokines, chemokines and reactive oxygen intermediates (Gantner *et al.*, 2003). The biological activity of (1→3)-α-D-glucan from *Ganoderma lucidum* was reported by Bao *et al.* (2001). The (1→4)-α-D-glucan from *Tinospora cordifolia* has been shown to possess immune-stimulatory properties (Nair *et al.*, 2006). It has been reported that the polysaccharides from *Strongylocentrotus nudus* eggs with α-(1→6) and α-(1→4) linkages act as promising anti-cancer agents (Liu *et al.*, 2007). The prebiotic β-glucan from probiotic bacterium *Lactobacillus plantarum* NRRL B-4496 showed antitumor activity (Haroun *et al.*, 2013). Shukla and Goyal (2013) reported the antitumor dextran from *Pediococcus pentosaceus* CRAG3 with α-(1→6) and α-(1→3) branched linkages (present work).

α-Glucans from various sources have been evaluated for *in vitro* cytotoxicity (Lin *et al.*, 2004; Patel *et al.*, 2010; Huang and Zhang, 2011; Shukla and Goyal, 2013a). α-Glucan from the lichen *Ramalina celastri* demonstrated to have cytotoxic effects against cervical cancer (HeLa) cells (Carneiro-Leao *et al.*, 1997). Patel *et al.* (2010) studied the cytotoxicity of biocompatible and non-toxic dextran from *Pediococcus pentosaceus* on HeLa cell line. The branched dextran from *Pediococcus pentosaceus* CRAG3 displayed anticancer activity against cervical cancer (HeLa) and colon cancer (HT29) cell line, however it did not exhibit any negative effect on macrophage cell line (Shukla and Goyal, 2013a).

1.6 *Pediococcus* as dextran producer

Pediococci are a group of homofermentative lactic acid bacteria (Benachour *et al.*, 1995). Till now this group includes *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). They were isolated from various places like soil (Patel and Goyal, 2010), plants (De Cagno *et al.*, 2009), wines (Beneduce *et al.*, 2004), cheese (Gurira and Buys, 2005), wheat kernels (Corsetti *et al.*, 2007), meat (Anastasiadou *et al.*, 2008) and sausages (Nieto-Lozano *et al.*, 2010). Very few reports are available showing dextran production by

Pediococcus genus (Smitinont *et al.*, 1999; Patel et al., 2010). Duenas-Chasco *et al.* (1997) and Duenas *et al.* (2003) reported a β-linked homopolymer of D-glucose from *Pediococcus damnosus* (strain 2.6) in semi defined medium which consists of trisaccharides repeating units as determined by ^{1}H, ^{13}C, 1D and 2D NMR analyses. The ropy EPS from the same strain was used as thickener in food systems (Lambo-Fodje *et al.*, 2007). *Pediococcus pentosaceus* strains AP-1 and AP-3 isolated from traditional thai fermented food produced EPS in high yield (Smitinont *et al.*, 1999). A substitute of (1→3)-β-D-glucan from *P. parvulus* 2.6 was isolated from cider (Martensson *et al.*, 2002). The (1→3)-β-D-glucans has been used for the production of a fermented oat product (Martensson *et al.*, 2002) and also in pharmaceutical and functional food industries (Zekovic *et al.*, 2005). The exopolysaccharides from *Pediococcus* (Shukla and Goyal., 2013a) and other LAB (Welman and Maddox, 2003; Liu *et al.*, 2011) have been successfully used as antitumoral, immunostimulator and blood cholesterol reducing agents. The β-D-glucan producing *Pediococcus* strain was used to immunomodulate macrophages (Fernández de Palencia *et al.*, 2009).

1.7 Probiotics

Probiotics are the live microorganisms, which contribute to the health and well being of the hosts by maintaining or improving their intestinal microbial balance (Asahara *et al.*, 2004). The mechanisms of probiotic action are not much studied, but colonization resistance or competitive exclusion are often used to explain their mode of action (Elo *et al.*, 1991). The effects of probiotics may be classified in three modes of action by Oelschlaeger (2010):

i. Controlling the host defence system which helps in prevention and therapy of infectious diseases and also for treatment of inflammation of the digestive tract.

ii. Direct effect on other microorganisms, commensal and/or pathogenic ones which shows its importance in prevention and therapy of infections and restoration of the microbial equilibrium in the gut.

iii. The probiotic effects based on actions affecting microbial products (such as toxins) and host products (like bile salts and food ingredients)due to which toxins get deactivated and detoxification of host components occurs in the gut.

For a bacterium to behave as probiotic and can benefit human health, it must fulfil several criteria such as activity and viability in products, adherence, invasive potential, resistance to low pH, gastric juice, bile acid and pancreatic juice, colonisation/survival *in vivo*, antagonism to pathogens and antimicrobial activity. There are various factors that affect the viability of probiotic bacteria until they reach the target site of the host (Fig. 1.6) (Lacroix and Yildirim, 2007).

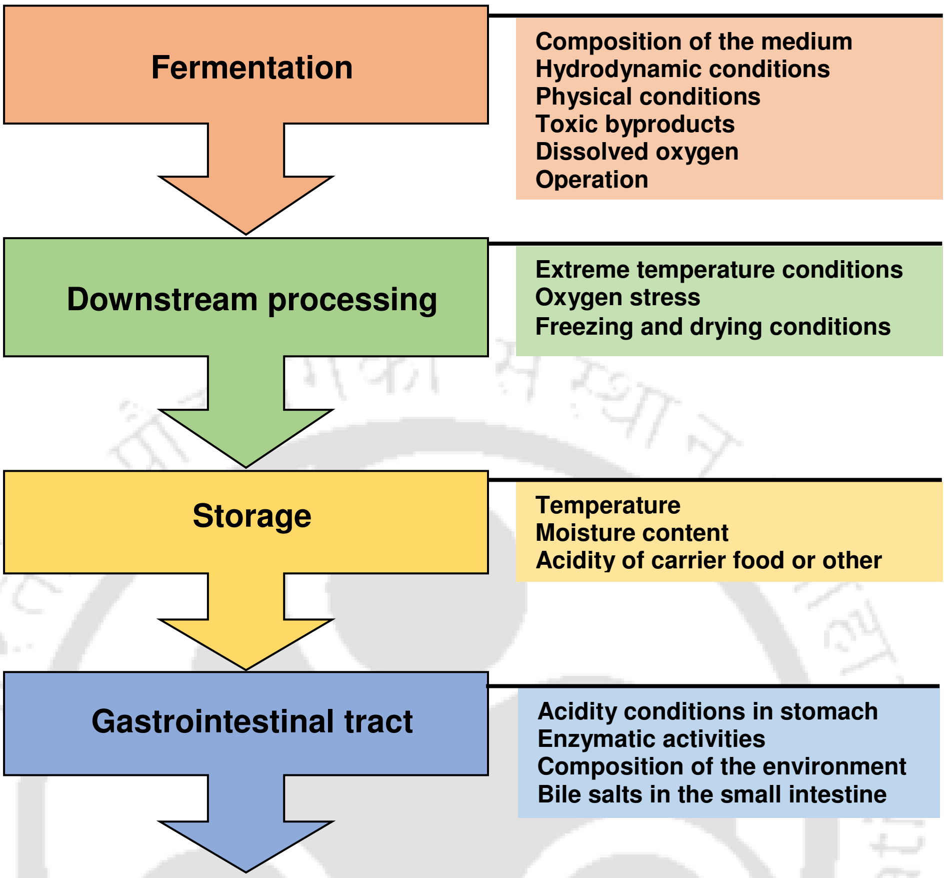

Fig. 1.6 Factors affecting the stability of probiotics during processing steps (Lacroix and Yildirim, 2007)

Probiotics may cause four types of side effects in susceptible individuals such as systemic infections, deleterious metabolic activities, excessive immune stimulation and gene transfer (Marteau, 2001; Marteau and Seksik, 2004). However, probiotic *Lactobacilli* and *Bifidobacteria* rarely cause infections in humans (Salminen *et al.*, 1998; Ishibashi and Yamazaki, 2001; Borriello *et al.*, 2003). Members of the genera *Lactococcus* and *Lactobacillus* are predominantly probiotic and given the GRAS (Generally recognised as safe) status. Currently most probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium* (Prasad *et al.* 1998). However, species belonging to the genera *Lactococcus*, *Enterococcus*, *Saccharomyces* (Salminen and von Wright 1998, Dunne *et al.*, 1999; Sanders and in't Veld, 1999) and

Propionibacterium (Grant and Salminen, 1998) are also considered as probiotic microorganisms. Even *Streptococcus thermophilus* (Cosson and Deschamps, 1994; Collins *et al.*, 1998; Naidu *et al.*, 1999; Sreekumar and Hosono, 2000) and *Lb. delbrueckii* subsp. *bulgaricus* (Bezkorovainy *et al.*, 1997; Naidu *et al.* 1999; Sreekumar and Hosono, 2000) are also considered probiotics. There are number of reports which demonstrate the probiotic properties of *Pediococcus* spp. (Benjamas *et al.*, 2008; Yuksekdag and Aslim, 2010; Sukumar and Ghosh, 2010; Shukla and Goyal, 2013c).

P. acidilactici has GRAS (Generally Recognized as Safe) status and is used as probiotic culture and nutritional enhancer in silage (O'Connor *et al.*, 2007; Kantor *et al.*, 1997). The members of the *Pediococcus* genus have great economic importance in the fermented food industry (Dellaglio and Torriani, 1986; Benachour *et al.*, 1995) and as starter cultures in fermentation processes of milk, meat, vegetable products and sausages (Dellaglio and Torriani, 1986; O'Connor *et al.*, 2007). The roles of probiotics include inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activity, increase of the immune response and reduction of cholesterol levels (Shornikova *et al.*, 1997; Du Toit *et al.*, 1998). They can also be serve in reducing diarrhea, decreasing pain and constipation associated with irritable bowel syndrome, reducing bloating and flatulence (Parvez et al., 2006; Candela et al., 2008).

Several *Pediococcus* strains produce bacteriocins thereby inhibiting the growth of major Gram-positive foodborne pathogens, as well as other food spoilage bacteria which display their role as potential probiotics (Kantor *et al.*, 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 and Dicks, 2009). With a compound annual growth rate of 4.3%, the global market of probiotic ingredients, supplements and food was around \$14.9 billion in 2007 and \$15.9 billion in 2008 and it was likely to reach 19.6 billion in 2013 (Agheyisi, 2008). The use of probiotics as functional foods has increased in recent times. Functional foods are defined as: 'foods which contain some health-promoting components along with traditional nutrients'. The addition of probiotics to foods is the one way to make these foods functional (FAO, 2002).

The growth of probiotic is influenced by the consumption of prebiotics which is non-digestible food ingredient that selectively stimulates the growth and activity of colon bacteria and thereby beneficially affects the host (Gibson and Roberfroid, 1995). Certain carbohydrates (or prebiotics), influence the composition of microflora in the large intestine by escaping the metabolism and adsorption in the small intestine (Loo *et al.*, 1999). The prebiotics identified so far are non-digestible carbohydrates, including lactulose, inulin, and a range of oligosaccharides especially fructooligosaccharides (Crittenden, 1999; Crittenden *et al.*, 2001). For an oligosaccharide or carbohydrate to behave as prebiotics it must be able to withstand digestive processes before it reach the colon and preferably persist throughout the large intestine such that benefits are apparent distally (Gibson *et al.*, 2004). The most important function of the prebiotic carbohydrates is to strengthen the resistance of body to invading pathogens and thereby prevent the chances of diarrhoea. The prebiotic must resist to gastric acidity, enzymatic hydrolysis by mammalian system and absorption in gastrointestinal tract. The prebiotics support the growth of an organism as compared to other organisms and growth on non-prebiotic substrates like glucose (Huebner *et al.*, 2007). Recently it has been reported that the glycans shape the composition of the gut microbiota and provides potential opportunities to intentionally influence their ecosystem for better health and nutrition (Koropatkin *et al.*, 2012).

1.8 Significance of the investigation

Lactic acid bacteria (LAB) comprise a group of Gram positive, non-spore forming, catalase negative cocci or rod shaped microorganisms having great importance in fermented food industries. Although there are reports available on isolation of LAB from different sources, only few emphasized on isolation from fruits and vegetables (Padmaja et al., 2011; Ravi et al., 2011). Cucumbers serve as traditional fermented foods in some areas although it is cultivated throughout the world (Chen et al., 2012). *Pediococci* are a group of homofermentative, coccus shaped, gram positive lactic acid bacteria that involve in the manufacturing of fermented foods (Semjonovs and Zikmanis, 2009). The members of the *Pediococcus* have great economic importance in fermented food industry (Dellaglio and Torriani, 1986) and as starter cultures in fermentation processes of milk, meat, vegetable products and sausages (O'Connor et al., 2007). Apart from various other industrial application *Pediococcus* spp. is known to produce exopolysaccharides. The interest in polysaccharides has arisen due to their biological activities such as immunomodulatory and antitumor effects. The earliest polysaccharide named Shear's polysaccharide having antitumor activity was isolated from the bacterium *Serratia marcescens* (Whistler et al., 1976), later some other bacterial polysaccharides with similar property were also reported (Umezava et al., 1983). The lactic acid bacterium *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber (Shukla and Goyal, 2013c). The dextransucrase produced by this strain catalyzed the formation of the branched dextran with α-(1→6) and α-(1→3) linkages. So this enzyme could be used in production of branched oligosaccharides having superior prebiotic applications. The glucooligosaccharides produced serve as excellent prebiotics as they escape from being digested by intestinal track however probiotic microflora present in gut can ferment these oligosaccharides and synthesizes various neutraceuticals. Prebiotic oligosaccharides have a beneficial impact on human health.

1.9 Objectives of the present study

The lactic acid bacteria (LAB) have been isolated from different sources, but only few emphasized on isolation from fruits and vegetables. The LAB from lettuce, cucumber and cabbage are not much studied. Fermented cucumber serves as traditional food in some parts of the world. Therefore the isolation and characterisation of industrially important LAB from fermented cucumber earns special attention.

The aim of the current study is to isolate novel and hyper dextransucrase and dextran producing lactic acid bacteria from fermented cucumber. Based on their higher enzyme activity, the bacterial colonies will be selected. The selected bacterium will be physiologically and biochemically characterized using Gram staining, catalase test, carbohydrate utilization pattern and antibiotic susceptibility experiments to distinguish the selected isolate from other closely related lactic acid bacteria. The isolated bacterium will be identified at species and genus level based on 16S rRNA gene sequencing. The culture conditions for maximum enzyme production will be optimized. The purification of dextransucrase from the isolate will be carried out using the polyethylene glycol fractionation and gel filtration. The purified dextransucrase will be characterized by non-denaturing SDS PAGE gel and zymogram analysis. The reaction conditions for enzyme activity such as sucrose concentration, temperature, pH and ionic strength of the buffer will be optimized. The effects of different salts, temperature and additives on the dextransucrase activity of the isolate will be studied.

The dextran will be synthesised using purified dextransucrase and will be purified by alcohol precipitation and lyophilized. The dextran will be structurally characterized by optical rotation, FT-IR, NMR and SEM studies. *In vitro* cytotoxicity assay of dextran will be performed using various mammalian cell lines to evaluate its biomedical applications. The production of dextransucrase and dextran from the natural isolate of lactic acid bacterium will be enhanced using statistical methods. The isolate will be explored for its probiotic properties such as cell adhesion, resistance to biological barriers, utilization of prebiotics and antibacterial activity.

1.10 Specific objectives of present study

1. Identification and biochemical characterization of *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber.
2. Production, purification and characterization of dextransucrase from *Pediococcus pentosaceus* CRAG3.
3. Purification and characterization of dextran from *Pediococcus pentosaceus* CRAG3.
4. Enhancement of production of dextransucrase and dextran from *Pediococcus pentosaceus* CRAG3 in bioreactor using Taguchi methodology.
5. Analysis of probiotic potential of *Pediococcus pentosaceus* CRAG3.

1.11 References

Abdel-Fattah Y.R., Saeed H.M., Gohar Y.M. and El Baz M.A. (2005) Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. Process Biochem. 40, 1707-1714.

Aderem, A. and Ulevitch, R.J. (2000) Toll-like receptors in the induction of the innate immune response. Nature. 406, 782-787.

Agheyisi, R. (2008) The probiotics market: Ingredients, supplements, foods, Report code: FOD035B, BCC Research, Wellesley, MA, USA (*http://www.bccresearch.com/report/FOD035B.html*).

Agrawal, 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., Hogg, 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.

Albenne, C., Skov, L. K. Mirza, O. Gajhede, M. Feller, G.’Amico, S. D Andre, G. Potocki-Veronese, G. van der Veen, B. A. Monsan, P. and Remaud-Simeon M. (2004) Molecular basis of the amylose-like polymer formation catalyzed by *Neisseria polysaccharea* amylosucrase. J. Biol. Chem. 279,726-734.

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

Aman, A., Siddiqui, N. N. and Qader, S. A. (2012) Characterization and potential applications of high molecular weight dextran produced by *Leuconostoc mesenteroides* AA1. Carbohydr. Polym. 87, 910-915.

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.

Arguello Morales, M. A., Remaud-Simeon, M. Willemot, R. M. Vignon, M. R. and Monsan. P. (2001) Novel oligosaccharides synthesized from sucrose donor and cellobiose acceptor by alternansucrase. Carbohydr. Res. 331, 403-411.

Arguello-Morales, M. A., Remaud-Simeon, M., Pizzut, S., Sarcabal, P. and Monsan, P. (2000) Sequence analysis of the gene encoding alternansucrase, a sucrose glucosyltransferase from *Leuconostoc mesenteroides* NRRL B-1355. FEMS Microbiol. Lett. 182, 81-85.

Asahara, T., Shimizu, K., Nomoto, K., Hamabata, T., Ozawa, A. and Takeda, Y. (2004) Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing *Escherichia coli* O157: H7. Infect. Immun. 72, 2240–47.

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. 59, 74-80.

Baba, M., De Clercq, E., Schols, D., Pauwels, R., Snoek, R., Van Boeckel, C., Van Dedem, G., Kraijeveld, N., Hobbelen, P., Ottenheijm, H. and Den Hollander, F. (1990) Novel sulfated polysaccharides: dissociation of anti-human immunodeficiency virus activity from antithrombin activity. J. Inf. Dis. 161, 208-213.

Bao, X., Duan, J., Fang, X. and Fang, J. (2001) Chemical modifications of the (1→3)-α-D-glucan from spores of *Ganoderma lucidum* and investigation of their physicochemical properties and immunological activity. Carbohydr. Res. 336, 127-140.

Barry T, Powell R, Gannon F. (1990) A general method to generate DNA probes for microorganisms. Biotechnol. 8, 233–236.

Bautista, M.C., Bomati-Miguel, O., Morales, M.D., *et al.* (2005) Surface characterisation of dextran-coated iron oxide nanoparticles prepared by laser pyrolysis and coprecipitation. J. Magn. Magn. Mater. 293, 20-27.

Beg, Q.K., Sahai, V. and Gupta, R. (2003) Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process Biochem. 39, 203-209.

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.

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.

Benjamas, J., 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.

Bezkorovainy, A., Miller-Catchpole, R. and Kot, E. (1997) Health benefits of bifidobacteria. Tecnologı´a La´ctea Latinoamericana, 10, 34-41.

Borriello, S. P., Hammes, W. P., Holzapfel, W., Marteau, P., Schrezenmeir, J., Vaara, M. and Valtonen, V. (2003) Safety of probiotics that contain lactobacilli or bifidobacteria, Clin. Infect. Dis. 36, 775-780.

Bottger, E.C. (1989) Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol. Lett. 65, 171-176.

Box, G. E. and Hunser, J. S. (1957) Multi-factor experimental designs for exploring response surfaces. Ann. Math. Stat. 28, 195-241.

Bozonnet, S., Dols-Laffargue, M., Fabre, E., Pizzut, S., Remaud-Simeon, M., Monsan, P. and Willemot, R. M. (2002) Molecular characterization of DSR-E, an alpha-1,2 linkage-synthesizing dextransucrase with two catalytic domains. J. Bacteriol. 184, 5753-5761.

Brison, Y., Pijning, T., Malbert, Y., Fabre, E., Mourey, L., Morel, S., Potocki-Véronèse, G., Monsan, P., Tranier, S., Remaud-Siméon, M. and Dijkstra, B.W. (2012) Functional and structural characterization of an α-(1-2) branching sucrase derived from DSR-E glucansucrase. J. Biol. Chem. 287, 7915-7924.

Cai, Y., Kumai, S., Ogawa, M., Benno, Y. and Nakase, T. (1999) Characterization and identification of *Pediococcus* species isolated from forage crops and their application for silage preparation. Appl. Environ. Microbiol. 2901-2906.

Campbell, J. A., Davies, G. J., Bulone, V. and Henrissat, B. (1997) A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. Biochem. J. 326, 929-939.

Cao, W., Li, X-Q., Liu, L., Yang, T-H., Li, C., Fan, H-T., *et al.* (2006) Structure of antitumor polysaccharide from *Angelica sinensis* (Oliv) Diels. Carbohydr. Polym. 66, 149-159.

Carneiro-Leao, A. M. A., Buchi, D. F., Iacomini, M., Gorin, P. A. J., Oliveira, M. B. M. (1997) Cytotoxic effect against HeLa cells of polysaccharides from the lichen Ramalina celastri. J. Submicrosc. Cytol. Pathol. 24, 1-5.

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

Cerning, J. (1995) Production of exopolysaccharides by lactic acid bacteria and dairy propionibacteria. Lait, 75, 463-472.

Chen, H. Y., Lin, L., Yu, X. Y., Qiu, K.Q., Lü, X. Y., Kuang, D. B. and Su, C. Y. (2013) Dextran based highly conductive hydrogel polysulfide electrolyte for efficient quasi-solid-state quantum dot-sensitized solar cells. Electrochim. Acta. 92, 117-123.

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.

Chen, K., Neimark, H., Rumore, P. and Steinman, C. R. (1989) Broad range DNA probes for detecting and amplifying eubacterial nucleic acids. FEMS Microbiol. Lett. 57, 19-24.

Chen, Y. S., Wu, H. C., Lo, H-Y, Lin, W-C, Hsu, W-H, Lin, C. W., Lin, P. Y. and Yanagida, F. (2012) Isolation and characterization of lactic acid bacteria from jiang-gua (fermented cucumbers), a traditional fermented food in Taiwan. J. Sci. Food Agric. 92, 2069-2075.

Chen, YS., Wu, H. C., Lo, H-Y., Lin, W-C., Hsu, W-H., Lin, C. W., Lin, P. Y. and Yanagida, F. (2012) Isolation and characterization of lactic acid bacteria from jiang-gua (fermented cucumbers), a traditional fermented food in Taiwan. J. Sci. Food Agric. 92, 2069-75.

Cobb, B. D. and Clarkson, J. M. (1994) A simple procedure for optimizing the polymerase chain reaction (PCR) using modified Taguchi methods. Nucleic Acids Res. 22, 3801-3805.

Collins, J. K., Thornton, G. and Sullivan, G. O. (1998) Selection of probiotic strains for human applications. Int. Dairy J. 8, 487-490.

Colson, P., Jennings, H. J. and Smith, I. C. P. (1974). Composition, sequence and conformation of polymers and oligomers of glucose as revealed by carbon-13 nuclear magnetic resonance. J. Am. Chem. Soc. 96, 8081-8087.

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. (2004) Temperature effect on dextransucrase production by *Leuconostoc mesenteroides* FT045B isolated from alcohol and sugar mill plant. Afr. J. Biotechnol. 4, 279-285.

Cosson, C. and Deschamps, A. M. (1994) Comportement de souches bacteriennes probiotiques en presence de bile et de sels biliaires. Microbiologie-Aliments-Nutrition, 12, 93-98.

Coutinho, P. M., Deleury, E., Davies, G. J. and Henrissat, B. (2003) An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. 328, 307-17.

Crittenden, R. G. (1999) Prebiotics. In: *Probiotics: a critical review.* (ed. Tannock, G.W.) Horizon Scientific Press, Wymondham, Norfolk, U.K. pp. 141-156.

Crittenden, R., Laitila A., Forssell P., Matto, J., Saarela, M., Mattila-Sandholm, T. and Myllarinen, P. (2001) Adhesion of bifidobacteria to granular starch and its implications in probiotic technologies. Appl. Environ. Micobiol. 67, 3469-3475.

Dasu V.V., Panda, T. and Chidambaram, M. (2003) Determination of significant parameters for improved griseofulvin production in a batch bioreactor by Taguchi's method. Process Biochem. 38, 877-880.

De 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.

De Roos, N. M. and Katan, M. B. (2000) Effects of probiotic bacteria on diarrhea, lipid metabolism, and carcinogenesis: a review of papers published between 1988 and 1998. Am. J. Clin. Nutr. 71, 405-411.

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

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.

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

Di Cagno, R., De Angelis, M., Limitone, A., Minervini, F., Carnevali, P., Corsetti, A., *et al.* (2006) Glucan and fructan production by sourdough *Weissella cibaria* and *Lactobacillus plantarum.* J. Agr. Food Chem. 54, 9873-9881.

Dinu, M. V., Perju, M. M., Dragan, E. S. (2011) Composite IPN ionic hydrogels based on polyacrylamide and dextran sulfate. React. Funct. Polym. 71, 881-890.

Ditson, S. L. and Mayer, R. M. (1984) Dextransucrase: the direction of chain growth during autopolymerisation. Carbohydr. Res. 126, 170-175.

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.

Duenas, M., Munduate, A., Perea, A. and 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.

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.

Dunne, C., Murphy, L., Flyin, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrisey, D., Thorton, G., Fitzgerald, G., Daly, C., Kiely, B., Quigley, E. M. M., O'Sullivan, G. C., Shanahan, F. and Collins, K. (1999) Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. Antonie van Leeuwenhoek, 76, 279-292.

Ebert, K. H. and Schenk, G. (1968) Mechanism of biopolymer growth: the formation of dextran and levan. Adv. Enzymol. 30, 179-210.

Ebert, K.H. and Schenk, G. (1968) Mechanism of biopolymer growth: the formation of dextran and levan. Adv. Enzymol. 30, 179-210.

Elo, S. Saxelin, M. and Salminen, S. (1991) Attachment of *Lactobacillus casei* strain GG to human colon carcinoma cell line Caco-2: Comparison with other dairy strains. Lett. Appl. Microbiol. 13 154-156.

Farwa, S., Qader, S. A., Aman, A. and Ahmed, N. (2008) Production and characterization of a unique dextran from an indigenous *Leuconostoc mesenteroides* CMG713. Int. J. Biol. Sci. 4, 379-386.

Fernández de Palencia, P., Werning, M. L., Sierra-Filardi, E., Due˜nas, M. T., Irastorza, A., Corbí, A. L., *et al.* (2009) Probiotic properties of the 2-substituted (1→3)-beta-D-glucan-producing bacterium *Pediococcus parvulus* 2.6. Appl. Environ. Microbiol. 75, 4887-4891.

Festl H., Ludwig, W. and Schleifer, K. H. (1986) DNA hybridization probe for the *Pseudomonas fluorescens* group. Appl. Environ. Microbiol. 52, 1190-1194.

Food and Agriculture Organization/World Health Organization (FAO/WHO), Guidelines for the evaluation of probiotics in food, Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London, Ontario, Canada (2002). (*http://ftp.fao.org/es/esn/food/wgreport2.pdf*).

Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S. and Underhill, D. M. (2003) Collaborative induction of inflammatory responses by Dectin-1 and Toll-like receptor 2. J. Exp. Med. 197, 1107-1117.

Garrity, G. M. and Holt, J. G. (2001) The road map to the manual. In: *Bergey's manual of systemic bacteriology*. (ed. Garrity, G. M.), Springer-Verlag, New York, N.Y. pp. 119-166.

Gibson, G. R. and Roberfroid, M. B. (1995) Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J. Nutr. 125, 1401-1412.

Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A. and Roberfroid, M. B. (2004) Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. Nutr. Res. Rev. 17, 259-275.

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

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

Grant, C. and Salminen, S. (1998) The potential of *Propionibacterium* spp. as probiotics. In: *Lactic acid bacteria. Microbiological and functional aspects* (ed. Salminen, S. and von Wright, A.) New York: Marcel Dekker, Inc. pp. 588-603.

Gruter, M., Leeflang, B. R. Kuiper, J. Kamerling, J. P. and Vliegenthart, J. F. (1992) Structure of the exopolysaccharide produced by *Lactococcus lactis* subspecies *cremoris* H414 grown in a defined medium or skimmed milk. Carbohydr. Res. 231, 273-291.

Gupta, A. and Prabhu, K. A. (1995) Immobilization and properties of dextransucrase from *Leuconostoc mesenteroides* culture LM1. J. Gen. Appl. Microbiol. 41, 399-407.

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.

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.

Hamada, S. and H. D. Slade. (1980) Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. 44, 331-384.

Han, J. J., Yang, T. H. and Rhee, J. S. (1998) Optimization of reaction variables for sucrose monoester production using lipase in a solvent free system by Taguchi's method. Biotechnol. Tech. 12, 295-299.

Harmsen, D. and Karch, H. (2004) 16S rDNA for diagnosing pathogens: a living tree. ASM News. 70, 19-24.

Haroun, B. M. Refaat, B. M., El- Menoufy, H. A., Amin H. A. and El-Waseif, A. A. (2013) Structure analysis and antitumor activity of the exopolysaccharide from probiotic *Lactobacillus plantarum* NRRL B- 4496 *in vitro* and *in vivo.* J. App. Sci. Res. 9, 425-434.

Hehre, E.J. (1946) Studies on the enzymatic synthesis of dextran from sucrose. J. Biol. Chem. 163, 221-233.

Hernandez, L., Arrieta, J., Menendez, C., Vazquez, R., Coego, A., Suarez, V., Selman, G., Petit-Glatron, M. F. and Chambert, R. (1995) Isolation and enzymic properties of levansucrase secreted by *Acetobacter diazotrophicus* SRT4, a bacterium associated with sugar cane. Biochem. J. 309, 113-118.

Holzapfel, W. H., Haberer, P, Geisen, R, Bjorkroth, J. and Schillinger, U. (2001) Taxonomy and important features of probiotic microorganisms in food nutrition. Am. J. Clin. Nutr. 73, 365S-373S.

Houng, J. Y., Liao, J. H., Wu, J. Y., Shen, S. C. and Hsu, H. F. (2006) Enhancement of asymmetric bioreduction of ethyl 4-chloro acetoacetate by the design of composition of culture medium and reaction conditions. Process Biochem. 42, 1-7.

Huang, Q. L. and Zhang L. N. (2011) Preparation, chain conformation and anti-tumor activities of water-soluble phosphated (1→3)-α-D-glucan from *Poria cocos* mycelia Carbohydr. Polym. 83, 1363-1369.

Huebner, J., Wehling, R. L., and Hutkins, R. W. (2007) Functional activity of commercial prebiotics. Int. Dairy J. 17, 770-775.

Ishibashi, N. and Yamazaki, S. (2001) Probiotics and safety. Am. J. Clin. Nutr. (Suppl.), 73 (2001) 465–470.

Ito, K., Ito, S., Shimamura, T., Weyand, S., Kawarasaki, Y., Misaka, T., Abe, K., Kobayashi, T., Cameron, A. D. and Iwata, S. (2011) Crystal structure of glucansucrase from the dental caries pathogen *Streptococcus mutans*. J. Mol. Biol. 408, 177-186.

Jeney, C., Dobay, O., Lengyel, A., Adam, E. and Nasz, I. (1999) Taguchi optimization of ELISA procedures. J. Immun. Meth. 223, 137-146.

Jensen, M. H., Mirza, O. Albenne, C. Remaud-Simeon, M. Monsan, P. Gajhede, M. and Skov, L. K. (2004) Crystal structure of the covalent intermediate of amylosucrase from *Neisseria polysaccharea*. Biochem. 43, 3104-3110.

Joseph, J. and Piganatiells, J.R. (1988) An overview of the strategy and tactics of Taguchi IIE Trans. 20, 247-253.

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.

Katina, K., Maina, N. H., Juvonen, R., Flander, L., Johansson, L., Virkki, L., Tenkanen, M. and Laitila, A. (2009) *In situ* production and analysis of *Weissella confusa* dextran in wheat sourdough. Food Microbiol. 26, 734-743.

Kattar M. M., Chavez J. F., Limaye A. P., Rassoulian- Barrett S. L., Yarfitz S. L., Carlson L. C., Houze Y., Swanzy S., Wood B. L. and Cookson B. T. (2001) Applicaton of 16S rRNA gene sequencing to identify Bordetella hinzii as the causative agent of fatal septicemia. J. Clin. Microbiol. 38, 789-794.

Kiho, T. Yoshida, I. Nagai, K. Ukai, S. and Hara, C. (1989) (1→3)-α-D-glucan from an alkaline extract of *Agrocybe cylindracea*, and antitumor activity of its *O*-(carboxymethyl)ated derivatives, Carbohydr. Res. 189, 273–279.

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

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

Koepsell, H. J. and Tsuchiya, H. M. (1952) Enzymatic synthesis of dextran. J. Bacteriol. 63, 293-295.

Kolbert, C. P. and Persing, D. H. (1999) Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Curr. Opin. Microbiol. 2, 299-305.

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.

Kothari, D., Tyagi, A., Patel, S. and Goyal A. (2011) Dextransucrase from the mutant of *Pediococcus pentosaceus* (PPm) is more stable than the wild type. 3 Biotech. 1, 199–205.

Kralj, S., Stripling, E., Sanders, P., van Geel-Schutten, G. H. and Dijkhuizen, L. (2005) A highly hydrolytic reuteransucrase from a probiotic *Lactobacillus reuteri* strain. Appl. Environ. Microbiol. 71, 3942-50.

Kralj, S., van Geel-Schutten, G. H. Rahaoui, H. Leer, R. J. Faber, E. J. van der Maarel, M. J. and Dijkhuizen, L. (2002) Molecular characterization of a novel glucosyltransferase from *Lactobacillus reuteri* strain 121 synthesizing a unique, highly branched glucan with alpha-(1→4) and alpha-(1→6) glucosidic bonds. Appl. Environ. Microbiol. 68, 4283-4291.

Kralj, S., van Geel-Schutten, G. H., Dondorff, M. M. G., Kirsanovs, S., van der Maarel, M. J. and Dijkhuizen, L. (2004) Glucan synthesis in the genus *Lactobacillus*: isolation and characterization of glucansucrase genes, enzymes and glucan products from six different strains. Microbiol. 150, 3681-3690.

Krishna Prasad K., Venkata Mohan S., Sreenivas Rao R., Ranjan Pati B. and Sarma P.N. (2005). Laccase production by *Pleurotus ostreatus* 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. Biochem. Eng. J. 24, 17-26.

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

Kyono, K., Yanase, H. Tonomura, K. Kawasaki, H. and Sakai, T. (1995) Cloning and characterization of *Zymomonas mobilis* genes encoding extracellular levansucrase and invertase. Biosci. Biotechnol. Biochem. 59, 289-293.

Lacroix, C. and Yildirim, S. (2007) Fermentation technologies for the production of probiotics with high viability and functionality, Curr. Opin. Biotechnol. 18, 176-183.

Lairson, L. L., Henrissat, B., Davies, G. J. and Withers, S. G. (2008) Glycosyltransferases: structures, functions, and mechanisms. Annu. Rev. Biochem. 77, 521-55.

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.

Lavi, I., Friesem, D., Geresh, S., Hadar, Y. and Schwartz, B. (2006) An aqueous polysaccharide extract from the edible mushroom *Pleurotus ostreatus* induces anti-proliferative and pro-apoptotic effects on HT-29 colon cancer cells. Cancer Lett. 244, 61-70.

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.

Li, B., Dobruchowska, J.M., Gerwig, G.J., Dijkhuizen, L. and Kamerling, J.P. (2013) Structural investigation of water-soluble polysaccharides extracted from the fruit bodies of *Coprinus comatus.* Carbohydr. Polym. 91, 314-321.

Li, Y., Triccas, J. A. and Ferenci. T. (1997) A novel levansucrase-levanase gene cluster in *Bacillus stearothermophilus* ATCC12980. Biochim. Biophys. Acta 1353, 203-208.

Lin, Y.L. Zhang, L.N. Chen, L. and Jin Y. (2004) Molecular mass and antitumor activities of sulfated derivatives of α-glucan from *Poria cocos* mycelia Int. J. Biol. Macromol. 34, 231–236.

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.

Liu, C.-F., Tseng, K.-C., Chiang, S.-S., Lee, B.-H., Hsua, W.-H. and Pan, T.-M. (2011) Immunomodulatory and antioxidant potential of Lactobacillus exopolysaccharides. J. Sci. Food Agr. 91, 2284-2291.

Loesche, W. J. (1986) Role of *Streptococcus* mutans in human dental decay. Microbiol. Rev. 50, 353-380.

Loo, J. V., Cummings, J., Delzenne, N., Englyst, H., Franck, A., Hopkins, M., Kok, N., Macfarlane, G., Newton, D., Quigley, M., Roberfroid, M., Vliet, T.V. and Heuvel, E. (1999) Functional food properties of non-digestible oligosaccharides: a consensus report from the ENDO project (DGXII AIRII-CT94-1095). Brit. J. Nutr. 81, 121-132.

Luzio, G. A., Mayer, R. M. (1983) The hydrolysis of sucrose by dextransucrase. Carbohydr. Res. 111, 311–318.

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

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.

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

Marteau, P. (2001) Safety aspects of probiotic products, Scand. J. Nutr. Näringsforskning, 45, 22-30.

Marteau, P. and Seksik, P. (2004) Tolerance of probiotics and prebiotics. J. Clin. Gastroenterol. 38, 67-69.

Martensson, O., Staaf, J., Dueñas-Chasco, M. T., Irastorza, A., Oste, R. and Holst, O. (2002) A fermented ropy non-dairy oat product based on the exopolysaccharides producing strain *Pediococcus damnosus* 2.6. Adv. Food Sci. 24, 4-11.

Michelena, G. L., Martínez, A., Bell, A., Carrera, E., Valencia, R. (2003) Scale-up of dextransucrase production by *Leuconostoc mesenteroides* in fed batch fermentation. Braz. Arch. Biol. Technol. 46, 455-459.

Miller, A. W. and Robyt, J. (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.

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

Monchois, V., Willemot, R. M. and Monsan, P. (1999) Glucansucrases: Mechanism of action and structure-function relationships. FEMS Microbiol. Lett. 23, 131-151.

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

Monsan, P., Remaud-Simeon, M., Andre, I. (2010). Transglucosidases as efficient tools for oligosaccharide and glucoconjugate synthesis. Curr. Opin. Microbiol. 13, 293-300.

Mooser, G. (1992) Glycosidases and glycosyltransferases. The Enzymes. Vol XX, Academic Press, New York. pp. 187-221.

Mooser, G. and Iwaoka, K.R. (1989) Sucrose 6-α-glucosyltransferase from *Streptococcus sobrinus*: characterization of a glucosyl-enzyme complex. Biochemistry 28, 443-449.

Mooser, G., Hefta, S. A., Paxton, R. J., Shively, J. E. and Lee, T. D. (1991) Isolation and sequence of an active-site peptide containing a catalytic aspartic acid from two *Streptococcus sobrinus* alpha-glucosyltransferases. J. Biol. Chem. 266, 8916-8922.

Naidu, A. S., Bidlack, W. R. and Clemens, R. A. (1999) Probiotic spectra of lactic acid bacteria (LB). Critical Rev. Food Sci. Nutr., 38, 13-126.

Nair, P. K., Melnick, S. J., Ramachandran, R., Escalon, E. and Ramachandran, C. (2006) Mechanism of macrophage activation by (1→4)-alpha-D-glucan isolated from *Tinospora cordifolia*. Int. Immunopharmacol. 6, 1815–1824.

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.

Nigam, M., Goyal, A. and Katiyar, S.S. (2006) High yield purification of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F by phase-partitioning. J. Food Biochem. 30, 12-20.

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 treptomycin resistance (aadE) gene to that found in *Campylobacter jejuni*. Plasmid. 58,115-126.

Oda, M., Hasegawa, S., Komatsu, S., Kambe, M. and Tsuchiya, M. (1983) Anti-tumor polysaccharide from *Lactobacillus* sp. Agric. Biol. Chem 47, 1623-1625.

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.

Olafsdottir, E. S. Ingolfsdottir, K., Barsett, H., Paulsen, B. S., Jurcic, K. and Wagner, H. (1999) Immunologically active (1→3)-(1→4)-α-D-glucan from *Cetraria islandica* Phytomed. 6, 33-39.

Olivares-Illana, V., Lopez-Munguia, A. and Olvera. C. (2003) Molecular characterization of inulosucrase from *Leuconostoc citreum*: a fructosyltransferase within a glucosyltransferase. J. Bacteriol. 185, 3606-3612.

Padmaja, G. A., Ramachandra, B., Manjunath, H., Prabh, R., Krishna, R., Shankar, P. A. (2011) Characterization of lactic acid bacteria isolated from fruits and vegetables for their antibacterial activity. J. Dairying Foods Home Sci. 30, 85-89.

Palys, T., Nakamura L. K. and Cohan F.M. (1997) Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int. J. Syst. Baceriol. 47, 1145-1156.

Patel, S. and Goyal, A. (2010) Isolation, characterization and mutagenesis of exopolysaccharide synthesizing new strains of lactic acid bacteria. Int. J. Microbiol. 8(1).

Patel, S., Kasoju, N., Bora, U., and Goyal, A. (2010). Structural analysis and biomedical applications of dextran produced by a new isolate *Pediococcus pentosaceus* screened from biodiversity hot spot Assam. Biores. Technol. 101, 6852-6855.

Patel, S., Kothari, D. and Goyal, A. (2011a) Purification and characterization of an extracellular dextransucrase from *Pediococcus pentosaceus* isolated from the soil of north east India. Food Technol. Biotechnol. 49 (3), 297-303.

Patel, S., Kothari, D., Shukla, R. and Goyal, A. (2011b) Scale up of dextran production from a mutant of *Pediococcus pentosaceus* (SPAm) using optimized medium in a bioreactor. Braz. Arch. Biol. Technol. 54(6), 1125-1133.

Patel, S., Majumder, A. and Goyal, A. (2012) Potentials of exopolysaccharides from lactic acid bacteria. Indian J. Microbiol. 52, 3-12.

Paul, F., Lopez-Munguia, A., Remaud-Simeon, M., Pelenc, V. and Monsan, P. (1992) Method for the production of α-1,2 oligodextrans using *Leuconostoc mesenteroides* NRRL B-1299. U.S. patent 5, 141-858.

Pepe, O., Ventorino, V., Cavella, S., Fagnano, M. and Brugno B. (2013) Prebiotic content of bread prepared with flour from immature wheat grain and selected dextran-producing lactic acid bacteria. Appl. Environ. Microbiol. 79, 3779-3785.

Perez, S., Kouwijzer, M., Mazeau, K. and Engelsen, S. B. (1996) Modelling polysaccharides: Present status and challenges, J. Mol. Graphics. 14, 307-321.

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.

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

Purama, R. K. and Goyal, A. (2007) Identification, purification and functional characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. Biores. Technol. 99, 3635-3642.

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

Purama, R. K., Agrawal, M. and Goyal A. (2010) Stabilization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. Ind. J. Microbiol. 50, 57–61.

Qader, S. A., Shireen, E., Aman, A., Iqbal, S. and Azhar, A. (2007) Production and characterization of dextransucrase from newly isolated strain of *Leuconostoc mesenteroides* EA-6. Int. J. Biotechnol. Biochem. 3, 117–129.

Rabelo, M. C., Fontes, C. M. and Rodrigues, S. (2011) Stability study of crude dextransucrase from *Leuconostoc citreum* NRRL B-742. Ind. J. Microbiol. 51, 164-170.

Rao, T. J. M. and Goyal, A. (2013) Purification, optimization of assay, and stability studies of dextransucrase isolated from *Weissella cibaria* JAG8. Prep. Biochem. Biotechnol. 43, 329-341.

Ravi, V., Prabhu, M. and Subramanyam, D. (2011) Isolation of bacteriocin producing bacteria from mango pulp and its antimicrobial activity. J. Microbiol. Biotechol. Res. 1, 54-63.

Relman D. A. (1999) The search for unrecognized pathogens. Science 284, 1308-1310.

Remaud-Simeon, M., Mungia, A.L., Pelenc, V., Paul, F. and Monsan, P.F. (1994) Production and use of glucosyltransferase from *Leuconostoc mesenteroides* NRRL B-1299 for the synthesis of oligosaccharides containing α-(1→2) linkages. Appl. Biochem. Biotechnol. 44, 101-107.

Roberfroid, M., Gibson, G. R. and Delzenne, N. (1993) The biochemistry of oligofructose, a nondigestible fiber: an approach to calculate its caloric value. Nutr. Rev. 51, 137-146.

Robert V. S. and Spencer J. W. (2009) Carbohydrates. Amsterdam; Elsevier, ISBN:978-0-240-52118-3 (StickWilliams)

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

Robyt, J. F. (1996) Mechanism and action of glucansucrase. In: *Enzymes for carbohydrate engineering.* (eds. Park, K. H., Robyt, J. F. and Choi Y. D.) Elsevier Science B.V., Amsterdam. pp. 1-22.

Robyt, J. F. and Martin, P. J. (1983) Mechanism of synthesis of D-glucans by D-glucosyltransferases from *Streptococcus mutans* 6715. Carbohydr. Res. 113, 301-315.

Robyt, J. F. and Taniguchi, H. (1976) The mechanism of dextransucrase action. Biosynthesis of branch linkages by acceptor reactions with dextran. Arch. Biochem. Biophys. 174, 129–135.

Robyt, J. F. and Walseth, T. F. (1978) The mechanism of acceptor reactions of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase. Carbohydr. Res. 61, 433-445.

Robyt, J. F., Kimble, B. K. and Walseth, T. F. (1974) The mechanism of dextransucrase action. Direction of dextran biosynthesis. Arch. Biochem. Biophys. 165, 634-640.

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

Rodrigues, S., Lona, L. M. F. and Franco, T. T. (2003) Effect of phosphate concentration on the production of dextransucrase by *Leuconostoc mesenteroides* B-512F. Bioprocess. Biosyst. Eng. 26, 57-62.

Russell, R. R. B. (1979) Purification of *Streptococcus mutans* glucosyltransferase by polyethylene glycol precipitation. FEMS Microbiol. Lett. 6, 197-199.

Sacchi, C. T., Whitney, A. M., Mayer, L. W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R. S. and Popovic, T. (2002a) Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis*. Emerg. Infect. Dis. 8, 1117-1123.

Sacchi, C. T., Whitney, A. M., Reeves, M. W., Mayer, L.W. and Popovic, T. (2002b) Sequence diversity of *Neissaria meningitides* 16S rRNA genesand use of 16S rRNA gene sequencing as a molecular subtyping tool. J. Clin. Microbiol. 40, 4520-4527.

Sahoo, S. K., Parveen, S., Panda, J.J. (2007) The present and future of nanotechnology in human health care. Nanomedicine. 3, 20–31.

Salminen, S. and von Wright, A. (1998) Current probiotics-safety assured?. Microb. Ecol. Health Dis. 10, 68-77.

Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W. M., Fondén, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S. E. and Mattila-Sandholm, T. (1998) Demonstration of safety of probiotics - A review, Int. J. Food Microbiol. 44, 93-106.

Sanders, M. E. and in't Veld, J. H. (1999) Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. Antonie van Leeuwenhoek. 76, 293-315.

Schiffrin, E. J., Rochat, F., Link-Amster, H., Aeschlimann, J. M. and Donnet-Hughes, A. (1995) Immunomodulation of human blood cells following the ingestion of lactic acid bacteria. J. Dairy Sci. 78, 491-497.

Semjonovs, P. and Zikmanis, P. (2009) Evaluation of novel lactose-positive and exopolysaccharideproducing strain of *Pediococcus pentosaceus* for fermented foods. Eur. Food Res. Technol. 227, 851-856.

Seymour, F. R., Knapp, R. D. and Jeanes, A. (1979c) Structural analysis of levans by use of ^{13}C-NMR. Spectroscopy. Carbohydr. Res. 72, 222-228.

Seymour, F. R., Knapp, R. D., Bishop, S. H. and Jeanes, A. (1979a) High temperature enhancement of ^{13}C-N.M.R. chemical shiftes of unusual dextrans and correlation with methylation structural analysis. Carbohydr. Res. 68, 123-140.

Seymour, F. R., Knapp, R. D., Chen, E. C. M., Jeanes, A. and Bishop, S. H. (1979b) Structural analysis of dextrans containing 2-O-α-D-glucosylated α-d-glucopyranosyl residues at the branch points by use of ^{13}C-nuclear magnetic resonance spectroscopy and gas-liquid chromatography- mass spectrometry. Carbohydr. Res. 71, 231-250.

Seymour, F. R., Knapp, R. D., Chen, E. C. M., Jeanes, A. and Bishop, S. H. (1979e) Structural analysis of dextrans containing 4-O-α-d-Glucosylated α-d-Glucopyranosyl residues at the branch points by use of ^{13}C-nuclear magnetic resonance spectroscopy and gas-liquid chromatography-mass spectrometry. Carbohydr. Res. 75, 275-294.

Seymour, F. R., Knapp, R. D., Chen, F. C. M., Bishop, S. H. and Jeanes, A. (1979d) Structural analysis of *Leuconostoc* dextrans containing 3-O-α-D-glucosylated α-d-glucosyl residues in both linear chain and branch-point positions by methylation and by ^{13}C-nuclear magnetic resonance spectroscopy and gas-liquid chromatography mass spectrometry. Carbohydr. Res. 74, 41-62.

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

Shornikova, A. V., Casas, I., Mykkänen, H., Salo, E. and Vesikari, T. (1997) Bacteriotherapy with *Lactobacillus reuteri* in rotavirus gastroenteritis. Pediatr. Infect. Dis. J. 16, 1103-1107

Shukla R. and Goyal, A. (2013a) Novel dextran from *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber with anticancer properties. Int. J. Biol. Macromol. 62, 352-357.

Shukla, R. and Goyal, A. (2012) Optimization and scale-up of fermentation of glucansucrase and branched glucan by *Pediococcus pentosaceus* CRAG3 using Taguchi methodology in bioreactor. J. Biosci. Biotechnol. 1, 73-82.

Shukla, R. and Goyal, A. (2013c) Probiotic potential of *Pediococcus pentosaceus* CRAG3 a new isolate from fermented cucumber. Probiotics Antimicrob. Proteins. DOI. 10.1007/s12602-013-9149-8.

Shukla, S. and Goyal, A. (2013b) Medium optimization of fermentation for enhanced dextran production from *Weissella confusa* Cab3 by statistical methods. Curr. Biotechnol. 2, 39-46.

Shukla, S., Shi, Q., Maina, N.H., Juvonen, M., Tenkanen, M. and Goyal, A. (2013) *Weissella confusa* Cab3 dextransucrase: properties and *in vitro* synthesis of dextran and glucooligosaccharides. Carbohydr. Polym. doi:10.1016/j.carbpol.2013.09.087.

Simms, P. J., Boyko, W. J. and Edwards, J. R. (1990) The structural analysis of a levan produced by *Streptococcus salivarius* SS2. Carbohydr. Res. 208, 193-198.

Singh, A. K. and Ramesh A (2008) Succession of dominant and antagonistic lactic acid bacteria in fermented cucumber: insights from a PCR based approach. Food Microbiol. 25, 278-287.

Sinnott, M. L. (1990) Catalytic mechanisms of enzymatic glycosyl transfer. Chem. Rev. 90, 1171-1202.

Smiderle, F. R., Ruthes, A. C., van Arkel, J., Chanput, W., Iacomini, M., Wichers, H. J., *et al.* (2011) Polysaccharides from *Agaricus bisporus* and *Agaricus brasiliensis* show similarities in their structures and their immunomodulatory effects on human monocytic THP-1 cells. BMC Complement. Altern. Med. 11, 58.

Smith, M. R., Wong, R. Y., Lundin, R. E. and Ahlgren, J. A. (1998) A mutant strain of *Leuconostoc mesenteroides* B-1355 producing a glucosyltransferase synthesizing α-(1-2) glucosidic linkages. J. Ind. Microbiol. Biotechnol. 21, 37-45.

Smitinont, T., Tansakul, C., Tanasupawat, S., Keeratipibul, S., Navarini, L., Bosco, M. and Cescutti, P. (1999) Exopolysaccharideproducing 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., Buchholz, K. and Vandamme, E. J. (1995) A wide range of carbohydrate modifications by a single microorganism: *Leuconostoc mesenteroides.* In: *Progress in Biotechnology* (eds. Peterson, S. B. Svensson B. and Pedersen S.) vol. 10. Elsevier, Amsterdam, The Netherlands. pp. 351-358.

Song, K. B., Joo, H. K. and Rhee S.K. (1993) Nucleotide sequence of levansucrase gene (levU) of *Zymomonas mobilis* ZM1 (ATCC10988). Biochim. Biophys. Acta, 1173, 320-324.

Soto, E., Kim, Y.S., Lee, J., Kornfeld, H. and Ostroff, G. (2010) Glucan particle encapsulated rifampicin for targeted delivery to macrophages. Polymers, 2, 681-689.

Sreekumar, O. and Hosono, A. (2000) Immediate effect of *Lactobacillus acidophilus* on the intestinal flora and fecal enzymes of rats and the *in vitro* inhibition of *Escherichia coli* in coculture. J. Dairy Sci. 83, 931-939.

Sreenivas, R. R., Prakasham, R. S., Krishna Prasad, K., Rajesham, S., Sarma, P. N. and Venkateswar Rao, L. (2004) Xylitol production by *Candida* sp.: parameter optimization using Taguchi approach. Process Biochem. 39, 951-956.

Stiles, M. E. and Holzapfel, W.H. (1997) Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36, 1-29.

Stone, R. A. and Veeves, A. (1994) The Taguchi influence on designed experiments. J Chemometrics. 8, 103-110.

Su, D. and Robyt, J.F. (1994) Determination of number of sucrose and acceptor binding sites for *Leuconostoc mesenteroides* B-512FM dextransucrase and the confirmation of the two-site mechanism of dextran synthesis. Arch. Biochem. Biophys. 2, 471-476.

Sukumar, G. and Ghosh, A. R. (2010) *Pediococcus* spp- *Pediococcus* spp.- A potential probiotic isolated from Khadi (an Indian fermented food) and identified by 16S rDNA sequence analysis. African J. Food Sci. 4, 597-602.

Sun, W. W., Yu, S. J., Zeng, X. A., Yang, X. Q. and Jia, X. (2011) Properties of whey protein isolate-dextran conjugate prepared using pulsed electric field. Food Res. Int. 44, 1052-1058.

Sutherland, I. W. (1972) Bacterial exopolysaccharides. Adv. Microb. Physiol. 8, 143-213.

Sutherland, I. W. (1998) Novel and established applications of bacterial polysaccharides. Trends Biotechnol. 16, 41-46.

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

Tallon, R., Bressollier, P. and Urdaci, M. C. (2003) Isolation and characterization of two exopolysaccharides produced by *Lactobaciilus plantarum* EP56. Res. Microbiol. 154, 705-712.

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. Process Biochem. 40, 365-370.

Tortoli E. (2003) Impact of genomic studies on mycobacterial taxonomy: the new mycobacteria of the 1990's. Clin. Microbiol. Rev. 16, 319-354.

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. (1955) Factors affecting molecular weight of enzymatically synthesized dextran. J. Am. Chem. Soc. 77, 2412-2419.

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.

Tsuda, H. and Miyamoto, T. (2010) Production of exopolysaccharide by *Lactobacillus plantarum* and the prebiotic activity of the exopolysaccharide. Food Sci. Technol. Res. 16, 87-92.

Uitdehaag, J. C., Mosi, R., Kalk, K. H., van der Veen, B. A., Dijkhuizen, L., Withers, S. G. and Dijkstra, B. W. (1999) X-ray structures along the reaction pathway of cyclodextrin glycosyltransferase elucidate catalysis in the alpha-amylase family. Nat. Struct. Biol. 6, 432-436.

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

Uzochukwu, S., Balogh, E., Loefler, R.T., & Ngoddy, P.O. (2001) Structural analysis by ^{13}C-nuclear magnetic resonance spectroscopy of glucans elaborated by gum-producing bacteria isolated from palm wine. Food Chem. 73, 225-223

Valette, P., Pelenc, V., Djouzy, Z., Andrieux, C., Paul, F., Monsan, P. and Szylit, O. (1993) Bioavailability of new synthesized glucooligosaccharides in the intestinal tract of gnotobiotic rats. J. Food Agric. Sci. 62, 121-127.

van der Vlist, J., Schonen, I., Loos, K. (2011). Utilization of glycosyltransferases for the synthesis of a densely packed hyperbranched polysaccharide brush coating as artificial glycocalyx. Biomacromolecules 12, 3728-3732.

van Geel-Schutten, G. H., Faber, E. J., Smit, E., Bonting, K., Smith, M. R., Ten Brink, B., Kamerling, J. P., Vliegenthart, J. F. and Dijkhuizen. L. (1999) Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. Appl. Environ. Microbiol. 65, 3008-3014.

van Geel-Schutten, G. H., Flesch, F., Ten Brink, B., Smith, M. R. and Dijkhuizen, L. (1998) Screening and characterization of *Lactobacillus* strains producing large amounts of exopolysaccharides. Appl. Microbiol. Biotechnol. 50, 697-703.

van Hijum, S. A., Bonting, K., van der Maarel, M. J. and Dijkhuizen, L. (2001) Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced. FEMS Microbiol. Lett. 205, 323-328.

van Hijum, S. A., van Greel-Schutten, H., Rahaoui, H., van der Maarel, M. and Dijkhuizen, L. (2002) Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesized high-molecular-weight inulin and inulin oligosacharides. Appl. Environ. Microbiol. 68, 4390-4398.

Vujicic-Zagar, A., Pijning, T., Kralj, S., López, C.A., Eeuwema, W., Dijkhuizen, L. and Dijk-stra, B.W. (2010) Crystal structure of a 117 kDa glucansucrase fragment provides insight into evolution and product specificity of GH70 enzymes. Proc. Natl. Acad. Sci. (USA), 107, 21406-21411.

Waldherr, F. W. and Vogel, R. F. (2009) Commercial exploitation of homo-exopolysaccharides in non-dairy food systems. In: *Bacterial polysaccharides: Current innovations and future trends* (ed. Ullrich, M.) Norfolk: Caister Academic Press. pp. 313–332

Wang, T., Li, D, Li, S. and Tan T. (2007) Structural characterization of a water-insoluble (1→3)-α-D-glucan isolated from the *Penicillium chrysogenum*. Carbohydr. Polym. 67, 133–137.

Welman, A. D. and Maddox, I. S. (2003) Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends Biotechnol. 21, 269-274.

Wiater, A., Paduch, R., Pleszczyńska, M., Próchniak, K., Choma, A., Kandefer-Szerszeń, M. and Szczodrak, J. (2010) α-(1→3)-D-Glucans from fruiting bodies of selected macromycetes fungi and the biological activity of their carboxymethylated products. Biotechnol. Lett. 33, 787-795.

Woese, C. R. (1987) Bacterial evolution. Microbiol. Rev. 51, 221-271.

Woese, C. R., Stackebrandt E., Macke T. J., and Fox, G. E. (1985) A phylogenetic definition of the major eubacterial taxa. Syst. Appl. Microbiol. 6, 143-151.

Yuksekdag, Z. N. and Aslim, B. (2010). Assessment of potential probiotic and starter properties of *Pediococcus* spp. isolated from Turkish-type fermented sausages (Sucuk) J. Microbiol. Biotechnol. 20, 161-168.

Zekovic, D. B., Kwlatkowski, S., Vrvic, M. M., Jakovijevic, D. and Moran, C. A. (2005) Natural and modified (1→3)-β-D-glucans in health promotion and disease alleviation. Crit. Rev. Biotech. 25, 205-230.

Zhang, J. B., Wu, N. N., Yang, X. Q., He, X. T. and Wang, L. J. (2012) Improvement of emulsifying properties of maillard reaction products from β-conglycinin and dextran using controlled enzymatic hydrolysis. Food Hydrocoll. 28, 301-312.

Zhang, L. Zhang, M. Zhou, Q. Chen, J. and Zeng, F. (2000) Solution properties of antitumor sulfated derivative of α-(1→3)-D-glucan from *Ganoderma lucidum*, Biosci. Biotechnol. Biochem. 64, 2172-2178.

Chapter 2

Identification and biochemical characterization of *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber

2.1 Introduction

Screening and isolation of microorganisms from naturally existing substances is important for obtaining useful cultures for scientific and commercial purposes (Vanden Berg *et al.*, 1993). Lactic acid bacteria (LAB), which are economically important microbes, are used throughout the world for manufacture of a wide variety of traditional fermented foods. They are designated as GRAS (Generally recognized as safe) organisms. LAB are gram positive, rods or cocci shaped bacteria which ferment carbohydrates into lactic acid and energy (Jay, 2000). They include genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* (Stiles and Holzapfel, 1997; Jay, 2000;

Holzapfel *et al.*, 2001). Depending on the metabolism of carbohydrates they can be of two types:

1. Homofermentive LAB in which the end product is lactic acid (Fig. 2.1.1)
2. Heterofermentive LAB which produce lactic acid, carbon dioxide, ethanol and/or acetic acid (Fig. 2.1.1).

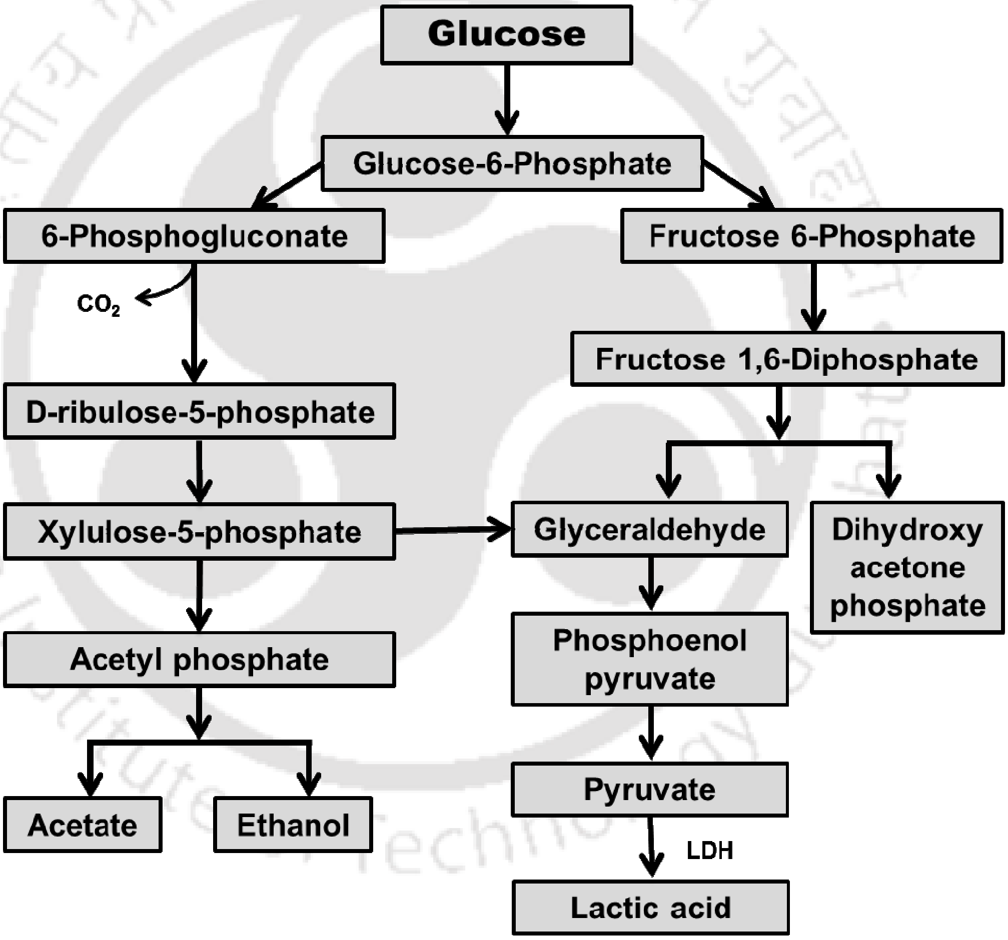

Fig. 2.1.1 Metabolism of glucose showing production of lactic acid (by homofermentive lactic acid bacteria) or of acetate and ethanol (by heterofermentive lactic acid bacteria

They can be isolated from fermented products such as meat and vegetables (Aukrust and Blom, 1992), sauerkraut (Harris *et al.*, 1992), sourdough (Gobbetti and

Corsetti, 1997) milk products (Jay, 2000), beverages (Lonvaud-Funel, 2001; Liu, 2003) and bakery products (O'Sullivan *et al.*, 2002). They can also be isolated from human milk (Martín *et al.*, 2003) and human vagina (Boris *et al.*, 1998; Ocaña *et al.*, 1999). Although there are reports available on isolation of LAB from different sources, only few emphasized on isolation from fruits (Ravi *et al.*, 2011) and vegetables (Padmaja *et al.*, 2011), the LAB from lettuce, cucumber and cabbage are not much studied. Fermented cucumbers serve as traditional food in some countries in Europe, China and India (Chen *et al.*, 2012). The fermentation in cucumbers is carried out by lactic acid bacteria as reported by Singh and Ramesh (2008).

Identification of LAB depends mostly on phenotypic analysis; however molecular based techniques are also available (Hertel *et al.*, 1993; Pot and Janssens, 1993; Vandamme *et al.*, 1996). Woese and his co-workers compared a stable part of the genetic code to determine phylogenetic relationships of bacteria and other life forms (Woese *et al.*, 1985; Woese, 1987). This included the genes that code for the 5S, the 16S (also known as small subunit), and the 23rRNA and the spaces between these genes. But now-a-days most commonly used part of DNA for bacterial taxonomy is the 16S rRNA gene (Bottger, 1989; Tortoli, 2003). The 16S rRNA gene is also designated as 16S rDNA, and the terms have been interchangeably used. The length of 16S rRNA is about 1,550 bp which is composed of both variable and conserved regions (Clarridge, 2004). Usually universal primers chosen are complementary to the conserved regions at the beginning of the gene and at either the 540 bp region or at the end of the whole sequence which is about 1,550 bp region and the sequence of the variable region in between is used for comparative taxonomy (Chen *et al.*, 1989). The 16S rRNA gene sequence has been determined for a large number of strains and this gene is universal amongst bacteria which help in measuring relationships among all bacteria (Woese *et al.*, 1985; Woese, 1987). In other words the 16S rRNA gene sequence comparison allows differentiating organisms at genus level and also classifying strains at species and subspecies level. Now it is also important to consider that is it necessary to sequence the whole 1.5 kbp length or whether the commonly reported shorter sequence can provide enough information. It has been reported that sometimes it is necessary to sequence the entire 1.5 kbp region for identification (Sacchi *et al.*, 2002a; Sacchi *et al.*, 2002b). Usually the entire 1.5 kbp sequence is required when describing a new species. However in case of many clinical bacteria the first 500 bp sequence provides sufficient differentiation for identification and in fact also provide a large difference between strains due to the presence of more diversity per kilobase sequence in region. Kattar *et al.* (2001) found in 16S rRNA of *Bordetella* species that there is 66% variability in initial 500 bp.

Pediococci are a group of homofermentative lactic acid bacteria (Benachour *et al.*, 1995) of great economic importance. *Pediococcus* group includes *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* sp. have been isolated from various places like soil (Patel and Goyal, 2010), wines (Beneduce *et al.*, 2004), cheese (Gurira and Buys, 2005), wheat kernels (Corsetti *et al.*, 2007), meat (Anastasiadou *et al.*, 2008) and sausages (Nieto-Lozano *et al.*, 2009).

The members of the *Pediococcus* genus have great economic importance in the fermented food industry and as starter cultures in fermentation processes of milk, meat, vegetable products and sausages (Dellaglio and Torriani, 1986; O’Connor *et al.*, 2007). *P. acidilactici* has GRAS (Generally Recognized as Safe) status and is used as probiotic culture and nutritional enhancer in silage (O'Connor *et al.*, 2007). Along with fermented foods, several *Pediococcus* strains produce bacteriocins which inhibit the growth of major Gram-positive foodborne pathogens, as well as other food spoilage bacteria (Kantor *et al.*, 1997). Bacteriocinogenic strains of *Pediococcus* genus have been found active against pathogenic bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Listeria innocua*, *Listeria ivanovii* and *Listeria monocytogenes* (Todorov and Dicks, 2009). A plasmid associated with glucan-production is reported in *P. damnosus* isolated from wine (Gindreau *et al.*, 2001). Ropy *Pediococcus damnosus* strain 2.6 was used for production of exopolysaccharide (EPS) in a semi-defined medium (Duenas *et al.*, 2003). In some Argentinian wines, the ropiness inducing lactic acid bacteria include *Pediococcus pentosaceus* (de Nadra and de Saad, 1995). No convincing proof is available showing dextran production by *Pediococcus* genus, except the report by Smitinont *et al.* (1999). The ability of *Pediococcus pentosaceus* to produce dextran was first reported by Patel *et al.* (2010). In the present study the isolation, 16S rRNA gene sequence based identification and characterization of a strain of lactic acid bacteria from fermented cucumber has been reported. The isolate was explored for its ability to produce dextransucrase and dextran.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

The ingredients required for the preparation of MRS medium and enzyme production medium (Tsuchiya *et al.*, 1952) such as sucrose, yeast extract, beef extract, peptone, tri-ammonium citrate, sodium chloride and dipotassium hydrogen orthophosphate were purchased from Hi-Media Pvt. Ltd., India. Sodium acetate, magnesium sulphate heptahydrate, manganese sulphate tetrahydrate, ferrous sulphate heptahydrate, calcium chloride dihydrate and Tween80 were purchased from Fisher Scientific Pvt. Ltd., India. The chemicals required for reducing sugar estimation (sodium carbonate anhydrous, sodium potassium tartrate, sodium bicarbonate, sodium sulphate anhydrous, cupric sulphate, ammonium molybdate and sodium arsenate), for protein estimation (sodium carbonate, sodium hydroxide, sodium potassium tartrate, cupric sulphate and phenol reagent) and buffer preparation were of high purity grade. PEG-400 from Qualigens Pvt. Ltd., India and PEG-1500 from BDH, U. K. were used for fractionation of dextransucrase.

2.2.2 Screening, isolation and culturing of microorganism

The freshly chopped cucumber (300 g) in 2.5% (w/v) NaCl solution in 1 litre air tight bottle was subjected to fermentation at 25°C for 5 days. The 1 g of fermented cucumber was homogeneously mixed in 10 ml of normal saline (0.85% w/v) in test tubes and the serial dilutions up to 10^{-7} were made. 100 µl from each dilution from 10^{0} to 10^{-7} was taken and spread plated on 1.7% (w/v) MRS agar plates (De Man *et al.*, 1960) as described later in Section 2.2.3. Petri plates were incubated at 28°C for 24 h. 43 colonies were picked from Petri plates of different dilutions. A loopful from each distinct colony was inoculated separately in 5 ml enzyme production medium (Tsuchiya *et al.*, 1952) as described later in Section 2.2.4 and incubated at 25°C and 180 rpm for 12 h. An aliquot (1 ml) from each culture was centrifuged at 10,000***g*** and 4°C for 10 min. The cell free supernatant from all the isolates were analysed for the enzyme activity as described in Section 2.2.5.1.3. The isolates displaying higher activity were also analysed for the total carbohydrate content as described in Section 2.2.6. The isolate CRAG3 (named after the source of isolation, initials of author's names and its colony number) was selected based on its highest dextransucrase activity and highest total carbohydrate content. The isolate was sub-cultured and propagated as stab in modified MRS agar medium (Goyal and Katiyar, 1996) as described later in Section 2.2.3, at 28°C and stored at 4°C. The isolate was preserved and maintained as glycerol stock (20%, v/v) in modified MRS medium at -80°C.

2.2.3 Medium for culturing

The culture was maintained in modified MRS agar medium (Goyal and Katiyar, 1996) as a stab at 4°C and sub-cultured every 15 days. The modified MRS agar medium composed of components given in Table 2.2.1. The pH of the medium was adjusted to 6.4 using glacial acetic acid (17.5 M). The medium was sterilized by autoclaving at 15 psi steam pressure and 121°C temperature for 20 min.

Table 2.2.1 Components for preparation of modified MRS medium.

Component	% (w/v)
Sucrose	2.00
Yeast extract	0.50
Beef extract	1.00
Peptone	1.00
Dipotassium hydrogen orthophosphate	0.20
Tri-ammonium citrate	0.20
Sodium acetate	0.50
Tween80 (%, v,v)	0.10
Magnesium sulphate ($MgSO_4.7H_2O$)	0.02
Manganese sulphate ($MnSO_4.4H_2O$)	0.02
Agar powder	1.70

2.2.4 Medium for enzyme production

The medium described by Tsuchiya *et al.* (1952) was utilized for enzyme production. Tsuchiya medium was prepared by dissolving the components as shown in Table 2.2.2. The pH of medium was adjusted to 6.9 using 0.1 M HCl solution followed by sterilization with autoclaving at 15 psi steam pressure and 121°C temperature for 20 min.

Table 2.2.2 Components of enzyme production medium (Tsuchiya *et al.*, 1952)

Component	g/l
Sucrose	20.0
Yeast extract	20.0
Dipotassium hydrogen orthophosphate (K_2HPO_4)	20.0
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.20
Manganese sulphate tetrahydrate ($MnSO_4.4H_2O$)	0.20
Ferrous sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$)	0.01
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.01
Sodium chloride (NaCl)	0.01

2.2.5 Enzyme assay

2.2.5.1 Reducing sugar estimation

2.2.5.1.1 Preparation of reagents for reducing sugar estimation

The reagents for reducing sugar estimation were prepared by the method described by Nelson (1944) and Somogyi (1945) (Table 2.2.3).

Table 2.2.3 Components for preparation of reagents for reducing sugar estimation

Reagents	Components	Amount	Procedure
Reagent A	Sodium carbonate anhydrous	2.5 g	The components were dissolved in MilliQ water and the volume made up to 100 ml. The solution was filtered with Whatman filter #1 and stored at 30-37°C.
	Sodium potassium tartrate	2.5 g	
	Sodium bicarbonate	2.0 g	
	Sodium sulfate anhydrous	20.0 g	
Reagent B	Cupric sulphate	15%, w/v	4.5 g cupric sulfate was dissolved in MilliQ water and 1-2 drops of conc. sulfuric acid added.
Reagent C	i. Ammonium molybdate	2.5 g	The components were dissolved in 45 ml of MilliQ water
	Conc. sulfuric acid	2.1 ml	
	ii. Sodium arsenate	3.0 g	Dissolved in 2.5 ml of water
			47.5 ml of solution (i) was mixed to 2.5 ml of solution (ii) kept at 37°C in the dark place for 24 h before use.
Reagent D	Freshly prepared by mixing Reagent A and Reagent B in the ratio 25:1.		

2.2.5.1.2 Standard plot of fructose for enzyme assay

The standard plot was prepared using varying concentrations (50-500 µg/ml) of fructose in 20 mM sodium acetate buffer (pH 5.4) for enzyme assay. The stock solution (5.0 mg/ml) of fructose was prepared to make different concentrations. To 100 µl of fructose solution, 100 µl Reagent D was added and the mixture was placed in boiling water bath for 20 min. The solution was cooled to 25°C and 100 µl Reagent

C was added with vigorous shaking. It was diluted to 1 ml by adding 700 µl deionized water and the absorbance was measured at 500 nm against blank on a UV-visible spectrophotometer (Varian, Cary 100 Bio). The absorbance (A_{500}) against the fructose concentration was plotted (Fig. 2.2.1).

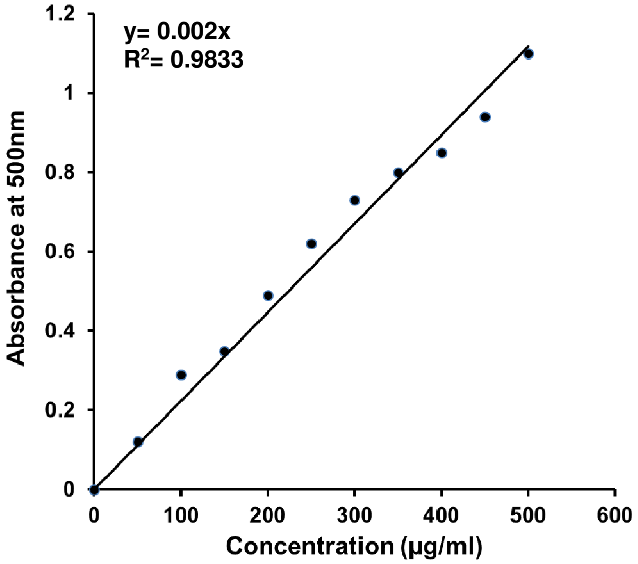

Fig. 2.2.1 Standard plot between varying concentration of fructose against absorbance (A_{500}) using Nelson and Somogyi's method for enzyme assay.

2.2.5.1.3 Enzyme assay method

The enzyme assay was carried out in 1 ml reaction mixture containing 5% (w/v) sucrose in 20 mM sodium acetate buffer (pH 5.4) and 20 µl cell free supernatant (as described earlier in Section 2.2.2). The enzymatic reaction was performed at 30°C for 15 min. An aliquot (100 µl) was taken from the reaction mixture to estimate reducing sugar by method of Nelson (1944) and Somogyi (1945). To 100 aliquot from the reaction mixture, 100 µl of reagent D was added, the solution was mixed on a vortex and heated in boiling water bath for 20 min. It was cooled to 25°C and 100 µl of reagent C was added due to which the colour developed with the evolution of carbon dioxide. It was diluted to 1 ml by adding 700 µl deionized water and the absorbance was measured at 500 nm on a UV-visible spectrophotometer (Varian, Cary 100 Bio). A standard plot was prepared using fructose as described earlier in Section 2.2.5.1.2 was used for determining the amount of reducing sugar. One unit (U) of dextransucrase activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar per min under optimum conditions of temperature and pH. The enzyme activity was calculated as follows:

$$\textbf{Enzyme activity (U/ml)} = \frac{A_{500} \times C \times V}{180 \times t \times v} \quad \textbf{(µmol/min/ml)}$$

where,

A_{500} = Absorbance at 500 nm
C = Concentration (mg/ml) of fructose at OD=1 from standard plot (500 µg/ml)
V = Volume (ml) of reaction mixture
t = Time of reaction (min)
v = Volume (ml) of the enzyme for estimation of reducing sugar
180 = Molecular mass of fructose

2.2.6 Analysis of total carbohydrate content

The isolates (as described in Section 2.2.2) were grown in 5 ml Tsuchiya medium (as described in Section 2.2.4) at 25°C and 180 rpm for 6 h. The culture broth from each isolate was centrifuged at 10,000***g*** and 4°C for 10 min. To 200 µl of cell free supernatant (as described earlier in Section 2.2.2) from each isolate, 3 volumes i.e. 600 µl of 95% (v/v) prechilled ethanol at 4°C was added and centrifuged at 13,000***g*** and at 25°C for 30 min. The process was repeated three times and finally the precipitated polysaccharide was dissolved in 200 µl deionised water. Its total carbohydrate content was determined by phenol-sulphuric acid method (Dubois *et al.*, 1956; Fox and Robyt, 1991). To 25 µl of cell free supernatant in a microtitre plate, 25 µl of 5% (w/v) phenol was added with slow mixing on a vortex for 30 s. The plate was then placed onto ice bath and 125 µl of concentrated sulphuric acid (18 M) was added to each well containing the sample and phenol. The plate was again shaken on vortex for 30 s and then incubated at 80°C in water bath for 30 min. It was then cooled and the absorbance was measured at 490 nm on a microplate reader (Tecan, Infinite 200 Pro). Standard curve was prepared using dextran T-40 (40 kDa, Sigma, St. Louis, MO, USA) in the concentration range 0.1- 1 µg/ml (Fig. 2.2.3).

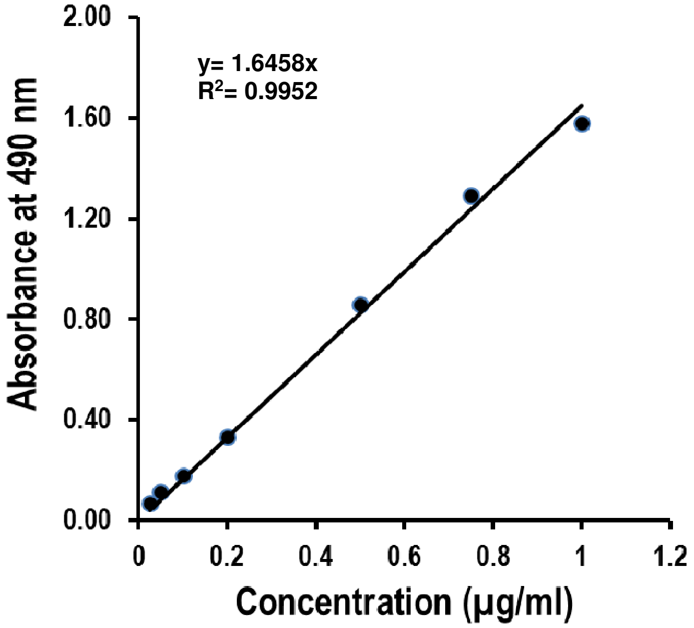

Fig. 2.2.3 Standard plot between varying concentrations of Dextran T-40 against absorbance (A_{490}) using phenol-sulphuric acid method for estimation of total carbohydrate content.

2.2.7 Morphological characterization of the isolate CRAG3

The morphology of CRAG3 grown in modified MRS medium (Goyal and Katiyar, 1996) overnight at 25°C was observed directly and by light microscopy. The Gram reaction was performed using method of Gram (1884). The cell morphology of selected isolate was examined by field emission scanning electron microscopy (Carl Zeiss, Model Sigma) operated at 10.0 kV. The sample of cells for scanning electron microscopy was prepared by taking 1 ml of 12 h old culture and centrifuged at 8,000***g*** for 10 min. The pellet containing cells was dissolved in 1 ml of saline solution (0.85%, w/v) and fixed with equal volume of gluteraldehyde (2.5%, v/v) for 5 h. Gluteraldehyde was removed by centrifuging at 8,000***g*** for 10 min and cells were dehydrated using different concentrations of acetone and finally dried in vacuum desiccator.

2.2.8 Biochemical characterization of the isolate CRAG3

The isolate was tested for its ability to ferment 21 different carbohydrates using the method described earlier (Kandler and Weiss, 1986). The MRS medium without glucose containing 1.8% (w/v) agar and phenol red (100 µg/ml) was first poured into petri-plate. The overnight grown culture (1%) of the isolate was seeded in MRS-soft agar (0.8%, w/v) containing phenol red (100 µg/ml) and overlaid in the petri-plate having a bottom layer of 1.8% (w/v) MRS agar. The plate was allowed to dry for 2-3 min. The carbohydrate discs were gently placed on to the surface of soft agar and the petri-plates were incubated at 28°C for 48 h to observe the colour change from red to yellow and formation of zone around carbohydrate discs.

Triple sugar iron (TSI) agar test was carried out using TSI agar slant (SL045, HiMedia) having sucrose, glucose and lactose as triple sugars for acid, H_2S and/or gas production. A loopful of overnight grown culture was streaked over the slant and then stabbed deep into the butt and incubated at 28°C for 48 h. The acid production was inferred by changing colour from red to yellow. The isolate was tested for its ability to display catalase activity using the method of Maehly and Chance (1954). To the 5 ml 12-16 h grown culture of isolate CRAG3 few drops of 3% (v/v) H_2O_2 was added and observed for bubble formation. *E. coli* was taken as positive control for catalase test. The motility test of CRAG3 was carried out by stabbing a loopful of culture deep into the Motility Indole Lysine Agar Slant (SL033, HiMedia).

The sensitivity of isolate against 42 antibiotics was evaluated using agar disc diffusion method (Barry and Thornsberry, 1980). The MRS medium containing 2% (w/v) glucose and 1.8% (w/v) agar was first poured into petri-plate and allowed to solidify. The overnight grown culture (1%) of the isolate was seeded in MRS-soft agar (0.8%, w/v) having 2%, w/v glucose and overlaid in the petri-plate having a bottom layer of 1.8% (w/v) MRS agar. The plate was allowed to dry for 2-3 min. The octodiscs were gently placed on to the surface of soft agar and the petri-plates were incubated at 28^{o}C for 24 h to observe the zone of inhibition around the discs.

2.2.9 16S rRNA gene sequence based identification of isolate CRAG3

2.2.9.1 Genomic DNA extraction of isolate CRAG3

1% (v/v) culture inoculum of CRAG3 was grown in 5 ml MRS medium (as described earlier in Section 2.2.3) at 28°C and 180 rpm for 14 h. The genomic DNA from CRAG3 was extracted by HiPurA Bacterial and Yeast Genomic DNA Purification Spin Kit (Himedia India Pvt. Ltd., India) as described below:

1. The 5 ml of broth was centrifuged at 12,000***g*** and 4°C for 2 min.
2. The supernatant was discarded, the cell pellet was re-suspended in 200 µl Gram positive lysis solution containing lysozyme (2.0 x10^5 U/ml, 2 mg/ml) and incubated at 37^{o}C for 30 min.

3. To the above lysate, 20 µl of the RNaseA solution (20 mg/ml) was added, mixed thoroughly and incubated for 2 min at 25°C.
4. 20 µl of proteinase K solution (20 mg/ml) was added to the above solution followed by 200 µl of lysis solution and mixed on vortex thoroughly for 15 sec and incubated at 55°C for 10 min, to obtain a clear lysate solution.
5. DNA was precipitated by adding 200 µl of absolute alcohol to the lysate and mixed on vortex thoroughly for 15 sec. for homogeneous mixing.
6. The homogeneous lysate was passed through HiElute miniprep spin column provided with the kit by centrifugation at 12,000***g*** for 10 min. The flow through liquid was discarded and the spin column was placed on a new 2.0 ml collection tube.
7. 500 µl of prewash solution was added to the column and centrifuged at 15,000***g*** for 3 min to dry the column. The column should be free of ethanol before eluting the DNA.
8. 50 µl of the elution buffer was added directly to the column without spilling to the sides and incubated for 1 min at 25°C. The column was then centrifuged at 8,000***g*** for 1 min to elute the DNA.

The extracted DNA was purified using QIAquick® Gel Extraction Kit (QIAGEN) as described later in Section 2.2.9.4. DNA concentrations were determined in duplicate using a UV-visible spectrophotometer (Varian, Carry 100). The quality of purified DNA was evaluated on 0.8% (w/v) agarose gel (as described later in Section 2.2.9.3).

2.2.9.2 PCR amplification of 16S rRNA gene

The genomic DNA of isolate CRAG3 was used as a template for amplification of 16S rRNA gene by polymerase chain reaction (PCR) using universal 16S rRNA oligonucleotide primers, forward primer 27F (5´- AGAGTTTGATCCTGGCTCAG-3´) and reverse primer 1492R (5´- GGTTACCTTGTTACGACTT-3´). The reaction mixture (50 µl) used in polymerase chain reaction (PCR) comprising of components are shown in Table 2.2.4. The conditions of PCR amplification are shown in Table 2.2.5. The PCR reactions were carried out on a Thermal Cycler (Applied Biosystems, model ABI 2720).

Table 2.2.4 Reaction mixture for polymerase chain reaction of 16S rDNA gene

PCR Components	Concentration	Volume (µl)	Final concentration
Reaction buffer	10x	5.0	1x
dNTP mix	100 mM	1.0	2.5mM
Forward primer	15 µM	1.7	0.5 µM
Reverse primer	15 µM	1.7	0.5 µM
Genomic DNA template	10 ng/µl	1.0	10 ng
Taq DNA polymerase	5 U/ µl	1.0	5 U
$MgCl_2$	1 M	2.5	50 mM
DNAse free water, pH 8.0 (Sigma, St. Louis, USA)		36.1	-
Total volume of reaction mixture		**50.0**	

Table 2.2.5 PCR conditions for amplification of 16S rRNA gene of isolate CRAG3.

Steps	PCR Conditions
1.	Denaturation of 94°C for 4 min
2.	30 cycles of Denaturation at 94°C for 0.5 min Annealing at 55°C for 1.0 min Extension at 72°C for 2.0 min
3.	Final extension at 72°C for 10 min

2.2.9.3 Analysis of PCR amplicon by Agarose gel electrophoresis

2.2.9.3.1 Preparation of electrophoresis buffer

A stock of 10x TAE buffer was prepared by adding 400 mM Tris-acetate and 10 mM EDTA (pH 8.0) as described by Sambrook and Russel (2001). The working solution (1x) was prepared by diluting stock solution. 1x TAE (Tris-acetate-EDTA) buffer was used for preparation of agarose gels and as electrophoresis running buffer to separate the PCR amplified DNA (Sambrook and Russel, 2001).

2.2.9.3.2 Preparation of agarose gel

The agarose gel (0.8%, w/v) was prepared by dissolving 400 mg agarose in 50 ml of 1x TAE buffer (pH 8.0) by heating in a microwave oven for few min to get a clear solution. It was allowed to cool at around 50ºC and then 5.0 µl ethidium bromide (5.0 mg/ml) was added. The solution was mixed and poured on the casting apparatus, comb was placed and the gel was allowed to set for 30 min. The DNA sample and DNA sample loading buffer (as described later in Section 2.2.9.3.3) were mixed in 4:1 ratio and the gel was run at constant 100 Volt for 2 h. The bands were then visualized under UV illumination on a gel documentation system (Gel doc EQ, Bio-Rad, USA).

2.2.9.3.3 Preparation of DNA sample loading buffer

The components and their final concentrations for the preparation of DNA sample loading buffer are shown in Table 2.2.6. A 5x stock solution of DNA loading buffer was prepared and the final pH adjusted to 8.0. The stock solution (5x) was mixed with 4 volumes of DNA to make it to 1x before loading on to the agarose gel.

Table 2.2.6 Components for preparation of 5x sample loading buffer for DNA.

Components	Final Concentration (5x)
Tris HCl	50 mM
Glycerol	25% (w/v)
EDTA	5.0 mM
Bromophenol Blue	0.2% (w/v)
Xylene Cynol	0.2% (w/v)

2.2.9.4 Extraction of DNA from agarose gel

The amplified 16S rRNA gene from the isolate was eluted and purified from the gel slice using the QIAquick® Gel Extraction Kit (QIAGEN) as described below:

DNA gel extraction protocol

1. 1.5 ml sterile, empty micro centrifuge tube was weighed and noted the weight.
2. The extracted genomic DNA or PCR amplified DNA was excised from gel using sharp sterile scalpel and transferred to pre-weighed empty microcentrifuge tube. The tubes were weighed again and the weight of excised gel was determined by subtracting the weight of empty tube (noted above).
3. Now, 3 volumes of buffer QG were added to every 1 volume of gel (taking 100 mg gel as ~ 100 µl).
4. The microcentrifuge tubes containing excised gel were incubated at 50°C for 10 min (or until the gel slice completely dissolved). The colour of the solution became yellow when the gel slice dissolved completely, (similar to the colour of buffer QG without dissolved agarose).
5. 1 gel volume of isopropanol was added to above yellow colour solution in case of PCR amplified DNA (For higher yield of DNA fragments <500 bp and >4000 bp).

6. QIAquick spin column (DNA binding column) was placed in 2 ml collection tube provided with the kit. The above solution containing extracted genomic DNA or PCR-amplified DNA (750 µl) was added to DNA binding columns and centrifuged at 17,900***g*** for 1 min at 25°C, and the flow through was discarded. If the volume was more than 750 µl, the remaining solution was centrifuged similarly and again the flow through was discarded.
7. 500 µl of buffer QG was added to each QIAquick spin column and the mixtures were centrifuged again at 17,900***g*** for 1 min at 25°C, and the flow through was discarded.
8. Now, 750 µl of buffer PE was added to each column containing extracted genomic DNA or PCR-amplified DNA and the mixture was centrifuged at 17,900***g*** for 1 min at 25°C. The flow through was discarded and the column was given an additional spin at 17,900***g*** for 1 min to completely remove the residual ethanol.
9. Now the column containing bound DNA was placed in a fresh 1.5 ml sterile microcentrifuge tube. 50 µl of DNAse free water (Sigma, St. Louis, MO, USA) or elution buffer (10 mM Tris-HCl, (pH 8.5) was added at the centre of the column. The column was incubated for 2 min at 25°C and centrifuged at 17,900***g*** for 1 min.
10. The extracted genomic DNA or PCR-amplified DNA was eluted from QIAquick spin column and collected in 1.5 ml sterile microcentrifuge tube. The extracted genomic DNA or PCR-amplified DNA (50 µl) was stored at -20°C for further use.

2.2.9.5 Ligation and transformation

The cloning of purified 16S rRNA gene was performed by using the method of Bharali *et al.* (2006). The purified DNA fragment was ligated to pGEM$_{\text{Teasy}}$ cloning vector (Promega, USA) using T4 DNA ligase (Promega, USA) and incubated at 16°C overnight. 200 µl of freshly prepared *E. coli* XL-10 competent cells were transformed using 10 µl of ligation mixture by subjecting to heat shock treatment at 42°C for 40 sec. The transformed cells were treated with 800 µl of SOC medium and incubated at 37ºC with shaking at 200 rpm for 1 h (Bharali *et al.*, 2006). The SOC medium was prepared as described later in Section 2.2.9.6. Cells were then centrifuged at 12,000***g*** for 5 min and 800 µl supernatant was discarded. The cell pellet was resuspended in the remaining 200 µl supernatant and plated on LB agar plate containing 100 µg/ml ampicillin (Himedia India Pvt. Ltd., India) and 2 µg/L 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). The positive, recombinant clones were differentiated and identified by white colour colonies against blue. The white colonies were picked for DNA isolation and for further screening. The pGEM$_{\text{Teasy}}$ plasmids containing the 16S rRNA gene were confirmed by digestion with *EcoR*I and *Sac*I restriction enzymes (New England Biolab Incorporation, USA). The PCR amplification of amplified 16S rRNA gene was also done using pGEM$_{\text{Teasy}}$ plasmid containing insert as a template and universal 16S rRNA oligonucleotide primers, forward primer 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and reverse primer 1492R (5´-GGTTACCTTGTTACGACTT-3´) to confirm the positive clones. The reaction mixture (50 µl) used in polymerase chain reaction (PCR) comprised of components as shown in Table 2.2.7. The conditions of PCR amplification are shown in Table 2.2.5.

The PCR reactions were carried out on a Thermal Cycler (Applied Biosystems, model ABI 2720).

Table 2.2.7 Reaction mixture for polymerase chain reaction of isolated DNA

PCR Components	Concentration	Volume (µl)	Final concentration
Reaction buffer	10x	5.0	1x
dNTP mix	100 mM	1.0	2.5 mM
Forward primer	15 µM	1.7	0.5 µM
Reverse primer	15 µM	1.7	0.5 µM
pGEM$_{\text{Teasy}}$ plasmid	10 ng/µl	1.0	10 ng
Taq DNA polymerase	5U/ µl	1.0	5 U
$MgCl_2$	1 M	2.5	50 mM
DNAse free water, pH 8.0 (Sigma, St. Louis, USA)		36.1	-
Total volume of reaction mixture		**50.0**	

2.2.9.6 Preparation of SOC (super optimal medium with catabolic repression) medium

The SOC (super optimal medium with catabolic repression) medium was prepared by modifying SOB medium with the addition of glucose (Hanahan, 1983). It is composed of components given in Table 2.2.8. The components such as bactotryptone, yeast extract and NaCl were autoclaved separately at 15 psi steam pressure and at 121°C for 20 min. However, 1 M stock solutions of KCl, $MgCl_2$, $MgSO_4$ and glucose were filter-sterilized and the desired amount was added to above solution in the laminar hood to finally make SOC medium. The pH of the medium was adjusted to 7.0.

Table 2.2.8 Components for preparation of SOC medium.

Component	Final Concentration
Bacto tryptone	2.0% (w/v)
Yeast extract	0.5% (w/v)
Sodium chloride (NaCl)	10 mM
Potassium chloride (KCl)	2.5 mM
Magnesium chloride ($MgCl_2$)	10 mM
Magnesium sulphate ($MgSO_4$)	10 mM
Glucose	20 mM

2.2.9.7 Sequencing and alignment of 16S rRNA gene

The sequencing of 16S rRNA gene was obtained from Xcelris Labs Limited, Ahmedabad, India. The forward and reverse DNA sequencing reaction of PCR amplicon using 8F and 1492R oligonucleotide primers was carried out in BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 1269 bp of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence of the isolate CRAG3 was used to carry out BLAST with the non-redundant database of NCBI GenBank database. First ten sequences, based on maximum identity score, were selected and aligned using ClustalW2, the multiple alignment software program. RDP database was used to generate distance matrix and the phylogenetic tree was constructed using MEGA4 (Tamura *et al.*, 2007).

2.3 Results and Discussion

2.3.1 Isolation of a dextransucrase and dextran producing bacterium from fermented cucumber

Out of selected 43 colonies, the isolate named CRAG3 showed maximum enzyme activity (2.7±0.08 U/ml) hence was selected for further characterization (Table 2.3.1). The isolated colonies displaying considerably higher enzyme activity were further investigated for the total carbohydrate content (Table 2.3.2). The isolate CRAG3 gave maximum carbohydrate content of 4.5±0.12 mg/ml (Table 2.3.2) and hence selected for further studies.

Table 2.3.1 Dextransucrase activity of 43 isolates from fermented cucumber.

S. No.	Isolate	Enzyme activity (U/ml)	S. No.	Isolate	Enzyme activity (U/ml)
1	C1	-	23	C23	0.09±0.01
2	C2	-	24	C24	0.65±0.08
3	C3	-	25	C25	1.11±0.11
4	C4	-	26	C26	0.56±0.05
5	C5	-	27	C27	1.02±0.06
6	C6	-	28	C28	1.30±0.07
7	C7	0.51±0.04	29	C29	0.93±0.05
8	C8	1.85±0.04	30	C30	0.06±0.00
9	C9	0.56±0.06	31	C31	0.37±0.01
10	C10	-	32	C32	0.46±0.02
11	C11	1.41±0.04	33	C33	-
12	C12	0.15±0.03	34	C34	0.37±0.01
13	C13	0.36±0.10	35	C35	
14	C14	0.52±0.02	36	C36	0.35±0.04
15	C15	-	37	C37	-
16	C16	0.14±0.01	38	C38	-
17	C17	0.63±0.08	39	C39	0.37±0.03
18	C18	0.74±0.10	40	C40	-
19	C19	0.23±0.05	41	C41	2.41±0.08
20	C20	-	42	C42	1.85±0.06
21	C21	0.63±0.03	**43**	**CRAG3**	**2.68±0.08**
22	C22	0.96±0.13			

Table 2.3.2 Total carbohydrate content of selected isolates from fermented cucumber.

S. No.	Isolate	Total carbohydrate content (mg/ml)	S. No.	Isolate	Total carbohydrate content (mg/ml)
1	C7	0.35±0.01	9	C25	1.16±0.09
2	C8	1.88±0.08	10	C26	0.47±0.07
3	C9	0.40±0.02	11	C28	1.23±0.08
4	C11	0.68±0.05	12	C29	1.09±0.08
5	C14	0.37±0.01	13	C41	2.97±0.09
6	C18	0.93±0.08	14	C42	2.35±0.10
7	C21	0.45±0.02	**15**	**CRAG3**	**4.52±0.14**
8	C22	1.09±0.09			

2.3.2. Morphological characterization of the isolate

The isolate CRAG3 appeared purple, reflecting its Gram positive nature as determined by the method of Gram (1884) (Fig. 2.3.1, Table 2.3.1).

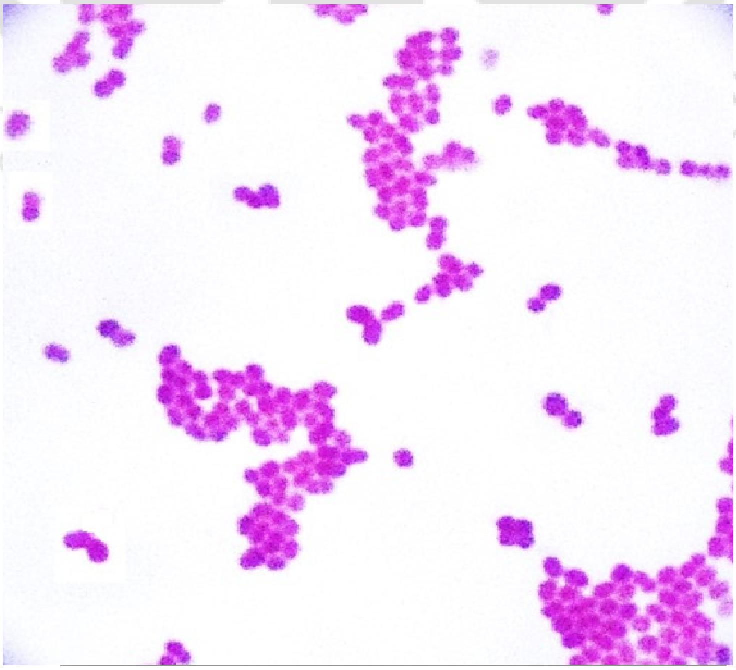

Fig. 2.3.1 Gram staining of the isolate CRAG3 showing purple color, indicating its Gram positive nature.

The scanning electron microscopic analysis showed that the isolate CRAG3 was cocci shaped arranged in pairs or chains with a diameter of 0.7- 0.9 µm (Fig. 2.3.2, Table 2.3.3). The colonies of the strain on MRS agar medium were opaque and circular and produced slimy layer when grown on MRS agar plate supplemented with sucrose due to the formation of dextran polymer.

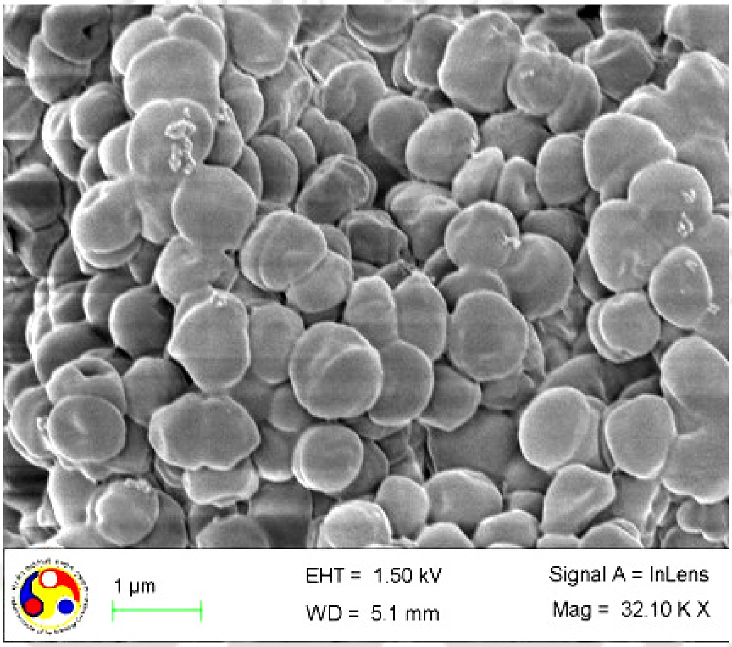

Fig. 2.3.2 Scanning electron micrograph of the isolate CRAG3.

Table 2.3.3 Morphological and biochemical characterization of the isolate CRAG3.

S. No.	Characteristic	Isolate CRAG3
1	Morphology	Coccus shaped
2	Gram reaction	Gram positive
3	Catalase activity	Negative
4	Triple sugar iron agar test	Ferment sucrose and glucose
5	Motility agar test	Non motile

2.3.3 Biochemical characterization of isolate

The ability of the isolate to ferment carbohydrates was evaluated by observing the change in colour of phenol red to yellow and due to production of lactic acid during fermentation. The isolate displayed yellow zone around cellobiose, dextrose, arabinose, rhamnose, sucrose, mannitol, fructose, maltose, trehalose, mannose, selicin and galactose, showing its ability to ferment these sugars (Fig. 2.3.3, Table 2.3.4). However, the isolate CRAG3 did not utilize raffinose, lactose, adonitol, sorbitol, inositol, inulin, mellibiose, xylose and dulsitol since no zone was been observed around these sugars. These results of CRAG3 corroborated with *Pediococcus pentosaceus* SPA (Patel and Goyal, 2010) with some variations (Table 2.3.4). Unlike *Pediococcus pentosaceus* SPA which weakly fermented lactose and did not utilize mannitol, the isolate CRAG3 was able to ferment mannitol but unable to utilize lactose (Table 2.3.4).

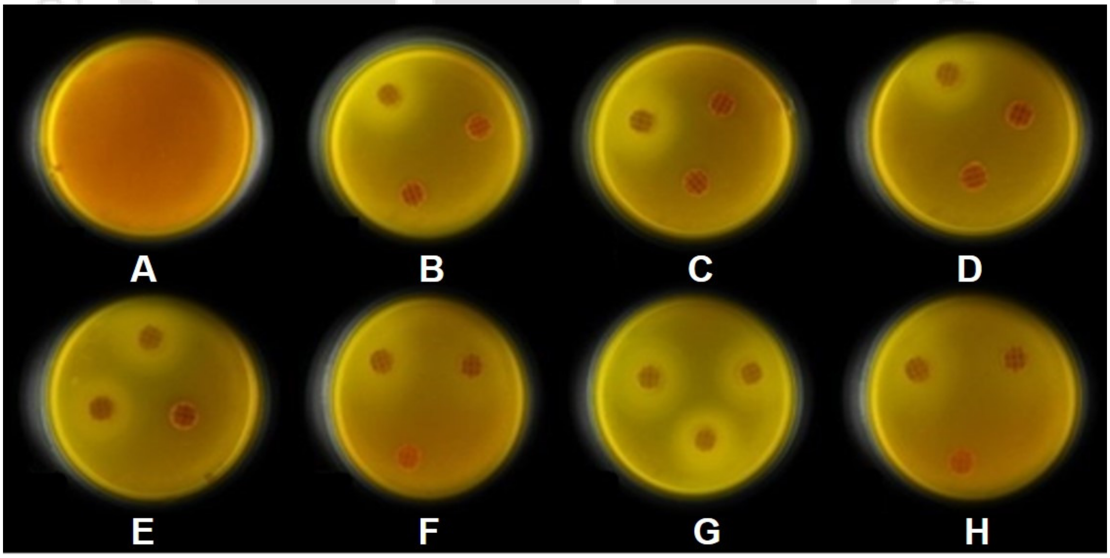

Fig. 2.3.3 Carbohydrate fermentation pattern of the isolate CRAG3 using discs of 21 sugars. The yellow zone around discs indicates sugar fermentation. **(A)** Control culture of isolate CRAG3 without any carbohydrate disc; and with carbohydrate discs containing **(B)** Dextrose, inulin and adonitol; **(C)** Maltose, inositol and raffinose; **(D)** Selicin, lactose and melibiose; **(E)** Fructose, trehalose and xylose; **(F)** Arabinose, rhamnose and dulsitol; **(G)** Mannose, cellobiose and sucrose; **(H)** Mannitol, galactose and sorbitol.

Table 2.3.4 Fermentation of different carbohydrates by isolate CRAG3.

S. No.	Carbohydrate	Isolate CRAG3	*P. pentosaceus* SPA*
1	Cellobiose	+++	+++
2	Dextrose	+++	+++
3	Arabinose	+++	Nd
4	Rhamnose	++	++
5	Sucrose	+++	+++
6	Mannitol	++	-
7	Fructose	++	+++
8	Maltose	+++	+++
9	Trehalose	+++	+++
10	Mannose	+++	Nd
11	Selicin	+++	Nd
12	Galactose	+++	+++
13	Raffinose	-	-
14	Lactose	-	++
15	Adonitol	-	Nd
16	Sorbitol	-	Nd
17	Inositol	-	Nd
18	Inulin	-	Nd
19	Mellibiose	-	+++
20	Xylose	-	+
21	Dulsitol	-	Nd

(+++) Strongly positive; (++) fairly positive; (+) weakly positive; (-) negative; Nd- not determined

**Patel and Goyal, 2010*

Triple sugar agar test also confirmed that the isolate CRAG3 was able to ferment sucrose and glucose since the colour of slant changed to yellow due to the formation of acid (Fig. 2.3.4, Table 2.3.3). The isolate CRAG3 was catalase negative since no bubble formation was observed on addition of H_2O_2 (Table 2.3.3). However, *E. coli* which was taken as positive control showed bubble formation (Fig. 2.3.5). The motility agar test displayed the non-motile nature of CRAG3 since it did not spread in agar medium like *E. coli* (Fig. 2.3.6, Table 2.3.3).

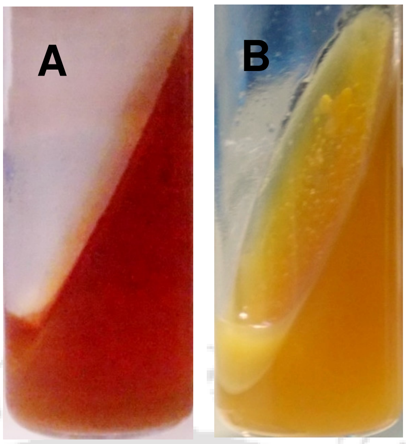

Fig. 2.3.4 Triple sugar iron agar slants **(A)** Blank slant (red); **(B)** With culture of the isolate CRAG3 showing colour change from red to yellow confirming the fermentation of sucrose and glucose.

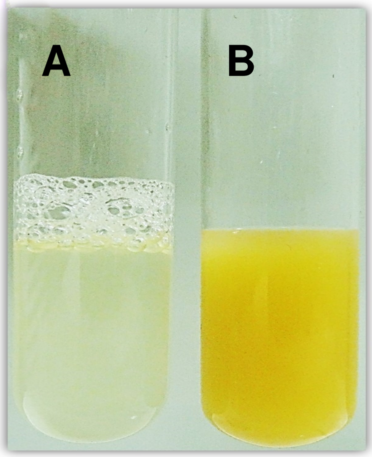

Fig. 2.3.5 Catalase activity test of **(A)** *E. coli* as a positive control and **(B)** isolate CRAG3 showing its catalase negative nature.

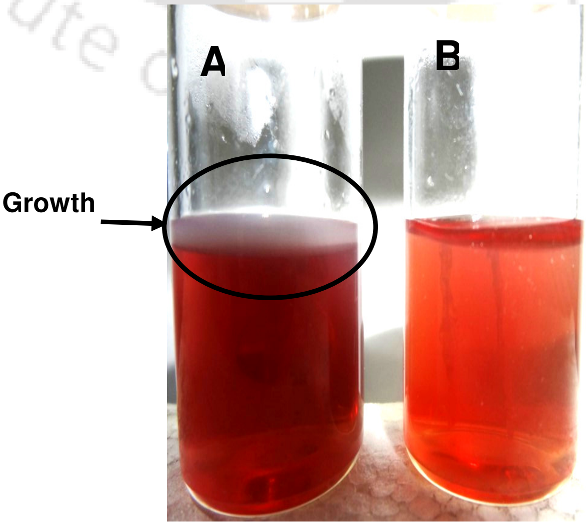

Fig. 2.3.6 Motility agar test of **(A)** *E. coli* as a positive control and **(B)** isolate CRAG3 showing its non-motile nature.

The antibiotic sensitivity of isolate CRAG3 was evaluated by measuring the diameter of zone of inhibition around the discs and comparing with those given in standard chart. Depending upon the diameter of zone, the isolate was categorized as resistant, moderate or susceptible. The isolate CRAG3 was resistant to cefixime, ciprofloxacin, co-trimoxazole, kanamycin, nalidixic acid, sulphamethoxazole, tobramycin, trimethoprim, vancomycin and gentamicin (Fig. 2.3.7, Table 2.3.5). However it displayed susceptibility to amoxycillin, amoxyclav, cefoperazone, cephaclor, cephalexin, cephalothin, clindamycin, erythromycin, imipenam, lincomycin, nitrofurantoin, novobiocin, oleandomycin, oxytetracycline, piperacilin, tetracycline, ticarcillin, carbenicillin cephotaxime, chloramphenicol, co-trimazine and oxacillin. Similar results with some exceptions were observed with *Pediococcus pentosaceus* SPA (Patel and Goyal, 2010) as listed in Table 2.3.5 and with *Leuconostoc mesenteroides* (Purama *et al.*, 2008). Unlike *Pediococcus pentosaceus* SPA, which was resistant to amikacin, ampicillin, gentamicin and norfloxacin, *Pediococcus pentosaceus* CRAG3 was moderately sensitive to these antibiotics. The resistance of *Pediococcus*, *Leuconostoc* and *Lactobacillus* spp. to vancomycin is a common trait amongst these lactic acid bacteria (Barton *et al.*, 2001).

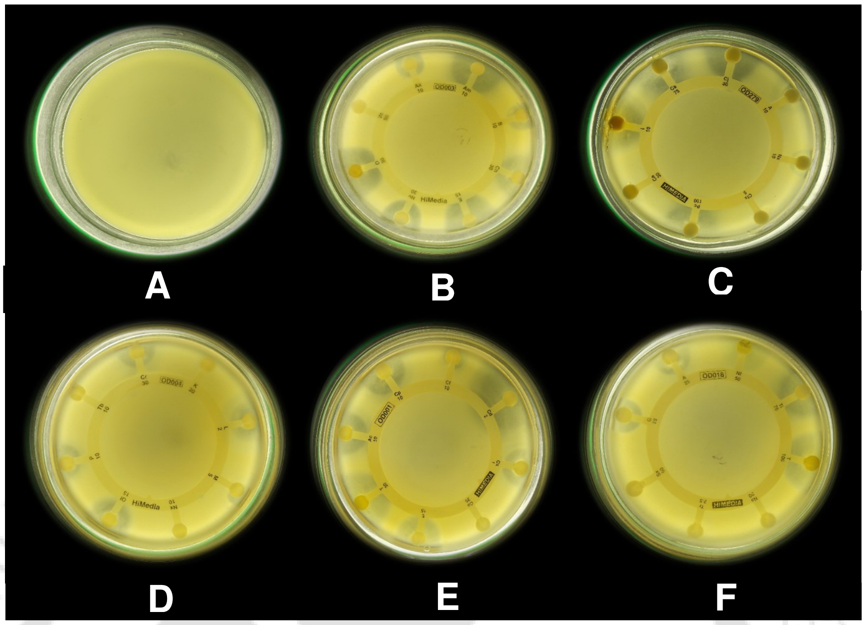

Fig. 2.3.7 Antibiogram of the isolate CRAG3 using antibiotic octodiscs on MRS agar plates. **(A)** Control culture of the isolate CRAG3. **(B-F)** Antibiotic treated culture of the isolate CRAG3.

Table 2.3.5 Antibiogram of isolate CRAG3 using antibiotic containing octodiscs on MRS agar plates.

S. No.	Antibiotic	Concentration (µg)	Isolate CRAG3	*P. pentosaceus* SPA*
1	Amikacin	10	M	R
2	Ampicillin	25	M	R
3	Bacitracin	10 U	M	M
4	Ceftazidime	30	M	nd
5	Cephaloridine	30	M	R
6	Cloxacillin	01	M	R
7	Gentamicin	10	M	R
8	Methicillin	05	M	S
9	Norfloxacin	10	M	R
10	Penicillin-G P	10 U	M	S
11	Cefixime	05	R	nd
12	Ciprofloxacin	10	R	R
13	Co-Trimoxazole	25	R	nd
14	Kanamycin	30	R	R
15	Nalidixic acid	30	R	nd
16	Sulphamethoxazole	50	R	nd
17	Tobramycin	10	R	R
18	Trimethoprim	2.5	R	nd
19	Vancomycin	30	R	R
20	Gentamicin	10	R	nd

S. No.	Antibiotic	Concentration (μg)	Isolate CRAG3	*P. pentosaceus* SPA*
21	Amoxycillin	10	S	S
22	Amoxyclav	10	S	M
23	Cefoperazone	75	S	nd
24	Cephaclor	30	S	nd
25	Cephalexin	10	S	M
26	Cephalothin	30	S	M
27	Clindamycin	02	S	M
28	Erythromycin	15	S	S
29	Imipenam	10	S	nd
30	Lincomycin	02	S	S
31	Nitrofurantoin	50	S	nd
32	Novobiocin	30	S	M
33	Oleandomycin	15	S	S
34	Oxytetracycline	30	S	S
35	Piperacilin	100	S	S
36	Tetracycline	100	S	nd
37	Ticarcillin	75	S	nd
38	Carbenicillin	100	S	S
39	Cephotaxime	30	S	M
40	Chloramphenicol	30	S	S
41	Co-trimazine	25	S	R
42	Oxacillin	05	S	M

Zone of inhibition of growth of microorganisms: 0-2 mm (R-Resistant); 3-6 mm (M- Moderate); 7-13 mm (S- Sensitive). nd- not determined

**Patel and Goyal, 2010*

2.3.4 16S rRNA gene sequence based identification of isolate CRAG3

2.3.4.1 Genomic DNA isolation

The genomic DNA from the isolate CRAG3 extracted and purified using QIAquick® Gel Extraction Kit (QIAGEN) as described earlier in Section 2.2.9.4 showed a single band of 19.1 kbp size on 0.8% (w/v) agarose gel (Fig. 2.3.8).

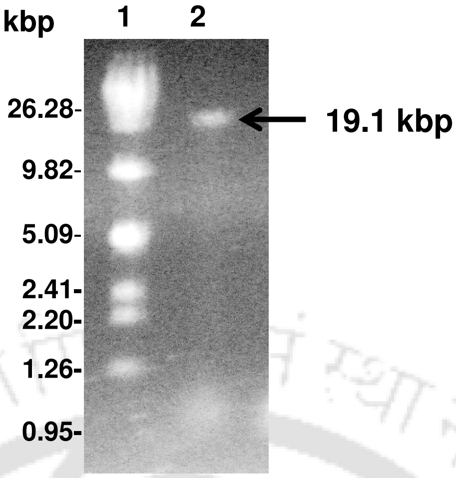

Fig. 2.3.8 Agarose gel (0.8%) showing isolated genomic DNA of isolate CRAG3. Lane 1: DNA Ladder; Lane 2: Genomic DNA of isolate CRAG3.

2.3.4.2 PCR amplification and cloning of 16S rRNA gene

The PCR amplified 16S rRNA gene using genomic DNA as template showed a single band of approximately, 1.5 kbp (Fig. 2.3.9).

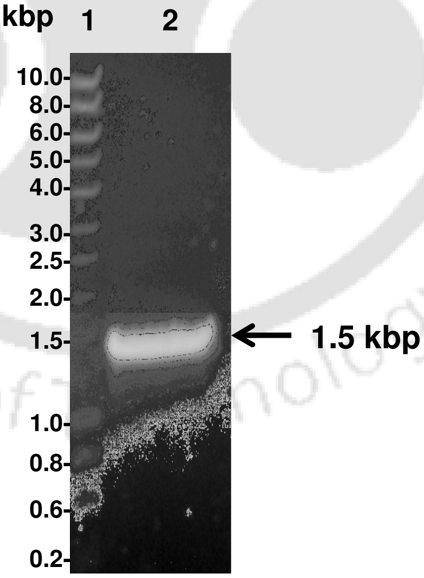

Fig. 2.3.9 Agarose gel (0.8%, w/v) showing PCR product of 16S rRNA gene of 1.5 kbp amplified from genomic DNA of isolate CRAG3. Lane 1: DNA Ladder; Lane 2: PCR amplicon of 16S rRNA gene.

The PCR amplicon of 16S rRNA gene was cloned into $\text{pGEM}_{\text{Teasy}}$ vector and the ligation was confirmed by digestion with *Eco*RI and *Sac*I restriction enzymes. The restriction digestion of $\text{pGEM}_{\text{Teasy}}$ plasmids containing the 16S rRNA gene with *Eco*RI enzyme showed a band of nearly 4,500 bp due to the linearization of plasmid while *Sac*I treated plasmid showed two bands, *viz.* 3,000 bp of $\text{pGEM}_{\text{Teasy}}$ plasmid and 1,500 bp of 16S rRNA gene (Fig. 2.3.10A). The PCR amplification using universal oligonucleotide primers and $\text{pGEM}_{\text{Teasy}}$ plasmid showed a single band of approximately 1,500 bp (Fig. 2.3.10B) confirmed the cloning of the 16S rRNA gene.

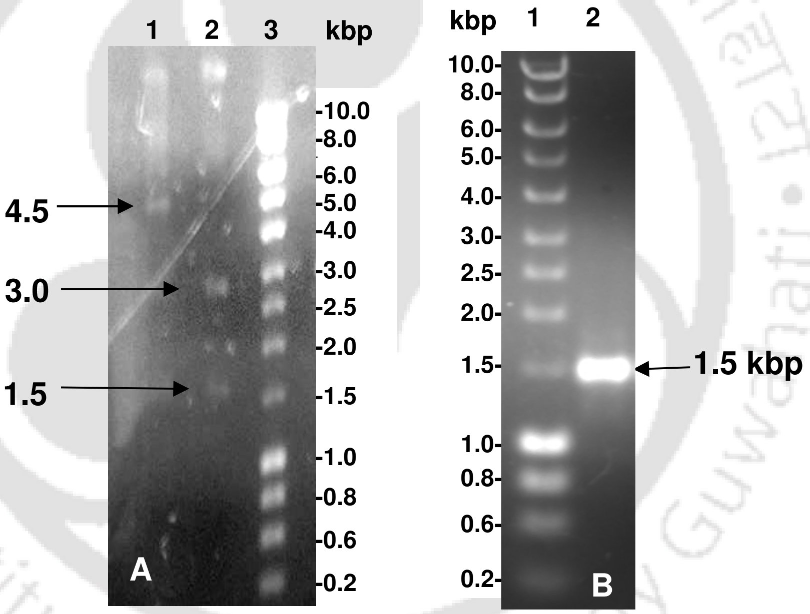

Fig. 2.3.10 Agarose gel (0.8%, w/v) showing confirmation of positive clone using restriction digestion. **(A)** Plasmid treated with; Lane 1. *Eco*RI showing a band of 4.5 kbp (plasmid + insert); Lane 2. *Sac*I showing two bands of 3.0 kbp (plasmid) and 1.5 kbp (insert); Lane 3. DNA ladder. **(B)** PCR amplification of 16S rDNA. Lane 1. DNA ladder; Lane 2. PCR amplicon.

2.3.4.3 Sequence analysis of 16S rRNA gene

After PCR amplification, 16S rDNA gene sequence analysis was performed to identify the isolate CRAG3. The profiles obtained by PCR amplification allowed for identification of the isolate at both genus and species levels. The consensus sequence of 1269 bp of 16S rDNA was generated from forward and reverse sequence data using aligner software (Fig. 2.3.11).

```
ATTATGACGTACTTGTACTGATTGAGATTTTAACACGAAGTGAGTGGCGAACGGGTGAGTAACAC
GTGGGTAACCTGCCCAGAAGTAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAATAGA
GAAAACCGCATGGTTTTCTTTTAAAAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGTA
TTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAAT
CGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
ACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAG
CTCTGTTGTTAAAGAAGAACGTGGGTAAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTT
ATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCGGCTCAACCGA
AGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCG
GTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGAC
GCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACG
ATGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGTAATCCGC
CTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGACAGTCT
AAGAGATTAGAGGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCATTAAGT
TGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAATCATC
ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAACGAGTCGCGAGACCGC
GAGGTTAAGCTAATCTCTTAAAACCATTCTCAGTTCGGACTGTAGGCTGCA
```

Fig. 2.3.11 Consensus sequence (1269 bp) of 16S rRNA gene of isolate CRAG3.

The 16S rRNA gene sequence was compared with reference bacteria from the National Centre for Biotechnological Information (NCBI) Genbank (http:// www.ncbi.nlm.nih.gov/) and Ribosomal Database Project (RDP). The sequence analysis using Blast program demonstrated 99%-100% homology with 16S rRNA gene sequence of members of *Pediococcus pentosaceus* (Table 2.3.6).

Table 2.3.6 Alignment results using combination of NCBI GenBank and RDP database

Accession	Description	Max score	Total score	Query coverage	E value	Max identity
DQ267152.1	*Pediococcus pentosaceus*	2344	2344	100%	0.0	100%
JN944736.1	*Pediococcus pentosaceus* strain ULAG75	2344	2344	100%	0.0	100%
JN944687.1	*Pediococcus pentosaceus* strain ULAG23	2338	2338	100%	0.0	99%
JN036551.1	*Pediococcus pentosaceus* strain R-P	2338	2338	100%	0.0	99%
JX232608.1	*Pediococcus pentosaceus* strain S6	2338	2338	100%	0.0	99%
HQ589248.1	*Pediococcus pentosaceus* strain Z1	2338	2338	100%	0.0	99%
HQ834496.1	*Pediococcus pentosaceus* strain SC1	2338	2338	100%	0.0	99%
HQ286591.1	*Pediococcus pentosaceus* strain PP strain MJK7	2338	2338	100%	0.0	99%
AB494722.1	*Pediococcus pentosaceus* strain: MJK7	2338	2338	100%	0.0	99%
AB481102.1	*Pediococcus pentosaceus*, strain: KT3CE27	2338	2338	100%	0.0	99%

Similarity searches were carried out using the BLAST algorithms available at (http://www.ncbi.nlm.nih.gov/blast). Multiple sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/clustalw2) (Casanellas and Fernandez-S´anchez, 2008). The alignment was checked visually and corrected manually using the sequence editor. The homology in sequences identified by the above methods was used to find out common ancestry. The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+ noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete-deletion option). There were a total of 1269 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007). The isolate CRAG3 was clustered with *Pediococcus pentosaceus* strain ULAG75 (GenBank Accession Number: JN944736) and *Pediococcus pentosaceus* (GenBank Accession Number: DQ267152) (Fig. 2.3.12) which confirmed the isolate to be *Pediococcus pentosaceus*. The isolate CRAG3 was identified as *Pediococcus pentosaceus* and its 16S rRNA gene sequence was submitted to the NCBI GenBank database. The isolate CRAG3 was assigned the Genbank accession number JX679020.

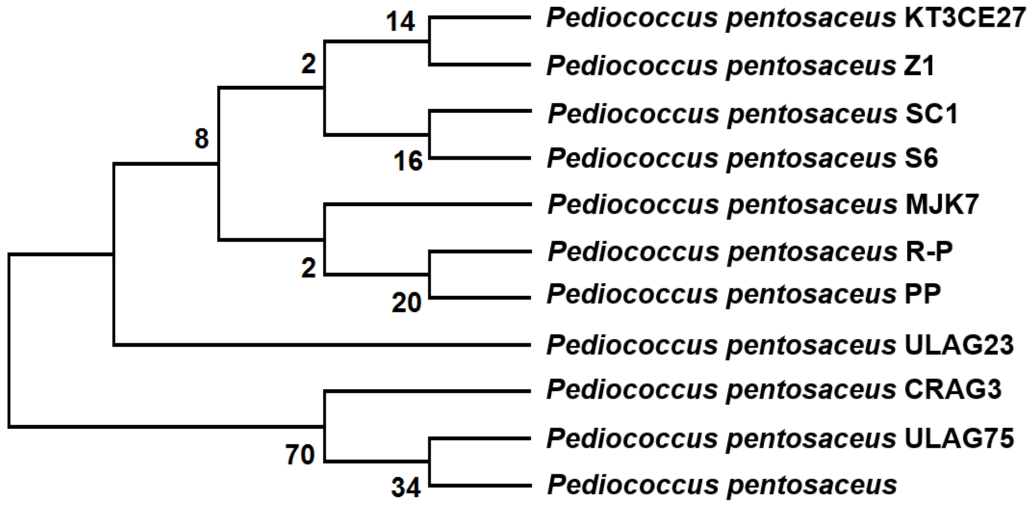

Fig. 2.3.12 Phylogenetic tree made in MEGA4 software using Neighbour Joining method demonstrating the relationship of isolate CRAG3 to other *Pediococcus* species from NCBI.

2.4 Conclusions

A strain of lactic acid bacteria was isolated from fermented cucumber on the basis of its ability to produce higher dextransucrase and dextran. The isolate CRAG3 displaying maximum enzyme activity (2.7 U/ml) and maximum total carbohydrate content (4.5 mg/ml) was selected for identification and further biochemical characterization. The morphological characteristics showed that the isolate was Gram positive and coccus shaped bacterium, forming round colonies.

The biochemical characterization of isolate CRAG3 displayed its ability to utilize carbohydrates such as cellobiose, dextrose, arabinose, sucrose, maltose, mannose and galactose. However, the isolate CRAG3 failed to utilize raffinose, inulin, lactose and mellibiose. Triple sugar iron agar test also confirmed that the isolate CRAG3 was able to ferment sucrose and glucose. The isolate CRAG3 was catalase negative since no bubble formation was observed on addition of H_2O_2. The motility agar test proved CRAG3 as a non-motile bacterium since it did not spread in motility indole lyine agar slant. The antibiotic sensitivity test showed that the isolate was resistant to cefixime, ciprofloxacin, co-trimoxazole, kanamycin, nalidixic acid, sulphamethoxazole, tobramycin, trimethoprim, vancomycin and gentamicin. The resistance of *Pediococcus pentosaceus* CRAG3 to vancomycin, showed its common characteristics of lactic acid bacteria.

Identification of isolate was done on the basis of 16S rRNA sequence analysis. Consensus sequence of 1,269 bp of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The isolate was clustered with *Pediococcus pentosaceus* strain ULAG75 (GenBank Accession Number: JN944736) and *Pediococcus pentosaceus* (GenBank Accession Number: DQ267152) which confirmed it as *Pediococcus pentosaceus*. The isolate CRAG3 was assigned the Genbank accession number JX679020. The members of *Pediococcus* genus have great economic importance in the fermented food industry, as starter cultures in fermentation processes of milk, meat, vegetable products and sausages and also as probiotics. Since the dextransucrase producing ability of *Pediococcus pentosaceus* has not been much explored, this strain could serve as good candidate for potential applications in food industries. The interest in studying applications of dextran is increasing day by day which shows the importance of exploring of novel strains of bacteria and characterization of their traits.

2.5 References

Albano, H., Todorov, S.D., van Reenen, C.A., Hogg, 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. Tech. 43, 448-454.

Aukrust, T. and Blom, H. (1992) Transformation of *Lactobacillus* strains used in meat and vegetable fermentations. Food Res. Int. 25, 253-261.

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

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.

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.

Bharali S., Purama R.K., Majumder, A., Fontes, C.M.G.A. and Goyal, A. (2006) Molecular characteristics of a recombinant, novel glycoside hydrolase of family 26 from *Clostridium thermocellum*. Ind. J. Microbiol. 46, 371-378.

Boris, S., Suárez, J.E., Vázquez, F. and Barbés, C. (1998) Adherence of human vaginal *lactobacilli* to vaginal epithelial cells and interaction with uropathogens. Infect. Immun. 66, 1985-1989.

Bottger, E.C. (1989) Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol. Lett. 65, 171-176.

Casanellas, M. and Fern´andez-S´anchez, J. (2008) Geometry of the Kimura 3-parameter model. Adv. Appl. Math. 41(3), 265-292.

Chen K., Neimark, H., Rumore, P. and Steinman, C.R. (1989) Broad range DNA probes for detecting and amplifying eubacterial nucleic acids. FEMS Microbiol. Lett. 57, 19-24.

Chen, Y. S., Wu, H. C., Lo, H-Y, Lin, W-C, Hsu, W-H, *et al.* (2012) Isolation and characterization of lactic acid bacteria from jiang-gua (fermented cucumbers), a traditional fermented food in Taiwan. J. Sci. Food Agr. 92, 2069-2075.

Clarridge, J. E. (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17, 840-862.

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 non0conventional flours. Syst. Appl. Microbiol. 30, 561-571.

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.

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.

De Man, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of *Lactobacilli*. J. Appl. Bacteriol. 23, 130-135

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

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.

Duenas, M., Munduate, A., Perea, A. and 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.

Felsenstein J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 39, 783-791.

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

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.

Gobbetti, M. and Corsetti, A. (1997) *Lactobacillus sanfrancisco* a key sourdough lactic acid bacterium: a review. Food Microbiol. 14, 175-187.

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

Gram, H.C (1884) Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten. Fortschritte der Medizin 2, 185–189.

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.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166, 557-580.

Harris, L.J., Fleming, H.P. and Klaenhammer, T.R. (1992) Characterization of two nisin producing *Lactococcus lactis* subsp. *lactis* strains isolated from a commercial sauerkraut fermentation. Appl. Environ. Microbiol. 58, 1477-1483.

Hertel, C., Ludwig, W., Pot, K., Kersters, K. and Schleifer, K.H. (1993) Differentiation of *lactobacilli* in fermented milk products by using oligonucleotide probes and electrophoretic protein profiles. Syst. Appl. Microbiol. 16, 463-467.

Holzapfel, W.H., Haberer, P., Geisen R., Bjorkroth, J. and Schillinger, U. (2001) Taxonomy and important features of probiotic microorganisms in food nutrition. Am. J. Clin. Nutr. 73, 365S-373S.

Jay, J.M. (2000) Fermentation and fermented dairy products. In: Modern Food Microbiology, 6th edition. An Aspen Publication, Aspen Publishers, Inc. Gaithersburg, USA. pp. 113-130.

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

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.

Kattar, M.M., Chavez, J.F., Limaye, A.P., Rassoulian-Barrett, S.L., Yarfitz, S.L., Carlson, L.C., Houze, Y., Swanzy, S., Wood, B.L. and Cookson, B.T. (2001) Applicaton of 16S rRNA gene sequencing to identify *Bordetella hinzii* as the causative agent of fatal septicemia. J. Clin. Microbiol. 38, 789-794.

Kimura, M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111-120.

Liu, S.Q. (2003) Review article: Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. Int. J. Food Microbiol. 83, 115-131.

Lonvaud-Funel, A. (2001) Biogenic amines in wines: role of lactic acid bacteria. FEMS Microbiol. Lett. 199, 9-13.

Maehly, A. and Chance, B. (1954) The assay of catalases and peroxidases. Meth Biochem. Anal. 1, 357-424.

Martín, R., Langa, S., Reviriego, C., Jimínez, E., Marín, M.L., Xaus, J., Fernández, L. and Rodríguez, J.M. (2003) Human milk is a source of lactic acid bacteria for the infant gut. J. Pediatr. 143, 754-758.

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

Nieto-Lozano, J.C., Reguera-Useros, J.I., Pelaez-Martinez, M.C., Sacristan-Perez-Minayo, G., Gutierrez-Fernandez, A. J. and de la Torre, A.H. (2009) The effect of the pediocin PA-1 produced by *Pediococcus acidilactici* against *Listeria monocytogenes* and *Clostridium perfringens* in Spanish dryfermented 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 treptomycin resistance (aadE) gene to that found in *Campylobacter jejuni*. Plasmid. 58, 115-126.

O'Sullivan, L., Ross, R.P. and Hill, C. (2002) Review: Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie. 84, 593-604.

Ocaña, V.S., Pesce de Ruiz Holgado, A.A. and Nader-Macías, M.E. (1999) Characterization of a bacteriocin-like substance produced by a vaginal *Lactobacillus salivarius* strain. Appl. Environ. Microbiol. 65, 5631-5635.

Padmaja, G. A., Ramachandra, B., Manjunath, H., Prabh, R., Krishna, R. and Shankar, P. A. (2011) Characterization of lactic acid bacteria isolated from fruits and vegetables for their antibacterial activity. J. Dairying Foods Home Sci. 30, 85-89

Patel, S. and Goyal, A. (2010) Isolation, characterization and mutagenesis of exopolysaccharide synthesizing new strains of lactic acid bacteria. Int. J. Microbiol. 8(1).

Patel, S., Kasoju, N., Bora, U. and Goyal, A. (2010) Structural analysis and biomedical applications of dextran produced by a new isolate *Pediococcus pentosaceus* screened from biodiversity hot spot Assam. Biores. Technol. 101, 6852-6855.

Pot, B. and Janssens, D. (1993) The potential of a culture collection for identification and maintenance of lactic acid bacteria. In: *The lactic acid bacteria*. Proceedings of the first Lactic Acid Bacteria Computer Conference (ed. Foo, E.L., Griffin, H.G., Mollby, R. and Heden, C.G.) Horizon Scientific Press, Norfolk, Va, pp 81-87.

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.

Ravi, V., Prabhu, M. and Subramanyam, D. (2011) Isolation of bacteriocin producing bacteria from mango pulp and its antimicrobial activity. J. Microbiol. Biotech. Res. 1, 54-63.

Sacchi, C.T., Whitney, A.M., Mayer L.W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R.S. and Popovic, T. (2002a) Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis*. Emerg. Infect. Dis. 8, 1117-1123.

Sacchi, C.T., Whitney, A.M., Reeves, M.W., Mayer, L.W. and Popovic, T. (2002b) Sequence diversity of *Neissaria meningitides* 16S rRNA genesand use of 16S rRNA gene sequencing as a molecular subtyping tool. J. Clin. Microbiol. 40, 4520-4527.

Saitou, N. and Nei, M. (1987) The neighbour-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

Sambrook, J. and Russell, D. (2001) In *Molecular Cloning: a Laboratory Manual,* 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Singh, A. K and Ramesh, A. (2008) Succession of dominant and antagonistic lactic acid bacteria in fermented cucumber: insights from a PCR based approach. Food Microbiol. 25, 278-287

Smitinont, T., Tansakul, C., Tanasupawat, S., Keeratipibul, S., Navarini, L., Bosco, M. and Cescutti, P. (1999) Exopolysaccharideproducing 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.

Stiles, M.E. and Holzapfel, W.H. (1997) Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36, 1-29.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596-1599.

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

Tortoli, E. (2003) Impact of genomic studies on mycobacterial taxonomy: the new mycobacteria of the 1990's. Clin. Microbiol. Rev. 16, 319-354.

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.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. and Swings, J., (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. 60, 407-438.

vanden Berg, D.J.C., Smits, A., Pot, B., Ledeboer, A.M., Kersters, K., Verbakel, J.M.A. and Verrips, C.T. (1993) Isolation, screening and identification of lactic acid bacteria from traditional food fermentation processes and culture collections. Food Biotechnol. 7, 189-205.

Woese, C.R. (1987) Bacterial evolution. Microbiol. Rev. 51, 221-271.

Woese, C.R., Stackebrandt, E., Macke, T.J. and Fox G.E. (1985) A phylogenetic definition of the major eubacterial taxa. Syst. Appl. Microbiol. 6, 143-151.

Chapter 3

Production, purification and characterization of dextransucrase from *Pediococcus pentosaceus* CRAG3

3.1 Introduction

Glucansucrases (GSs), also known as glucosyltransferases (GTFs), are large size extracellular enzymes that catalyse the synthesis of different types of α-glucans such as dextran, mutan, alternan and reuteran using sucrose as a substrate. Depending upon the nature of synthesized product they are categorize as dextransucrase (EC 2.4.1.5), alternansucrase (EC 2.4.1.140), mutansucrase (EC 2.4.1.5) and reuteransucrase (EC 2.4.1.5) (Leemhuis *et al.*, 2013). Glucansucrases have been identified in lactic acid bacteria (LAB).

The high molecular weight of dextransucrase is due to its aggregated form in presence of dextran (Majumder *et al.*, 2007). Dextransucrase consists of two domains; N-terminal catalytic and C-terminal glucan-binding domain containing chain of direct repeating units (Funane *et al.*, 1998). The major dextransucrase producers belong to families *Lactobacillaceae* and *Streptococcaceae*, especially the genera *Lactobacillus*, *Leuconostoc* and *Streptococcus*. Most of the studies on dextran and dextransucrase production have been conducted on *Leuconostoc mesenteroides* in which the dextransucrases are produced by sucrose induction (Neely and Nott, 1962), with few exceptions where they are constitutively produced (Kim and Robyt, 1994a; Kim and Robyt, 1994b). The dextran production by *Pediococcus* genus had not been much studied (Smitinont *et al.,* 1999; Patel *et al.*, 2010). There are number of factors such as temperature, medium components and shaking conditions that affect production of dextransucrase (Tsuchiya *et al.*, 1952; Lazic *et al.*, 1993; Goyal *et al.*, 1995; Rodrigues *et al.*, 2003; Cortezi *et al.*, 2004). The optimum temperature for production of dextransucrase from *Leuconostoc* spp ranging from 20 to 30^{o}C (Tsuchiya *et al.*, 1952; Barker and Ajongwen, 1991; Veljkovic *et al.*, 1992; Goyal and Katiyar, 1997; Dols *et al.*, 1997; Cortezi *et al.*, 2004). The shaken and static flask cultures also affect the production of dextransucrase (Tsuchiya *et al.*, 1952; Veljkovic *et al.*, 1992; Goyal *et al.*, 1995).

Since the dextransucrase production is induced by sucrose, there is continuous production of dextran in culture medium which makes it viscous and thereby the purification of enzyme becomes unwieldy. The purification of dextransucrase can be done by ultra-filtration, salt and PEG precipitation, chromatography and phase-partitioning, alone or in combination (Majumder *et al.*, 2007). However the simplest, effective and one step purification method amongst them is fractionation by polyethylene glycol (PEG) (Russell, 1979; Goyal and Katiyar, 1994; Purama and Goyal, 2008). It involves the precipitation of high molecular weight proteins which occur in aggregated forms (Miekka and Ingham, 1978). Due to their large molecular weight and aggregate forming tendency in solution of dextransucrase, nonionic hydrophilic polymer PEG is used for purification (Goyal and Katiyar, 1994; Purama and Goyal, 2008; Nigam *et al.*, 2006).

Dextransucrase has been reported to exist in single or multiple forms having molecular weight in the range 64,000-245,000 (Kobayashi and Matsuda, 1980; Willemot *et al.*, 1988; Goyal and Katiyar, 1994; Purama and Goyal, 2008). *Leuconostoc mesenteroides* NRRL B-512F is a widely studied dextransucrase producing strain (Fu and Robyt 1990; Goyal *et al.*, 1994; Goyal and Katiyar, 1994). In one study it has been reported to produce two main forms of dextransucrase of molecular weight nearly 177 and 158 kDa as determined by SDS-PAGE (Miller and Robyt, 1986), however there are reports on the existence of multiple molecular forms of the same enzyme (Kobayashi and Matsuda, 1986; Goyal and Katiyar, 1994; Quirasco *et al.*, 1999). The four active forms of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F with molecular weights 170, 160, 116 and 97 kDa were characterized by Quirasco *et al.* (1999). The dextransucrase from *Leuconostoc mesenteroides* NRRL B-512FMC and *Leuconostoc mesenteroides* NRRL B-512F were found to be was 184 kDa (Kim and Robyt, 1994a) and 188 kDa (Goyal and Katiyar, 1994), respectively while that from *Pediococcus pentosaceus* was 180 kDa (Patel *et al.*, 2011).

The effects of different metal ions on dextransucrase from *Pediococcus pentosaceus* (Patel *et al.*, 2011) and *Leuconostoc mesenteroides* NRRL B-1299 (Kobayashi and Matsuda, 1976) showed that Ca^{2+} and Mg^{2+} enhanced the activity of both bacteria. The divalent ions such as Ca^{2+} and Mg^{2+} enhanced the activity of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1146 (Majumder *et al.*, 2008). The addition of $CaCl_2$ with purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F increased its activity (Lopez and Monsan, 1980; Goyal *et al.*, 1995).

The present study describes the purification of dextransucrase from *Pediococcus pentosaceus* CRAG3 using fractionation and gel filtration. The purified enzyme was identified and confirmed as dextransucrase by Periodic Acid Schiff's (PAS) staining procedure using sucrose and raffinose. The biochemical properties of purified dextransucrase were analysed.

3.2 Materials and Methods

3.2.1 Chemicals, reagents and microorganism

The bacterial strain *Pediococcus pentosaceus* CRAG3 (Genbank Accession Number JX679020) was isolated from fermented cucumber as described earlier in Chapter 2, Section 2.2.2. It was propagated in modified MRS agar medium (Goyal and Katiyar, 1996) as a stab incubated at 30°C, stored at 4°C and subcultured every 15 days. The modified MRS agar medium was prepared as described in Chapter 2, Section 2.2.3. The chemicals required for protein estimation such as sodium carbonate, sodium hydroxide, sodium potassium tartrate, cupric sulphate and phenol reagent and buffer preparation were of highest purity grade. PEG-400 from Qualigens Pvt. Ltd., India and PEG-1500 from BDH, U.K. were used for fractionation of dextransucrase. The chemicals required for Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma, St. Louis, MO, USA. The protein ladder (40-300 kDa) for SDS-PAGE was purchased from Fermentas, USA. Tween 80, magnesium sulphate heptahydrate, manganese sulphate tetrahydrate, ferrous sulphate heptahydrate and calcium chloride dihydrate were purchased from Fisher Scientific Pvt. Ltd., India. The semi-skimmed milk was purchased from Anand Milk Union Limited (AMUL), India.

3.2.2 Protein estimation method

3.2.2.1 Preparation of reagents for protein estimation

The reagents for estimation of protein content were prepared by the method of Lowry *et al.* (1951) and as described in Table 3.2.1.

Table 3.2.1 List of components for preparation of reagents for protein estimation.

Reagents	Components	Amount	Procedure
Reagent A	Sodium carbonate	2.0 g	Both the components were dissolved in deionized water and volume was made to 100 ml.
	Sodium hydroxide	0.4 g	
Reagent B1	Sodium potassium tartrate	2% (w/v)	1 g was dissolved in 50 ml deionized water.
Reagent B2	Cupric sulphate	1% (w/v)	0.5 g was dissolved in 50 ml in deionized water.
Reagent C	Freshly prepared by mixing Reagent B1, Reagent A and Reagent B2 in the ratio 1:100:1.		
Phenol Reagent	Phenol reagent (1N)		3 N Phenol reagent (10 ml) was dissolved in 20 ml deionized water.

3.2.2.2 Standard plot of BSA for protein estimation

A standard plot was prepared by method of Lowry *et al.* (1951) with certain modifications using Bovine serum albumin (BSA) for protein estimation. The absorbance at 660 nm (A_{660}) was taken. The stock solution of BSA (5.0 mg/ml) was prepared from which dilutions varying between 50-500 μg/ml were made. To the BSA solution (200 μl), 1 ml of reagent C was added and incubated for 15 min at 25°C. Then 100 μl of 1N phenol reagent was added and the solution (1.3 ml) was further incubated for 30 min. For blank, instead of BSA solution 200 μl of distilled water was used. The absorbance at 660 nm (A_{660}) of samples was taken against the blank. The A_{660} was plotted against each concentration of BSA (Fig. 3.2.1).

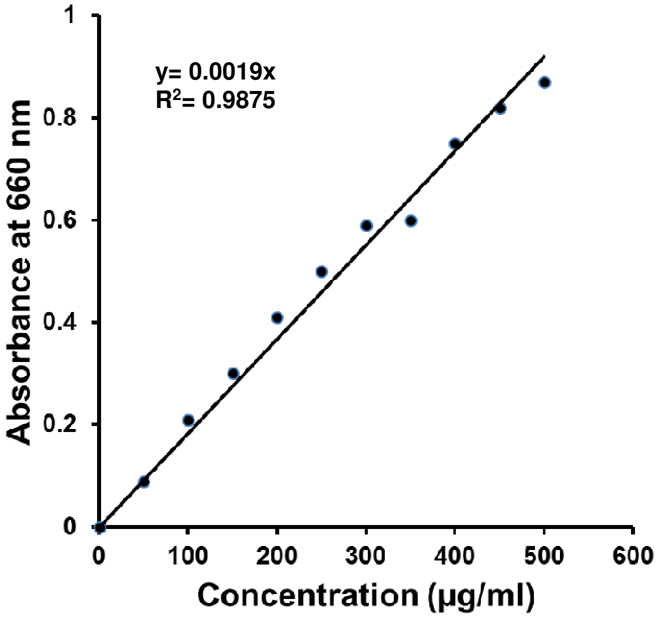

Fig. 3.2.1 Standard plot between varying concentrations of bovine serum albumin against absorbance (A_{600}) using Lowry's method for protein estimation.

3.2.2.3 Procedure for protein estimation

To 200 µl of sample containing protein (as described later in Section 3.2.5), 1 ml of reagent C was added. After 15 min, 100 µl of phenol reagent was added and the solution (1.3 ml) was mixed. The absorbance at 660 nm (A_{660}) was measured after 30 min against a blank and the protein concentration was calculated as follows:

$$\text{Protein concentration} = \frac{A_{660} \times C \times 10^{-3} \times V}{v} \quad \text{(mg/ml)}$$

where,

A_{660} = Optical density of protein sample at 660 nm
C= Amount (µg/ml) of BSA at OD=1 from standard plot (526 µg/ml)
V= Volume (ml) of reaction mixture
v = Volume (ml) of the protein sample

3.2.3 Calculation of specific activity of enzyme

Specific activity is defined as the amount of substrate which an enzyme converts per mg of protein, per unit of time and expressed in U/mg. It measures the purity of enzyme. The larger the value of specific activity the more pure the enzyme, since the amount of protein (mg) is less, but the rate of reaction stays the same. The specific activity of an enzyme was calculated using enzyme activity (as described earlier in Chapter 2, Section 2.2.5.1.3) and protein concentration (as described in Section 3.2.2.3) as follows:

$$\text{Specific activity} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein concentration (mg/ml)}} = \text{U/mg}$$

3.2.4 Optimization of culture conditions for dextransucrase and dextran production

3.2.4.1 Effect of shake flask culture on dextransucrase and dextran production

The enzyme production medium (Tsuchiya *et al.*, 1952) as described earlier in Chapter 2, Section 2.2.4 was used for dextransucrase and dextran production. Fermentations were carried out with 1% (v/v) inoculum of overnight grown culture of *Pediococcus pentosaceus* CRAG3 in 100 ml enzyme production medium each in a 250 ml Erlenmeyer flask and incubated under shaking (180 rpm) and static condition at 28°C. 1.0 ml samples were withdrawn at regular time intervals and centrifuged at 8,000***g*** and at 4°C for 10 min. The cell pellet was discarded and the cell free supernatant was analyzed for enzyme activity and total carbohydrate content as described in Chapter 2, Section 2.2.5.1.3 and Section 2.2.6, respectively.

3.2.4.2 Effect of temperature on dextransucrase and dextran production

The effect of temperature on dextransucrase and dextran was determined by growing *Pediococcus pentosaceus* CRAG3 at various temperatures. 1% (v/v) inoculum of overnight grown culture in 100 ml enzyme production medium (Tsuchiya *et al.*, 1952) as described earlier in Chapter 2, Section 2.2.4 in a 250 ml Erlenmeyer flask was incubated at different temperatures (10°C, 20°C, 25°C, 28°C, 30°C, 37°C, and 45°C) at 180 rpm in a shaking incubator. Aliquot (1.0 ml) of culture was withdrawn at regular time intervals and centrifuged at 8,000***g*** and at 4°C for 10 min to pellet the cells. The cell free supernatant was analyzed for enzyme activity and total carbohydrate content as described earlier in Chapter 2, Section 2.2.5.1.3 and Section 2.2.6, respectively.

3.2.5 Production and purification of dextransucrase

The enzyme production medium (Tsuchiya *et al.*, 1952) as described earlier in Chapter 2, Section 2.2.4 was used for dextransucrase production. Fermentation was carried out with 1% (v/v) inoculum of overnight grown culture of *Pediococcus pentosaceus* CRAG3 in 50 ml enzyme production medium in a 150 ml Erlenmeyer flask and incubated at optimized conditions of 25°C and 180 rpm for 6 h. The cell pellet was removed by centrifuging at 8,000***g*** for 10 min at 4°C and the cell free supernatant was used for purification of enzyme as described later in Section 3.2.5.1.

3.2.5.1 Purification by fractionation with polyethylene glycol

Purification of dextransucrase was done by using graded concentrations of polyethylene glycols (PEGs) of different molecular weights. Pre-chilled PEG-400 was added to 50 ml cell free supernatant in a manner that final concentration reached between 15-40% (v/v). Solution containing 50% (w/v) of PEG-1500 was prepared in distilled water and added to 50 ml cell free supernatant to make final concentration between 5-25% (v/v). The dextransucrase was allowed to fractionate by incubating the solutions at 4^{o}C for 12 h. The fractionated dextransucrase was centrifuged at 13,000***g*** for 30 min at 4^{o}C and pellet containing enzyme was dissolved in minimum volume of 20 mM sodium acetate buffer (pH 5.4). Dialysis of re-suspended enzyme was carried out using 14 kDa cut off membrane (Himedia Pvt. Ltd. India) using 20 mM sodium acetate buffer (pH 5.4). The samples of purified dextransucrase were analysed for enzyme activity by the method as described earlier in Chapter 2, Section 2.2.5.1.3. The protein concentration was determined by the method as described earlier in Section 3.2.2.3.

3.2.5.2 Purification of dextransucrase using gel filtration

The enzyme partially purified with 15% (v/v) PEG-1500 with 20 U/mg specific activity was further purified using gel filtration. The glass column (1.5 x 50 cm) (Bio-Rad Laboratories, USA) was packed with Sephacryl S-300HR matrix (Sigma, St. Louis, MO, USA) of bed volume 80 ml. The column was pre-equilibrated with 20 mM sodium acetate buffer (pH 5.4). 2 ml dialyzed enzyme (0.3 mg/ml) with 20 U/mg specific activity was loaded on column which was connected to chromatography system with UV detector and fraction collector (Biologic LP, Bio-

Rad Labs, USA). Total 50 fractions (3 ml each) were eluted using 20 mM sodium acetate buffer (pH 5.4) at a flow rate of 0.5 ml/min and those having high absorbance at 280 nm were analysed for enzyme activity as described earlier in Chapter 2, Section 2.2.5.1.3 and protein concentration as described earlier in Section 3.2.2.3. The fractions with considerable higher enzyme activity were pooled, lyophilized and run on SDS-PAGE (as described later in Section 3.2.6) to analyse the purity and size of the enzyme. *In situ* activity of dextransucrase was done by periodic acid Schiff's base (PAS) protocol as discussed later in Section 3.2.8. The molecular mass of protein band was determined with the help of Hedrick's plot (Hedrick and Smith, 1968).

3.2.6 Electrophoretic analysis of purified dextransucrase

The electrophoretic analysis of purified dextransucrase from *Pediococcus pentosaceus* CRAG3 was carried out using Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (1970). The proteins are separated on SDS-PAGE on the basis of their molecular sizes (Laemmli, 1970; Sambrook *et al.*, 1989). To determine the molecular mass of dextransucrase from *Pediococcus pentosaceus* CRAG3, SDS-PAGE was run under denaturing condition as described later in Section 3.2.6.1. The stock reagents for SDS-PAGE were prepared as described in Table 3.2.2. The components for preparing running buffer for electrophoresis and sample loading buffer are given in Table 3.2.3. The resolving gel and stacking gel were prepared using the components described in Table 3.2.4 and 3.2.5, respectively. All the solutions were prepared in deionized water. The dextransucrase sample purified by 10% (v/v) PEG 1500 was also run on native-PAGE as described later in Section 3.2.6.2 to check its purity. However, *in situ* activity of dextransucrase was determined by running the gel under non denaturing condition as described later in Section 3.2.6.3.

Table 3.2.2 Components for the preparation of stock reagents for SDS-PAGE.

Stock reagent	Preparation
Acrylamide solution (30%)	0.8 g of N'N'-methylene-bis acrylamide was dissolved in 50 ml ultra-pure deionized water collected at 18 MΩcm (Millipore, Milli-Q water purification system) in amber colored bottle. On complete dissolving, 29.2 g acrylamide was added to it and stirred on a magnetic stirrer till the clear solution was formed. The final volume was adjusted to 100 ml. The solution was filtered (Whatman No. 1) and stored at 4°C in dark.
Tris HCl (1.5 M, pH 8.8)	54.45 g Tris base was dissolved in 150 ml deionized water. The pH of solution was adjusted to 8.8 using HCl and volume made to 300 ml. It was stored at 4°C.
Tris HCl (0.5 M, pH 6.8)	6 g Tris base was dissolved in 60 ml deionized water. The pH of solution was adjusted to 6.8 using HCl and volume made to 100 ml. It was stored at 4°C.
SDS (10%, w/v)	10 g sodium dodecyl sulfate (SDS) was dissolved in 60 ml deionized water. The volume made to 100 ml.
APS (10%, w/v)	100 mg ammonium per sulfate (APS) was dissolved in 1 ml deionized water.

Table 3.2.3 Components for the preparation of 5x running buffer and 5x sample loading buffer.

Solution	Preparation
5x running buffer for electrophoresis	15 g Tris base, 5 g 10% (w/v) SDS and 72 g glycine were dissolved in 800 ml of deionized water. The pH was adjusted to 8.3 and volume was adjusted to 1000 ml. The solution was filtered (Whatman, Filter No. 1) and stored at 4°C. The buffer (5x) was diluted to 1x and prewarmed at 37°C before use.
5x sample loading buffer	The sample loading buffer was prepared by the method of Laemmli, (1970). 1.0 ml 0.5 M Tris (pH 6.8), 1.6 ml 10% SDS, 1.0 ml glycerol, 0.4 ml β-mercaptoethanol 0.4 ml 0.5% (w/v) bromophenol blue were dissolved in 3.0 ml deionized water and pH was adjusted to 6.8. The final concentration of buffer was 1x by mixing 1 volume of 5x sample loading buffer to 4 volumes of sample (protein) before loading in gel.

Table 3.2.4 Components for the preparation of resolving gel.

Component	7.0% gel Volume (ml)	7.5% gel Volume (ml)
30% acrylamide solution	2.30	2.50
10% (w/v) SDS solution	1.00	1.00
50% (v/v) glycerol	1.00	1.00
1.5 M Tris (pH 8.8)	3.30	3.30
10% (w/v) APS solution	0.10	0.10
TEMED	0.01	0.01
Deionized water	2.30	2.10
Total volume (ml)	10.00	10.00

Table 3.2.5 Components for the preparation of 4% stacking gel.

Component	Volume (ml)
30% acrylamide-bisacrylamide solution	0.70
10% (w/v) SDS solution	0.50
0.5 M Tris (pH 6.8)	1.00
10% (w/v) APS solution	0.05
TEMED	0.01
Deionized water	2.74
Total volume (ml)	5.00

3.2.6.1 Denaturing SDS-PAGE

The 40 µl protein sample was mixed with 10 µl 5x sample loading buffer (Table 3.2.3) along with protein molecular mass marker and boiled for 2 min in boiling water bath and loaded on the 7.0% gel. The electrophoresis was carried out in 1x running buffer (as described in Section 3.2.6) with a constant current of 2 mA per lane using vertical gel electrophoresis unit (Biorad, Mini-PROTEAN Tetra Cell).

3.2.6.2 Native-polyacrylamide gel electrophoresis of purified dextransucrase

The native-polyacrylamide gel electrophoresis (PAGE) of dextransucrase was performed using 7% (w/v) gel using the method described earlier (Purama and Goyal, 2008). The acrylamide gel (1.5 mm thick) was prepared in 1 M Tris-HCl pH 8.8. The protein samples were prepared in 5x sample loading buffer as described in Table 3.2.3, but without 2-mercaptoethanol and SDS. The enzyme samples were loaded without heating. The electrophoresis was carried out using running buffer for electrophoresis as described in Table 3.2.3 but without SDS, with a constant current of 2 mA per lane using a vertical slab mini gel unit (Mini-PROTEAN Tetra Cell BioRad, USA). After run the protein bands were fixed with (5%, v/v) acetic acid for 5 min and stained with 0.25% (w/v) Coomassie Brilliant Blue for 30 min. The gel was de-stained by repeated washing using a solution containing 40% methanol and 10% (v/v) acetic acid.

3.2.6.3 Non-denaturing SDS-PAGE

Non-denaturing SDS-PAGE analysis of purified enzyme was carried out to analyse the *in situ* activity of dextransucrase. The samples were prepared by mixing 40 µl enzyme samples and 10 µl 5x sample loading buffer without 2-mercaptoethanol and the samples loaded without boiling. The samples were loaded in 7% gel and the electrophoresis was carried out in 1x running buffer (as described in Section 3.2.6) with a constant current of 2 mA per lane using vertical gel electrophoresis unit (Mini-PROTEAN Tetra Cell, Biorad, USA).

3.2.7 Staining of dextransucrase using Coomassie brilliant blue dye

After the electrophoresis the protein bands were fixed with solution containing 5% (v/v) acetic acid for 5 min and visualized using a staining solution containing 0.25% (w/v) Coomassie brilliant blue (CBB) R-250 dye in 10% (v/v) acetic acid and 90% methanol:water (in 1:1 ratio) after incubation for 30 min. The gel was then destained by repeated washing using a destaining solution containing 40% (v/v) methanol and 10% (v/v) acetic acid till the protein bands were visible.

3.2.8 Activity staining of dextransucrase using Periodic Acid Schiff's base

3.2.8.1 Preparation of solutions for Periodic Acid Schiff's staining

The components and their composition, used in the preparation of solutions for periodic acid Schiff's base (PAS) staining are shown in Table 3.2.6.

Table 3.2.6 List of components for preparation of solutions for PAS staining

S. No.	Solutions and their composition
1.	0.1% (v/v) Triton X-100 and 0.005% (w/v) $CaCl_2$ in 20 mM sodium acetate buffer (pH 5.4)
2.	10% sucrose (w/v) in 20 mM sodium acetate buffer (pH 5.4)
3.	75% (v/v) ethanol
4.	0.7% (w/v) periodic acid and 5% (v/v) acetic acid in deionized water
5.	0.2% (w/v) sodium metabisulfite and 5% (v/v) acetic acid in deionised water
6.	Schiff's reagent 0.5% (w/v) basic fuschin and 1% (w/v) sodium metabisulfite in 0.1 N HCl

3.2.8.2 *Periodic Acid Schiff's staining method*

In situ activity of dextransucrase run on 7% SDS-PAGE under non denaturing conditions (as described earlier in Section 3.2.6.2) was detected by periodic acid Schiff's base (PAS) staining using the protocol of Holt *et al.* (2001). After the electrophoresis run the gel containing identical enzyme samples was cut into two parts. The SDS of both gel parts was removed from gel by washing thrice by a solution containing 20 mM sodium acetate buffer (pH 5.4), 0.1% (v/v) TritonX-100 and 0.005% (w/v) calcium chloride for 20 min. Dextransucrase activity was detected by incubating one gel part in 5% (w/v) sucrose prepared in 20 mM sodium acetate buffer (pH 5.4) at 30ºC for 12 h (Shukla *et al.*, 2010). The presence of fructansucrase was ruled out by incubating second part of gel in 2% (w/v) raffinose in 20 mM sodium acetate buffer (pH 5.4) at 30ºC for 12 h. After incubation, both the gel parts were washed twice with 75% (v/v) ethanol for 20 min each time and incubated in solution with 0.7% (w/v) periodic acid in 5% (v/v) acetic acid for 20 min at 25ºC. Both the parts of gel were then washed thrice with 0.2% (w/v) sodium bisulphate in 5% (v/v) acetic acid solution and finally stained with Schiff's reagent (0.5%, w/v basic Fuschin, 1%, w/v sodium bisulphite and 0.1 N HCl) until the discrete magenta bands appeared within the gel, which confirmed dextransucrase activity.

3.2.9 Optimization of assay conditions for dextransucrase

3.2.9.1 *Effect of pH on dextransucrase activity*

The effect of pH on dextransucrase activity was determined by varying the pH of the reaction mixture from 4.0-7.0. The reaction mixture (1 ml) comprising 5% (w/v) sucrose in 20 mM sodium acetate buffer of varying pH (4.0-7.0) and 20 µl purified enzyme (20.0 U/mg, 0.3 mg/ml) was incubated at 30°C in waterbath for 15 min. The enzyme activity was determined by taking 100 µl of reaction mixture using the method as described in Chapter 2, Section 2.2.5.1.3.

3.2.9.2 Effect of temperature on dextransucrase activity

The reaction was carried out at various temperatures ranging from 10ºC-50ºC to determine the effect of temperature on dextransucrase activity. The reaction mixture (1 ml) contained 5% (w/v) sucrose and 20 µl partially purified dextransucrase (20.0 U/mg, 0.3 mg/ml) in 20 mM sodium acetate buffer (pH 5.4). The reaction was performed at varying temperatures (10ºC, 20ºC, 30ºC, 40ºC and 50ºC) for 15 min and the enzyme activity was determined by the method as described earlier in Chapter 2, Section 2.2.5.1.3.

3.2.9.3 Effect of ionic strength on dextransucrase activity

The reaction mixture (1 ml) containing varying concentration (5-100 mM) of sodium acetate buffer (pH 5.4), sucrose (5%, w/v) and dextransucrase (20 µl, 20.0 U/mg, 0.3 mg/ml) was used for determining the effect of ionic strength on enzyme activity. The reaction was carried out at 30ºC in waterbath for 15 min. The enzyme activity was determined by the method described earlier in Chapter 2, Section 2.2.5.1.3.

3.2.9.4 Effect of sucrose concentration on dextransucrase activity

The reaction mixture with varying concentrations of sucrose between 2-500 mM (0.1-15%, w/v) was used to analyse its effect on enzyme activity and to determine the kinetic parameters. The reaction mixture (1 ml) contained varying concentrations of sucrose in 20 mM sodium acetate buffer (pH 5.4), 20 µl purified dextransucrase (20.0 U/mg, 0.3 mg/ml). The mixture was kept at 30°C in waterbath for 15 min. The enzyme activity was determined by the method as described earlier in Chapter 2, Section 2.2.5.1.3.

3.2.10 Effects of salts on dextransucrase activity

The effects of various salts such as $MgCl_2$, $CaCl_2$, $CoCl_2$, $NiCl_2$, $ZnCl_2$ and $MnCl_2$ on dextransucrase activity were studied. The salt solutions were prepared in 20 mM sodium acetate buffer (pH 5.4) to make their final concentration between 0-10 mM. The reaction was carried out in 1 ml mixture containing 5% (w/v) sucrose in 20 mM sodium acetate buffer (pH 5.4), 20 µl of enzyme (20.0 U/mg, 0.30 mg/ml) and the salt. The reaction mixture was kept at 30°C in waterbath for 15 min. The enzyme activity was determined as described earlier in Chapter 2, Section 2.2.5.1.2.

3.2.11 Thermostability studies of dextransucrase

The thermostability of purified dextransucrase (20.0 U/mg, 0.3 mg/ml) was determined by incubating it at various temperatures (10-60°C) for 1 h. The reaction mixture (1 ml) contained 5% (w/v) sucrose solution in 20 mM sodium acetate buffer (pH 5.4) and aliquot (20 µl) of enzyme from different temperatures. The mixture was kept at 30°C in waterbath for 15 min and the residual activity was determined by following the method as described in Chapter 2, Section 2.2.5.1.3.

3.2.12 Effect of pH on stability of dextransucrase

The effect of pH on dextransucrase was studied by incubating it at different pH (3.5-7.0). The lyophilised dextransucrase (1 mg) was dissolved in 1 ml each of 20 mM sodium acetate buffer of varying pH (3.5-7.0). The aliquots (20 µl) were taken at regular time interval to determine the residual enzyme activity. The reaction mixture (1 ml) containing 5% sucrose in 20 mM sodium acetate buffer (pH 5.4) and 20 µl enzyme was incubated at 30°C for 15 min and the residual activity was determined as described earlier in Chapter 2, Section 2.2.5.1.2.

3.2.13 Effects of storage temperatures on dextransucrase activity

The storage stability of dextransucrase was studied by incubating the dextransucrase (20 U/mg, 0.3 mg/ml) at different temperatures (0°C, 4°C and -20°C). The aliquots (20 µl) were taken at regular time interval to determine the residual enzyme activity. The reaction mixture (1 ml) containing 5% sucrose in 20 mM sodium acetate buffer (pH 5.4) and 20 µl enzyme was incubated at 30°C for 15 min and the residual activity was determined as described earlier in Chapter 2, Section 2.2.5.1.2. The residual activity and half-life (*t1/2*) of dextransucrase were measured at different temperatures with respect to time by assuming that the decay followed first order kinetics following the method of Naidu *et al.* (2003). According to single step two-stage theory of Sadana (1995) the active enzyme state *E* is supposed to be directly converted to inactive state *E*d without providing any significant amount of intermediates. The two-stage mechanism is as follows:

$$E \rightarrow E\mathrm{d} \qquad \text{...(1)}$$

The first-order deactivation can be represented as:

$$dE/dt = -k_d[E] \qquad ...(2)$$

Integration of Eq. (2) leads to:

$$\alpha = \exp(-k_d t) \qquad ...(3)$$

where, $\alpha = E_d/E$.

From the plot of $\ln(\alpha)$ versus t, the slope gives the value of deactivation rate constant k_d. The half-life of an enzyme is defined as the time required by the enzyme to lose half of its initial activity. After solving equation 3, for $\alpha = 1/2$ and $t = t_{½}$ we get:

$$t_{½} = \ln 2/k_d \qquad ...(4)$$

where,

$t_{½}$ = half-life of enzyme (time).

k_d = deactivation rate constant (time^{-1})

3.2.14 Effects of additives on dextransucrase activity

The effects of various additives such as dextran (500 kDa), PEG-8000, glutaraldehyde, glycerol and Tween 80 on the stability of dextransucrase at 30°C were determined. The aqueous solutions (1 ml) of additives containing dextransucrase (20.0 U/mg, 0.3 mg/ml) in 20 mM sodium acetate buffer (pH 5.4) with final concentrations of 2 µg/ml (w/v) dextran (500 kDa), 10 µg/ml (w/v) PEG-8000, 0.1% (v/v) glutaraldehyde, 0.5% (v/v) glycerol and 0.1% (v/v) Tween 80 were incubated at 30ºC for 15 min and the residual activity was determined as described earlier in Chapter 2, Section 2.2.5.1.2. The half-life (*t1/2*) of dextransucrase treated with different additives was determined at 30°C as described in Section 3.2.13.

3.2.15 Application of dextransucrase on the solidification of sucrose-supplemented milk

The effect of dextransucrase from *Pediococcus pentosaceus* CRAG3 on sucrose-supplemented semi-skimmed milk was analysed by using the method of Bejar *et al.* (2013). To the semi-skimmed milk (20 ml), sucrose was added at final concentration of 2 and 5% (w/v). The partially purified dextransucrase (20 U/mg, 0.3 mg/ml, 4 ml) from *Pediococcus pentosaceus* CRAG3 (to make final concentration of 5 U/ml) was added to each of the sucrose-supplemented milk samples. The mixtures were incubated at 30°C for 20 h. The solidified milk was then visually compared with the control (milk without enzyme).

3.3 Results and Discussion

3.3.1 Effect of culture conditions on dextransucrase and dextran production from *Pediococcus pentosaceus* CRAG3

The effect of temperature on dextransucrase production from *Pediococcus pentosaceus* CRAG3 is shown in Fig. 3.3.1. The maximum enzyme activities at different temperatures were observed at 6 h. The enzyme activities at 10°C, 20°C, 25°C, 28°C, 32°C, 37°C and 45°C were 0.98, 2.96, 4.32, 2.96, 1.50, 0.77 and 0.17 U/ml, respectively (Fig. 3.3.1). The isolate showed maximum dextransucrase activity at 25°C and 180 rpm. At temperature lower than 25°C, the enzyme activity was low that might be due to the slow cell growth. At higher temperatures (>25°C) the deactivation of the enzyme might be the cause for decreased enzyme activity.

The effect of shaking and static condition on production of dextransucrase showed that under shaking condition (180 rpm) the enzyme activity was 2.4 fold higher (4.35 U/ml) as compared to that observed at static condition (1.8 U/ml) (Fig. 3.3.2). This showed that shaking is required for microaerophillic *Pediococcus pentosaceus* CRAG3 resulting in higher dextransucrase activity as compared with static condition. Similar results were observed in case of *Pediococcus pentosaceus* SPA (Patel and Goyal, 2010). The dextran concentration of crude cell free supernatant from the isolate was determined under shaking and static condition at 25°C. The dextran concentration at 180 rpm (4.5 mg/ml) was 20% higher than that observed under static condition (3.6 mg/ml) (Fig. 3.3.3).

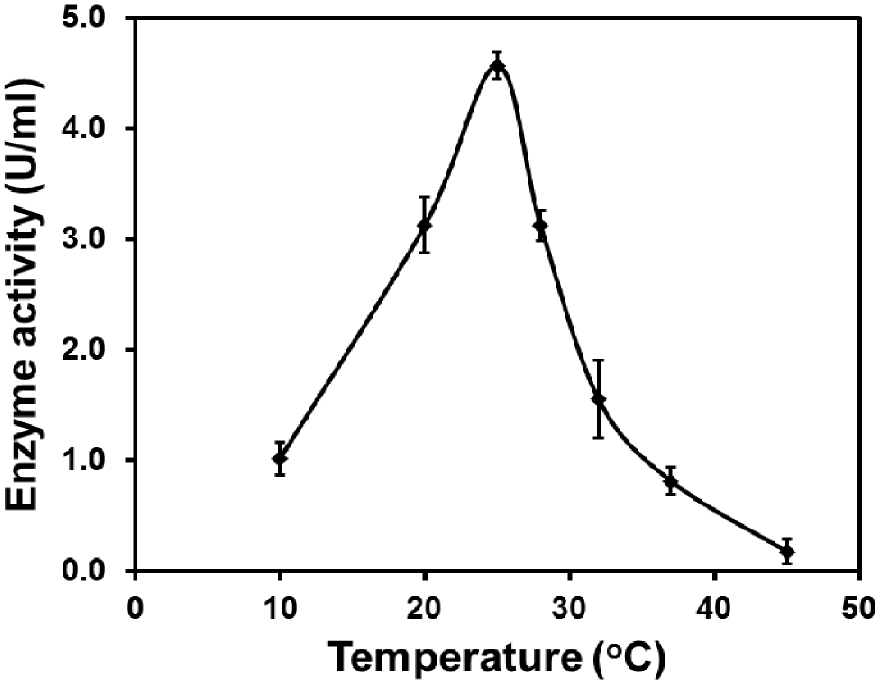

Fig. 3.3.1 Effect of temperatures on enzyme activity of *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) at 180 rpm after 6 h.

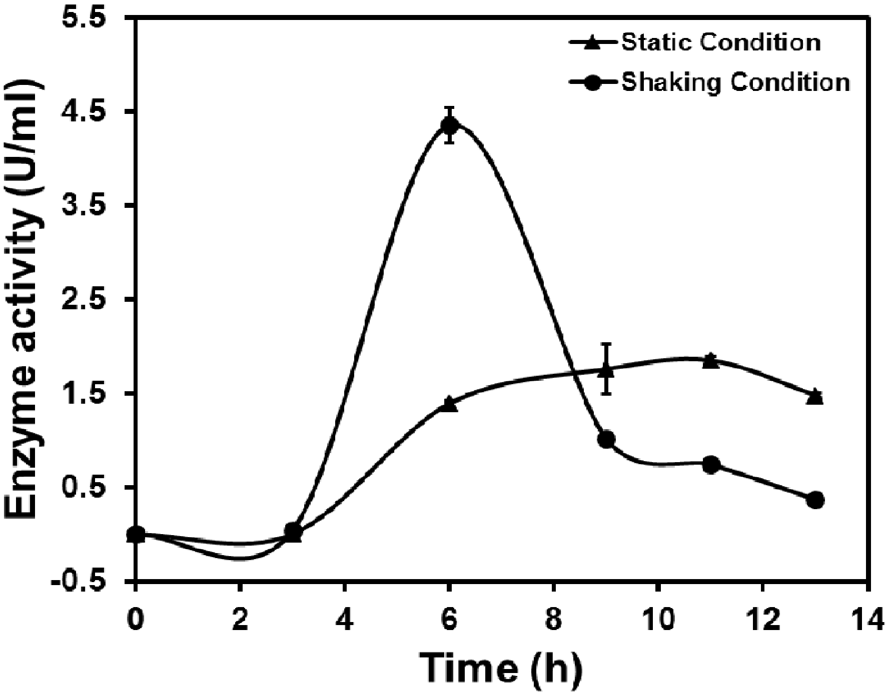

Fig. 3.3.2 Effect of shaking and static condition on enzyme activity of *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) at 25°C.

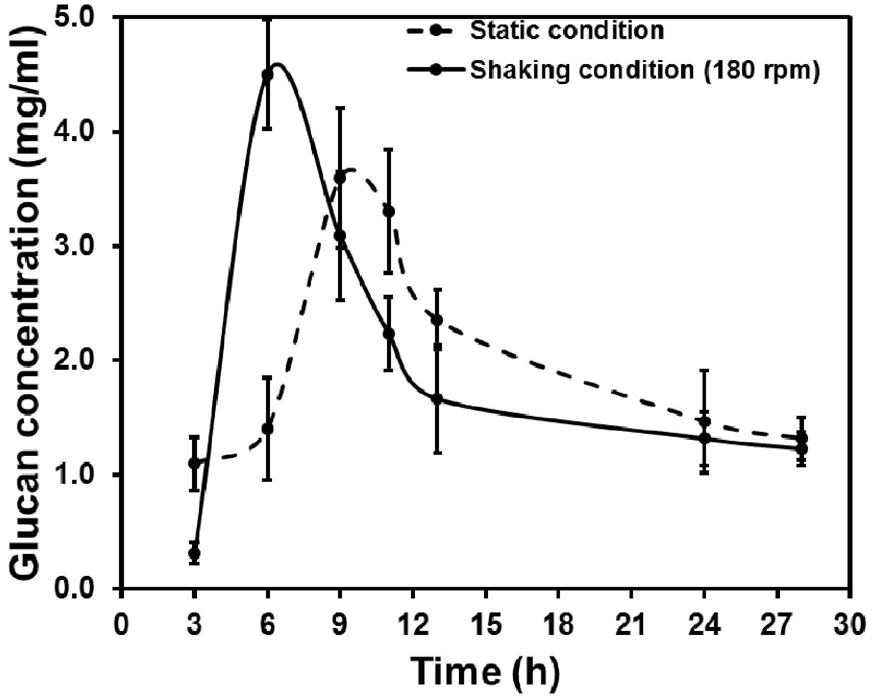

Fig. 3.3.3 Effect of shaking and static condition on dextran concentration (mg/ml) of *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) at 25°C.

3.3.2 Purification of dextransucrase from *Pediococcus pentosaceus* CRAG3

3.3.2.1 PEG fractionation

The cell free supernatant containing dextransucrase (0.67 U/mg, 5.2 mg/ml) was subjected to fractionation with different concentrations of PEG-400 and PEG-1500. The purification data including maximum specific activity, fold purification and percent yield of dextransucrase obtained with both PEGs are shown in Table 3.3.2. Dextransucrase showed maximum specific activity of 13.0 U/mg with 20 fold purification with 5% yield at 33% (v/v) of PEG-400. The purification of dextransucrase with other concentrations of PEG-400 showed lower specific activities (Fig. 3.3.4). Unlike PEG-6000 which precipitates other non-dextransucrase proteins also, PEG-400 is more specific towards dextransucrase and can be easily removed by dialysis (Russell, 1979). Purification of dextransucrase with PEG-1500 was performed in a concentration range of 10-25% (Fig. 3.3.4). The maximum specific activity of 20.0 U/mg with 30 fold purification and 15.6% overall yield was obtained at 10% (v/v) PEG-1500 (Table 3.3.1). The increase in concentration above 10% (w/v) of PEG-1500 did not favour the purification of dextransucrase with higher enzyme activity. Similar results were reported on dextransucrase purification from other lactic acid bacteria such as *Leuconostoc mesenteroides* NRRL B-512F (Goyal and Katiyar, 1994) and *Leuconostoc mesenteroides* NRRL B-640 Purama and Goyal, 2008). Dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 purified by 10% (v/v) PEG-1500 showed maximum specific activity of 23.0 U/mg with 40 fold purification (Purama and Goyal, 2008).

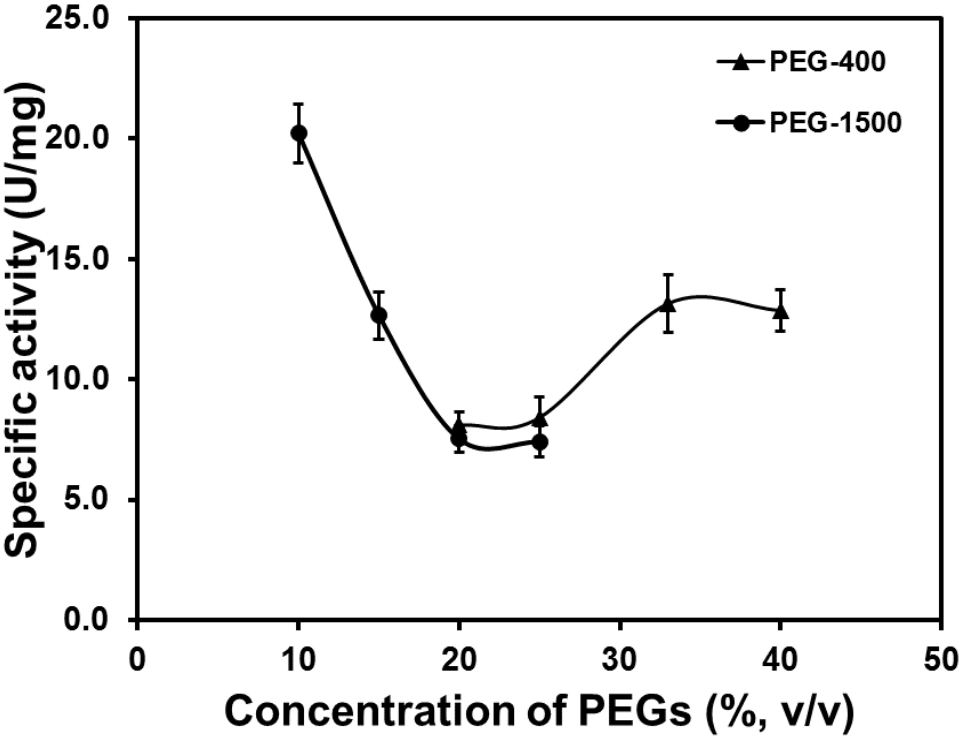

Fig. 3.3.4 Purification of dextransucrase from *Pediococcus pentosaceus* CRAG3 by polyethylene glycol fractionation using PEG-400 and PEG-1500.

Table 3.3.1 Purification of dextransucrase by fractionation with polyethylene glycol.

Sample	Volume (ml)	Enzyme Activity (U/ml)	Overall % Yield	Protein (mg/ml)	Specific Activity (U/mg)	Fold Purification
Cell free supernatant	30	3.50	-	5.20	0.67	-
PEG-400						
20% (v/v)	2.0	6.50	12.38	0.80	8.09	12.0
25% (v/v)	2.0	1.85	3.60	0.22	8.41	13.0
33% (v/v)	1.7	5.00	8.10	0.38	13.2	19.0
40% (v/v)	1.0	5.30	5.04	0.41	12.9	20.0
PEG-1500						
10% (v/v)	3.0	5.46	15.60	0.27	20.21	30.0
15% (v/v)	3.9	4.80	17.82	0.38	12.66	19.0
20% (v/v)	2.0	1.20	2.30	0.16	7.52	11.0
25% (v/v)	2.0	1.11	2.10	0.15	7.40	11.0
Sephacryl S300 HR	9.0	1.52	13.03	0.033	46.0	69.0

3.3.2.2 Electrophoretic analysis of PEG purified dextransucrase

The electrophoretic analysis of dextransucrase purified with 10% (w/v) PEG-1500, on SDS-PAGE gels under denaturing conditions showed two bands (Fig. 3.3.5 A, Lane 1). The denatured dextransucrase gave bands of approximately, 224 and 188 kDa molecular size as determined by Hedrick's plot (Hedrick and Smith, 1968) (Fig. 3.3.6). This might be due to the denaturation of dextransucrase run under denaturing conditions as also reported earlier (Purama and Goyal, 2009). However, the same enzyme sample showed predominantly a single, intact and homogeneous protein band on native-PAGE (Fig. 3.3.5B, Lane 2). This showed that dextransucrase remains in single molecular form in native state after purification and dialysis but shows two bands only in under denaturing conditions. After PAS staining, on incubating the gel with 5% sucrose for 16 h, single magenta colour band appeared which corresponded well with the 224 kDa protein band observed in non-denaturing SDS-PAGE. This was due to the formation of polysaccharide on polyacrylamide gel confirming the presence of dextransucrase (Fig. 3.3.5C, Lane 3). Magenta colour band on sucrose-incubated gel showed the native and active form of the purified dextransucrase. The absence of band on the gel incubated with 2% raffinose ruled out the presence of fructansucrase (Fig. 3.3.5C, Lane 4).

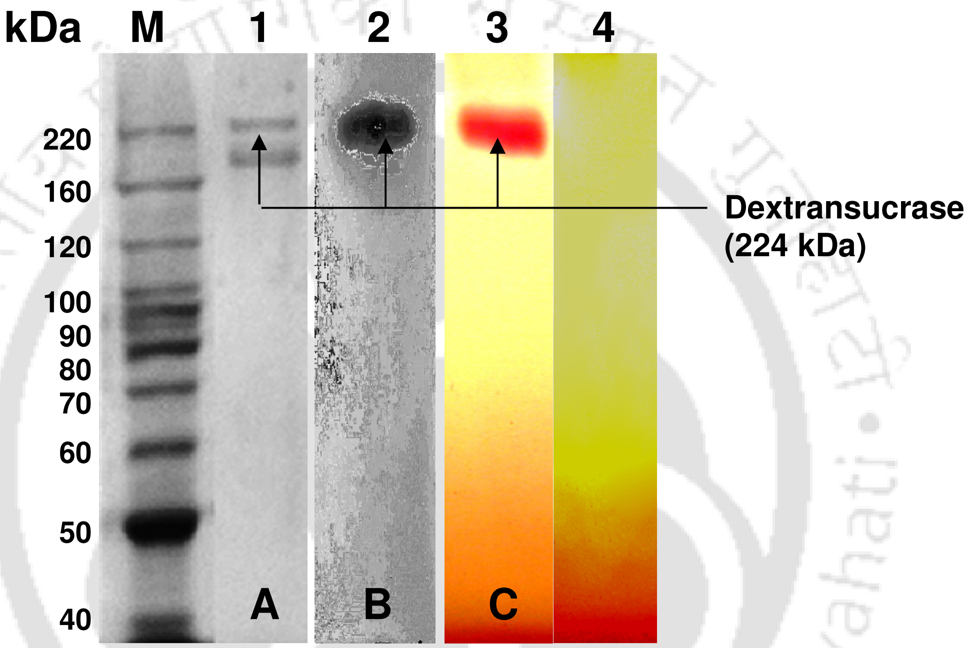

Fig. 3.3.5 Polyacrylamide gel (7%) electrophoresis of dextransucrase and its confirmation by PAS staining method. **(A)** Denaturing SDS-PAGE: Lane: (M) Protein molecular mass marker: **40-**220 kDa; (1) 10% (v/v) PEG-1500 fraction stained with CBB R250; **(B)** Native PAGE: (2) 10% (v/v) PEG-1500 fraction stained with CBB R250; **(C)** Non-denaturing SDS-PAGE: 10% (v/v) PEG-1500 fraction stained with PAS staining using (3) Sucrose as substrate; (4) Raffinose as substrate.

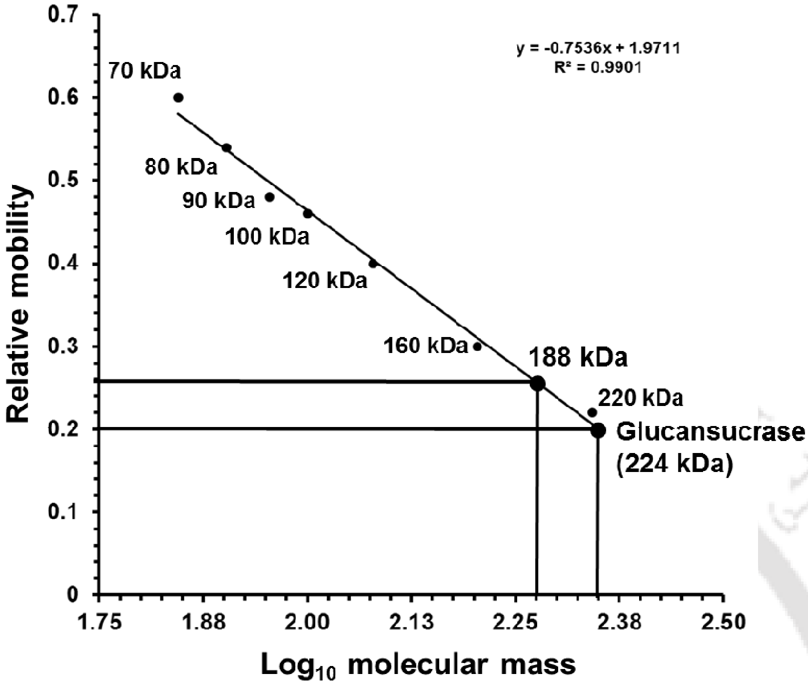

Fig. 3.3.6 Hedrick's plot of Log_{10} molecular mass and relative mobility of proteins, showing two forms 188 kDa and 224 kDa of PEG-1500 purified PEG-1500 dextransucrase.

3.3.2.3 Purification of dextransucrase by gel filtration

The dextransucrase partially purified with 10% PEG-1500 having specific activity of 20.0 U/mg in a single step was further purified using gel filtration. The purification profile of dextransucrase showing relation between fraction number and their corresponding specific activity and protein (absorbance, A_{280}) is shown in Fig. 3.3.7. The profile showed a single peak of specific activity and protein (absorbance, A_{280}) from fraction number 10-15. The fractions with high specific activities (fraction number 11-13) were pooled. The pooled enzyme showed specific activity of 46 U/mg with 13% overall yield and 69 fold purification. The similar results, but with lesser activity and fold purification, were observed with dextransucrase of *Leuconostoc mesenteroides* NRRL B-640. The dextransucrase from *Leuconostoc mesenteroides*

NRRL B-640 partially purified with 10% PEG-1500 gave 23.0 U/mg specific activity with 40 fold purification while further purification by gel filtration showed a specific activity of 35.0 U/mg with 61 fold purification (Purama and Goyal, 2008).

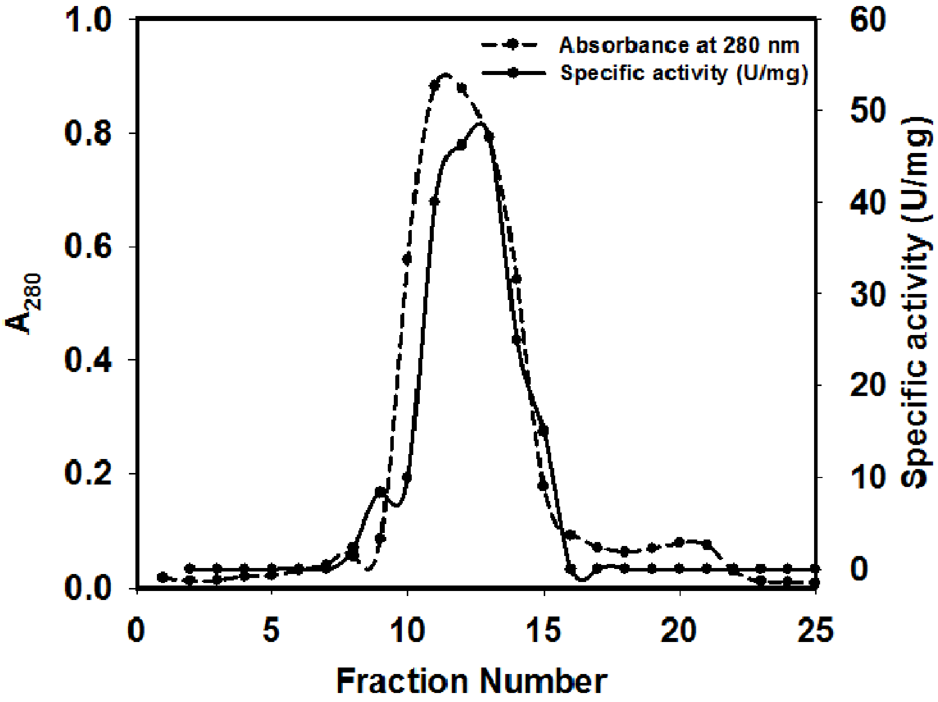

Fig. 3.3.7 Purification of dextransucrase from *Pediococcus pentosaceus* CRAG3 by gel filtration using Sephacryl S300HR column.

3.3.2.4 Electrophoretic analysis of dextransucrase purified by gel filtration

The pooled dextransucrase (46 U/mg) from *Pediococcus pentosaceus* CRAG3 (as described in Section 3.3.2.3) was run on denaturing SDS-PAGE using 7% gel. After gel filtration a prominent band of 224 kDa and a light band of 188 kDa (Fig. 3.3.8, Lane 1) was observed which might be due to the denaturation of dextransucrase as also reported earlier (Purama and Goyal., 2009). The presence of single 224 kDa magenta color band after PAS staining, on gel incubating with 5% sucrose in non-denaturing SDS-PAGE, confirmed the presence of dextransucrase (Fig. 3.3.8, Lane

2). However, the absence of band on the gel incubated with 2% raffinose ruled out the presence of fructansucrase (Fig. 3.3.8, Lane 3). Dextransucrase from *Leuconostoc dextranicum* NRRL B-1146 and *Leuconostoc mesenteroides* NRRL B-640 showed similar molecular size of approximately, 205 kDa (Majumder *et al.*, 2008) and 180 kDa (Purama and Goyal, 2008), respectively.

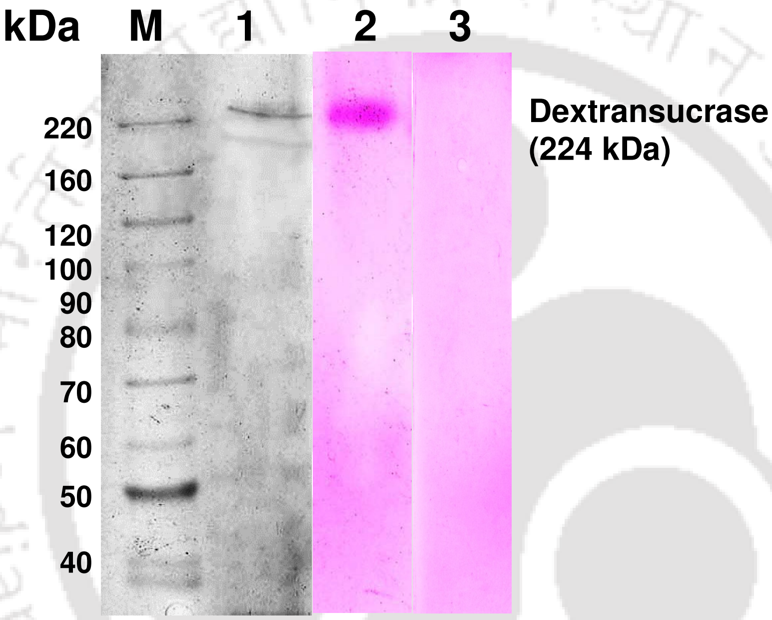

Fig. 3.3.8 SDS-polyacrylamide gel (7%) electrophoresis of dextransucrase purified by gel filtration. Lane: **(M)**-Protein molecular weight marker: 10-220 kDa; Sephacryl S-300HR pooled fraction: **(1)** Stained with CBB R250, **(2)** Stained with PAS staining using sucrose as substrate and **(3)** Stained with PAS staining using raffinose as substrate.

3.3.3 Optimization of assay conditions for dextransucrase activity

The effects of pH, ionic strength of buffer and temperature on dextransucrase activity are shown in Fig. 3.3.9. The results showed that 20 mM sodium acetate buffer, pH 5.4 and 30ºC gave maximum enzyme activity (~20.0 U/mg) (Fig. 3.3.9). The effect of sucrose concentration on dextransucrase activity is shown in Fig. 3.3.10. The rate of reaction increased with increase in the concentration of sucrose up to 146 mM (5%, w/v) but decreased thereafter, which might be due to substrate inhibition. This showed that dextransucrase from *Pediococcus pentosaceus* CRAG3 did not follow classical Michaelis-Menten kinetics (Fig. 3.3.10A). Similar pattern was observed with dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F (Goyal *et al.*, 1995). However, dextransucrase from *Leuconostoc dextranicum* NRRL B-1146 exhibited Michaelis-Menten kinetics (Majumder *et al.*, 2008). The kinetic parameters using sucrose were determined as K_m = 15.4 ±1.4 mM and V_{max} 20.9 ±1.2 U/mg (Fig. 3.3.10B). The turn over number (K_{cat}) and the catalytic efficiency (K_{cat} / Km) of dextransucrase were 20.29 min^{-1} and 1.32 mM^{-1} min^{-1}, respectively.

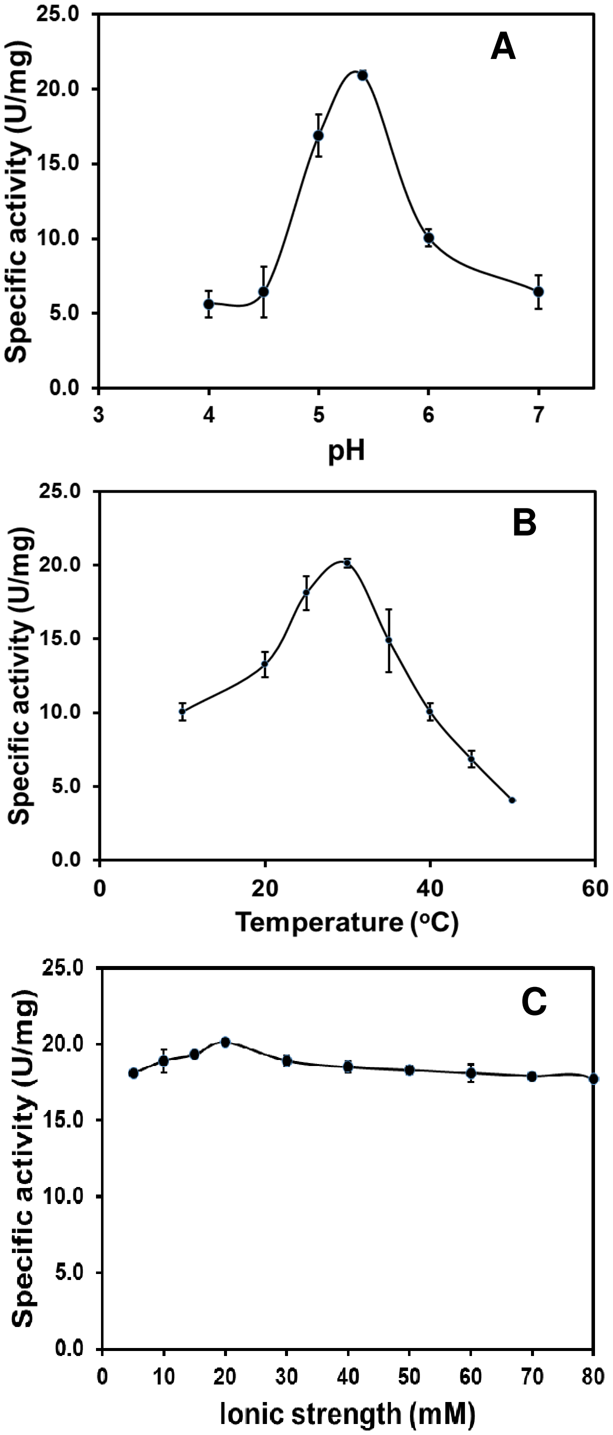

Fig. 3.3.9 Effects of assay conditions on dextransucrase activity in a reaction mixture (1 ml) containing sucrose (5%, w/v) and dextransucrase (20 µl, 20.0 U/mg, 0.3 mg/ml) with varying pH, temperature and ionic strength of buffer. Effect of **(A)** pH in reaction mixture containing 20 mM sodium acetate buffer of varying pH at 30ºC. **(B)** Temperature in reaction mixture containing 20 mM sodium acetate buffer (pH 5.4) and reaction was carried out at various temperatures and **(C)** Varying ionic strength of sodium acetate buffer in reaction mixture (pH 5.4) at 30ºC.

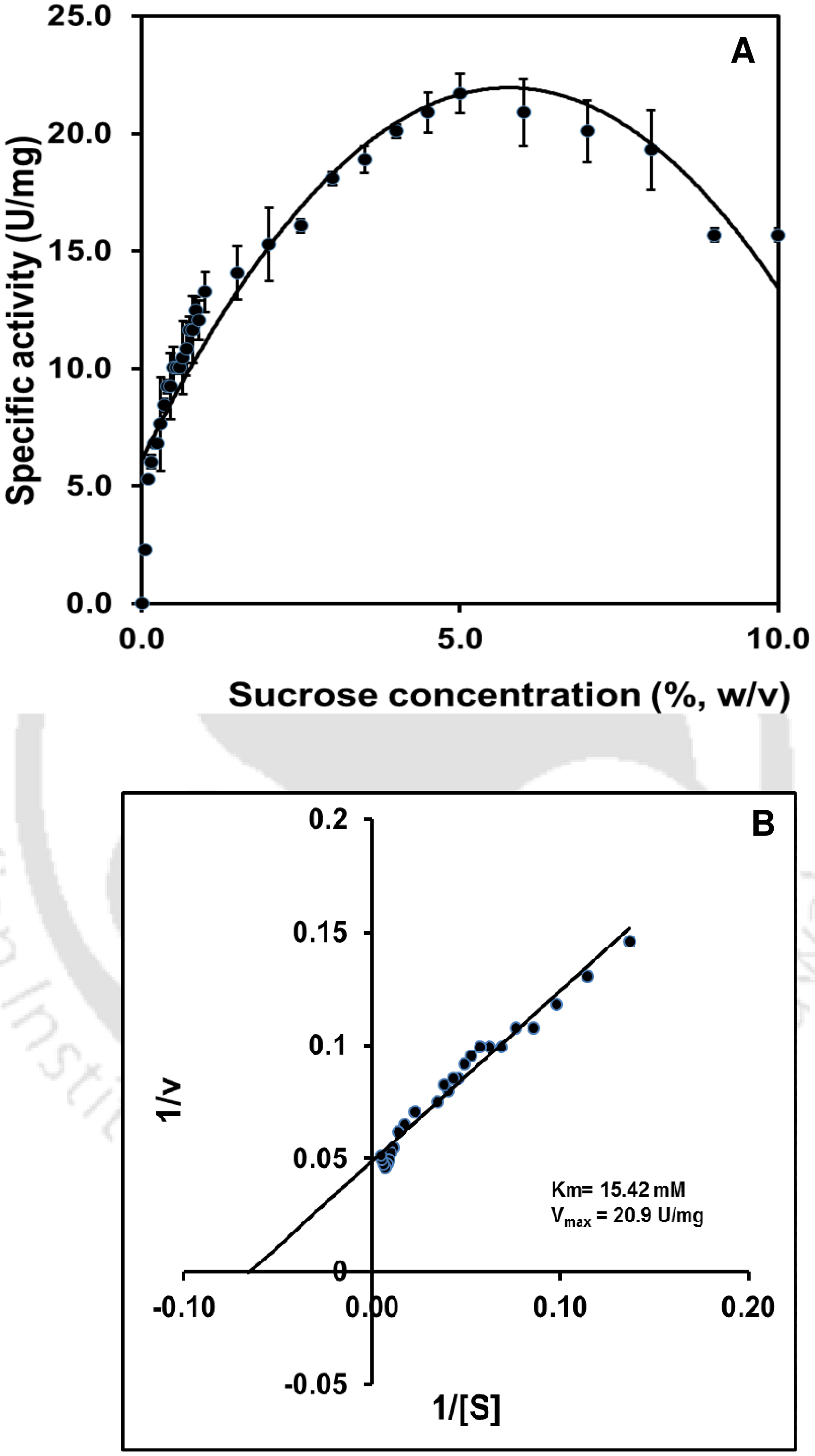

Fig. 3.3.10 **(A)** Effect of sucrose concentration on dextransucrase activity. **(B)** Lineweaver-Burk plot for determination of kinetic parameters of purified dextransucrase.

3.3.4 Effects of salts on dextransucrase activity

The effects of different metal ions on dextransucrase activity were studied. Forman and Kennedy (1977) reported that the salts affect water structure of enzymes thereby affecting their solubility and activity. The effects of various metal ions on activity of dextransucrase from *Pediococcus pentosaceus* showed that $MgCl_2$, $CaCl_2$, $CoCl_2$ and $ZnCl_2$ enhanced the activity while $NiSO_4$ and $MnSO_4$ decreased the enzyme activity as compared to control (Fig. 3.3.11, Fig. 3.3.12 and Fig. 3.3.13, Table 3.3.2). The Co^{2+} and Ca^{2+} were most effective metal ions which displayed enhancement of the dextransucrase activity. The activity was increased by 56% and 44% in presence of 4 mM $CoCl_2$ (Fig. 3.3.11A) and 2 mM $CaCl_2$ (Fig. 3.3.11B), respectively (Table 3.3.2). The dextransucrase activity was enhanced by 14% in presence of 2 mM $MgCl_2$ (Fig. 3.3.12A, Table 3.3.2). It has been reported that Mg^{2+} and Ca^{2+} salts enhance the catalytic activity of enzyme by stabilizing the three-dimensional protein structure (Goyal *et al.*, 1995). Miller and Robyt, (1986) reported that the Ca^{2+} has been associated with catalytic sites of dextransucrase. The similar results of increasing dextransucrase activity in presence of Ca^{2+} ion were reported in *Leuconostoc mesenteroides* NRRL B-640 (4 mM, 108% enhancement) (Purama *et al.* 2010) and *Pediococcus pentosaceus* (6 mM, 150% enhancement) (Patel *et al.*, 2011). The $ZnCl_2$ at its final concentration of 1 mM resulted in 11% enhanced dextransucrase activity (Fig. 3.3.12B, Table 3.3.2). On further increasing the concentration above 1 mM the enzyme activity decreased gradually. The salts such as $MnSO_4$ and $NiSO_4$ negatively affected the dextransucrase activity. On addition of 2 mM $MnSO_4$ and 1 mM $NiSO_4$ the enzyme activity decreased by 18% and 12%, respectively (Fig. 3.3.13A and 3.3.13B, Table 3.3.2). On further increasing the concentration of $MnSO_4$ and $NiSO_4$ to 8 mM the enzyme activity lost by 89% and 90%, respectively.

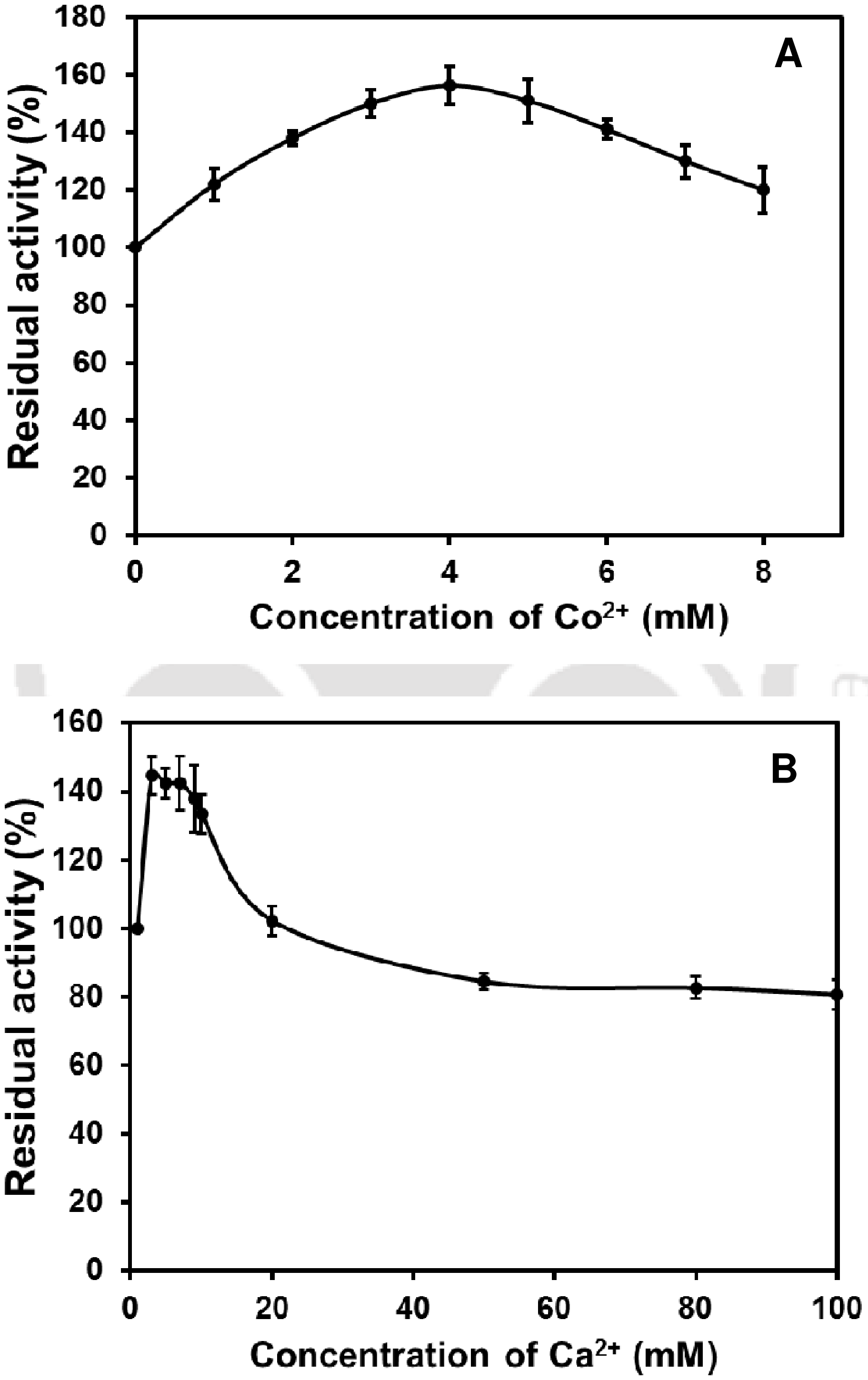

Fig. 3.3.11 Effects of Co^{2+} ions **(A)** and Ca^{2+} ions **(B)** on activity of purified dextransucrase from *Pediococcus pentosaceus* CRAG3.

Table 3.3.2 Effects of various metal ions on dextransucrase from *Pediococcus pentosaceus* CRAG3.

Dextransucrase + Metal ions	Relative enzyme activity (%)
Control	100
$CoCl_2$ (4 mM)	156
$CaCl_2$ (2 mM)	144
$MgCl_2$ (2 mM)	114
$ZnCl_2$ (1 mM)	112
$MnSO_4$ (8 mM)	11
$NiSO_4$ (8 mM)	10

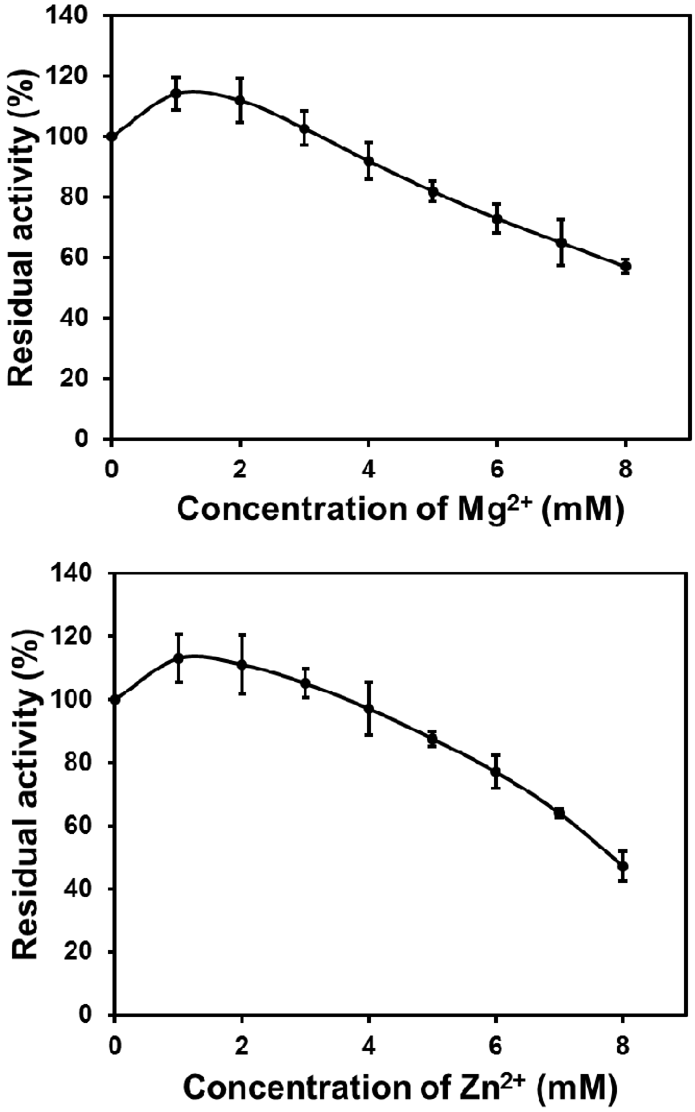

Fig. 3.3.12 Effects of Mg^{2+} ions **(A)** and Zn^{2+} ions **(B)** on activity of purified dextransucrase from *Pediococcus pentosaceus* CRAG3.

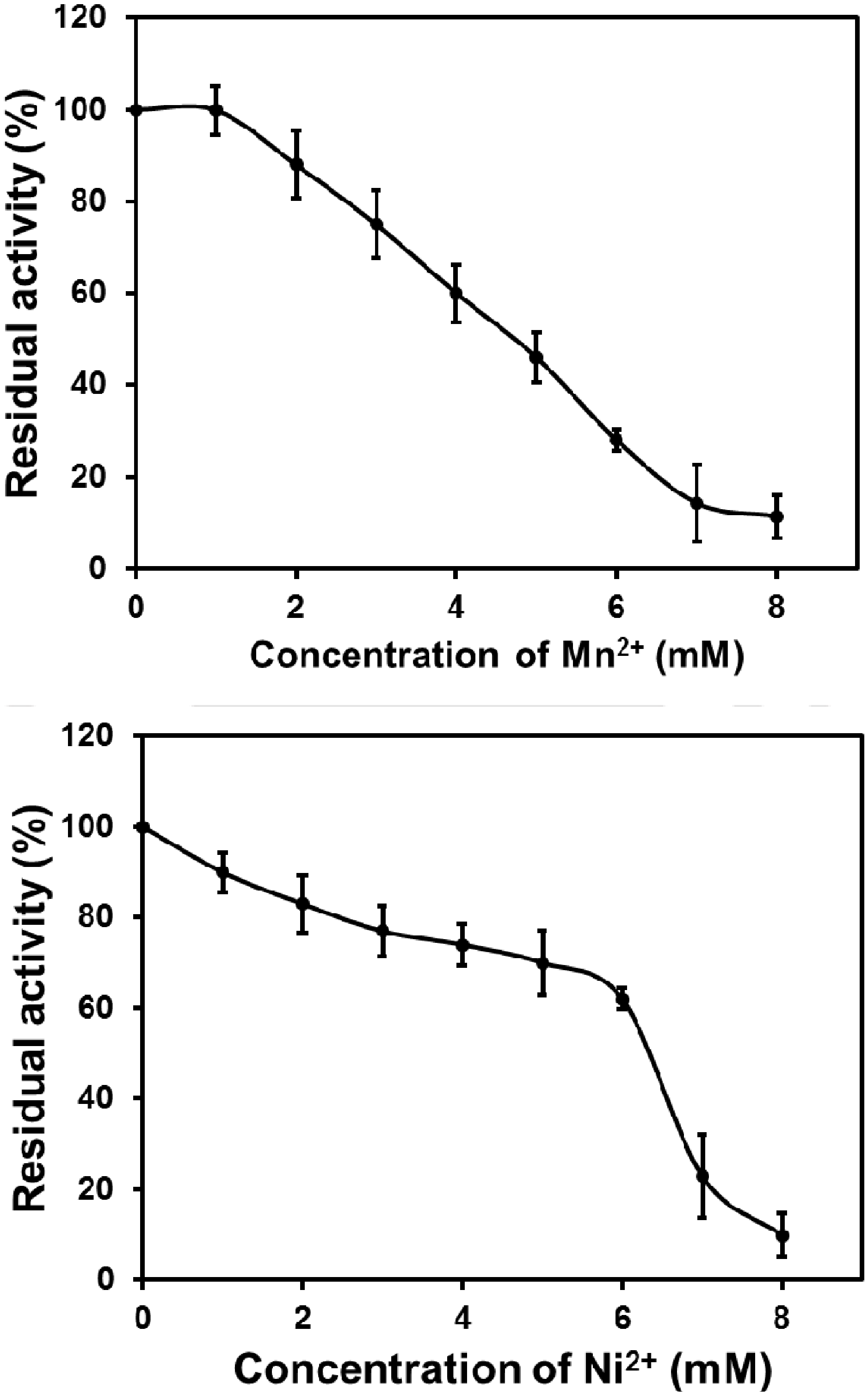

Fig. 3.3.13 Effects of Mn^{2+} ions **(A)** Ni^{2+} ions **(B)** on activity of purified dextransucrase from *Pediococcus pentosaceus* CRAG3.

3.3.5 Thermostability studies of dextransucrase from *Pediococcus pentosaceus* CRAG3

The thermostability studies of dextransucrase from *Pediococcus pentosaceus* CRAG3 at different temperatures displayed its mesophilic nature. The enzyme was stable up to 40ºC when incubated for 1 h (Fig. 3.3.14). Above 40ºC the enzyme activity was rapidly lost. The optimum temperature for assay was also 30ºC. It proved to be better thermostable enzyme than dextransucrase from other lactic acid bacterium such as *Leuconostoc mesenteroides* NRRL B-640 which lost its activity above 30°C on incubating for 10 min (Purama *et al.*, 2010).

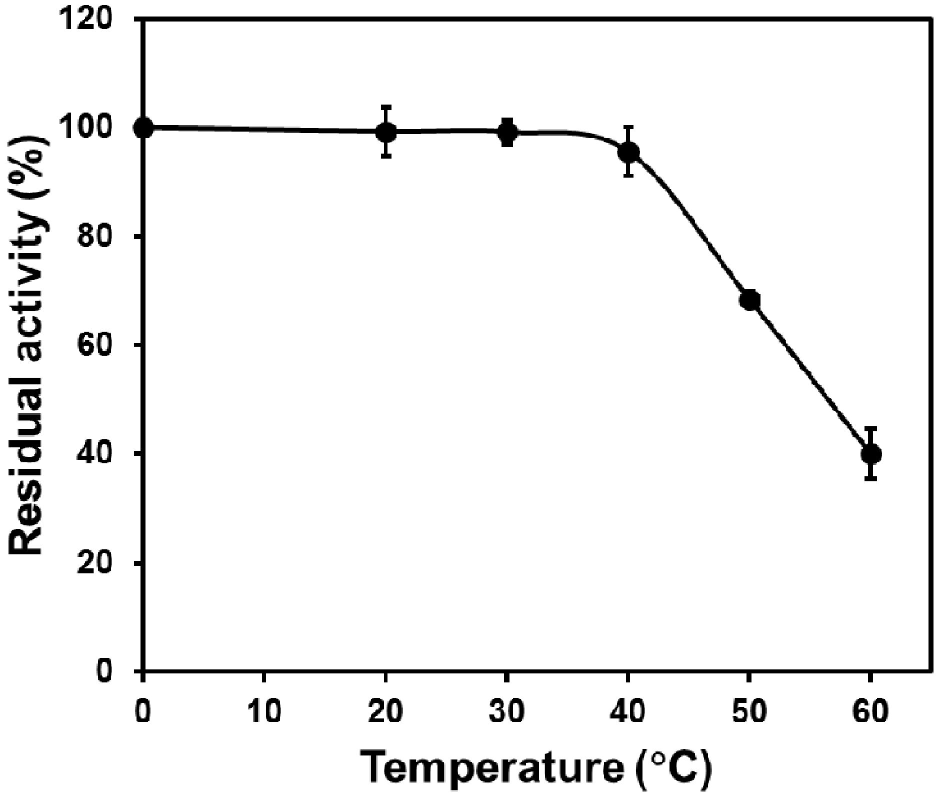

Fig. 3.3.14 Effect of temperature on stability of dextransucrase from *Pediococcus pentosaceus* CRAG3. The enzyme was incubated at different temperatures for 1 h and assayed at 30°C to determine its thermostability.

3.3.6 Effects of pH on stability of dextransucrase

The effect of different pH on stability of dextransucrase from *Pediococcus pentosaceus* CRAG3 is shown in Fig. 3.3.15. The dextransucrase was more stable at higher pH (5.4 – 7.0) when incubated at 30ºC. The residual activity of dextransucrase at pH 5.4 was 82% after 3 h (Fig. 3.3.15). The activity greatly reduced to 12% and 21% at pH 3.5 and 4.0, respectively (Fig. 3.3.15). Similar results were observed in fusion enzyme of endodextranase and dextransucrase by Kim *et al.* (2008). The dextransucrase from *Leuconostoc mesenteroides* was stable between pH 5.0-6.5 (Kaboli and Reilly) and that from *Leuconostoc mesenteroides* B-742 was stable at 6.5 (Rabelo *et al.*, 2011).

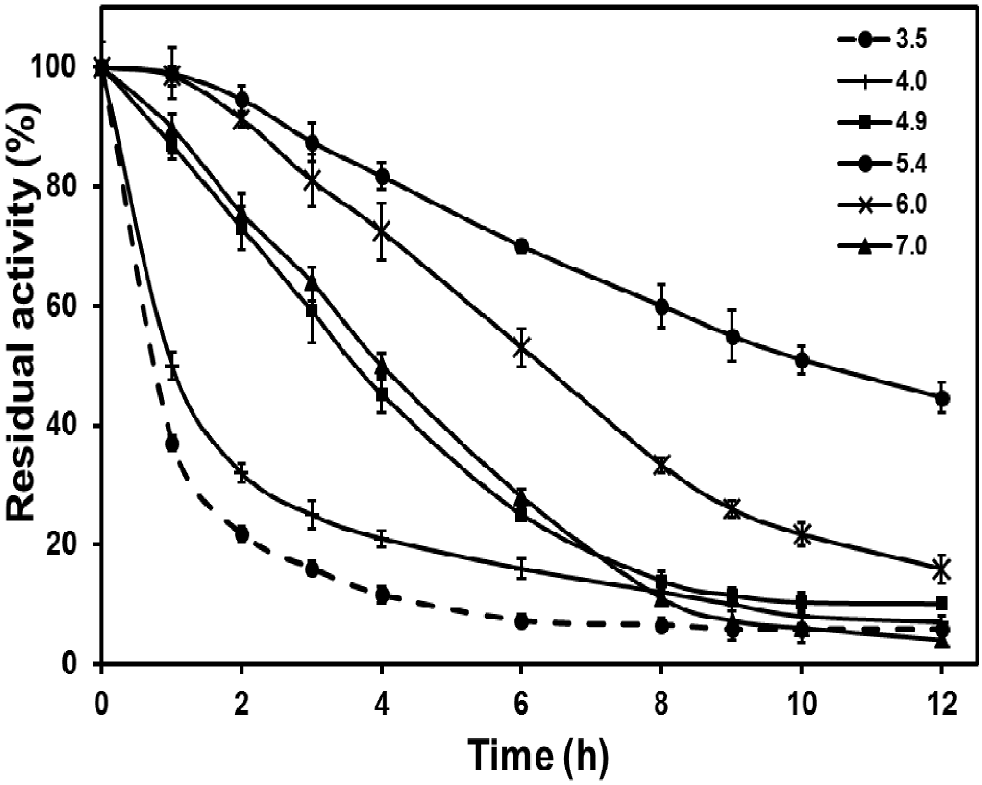

Fig. 3.3.15 Effect of pH on stability of dextransucrase. The enzyme (20 U/mg, 0.30 mg/ml) in 20 mM sodium acetate buffer (pH 5.4) was pre-incubated in same buffer of varying pH. The aliquots were assayed for dextransucrase activity at 30°C at different time intervals.

3.3.7 Effects of storage temperatures on dextransucrase activity

The residual activity of dextransucrase from *Pediococcus pentosaceus* CRAG3 at various storage temperatures was also studied. The enzyme activity profile at -20ºC, 4ºC and 30ºC is shown in Fig. 3.3.16. The dextransucrase lost only 5% and 11% activity on incubating at -20ºC and 4ºC, respectively after 21 days which showed that -20ºC is suitable temperature for storing dextransucrase (Fig. 3.3.16). The residual activity of dextransucrase at 30ºC was 35% after 24 h (Fig. 3.3.16). The similar results were observed in previously reported *Pediococcus pentosaceus* and its mutant where the residual activity was 26% and 41%, respectively (Kothari *et al.*, 2011). The half-life ($t_{1/2}$) of dextransucrase alone and with additives was calculated assuming that its decay followed first order kinetics (Table 3.3.3). On storing the dextransucrase at -20ºC and 4ºC the $t_{1/2}$ increased to 306.9 days and 128.6 days, respectively (Table 3.3.3). Thus, -20ºC was selected for storage of dextransucrase.

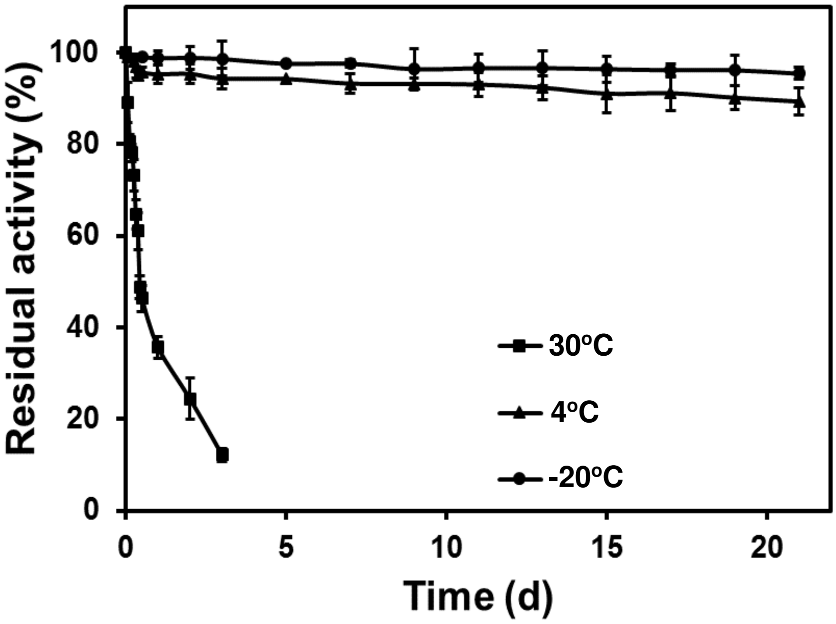

Fig. 3.3.16 Effect of temperature on storage stability of dextransucrase. The enzyme (20 U/mg, 0.30 mg/ml) in 20 mM sodium acetate buffer (pH 5.4) was incubated at 30°C, 4°C and -20°C. Aliquots were assayed for residual dextransucrase activity at 30°C at 1 day intervals.

Table 3.3.3 Effect of additives on dextransucrase from *Pediococcus pentosaceus* CRAG3

Dextransucrase with additive	**Half-life ($t_{1/2}$)**		
	30°C	**4°C**	**-20°C**
Dextransucrase	06.90 h	128.6 d	306.9 d
Dextransucrase + Tween 80 (0.1%)	85.48 h	Nd	Nd
Dextransucrase + Glycerol (0.5%)	15.53 h	Nd	Nd
Dextransucrase + Dextran (2 µg/ml)	06.32 h	Nd	Nd
Dextransucrase + Glutaraldehyde (0.1%)	05.08 h	Nd	Nd
Dextransucrase + PEG-8000 (10 µg/ml)	08.83 h	Nd	Nd

Nd- not determined

3.3.8 Effects of additives on dextransucrase activity

The effect of various additives on dextransucrase activity is shown in Fig. 3.3.17. The residual activity of dextransucrase at 30ºC in presence of Tween 80 after 12 h was 90% as compared to control (46%), which showed that it was most effective stabiliser for dextransucrase (Fig. 3.3.17). In presence of glycerol the residual activity was 83% after 9 h of incubation at 30ºC. PEG-8000 moderately stabilized the enzyme since the residual activity of dextransucrase in its presence after 12 h was 39% (Fig. 3.3.17). However, the other additives did not stabilize the enzyme. The residual activity of dextransucrase in presence of dextran (500 kDa) and gluteraldehyde after 12 h was only 27%, and 19%, respectively (Fig. 3.3.17). The residual activity of *Pediococcus pentosaceus* and its mutant in presence of Tween 80, PEG-8000, dextran (100 kDa), glycerol and without any stabilizer at 30ºC was 66, 24, 26, 19 and 26% and 68, 28, 45, 38 and 41%, respectively (Kothari *et al.*, 2011). Similar results were observed with dextransucrase of *Leuconostoc mesenteroides* NRRL B-640 where residual activity at 30°C was 92%, 44%, 38%, 36%, 32% and 6% with Tween 80, glycerol, PEG-8000, dextran (500 kDa), control and glutaraldehyde, respectively at 20h (Purama *et al.*, 2010).

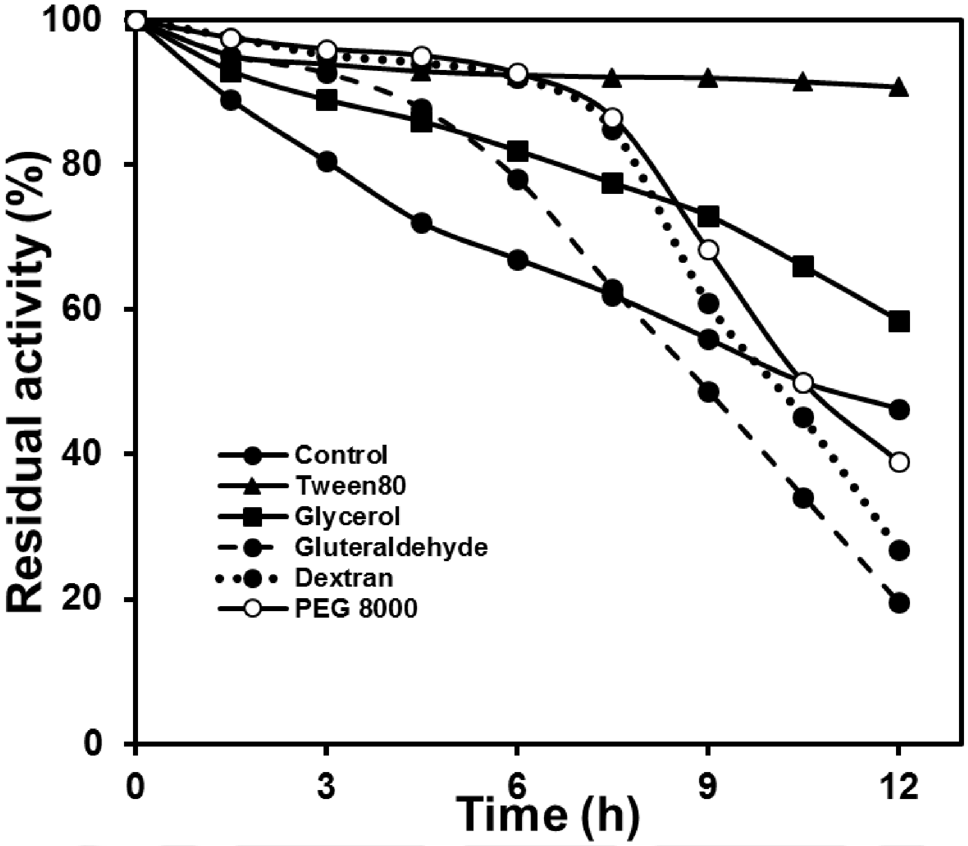

Fig. 3.3.17 Effect of various additives on stability of dextransucrase. The enzyme (20 U/mg, 0.30 mg/ml) in 20 mM sodium acetate buffer (pH 5.4) was pre-incubated with additive at 30°C.

Amongst various additives used Tween 80 was proved to be better stabiliser giving stabilization of dextransucrase with $t_{1/2}$ of 85.48 h at 30°C as compared to control having $t_{1/2}$ of 6.9 h (Table 3.3.3). The presence of glycerol and PEG-8000 along with dextransucrase resulted in enhanced half-life to 15.53 h and 8.83 h as compared to control (6.9 h). However, as dextran and gluteraldehyde negatively affected the stability of dextransucrase with $t_{1/2}$ of 6.32 h and 5.08 h, respectively.

3.3.9 Application of dextransucrase on the solidification of sucrose-supplemented milk

Varying degrees of solidification was achieved on addition of sucrose at concentrations 2% and 5% (w/v) as displayed in Fig. 3.3.18. The solidification of sucrose supplemented milk due to addition of dextransucrase was accredited to the formation of dextran. The application of dextransucrase from *Weissella* sp. TN610 in solidification of milk was observed by Bejar *et al.* (2013). The similar results have also been observed in dextran from *W. hellenica* SKKimchi3 (Kim *et al.,* 2008) where the bacterial strain (and not the dextransucrase enzyme as in our case) was cultivated in skim milk supplemented with 10% (w/v) sucrose to produce *in vivo* dextran. This property served application of dextransucrase from *Pediococcus pentosaceus* CRAG3 as a food additive to improve the texture of sucrose-supplemented milk based products. The modification in texture of milk was due to the interaction between synthesised polymer and proteins present in milk. This property depends on various parameters such as physical characteristics of dextran (the nature of glucosidic linkages, charge, branching linkages, and molecular mass), proteins present in the milk and on dextran/milk protein ratio (Ayala-hernandez *et al.*, 2008). These properties showed the importance of dextransucrase in food industry to enhance the rheological properties of dairy and bakery products (Katina *et al.*, 2009; Behare *et al.*, 2010).

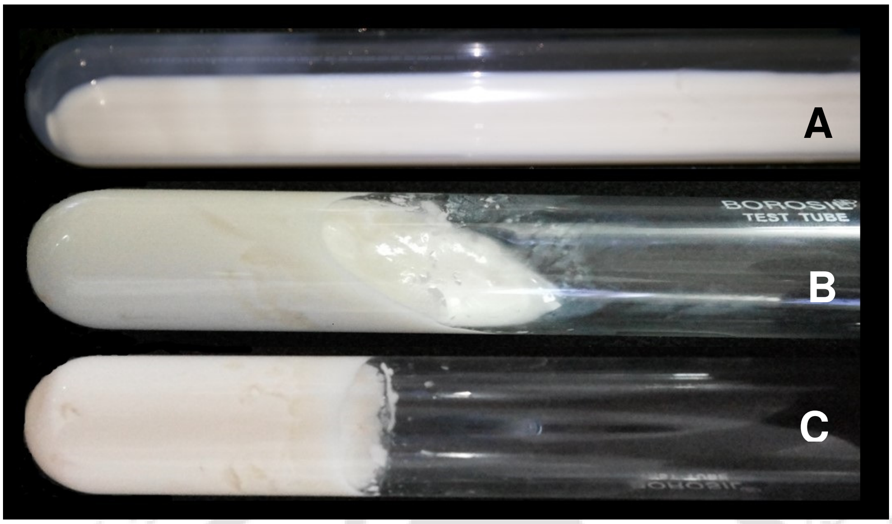

Fig. 3.3.18 Application of the dextransucrase from *P. pentosaceus* CRAG3 for the solidification of sucrose-supplemented milk. Effect of dextransucrase (5 U/ml) on the solidification of semi-skimmed milk supplemented with **(A)** 0% (Control milk without sucrose and enzyme), **(B)** 2% and **(C)** 5% sucrose after incubation at 30ºC for 18 h.

3.4 Conclusions

The culture supernatant of *Pediococcus pentosaceus* CRAG3 showed maximum dextransucrase activity (4.35 U/ml) and total carbohydrate content (4.5 mg/ml) at optimum temperature and shaking condition of 25ºC and 180 rpm, respectively. The crude dextransucrase (0.67 U/mg) was purified by PEG fractionation and gel filtration. The dextransucrase after fractionation with PEG-1500 (10%, w/v) gave 20.0 U/mg specific activity with 30 fold purification. The enzyme purified with PEG-1500 gave two bands of 224 and 188 kDa on SDS-PAGE under denaturing condition. However, the same enzyme sample gave single band of 224 kDa when run on native-PAGE and also on non-dentauring SDS-PAGE after PAS staining with sucrose, which confirmed a single molecular and pure form of dextransucrase. No band on gel incubated with raffinose was obtained that ruled out the presence of fructansucrase. The dextransucrase purified by PEG-1500 (10%, w/v) was further purified by gel-filtration using Sephacryl S-300HR column which gave the specific activity of 46.0 U/mg with 69 fold purification. The enzyme purified by gel filtration also showed 224 kDa band of dextransucrase on denaturing SDS-PAGE. The presence of single magenta color band after PAS staining confirmed the presence of dextransucrase. The dextransucrase partially purified with 10% PEG-1500 was further biochemically characterized. The assay conditions for dextransucrase activity such as pH, temperature, sucrose concentration and ionic strength of buffer, were optimized. The enzyme showed maximum activity (20.0 U/mg) in 5% sucrose in 20 mM sodium acetate buffer (pH 5.4) at 30ºC. The effect of sucrose concentration on dextransucrase activity showed that the specific activity increased with increasing sucrose concentration to 5% but decreased thereafter, which suggested that it did not completely follow classical Michaelis Menton kinetics. The purified dextransucrase showed K_m of 15.42 mM and V_{max} of 20.9 U/mg. The K_{cat} and the catalytic efficiency (K_{cat} / Km) of dextransucrase were 20.29 min^{-1} and 1.32 mM^{-1} min^{-1}, respectively.

The effects of various metal ions on dextransucrase activity showed that Co^{2+} (4 mM), Ca^{2+} (2 mM), Mg^{2+} (2 mM) and Zn^{2+} (1 mM) enhanced the activity by 56%, 44%, 14% and 12%, respectively. This displayed the importance of these metal ions for the catalytic efficiency of dextransucrase from *Pediococcus pentosaceus* CRAG3. However, the dextransucrase activity was decreased by 89% and 90% in presence of Mn^{2+} (8 mM) and Ni^{2+} (8 mM) respectively. The dextransucrase was stable up to 40ºC when incubated for 1 h which showed its superior property over other previously reported dextransucrases which were stable up to 30ºC. The storage stability of dextransucrase was also studied at different temperatures and pH. The dextransucrase was most stable at -20ºC with half-life of 307 days, hence -20ºC was chosen to store dextransucrase. It was found to be stable at pH range of 5.4-7.0 where the residual activity of dextransucrase at pH 5.4 was 82% after 3 h. The additives such as Tween80 and glycerol enhanced the stability of dextransucrase at 30ºC with half-life ($t_{1/2}$) of 85.5 h and 15.53 h, respectively as compared to control (6.9 h). The effect of purified dextransucrase on sucrose supplemented milk showed the solidification of milk due to formation of dextran, signifying its importance in improving the texture of dairy and bakery products. The purified dextransucrase from *Pediococcus pentosaceus* CRAG3 with higher activity and stability can serve as a potential candidate for industrial applications.

3.5 References

Ayala-hernandez, I., Goff, H.D. and Corredig, M. (2008) Interactions between milk proteins and exopolysaccharides produced by *Lactococcus lactis* observed by scanning electron microscopy. J. Dairy Sci. 91, 2583-2590.

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

Behare, P.V. Singh, R. Tomar, S.K. Nagpal, R. Kumar, M. and Mohania, D. (2010) Effect of exopolysaccharide-producing strains of *Streptococcus thermophilus* on technological attributes of fat-free lassi. J. Dairy Sci. 93, 2874-2879.

Cortezi, M., Monti, R. and Contiero, J. (2004) Temperature effect on dextransucrase production by *Leuconostoc mesenteroides* FT 045 B isolated from alcohol and sugar mill plant. Afr. J. Biotechnol. 4, 279-285.

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

Fu, D. and Robyt, J.F. (1990) A facile purification of *Leuconostoc mesenteroides* B-512FM dextransucrase. Prep. Biochem. 20, 93-106.

Funane, K., Ookura, T. and Kobayashi, M. (1998) Glucan binding regions of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F. Biosci. Biotechnol. Biochem. 62, 123-127.

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

Goyal, A. and Katiyar, S.S. (1996) Regulation of dextransucrase productivity of *Leuconostoc mesenteroides* NRRL 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.

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.

Hedrick, J.L. and Smith, A.J. (1968) Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. 126, 155-164.

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

Kaboli, H. and Reilly, P.J. (1980) Immobilization and properties of *Leuconostoc mesenteroides* dextransucrases. Biotechnol. Bioeng. 22, 1055-1069.

Katina, K., Maina-Ndegwa, H., Juvonen, R., Flander, L., Johansson, L. Virkki, L., Tenkanen, M. and Laitila, A. (2009) *In situ* production and analysis of *Weissella confusa* dextran in wheat sourdough. Food Microbiol. 26, 734‑743.

Kim, D. and Robyt, J.F. (1994a) Production and selection of mutants of *Leuconostoc mesenteroides* constitutive for glucansucrases. Enz. Microbiol. Technol. 16, 659-664.

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

Kim, M.J., Seo, H.N., Hwang, T.S., Lee, S.H. and Park, D.H. (2008) Characterization of exopolysaccharide (EPS) produced by *Weissella hellenica* SKkimchi3 isolated from kimchi. J. Microbiol, 46, 535‑541.

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

Kothari, D., Tyagi, A., Patel, S. and Goyal A. (2011) Dextransucrase from the mutant of *Pediococcus pentosaceus* (PPm) is more stable than the wild type. 3 Biotech. 1, 199-205.

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

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.

Leemhuis, H., Pijningb, T., Dobruchowskaa, J. M. van Leeuwena, S. S., Kralj, S., Dijkstrab, B. W. and Dijkhuizen L. (2013) Glucansucrases: Three-dimensional structures, reactions, mechanism, α-glucan analysis and their implications in biotechnology and food applications. J. Biotechnol. 163, 250-272.

Lopez, A. and Monsan, P. (1980) Dextran synthesis by immobilized dextransucrase. 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., 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.

Majumder, A., Purama, R.K. and Goyal, A. (2007) An overview of purification methods of glycoside hydrolase family 70 dextransucrase. Ind. J. Microbiol. 47, 252-263.

Miekka, S. and Ingham, K.C. (1978) Influence of self-association of proteins on their precipitation by poly(ethylene glycol). Arch. Biochem. Biophys. 191, 525-536.

Miller, A.W. and Robyt, J. (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.

Naidu, G.S.N. and Panda, T. (2003) Studies on pH and thermal deactivation of pectolytic enzymes from *Aspergillus niger*. Biochem. Eng. J. 16, 57-67.

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

Nigam, M., Goyal, A. and Katiyar, S.S. (2006) High yield purification of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F by phase-partitioning. J. Food Biochem. 30, 12–20.

Patel, S. and Goyal, A. (2010) Isolation, characterization and mutagenesis of exopolysaccharide synthesizing new strains of lactic acid bacteria. Int. J. Microbiol. 8(1).

Patel, S., Kasoju, N., Bora, U. and Goyal, A. (2010) Structural analysis and biomedical applications of dextran produced by a new isolate *Pediococcus pentosaceus* screened from biodiversity hot spot Assam. Biores. Technol. 101, 6852-6855.

Patel, S., Kothari, D. and Goyal, A. (2011) Purification and characterization of an extracellular dextransucrase from *Pediococcus pentosaceus* isolated from the soil of North East India. Food Technol. Biotechnol. 49, 297-303.

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

Purama, R.K. and Goyal A. (2009) 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., Agrawal, M. and Goyal A. (2010) Stabilization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-640. Ind. J. Microbiol. 50, 57-61.

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

Rabelo, M.C., Fontes, C.M.L. and Rodrigues, S. (2011) Stability study of crude dextransucrase from *Leuconostoc citreum* NRRL B-742. Ind. J. Microbiol. 51, 164-170.

Rodrigues, S., Lona, L.M.F. and Franco, T.T. (2003) Effect of phosphate concentration on the production of dextransucrase by *Leuconostoc mesenteroides* B-512F. Bioproc. Biosyst. Eng. 26, 57-62.

Russell, R.R.B. (1979) Purification of *Streptococcus mutans* glucosyltransferase by polyethylene glycol phase precipitation. FEMS Microbiol. Lett. 6, 197-199.

Sadana, A. (1995) Biocatalysis: fundamentals of deactivation kinetics, Prentice-Hall, Englewood Cliffs.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) In (2nd ed) *Molecular Cloning: A Laboratory Manual*, Vol. 1. Plainview, Cold Spring Harbor Laboratory Press, Woodbury, New York.

Shukla, R., Iliev, I. and Goyal, A. (2010) Purification and characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1149. Biotechnol. Biotechnol. Equip. 24, 576–580.

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.

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.

Veljkovic, V.B., Lazic, M.L., Rutic, D.J., Jovanovic, S.M. and Skala, D.U. (1992) Effects of aeration on extracellular dextransucrase production by *Leuconostoc mesenteroides*. Enz. Microb. Technol. 14, 665-668.

Waldherr, F.W., Doll, V.M., Meissner, D., Vogel, R.F. (2010) Identification and characterization of a glucan-producing enzyme from *Lactobacillus hilgardii* TMW 1.828 involved in granule formation of water kefir. Food Microbiol. 27, 672-678.

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

Chapter 4

Production, purification and characterization of dextran from *Pediococcus pentosaceus* CRAG3

4.1 Introduction

Polysaccharides, one of the most important bio-macromolecules, exist extensively in all living organisms performing various biological roles (Pe´rez *et al.*, 1996). The exopolysaccharides (EPSs) produced by lactic acid bacteria (LAB) have proven useful for several industrial applications such as safe food additive for improving the textural properties of sucrose-supplemented dairy products (Bejar *et al.*, 2013) and as ingredients in food industry (Anwar *et al.*, 2010). α-Glucans are the EPSs produced by lactic acid bacteria that have immense applications as viscosifier and water-binding agents in food and non-food industries (Badel *et al.*, 2011). The research has been driven on the production of EPSs from lactic acid bacteria with particular structure and size, which instruct preferred functional properties (De Vuyst and Degeest, 1999; Welman and Maddox, 2003). Depending upon the localization, EPS are of two types; one type is the polysaccharides which are not or loosely attached to the cells due to which they form slimy or ropy colonies and the other type is capsular polysaccharides that are resolutely adhered to the cells (Hassan, 2008). However, depending upon the composition, polysaccharides are of two types; homopolysaccharides and heteropolysaccharides. The former is composed of similar monosaccharides such as α-D-glucan or β-D-glucan (composed of glucose monomers), fructan (composed of fructose monomers) while the latter comprise different monosaccharides units and may contain acetamido sugars, phosphates, or acetyl groups (De Vuyst and Degeest, 1999; Monsan *et al.*, 2001). There are reports on the production of both types of polysaccharides by LAB (De Vuyst and Degeest, 1999; Monsan *et al.*, 2001). Dextrans, the homopolysaccharides with glucose units, are chiefly produced by *Leuconostoc sp* (Jeanes *et al.*, 1954; Slodki *et al.*, 1986). However, other LAB like *Streptococcus*, *Lactobacillus* (Sidebotham, 1974), *Weissella* species (Collins *et al.*, 1993) and *Pediococcus* sp. (Patel *et al.*, 2010) also produce dextran. The dextran production by *Pediococcus* genus have not been much studied (Smitinont *et al.,* 1999; Patel *et al.*, 2010).

Now-a-days the interest in studying and modifying polysaccharides has arisen owing to their medicinal and bioactive properties. Bao *et al.* (2001) isolated (1→3)-α-D-glucan from cell wall of *Ganoderma lucidum* to investigate its physicochemical properties and immunological activity. The (1→3)-β-D-glucan from *Chaetoceros mulleri* showed immunomodulatory properties like adjuvant, antimicrobial, antitumor and radiation protective activities (Storseth *et al.*, 2004). The polysaccharides especially β-glucans (Chen *et al.*, 2010) and α-glucans (Wiater *et al.*, 2010) serve as potential immuno-stimulator, immuno-modulator and anti-cancer agents. As compared to β-glucans there are very few reports on anticancer properties or immuno-stimulatory property of α-glucans. There are reports available on anticancer α-glucans but only after their chemical modification such as sulfation (Wiater *et al.*, 2010) and carboxymethylation (Bao *et al.*, 2001). It has been reported that the polysaccharides from *Strongylocentrotus nudus* eggs with α-(1→6) and α-(1→6) linkages act as promising anti-cancer agents (Liu *et al.*, 2007).

In the present study purification and characterization of dextran produced from *Pediococcus pentosaceus* CRAG3 a new isolate from fermented cucumber is described. The dextran structure was analysed and its effect on mammalian cancer lines was studied.

4.2 Materials and Methods

4.2.1 Microorganism, cell lines, chemicals and reagents

The bacterial strain *Pediococcus pentosaceus* CRAG3 (Genbank Accession Number JX679020) was isolated from fermented cucumber as described in Chapter 2, Section 2.2.2. It was propagated in modified MRS agar medium (Goyal and Katiyar, 1996) as a stab at 30°C, stored at 4°C and subcultured every 15 days. The modified MRS agar medium was prepared as described in Chapter 2, Section 2.2.3.

The chemicals required for molecular weight determination by copper (Cu) bicinchoninate method such as disodium 2,2’-bicinchoninate, sodium bicarbonate and L-serine were purchased from Sigma, St. Louis, MO, USA and cupric sulphate pentahydrate and sodium carbonate were purchased from Himedia India Pvt. Ltd., India. The mammalian cell lines for *in vitro* cytotoxicity test of dextran such as cervical cancer (HeLa) cell line, colon cancer (HT29) cell line and murine macrophage (J774A.1) line were purchased from National Centre for Cell Science (NCCS), Pune, India. The ingredients required for preparation of cell culture medium such as Dulbecco’s Modified Eagle’s Medium (DMEM) containing 4.0 mM L-glutamine and 110 mg/L sodium pyruvate, fetal bovine serum (FBS) and sodium bicarbonate were purchased from Sigma, St. Louis, MO, USA, while W/5000 units Penicillin and Streptomycin (5 mg/ml in 0.9% normal saline) were purchased from Himedia India Pvt. Ltd., India. 1x trypsin-EDTA solution (0.25% trypsin and 0.2% EDTA) and cell culture grade phosphate buffered saline (PBS) (pH 7.4) were purchased from Himedia India Pvt. Ltd., India. The chemicals required for 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) assay like MTT was purchased from Sigma, St. Louis, MO, USA. Dimethyl sulfoxide (DMSO) and phosphate buffered saline (PBS) were purchased from Himedia India Pvt. Ltd., India. The ingredients required for cryopreservative medium preparation like Fetal Bovine Serum (FBS) were purchased from Sigma, St. Louis, MO, USA and DMSO (cell culture grade) was purchased from Himedia India Pvt. Ltd., India. 0.4% Trypan blue for counting of cells was purchased from Sigma, St. Louis, MO, USA. DMEM, MTT and PBS were stored at 4^{o}C while FBS, trypsin and penicillin-streptomycin solution were stored at -20^{o}C.

4.2.2 Enzymatic synthesis of dextran and its purification

The dextran from *Pediococcus pentosaceus* CRAG3 was produced enzymatically by dextransucrase which was partially purified by 10% (v/v) PEG-1500 as described earlier in Section 3.2.5.1. The reaction mixture (50 ml) containing 10 ml of partial purified dextransucrase (20.0 U/mg, 0.27 mg/ml), 5% (w/v) sucrose, 0.3 mM $CaCl_2$ and 15 mM sodium azide in 20 mM sodium acetate buffer (pH 5.4) was incubated at 30^{o}C and 180 rpm for 48 h. After incubation, the viscous solution containing dextran was mixed with 65% (v/v) ethanol as final concentration and centrifuged at 12,000***g*** and 25°C for 30 min. The supernatant part was removed and pellet containing dextran was again mixed with 65% ethanol and centrifuged at 12,000***g*** and 25°C for 30 min. This was repeated two times and finally the pellet was dissolved in water and lyophilized. The lyophilised powder of dextran was used for structural characterization. The purity of dextran sample was also checked by analysing its protein content using the method as described earlier in Chapter 3, Section 3.2.2.3.

4.2.3 Structural characterization of dextran

4.2.3.1 Determination of number average molecular weight of dextran

4.2.3.1.1 Preparation of reagents

Two stock solutions were prepared: Solution A consisted of 97.1 mg of disodium 2,2'-bicinchoninate dissolved in 45 ml of water containing 3.2 g of sodium carbonate monohydrate and 1.2 g of sodium bicarbonate. The final volume was adjusted to 50 ml. Solution B consisted of 62 mg of copper sulfate pentahydrate and 63 mg of L-serine dissolved in 45 ml of water, and the final volume was adjusted to 50 ml. The working reagent was freshly prepared by mixing equal volumes of Solutions A and B. Reducing sugar standard consisted of 1-20 pg/ml of maltose.

4.2.3.1.2 Standard plot for determination of reducing value

The stock solution (25.0 µg/ml) of maltose was prepared to make different concentrations. The standard plot was prepared by varying concentrations (0-20 µg/ml) of maltose in deionised water. To 100 µl of maltose solution, 100 µl of working reagent containing equal volumes of Solutions A and B was added and the mixture was placed in water bath at 80°C for 35 min. The solution was cooled to 25°C and the absorbance was measured at 560 nm against blank on a UV-visible spectrophotometer (Varian, Cary 100 Bio). The absorbance (A_{560}) against the maltose concentration was plotted (Fig. 4.2.1).

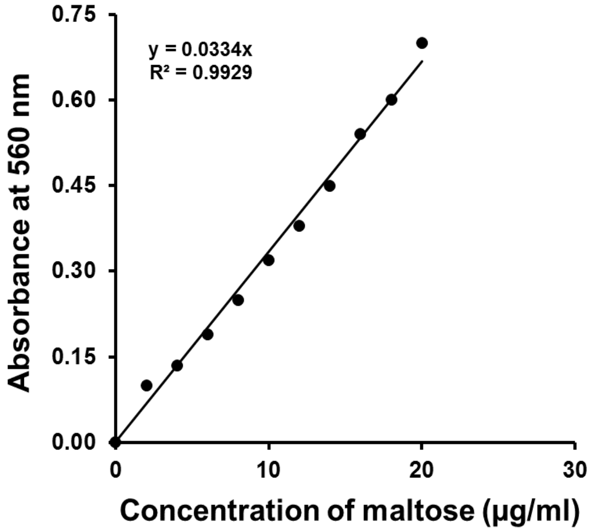

Fig. 4.2.1 Standard plot between varying concentrations of maltose against absorbance (A_{560}) using copper (Cu) bicinchoninate method for estimation of reducing value.

4.2.3.1.3 Method for number average molecular weight determination

The number average molecular weight of purified dextran from *Pediococcus pentosaceus* CRAG3 was determined by copper (Cu) bicinchoninate method (Fox and Robyt, 1991). The concentration of maltose in purified dextran was determined using standard plot (as described earlier in Section 4.2.3.1.2.) and taken as reducing value to determine number average degree of polymerisation. The procedure consisted of adding 100 µl of reducing dextran sample (20 µg/ml) to 100 µ1 of working reagent (as described earlier in Section 4.2.3.1.1) in the wells of a 96-sample microtiter plate. The plate was covered with cling film and incubated in a water bath at 80°C for 35 min. The plate was then cooled to 25ºC for 15 min and the absorbance measured at 560 nm. The number-average degree of polymerization (DPn) and average molecular weight (MW) were determined by following equations as described by Fox and Robyt, (1991):

$$\mathbf{DPn = (Cc / Cm) \times 1.9}$$

Cc = Concentration of carbohydrate sample (mg/ml)

Cm = Concentration of maltose as determined by reducing value (mg/ml)

$$\mathbf{MW = [DPn \times 162] + 18}$$

4.2.3.2 Monosaccharide analysis of purified dextran

The monosaccharide analysis of dextran from *Pediococcus pentosaceus* CRAG3 was carried out using High Performance Anion Exchange Chromatography (HPAEC). 50 mg of lyophilised and purified dextran sample was hydrolysed by 1 ml of 50 mM sulphuric acid in 2.0 ml microcentrifuge tube at 100ºC on dry block heater (Labtech, model LBH T01) for 6 h. The hydrolysed dextran was neutralized by 1 ml 100 mM Na_2CO_3 and filtered through 0.2 µm membrane. The sample was analysed by HPAEC ICS-3000 system (Dionex Corporation, USA) using CarboPac PA-20 analytical column (3x150 mm) and CarboPac PA-20 guard column (3x30 mm). The temperature of the column was adjusted to 30°C and the injection volume was 25 µl. The eluent used was 250 mM NaOH at flow rate of 0.2 ml/min. Glucose (20 µg/ml) and fructose (20 µg/ml) were used as standard monosaccharides.

4.2.3.3 Field Emission Scanning Electron Microscopic analysis of dextran

The Field Emission Scanning Electron Microscopic (FESEM) analysis of dried dextran was done by fixing it to the FESEM stub with a double-sided carbon tape. A dried sample of dextran was then coated with ~ 10 nm Au in a sputter coater (Polaron, Model SC7620). The surface of the dry powder was viewed in Field

Emission Scanning Electron Microscope (Carl Zeiss, Model Sigma) operated at 10.0 kV.

4.2.3.4 Optical rotation

The optical rotation of purified dextran was determined using a polarimeter (Perkin-Elmer Instruments, Model 343 Polarimeter). 10 ml of 0.2% (w/v) dextran in distilled water was prepared and analysed using sodium D-line (589 nm) at 25ºC.

4.2.3.5 Fourier Transform Infrared Spectrometry

The sample for Fourier Transform Infrared (FTIR) Spectrometry was prepared by taking 1-2% (w/w) dried powder of purified dextran in potassium bromide (KBr) into a mortar. It was mixed and grind to a fine powder. The sample was very finely ground to reduce scattering losses and absorption band distortions. Now, the KBr-dextran powder was placed in between two stainless steel disks in KBr pellet holder. The whole assembly was placed onto the pistil in the hydraulic press. The pressure of hydraulic press was increased to around 20,000 prf (pulse repeatition frequency) with the help of pumping movement of hydraulic pump handle. The assembly was left for 3 min and the pressure was then released. The disks were removed and homogenous and transparent film of dextran sample was placed in IR sample holder. The FTIR spectrum of purified sample was recorded by FTIR Spectrometer (Perkin-Elmer Instruments, Model Spectrum One).

4.2.3.6 *Nuclear Magnetic Resonance Spectroscopy*

The vacuum dried dextran sample was dissolved in D_2O (99.6% atom ^{2}H, Sigma, St. Louis, MO, USA) and used for recording the spectrum. 15 mg and 30 mg of purified dextran powder was dissolved in 0.4 ml of D_2O for ^{1}H-NMR and ^{13}C-NMR spectroscopic analysis, respectively. NMR spectroscopy was performed on a spectrometer (Varian, Model AS400). 1D ^{1}H-NMR and ^{13}C-NMR spectra of sample were recorded at a base frequency of 100 MHz for analysing the linkage composition. Tetramethyl silane (TMS) was used as an internal reference.

4.2.4 *In vitro* analysis of dextran effect on mammalian cancer cell lines

4.2.4.1 *Preparation of medium for cell culture*

The incomplete DMEM was prepared by adding 13.4 g dry powder of DMEM containing 4.0 mM L-glutamine and 110 mg/L sodium pyruvate and 3.7 g sodium bicarbonate in autoclaved double distilled water and volume was adjusted to 1L. The pH of medium was adjusted to 7.0 using 1 N NaOH or 1 N HCl solution. The medium was filtered using 0.2 µm membrane and stored at 4°C. The complete DMEM was prepared by adding incomplete DMEM supplemented with 10% (v/v) fetal bovine serum, 50 µg/ml streptomycin and 50 IU/ml penicillin. It was stored at 4°C.

4.2.4.2 *Maintenance of culture*

All three mammalian cell lines (cervical cancer (HeLa), colon cancer (HT29) cell line and murine macrophage (J774A.1) line) were adherent cell lines which were grown on the surface of tissue culture flask (25 cm^2). The tissue culture flasks containing each of three cell lines in complete DMEM medium (as described in

Section 4.2.4.1) were incubated at 37ºC in 5% CO_2 atmosphere. The exponentially growing cells of each cell line were observed in inverted microscope (Nikon, model PS100-F) under phase contrast optics. At post confluence, the DMEM medium from all flasks was carefully aspirated without disturbing the surface of cells and the cell surface was washed with 5 ml sterile 150 mM PBS twice to remove dead cells. 1x trypsin-EDTA solution was slowly applied to the surface of tissue culture flasks containing adherent cells sufficient to cover the monolayer of cells and the flasks were incubated at 37ºC in 5% CO_2 atmosphere for 4-5 min. Each flask was gently tapped and again observed in inverted microscope to check the running monolayer. The trypsinized cells were resuspended in 5 ml complete DMEM medium (as described in Section 4.2.4.1) (pre-warmed at 37ºC) to inactivate trypsin and centrifuged in 15 ml centrifuge tubes at 300***g*** and 4ºC for 5 min. The supernatant containing medium and trypsin was removed and the cells were resuspended in fresh complete DMEM medium. The cells were counted as described later in Section 4.2.4.4. The cultures were seeded in new tissue culture flasks (25 cm^2) at a split ratio of 1:3 for HeLa cell line (3 ml of cell suspension in 9 ml complete DMEM medium), 1:8 for HT29 cell line (1 ml of cell suspension in 8 ml complete DMEM medium) in flask and 1:5 for J774A.1 cell line (2 ml of cell suspension in 10 ml complete DMEM medium) and incubated at 37ºC in 5% CO_2 atmosphere. The medium was regularly changed when its colour started changing from red to yellow.

4.2.4.3 Preservation and storage of cells

The cells of each of three cell lines (HeLa, HT29 and J774A.1) were trypsinized as described in Section 4.2.4.2. After trypsinization the cells were resuspended in complete DMEM medium at high cell density ($\geq 2 \times 10^6$ cells) and centrifuged at 300***g*** and 4°C for 5 min. The supernatant was discarded and the cells were resuspended separately in cryopreservative medium consisted of 10% (v/v) DMSO and 40% DMEM complete medium in FBS and stored at -80°C up to 6 months.

4.2.4.4 Counting of cells using dye-exclusion haemocytometer procedure

The haemocytometer is a thick glass microscope slide with a rectangular notch that creates a chamber and is used to count the cells in a definite volume of suspension. The number of cells were counted using haemocytometer as described below. 10 µl of cell suspension was mixed with 10 µl 0.4% (w/v) trypan blue dye. 10 µl from this mixture was dropped at the edge of coverslip placed on haemocytometer. The slide was placed in inverted microscope under phase contrast optics and the cells of each of three cell lines (HeLa, HT29 and J774A.1) were counted in four squares of haemocytometer. The total number of cells present per ml of suspension was calculated as

$$\textbf{Total number of viable cells/ml} = \frac{\textbf{Number of cells in four squares}}{\textbf{4}} \textbf{ x dilution x } \mathbf{10^4}$$

4.2.4.5 MTT cytotoxicity assay

The lyophilized powder of dextran from *Pediococcus pentosaceus* CRAG3 was used for *in vitro* MTT cytotoxicity assay. Different concentrations of dextran in incomplete DMEM medium were prepared for cytotoxicity assay and filtered through

0.22 µm membrane. The effects of dextran on HeLa, HT29 and J774A.1 cells were determined using the colorimetric MTT assay method as described earlier (Mosmann, 1983). The number of cells of each of HeLa, HT29 and J774A.1 cell lines were counted as described in Section 4.2.4.4. Each of the three cell lines at cell density approximately, 1.2×10^4 cells per well were seeded in a 100 µl DMEM complete medium in three separate 96-well plates. The plates were covered by lid and incubated at 37°C in 5% CO_2 atmosphere for 12-14 h to allow the cells to attach on surface of cluture flasks. After incubation, the medium was removed from all plates and dextran solutions in a concentration range from 1-1000 ng/ml for HeLa cell line, from 0.1-500 µg/ml for HT29 cell line and from 1-1000 µg/ml for J774A.1 cell line was added in the wells. The medium without dextran was used as negative control. All the experiments were performed in quadruples and the average value was taken. All the plates were incubated at 37°C in 5% CO_2 atmosphere for 48 h. After 48 h whole medium was removed and 100 µl MTT solution (500 µg/ml) was added to each well. The plates were further incubated at 37°C in 5% CO_2 atmosphere for 4 h. The MTT solution was removed, 100 µl DMSO was added to each well to solubilize formazan crystals and absorbance was measured at 570 nm by a 96-well microplate reader (Tecan, Model Infinite 200 Pro). The statistical analysis of data was performed by Student's t-test and a value of $p < 0.05$ was considered as significant.

4.3 Results and Discussion

4.3.1 Production and purification of *Pediococcus pentosaceus* CRAG3 dextran

The dextran was purified by precipitating with 99% ethanol. The purified dextran (35.0 mg/ml) from *Pediococcus pentosaceus* CRAG3 showed negligible (<0.5%) protein content (8.5 µg/ml) which might be due to protein associated with it.

4.3.2 Structural characterization of purified dextran

4.3.2.1 Molecular weight determination of dextran

The number average degree of polymerization (DPn) of purified dextran as determined by Cu bicinchoninate method was 1809. The number average molecular weight of dextran from *Pediococcus pentosaceus* CRAG3 as calculated using DPn was 2.93×10^5. The molecular size of commercially used dextran from *Leuconostoc mesenteroides* B-512FMC ranges from 21×10^3 to $1{,}650 \times 10^3$ Da (Falconer *et al.*, 2011). It has been reported that European Union approved high molecular weight glucans (1×10^6-2×10^6) as food ingredients in bakery products (Naessens *et al.*, 2005). Cross linkages of high molecular weight dextrans can be used in gel filtration material like Sephadex products (Falconer *et al.*, 2011). Commercial dextrans are available in different molecular sizes (5,000-5,00,000 Da) which have broader range of applications in food and pharmaceutical industries (Falconer *et al.*, 2011).

4.3.2.2 Monosaccharides analysis of purified dextran

The chromatogram of hydrolyzed sample of purified dextran from *Pediococcus pentosaceus* CRAG3 is shown in Fig. 4.3.1. The chromatogram of hydrolyzed dextran sample was compared with that of fructose and glucose standards

(Fig. 4.3.1A and 4.3.1B, respectively). The hydrolyzed sample of dextran showed a peak at retention time of 7.25 min (Fig. 4.3.1C) similar to the peak at 7.22 min for glucose (Fig. 4.3.1B), whereas fructose showed the peak at 7.55 min (Fig. 4.3.1A). These results displayed the presence of only glucose monomers in the hydrolysed dextran sample.

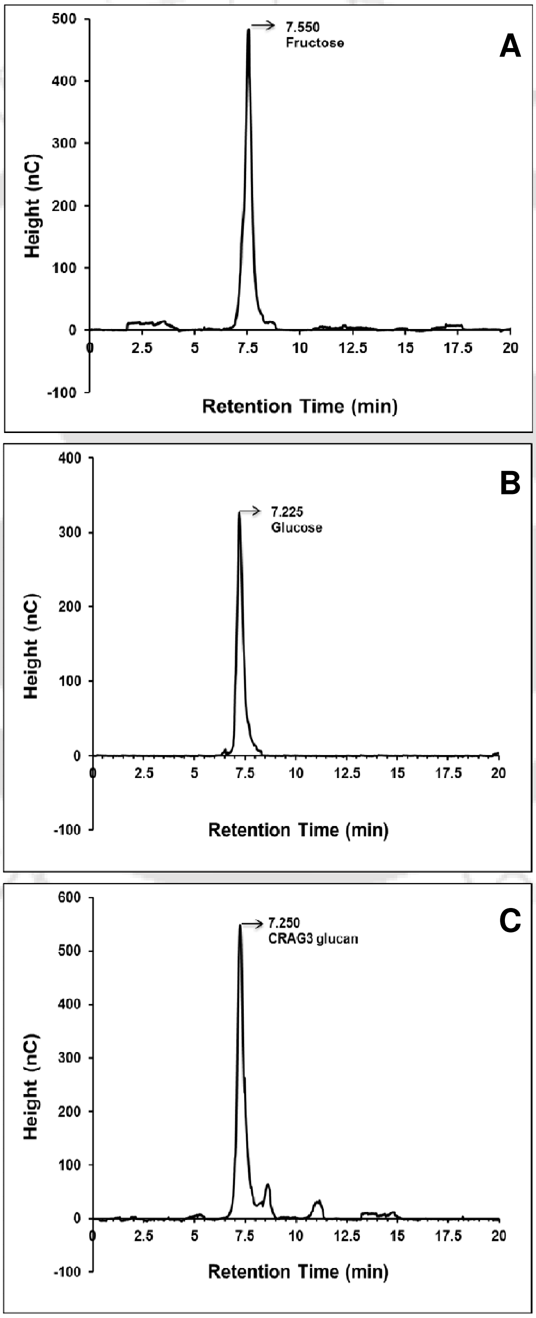

Fig. 4.3.1 HPAEC analysis of hydrolyzed dextran from *Pediococcus pentosaceus* CRAG3. Different sugar samples in 250 mmol/L sodium hydroxide **(A)** Fructose standard **(B)** Glucose standard and **(C)** Hydrolyzed dextran from *Pediococcus pentosaceus* CRAG3.

4.3.2.3 FESEM analysis of Pediococcus pentosaceus CRAG3 dextran

The surface morphology of powdered dextran was analysed by field emission scanning electron microscopy (FESEM). The FESEM image of dextran revealed its porous structure (Fig. 4.3.2). It has been reported that the polysaccharides can also be used in foods as thickening, gelling, stabilizing, emulsifying and water-binding agents (Khan *et al.*, 2007). The porous glucan particles from *Saccharomyces cerevisiae* are known to be used in macrophage targeted delivery of nanoparticles (Soto *et al.*, 2011). Soto *et al.*, 2010 also reported the application of glucan particle encapsulated rifampicin in delivery of nanoparticles to macrophages. Due to its high molecular weight and porosity, dextran from *Pediococcus pentosaceus* CRAG3 could be used for pharmaceutical and industrial applications.

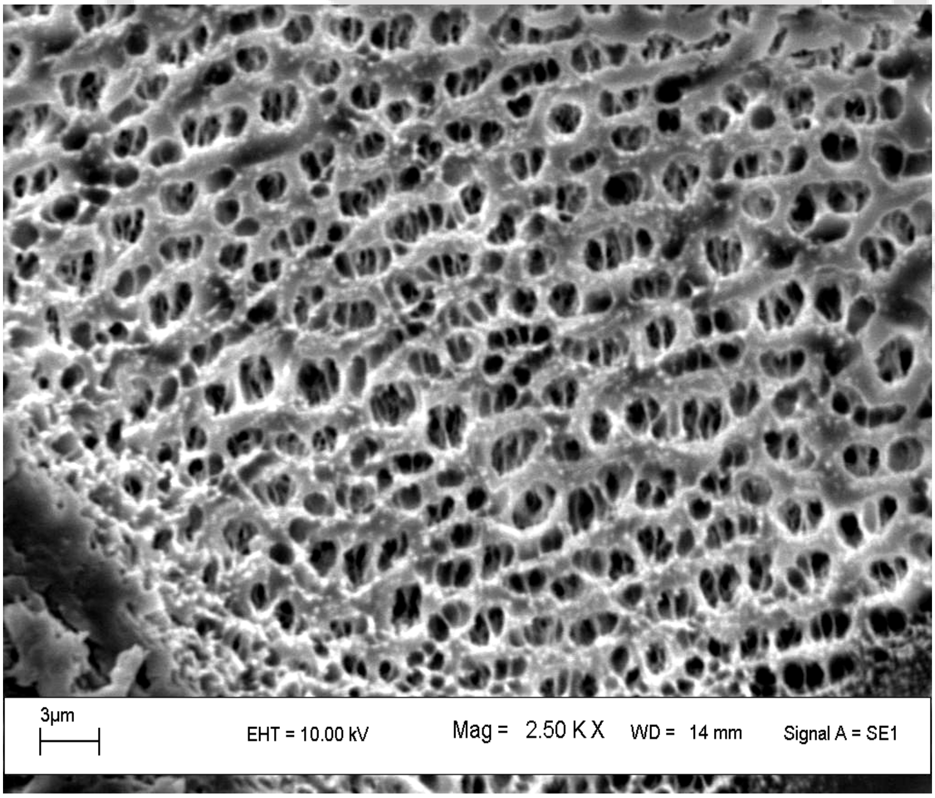

Fig. 4.3.2 Scanning electron microscopic image of purified dextran from *Pediococcus pentosaceus* CRAG3 at 2.50 KX magnification.

4.3.2.4 Optical Rotation and FTIR Spectrometry

The dextran from *Pediococcus pentosaceus* CRAG3 showed optical rotation $[\alpha_D]^{20}$ of +153° at $C^{0.2}$ indicating the presence of D-configuration of glucosyl residues. The result was in good agreement with earlier reports (Purama *et al.*, 2009; Liu *et al.*, 2007). The FTIR spectrum of purified dextran from *Pediococcus pentosaceus* CRAG3 is shown in Fig. 4.3.3. The band in the 3421 cm^{-1} region represented the hydroxyl stretching vibration of polysaccharide while the presence of band in 2927 cm^{-1} and 1639 cm^{-1} region showed C-H stretching and the feature signal for the presence of bound water, respectively (Table 4.3.1). These results were in agreement with earlier reports (Park, 1971; Shingel, 2002; Chiu *et al.*, 2004; Cao *et al.*, 2006; Liu *et al.*, 2007). The presence of glycosidic bond was confirmed by the absorption peak at 914 cm^{-1}. The bands at 1152, 1104 and 1029 cm^{-1} in FTIR spectrum of *Pediococcus pentosaceus* CRAG3 dextran were the main characteristic bands due to the valent vibrations of C-O and C-C bonds and deformational vibrations of the CCH, COH and HCO bonds. The band at 1152 cm^{-1} was assigned to valent vibrations of C-O-C bond and glycosidic bridge. The peak at 1104 cm^{-1} showed vibration of the C-O bond at the C-4 position of glucose residue and the presence of peak at 1029 cm^{-1} was due to the flexibility in main chain present in dextran around the α-(1→6) glycosidic bonds. These results were in accordance with the commercially available dextran T-500 of Shingel (2002). The peak at 914 cm^{-1} showed the characteristic peak of (1→3)-α-D-glucan. This was supported by the work of Seymour *et al.* (1980) and Zhang *et al.* (1999). Several other workers reported the use of FTIR spectral data for the characterization of glucans from *Angelica sinensis* (Oliv) Diels and *Strongylocentrotus nudus* eggs with α-(1→6) and α-(1→4) linkages having anticancer properties (Cao *et al.*, 2006; Liu *et al.*, 2007). FTIR spectral analysis of dextran from *Pediococcus pentosaceus* CRAG3 showed the presence of α-(1→6) and α-(1→3) linkages which was further confirmed by NMR spectroscopic analysis.

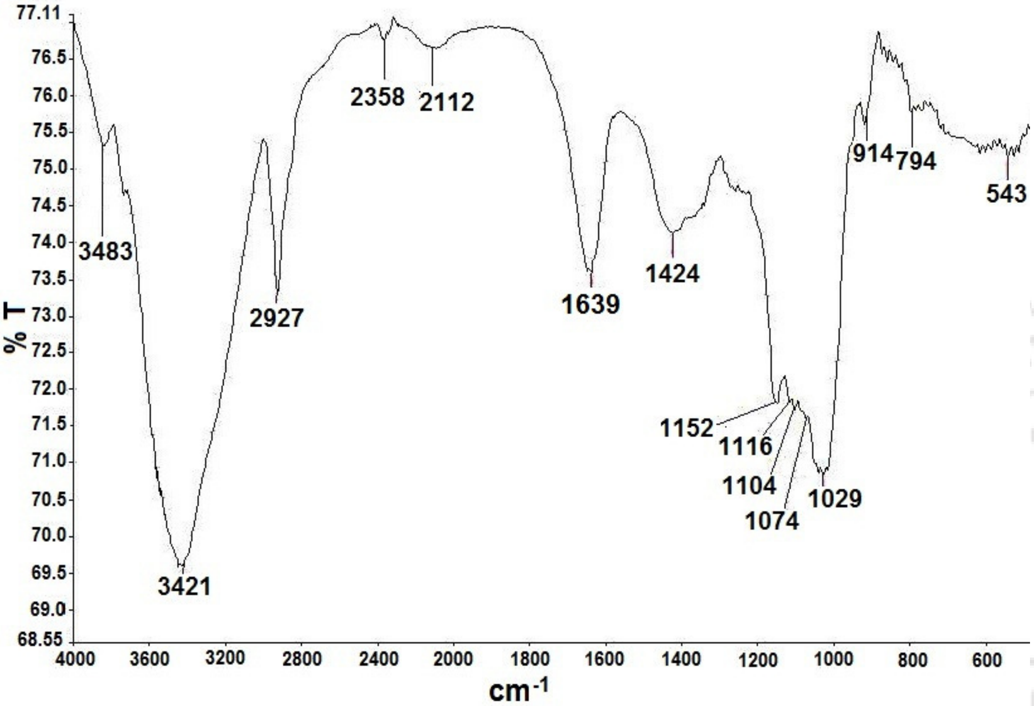

Fig. 4.3.3 FTIR spectrum of dextran from *Pediococcus pentosaceus* CRAG3.

Table 4.3.1 FTIR analysis of dextran from *Pediococcus pentosaceus* CRAG3.

Band (cm^{-1})	Functional group
3421	Represents hydroxyl stretching vibration
2927	C-H group stretching vibration
1639	Bound water
1152, 1104	C-O-C bond and glycosidic linkage
1029	C-O-C, α-pyranose of glucose
914	α-Glycosidic linkage and 1,3-α-D-dextran

4.3.2.5 NMR Spectroscopy of purified dextran

4.3.2.5.1 ^{1}H-NMR Spectroscopy

The linkages and branching in purified dextran from *Pediococcus pentosaceus* CRAG3 were determined by ^{1}H-NMR spectroscopic analysis. The ^{1}H-NMR spectrum of dextran from CRAG3 is presented in Fig. 4.3.4. It showed five spectral resonances at 3.58, 3.75, 3.47, 3.92 and 4.01 ppm corresponded to C-2, C-3, C-4, C-5 and C-6, respectively and two anomeric resonance peaks at 4.99 ppm and 5.33 ppm corresponding to α-(1→6) and α-(1→3) linkages, respectively. These findings were in agreement with previous reported data (Sidebotham, 1974; Maina *et al.*, 2008). The assignment of various resonances of ^{1}H-NMR of dextran from CRAG3 are shown in Table 4.3.2. The ^{1}H-NMR spectrum of dextran from CRAG3 showed intense anomeric resonance peak at 4.99 ppm and a low intense branching point resonance peak at 5.33 ppm. The integration of anomeric signals in 1D ^{1}H-NMR spectrum of dextran showed 75% α-(1→6) linked glucose (δ_{H-1}~4.99) and 25% α-(1→3) linked glucose (δ_{H-1} ~ 5.33). The absence of any peak at δ_{H-5} 5.40 implied the absence of α-(1→4) linkage. The 75% α-(1→6) linked linear chain with 25% branching in CRAG3 glucan confirmed it as dextran. The percentage of branching in exopolysaccharide (EPS) from *Weissella confusa* E392 using integration of ^{1}H-NMR spectrum showed that it contained 97.3% of α-(1→6) linked linear chain with 2.7% of α-(1→3) linked branches (Maina *et al.*, 2008). The dextran from *L. dextranicum* NRRL B-1146 showed α-(1→6) linear and α-(1→4) branched linkages (Purama *et al.*, 2009). The dextran containing branches can be used for production of branched oligosaccharides which have superior prebiotic properties over linear oligosaccharides such as resistance to glycolytic digestive enzymes and ability to get readily fermented by intestinal microflora (Remaud-Simeon *et al.*, 1994; Dols *et al.*, 1998, Vasileva et al., 2010).

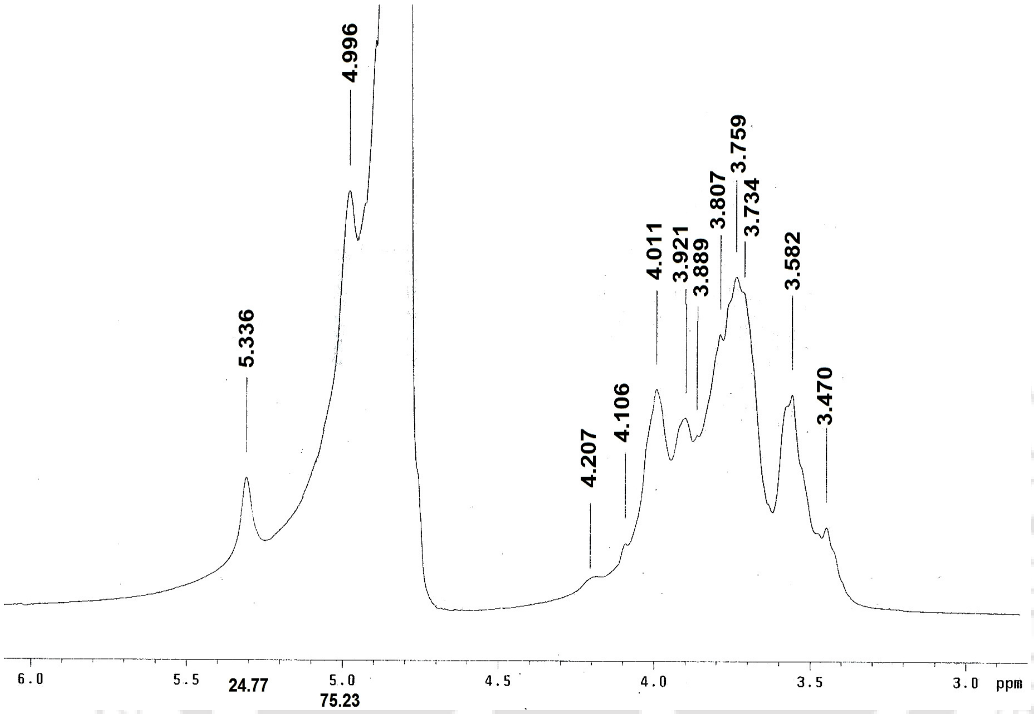

Fig. 4.3.4 ^{1}H-NMR spectrum of dextran from *Pediococcus pentosaceus* CRAG3.

Table 4.3.2 ^{1}H- and ^{13}C-NMR chemical shifts of dextran from *Pediococcus pentosaceus* CRAG3.

Resonances (in ppm)							
	Linear linkages						Branching
Atoms	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6	α-(1→3) linkage
^{1}H	4.99	3.58	3.75	3.47	3.92	4.01	5.33
^{13}C	97.96	71.94	73.61	69.72	70.32	60.57	80.68

4.3.2.5.2 ^{13}C-NMR Spectroscopy

The ^{13}C-NMR spectrum of *Pediococcus pentosaceus* CRAG3 dextran is shown in Fig. 4.3.5. It showed peaks at 73.61, 71.94, 70.32 and 69.72 ppm representing the chemical shifts for C-2, C-3, C-4 and C-5, respectively (Fig. 4.3.5, Table 4.3.2) similar to previously reported data (Gorin, 1981). The peak at 97.96 ppm rather than at 90 ppm showed that branch terminating C-1 was linked and at 60.57 ppm peak suggested the linked nature of C-6 also (Fig. 4.3.5). These results were in accordance with report of Seymour *et al.* (1976). The spectrum also contained two other prominent, though less-intense peaks at 99.51 ppm and 80.68 ppm. The peak at 80.68 ppm in the spectrum of *Pediococcus pentosaceus* CRAG3 dextran was indicative of branching and was diagnostic of the presence of α-(1→3) linkages (Table 4.3.1). Similar results were also reported by Uzochukwu *et al.* (2001). The other peak at 99.51 ppm indicated that the branching in dextran occurred through 3,6-di-O-substituted residues similar to those observed by Uzochukwu *et al.* (2001).

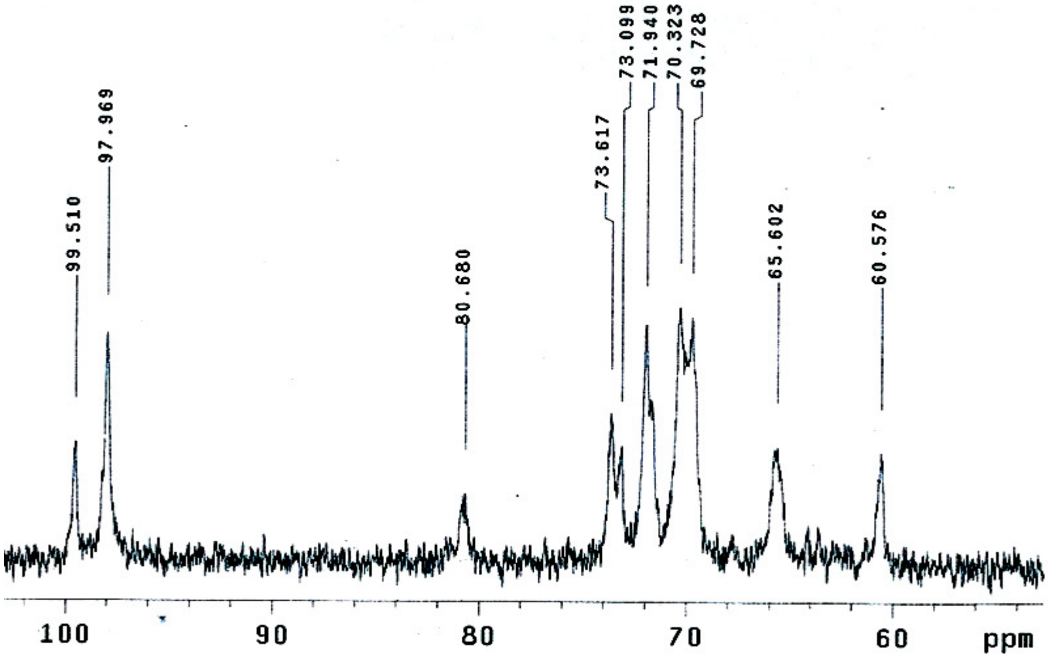

Fig. 4.3.5 ^{13}C-NMR spectrum of dextran from *Pediococcus pentosaceus* CRAG3.

The degree of linearity or relative concentration of linkages in *Pediococcus pentosaceus* CRAG3 dextran was determined by the concept of anomeric ratio which is used to determine the value n (n= number of chain extending residue per branch point residue) (Uzochukwu *et al.*, 2001; Seymour *et al.*, 1979a; Seymour *et al.*, 1979b). The value of n in CRAG3 dextran was 4.31 for α-(1→3) branching which indicated that the branching of α-(1→3) linkage occurred once every five glucose residues.

4.3.3 *In vitro* cytotoxicity assay of dextran from *Pediococcus pentosaceus* CRAG3

4.3.3.1 Cytotoxic effects of dextran on HeLa and HT29 cell lines

The dextran from *Pediococcus pentosaceus* CRAG3 showed anticancer activity against both cervical cancer (HeLa) and colon cancer (HT29) cell lines. The concentration dependent effects of dextran on cervical cancer (HeLa) and colon cancer (HT29) cell lines are shown in Fig. 4.3.6A and 4.3.6B, respectively. The decrease in viability of HeLa cells was observed with increasing concentration from 0.05 to 1.0 µg/ml (Fig. 4.3.6A). At the concentrations from 0.1 to 1.0 µg/ml the dextran significantly inhibited HeLa cells proliferation ($p<0.05$). However, at concentrations higher than 1 µg/ml, no clear relationship between dextran concentration and its effect on the HeLa cell line could be observed. The viability of HT29 cells decreased when the dextran concentration increased from 0.1-500 µg/ml (Fig. 4.3.6B). The significant decrease in viability of HT29 cells was observed at the dextran concentration range from 5 to 500 µg/ml ($p<0.05$). At highest concentration of dextran (1 µg/ml) the percent survival of HeLa cells was only 63.3±3.2% while the percent survival of HT29 cells was only 34.24±0.54% at 500 µg/ml dextran concentration. These results showed that the dextran from *Pediococcus pentosaceus* CRAG3 displayed reduced viability of both HeLa and HT29 cell lines which might be due to the ability of dextran to modify membrane surface proteins in tumour cells which may lead to adhesion disturbances. Thus, it was concluded that the branched dextran from *Pediococcus pentosaceus* CRAG3 reduced the cell viability of HeLa and HT29 cell lines. Zhang and Cheung (2002) also observed similar results with sulphated α-glucans. As compared to β-glucans the biological activity of α-glucans are reported, especially after their chemical or structural modification (carboxymethylation, sulfation, aminopropylation, or hydroxyethylation). α-Glucan from a lichen *Ramalina celastri* showed cytotoxic effect against HeLa cells (Carneiro *et al.*, 1997). α-Glucan from edible mushroom *Pleurotus ostreatus* showed promising anti-tumorigenic properties against HT29 colon cancer cell growth (Lavia *et al.*, 2006). The anticancer effect of biomacromolecules could be due to the stimulation of cell-mediated immune response (Li *et al.*, 2008).

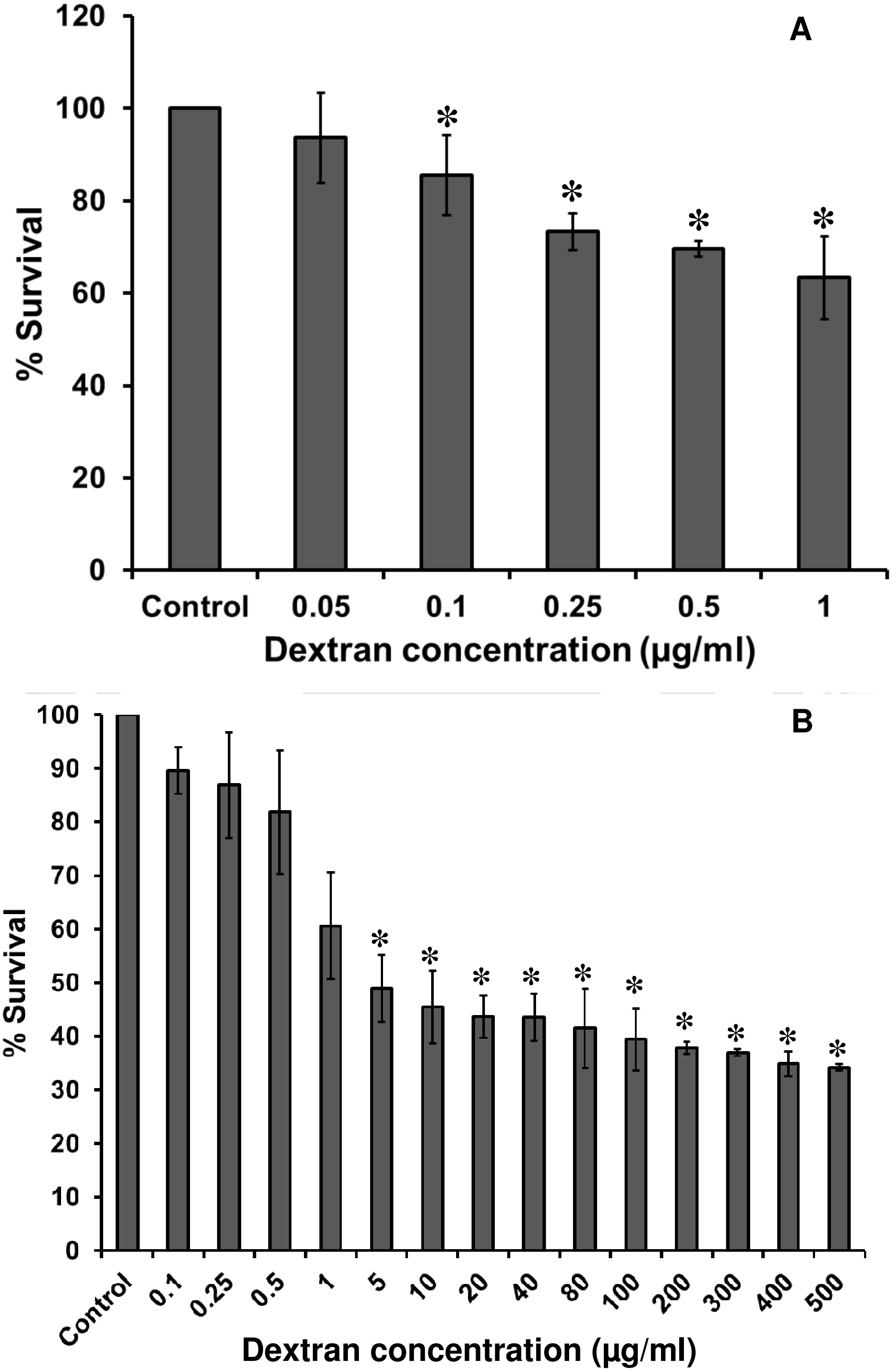

Fig. 4.3.6 Effect of dextran from *Pediococcus pentosaceus* CRAG3 on (A) Cervical cancer (HeLa) cell line and (B) Colon cancer (HT29) cell line (*p< 0.05)

4.3.3.2 Cytotoxic effects of dextran on murine macrophages line

The effect of dextran (1-1000 µg/ml) on murine macrophage line showed increased growth of macrophages (Fig. 4.3.7). Dextran from *Pediococcus pentosaceus* CRAG3 at 5.0 µg/ml concentration showed significant 20% increase ($p<0.05$) in growth of macrophages. However, increase in dextran concentration beyond 5.0 µg/ml did not increase further the growth of macrophages, rather it remained constant but higher than the control even up to 1000 µg/ml. The enhanced growth of murine macrophage line due to CRAG3 dextran might be beneficial in mediating immunity in vertebrates since the macrophages along with neutrophils and Natural Killer (NK) cells form first line of host defense and mediate innate immunity. The activated macrophages over-produce inducible nitric oxide synthase having NADPH diphorase activity which reduces nitroblue tetrazolium (NBT) salts to formazan blue resulting in overestimation of MTT assay (Pozzolini *et al.*, 2003). Although β-glucans are the potent stimulator of innate immunity, the immunostimulating effects of α-glucans are unexplored. The β-glucans activate macrophages by interacting with cognate receptors on namely complement receptor 3 (CD11b/CD18 or CR3), Dectin-1 and Toll-like receptors (TLRs) 2 and 6 releasing cytokines, chemokines and reactive oxygen intermediates (Gantner *et al.*, 2003). The biomacromolecules such as β-glucans are also involved in triggering of granulocytes, monocytes, macrophages and NK-cells to secrete IFN-g, IL-6, IL-8 and IL-12 from macrophages, neutrophils and NK-cells (Ladanyi *et al.*, 1993). Many of those natural biomacromolecules also showed antibacterial and antitumor activities (Wang *et al.*, 1996; Leung *et al.*, 1997) which might be due to activation of macrophages.

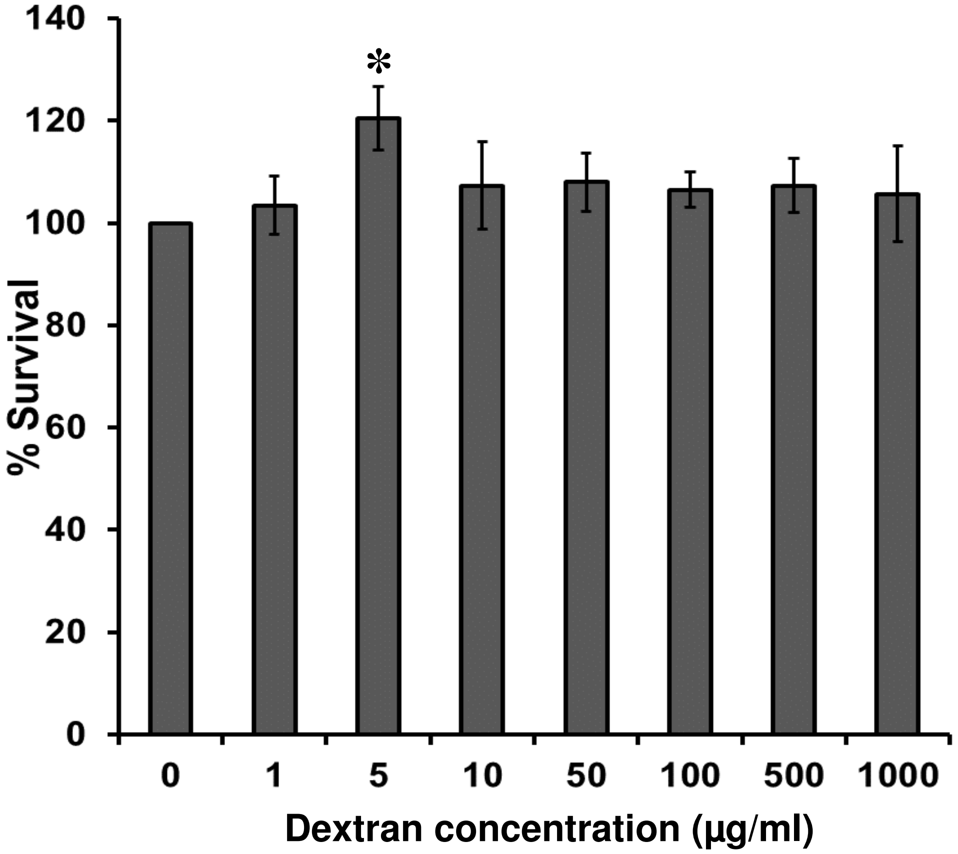

Fig. 4.3.7 Effect of dextran from *Pediococcus pentosaceus* CRAG3 on murine macrophages (J774A.1) line. ($p<0.05$)

4.4 Conclusions

The dextran produced by dextransucrase from *Pediococcus pentosaceus* CRAG3 (GenBank ID: JX679020) was purified and its structure and properties were characterised. The purified dextran displayed number average molecular weight, $2.93x10^5$. The high molecular weight dextran can be used as food ingredient in bakery products or in gel filtration material like Sephadex products. The SEM analysis of dextran revealed porous structure, which accounts for its importance as a carrier for drug delivery. The monosaccharide analysis of dextran confirmed the presence of only glucose subunits. The FTIR spectrum showed the presence of α-(1→6) and α-(1→3) linkages which were further confirmed by NMR spectroscopy. The ^{1}H-NMR spectrum confirmed the presence of α-(1→6) linear chain and α-(1→3) branching. The dextran possesses 75% α-(1→6) linear linkage and 25% α-(1→3) branched linkages. The ^{13}C-NMR spectrum of dextran also confirmed the presence of α-(1→6) and α-(1→3) linkages. The dextran with branching can be used for production of branched oligosaccharides which have superior prebiotic properties over linear oligosaccharides. The *in vitro* cytotoxicity effect of *Pediococcus pentosaceus* CRAG3 dextran on HeLa and HT29 cell lines showed its anti-cancer activity against both cell lines. Dextran at 1 µg/ml for HeLa cells and 500 µg/ml for HT29 cells, displayed per cent survival of 63.3±3.2% and 34.24±0.54%, respectively. The polysaccharides with anti-cancer activity have been shown to activate macrophages. The effect of dextran on murine macrophages (J774A.1) line showed increase in macrophage growth by 20%. It was concluded that the anti-cancer activity of dextran from *Pediococcus* *pentosaceus* CRAG3 might be due to macrophage activation. This branched dextran from *Pediococcus pentosaceus* CRAG3 may potentially be used for clinical purposes.

4.5 References

Anwar, M.A., Kralj, S., Pique, A.V., Leemhuis, H., van der Maarel, M.J.E.C., Dijkhuizen, L. (2010) Inulin and levan synthesis by probiotic *Lactobacillus gasseri* strains: characterization of three novel fructansucrase enzymes and their fructan products. Microbiol. 156, 1264-1274.

Badel, S., Bernardi, T. and Michaud, P. (2011) New perspectives for *Lactobacilli* exopolysaccharides. Biotechnol. Adv. 29, 54–66.

Bao, X., Duan, J., Fang, X. and Fang, J. (2001) Chemical modifications of the (1 →3)-α-D-glucan from spores of *Ganoderma lucidum* and investigation of their physicochemical properties and immunological activity. Carbohydr. Res. 336, 127-140.

Bejar, W., Gabriel, V., Amari, M., Morel, S., Mezghani, M., Maguin, E., Fontagne-Faucher, C., Bejar, S., Chouayekh, H. (2013) Characterization of glucansucrase and dextran from *Weissella* sp. TN610 with potential as safe food additives. Int. J. Bio. Macromol. 52, 125-132.

Cao, W., Li, X-Q., Liu, L., Yang, T-H., Li, C., Fan, H-T., *et al.* (2006) Structure of antitumor polysaccharide from *Angelica sinensis* (Oliv) Diels. Carbohydr. Polym. 66, 149-159.

Carneiro-Leao, A.M.A., Buchi, D.F., Iacomini, M., Gorin, P.A.J. and Oliveira, M.B.M. (1997) Cytotoxic effect against HeLa cells of polysaccharides from the lichen *Ramalina celastri*. J. Submicro. Cytol. Pathol. 24, 1-5.

Chen, X. Zhang, L. and Cheung, P.C.K. (2010) Immunopotentiation and anti-tumor activity of carboxymethylated-sulfated β-(1→3)-D-glucan from *Poria cocos*. Int. Immunopharmacol. 10, 398-405.

Chiu, H.C., Hsiue, T. and Chen, W.Y. (2004) FTIR-ATR measurements of the ionization extent of acrylic acid within copolymerized methacrylated dextran/acrylic acid networks and its relation with pH/salt concentration-induced equilibrium swelling. Polymer, 45, 1627-1636.

Collins, M. D., Samelis, J., Metaxopoulos, J. and Wallbanks, S. (1993) Taxonomic studies on some *Leuconostoc*-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. J. Appl. Bacteriol. 75(6), 595-603.

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

Dols, M., Simeon, M.R., Willemot, R.M., Vignon, M.R. and Monsan, P.F. (1998) Structural characterization of the maltose acceptor-products synthesized by *Leuconostoc mesenteroides* NRRL B-1299 dextransucrase. Carbohydr. Res. 305, 549-559.

Falconer, D.J., Mukerjea, R. and Robyt, J.F. (2011) Biosynthesis of dextrans with different molecular weights by selecting the concentration of *Leuconostoc mesenteroides* B-512FMC dextransucrase, the sucrose concentration, and the temperature. Carbohydr. Res. 346, 280-284.

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

Gantner, B. N., Simmons, R. M., Canavera, S.J., Akira, S. and Underhill, D. M. (2003) Collaborative induction of inflammatory responses by Dectin-1 and Toll-like receptor 2. J. Exp. Med. 197, 1107-1117.

Gorin, P.A.J. (1981) Carbon-13 nuclear magnetic resonance spectroscopy of polysaccharides. Adv. Carbohydr. Chem. Biochem. 38, 13-104.

Hassan, A.N. (2008) ADSA foundation scholar award: possibilities and challenges of exopolysaccharide-producing lactic cultures in dairy foods. J. Dairy Sci. 91, 1282–1298.

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

Khan, T., Park, J.K. and Kwon, J.H. (2007) Functional biopolymers produced by biochemical technology considering applications in food engineering. Korean J. Chem. Eng. 24, 816-826.

Ladanyi, A., Timar, J. and Lapis, K. (1993) Effect of lentinan on macrophage cytotoxicity against metastatic tumor cells. Cancer Immunol. Immunother. 36, 123-126.

Lavia, I., Friesemb, D., Gereshc, S., Hadarb, Y. and Schwartza, B. (2006) An aqueous polysaccharide extract from the edible mushroom *Pleurotus ostreatus* induces anti-proliferative and pro-apoptotic effects on HT-29 colon cancer cells. Cancer Lett. 244, 61-70.

Leung, M. Y. K., Fung, K. P. and Choy, Y. M. (1997) The isolation and characterization of an immunomodulatory and anti-tumor polysaccharide preparation from *Flammulina* velutipes. Immunopharmacol. 35, 255-263.

Li, S. G., Wang, D. G., Tian, W., Wang, X. X., Zhao, J. X., Liu, Z. and Chen, R. (2008) Characterization and anti-tumor activity of a polysaccharide from *Hedysarum polybotrys* Hand.-Mazz. Carbohydr. Polym. 73, 344-350.

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

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

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

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Meth. 65, 55-63.

Naessens, M., Cerdobbel, A., Soetaert, W. and Vandamme, E. J. J. (2005) *Leuconostoc* dextransucrase and dextran: production, properties and applications. Chem. Technol. Biotechnol. 80, 845-860.

Park, F.S. (1971) Application of I.R. spectroscopy in biochemistry, biology, and medicine. New York: Plenum. pp. 100-140.

Patel, S., Kasoju, N., Bora, U. and Goyal, A. (2010) Structural analysis and biomedical applications of dextran produced by a new isolate *Pediococcus pentosaceus* screened from biodiversity hot spot Assam. Biores. Technol. 101(17), 6852-6855.

Pe´rez, S., Kouwijzer, M., Mazeau, K., and Engelsen, S. B. (1996) Modeling polysaccharides: Present status and challenges. J. Mol. Graphics. 14, 307-321.

Pozzolini, M., Scarf, S., Benatti, U. and Giovineb, M. (2003) Interference in MTT cell viability assay in activated macrophage cell line. Anal. Biochem. 313, 338-341.

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.

Remaud-Simeon, M., Lopez-Munguia, A., Pelec, V., Paul, F. and Monsan, P. (1994) Production and use of glucosyltransferases from *Leuconostoc mesenteroides* NRRL B-1299 for the synthesis of oligosaccharides containing α-1,2 linkages. Appl. Biochem. Biotechnol. 44, 101-117.

Seymour, F.R., Julian, R.L., Jeanes, A. and Lamberts, B.L. (1980) Structural analysis of insoluble D-glucans by Fourier transform infrared difference-spectrometry: Correlation between structures of dextrans from strains of *Leuconostoc mesenteroides* and of D-glucans from strains of *Streptococcus mutans*. Carbohydr. Res. 86, 227-246.

Seymour, F.R., Knapp, R.D. and Bishop, S.H. (1976) Determination of the structure of dextrans by ^{13}C-nuclear magnetic resonance spectroscopy. Carbohydr. Res. 51, 171-194.

Seymour, F.R., Knapp, R.D., Chen, E.C.M., Jeanes, A. and Bishop, S.H. (1979a) Structural analysis of dextrans containing 2-O-α-D-glucosylated α-D-glucopyranosyl residues at the branch points by use of ^{13}C-nuclear magnetic resonance spectroscopy and gas-liquid chromatography- mass spectrometry. Carbohydr. Res. 71, 231-250.

Seymour, F.R., Knapp, R.D., Chen, F.C.M., Bishop, S.H. and Jeanes, A. (1979b) Structural analysis of *Leuconostoc* dextrans containing 3-O-α-D-glucosylated α-

D-glucosyl residues in both linear chain and branch-point positions by methylation and by ^{13}C-nuclear magnetic resonance spectroscopy and gas-liquid chromatography mass spectrometry. Carbohydr. Res. 74, 41-62.

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.

Slodki, M.E., England, R.E., Plattner, R.D. and Dick, W.E. (1986) Methylation analyses of NRRL dextrans by capillary gas-liquid chromatography. Carbohydr. Res. 156, 199-206.

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.

Soto, E., Kim, Y.S., Lee, J., Kornfeld, H. and Ostroff, G. (2010) Glucan particle encapsulated rifampicin for targeted delivery to macrophages. Polymers, 2(4), 681-689.

Soto, E.R., Caras, A.C., Kut, L.C., Castle, M.K. and Ostroff, G.R. (2011) Glucan particles for macrophage targeted delivery of nanoparticles. J. Drug Deliv. 2012 (2012), Article ID 143524, 13 pages, doi:10.1155/2012/143524.

Storseth, T.R., Hansen, K., Skjermo, J. and Krane, J. (2004) Characterization of a β-D-(1→3)-glucan from the marine diatom *Chaetoceros mulleri* by high-resolution magic-angle spinning NMR spectroscopy on whole algal cells. Carbohydr. Res. 339, 421-424.

Uzochukwu, S., Balogh, E., Loefler, R.T. and Ngoddy, P.O. (2001) Structural analysis by ^{13}C-nuclear magnetic resonance spectroscopy of glucans elaborated by gum-producing bacteria isolated from palm wine. Food Chem. 73, 225-223.

Vasileva, T. Ivanova, I. and Iliev I. (2010) Glucooligosaccharides synthesized by glycosyltransferases from mutant strain *Leuconostoc mesenteroides* M2860 and their prebiotic potential. Biotechnol. Biotechnol. Equip. 24, 553-557.

Wang, H. X., Liu, W. K., Ng, T. B., Ooi, V. E. C. and Chang, S. T. (1996) The immunomodulatory and antitumor activities of lectins from the mushroom *Tricholoma mongolicum*. Immunopharmacol. 31, 205-211.

Welman, A. and Maddox, I. (2003) Exopolysaccharides from lactic acid bacteria: perspectives and challenges. Trends Biotechnol. 21, 269-274.

Wiater, A., Paduch, R., Pleszczyńska, M., Próchniak, K., Choma, A., Kandefer-Szerszeń, M. and Szczodrak, J. (2010) α-(1→3)-D-Glucans from fruiting bodies of selected macromycetes fungi and the biological activity of their carboxymethylated products. Biotechnol. Lett. 787-795, DOI:10.1007/s10529-010-0502-7.

Zhang, P. and Cheung, P.C.K. (2002) Evaluation o-sulfated *Lentinus edodes* α-(1→3)-D-glucan as a potential antitumor agent. Biosci. Biotechnol. Biochemi. 66, 1052-1056.

Zhang, P., Zhang, L. and Cheng, S. (1999) Chemical structure and molecular weights of α-(1→3)-D-glucan from *Lentinus edodes*. Biosci. Biotechnol. Biochem. 63, 1197-1202.

Chapter 5

Enhanced production of dextransucrase and dextran from *Pediococcus pentosaceus* CRAG3 in bioreactor using Taguchi methodology

5.1 Introduction

Dextransucrases are large size extracellular enzymes which use sucrose as substrate to form dextran, a polymer of glucose (Purama and Goyal, 2005). Dextrans are the exopolysaccharides (EPS) excreted by lactic acid bacteria (LAB) into their surroundings. The dextransucrases and dextrans are primarily produced by four genera of lactic acid bacteria (LAB); *Streptococcus*, *Leuconostoc*, *Weissella* and *Lactobacillus* (van Geel-Schutten *et al.*, 1998; Monchois *et al.*, 1999; Kralj *et al.*, 2002; Tieking *et al.*, 2003; Kralj *et al.*, 2004; Kralj *et al.*, 2005; Purama and Goyal, 2005). Exopolysaccharides play an important role like as adhesives (Costerton *et al.*, 1987); in cellular recognition processes as slime forming agents for protection against dehydration, phagocytosis or toxins (Ceri *et al.*, 1986) and as thickeners, stabilizers, and gelling agents in food and dairy industry (Sandford and Baird, 1983). They have also potential applications in pharmaceutical, food, agriculture and fine chemical industries (Sutherland, 1998). Korakli and Vogel (2006) studied their role as potential therapeutic agents and also as viscosifying, stabilizing, emulsifying, sweetening, gelling, or water-binding agents, in the food as well as in the non-food industries. It had been reported that polysaccharides with α-(1→3) and α-(1→4) linked branches have anti-cancerous properties (Cao *et al.*, 2006; Liu *et al.*, 2007). The dextran with α-(1→6) and α-(1→4) linkages has also been reported from *L. dextranicum* isolated from palm wine (Uzochukwu *et al.*, 2002) and from *Lactobacillus reuteri* (Kralj *et al.*, 2005).

The dextransucrase production is affected by numerous factors like temperature, aeration, medium components (Tsuchiya *et al.*, 1952; Lazic *et al.*, 1993; Goyal *et al.*, 1995; Rodrigues *et al.*, 2003; Cortezi *et al.*, 2005). Hence, there is a need of developing proper fermentation medium to improve its efficient utilization in fermentation technology. The initial step in the process of medium optimization is the screening of significant factors affecting the production. However, alteration of one factor at a time, for conventional optimization of product formation, is a time and labor consuming process and also it does not effectively provide the combinatorial effect of studied factors (Beg *et al.*, 2003). Hence, to overcome such situation, statistical approaches have been developed using a combination of mathematical and analytical tools (Box and Hunter, 1975). In this respect, recently developed orthogonal array (OA) optimization methodology provides offline quality control of system, parameter, and tolerance designs that help in the identification of key factors and their levels for best performance (Rao *et al.*, 2004). For this, simultaneous study of various control factors and their optimization by statistical experimental designs had been done (Stone and Veevers, 1994; Abdel-Fattah *et al.*, 2005).

Taguchi experimental design is reported to be better over other statistical methods as it allows simultaneous examination of many factors and also allows the extraction of more quantitative information by employing only a few experiments (Cobb and Clarkson, 1994; Houng *et al.*, 2006). Taguchi method examines the effects of many variables and identifies only those which have major effect on the process and it involves large number of experimental situation known as orthogonal arrays (OA) and reduces experimental errors and enhances the efficiency and reproducibility of the experiments (Prasad *et al.*, 2005). Taguchi's method has been applied successfully on production of monoesters from lipase (Han *et al.*, 1998), optimization of ELISA procedures (Jeney *et al.*, 1999) and production of griseofulvin from *P. griseofulvin* (Dasu *et al.*, 2003).

The present study deals with optimization strategy for enhancement of production of dextransucrase and dextran from *Pediococcus pentosaceus* CRAG3 using statistical method. One Variable at a Time (OVAT) approach and Taguchi orthogonal array design was used to analyze the effect of various medium components on their production. The validation of data was done at flask and bioreactor level. The optimized medium gave a significant enhancement in both dextransucrase activity and dextran concentration by *Pediococcus pentosaceus* CRAG3.

5.2 Materials and Methods

5.2.1 Culturing and maintenance of microorganism

The bacterial strain *Pediococcus pentosaceus* CRAG3 (Genbank accession number JX679020) was isolated from fermented cucumber as described in Chapter 2, Section 2.2.2. It was propagated in modified MRS agar medium (Goyal and Katiyar, 1996) as a stab incubated at 30°C, stored at 4°C and subcultured every 15 days. The modified MRS agar medium was prepared as described in Chapter 2, Section 2.2.3.

5.2.2 Production of dextransucrase and dextran

The enzyme production medium (Tsuchiya *et al.*, 1952) as described earlier in Chapter 2 Section 2.2.4 was used for production of dextransucrase and dextran. One loopful culture from stab was inoculated in 5 ml enzyme production medium and incubated at 25°C and 180 rpm for 12 h. 1% (v/v) from this broth was transferred to 100 ml sterile enzyme production medium in 250 ml Erlenmeyer flask and incubated at 25°C and 180 rpm for 6 h. The cells were removed by centrifuging at 8,000***g*** and 4°C for 10 min and culture supernatant was analyzed for dextransucrase activity and dextran concentration as described in Chapter 2, Section 2.2.5.1.3 and 2.2.6, respectively.

5.2.3 Enzyme assay

The enzyme assay was carried out in 1 ml reaction mixture containing 5% (w/v) sucrose in 20 mM sodium acetate buffer (pH 5.4) and 20 µl cell-free supernatant. The enzyme activity was determined by the estimation of released reducing sugar as described in Chapter 2, Section 2.2.5.1.3 (Nelson, 1944; Somogyi; 1945)

5.2.4 Analysis of dextran concentration of the isolate

The dextran concentration of cell free supernatant from *Pediococcus pentosaceus* CRAG3 was determined by phenol-sulphuric acid method described by Fox and Robyt (1991) in microtiter plate (Dubois *et al.*, 1956) as described in Chapter 2, Section 2.2.6.

5.2.5 Sucrose estimation

5.2.5.1 Preparation of DNS reagent for reducing sugar estimation

3,5-Dinitrosalycylic acid (DNS) reagent was prepared as per the method as described by Sumner and Sisler, 1944. Potassium sodium tartrate (Rochelle salt, 183 g) was dissolved in 500 ml of hot water and mixed with 262 ml of 2 N NaOH. DNS (6.3 g) was slowly added to above mixture and stirred continuously. Redistilled phenol (5.0 g) and anhydrous sodium metabisulphite (5.0 g) were added to the above reagent mixture and cooled to room temperature. The volume was made up to 1.0 l and stored in an amber colour bottle.

5.2.5.2 Estimation of sucrose

To the 0.5 ml of cell free supernatant, 1 ml 1 N HCl was added and heated in a boiling water bath for 10 min. After cooling to room temperature, 1 ml of 1 N NaOH and 3 ml of DNS reagent were added. The mixture was diluted by adding 9.5 ml distilled water. The absorbance was taken at 540 nm using UV-Visible spectrophotometer (Varian, Cary 100). The fructose in the range of 0.1 to 1.0 mg/ml was used to plot standard.

5.2.6 Screening of nutrients for dextransucrase and dextran production from *Pediococcus pentosaceus* CRAG3 using One Variable at a Time Approach

5.2.6.1 Effect of carbon source on dextransucrase and dextran production

The enzyme production medium (Tsuchiya *et al.*, 1952) as described earlier in Chapter 2, Section 2.2.4 (but with varying concentrations of sucrose) was used to study the effects of carbon source (sucrose) on dextransucrase and dextran production from *Pediococcus pentosaceus* CRAG3. 1% of overnight grown culture of *Pediococcus pentosaceus* CRAG3 was inoculated in 50 ml enzyme production medium containing various concentrations of sucrose from 1-8% (w/v) and incubated at 25°C and 180 rpm for 6 h. The enzyme production medium containing 2% (w/v) sucrose was taken as control. The aliquot (1 ml) from samples were withdrawn from each concentration. The cells were removed by centrifuging at 10,000***g*** and 4°C for 10 min and the cell free supernatants were analyzed for their dextransucrase activity and dextran concentration as described earlier in Chapter 2, Section 2.2.5.1.3 and 2.2.6, respectively. All the experiments were performed in triplicates and the average values were taken.

5.2.6.2 Effect of nitrogen sources on dextransucrase and dextran production

The enzyme production medium (Tsuchiya *et al.*, 1952) as described earlier in Chapter 2, Section 2.2.4 supplemented with varying concentrations of yeast extract (0.2-4%, w/v) was used to determine its effects on dextransucrase and dextran production. However, to determine the effects of beef extract and peptone on dextransucrase and dextran production, the enzyme production medium (which already contained 2%, w/v yeast extract) supplemented with varying concentrations of beef extract (0.1-2%, w/v) and peptone (0.5-4%, w/v) were used. 1% of overnight grown culture of *Pediococcus pentosaceus* CRAG3 was inoculated in 50 ml of enzyme production medium supplemented with variable concentrations of yeast extract, beef extract or peptone. All sets of media were incubated at 25°C and 180 rpm for 6 h. The enzyme production medium (containing 2% yeast extract) was taken as control. After incubation the 1 ml aliquots were withdrawn and centrifuged at 10,000***g*** and 4°C for 10 min and the cell free supernatants were analyzed for dextransucrase activity and dextran concentration as described earlier in Chapter 2, Section 2.2.5.1.3 and 2.2.6, respectively. All the experiments were conducted in triplicates and the average values were taken.

5.2.6.3 Effect of K_2HPO_4 on dextransucrase and dextran production

The effect of K_2HPO_4 on dextransucrase and dextran production was determined by varying its concentration from 0.1-4% (w/v) by keeping the concentration of other components constant in enzyme production medium as described earlier in Chapter 2, Section 2.2.4. 1% of overnight grown culture of *Pediococcus pentosaceus* CRAG3 was inoculated in 50 ml enzyme production medium containing varying concentrations of K_2HPO_4 and incubated at 25°C and 180 rpm for 6 h. After incubation, the cells were removed by centrifuging at 10,000***g*** and 4°C for 10 min and the cell free supernatants from all samples were analyzed for their dextransucrase activity and dextran concentration as described earlier in Chapter 2, Section 2.2.5.1.3 and 2.2.6, respectively. The enzyme production medium containing 2% (w/v) K_2HPO_4 as described earlier in Chapter 2, Section 2.2.4 was taken as control. All the experiments were conducted in triplicates and the average values were taken.

5.2.6.4 Effect of Tween 80 on dextransucrase and dextran production

The effect of Tween 80 on dextransucrase and dextran production was determined by varying their concentrations from 0.05-2% (w/v). 1% of overnight grown culture of *Pediococcus pentosaceus* CRAG3 was inoculated in 50 ml enzyme production medium as described earlier in Chapter 2, Section 2.2.4 supplemented with variable concentrations of Tween 80 and incubated at 25ºC and 180 rpm for 6 h. After incubation, the cells were removed by centrifuging at 10,000***g*** and 4ºC for 10 min and the cell free supernatants from all samples were analyzed for their dextransucrase activity and dextran concentration as described earlier in Chapter 2, Section 2.2.5.1.3 and 2.2.6, respectively. The enzyme production medium as described earlier in Chapter 2, Section 2.2.4 was taken as control. All the experiments were conducted in triplicates and the average values were taken.

5.2.7 Optimization of dextransucrase and dextran production from *Pediococcus pentosaceus* CRAG3 by Taguchi's orthogonal array methodology

Taguchi methodology was performed to statistically optimize the medium for dextransucrase and dextran production by *Pediococcus pentosaceus* CRAG3 by the method described by Prakasham *et al.* (2005). It consisted of four interconnected sequential phases:

i) Planning: It included selection of factors.

ii) Conducting: It comprised of designing of experiment and defining of data analysis procedure.

iii) Analysis of data by fitting the results in Taguchi software and

iv) Validation: It included the use of optimized parameters in experiment.

In the present Taguchi design of experiment (DOE), six fermentation factors sucrose, yeast extract, beef extract, peptone, dipotassium hydrogen orthophosphate (K_2HPO_4) and Tween 80 were selected to analyze their effect on dextransucrase and dextran production. Based on the results obtained by OVAT approach, three levels of factor variations were considered. An L27 orthogonal array (OA) was designed consisting of 27 different experimental trials. The total degree of freedom available in an OA was equal to the number of trials minus one. Fermentations were carried out by using 12 h old culture of *P. pentosaceus* CRAG3 grown in enzyme production medium described by Tsuchiya *et al.* (1952). 1% (v/v) culture was inoculated in each of 27 different compositions of media and incubated at 25°C and 180 rpm for 6 h. After incubation all media were analyzed for their dextransucrase activity and dextran concentration as described earlier in Chapter 2, Section 2.2.5.1.3 and 2.2.6, respectively. All experiments were performed in triplicates. The design for the L27 orthogonal array was developed and analyzed using "MINITAB 15" software for predicted values, individual and interactive influences, ANOVA and to determine the contribution of each selected factor in dextransucrase and dextran production by the isolate. The validation of data was done by using optimized parameters of fermentation media components and levels by software in shake flask.

5.2.8 Scale up of dextransucrase and dextran production from *Pediococcus pentosaceus* CRAG3 at bioreactor level

The statistically optimized medium for dextransucrase and dextran from *P. pentosaceus* CRAG3 using Taguchi's orthogonal methodology was scaled up at bioreactor level using a 3L bioreactor (Applikon, model Bio Console ADI 1025) with 1L working volume. The bioreactor containing statistically optimized medium for dextransucrase or dextran was autoclaved at 121°C, 15 lb/in^2 pressure for 20 min and allowed to cool at room temperature. The dissolved oxygen (DO) was adjusted to 100% before inoculation. 1% inoculum from 12 h grown culture was inoculated in each bioreactor. The pH and temperature were kept constant at 7.0 (using 2M NaOH and 2M HCl) and 25°C, respectively throughout the fermentation process. The agitation was set to 180 rpm at the beginning of the run but changed accordingly to keep the DO above 40%. During fermentation, the parameters like cell absorbance, enzyme activity, sucrose concentration and dextran concentration were analyzed at every 2 h interval. The cell growth was observed by measuring the absorbance at 600 nm (A_{600}). The optimized medium without culture was used as blank. The enzyme activity of cell free supernatant was analyzed by the method as discussed earlier in Chapter 2, Section 2.2.5.1.3. The sucrose profile of media was observed by estimating the reducing sugars by the method of Sumner and Sisler (1944) as described earlier in Section 5.2.5.2. The dextran concentration of culture supernatant was measured as described earlier in Chapter 2, Section 2.2.6.

5.3 Results and Discussion

5.3.1 Estimation of dextransucrase activity and dextran concentration of isolate

The dextransucrase activity and dextran concentration of the isolate *Pediococcus pentosaceus* CRAG3 in enzyme production medium (Tsuchiya *et al.,* 1952) at 25°C and 180 rpm were determined. The maximum dextransucrase activity showed by the isolate after 6 h was 4.5 U/ml while the maximum dextran concentration was determined to be 4.5 mg/ml.

5.3.2 Screening of nutrients for dextransucrase production from *Pediococcus pentosaceus* CRAG3 using One Variable at a Time Approach (OVAT)

5.3.2.1 Effects of carbon source on dextransucrase production

The effects of sucrose on dextransucrase production is shown in Fig. 5.3.1, Table 5.3.1. The dextransucrase activity increased from 4.5 U/ml to 8.0 U/ml with increasing sucrose concentration from 1-3.5% (w/v) but decreased thereafter. The similar results have been observed in dextransucrase from *Leuconostoc mesenteroides* B-512F where the maximum enzyme production was achieved at lower sucrose concentration and decreased on further increasing the concentration (Goyal and Katiyar, 1997; Lopretti *et al*., 1999). It has been reported that the dextransucrase from *Leuconostoc* spp. is induced by sucrose which leads to synthesis of dextran during production of enzyme (Majumder *et al*., 2007).

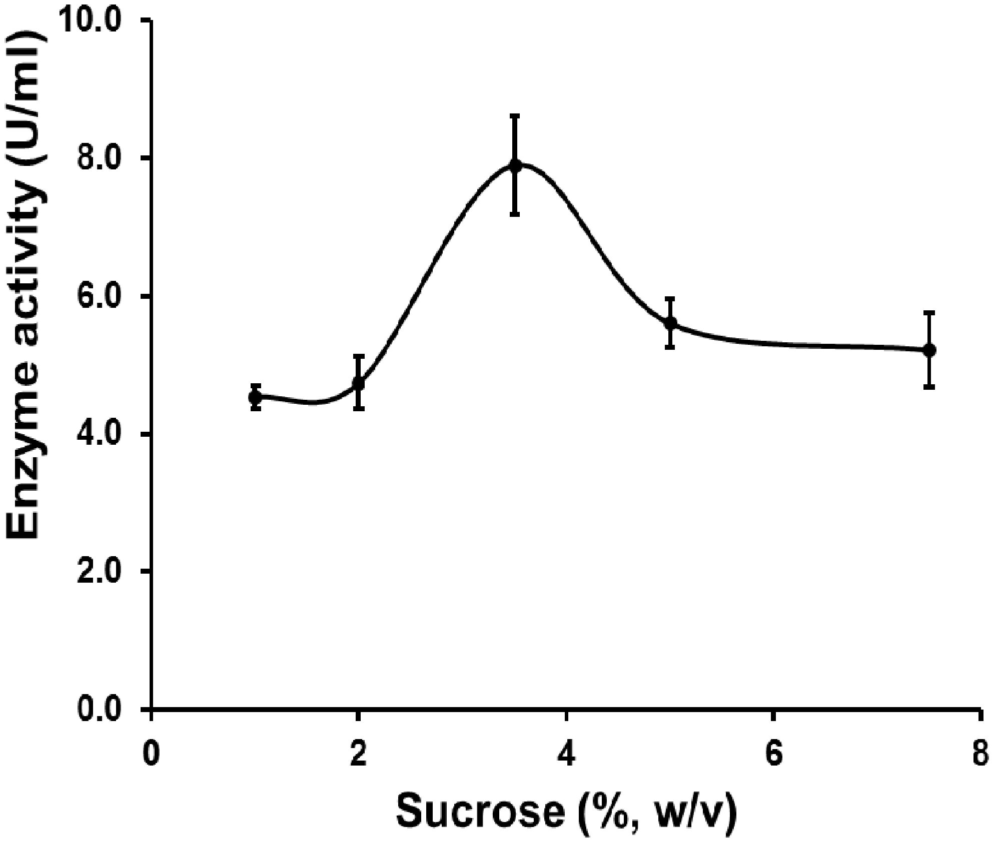

Fig. 5.3.1 Effect of sucrose on dextransucrase production from *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) at 25°C grown for 6 h.

Table 5.3.1 Effects of various nutrients on dextransucrase production from *Pediococcus pentosaceus* CRAG3 using OVAT approach

Nutrient (%, w/v)	Dextransucrase activity (U/ml)	Relative activity (%)
Control medium[a]	4.50	100
Sucrose (3.5)[b]	7.90	175
Yeast extract (1.0)[c]	6.50	144
Beef extract (1.0)[d]	7.35	163
Peptone (1.0)[d]	8.30	184
K_2HPO_4 (1.0)[e]	6.20	138
Tween 80 (1.0, v/v)	9.00	200

[a] Tsuchiya *et al.* (1952) as described in Chapter 2, Section 2.2.4

[b] Enzyme production medium (Tsuchiya *et al.*, 1952), with varying concentrations of sucrose.

[c] Medium contained only yeast extract as nitrogen source.

[d] Medium also contained 2% (w/v) yeast extract along with beef extract or peptone.

[e] Enzyme production medium (Tsuchiya *et al.*, 1952), with varying concentrations of K_2HPO_4.

5.3.2.2 Effects of nitrogen sources on dextransucrase production

The three nitrogen sources; yeast extract, beef extract and peptone were used to determine the effects of nitrogen source on dextransucrase activity. On increasing the concentration of yeast extract from 0.2-1% (w/v) the dextransucrase activity increased from 5.9 to 6.5 U/ml (Fig. 5.3.2A, Table 5.3.1). However, on further increase in yeast extract concentration the activity decreased, which showed that the higher concentration of yeast extract did not support the enzyme production. The similar results were observed in *Leuconostoc mesenteroides* B-640 (Purama and Goyal, 2008) and *Leuconostoc dextranicum* B-1146 (Majumder and Goyal, 2008) where the low concentration of yeast extract favoured dextransucrase production. The effects of beef extract and peptone on dextransucrase activity is shown in Fig. 5.3.2B and 5.3.2C, respectively. The increase in beef extract concentration upto 1% caused enhancement in dextransucrase activity to 7.3 U/ml as compared to control (4.5 U/ml) but decreased thereafter (Fig. 5.3.2B, Table 5.3.1). Similarly, on increasing peptone concentration to 1% the enzyme activity increased 8.3 U/ml but further increase in concentration did not support the dextransucrase production (Fig. 5.3.2C, Table 5.3.1). The decrease in activity might be due to the effect of trace elements present in beef extract and peptone (Majumder and Goyal, 2008). These results were supported by previous reports on lactic acid bacteria; *Leuconostoc mesenteroides* B-640 (Purama and Goyal, 2008) and *Leuconostoc dextranicum* B-1146 (Majumder and Goyal, 2008). The increase in dextransucrase production in medium supplemented with peptone and $CaCl_2$ along with yeast extract and other higher phosphate was also observed by Qader *et al.* (2003), which concluded that peptone plays an important role in dextransucrase production.

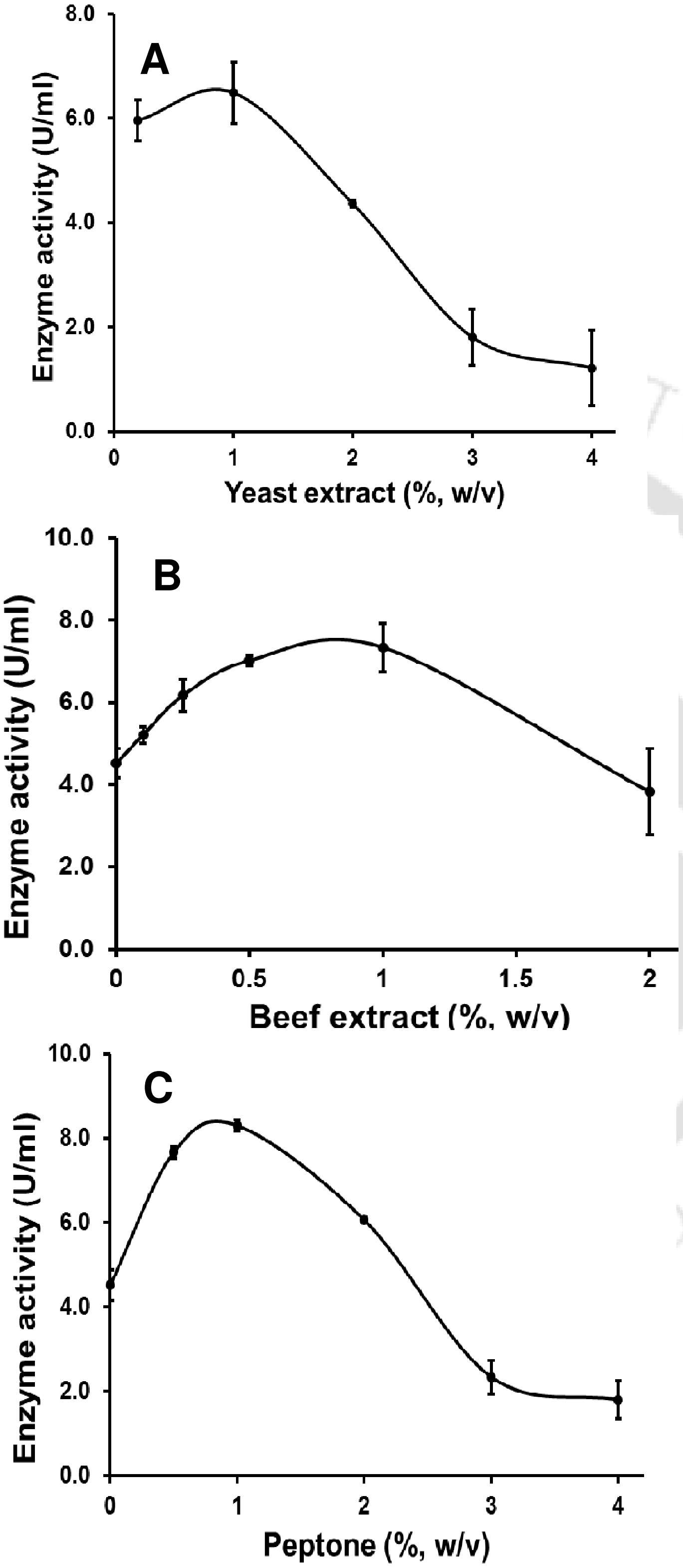

Fig. 5.3.2 Effect of various nitrogen sources on dextransucrase production from *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) at 25°C. **(A)** Yeast extract; **(B)** Beef extract and **(C)** Peptone.

5.3.2.3 Effects of K_2HPO_4 on dextransucrase production

K_2HPO_4 acts as buffering agent for lactic acid produced during fermentation (Tsuchiya *et al.*, 1952; Rodrigues *et al.*, 2003). On increasing the concentration of K_2HPO_4 up to 0.2%, the dextransucrase activity increased to 6.2 U/ml but decreased thereafter (Fig 5.3.3, Table 5.3.1). The similar result was observed in *Leuconostoc mesenteroides* B-512F by Rodrigues *et al.* (2003).

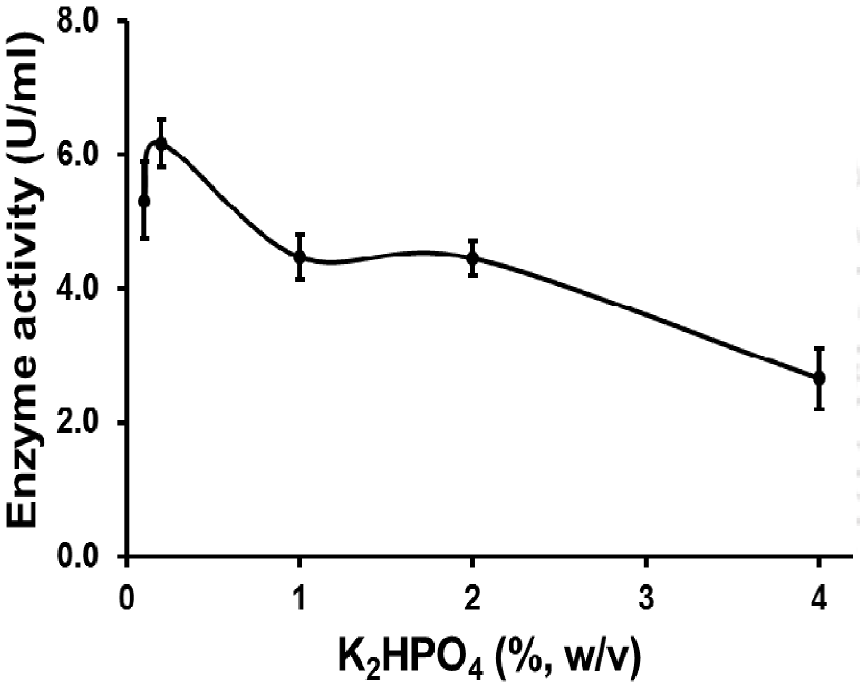

Fig. 5.3.3 Effect of K_2HPO_4 on dextransucrase production from the isolate *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) containing varying concentrations of K_2HPO_4. The culture was grown at 25°C and 180 rpm for 6 h.

5.3.2.4 Effects of Tween 80 on dextransucrase production

K_2HPO_4 acts as buffering agent for lactic acid produced during fermentation (Tsuchiya *et al.*, 1952; Rodrigues *et al.*, 2003). On increasing the concentration of K_2HPO_4 up to 0.2%, the dextransucrase activity increased to 6.2 U/ml but decreased thereafter (Fig 5.3.3A, Table 5.3.1). The similar result was observed in *Leuconostoc mesenteroides* B-512F by Rodrigues *et al.* (2003). The effects of Tween 80 on dextransucrase production by *Pediococcus pentosaceus* CRAG3 is shown in Fig. 5.3.3B. The dextransucrase activity increased to 9.0 U/ml with increasing concentration of Tween 80 up to 1% (v/v). However on further increasing concentration saturation reached and no effect on activity was observed (5.3.4, Table 5.3.1). These results corroborate the earlier findings where the enhancement of dextransucrase activity of *Leuconostoc dextranicum* B-1146 has been observed on increasing Tween 80 up to 1% and saturation was observed between 0.8-1% (Majumder and Goyal, 2008). Tween 80 is an industrial surfactant which helps in secretion of dextransucrase by altering the composition of fatty acids of membrane (Sato *et al.*, 1989; Goyal and Katiyar, 1997). It also facilitates the transfer of nutrients and oxygen to microorganism.

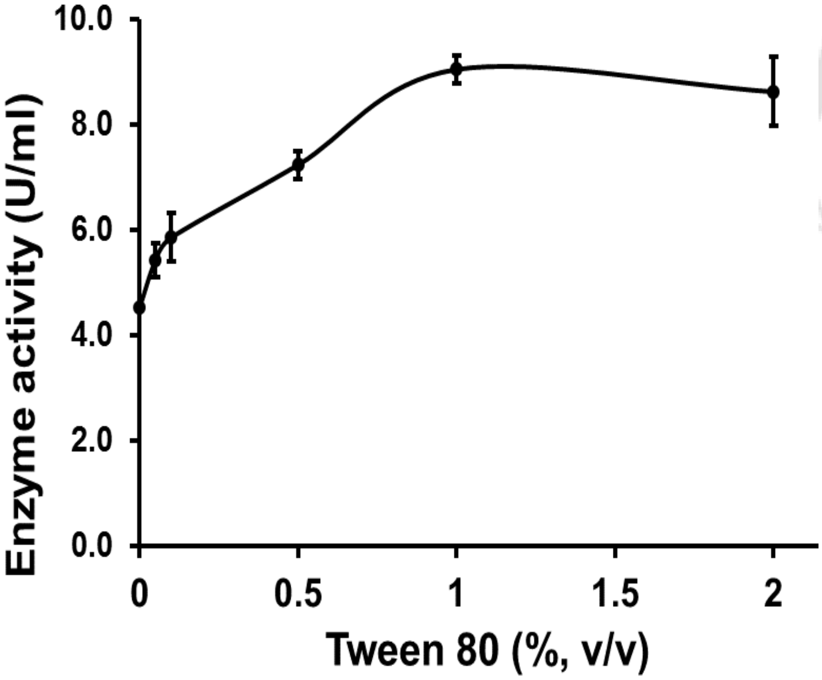

Fig. 5.3.4 Effect of Tween 80 on dextransucrase production from the isolate *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) at 25°C.

5.3.3 Screening of nutrients for dextran production from *Pediococcus pentosaceus* CRAG3 using One Variable at a Time Approach (OVAT)

5.3.3.1 Effects of carbon source on dextran production

Among various nutrients, the sucrose was taken as carbon source to analyse its effect on dextran production, since it acts as substrate for dextransucrase to produce dextran. On increasing the sucrose concentration from 1-4% the dextran concentration increased from 2.0 to 12.6 mg/ml but decreased thereafter (Fig. 5.3.5, Table 5.3.2). Due to high production of dextran the medium became viscous. The decrease in dextran concentration above 4% sucrose concentration might be due to the inhibitory effect of higher sucrose concentration on the dextransucrase.

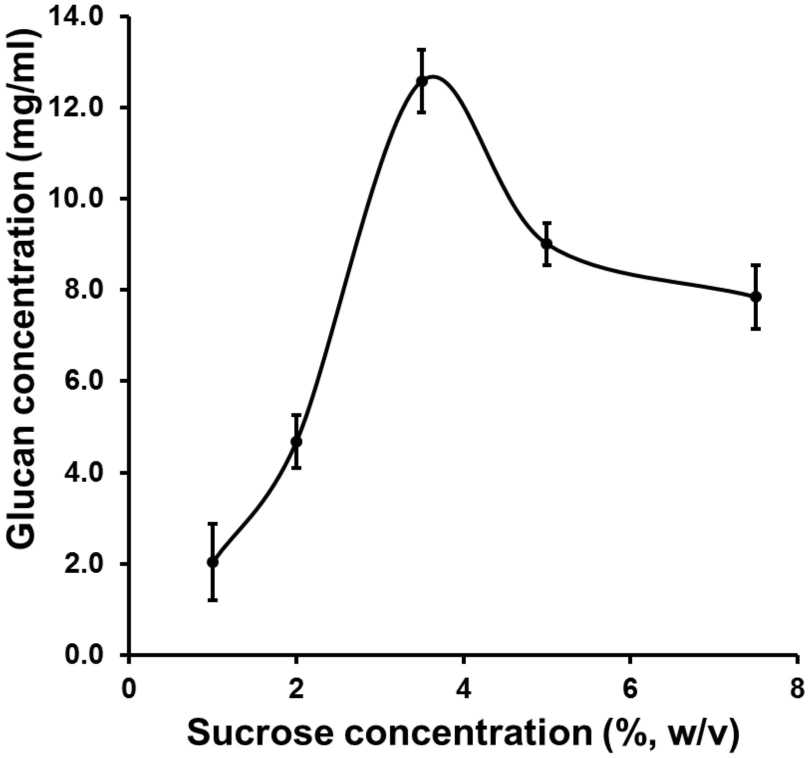

Fig. 5.3.5 Effect of sucrose on dextran production from *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) containing varying concentrations of sucrose. The culture was grown at 25°C and 180 rpm for 6 h.

Table 5.3.2 Effects of various nutrients on dextran production from *Pediococcus pentosaceus* CRAG3 using OVAT approach

Nutrient (%, w/v)	Dextran concentration (mg/ml)	Relative dextran concentration (%)
Control medium[a]	4.50	100
Sucrose (3.5)[b]	12.6	280
Yeast extract (0.2)[c]	5.70	127
Beef extract (0.1)[d]	3.75	83
Peptone (1.0)[d]	4.75	106
K_2HPO_4 (1.0)[e]	12.5	278
Tween 80 (0.05, v/v)	3.60	80

[a] Tsuchiya *et al.* (1952) as described in Chapter 2, Section 2.2.4
[b] Enzyme production medium (Tsuchiya *et al.*, 1952), with varying concentrations of sucrose.
[c] Medium contained only yeast extract as nitrogen source.
[d] Medium also contained 2% (w/v) yeast extract along with beef extract or peptone.
[e] Enzyme production medium (Tsuchiya *et al.*, 1952), with varying concentrations of K_2HPO_4.

5.3.3.2 Effects of nitrogen sources on dextran production

The effects of yeast extract, beef extract and peptone were analyzed on dextran production from *Pediococcus pentosaceus* CRAG3. Amongst three nitrogen sources, yeast extract was most effective for dextran production from *Pediococcus pentosaceus* CRAG3. At 0.2% (w/v) yeast extract, the dextran concentration was 5.7 mg/ml (27% higher) as compared to control (4.5 mg/ml) containing 2% (w/v) yeast extract (Fig. 5.3.6A, Table 5.3.2). The dextran concentration decreased on further increasing the yeast extract concentration (Fig. 5.3.6A). However, beef extract showed negative effect on dextran production. On increasing the concentration of beef extract from 0-2% (w/v), the dextran concentration decreased from 4.5 mg/ml to 0.9 mg/ml (Fig. 5.3.6B). The peptone at its lower concentrations showed only 6% increase in dextran production but at its higher concentration it negatively affected the dextran production from *Pediococcus pentosaceus* CRAG3 (Fig. 5.3.6C, Table 5.3.2).

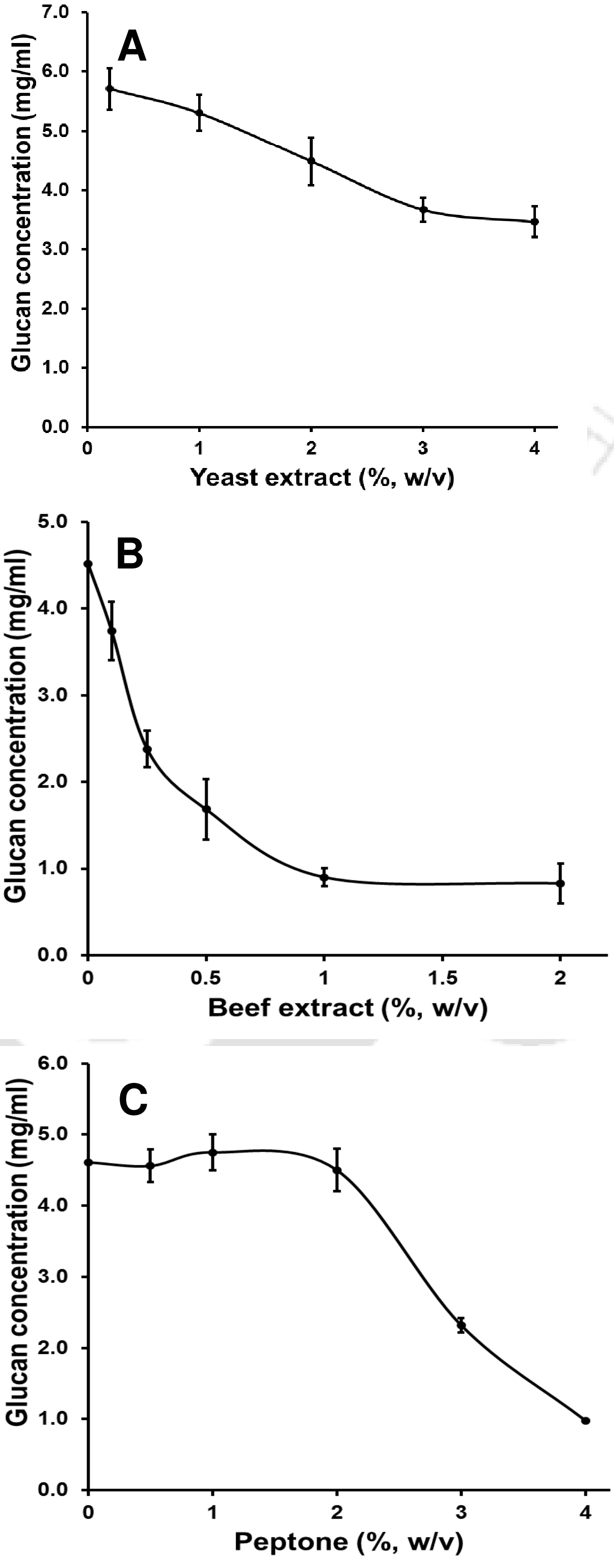

Fig. 5.3.6 Effect of various nitrogen sources on dextran production from *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) at 25°C. **(A)** Yeast extract; **(B)** Beef extract and **(C)** Peptone.

5.3.3.3 *Effects of K_2HPO_4 on dextran production*

The additive K_2HPO_4 acts as buffering agent. On increasing the concentration of K_2HPO_4 to 1% (mg/ml) the dextran concentration increased to 12.4 mg/ml as compared to control (4.5 mg/ml) containing 2% (mg/ml) K_2HPO_4 (Fig. 5.3.7, Table 5.3.2). Similar results have been observed in dextran production from *Weissella confusa* Cab3 (Shukla and Goyal, 2011).

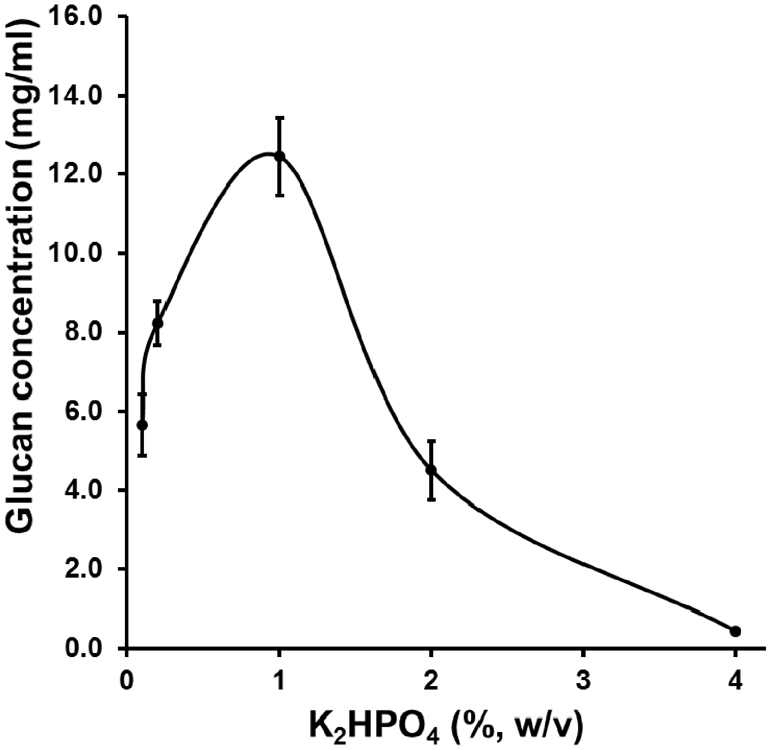

Fig. 5.3.7 Effect of K_2HPO_4 on dextran production from *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) supplemented with varying concentrations of K_2HPO_4. The culture was grown at 25°C and 180 rpm for 6 h.

5.3.3.4 *Effects of Tween 80 on dextran production*

Tween 80 showed negative effect on dextran production. On increasing the concentration above 0.05% (v/v) the dextran content started decreasing and reached to

0.34 mg/ml as compared to control (4.5 mg/ml) (Fig. 5.3.8, Table 5.3.2). These results were not in accordance with earlier findings (Sato *et al.*, 1989; Goyal and Katiyar, 1997; Purama and Goyal, 2008).

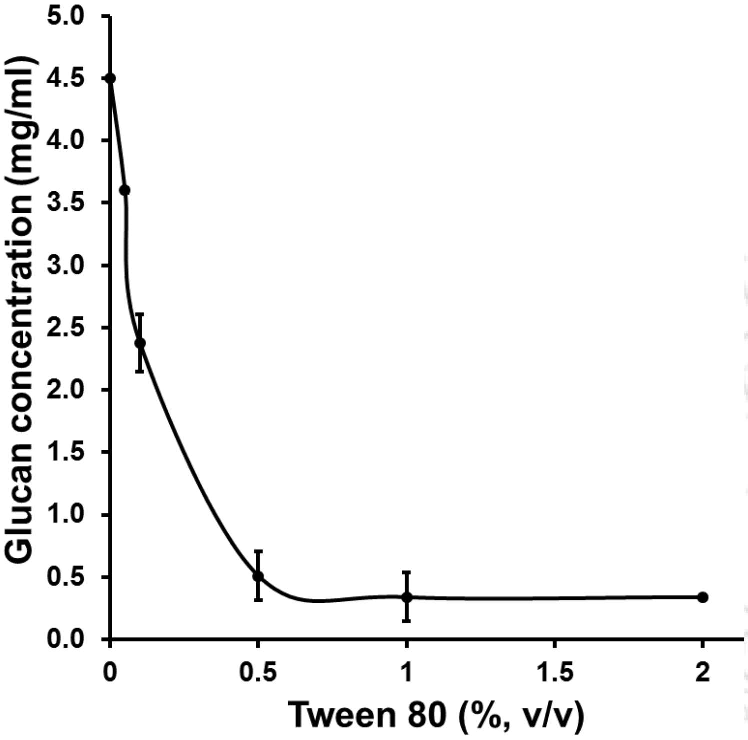

Fig. 5.3.8 Effect of Tween 80 on dextran production from *Pediococcus pentosaceus* CRAG3 in enzyme production medium (pH 6.9) at 25°C.

5.3.4 Taguchi orthogonal array design for dextransucrase production

5.3.4.1 Screening of medium components

The selected fermentation factors and their levels for optimization of dextransucrase production by CRAG3 are presented in Table 5.3.3.

Table 5.3.3 Selected fermentation factors and their assigned levels on dextransucrase and dextran production by *Pediococcus pentosaceus* CRAG3.

S. No.	Factor	Level 1	Level 2	Level 3
1.	**Sucrose**	2.0	3.5	5.0
2.	**Yeast Extract**	0.2	1.0	2.0
3.	**Beef Extract**	0.1	0.5	1.0
4.	**Peptone**	1.0	2.0	3.0
5.	**K_2HPO_4**	0.2	2.0	4.0
6.	**Tween 80**	0.1	0.5	1.0

The combination of concentrations of selected fermentation factors and their effect on dextransucrase activity is shown in Table 5.3.4. A total 27 sets of experiments were performed and variations in dextransucrase activity were observed. Wide variation in dextransucrase activity (from minimum 0.81 U/ml to maximum 10.1 U/ml) was observed in L27 orthogonal array experiments (Table 5.3.4), which showed the importance of optimization for attaining maximum production. The software predicted values were almost similar to experimental values for dextransucrase production, which showed the accuracy in experiment (Table 5.3.4).

Table 5.3.4 Fractional factorial design of L-27 (63) orthogonal array used for dextransucrase activity and dextran production optimization by *Pediococcus pentosaceus* CRAG3.

Run	Suc*	YE*	BE*	Pep*	K_2HPO_4	T80*	Dextransucrase (U/ml)		Dextran (mg/ml)	
	Concentration (%, w/v)						Experi-mental	Predic-ted	Experi-mental	Predic-ted
1	2.0	0.2	0.1	1.0	0.2	0.1	2.90±0.26	2.92	8.54±0.05	7.75
2	2.0	0.2	0.1	1.0	2.0	0.5	1.23±0.39	1.05	3.96±0.18	4.17
3	2.0	0.2	0.1	1.0	4.0	1.0	3.08±0.52	3.25	0.31±0.07	0.89
4	2.0	1.0	0.5	2.0	0.2	0.1	2.90±0.80	3.59	7.02±0.53	7.25
5	2.0	1.0	0.5	2.0	2.0	0.5	2.71±0.92	1.73	3.05±1.13	3.66
6	2.0	1.0	0.5	2.0	4.0	1.0	3.64±1.44	3.93	1.22±0.31	0.380
7	2.0	2.0	1.0	3.0	0.2	0.1	3.27±0.42	3.41	6.41±0.30	6.45
8	2.0	2.0	1.0	3.0	2.0	0.5	1.79±0.65	1.54	1.83±0.00	2.87
9	2.0	2.0	1.0	3.0	4.0	1.0	3.64±1.12	3.74	0.67±0.12	-0.41
10	3.5	0.2	0.5	3.0	0.2	0.5	7.71±0.26	7.34	8.54±0.18	8.54
11	3.5	0.2	0.5	3.0	2.0	1.0	7.52±0.39	7.50	8.84±0.77	6.17
12	3.5	0.2	0.5	3.0	4.0	0.1	1.79±0.39	2.18	1.22±0.00	3.89
13	3.5	1.0	1.0	1.0	0.2	0.5	5.67±0.39	6.04	12.51±0.47	11.57
14	3.5	1.0	1.0	1.0	2.0	1.0	6.41±0.38	6.20	10.06±0.77	9.20
15	3.5	1.0	1.0	1.0	4.0	0.1	1.05±0.26	0.89	5.12±0.10	6.92
16	3.5	2.0	0.1	2.0	0.2	0.5	5.12±0.37	5.67	12.51±0.47	12.09
17	3.5	2.0	0.1	2.0	2.0	1.0	4.75±0.38	5.83	6.71±0.00	9.73
18	3.5	2.0	0.1	2.0	4.0	0.1	2.16±0.26	0.52	10.06±0.47	7.45
19	5.0	0.2	1.0	2.0	0.2	1.0	9.90±0.25	8.95	11.29±0.18	11.66
20	5.0	0.2	1.0	2.0	2.0	0.1	0.81±0.03	1.59	10.68±0.47	10.29
21	5.0	0.2	1.0	2.0	4.0	0.5	1.60±0.26	1.77	5.80±0.61	5.79
22	5.0	1.0	0.1	3.0	0.2	1.0	10.1±1.31	9.59	10.06±0.47	8.82
23	5.0	1.0	0.1	3.0	2.0	0.1	2.72±0.39	2.23	7.02±0.35	7.45
24	5.0	1.0	0.1	3.0	4.0	0.5	1.42±0.65	2.40	2.14±0.30	2.95
25	5.0	2.0	0.5	1.0	0.2	1.0	9.56±0.26	9.59	12.20±0.53	13.55
26	5.0	2.0	0.5	1.0	2.0	0.1	1.97±0.22	2.23	16.05±0.63	15.98
27	5.0	2.0	0.5	1.0	4.0	0.5	2.71±0.39	2.41	10.37±0.18	9.05

*Suc- Sucrose, YE- Yeast Extract, BE- Beef Extract, Pep- Peptone, T80- Tween 80

Table 5.3.5 shows the response table of the selected factors on dextransucrase production. The effect of factors on response (dextransucrase production) was determined by their delta value and corresponding rank (Table 5.3.5). The delta value was calculated by taking the difference between the highest and lowest characteristic average for a factor. Higher delta value means higher effect of that component on response. In present study, the order of medium components for their effect on dextransucrase production was Tween 80 > K_2HPO_4 > Sucrose > Beef extract >

Peptone > Yeast extract, which suggested that Tween 80 had an ample effect and the yeast extract had least effect on dextransucrase production by *Pediococcus pentosaceus* CRAG3.

Table 5.3.5 Response table of means for dextransucrase production by *Pediococcus pentosaceus* CRAG3.

Level	**Sucrose**	**YE***	**BE***	**Peptone**	**K_2HPO_4**	**Tween 80**
1	2.797	4.062	3.721	3.846	6.348	2.174
2	4.688	4.070	4.503	3.733	3.326	3.332
3	4.533	3.887	3.795	4.440	2.345	6.513
Delta	1.891	0.183	0.783	0.707	4.003	4.338
Rank	**3**	**6**	**4**	**5**	**2**	**1**

*YE- Yeast Extract, BE- Beef Extract

ANOVA data indicated the significance of selected factors on enzyme production, which varied with factor to factor. Table 5.3.6 showed that sucrose, Tween 80 and K_2HPO_4 having least P-value were most significant factors for dextransucrase production. The experimental data revealed that out of the 3 selected levels, level 1 value of yeast extract and K_2HPO_4, level 2 value of beef extract and sucrose, and level 3 value of peptone and Tween 80 in the medium were optimal for dextransucrase production (Fig. 5.3.9A-F).

The optimum concentrations for maximum dextransucrase production are shown in Table 5.3.7. The sucrose with a final concentration of 3.5% (w/v) showed maximum dextransucrase activity. Increasing further the sucrose concentration leads to decreased activity of the enzyme (Fig. 5.3.9A). This is in agreement with previous reports where the strains of *Leuconostoc mesenteroides* showed maximum activity at lower concentration of sucrose and no further enhancement in activity was observed above 4% (Goyal and Katiyar, 1997; Lopretti *et al.*, 1999). K_2HPO_4 acts as a buffering agent to the lactic acid produced during the fermentation (Tsuchiya *et al.*, 1952; Rodrigues *et al.*, 2003). The increase in concentration of K_2HPO_4 above 0.2% (w/v) resulted in decreased dextransucrase activity (Fig. 5.3.9E). 1% (v/v) Tween 80 gave maximum dextransucrase activity (Fig. 5.3.9F). Tween 80 is used as a surfactant in industries and its presence in medium enhances secretion of dextransucrase from cells by altering the fatty acid composition of cell membrane (Sato *et al.*, 1989; Goyal and Katiyar, 1997). The other medium components such as yeast extract (Fig. 5.3.9B), beef extract (Fig. 5.3.9C) and peptone (Fig. 5.3.9D) at their final concentration of 0.2% (w/v) 0.5% (w/v) and 3.0% (w/v), respectively resulted in maximum dextransucrase activity.

Table 5.3.6 Analysis of variance (ANOVA) of experimental data on dextransucrase production by *Pediococcus pentosaceus* CRAG3.

Source	**DF***	**Seq SS***	**Adj SS***	**Adj MS***	**F**	**P***
Sucrose	2	19.843	19.8427	09.9213	14.55	0.000
Yeast extract	2	0.193	0.1934	0.0967	0.14	0.869
Beef extract	2	03.360	03.3603	01.6801	2.46	0.121
Peptone	2	02.596	02.5963	01.2981	1.90	0.186
K_2HPO_4	2	78.377	78.3767	39.1884	57.49	0.000
Tween 80	2	90.841	90.8411	45.4206	66.63	0.000
Residual error	14	9.543	9.543	0.6817		
Total	26	204.754				

Table 5.3.7 Optimized fermentation components for dextransucrase and dextran production by *Pediococcus pentosaceus* CRAG3.

Components (%)	Sucrose	Yeast extract	Beef extract	Peptone	K_2HPO_4	Tween 80
Dextransucrase activity (U/ml)	3.5	0.2	0.5	3.0	0.2	1.0
Dextran (mg/ml)	5.0	2.0	0.5	1.0	0.2	0.1

*DF- Degree of Freedom, Seq SS- Sequential Sum of Squares, Adj SS- Adjusted Sum of Squares, Adj MS- Adjusted Mean of Squares, P- Probability

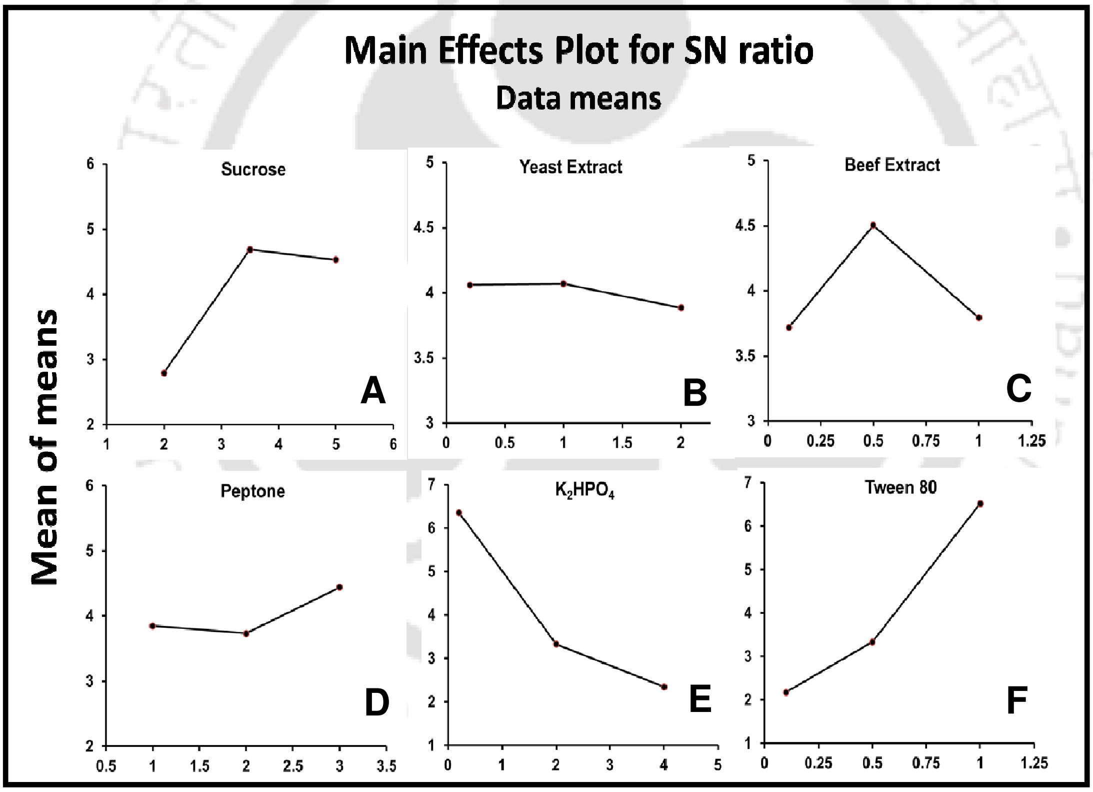

Fig. 5.3.9 Impact of selected fermentation factors and their assigned level on dextransucrase production by *Pediococcus pentosaceus* CRAG3. X-axis represents assigned levels of selected factor (%, w/w or w/v) and Y-axis represents dextransucrase activity (U/ml). **(A)** Sucrose, **(B)** Yeast extract, **(C)** Beef extract, **(D)** Peptone, **(E)** K_2HPO_4 and **(F)** Tween 80.

5.3.4.2 Experimental validation of the model in flask

The validation of data was done in triplicate at flask level with 100 ml medium in which the activity observed was 10.1 U/ml that was in good agreement with the predicted results (10.5 U/ml) of software. This showed a significant 2 folds increase in dextransucrase activity in optimized medium than in unoptimised medium (4.5 U/ml) (Table 5.3.8). The dextransucrase production was further confirmed and scaled up by performing the experiment at bioreactor level.

Table 5.3.8 Comparison of dextransucrase activity and dextran production by *Pediococcus pentosaceus* CRAG3 in un-optimized and optimized media at flask and bioreactor level.

Medium	Dextransucrase activity (U/ml)	Dextran concentration (mg/ml)
Unoptimized	4.5	4.50
Taguchi predicted value	10.5	15.98
Optimized (in flask)	10.1	16.05
Optimized (in bioreactor)	10.2	24.24

5.3.4.3 Experimental validation of the model in bioreactor

The optimized medium for dextransucrase production contained (%, w/v): sucrose, 3.5%; yeast extract, 0.2%; beef extract, 0.5%; peptone, 3%; $MgSO_4.7H_2O$, 0.02%; $MnSO_4.4H_2O$, 0.001%; $FeSO_4.7H_2O$, 0.001%; $CaCl_2.2H_2O$, 0.001%; NaCl 0.001%; K_2HPO_4, 0.2% and Tween 80, 1% (v/v). The dextransucrase production by *Pediococcus pentosaceus* CRAG3 in optimized medium was compared with that of unoptimized medium. The fermentation profile is shown in Fig. 5.3.10. The maximum dextransucrase activity (10.2 U/ml) was observed at 8th hour of fermentation. It was also in good agreement with predicted values of dextransucrase activity. The sucrose profile showed that it started being consumed from the 1st hour of fermentation and less than 10% sucrose remained in medium after 12 h. The pH and temperature remained constant throughout the fermentation while DO was controlled by varying the agitation speed.

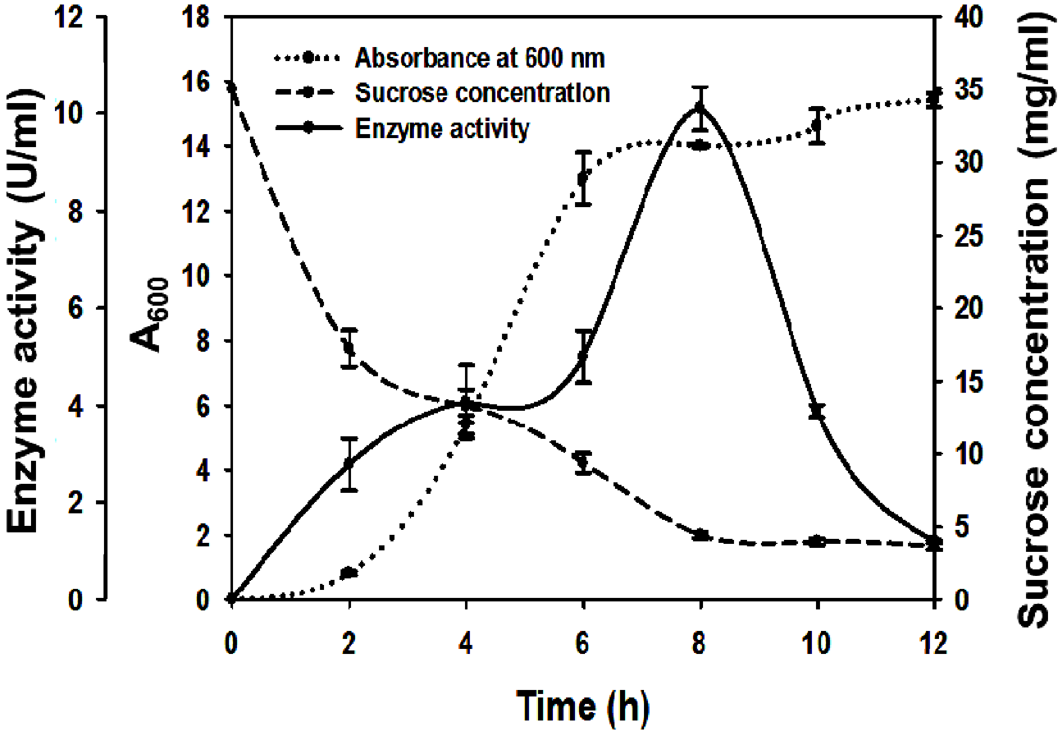

Fig. 5.3.10 Validation of data for dextransucrase production in bioreactor. Variation of dextransucrase activity (U/ml), sucrose concentration (mg/ml) and cell absorbance (A_{600}) during batch fermentation of *Pediococcus pentosaceus* CRAG3 in bioreactor.

5.3.5 Taguchi orthogonal array design for dextran production

5.3.5.1 Screening of medium components

The selected fermentation factors and their levels for optimization of dextran production by CRAG3 are shown in Table 5.3.3. The total 27 sets of experiments were performed and dextran concentration of each set was determined. Broad variation in dextran production (from minimum 0.3 mg/ml to maximum 14.9 mg/ml) was observed in L27 orthogonal array experiments (Table 5.3.4). The software predicted and experimental values for dextran production were in good agreement. Table 5.3.9 shows response table of selected factors for dextran production. The order of medium components for their effect on dextran production was K_2HPO_4 > Sucrose > Peptone > Yeast extract > Beef extract >Tween 80, which suggested that K_2HPO_4 showed most significant effect and Tween 80 had least effect for dextran production by *Pediococcus pentosaceus* CRAG3.

ANOVA data revealed that sucrose, peptone and K_2HPO_4 had least P-value hence were found to be most significant factors for dextran production (Table 5.3.10). The experimental data showed that selected level 1 value of peptone, Tween 80 and K_2HPO_4; level 2 value of beef extract and level 3 value of sucrose and yeast extract in the medium, were optimal for maximum dextran production by CRAG3 (Fig. 5.3.11A-F). Table 5.3.7 showed the optimum medium for maximum dextran production. The peptone in its final concentration range from 1 to 3% (w/v) was checked for its effect on dextran production. It was observed that 1% (w/v) peptone gave maximum dextran concentration, which further decreased after increasing the concentration (Figure 5.3.11D). The effects of sucrose and K_2HPO_4 on dextran concentration showed that 5% sucrose and 0.2% (w/v) K_2HPO_4 gave maximum dextran concentration (Fig. 5.3.11A and 5.3.11E). However, other medium components such as yeast extract (Fig. 5.3.11B), beef extract (Fig. 5.3.11C) and Tween 80 (Fig. 5.3.11F) at their final concentration of 2.0% (w/v) 0.5% (w/v) and 0.1% (v/v), respectively displayed maximum dextran concentration.

5.3.5.2 Experimental validation of the model in flask

The validation of data was done in triplicate at flask level and the maximum dextran concentration of 16.05 mg/ml was obtained using the statistically optimized medium. This was in good agreement with the predicted results (15.98 mg/ml) of software. The dextran concentration in optimized medium was 3.6 fold higher (16.05 mg/ml) than in unoptimized medium (4.5 mg/ml) (Table 5.3.8). This was further validated and scaled up at bioreactor level.

Table 5.3.9 Response table of means for dextran production by *Pediococcus pentosaceus* CRAG3.

Level	Sucrose	Yeast extract	Beef extract	Peptone	K_2HPO_4	Tween 80
1	3.667	6.574	6.812	8.668	9.896	7.889
2	8.397	6.466	7.489	7.591	7.456	6.744
3	9.387	8.411	7.15	5.192	4.1	6.818
Delta	5.720	1.946	0.678	3.477	5.795	1.145
Rank	**2**	**4**	**5**	**3**	**1**	**6**

Table 5.3.10 Analysis of variance (ANOVA) of experimental data on dextran production by *Pediococcus pentosaceus* CRAG3.

Source	DF*	Seq SS*	Adj SS*	Adj MS*	F	P*
Sucrose	2	168.242	168.242	84.121	22.04	0.000
Yeast extract	2	21.514	21.514	10.757	2.82	0.094
Beef extract	2	2.067	2.067	1.034	0.27	0.767
Peptone	2	57.011	57.011	28.505	7.47	0.006
K_2HPO_4	2	152.399	152.399	76.200	19.97	0.000
Tween 80	2	7.387	7.387	3.694	0.97	0.404
Residual error	14	53.427	53.427	3.816		
Total	26	462.048				

*DF- Degree of Freedom, Seq SS- Sequential Sum of Squares, Adj SS- Adjusted Sum of Squares, Adj MS- Adjusted Mean of Squares, P- Probability

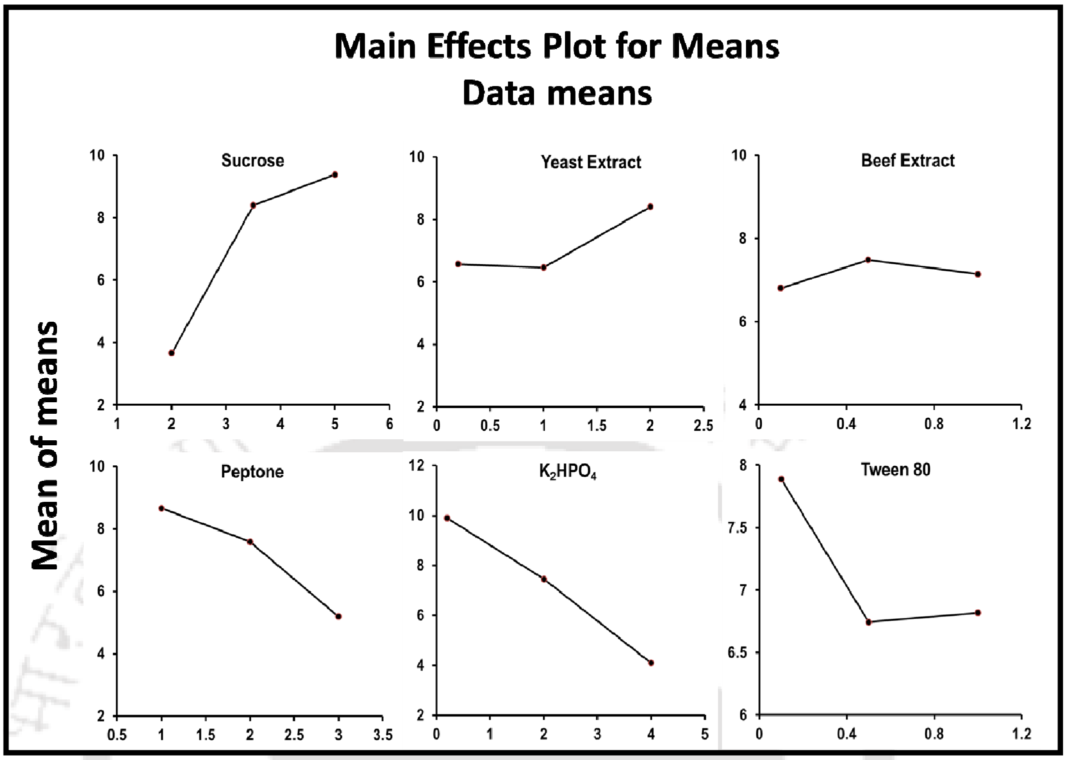

Fig. 5.3.11 Impact of selected fermentation factors and their assigned level on dextran production by *Pediococcus pentosaceus* CRAG3. X-axis represents assigned levels of selected factor (%, w/w or w/v) and Y-axis represents dextransucrase activity (U/ml). **(A)** Sucrose, **(B)** Yeast extract, **(C)** Beef extract, **(D)** Peptone, **(E)** K_2HPO_4 and **(F)** Tween80.

5.3.5.3 Experimental validation of the model in bioreactor

The optimized medium for dextran production consisted (%, w/v): sucrose, 5%; yeast extract, 2%; beef extract, 0.5%; peptone, 1%; K_2HPO_4, 0.2%; $MgSO_4.7H_2O$, 0.02%; $MnSO_4.4H_2O$, 0.001%; $FeSO_4.7H_2O$, 0.001%; $CaCl_2.2H_2O$, 0.001%; NaCl 0.001% and Tween 80, 0.1%. Comparing the dextran production by *Pediococcus pentosaceus* CRAG3 in optimized medium (24.24 mg/ml) with unoptimized medium (4.5 mg/ml), an increase of 5 fold was observed. The fermentation profile showed that the dextran concentration reached to its maximum level (24.24 mg/ml) during 6th-8th hour of fermentation (Fig. 5.3.12). The dextran concentration in bioreactor was 1.5 fold higher than that observed at flask level (16.05 mg/ml). The increase in concentration might be due to more growth of cells in medium (Fig. 5.3.12). The sucrose profile showed that it started getting consumed from 1st hour of fermentation and after 4th hour almost 50% of sucrose was consumed. 2-3% (w/v) sucrose remained in medium after 12 h. The pH and temperature remained constant throughout the fermentation while DO was controlled by varying the agitation speed.

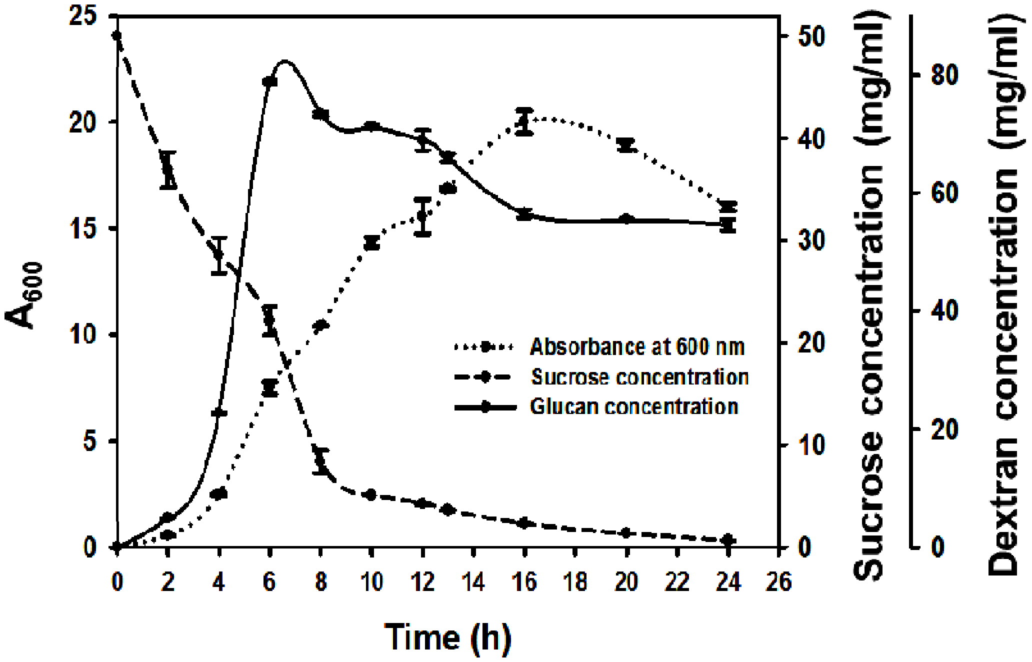

Fig. 5.3.12 Validation of data for dextran production in bioreactor. Variation of dextran concentration (mg/ml), sucrose concentration (mg/ml) and cell absorbance (A_{600}) during batch fermentation of *Pediococcus pentosaceus* CRAG3 in bioreactor.

5.4 Conclusions

The branched dextran producer strain of *Pediococcus pentosaceus* CRAG3 was selected for optimization of media components for enhanced dextransucrase and dextran production using One Variable at a Time (OVAT) approach and Taguchi orthogonal array design. The effects of sucrose, yeast extract, beef extract, peptone, K_2HPO_4 and Tween 80 on dextransucrase and dextran production were analysed. The dextransucrase activity and dextran concentration in control medium was 4.5 U/ml and 4.5 mg/ml, respectively. The sucrose at a concentration of 3.5% (w/v) resulted in 1.75 fold increase in dextransucrase activity (7.9 U/ml) with respect to control. 1% (w/v) yeast gave 6.5 U/ml dextransucrase which was 44% higher as compared to the control containing 2% (w/v) yeast extract. However, the addition of beef extract (1%, w/v) or peptone (1%, w/v) to the control medium increased the dextransucrase activity to 7.35 U/ml and 8.3 U/ml, respectively. There was an increase in dextransucrase activity with increase in concentrations of K_2HPO_4 and Tween 80. The increase in activity was 38% (6.2 U/ml) and 100% (9.0 U/ml) at 1% (w/v) K_2HPO_4 and 1% (v/v) Tween 80, respectively.

The effects of nutrients on dextran production showed that sucrose (3.5%, w/v), yeast extract (1%, w/v), peptone (1%, w/v) and K_2HPO_4 (1%, w/v) favoured its production with 180%, 27%, 6% and 178% increase in dextran concentration, respectively. However, beef extract (0.1%, w/v) and Tween 80 (0.05%, w/v) negatively affected dextran production with 17% and 20% decrease in dextran concentration.

Based on the results of OVAT, the different levels of medium components were selected for medium optimization of dextransucrase and dextran production using Taguchi methodology. The effect of factors on response showed that Tween 80 had an ample effect and yeast extract had least effect on dextransucrase production by *P. pentosaceus* CRAG3. However, K_2HPO_4 showed most significant effect and Tween 80 showed least effect on dextran production by *P. pentosaceus* CRAG3. ANOVA data showed significant effect of sucrose, Tween 80 and K_2HPO_4 on dextransucrase production. On the other hand, sucrose, peptone and K_2HPO_4 were found to be most significant for dextran production.

The optimized medium for dextransucrase production contained (%, w/v): sucrose, 3.5%; yeast extract, 0.2%; beef extract, 0.5%; peptone, 3%; $MgSO_4.7H_2O$, 0.02%; $MnSO_4.4H_2O$, 0.001%; $FeSO_4.7H_2O$, 0.001%; $CaCl_2.2H_2O$, 0.001%; NaCl 0.001%; K_2HPO_4, 0.2% and Tween 80, 1% (v/v). However, the optimized medium for dextran production consisted (%, w/v): sucrose, 5%; yeast extract, 2%; beef extract, 0.5%; peptone, 1%; K_2HPO_4, 0.2%; $MgSO_4.7H_2O$, 0.02%; $MnSO_4.4H_2O$, 0.001%; $FeSO_4.7H_2O$, 0.001%; $CaCl_2.2H_2O$, 0.001%; NaCl 0.001% and Tween 80, 0.1%. Significant increase in dextransucrase activity and dextran concentration was observed in optimized medium (10 U/ml and 16 mg/ml, respectively) as compared to unoptimized medium (4.5 U/ml and 4.5 mg/ml, respectively). Validation of data was done at flask and bioreactor levels. The maximum dextransucrase activity achieved in shake flask and bioreactor level was 10.1 U/ml 10.2 U/ml, respectively which was in good agreement with Taguchi predicted result (10.5 U/ml). The maximum dextran concentration at shake flask level was 16 mg/ml which was also in accordance with Taguchi predicted value (15.98 mg/ml). Interestingly, the enhancement in dextran concentration (24 mg/ml) was observed at bioreactor level which might be due to higher growth of cells.

5.5 References

Abdel-Fattah, Y.R., Saeed, H.M., Gohar, Y.M. and El-Baz, M.A. (2005) Improved production of *Pseudomonas aeruginosa* uricase by optimization of process parameters through statistical experimental designs. Process Biochem. 40, 1707-1714.

Beg, Q.K., Sahai, V. and Gupta, R. (2003) Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process Biochem. 39, 203-209.

Box, G.E. and Hunter, J.S. (1975) Multi-factor experimental designs for exploring response surfaces. Ann. Math. Stat. 28, 195-241.

Cao, W., Li, X.Q., Liu, L., Yang, T.H., Li, C., Fan, H.T., *et al.* (2006) Structure of anti-tumor polysaccharide from *Angelica sinensis* (Oliv) Diels. Carbohydr. Polym. 66, 149-159.

Ceri, H., McArthur, H.A. and Whitfield, C. (1986) Association of alginate from *Pseudomonas aeruginosa* with two forms of heparin-binding lectin isolated from rat lung. Infect. Immun. 51, 1-5.

Cobb, B.D. and Clarkson, J.M. (1994) A simple procedure for optimizing the polymerase chain reaction (PCR) using modified Taguchi methods. Nucleic Acids Res. 22, 3801-3805.

Cortezi, M., Monti, R. and Contiero, J. (2005) Temperature effect on dextransucrase production by *Leuconostoc mesenteroides* FT045B isolated from Alcohol and Sugar Mill Plant. Afr. J. Biotechnol. 4, 279-285.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987) Bacterial biofilms in nature and disease. Annu. Rev.

Microbiol. 41, 435-464.

Dasu, V.V., Panda, T. and Chidambaram, M. (2003) Determination of significant parameters for improved griseofulvin production in a batch bioreactor by Taguchi's method. Process Biochem. 38, 877-880.

DeMan, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of *Lactobacilli*. J. Appl. Bacteriol. 23, 130-135

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 a microsample plate reader. Anal. Biochem. 195, 93-96.

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.

Goyal, A., Nigam, M. and Katiyar, S.S. (1995) Optimal conditions for the production of dextransucrase from *Leuconostoc mesenteroides* NRRL 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* NRRL B-512F by the maintenance media. J. Gen. Appl. Microbiol. 42, 81-85.

Han, J.J., Yang, T.H. and Rhee, J.S. (1998) Optimization of reaction variables for sucrose monoester production using lipase in a solvent free system by Taguchi's method. Biotechnol. Tech., 12, 295-299.

Houng, J.Y., Liao, J.H., Wu, J.Y., Shen, S.C. and Hsu, H.F. (2006) Enhancement of asymmetric bioreduction of ethyl 4-chloro acetoacetate by the design of composition of culture medium and reaction conditions. Process Biochem. 42, 1-7.

Jeney, C., Dobay, O., Lengyel, A., Adam, E. and Nasz, I. (1999) Taguchi optimization of ELISA procedures. J. Immunol. Meth. 223, 137-146.

Korakli, M. and Vogel, R.F. (2006) Structure/function relationship of homopolysaccharide producing glycansucrases and therapeutic potential of their synthesized glycans. Appl. Microbiol. Biotechnol. 71, 790-803.

Kralj, S., van Geel-Schutten, G. H., Rahaoui, H., Leer, R. J., Faber, E. J., van der Maarel, M. J. and Dijkhuizen. L. (2002) Molecular characterization of a novel glucosyltransferase from *Lactobacillus reuteri* strain 121 synthesizing a unique, highly branched glucan with alpha-(1→4) and alpha-(1→6) glucosidic bonds. Appl. Environ. Microbiol. 68, 4283-4291.

Kralj, S., van Geel-Schutten, G.H., Dondorff, M.M.G., Kirsanovs, S., van der Maarel, M.J. and Dijkhuizen, L. (2004) Glucan synthesis in the genus *Lactobacillus*: isolation and characterization of glucansucrase genes, enzymes and glucan products from six different strains. Microbiol. 150, 3681-3690.

Kralj, S.E., Stripling, P., Sanders, G.H., van Geel-Schutten, and Dijkhuizen, L. (2005) Highly hydrolytic reuteransucrase from probiotic *Lactobacillus reuteri* strain ATCC 55730. Appl. Environ. Microbiol. 71, 3942-3950.

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*. Enzyme Microbiol. Technol. 15, 334-338.

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.

Lopretti, M., Martinez, E., Torres, L., Perdomo, R., Santos, M. and Rodrigues, A.E. (1999) Influence of nitrogen/carbon ratio and complementary sugars on dextransucrase production by *Leuconostoc mesenteroides* B-512F. Process Biochem. 34, 879-884.

Majumder, A. and Goyal A. (2008) Optimization of culture conditions of a novel glucan producing glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. Curr. Trends Biotechnol. Pharma.2, 260-268.

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

Monchois, V., Willemot, R. M. and Monsan, P. (1999) Glucansucrases: mechanism of action and structure-function relationships. FEMS Microbiol. Rev. 23, 131-151.

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

Prakasham, R.S., Subba Rao, C.H., Sreenivas Rao, R. and Sarma, P.N. (2005) Alkaline protease production by an isolated *Bacillus circulans* under solid state fermentation using agroindustrial waste: process parameter optimization. Biotechnol. Prog. 21, 1380-1388.

Prasad, K.K., Mohan, V.S., Rao, S.R., Pati, R.B. and Sarma, P.N. (2005) Laccase production by *Pleurotus ostreatus* 1804: Optimization of submerged culture conditions by Taguchi DOE methodology. Biochem. Eng. J. 24, 17-26.

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) Screening and optimization of nutritional factors for higher dextransucrase production by *Leuconostoc mesenteroides* NRRL B-640 using statistical approach. Biores. Technol. 99, 7108-7114.

Rao, R.S., Prakasham, R.S., Prasad, K.K., Rajesham, S., Sarma, P.N. and Rao, L.V. (2004) Xylitol production by *Candida* sp.: parameter optimization using Taguchi approach. Process Biochem. 39, 951-956.

Rodrigues, S., Lona, L.M.F. and Franco, T.T. (2003) Effect of phosphate concentration on the production of dextransucrase by *Leuconostoc mesenteroides* NRRL B-512F. Bioproc. Biosyst. Eng. 26, 57-62.

Sandford, P.A. and Baird, J. (1983) Industrial utilization of polysaccharides. In: Aspinall G.O. (ed), The polysaccharides. New York: Academic Press, 2, pp. 411-490.

Sato, M., Tsuchiya, H., Kato, M., Yamamoto, K., Nakazato, G., Tagaki, G. and Namikawa, I. (1989) Effects of Tween 80 and sodium fluoride on extracellular glucosyltransferase. Int. J. Biochem. 21, 751-754.

Shukla, S and Goyal, A. (2011) Optimization of fermentation medium for enhanced glucansucrase and glucan production from *Weissella confusa*. Braz. Arch. Biol. Technol. 54, 1117-1124.

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

Stone, R.A. and Veevers, A. (1994) The Taguchi influence on designed experiments. J. Chemometr. 8, 103-110.

Sumner, J.B. and Sisler, E.B. (1944) A simple method for blood sugar. Arch.

Biochem. 4, 433-436.

Sutherland, I.W. (1998) Novel and established applications of bacterial polysaccharides. Trends Biotechnol. 16, 41-46.

Tieking, M., Korakli, M., Ehrmann, M.A., Gänzle, 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.

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.

Uzochukwu, S., Balogh, E., Loefler, R.T., Ngoddy, P.O. (2002) Structural analysis by 13C-nuclear magnetic resonance spectroscopy of glucan extracted from natural palm wine. Food Chem. 76, 287-291.

Van Geel-Schutten, G.H., Flesch, F., Ten Brink, B., Smith, M.R. and Dijkhuizen, L. (1998) Screening and characterization of *Lactobacillus* strains producing large amounts of exopolysaccharides. Appl. Microbiol. Biotechnol. 50, 697-703.

Chapter 6

Probiotic potential of *Pediococcus pentosaceus* CRAG3

6.1 Introduction

The lactic acid bacteria (LAB) are a group of Gram-positive rod and coccus shaped, non-spore forming, non-motile microorganisms. The major product formed during fermentation of carbohydrates is lactic acid. This group comprises the genera such as *Carnobacterium, Enterococccus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella* (Stiles and Holzapfel, 1997; Holzapfel *et al.*, 2001). Many lactic acid bacteria served as better probiotics. Probiotics are live microbial feed supplements that positively affect the host animal by improving its intestinal microbial balance (Fuller, 1989). This has become the most widely accepted definition (Fooks *et al.*, 1999). Currently, most probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium* (Prasad *et al.*, 1998). However, species belonging to the genera *Lactococcus*, *Enterococcus*, *Saccharomyces* (Salminen and von Wright

1998, Dunne *et al.*, 1999; Sanders and in't Veld, 1999) and *Propionibacterium* (Grant and Salminen, 1998) are also considered as probiotic microorganisms. Even *Streptococcus thermophilus* (Cosson and Deschamps, 1994; Collins *et al.*, 1998; Naidu *et al.*, 1999, Sreekumar and Hosono, 2000) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Bezkorovainy *et al.*, 1997; Naidu *et al.*, 1999; Sreekumar and Hosono, 2000) are considered probiotics. There are number of reports demonstrating the probiotic properties of *Pediococcus spp.* (Jonganurakkun *et al.*, 2008; Yuksekdag and Aslim 2010; Sukumar and Ghosh, 2010) and *Leuconostoc spp.* (Kekkonen *et al.*, 2008; Allameh *et al.*, 2012). *P. acidilactici* has GRAS (Generally Recognized as Safe) status and is used as probiotic culture and nutritional enhancer in silage (O'Connor *et al.*, 2007; Kantor *et al.*, 1997). Along with fermented foods, several *Pediococcus* strains produce bacteriocins which inhibit the growth of major Gram-positive foodborne pathogens, as well as other food spoilage bacteria (Kantor *et al.*, 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 and Dicks, 2005). In the present study a number of *in vitro* tests were performed to screen *Pediococcus pentosaceus* CRAG3 for its probiotic properties such as cell adhesion property, resistance to biological barriers, deconjugation of bile salts, antimicrobial assay and prebiotic utilization.

6.2 Materials and Methods

6.2.1 Chemicals and reagents

The chemicals required for evaluating probiotic properties of strains such as Giemsa stain, immersion oil, dipotassium hydrogen orthophosphate, phosphate buffer saline, raffinose, inulin, yeast extract, beef extract, peptone, sodium chloride and sodium hydroxide were purchased from Himedia Pvt. Ltd., India. The chemicals such as lysozyme, pepsin, Ringer solution, sodium chloride, calcium chloride, potassium chloride, sodium bicarbonate, taurodeoxycholic acid, and bile salts were purchased from Sigma Chemical Co., St. Louis, MO, USA. Taurine was purchased from SRL Pvt. Ltd., India. Ninhydrin was purchased from Fisher Scientific Pvt. Ltd., India and Trichloro acetic acid, citric acid, Giemsa stain and sodium citrate heptahydrate were purchased from Merck, India.

6.2.2 Maintenance of culture

Pediococcus pentosaceus CRAG3 (Genbank accession number JX679020) was isolated from fermented cucumber as described in Chapter 2 Section 2.2.2. It was propagated in modified MRS agar medium (Goyal and Katiyar, 1996) as a stab incubated at 30°C, stored at 4°C and sub-cultured every 15 days. The modified MRS agar medium was prepared as described in Chapter 2, Section 2.2.3. The cervical cancer (HeLa) cell line and murine macrophage (J774A.1) cell line were used for *in vitro* cell adhesion assay of *Pediococcus pentosaceus* CRAG3. The cell lines were maintained in DMEM medium as described earlier in Chapter 4, Section 4.2.4.2.

6.2.3 Adhesive capacity and cell surface traits

6.2.3.1 Hydrophobicity

The hydrophobic behavior of the isolate *Pediococcus pentosaceus* CRAG3 was determined by the method as described by Perez *et al.* (1998) with modification. n-Hexadecane was used in place of xylene. Kiely and Olson (2000) reported the use of n-hexadecane to qualitatively determine the adhesion property of bacteria. The cells of *Pediococcus pentosaceus* CRAG3 overnight grown in 5 ml modified MRS medium (as described earlier in Chapter 2, Section 2.2.3) at 25°C and 180 rpm were harvested by centrifugation at 12,000***g*** for 5 min at 4°C. The cell pellet was washed twice with 5 ml 50 mM K_2HPO_4 buffer, pH 6.5 and finally re-suspended in 5 ml same buffer. The absorbance at 600 nm (A_{600}) of cell suspension was taken and adjusted to value of 1.0 with the same buffer. To 3 ml of the bacterial suspension, 0.6 ml of n-hexadecane was added and mixed well on vortex for 2 min. The two phases were allowed to separate at 37°C for 20 min. The aqueous phase was removed cautiously and the absorbance at 600 nm was taken. The cell surface hydrophobicity (H%) was calculated by observing the drop in absorbance of the aqueous phase using the following formula:

$$\mathbf{H\% = [(A_0 - A)/A_0] x 100}$$

Where, A_0 and A are the absorbance at 600 nm (A_{600}) before and after extraction with n-hexadecane, respectively.

6.2.3.2 Autoaggregation assay

The autoaggregation assay was performed by method as described by Del Re *et al.* (2000) with following modifications; the incubation time for autoaggregation was 5 h instead of 2 h. The culture (4 ml) of *Pediococcus pentosaceus* CRAG3 containing approximately 4x10^8 CFU was centrifuged at 12,000***g*** and 4°C for 10 min. The cell pellet was re-suspended in 4 ml 150 mM phosphate buffer saline (PBS, pH 7.0) and allowed to aggregate by further incubating at 37°C for 5 h. Aliquots of 100 µl were withdrawn at regular interval of 1 h from upper part of suspension and mixed with 3.9 ml 150 mM PBS (pH 7.0) and absorbance at 600 nm (A_{600}) was measured. The percent autoaggregation (A_a%) was calculated by using following equation (Del Re *et al.*, 2000):

$$\mathbf{A_a\% = 1 - (A_t / A_0) \times 100}$$

Where, A_t= absorbance at time t=1, 2,…., 5 h and A_0= absorbance at t=0 h.

The percentage of autoaggregation was expressed as a function of time, until 5 h.

6.2.3.3 Co-aggregation assay

The coaggregation assay was performed by using the method described by Kos *et al.* (2003). The cultures (4 ml) of *Pediococcus pentosaceus* CRAG3 and *E. coli* containing approximately, 2x10^8 CFU each were centrifuged at 12,000***g*** and 4°C for 10 min. The cell pellet of both cultures was re-suspended separately in 2 ml 0.1 M sodium phosphate buffer, pH 7.0. Equal volumes (2 ml) of both cell suspensions were mixed together and allowed to aggregate by incubating at 37°C for 5 h. The tubes containing 4 ml of each bacterial suspension was taken as control. Aliquots of 100 µl were withdrawn at regular interval of 1 h from upper part of suspension and mixed with 3.9 ml of same buffer and absorbance at 600 nm (A_{600}) was measured. The percentage of coaggregation (C_a%) was calculated by using the equation of Handley *et al.* (1987):

$$\mathbf{C_a\% = \frac{(Ax + Ay)/2 - (Ax + Ay)}{(Ax + Ay)/2} \times 100}$$

Where, Ax and Ay represent absorbance at 600 nm (A_{600}) of each of the two strains in the control tubes and A(x + y) the absorbance of mixture.

6.2.3.3 In vitro cell adhesion assay

The adhesive capacity of *Pediococcus pentosaceus* CRAG3 was studied using cervical cancer (HeLa) cell line and murine macrophage (J774A.1) cell line using the method as reported earlier (Chauviere *et al.*, 1992). The cell lines were grown separately in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 50 µg/ml streptomycin and 50 IU/ml penicillin at 37°C in 5% CO_2 atmosphere. The monolayers of each cell line were prepared on separate glass coverslips in six well tissue culture plates. Each of HeLa and J774A.1 cells were seeded at a concentration of 4×10^4 cells/cm^2 in 96 well plate and were incubated at 37°C in a 5% CO_2 incubator. The cells of *Pediococcus pentosaceus* CRAG3 from overnight grown culture in modified MRS medium at 25°C and 180 rpm were counted and approximately 10^8 CFU were added over the monolayer of each cell line at post confluence. The plates were incubated at 37°C under 5% CO_2 atmosphere for 90 min to allow them to adhere onto the surface of monolayers. The unbound bacteria were removed from monolayers by washing twice with sterile 1x PBS (150 mM, pH 7.0), followed by fixing with methanol and finally stained with 1%

(w/v) Giemsa solution. The adhered bacteria were observed under light microscope (Zeiss, Primo star) using oil immersion (100x) at 20 random microscopic fields per glass coverslip monolayer.

6.2.4 Resistance to biological barriers

6.2.4.1 Lysozyme resistance

The tolerance of *Pediococcus pentosaceus* CRAG3 against lysozyme was determined by method of Zago *et al.* (2011). The culture was grown in 10 ml modified MRS medium (as described earlier in Chapter 2, Section 2.2.3) at 25°C and 180 rpm for overnight. The cells were centrifuged at 12,000***g*** and 4°C for 10 min. The cell pellet was washed twice with 5 ml of 0.1 M potassium phosphate buffer (pH 7.0) and re-suspended in 2 ml of Ringer solution (Zago *et al.*, 2011). It is a commercially available medium for *in vitro* culture of preimplantation stage embryos. It is a modified Krebs-Ringer bicarbonate solution, which is very similar to Whitten′s Medium and contains pyruvate and lactate as energy sources since pre-implantation embryos cannot utilize glucose efficiently. 0.5 ml suspension of culture from above solution was inoculated in 5 ml sterile electrolyte solution (SES; 0.22 g/l $CaCl_2$, 6.2 g/l NaCl, 2.2 g/l KCl, 1.2 g/l $NaHCO_3$) containing 100 mg/l of lysozyme and incubated at 37°C for 2 h. The bacterial suspension in SES without lysozyme was taken as control. The aliquot (10 µl) from each sample was spread on petri-dish containing modified MRS medium and incubated at 25°C for 48 h. The microbial colonies grown on petri-dishes were counted and the survival rate was calculated as percent of CFU/ml (colony forming unit per ml) obtained from lysozyme treatment with respect to the control (without lysozyme treatment). The experiment was conducted in triplicate and the average value was taken.

6.2.4.2 Tolerance to simulated gastric juice

The resistance of *Pediococcus pentosaceus* CRAG3 against simulated gastric juice was determined by the method of Charteris *et al.* (1998). Two separate solutions of simulated gastric juice (each 10 ml) were prepared by mixing pepsin (1000 U/mg, 3 mg/ml) and NaCl (0.5% w/v) in water and pH of one solution was adjusted to 2 and of the other solution to pH 3. 30 ml overnight grown culture of *Pediococcus pentosaceus* CRAG3 was centrifuged at 6,000***g*** and 4°C for 20 min. After removing the supernatant, the bacterial cells were washed twice with 10 ml 50 mM K_2HPO_4 (pH 6.5) and re-suspended in 3 ml of the same solution. 1 ml each of cell suspension was added to 9 ml each of gastric solution of pH 2 and 3 and incubated at 37°C for 3 h. The total viable cells (CFU/ml) were counted by using the plate count method, before and after incubation period and expressed as the difference in colony counts. The aliquots (50 µl) from each solution were diluted to 10^{-6} dilution factor and spread on petri-dish containing MRS medium. The petri-dishes were incubated at 30°C for 24 h and the total viable cells CFU/ml was calculated. The experiment was performed in triplicate and the average value was taken.

6.2.4.3 Resistance to bile salts

The ability of the isolate *Pediococcus pentosaceus* CRAG3 to tolerate different concentrations of bile salts was evaluated by method of Walker and Gilliland (1993) with following modifications; instead of MRS-THIO medium (containing

0.2% sodium thioglycolate) modified MRS medium (as described earlier in Chapter 2, Section 2.2.3) was used. The overnight grown culture of *Pediococcus pentosaceus* CRAG3 (2%, v/v) was inoculated into 10 ml modified MRS medium (pH 6.4) containing 0.3, 0.5 or 1% (w/v) of bile salts. The cultures were incubated at 25°C and after 24 h absorbance at 600 nm (A_{600}) was measured and compared with a control culture (without bile salts). The results were expressed as the percent of growth (A_{600} nm) in the presence of bile salts with respect to the control. The experiment was conducted in triplicate and the average value was taken.

6.2.5 Bile salt hydrolase activity

6.2.5.1 Bile salt deconjugation

Bile salt hydrolase (BSH) activity of the isolate was determined according to the method of Taranto *et al.* (1995). The petri-dish containing modified MRS medium (pH 6.4) (Goyal and Katiyar, 1996 and as described earlier in Chapter 2, Section 2.2.3) supplemented with 1.7% (w/v) agar and 0.5% (w/v) sodium salt of taurodeoxycholic acid were made. The medium was autoclaved (121°C, 15 min, 15 psi) and immediately used. 12 h old culture of *Pediococcus pentosaceus* CRAG3 in modified MRS medium (Goyal and Katiyar, 1996) was streaked on petri-dish and was incubated anaerobically in air-tight gas jar containing the gas-pak sachet at 37°C for 72 h. In this process the amide bond of bile salts is hydrolyzed liberating glycine/taurine moiety from the steroid core leaving behind the deconjugated (unconjugated) bile acids (Fig. 6.2.1). The process of deconjugation is catalyzed by Bile salts hydrolase (BSH) enzyme (EC 3.5.1.24).

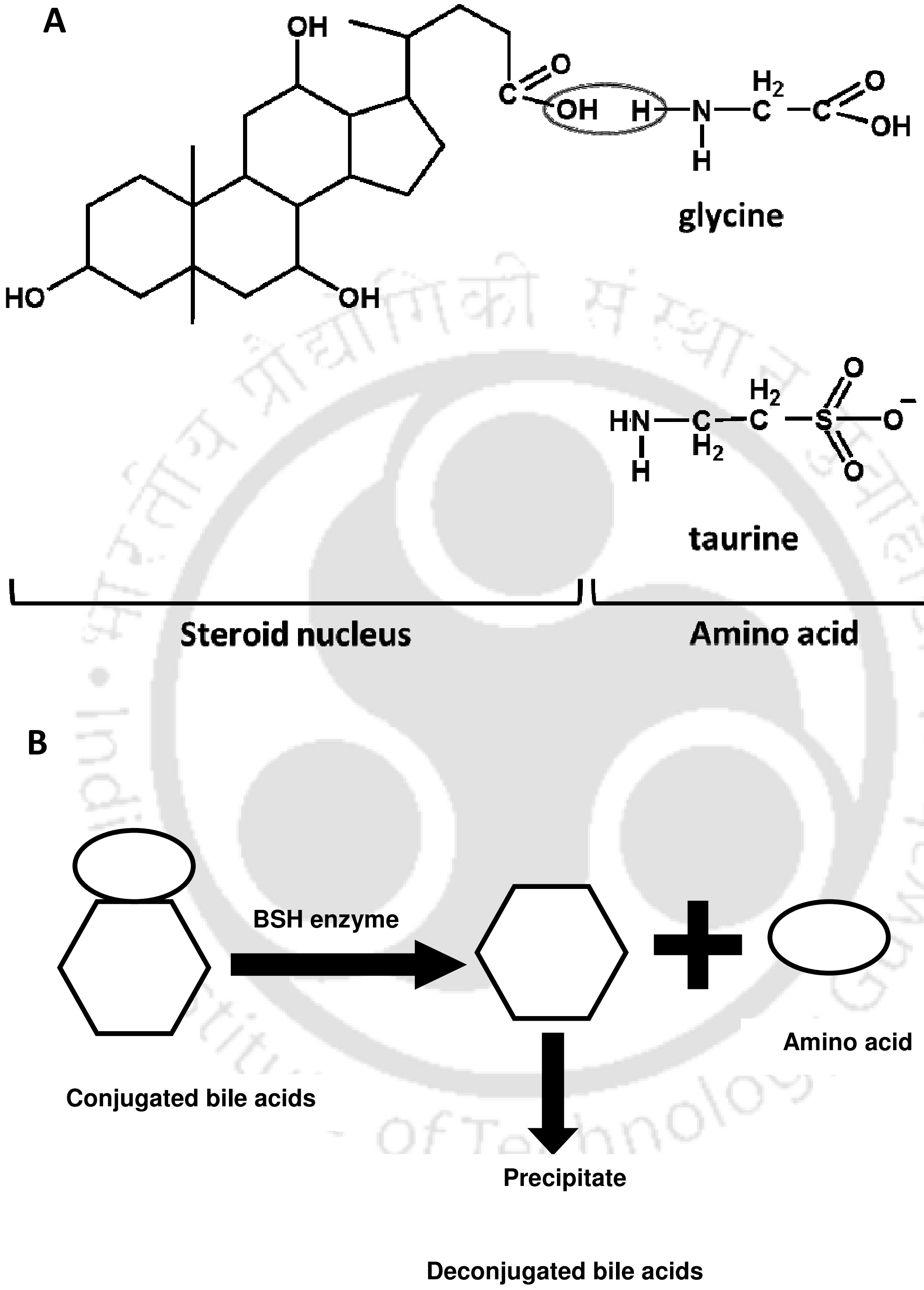

Fig. 6.2.1 **(A)** Chemical structure of bile acids. The carboxy goup of primary bile acids linked with the amino group of either glycine or taurine by amide bond to form conjugated bile acids prior to secretion. **(B)** The deconjugation reaction catalyzed by bile salts hydrolase enzyme to form deconjugated bile acids as precipitate.

6.2.5.2 Bile salt hydrolase activity assay

BSH activity assay was carried out using the method as described by Tanaka *et al.* (2000) with following modifications. The overnight grown culture (5 ml) of *Pediococcus pentosaceus* CRAG3 in modified MRS medium (Goyal and Katiyar, 1996 and as described earlier in Chapter 2, Section 2.2.3) was centrifuged at 10,000***g*** and 4°C for 10 min. The cells were washed twice with 0.1 M sodium phosphate buffer (pH 7.0) and finally resuspended in 5 ml of same buffer. The concentration of cell suspension was adjusted to absorbance value to 1 at 600 nm. The cell suspension was sonicated by using ultrasonic processor (Sonics, Vibra cell) for 5 min (with pulse rate of 15 sec On and 10 sec Off) with constant cooling on ice and centrifuged at 10,000***g*** and 4°C for 10 min. The cell pellet was discarded and 0.2 ml of supernatant was mixed with 0.2 ml 0.1 M sodium phosphate buffer, pH 7.0 and 0.1 ml of 6 mM taurodeoxycholic acid. The solution was incubated at 37°C for 30 min and the reaction was stopped by adding 0.5 ml of 15% (w/v) trichloroacetic acid. The mixture was centrifuged at 10,000***g*** and 4°C for 10 min and to the 0.4 ml of supernatant, 0.4 ml of ninhydrin reagent (0.2%, w/v ninhydrin in 0.5 M citrate buffer, pH 5.5) was added. The mixture was put in boiling water bath for 15 min and cooled to room temperature (25°C). After cooling, 0.2 ml deionized water was added and absorbance (A_{570}) at 570 nm was measured. The standard plot was prepared using taurine (20-200 µg/ml) (Fig. 6.2.2) and the BSH activity was measured by determining the amount of amino acids liberated from conjugated bile salts by *Pediococcus pentosaceus* CRAG3. One unit of BSH activity was defined as the amount of enzyme that liberated 1 µmol of amino acid from substrate per min.

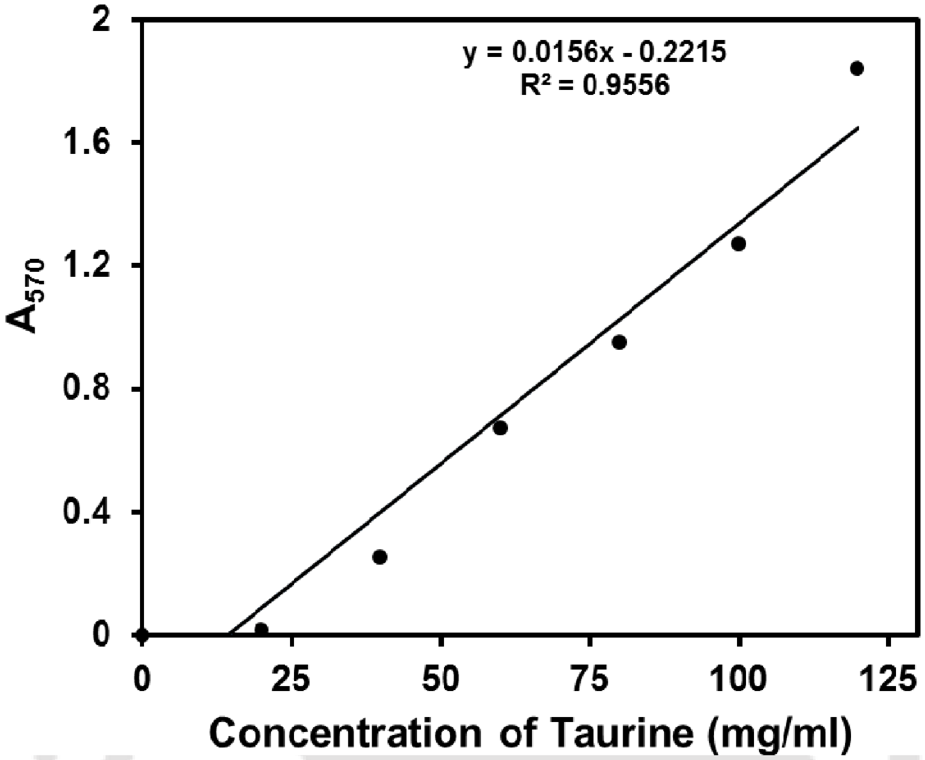

Fig. 6.2.2 Standard plot between concentrations of taurine against absorbance at 570 nm (A_{570}) for bile salt hydrolase activity assay.

6.2.6 Antibacterial activity of *Pediococcus pentosaceus* CRAG3

6.2.6.1 Nutrient medium preparation

The nutrient medium for culturing of test microorganisms (*Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*) was prepared using method as described earlier (Lapage *et al.*, 1970). The components for nutrient medium are given in Table 6.2.1. The pH of the medium was adjusted to 6.8 using 1N HCl or 1N NaOH. To prepare the nutrient agar medium, 1.7% (w/v) agar was added to nutrient broth. The medium was sterilized by autoclaving at 15 psi steam pressure and 121°C temperature for 20 min.

Table 6.2.1 Components for preparation of nutrient agar medium.

Component	% (w/v)
Yeast extract	0.2
Beef extract	0.1
Peptone	0.5
Sodium chloride	0.5

6.2.6.2 Analysis of antibacterial activity

The ability of *Pediococcus pentosaceus* CRAG3 to exhibit antibacterial activity was determined by the method of Yuksekdag and Aslim (2010). Test microorganisms (*Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*) were grown in 5 ml nutrient broth as described earlier in Section 6.2.6.1 at 37°C for 18-20 h. *Pediococcus pentosaceus* CRAG3 was grown in 5 ml of modified MRS medium as described earlier in Chapter 2, Section 2.2.3 at 25°C for 18-20 h. The cells of *Pediococcus pentosaceus* CRAG3 were removed by centrifugation at 8,000***g*** and 4°C for 10 min and the cell free supernatant was sterilized by filtering through 0.22 µm membrane. The petri-dishes (90 mm) containing nutrient agar previously inoculated with 1% each of test microorganisms were prepared. The wells (6 mm) were made in each petri-dish using sterile micro-tips and 100 µl cell-free filtrate of *Pediococcus pentosaceus* CRAG3 was added to each well. In another set of experiment the cell-free filtrate was neutralised using 2N NaOH and 100 µl was added to each well in petri-dishes containing test organisms. The plates were incubated at 37°C for 24 h and the zone of inhibition was measured.

6.2.7 Estimation of lactic acid production

The isolate *Pediococcus pentosaceus* CRAG3 was analyzed for its ability to produce lactic acid. The concentration of lactic acid was measured by using the method as described by Mirdamadi *et al.* (2002) with certain modifications. The isolate was grown in 5 ml modified MRS medium (Goyal and Katiyar, 1996) as described in Chapter 2, Section 2.2.3 at 25°C and 180 rpm for 24 h. The cells were removed by centrifugation at 10,000***g*** and 4°C for 10 min and the cell free supernatant was used for determination of lactic acid concentration. To 0.5 ml of cell free supernatant, 3.0 ml concentrated sulphuric acid (18 M) was mixed and heated in boiling water bath for 10 min. The mixture was cooled to 25°C and 50 µl of cupric sulphate (4%, w/v in water) and 100 µl phenolphthalein (1.5%, w/v in 95%, v/v ethanol) were added. The absorbance of mixture (3.65 ml) at 570 nm was read against distilled water as blank. The standard graph was plotted using lactic acid in the range 10-150 mg/ml (Fig. 6.2.3).

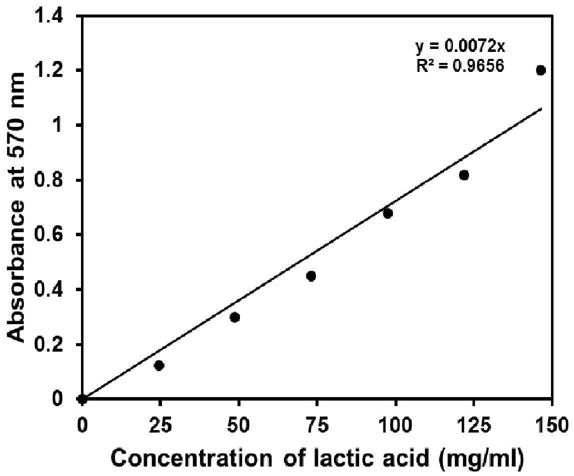

Fig. 6.2.3 Standard plot between concentrations of lactic acid against absorbance at 570 nm (A_{570}) for estimation of lactic acid.

6.2.8 Utilization of prebiotics by *Pediococcus pentosaceus* CRAG3

The isolate *Pediococcus pentosaceus* CRAG3 was explored for its ability to utilize prebiotics such as raffinose and inulin as described earlier (Zago *et al.*, 2011). The MRS medium (20 ml) containing raffinose or inulin (2%, w/v) were prepared separately. MRS containing that contains 2% (w/v) glucose was taken as positive control. The overnight grown culture of *Pediococcus pentosaceus* CRAG3 (1%) was inoculated in each of 20 ml MRS media and incubated at 37°C and 180 rpm for 24 h. The percentage of growth of *Pediococcus pentosaceus* CRAG3 in the presence of each prebiotic was calculated as follows (Zago *et al.*, 2011).

$$[((MRS_p - MRS_b)x100) / (MRS_g - MRS_b)]$$

where, MRS_p, MRS_b and MRS_g are absorbance at 600 nm (A_{600}) of culture grown in MRS medium supplemented with prebiotic, without carbon source and with glucose, respectively. MRS medium without carbon source as negative control and MRS medium with glucose as positive control were used.

6.3 Results and Discussion

6.3.1 Cell surface traits and adhesive capacity of the isolate

6.3.1.1 Hydrophobicity

Pediococcus pentosaceus CRAG3 showed 33% hydrophobicity (H%) after 20 min, which was similar to other reported probiotic bacteria such as *Lactococcus acidophilus* (38.1%), *Lactococcus casei* (24.1%) and *Lactococcus lactis* (31.3%) (Vinderola and Reinheimer, 2003). Hydrophobic interaction plays the initial significant step in the microbial adhesion between the bacterial cell surface and the contact substratum (Busscher and Weerkamp, 1987).

6.3.1.2 Autoaggregation

The auto-aggregation (%) of *Pediococcus pentosaceus* CRAG3 increased with increasing time and showed 43% auto-aggregation after 5 h implying its ability to form clumps (Fig. 6.3.1). Hydrophobicity and autoaggregation of microorganism are phenotypically related to its adhesion capacity (Perez *et al.*, 1998; Del Re *et al.*, 2000). It is the hydrophobic nature of outer layer of microbe which helps in attachment to the mammalian cell surface (Rosenberg *et al.*, 1980; Zago *et al.*, 2011). The autoaggregation is an important property of probiotic through which it colonizes in intestine.

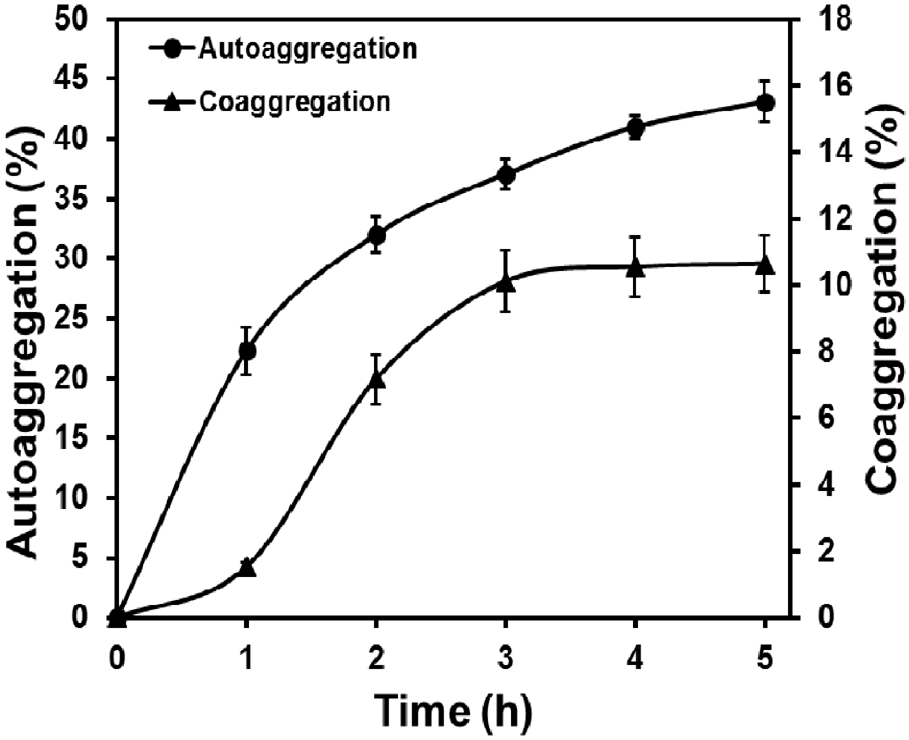

Fig. 6.3.1 Autoaggregation and coaggregation (with *E. coil*) property displayed by *Pediococcus pentosaceus* CRAG3.

6.3.1.3 Coaggregation

Pediococcus pentosaceus CRAG3 showed 10.65±.84% coaggregation with *E. coli* after 5 h (Fig. 6.3.1) similar to probiotic strain *Lactobacillus acidophilus* M92 which showed 4.36% and 15.11% coaggregation with *Lactobacillus plantarum* L4 and *Escherichia coli* 3014, respectively (Kos *et al.*, 2003). The coaggregation assay was performed to determine the interbacterial adherence between *Pediococcus pentosaceus* CRAG3 and *E. coli* which could increase its colonization potential if it was to be used in mixed culture as probiotics. It has been reported that the inhibitor producing bacteria may constitute an important host defence mechanism against infection in the gastrointestinal tract (Spencer and Chesson 1994) and also in intestine

(Suskovic *et al.*, 1997). *Pediococcus pentosaceus* CRAG3 is a potent producer of lactic acid therefore, it may also help in host defence mechanism.

6.3.1.4 In vitro cell adhesion assay

The adhesion of *P. pentosaceus* CRAG3 on HeLa and J774A.1 cell lines is shown in Fig. 6.3.2. The microscopic examination of adhesion between mammalian cell lines and the isolate showed that *Pediococcus pentosaceus* CRAG3 displayed adhesion property with all cell lines (Fig. 6.3.2A-D). The cells of *P. pentosaceus* CRAG3 adhered to both cell HeLa cell line (Fig. 6.3.2B) and J774A.1 cell line (Fig. 6.3.2D) indicating good adhesion property of the isolate. The adhesion capacity is a key property of probiotic through which competes with pathogenic strains. The adhesion property of a probiotic microbe supports in competing with other microorganisms in gastrointestinal tract (Naidu *et al.*, 1999). The characterization of many probiotic bacteria has been done on the basis of their adhesion capacity (Del Re *et al.*, 2000). It has been reported that the bacteria having high autoaggregation capacity also show good adhesion capacity (Del Re *et al.*, 2000).

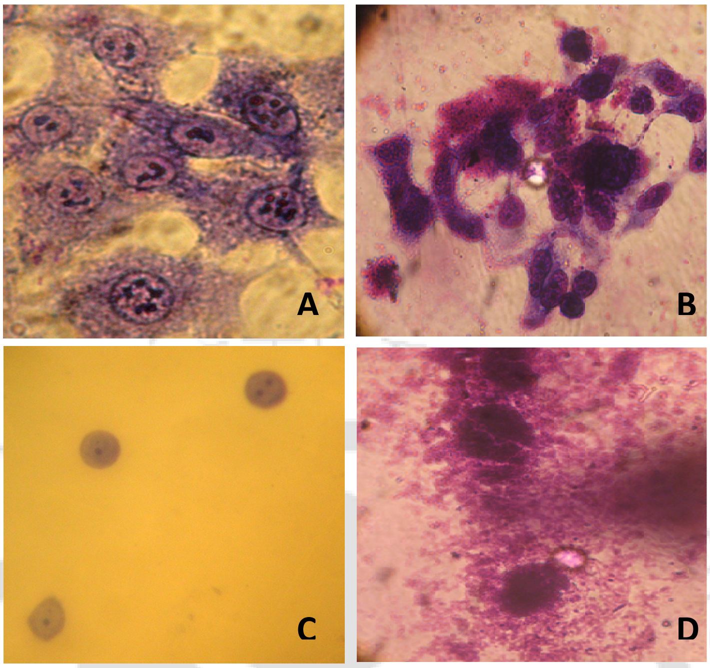

Fig. 6.3.2 Determination of adhesive capacity of *Pediococcus pentosaceus* CRAG3 on cervical cancer HeLa and murine macrophage J774A.1 cell lines. **(A)** Untreated HeLa cells (control); **(B)** Adherence of *Pediococcus pentosaceus* CRAG3 on HeLa cells; **(C)** Untreated J774A.1 cells (control); **(D)** Adherence of *Pediococcus pentosaceus* CRAG3 on J774A.1 cells.

6.3.2 Resistance to biological barriers

6.3.2.1 Resistance to lysozyme

The overall resistance of *P. pentosaceus* CRAG3 to lysozyme was expressed in terms of percent survival. The isolate showed resistance to 100 µg/ml of lysozyme with 75% (6.0 x 10^5 CFU/ml) survival after 2 h as compared to control having 7.95 x 10^5 CFU/ml (Fig. 6.3.3). The similar results were observed in other probiotic bacteria such as *Lactobacillus plantarum* Lp751 (72.52%), *Lactobacillus plantarum* Lp803 (72.64) and *Lactobacillus plantarum* Lp813 (74.04%) (Zago *et al.*, 2011). The swallowed probiotic microorganisms generally encounter first biological barrier of lysozyme of saliva in the mouth. The next barrier is gastric juice in stomach where the pH is between 1.5 and 3.0 and the upper part of small intestine, which contains bile (Corzo and Gilliland, 1999).

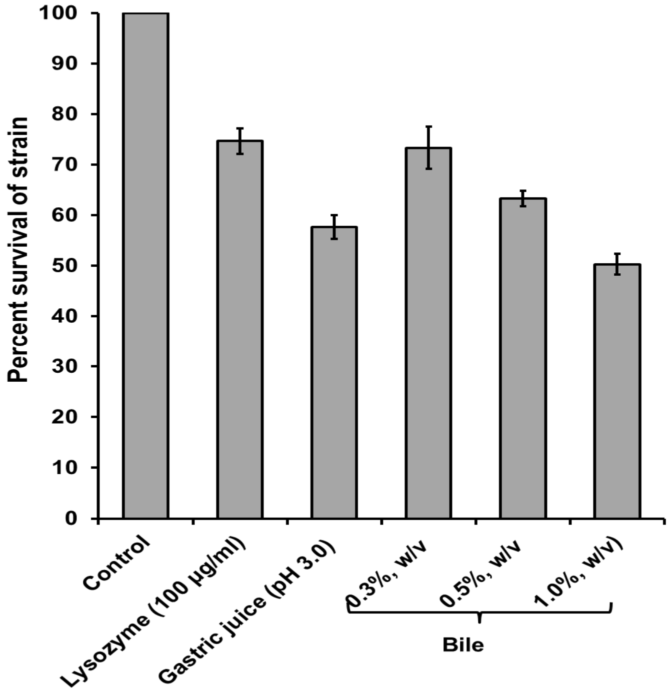

Fig. 6.3.3 Resistance of *Pediococcus pentosaceus* CRAG3 against biological barriers. Percent growth of the isolate in presence of 100 µg/ml lysozyme after 2 h, simulated gastric juice (pH 3.0) and bile salts at 0.3%, 0.5% and 1.0% (w/v) concentration.

6.3.2.2 Resistance to gastric juice

The resistance of *P. pentosaceus* CRAG3 towards gastric juice is shown in Fig. 6.3.3. The number of CFU/ml before incubation with gastric juice of pH 2 and pH 3 were 4 x 10^8 and 12.6 x 10^8, respectively. It showed resistance to simulated gastric juice of pH 3.0 with 57.68±2.4% survival (7.2 x 10^8 CFU/ml) but no survival was observed in pH 2.0. A lactic acid bacterium strain, *Leuconostoc mesenteroides* isolated from intestine of snakehead fish (*Channa striatus*) showing growth at pH 3 to 7 was considered as probiotic (Allameh *et al.*, 2012). According to Erkkilä and Petäjä

(2000) the strains of *Pediococcus acidilactici* (P2), *Lactobacillus curvatus* (RM10), and *P. pentosaceus* (FF) showed tolerance to acid (pH 3.0). The survival of *P. pentosaceus* CRAG3 at pH 3.0 indicated that it could transit through the stomach.

6.3.2.3 Resistance to bile salts

P. pentosaceus CRAG3 showed 73.33±4.2% survival in presence of 0.3% bile salts, 63.3±1.5% survival in 0.5% and 50.0±2.1% survival in presence of 1.0% bile salt concentration (Fig. 6.3.3). *P. pentosaceus* CRAG3 showed 8% higher survival than *P. pentosaceus* Z13P, which showed 65% viable cells at 0.3% bile salt concentration as reported by Yuksekdag and Aslim (2010). Bile composed of bile acids, cholesterol, phospholipids and biliverdin (Carey and Duane 1994; Hofmann, 1994) and plays vital role in fat digestion and also as potent antimicrobial agent. The human body contains bile salts in large intestine and the relevant physiological concentrations of human bile range from 0.3% (Umezawa *et al.*, 1983) to 0.5% (Zavaglia *et al.*, 1998). Thus, it is generally considered necessary to evaluate the ability of potentially probiotic bacteria to resist the effects of bile acids (Collins *et al.*, 1998).

6.3.3 Bile salt hydrolase activity

6.3.3.1 Bile salt deconjugation

The appearance of halos (nearly 6 mm) around colonies after growth in MRS-TDCA medium validated the ability of *Pediococcus pentosaceus* CRAG3 to hydrolyze sodium salt of taurodeoxycholate (Fig. 6.3.4). The presence of precipitated bile acid around colonies (opaque halo) confirmed the hydrolysis of bile salts.

Normally the bile acids are conserved by enterohepatic recirculation. The deconjugation of bile salts is an essential process only after which they can be modified for further use (Batta *et al.*, 1990). The inhibition of common intestinal bacteria is related to the presence of free (deconjugated) bile acids rather than conjugated ones (De Smet *et al.*, 1995; Grill *et al.*, 2000). The ability to hydrolyze bile salts could help the microorganism in sustaining the balance of the gut microflora (Rosenberg *et al.*, 1980; Taranto *et al.*, 1996). It has been suggested that bile salt hydrolase (BSH) enzyme might be a detergent shock protein that enables LAB to survive the intestinal bile stress (De Smet *et al.*, 1995).

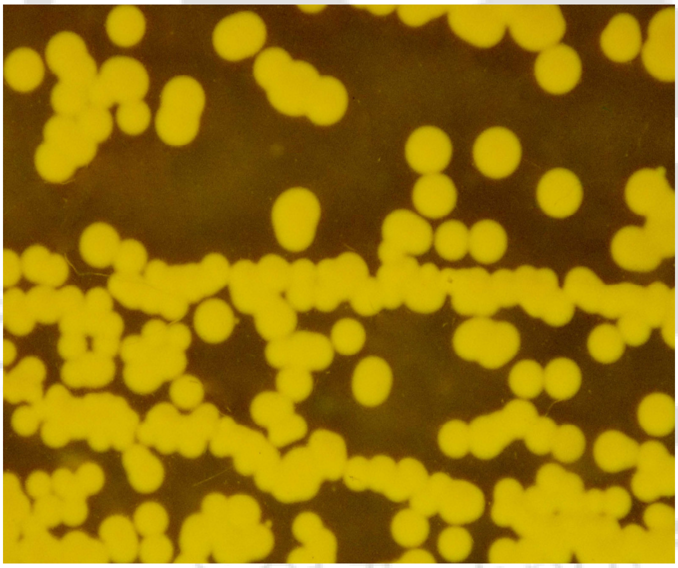

Fig. 6.3.4 Colonies of *Pediococcus pentosaceus* CRAG3 on MRS agar supplemented with 0.5% sodium salt of taurodeoxycholic acid showing bile salts hydrolase activity.

6.3.3.2 Bile salt hydrolase activity assay

The bile salt hydrolase (BSH) activity obtained from cell extract of *Pediococcus pentosaceus* CRAG3 was 1.04 U/ml which was in good agreement with previously reported probiotic bacteria. This is also an important property exhibited by a probiotic bacterium which signifies that barely soluble free bile acids formed by the deconjugation of conjugated bile salts are hardly reabsorbed by the intestinal lumen as compared to their conjugated counterpart. These bile salts are lost from the human body through faeces (Center, 1993) and result in increased metabolism of cholesterol due to which serum cholesterol reduces (Reynier *et al.*, 1981). The probiotic bacteria *L. acidophilus* ATCC 4356 and *L. casei* ASCC 290 showed 1.16 U/ml and 1.06 U/ml BSH activity towards sodium taurocholate (Liong and Shah, 2005). The BSH activity has also been reported in many probiotic routinely used strains such as *Lactobacillus* (Batta *et al.*, 1990; Bateup *et al.*, 1995; De Smet *et al.*, 1995; Elkins *et al.*, 2001) and *Bifidobacterium* (Grill *et al.*, 1995; Kim *et al.*, 2004; Kim *et al.*, 2005).

6.3.4 Antibacterial assay

Pediococcus pentosaceus CRAG3 displayed antibacterial activity against all three test microorganisms. The zone of inhibition observed by cell-free filtrate of *Pediococcus pentosaceus* CRAG3 without neutralizing around *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* was 12±0.2 mm, 7±0.1 mm and 15±0.2 mm, respectively (Fig. 6.3.5). Similar results were observed in *Pediococcus spp.* isolated from Turkish type fermented sausages which showed antibacterial activity against *Listeria monocytogenes*, *E. coli* and *Micrococcus flavus* (Yuksekdag and Aslim, 2010). No zone of inhibition was observed around wells containing neutralized cell-free filtrate of *Pediococcus pentosaceus* CRAG3 (Fig. 6.3.5). This might be due to the inability of the isolate to form any bacteriocin. The antibacterial activity exhibited by *Pediococcus pentosaceus* CRAG3 might be due to the production of lactic acid, hydrogen peroxide or other acids. The production of lactic acid by *Pediococcus pentosaceus* CRAG3 was analysed using the method as described in Section 6.2.7. The isolate produced 145±3.27 mg lactic acid per ml of cell free supernatant. Similar results were reported for *Pediococcus* spp. GS4 which produced 130 mg/ml lactic acid (Sukumar and Ghosh, 2010). Lactic acid bacteria have ability to produce antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins to inhibit the growth of pathogenic and spoilage microorganisms (Herreros *et al.*, 2005).

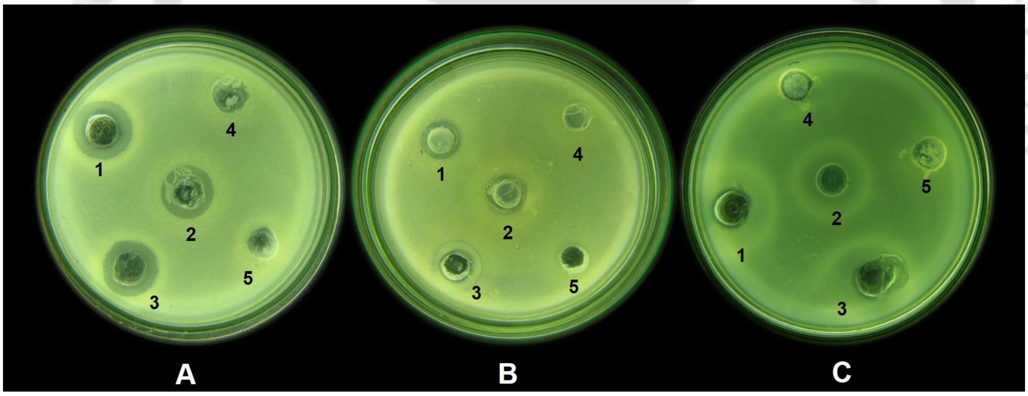

Fig. 6.3.5 Antibacterial activity of cell-free supernatant from *Pediococcus pentosaceus* CRAG3. The wells contain cell-free filtrate from *Pediococcus pentosaceus* CRAG3 without neutralizing (1, 2 and 3) and after neutralizing (4 and 5); against (A) *E. coli*; (B) *B. subtilis*; (C) *S. aureus.*

6.3.5 Utilization of prebiotics

Prebiotics are non-digestible carbohydrates which selectively stimulate the growth and/or activity of probiotic bacteria in the colon and thus improving the host health (Gibson and Roberfroid, 1995). The growth profile of *Pediococcus pentosaceus* CRAG3 in presence of different prebiotics is shown in Fig. 6.3.6. *Pediococcus pentosaceus* CRAG3 utilized inulin with 37.5% relative cell growth as compared with glucose at 24 h. It proved to be better than previously reported probiotic strains of *Lactobacillus plantarum* which were not capable of using inulin within 24 h (Zago *et al.*, 2011). However, it showed only 6% relative cell growth in presence of raffinose as carbon source (Fig. 6.3.6). Metabolism of prebiotics is an important feature observed in probiotics. The probiotic strains utilize different combinations of prebiotic in a strain specific manner. This selective utilization of the prebiotic by the putative probiotic strains, enhance their physiological benefits (Zago *et al.*, 2011).

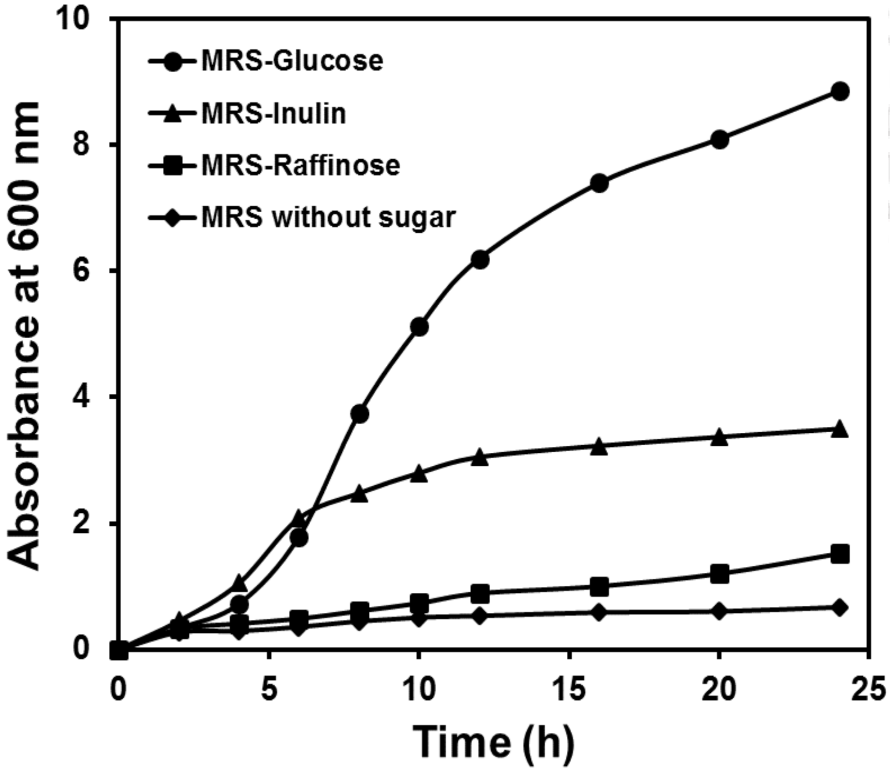

Fig. 6.3.6 Growth profile of *Pediococcus pentosaceus* CRAG3 in MRS medium containing inulin, raffinose or glucose as carbon source at 37°C. The MRS medium without any carbon source was taken as negative control

6.4 Conclusions

The new isolate *Pediococcus pentosaceus* CRAG3 (GenBank accession no. JX679020) from fermented cucumber was evaluated for probiotic properties by *in vitro* studies. The probiotic potential of *Pediococcus pentosaceus* CRAG3 was determined by analysing surface adhesive property, hydrophobicity and resistance to biological barriers. The isolate showed 33% hydrophobicity after 20 min which signifies the hydrophobic nature of its cell surface and significant factor in microbial adhesion. It displayed 43% autoaggregation and 10% coaggregation with *E. coli* after 5 h which are also related to cell adhesion properties of a probiotic microorganism. The coaggregation of *Pediococcus pentosaceus* CRAG3 with other microbes could increase its colonization potential if it was to be used in mixed culture as probiotics. It may also constitute an important host defence mechanism against infection, since it is a potent producer of lactic acid (an inhibitor to pathogenic bacteria). It displayed good adhesion property with mammalian cell lines (HeLa and J774A.1) in *in vitro* conditions which confirmed its ability to adhere with mammalian cells signifying in competing with other microorganisms in gastrointestinal tract. The isolate also showed ability to resist 100 µg/ml lysozyme for 2 h with 75.0% survival showing its ability to resist the first biological barrier in mouth. *P. pentosaceus* CRAG3 displayed tolerance against simulated gastric juice (pH 3.0) with 58% survival showing its ability to transit through high acidic environment of stomach. It possessed tolerance (73%, 63% and 50% survival) in presence of 0.3%, 0.5% and 1.0% (w/v) bile salts, respectively, which are potent antimicrobial agents. *Pediococcus pentosaceus* CRAG3 also showed 1.04 U/ml bile salts hydrolase (BSH) activity with yellow halo (6 mm) around colonies. It exhibited antibacterial activity against *E. coli*, *S. aureus* and *B.*

subtilis which might be due to the production of lactic acid signifying its capability to kill pathogenic microorganisms. *Pediococcus pentosaceus* CRAG3 displayed ability to utilize prebiotics such as inulin and raffinose with 37.5% and 6%, respectively relative cell growth which is also one of the important properties of a microorganism to behave as probiotic. Based on above all results it could be concluded that *Pediococcus pentosaceus* CRAG3 can serve as probiotic bacterium and with glucansucrase and glucan producing ability could play potential role for functional food applications.

6.5 References

Allameh, S. K., Daud, H., Yusoff, F. M., Saad, C. R., and Ideris, A. (2012) Isolation, identification and characterization of *Leuconostoc mesenteroides* as a new probiotic from intestine of snakehead fish (*Channa striatus*). Afr. J. Biotechnol. 11, 3810-3816.

Bateup, J.M., McConnell, M.A., Jenkinson, H.F. and Tannock, G.W. (1995) Comparison of *Lactobacillus* strains with respect to bile salt hydrolase activity, colonization of the gastrointestinal tract, and growth rate of the murine host. Appl. Environ. Microbiol. 61, 1147-1149.

Batta, A.K., Salen, G., Arora, R., Shefer, S., Batta, M. and Perseon, A. (1990) Side chain conjugation prevents bacterial 7-dehydroxylation of bile acids. J. Biol. Chem. 265, 10925-10928.

Bezkorovainy, A., Miller-Catchpole, R. and Kot, E. (1997) Health benefits of bifidobacteria. Tecnologı´a La´ctea Latinoamericana, 10, 34-41.

Busscher, H. J. and Weerkamp, A. H. (1987) Specific and non-specific interactions in bacterial adhesion to solid substrata. FEMS Microbiol. Rev. 46, 165-173.

Carey, M. C. and Duane, W. C. (1994) Enterohepatic circulation, In: *The liver: biology and pathobiology.* (ed. Arias, I. M., Boyer, N., Fausto, N., Jackoby, W. B., Schachter, D. A. and Shafritz, D. A.). Raven Press, Ltd., New York, N.Y. pp 719-738.

Center, S. A. (1993) Serum bile acid in companion animal medicine. In: *Gastroenterology*. (ed. Micheal, S. L.) The 1990s. Philadelphia: Saunders. pp. 625–657.

Charteris, W. P., Kelly, P. M., Morelli, L. and Collins, J. K. (1998) Development and application of an *in vivo* methodology to deter mine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. J. Appl. Microbiol. 84, 759-768.

Chauviere, G., Coconnier, M. H., Kerneis, S., Fourniat, J. and Servin, A. L. (1992) Adhesion of human *Lactobacillus acidophilus* strain LB to human enterocyte-like Caco-2 cells. J. Gen. Microbiol. 138, 1689-1696.

Collins, J. K., Thornton, G. and Sullivan, G. O. (1998) Selection of probiotic strains for human applications. Int. Dairy J. 8, 487-490.

Corzo, G. and Gilliland, S.E. (1999) Bile salt hydrolase activity of three strains of *Lactobacillus acidophilus*. J. Dairy Sci. 82, 472-480.

Cosson, C. and Deschamps, A.M. (1994) Comportement de souches bacteriennes probiotiques en presence de bile et de sels biliaires. Microbiologie-Aliments-Nutrition, 12, 93-98.

De Smet, I., Van Hoorde, L. Vande Woestyne, M. Christiaens, H. and Verstraete, W. (1995) Significance of bile salt hydrolytic activities of lactobacilli. J. Appl. Bacteriol. 79, 292-301.

Del Re, B., Sgorbati, B., Miglioli, M. and Palenzona, D. (2000) Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Lett. App. Microbiol. 31, 438-442.

Dunne, C., Murphy, L., Flyin, S., O'Mahony, L., O'Halloran, S.,Feeney, M., Morrisey, D., Thorton, G., Fitzgerald, G., Daly, C.,Kiely, B., Quigley, E. M. M., O'Sullivan, G. C., Shanahan, F. and Collins, K. (1999) Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. Antonie van Leeuwenhoek. 76, 279-292.

Elkins, C.A., Moser, S. A. and Savage, D. C. (2001) Genes encoding bile salt hydrolases and conjugated bile salt transporters in *Lactobacillus johnsonii* 100-100 and other *Lactobacillus* species. Microbiol. 147, 3403–3412.

Erkkilä, S. and Petäjä, E. (2000) Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. Meat Sci. 55, 297-300.

Fooks, L. J., Fuller, R. and Gibson, G. R. (1999) Prebiotics, probiotics and human gut microbiology. Int. Dairy J. 9, 53–61.

Fuller, R. 1989. Probiotics in man and animals. A review. J. Appl. Bacteriol. 66, 365.

Gibson, G. R. and Roberfroid, M. B. (1995) Dietary modulation of the human colonic microflora: introducing the concepts of prebiotics. J. Nutr. 125, 1401-1412.

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

Grant, C., and Salminen, S. (1998). The potential of Propionibacterium spp. ss probiotics. In S. Salminen, and A. von Wright (Eds.), Lactic acid bacteria. microbiological and functional aspects (pp. 588–603). New York: Marcel Dekker, Inc.

Grill, J. P., Cayuela, C., Antoine, J. M. and Schneider, F. (2000) Isolation and characterization of a *Lactobacillus amylovorus* mutant depleted in conjugated bile salt hydrolase activity: relation between activity and bile salt resistance. J. App. Microbiol. 89, 553-563.

Grill, J. P., Schneider, F. Crociani, J. and Ballongue, J. (1995) Purification and characterization of conjugated bile salt hydrolase from *Bifidobacterium longum* BB536. Appl. Environ. Microbiol. 61, 2577-2582.

Herreros, M. A., Sandoval, H., Gonzalez, L., Csatro, J. M., Fresno, J. M. and Tornadijo, M. E. (2005) Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). Food Microbiol. 22, 455-459.

Hofmann, A. F. (1994) Bile acids. In: *The liver: biology and pathobiology.* (ed. Arias, I.M. Boyer, J.L. Fausto, N. Jackoby, W.B. Schachter, D.A. and Shafritz D.A.). Raven Press, Ltd., New York, N.Y. pp 677-718.

Holzapfel, W. H., Haberer, P., Geisen, R., Bjorkroth, J. and Schillinger, U. (2001) Taxonomy and important features of probiotic microorganisms in food nutrition. Am. J. Clin. Nutr. 73, 365S-373S.

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.

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.

Kekkonen, R. A., Kajasto, E., Miettinen, M., Veckman, V., Korpela, R. and Julkunen, I. (2008) Probiotic *Leuconostoc mesenteroides* ssp. cremoris and *Streptococcus thermophilus* induce IL-12 and IFN-γ production. World J. Gastroenterol. 14, 1192-1203.

Kiely, L. J. and Olson, N.F. (2000) The physicochemical surface characteristics of *Lactobacillus casei*. Food Microbiol. 17, 277–291.

Kim, G. B., Brochet, M. and Lee. B. H. (2005) Cloning and characterization of a bile salt hydrolase (bsh) from *Bifidobacterium adolescentis*. Biotechnol. Lett. 27, 817-822.

Kim, G. B., Miyamoto, C. M. Meighen, E. A. and Lee, B. H. (2004) Cloning and characterization of the bile salt hydrolase genes (bsh) from *Bifidobacterium bifidium* strains. Appl. Environ. Microbiol. 70, 5603-5612.

Kos, B., Suskovic, J., Vukovic, S., Simpraga, M., Frece, J. and Matosic, S. (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J. App. Microbiol. 94, 981-987.

Lapage, S., Shelton, J. and Mitchell, T. (1970) In: *Methods in Microbiology*, (ed. Norris, J. and Ribbons, D.) Vol. 3A, Academic Press, London.

Liong, M. T. and Shah, N. P. (2005) Bile salt deconjugation ability, bile salt hydrolase activity and cholesterol co-precipitation ability of lactobacilli strains. Int. Dairy J. 15, 391-398.

Mirdamadi, S., Sadeghi, H., Sharafi, N., Fallahpour, M., Mohseni, F. A. and Bakhtiari, M.R. (2002) Comparison of lactic acid isomers produced by fungal and bacterial strains. Iran Biomed. J. 6, 69-75.

Naidu, A. S., Bidlack, W. R., and Clemens, R. A. (1999) Probiotic spectra of lactic acid bacteria (LB). Crit. Rev. Food Sci. Nutr. 38, 113-126.

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 treptomycin resistance (aadE) gene to that found in *Campylobacter jejuni*. Plasmid, 58,115-126.

Perez, P. F., Minnaard, Y., Disalvo, E. A., and de Antoni, G. (1998) Surface properties of bifidobacterial strains of human origin. App. Environ. Microbiol. 64, 21-26.

Prasad, J., Gill, H., Smart, J. and Gopal, P. K. (1998) Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. Int. Dairy J. 8, 993-1002.

Reynier, M. O., Montet, J. C., Gerolami, A., Marteau, C., Crotte, C., Montet, A. M. and Mathieu, S. (1981) Comparative effects of cholic, chenodeoxycholic & ursodeoxycholic acids on micellar solubilization and intestinal absorption of cholesterol. J. Lipid Res. 22, 467-473.

Rosenberg, M., Gutnick, D. and Rosenberg, E. (1980) Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. 9, 29-33.

Salminen, S. and von Wright, A. (1998). Current probiotics-safety assured? Microbial Ecology in Health and Disease, 10, 68-77. Scandinavian University Press.

Sanders, M. E. and in't Veld, J. H. (1999) Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. Antonie van Leeuwenhoek, 76, 293-315.

Spencer, R. J. and Chesson, A. (1994) The effect of *Lactobacillus* spp. on the attachment of enterotoxigenic *Escherichia coli* to isolated porcine enterocytes. J. App. Bacteriol. 77, 215-220.

Sreekumar, O. and Hosono, A. (2000) Immediate effect of Lactobacillus acidophilus on the intestinal flora and fecal enzymes of rats and the in vitro inhibition of Escherichia coli in coculture. J. Dairy Sci. 83, 931-939.

Stiles, M. E. and Holzapfel, W. H. (1997) Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36, 1-29.

Sukumar, G. and Ghosh, A. R. (2010) *Pediococcus* spp- *Pediococcus* spp. A potential probiotic isolated from Khadi (an Indian fermented food) and identified by 16S rDNA sequence analysis. Afr. J. Food Sci. 4, 597-602.

Suskovic, J., Brkic, B., Matosic, S. and Maric, V. (1997) *Lactobacillus acidophilus* M92 as potential probiotic strain. Milchwissenschaft 52, 430-435.

Tanaka, H., Hashiba, H., Kok, J. and Mierau, I. (2000) Bile salt hydrolase of Bifidobacterium longum-biochemical and genetic characterization. Appl. Environ. Microbiol. 66, 2502–2512.

Taranto, M. P., de Ruiz Holgado, A. P. and de Valdez, G. F. (1995) Bile salt hydrolase activity in *Enterococcus faecium* strains. Microbiologie-Aliments-Nutrition, 13, 375-379.

Taranto, M. P., Gonza´ lez de Llano, D., Rodrı´guez, A., Pesce de Ruiz Holgado, A. and Font de Valdez, G. (1996) Bile tolerance and cholesterol reduction by *Enterococcus faecium*, a candidate microorganism for the use as dietary adjunct in milk products. Milchwissenschaft, 51, 383-385.

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. Process Biochem. 40, 365-370.

Umezawa, H., Okami, Y., Kurasawa, S., Ohnuki, T., Ishizuka, M., Takeuchi, T., Shiio, T. and Yugari, Y. (1983) Marinactan, antitumor polysaccharide produced by marine bacteria. J. Antibiot. 5, 471-477.

Vinderola, C. G. and Reinheimer, J.A. (2003) Lactic acid starter and probiotic bacteria: a comparative "*in vitro*" study of probiotic characteristics and biological barrier resistance. Food Res. Int. 36, 895-904.

Walker, D. K. and Gilliland, S.E. (1993) Relationships among bile tolerance, bile salt deconjugation and assimilation of cholesterol by *Lactobacillus acidophilus*. J. Dairy Sci. 76, 956-961.

Yuksekdag, Z. N. and Aslim, B. (2010). Assessment of potential probiotic and starter properties of *Pediococcus* spp. isolated from Turkish-type fermented sausages (Sucuk) J. Microbiol. Biotechnol. 20, 161-168.

Zago, M., Fornasari, M. E., Carminati, D., Burns, P., Suàrez, V., Vinderola, G., Reinheimer, J. and Giraffa, G. (2011) Characterization and probiotic potential of *Lactobacillus plantarum* strains isolated from cheeses. Food Microbiol. 28, 1033-1040

Zavaglia, A.G., Kociubinsky, G., Perez, P. and de Antoni, G. (1998) Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. J. Food Protect. 61, 865-873.

List of publications

Published/accepted/submitted

1. **Rishikesh Shukla** and Arun Goyal **(2013)** Novel dextran from *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber with anticancer properties. *International Journal of Biological Macromolecules, 62,* 352– 357.
2. **Rishikesh Shukla** and Arun Goyal **(2013)** Probiotic potential of *Pediococcus pentosaceus* CRAG3 a new isolate from fermented cucumber. *Probiotics and Antimicrobial Proteins,* DOI: 10.1007/s12602-013-9149-8.
3. Veselin Bivolarski, Tonka Vasileva, **Rishikesh Shukla**, Arun Goyal and Ilia Iliev **(2012)** Physiological studies of *Leuconostoc mesenteroides* strain NRRL B-1149 during cultivation on glucose and fructose media. *Journal of Bioscience and Biotechnology, 1(3)*, 235-240.
4. **Rishikesh Shukla** and Arun Goyal **(2012)** Optimization and scale-up of fermentation of glucansucrase and branched glucan by *Pediococcus pentosaceus* CRAG3 using Taguchi methodology in bioreactor. *Journal of Bioscience and Biotechnology, 1*, 73-82.
5. Seema Patel, Damini Kothari, **Rishikesh Shukla**, Debasish Das and Arun Goyal **(2011)** Scale up of dextran production from a mutant of *Pediococcus pentosaceus* (SPAm) using optimized medium in a bioreactor. *Brazilian Archives of Biology and Technology, 54(6)*, 1125-1133.
6. Mayur Agrawal, **Rishikesh Shukla** and Arun Goyal **(2011)** UV Mutagenesis of *Leuconostoc mesenteroides* NRRL B-640 for generation of a mutant (B-640M) with hyper-producing dextransucrase activity. *Current Trends in Biotechnology and Pharmacy, 5 (4)*, 1445-1453.
7. **Rishikesh Shukla,** Shraddha Shukla, Vaselin Bivolarski, Ilia Iliev, Iskra Ivanova and Arun Goyal (**2011**) Production and structural characterization of insoluble dextran produced in the presence of maltose from *Leuconostoc mesenteroides* NRRL B-1149. Food Technol. Biotechnol. 49(3), 291-296.
8. **Rishikesh Shukla,** Ilia Iliev and Arun Goyal (**2010**) Purification and characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1149. Biotechnol. Biotechnol. Eq. 24(SE); 576-580.
9. 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.

Submitted

10. **Rishikesh Shukla,** Ilia Iliev and Arun Goyal **(2013)** *Leuconostoc mesenteroides* NRRL B-1149 as probiotic and its dextran with anticancer properties. Journal of Bioengineering and Biotechnology, (*submitted*).
11. **Rishikesh Shukla** and Arun Goyal (**2013**) Purified dextransucrase from *Pediococcus pentosaceus* CRAG3 as food additive. Indian Journal of Experimental Biology, (*submitted*).

List of conference papers

International

1. **Rishikesh Shukla,** Ilia Iliev, Iskra Ivanova and Arun Goyal (2012) *Leuconostoc mesenteroides* NRRL B-1149 as probiotic and its dextran displaying anti-cancer properties. 6th Annual Convention of Association of Biotechnology and Pharmacy (ABAP), ICEHT-2012, December 20-22, 2012; Sri Venkateswara University, Tirupati, A.P., India.
2. Veselin Bivolarski, Tonka Vasileva, **Rishikesh Shukla**, Arun Goyal and Ilia Iliev (2012) Physiological studies of strain *Leuconostoc mesenteroides* NRRL B-1149 during cultivation on glucose and fructose media. National Youth Conference, Oct 19-20, 2012; Plovdiv University Plovdiv, Bulgaria.
3. **Rishikesh Shukla** and Arun Goyal (2012) Purification and characterization of glucansucrase and glucan by *Pediococcus pentosaceus* CRAG3. 18th International Conference (Post ISCBC-2012), January 28-30, 2012; Institute of Advanced Study in Science and Technology (IASST), Guwahati, Assam, India.
4. **Rishikesh Shukla**, Rwivoo Baruah and Arun Goyal (2012) Molecular identification of a probiotic strain *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber. 18th International Conference (Post ISCBC-2012), January 28-30, 2012, Institute of Advanced Study in Science and Technology (IASST), Guwahati, Assam, India.
5. **Rishikesh Shukla** and Arun Goyal (2011) Probiotic potential and biochemical characterization of glucan producing *Lactobacillus plantarum* (RS3) isolated from fermented cucumber. 52nd Annual Conference of Association of Microbiologists of India (AMI), November 03-06, 2011; Panjab University, Chandigarh, India.
6. **Rishikesh Shukla**, Arun Dhillon and Arun Goyal (2011) Purification and characterization of *Leuconostoc mesenteroides* NRRL B-1149 sucrose hydrolyzing enzymes and enzyme synthesized oligosaccharides. 52nd Annual Conference of Association of Microbiologists of India (AMI), November 03-06, 2011; Panjab University, Chandigarh, India.

7. **Rishikesh Shukla**, Rwivoo Baruah and Arun Goyal (2011) Production, purification and characterization of polysaccharides and oligosaccharides produced by hydrolysis from *Leuconostoc mesenteroides* NRRL B-1149”; 26th Carbohydrate Conference; November 23-25, 2011; Indian Institute of Chemical Biology, Kolkata, West Bengal, India.

8. Tonka Vasileva, **Rishikesh Shukla**, Iskra Ivanova, Arun Goyal and Ilia Iliev (2011) Acceptor reactions of mannitol and lactitol with glucosyltransferases from *Leuconostoc mesenteroides* B-1149 and *Leuconostoc mesenteroides* URE 13. 9th Carbohydrate Bioengineering Meeting, May 15-18, 2011; Technical University of Lisbon, Lisbon.

9. **Rishikesh Shukla,** Seema Patel, Damini Kothari, Soumyadeep Chakraborty, Debasish Das and Arun Goyal; “Combined effects of pH and dissolved oxygen on dextran production from a mutant of soil isolate *Pediococcus pentosaceus* (SPAm), “International Symposium on Recent Advances in Cross Disciplinary Microbiology: Avenues and Challenges & International Workshop on rRNA sequencing, phylogeny & Next Generation Genome Sequencing”, 51st Annual Conference of Association of Microbiologists of India (AMI); December 14-17, 2010; Ranchi, Jharkhand, India.

10. **Rishikesh Shukla,** Seema Patel, Damini Kothari, Debasish Das and Arun Goyal; “Combined effects of freely available nitrogen substrates and carbon source on dextransucrase production from a mutant of soil isolate *Pediococcus pentosaceus* (SPAm)”; 7th BRSI Convention and International Conference on Genomic Sciences & Indo-Italian Workshop on Industrial and Pharmaceutical Biotechnology November 12-14, 2010; Madurai Kamraj University, Madurai, India.

11. **Rishikesh Shukla**, Shraddha Shukla, Ilia Iliev and Arun Goyal (2010) Production and structural characterization of insoluble glucan produced in the presence of maltose from *Leuconostoc mesenteroides* NRRL B-1149. 4th International Congress on Bioprocess in Food Industries, October 5-8, 2010; Curitiba, Brazil.

12. **Rishikesh Shukla**, Ilia Iliev and Arun Goyal (2010) Purification and characterization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1149. 2nd Balkan Conference on Biology, May 21-23, 2010; Plovdiv University, Plovdiv, Bulgaria.

13. **Rishikesh Shukla**, Mayur Agrawal and Arun Goyal; “Structural characterization of the two mutants B-640M1 and B-640M2 of *Leuconostoc mesenteroides* NRRL B-640 for the enhanced production of dextran”; 6th BRSI Convention and International Conference on Emerging Trends in Biotechnology; December 4-6, 2009 BHU, Varanasi, India.

14. Shadab Ahmed, **Rishikesh Shukla** and Arun Goyal; "Purification and biochemical characterization of bi-functional recombinant derivative (Lic26A-GH5) of cellulosomal cellulase from *Clostridium thermocellum*"; 5th BRSI Convention and International Conference on Bioprocesses in Food Industries; November 6-8, 2008; Osmania University, Hyderabad, India.

National

15. Seema Patel, **Rishikesh Shukla** and Arun Goyal; "Antibiotic sensitivity profiling and characterization of two new bacterial isolates of *Leuconostoc* sp"; North-East Conference of Medical Microbiologists; April 25-26, 2009, Down Town Hospital Complex, Guwahati, India.

VITAE

The author was born on January 19, 1983 in Gwalior, Madhya Pradesh, India. He passed the Secondary School Examination conducted by Board of Secondary Education, Madhya Pradesh, in 1998, and Board of Higher Secondary Education, Madhya Pradesh, in 2000. He completed B.Sc. with Microbiology, Chemistry and Botany as major subjects from Shrimant Madhavrao Scindia Government Model Science College, Gwalior, Madhya Pradesh, India, in 2005. He did his M.Sc. in Microbiology from Jiwaji University, Gwalior, Madhya Pradesh, India in 2007.

Mr. Rishikesh Shukla joined the Ph.D. program in July 2008 at the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781 039, Assam, India after selection by the Department based on his Graduate Aptitude Test in Engineering (GATE) score and performance at the interview. He received Junior and Senior Research Fellowships through (GATE) under the scheme run by the Ministry of Human Resource and Development (MHRD), India. He successfully completed the course work with 9.0/10 Cumulative Point Index (CPI). He gave the Open (PhD Synopsis) Seminar on Jan 03, 2014 and presented his thesis work before the Doctoral Committee and his performance was satisfactory. He submitted the PhD thesis in Jan 2014.