Bacteriocin, antioxidant and novel glucan production from probiotic *Lactobacillus plantarum* DM5 isolated from Marcha of Sikkim

A Thesis

Submitted in Partial Fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

by

DEEPLINA DAS

Under supervision of

Professor Arun Goyal

January 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, Assam, 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.

January, 2014 DEEPLINA DAS

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

It is certified that the work described in this thesis entitled "**Bacteriocin, antioxidant and novel glucan production from probiotic *Lactobacillus plantarum* DM5 isolated from Marcha of Sikkim"** by Deeplina Das 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, Assam, 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
(Thesis Supervisor)
Department of Biotechnology
Indian Institute of Technology Guwahati
Guwahati, 781 039, India

ACKNOWLEDGEMENTS

The findings I have embodied in this thesis would not have been possible without the kind support, active guidance, assistance and the encouragement of several individuals. Therefore, I take the pleasant opportunity to express my sincere gratitude and heartfelt obligation towards all the personages who have helped me in this endeavor.

My first and sincere gratitude with a deep sense of reverence goes to my supervisor Professor Arun Goyal, Department of Biotechnology, IIT Guwahati, for his excellent guidance, caring, patience and providing me with an excellent atmosphere for doing research. I am indebted to him for introducing me into the realm of this interesting work, training me with the techniques, giving me the freedom in designing and conducting the experiments. As my supervisor, he has constantly encouraged and motivated me to remain focused on achieving my goal. His advices and insight was invaluable to me. He has not only guided me in research, also his invaluable suggestion helped me to become a stronger person in life. I will remain grateful to him for all that he did for me.

I would also like to express my sincere gratitude to all my doctoral committee members Dr.(Mrs.) G.K. Saini, Dr. V.K. Dubey and Prof. T. Punniyamurthy for their continuous support, encouragement and valuable suggestions during my seminars and progress reviews that has led to the successful completion of my thesis. I extended my heartfelt gratitude to Dr. Debashish Das, for his help and support in materialization of some of my experimental endeavors.

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

I am thankful to the Indian Institute of Technology Guwahati for providing me with the state of the art infrastructure for advance level of research. The financial support for scholarship from Ministry of Human Resource Development (MHRD), India is duly acknowledged. I take this opportunity to sincerely acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi, India for providing financial assistance in the form of Senior Research Fellowship which buttressed me to perform my work comfortably.

I remain thankful to my seniors Dr. Seema Patel, Dr. Deepmoni Deka and Dr. Shadab Ahmed for their guidance and training. My completion of this study could not have been accomplished without the support of my research group members and I am immensely thankful to all of them. I am grateful to all my lab members of Department of Biotechnology for their cooperation and support.

My greatest appreciation and friendship goes to my closest friends, Priyanka, Aditi, and Sushant who were always a great support in all my struggles and frustrations during my stay in IIT Guwahati. They were always cheering me up and stood by me through the good times and bad times. I am also thankful to Himangshu Sonowal for his valuable suggestions and untried help during FACS analysis. I heartily thank my Hostel friends Chandani, Kasturi, Souvika, Sangeeta, Damini, Shuchi, Ruchi and Aruna for the humour we shared and the love they showered on me.

Lastly I am thankful to my family members whose continuous encouragement, support and blessings helped me in successful completion of my research work. I owe my achievements to my parents, elder brother and sister-in-law for their motivation. It is also pleasure to mention my nephew, Ribhu, for his immense love which gives me a moral support in achieving my goal.

Deeplina Das
January, 2014

SYNOPSIS

Lactic acid bacteria (LAB) are industrially important and beneficial microbes that are gram positive, non motile and catalase negative. They all produce lactic acid as an end product of the fermentation process. Lactic acid bacteria are commercially used in the preparation of fermented food products to improve the nutritional value and shelf life of the fermented food. They are also used for pickling of vegetables, baking, wine-making, curing fish, meats and sausage. LAB are potentially used as starter cultures for manufacture of dairy-based probiotic food. Lactic acid bacteria (LAB), as probiotic have been proven effective against diarrhoea, irritable bowel disorder, allergies and lactose intolerance. In addition to theses, LAB are involved in the production of several metabolites such as bacteriocin, bioactive peptides, antioxidant, vitamins, oligosaccharides and exopolysaccharide that can act as nutraceuticals. The isolation of LAB strains as bioprotective cultures from different fermented food products could be useful as LAB inhibit other spoilage and pathogenic microorganisms. The north eastern region comprising eight states *viz.* Assam, Arunachal Pradesh, Sikkim, Manipur, Mizoram, Meghalaya, Tripura and Nagaland are rich in numerous endemic species of microorganisms and consider as biodiversity hot spot region. In the north east India the rural population use various traditional fermented food products enriched with nutritional and medicinal properties. Therefore the fermented food products of north east India are promising for isolating commercially important lactic acid bacteria.

With this objective, a novel strain *Lactobacillus plantarum* DM5 was isolated from an ethnic fermented beverage Marcha of Sikkim, India and was explored for its prospective application in food industry as bio-preservant probiotic. The isolate *Lb. plantarum* DM5 was also examined for its *in vitro* antioxidant activities including inhibition of ascorbate auto-oxidation and scavenging activity of DPPH radicals, superoxide anion radicals and hydroxyl radicals. GABA producing ability of *Lb. plantarum* DM5 was also explored for further establishing it as an exclusive probiotic anti-oxidative strain. This novel strain of lactic acid bacteria was also explored for industrially useful glucan producing probiotic strain. The production, purification and characterization of glucan from *Lactobacillus plantarum* DM5 was carried out. The effect of purified glucan was evaluated for cytotoxicity tests on Human embryonic kidney (HEK 293) and Human cervical cancer (HeLa) cell lines. This work reports for the first time, production and purification of glucan from probiotic *Lactobacillus plantarum*.

Present work

The present investigations are carried out on the "**Bacteriocin, antioxidant and novel glucan from probiotic *Lactobacillus plantarum* DM5 isolated from Marcha of Sikkim**". The thesis work comprises 6 Chapters.

Chapter 1 is the General Introduction which represents the brief review of literature dedicated to the industrial applications of lactic acid bacteria and the importance of isolation of new strains from the biodiversity hot spot north east region of India. The chapter portrays detailed application of lactic acid bacteria as probiotic and the *in vitro* selection criteria of a novel putative probiotic strain. The chapter represents detailed review of production of γ-amminobutyric acid and antioxidative activity of lactic acid bacteria. The chapter also elaborates the potential applications of the bacteriocin, glucansucrase, glucan and their characterization. In addition, the chapter describes the molecular architecture, three dimensional structure of glucansucrase and the mechanism of glucan production using sucrose as substrate. The detailed production, purification and characterization methods of the bacteriocin, glucansucrase and the glucan have been discussed in this chapter. Various applications of *Lactobacillus plantarum* in food and pharmaceutical industry are extensively reviewed.

Chapter 2 describes the detailed protocol of screening of the natural isolate of lactic acid bacterium from Marcha, an ethnic fermented beverage of Sikkim on the basis of glucansucrase activity as well as bacteriocin activity. The isolate DM5 showed maximum zone of inhibition (>15 mm) around the well against indicator strain *Escherichia coli* DH5α and glucansucrase activity of 1.8 U/ml and was selected for further characterization. Based on biochemical and physiological studies the isolate showed its relation with other *Lactobacillus* spp. Antibiotic susceptibility test showed its resistance to the antibiotics cloxacillin, cefexime, trimethoprim, nalidixic acid and vancomycin. The isolate DM5 could efficiently utilize arabinose, cellibiose, fructose, glucose, galactose, inulin, lactose, maltose, mellibiose, mannitol, raffinose, sucrose

and trehalose like other *Lactobacillus plantarum* strains. The plasmid DNA profile of isolate DM5 showed two plasmids of molecular sizes 19.6 kb and 2.3 kb. Identification of isolate DM5 was carried out on the basis of 16S rRNA and *rpoA* sequence analysis. The 16S rRNA gene sequence analysis revealed the identity of the isolate DM5 as *Lactobacillus plantarum* (Genbank Accession Number KC020195). As the 16S rRNA gene sequence data do not allow the identification of closely related species, an alternative identification was done by using the *rpoA* partial gene sequence specific primers, which also revealed the isolate as *Lactobacillus plantarum* (Genbank Accession Number KF286000). The strain *Lb. plantarum* DM5 displayed a broad range of antimicrobial activity against both members of gram positive and gram negative bacteria. The presence and action of bacteriocin of the isolate DM5 was inferred by developing zones of inhibition around the well against the food spoilage bacteria *Staphylococcus aureus* and *Escherichia coli*.

Chapter 3 describes the potential probiotic attributes and antioxidative activity of isolate *Lb. plantarum* DM5. The isolate *Lb. plantarum* DM5 showed adequate level of survival to the harsh conditions of the gastrointestinal tract and survived low acidic pH 2.5 for 5 h. Artificial gastric juice and intestinal fluidic environment decreased the initial viable cell population of isolate DM5 only by 7% and 13%, respectively while lysozyme (200 µg/ml) and bile salt (0.5%) enhanced its growth. It possessed the bile salt hydrolase activity (1.18 U/mg) and was found to deconjugate taurodeoxycholic acid, indicating its potential to cause hypercholesterolemia. Isolate *Lb. plantarum* DM5 demonstrated cell surface hydrophobicity of 53% and auto-aggregation of 54% which are the prerequisite for adhesion to epithelial cells and colonization to host. The

adhesion ability of isolate DM5 was confirmed by a good adhesion ratio of 8.63% with HT-29 cell line. The anti-oxidative activity of isolate *Lb. plantarum* was evaluated. A concentration of 10^{10} CFU/ml of *Lb. plantarum* DM5 demonstrated the hydroxyl radical, superoxide anion radical and DPPH scavenging activities of 49%, 48%, and 55%, respectively and reducing activity of 149 µM (cysteine equivalents). *Lactobacillus plantarum* DM5 showed 38% and 20% higher hydroxyl radical, 31% and 22% higher superoxide anion radical and 43% and 33% higher DPPH scavenging activities than two standard strain *Lactobacillus plantarum* NRRL B-4496 and *Lactobacillus acidophilus* NRRL B-4495, respectively. Probiotic *Lb. plantarum* DM5 has the ability to produce bioactive γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in mammalian brain.

Chapter 4 deals with the production, purification and characterization of bacteriocin from isolate *Lb. plantarum* DM5. The cell free supernatant of *Lb. plantarum* DM5 showed bacteriocin activity of 6400 AU/ml in MRS medium (pH 6.0) against against gram positive indicator strain *S. aureus* 737 and gram negative indicator strain *E. coli* DH5α at 37ºC under static condition. The maximum bacteriocin activity was observed with an initial medium pH of 6.0 to 6.5 and the bacteriocin activity was lost by 91% and 87% at initial medium pH of 4.0 and 4.5, respectively. Growth of *Lb. plantarum* DM5 in presence of 20 g/L glucose and 20 g/L maltose yielded the same activity of 6400 AU/ml but in case of 20g/L of maltose the specific activity was higher by 3% with ± 0.8% standard error. The bacteriocin was heat stable (60 min at 100ºC) and 75% activity still remained after a heat treatment at 100ºC for 90 min. The antimicrobial activity of *Lb. plantarum* DM5 was stable in a wide pH range from 4-8

while 100% activity was recorded between pH 5 to 7. No change in antimicrobial activity of cell free supernatant of *Lb. plantarum* was recorded when it was treated with catalase, α-amylase and lysozyme; however the antimicrobial activity was completely lost when treated with proteolytic enzymes such as pepsin, trypsin, proteinase K. Hence, the antimicrobial compound present in the cell free supernatant of isolate *Lb. plantarum* DM5 was identified as bacteriocin due to its proteinaceous nature. The bacteriocin from *Lb. plantarum* showed bactericidal effect on *S. aureus, E. coli* and *L. monocytogenes* by causing 99% of cell lysis. The cell morphology of the treated *S. aureus, E. coli* and *L. monocytogenes* was completely deformed as revealed by scanning electron microscopy, suggesting the high potential of *Lb. plantarum* DM5 as natural preservatives in food industry. The bactericidal action of bacteriocin from isolate was also analyzed by flow cytometry. The antimicrobial compound was purified by 80% ammonium sulphate precipitation and showed antimicrobial activity of 2196 AU/mg with 5.3 fold purification. The bacteriocin from *Lb. plantarum* DM5 was further purified by cation exchange chromatography and gel filtration, which showed single distinct band of molecular mass of 15.2 kDa on 15% SDS PAGE, confirming the homogeneity of the protein. The result was also confirmed by an *in situ* agar gel overlay method which gave a prominent inhibition zone of *S. aureus* MTCC 737 in agar plate. Bactertiocin from *Lb. plantarum* DM5 was subjected to cytotoxicity assay using HEK-293 and HeLa cells and the *in vitro* cytotoxicity data revealed the biocompatible nature of bacteriocin.

Chapter 5 deals with the production, purification and functional characterization of glucansucrase from isolate *Lb. plantarum* DM5. The cell free supernatant of *Lb.*

plantarum DM5 displayed glucansucrase activity of 2.7 U/ml (0.48 U/mg) at 27°C under static condition. The medium compositions for glucansucrase production were optimized and was found that K_2HPO_4 (2.5%, w/v), yeast extract (2.5%, w/v), Tween 80 (0.6%, v/v), and sucrose (5%, w/v) enhanced the activity by 20%, 22%, 68% and 230%, respectively. The cell free supernatant of *Lb. plantarum* DM5 containing extracellular glucansucrase was purified using polyethylene glycol 400 and 1500 fractionation followed by gel filtration using sephacryl S-300HR. The cell free supernatant (0.48 U/mg, 5.7 mg/ml) was subjected to fractionation with various concentrations of PEG-400 and PEG-1500. The maximum specific activity of 6.7 U/mg and 10.1 U/mg was achieved at 36%, (v/v) PEG-400 and 15%, (w/v) PEG-1500, respectively. The glucansucrase purified by 15%, (w/v) PEG-1500 was subjected to next step of purification by gel filtration using Sephacryl S-300HR. The column purified glucansucrase gave enhanced specific activity of 18.7 U/mg with 40-fold purification and showed molecular mass of approximately, 148 kDa. The zymogram analysis of purified enzyme confirmed that it was glucansucrase as it showed magenta colour band only in presence of sucrose not in presence of raffinose. The purified glucansucrase was maximally active at 30ºC and pH 5.4 and was stable at acidic pH and low temperature. The V_m and K_m of purified glucansucrase for sucrose as substrate was 19.6 µmoles/mg/min and 4.5 mM. Divalent cations Mg^{2+}, Ca^{2+} and Co^{2+} enhanced glucansucrase activity by 16%, 18% and 19%, respectively, whereas Hg^{2+} and Mn^{2+} decreased the enzyme activity by 81% and 79%, respectively when assayed in presence of sucrose. Among stabilizers, dextran T-40, PEG 6000, PEG 8000, glutaraldehyde, glycerol, Tween 80 and acetonitrile, Tween 80 provided maximum stabilization with half life ($t_{1/2}$) of 86 days as compared with the enzyme ($t_{1/2}$= 65 days)

without any added stabilizer at -20ºC. The overall biochemical characterization reveals a promising novel glucansucrase which can compensate the increasing demand of glucan as viscosifier and stabilizer in food industry.

Chapter 6 describes the characterization and biocompatibility of glucan, a safe food additive from probiotic *Lb. plantarum* DM5. The exopolysaccharide (1.48 mg/ml) synthesized by *Lb. plantarum* DM5 was purified by ethanol precipitation and gel filtration using Sepharose CL-6B. Monosaccharide analysis of purified exopolysaccharide showed only glucose residues as building blocks and thus confirmed its glucan nature. The gel permeation chromatography of glucan revealed an average molecular mass of $1.11x10^6$ Da. The structural characterization of purified glucan was carried out using FTIR, ^{1}H-NMR and ^{13}C-NMR techniques. The peak at 1024 cm^{-1} in the FTIR spectrum indicated the presence of α(1→6) linkages and the peak at 1102 cm^{-1} corresponded to the α-(1→3) branched linkages. ^{1}H-NMR and ^{13}C-NMR data confirmed the abundance of α-(1→6) glycosidic bonds in the backbone of the glucan, with α-(1→3) linkage. The integration analysis of ^{1}H NMR revealed 86.5% of α-(1→6) and 13.5% α-(1→3) branching with average chain length of 7 glucose units between branch linkages. The rheological study of glucan revealed that the viscosity was decreased with the increase in shear rate exhibiting a typical non-Newtonian pseudoplastic behaviour. The surface morphology showed that it has porous structure and may be used as thickening and gelling agent in dairy and bakery industry. The glucan polymer displayed 21.6% solubility and 317% water holding capacity. It also showed flocculation activity and emulsification activity of 80.6% and 86.2% respectively and can be potentially used as bio-flocculent and emulsifier in

food as well as non-food industry. The glucan polymer revealed excellent thermal stability with degradation temperature (T_d) of 292.2ºC, which indicates that the polymer can able to withstand high temperature during food processing and manufacturing. The glucan exerted considerable nontoxic effect on HEK 293 and HeLa cell lines, displaying its biocompatible nature. This is the first report on the structure and biocompatibility of homopolysaccharide α-D-glucan from probiotic *Lactobacillus plantarum* strain with its unique physical and rheological properties that can facilitate its application in food industry as viscosifying and gelling agent.

CONTENTS

Chapter 3. Probiotic attributes and antioxidative activity of *Lactobacillus plantarum* DM5

Chapter 5. Production, purification and functional characterization of glucansucrase from *Lactobacillus plantarum* DM5

Chapter 1

General Introduction

1.1 Introduction

Lactic acid bacteria (LAB) are industrially important and beneficial microbes that have similar properties (gram positive and catalase negative) and all produce lactic acid as an end product of the fermentation process. The genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* are recognized as LAB (Ercolini *et al*., 2001; Holzapfel *et al.,* 2001). They are widespread in nature and are also found in our digestive systems. There are several potential health and nutritional benefits possible from several species of lactic acid bacteria, among these are: improved nutritional value of food, control of intestinal infections, improved digestion of lactose, control of some type of cancer and control of serum cholesterol level (Holzapfel *et al.,* 2001; Galvez *et al*., 2007; Bao *et al*., 2012). Although they are best known for their role in the preparation of fermented dairy products, they are also used for pickling of vegetables, baking, wine-making, curing fish, meats and sausages. *Lactobacillus plantarum* and

Lactobacillus sanfrancisco are commercially available and widely used especially for the production of fermented milk products and for the preparation of sourdough (Leroy and De vuyst, 2004). LABs are also regarded as a major group of probiotic bacteria (Schrezenmeir and de Vrese, 2001; Williams, 2010) that is used as live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Forssten *et al*., 2011). LAB produces various types of exopolysaccharide which has numerous applications in food and pharmaceutical industry (Purama and Goyal, 2005; Das and Goyal, 2012). The various applications of LAB in food and fermentation industry are shown in Fig.1.1.

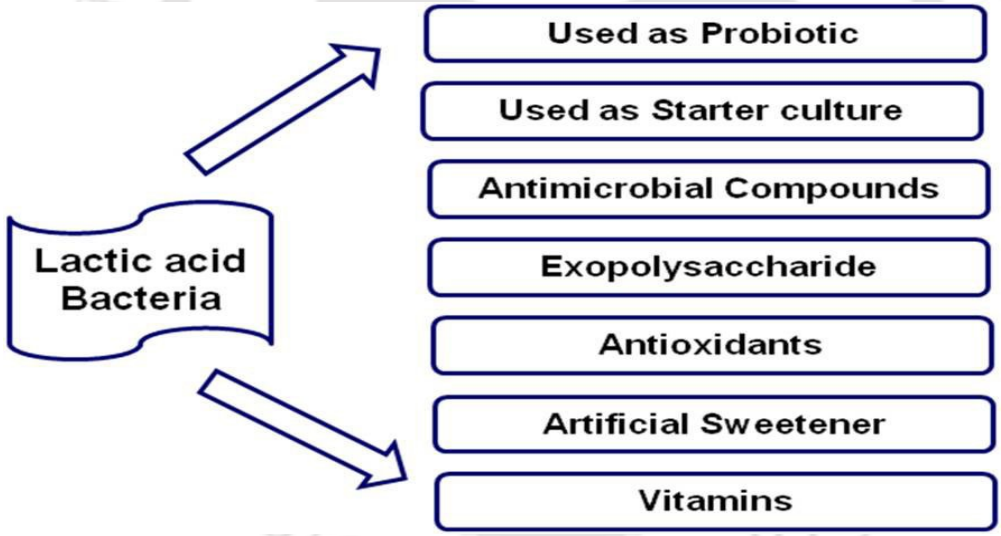

Fig. 1.1 Applications of Lactic acid bacteria in food and fermentation industry.

Lactic acid bacteria are either homofermentative or heterofermentative based on the organism's metabolic pathway. Homofermentative bacteria such as *Lactococcus and Streptococcus* followed Embden Meyerhof Parnas pathway (EMP, or glycolysis) and yielded two lactate from one glucose molecule, whereas the heterofermentative bacteria such as *Leuconostoc* and *Weissella* transform a glucose

molecule into lactate, ethanol and carbon dioxide (Kandler and Wiess, 1986; Fugelsang and Edward, 2007) by following the pentose phosphate pathway. The most diverse group of LAB is *Lactobacillus* species and according to the carbohydrate metabolism (Kandler and Wiess, 1986; Todorov and Franco, 2010), it can be divided in three groups;

Group I	:	Obligate homofermentative; includes *Lactobacillus delbrüeckii* and *Lactobacillus acidophilus*.
Group II	:	Facultative heterofermentative; includes *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus sakei*.
Group III	:	Obligate heterofermentative: *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus reuteri.*

1.2 Lactic acid bacteria in food industry

Lactic acid bacteria is used throughout the world for the production of specialty foods, particularly fermented milk products, including yoghurt, cheese, butter, buttermilk and kefir (Leroy and De vuyst, 2004). Some of the traditional fermented food products and the associated lactic acid bacteria are listed in Table 1.1 (Das and Goyal, 2012). Lactic acid bacteria also produce acetic acid, aroma compounds, bacteriocins and exopolysaccharides and several important enzymes. For example, acetaldehyde provides the characteristic aroma of yoghurt (Leroy and De vuyst, 2004), while diacetyl imparts a buttery taste to other fermented milk which improve the taste and quality (Leroy and De vuyst, 2004; Todorov and Franco, 2010) The fermentation by lactic acid bacteria also produces lactic acid in fermented milk which gives slightly tart taste (Todorov and Franco, 2010). Several metabolites produced by LAB such as organic acids, fatty acids, hydrogen peroxide, carbon

dioxide and bioactive peptides have antimicrobial effects (Galvez *et al*., 2007). As biopreservatives are preferred over chemical preservatives, there is an increased interest in prevention of food from spoilage, through these metabolites produced by LAB because of their safe association with human fermented foods.

Table 1.1 Fermented food products and associated bacteria (Das and Goyal, 2012).

Fermented food	Lactic acid bacteria	Country	Sources
Kimchi	*Leuconostoc mesenteroides, Lactobacillus curvatus, Lactobacillus brevis, Lactobacillus sakei* and *Lactobacillus plantarum*	Korea	Fermented mixture of Chinese cabbage, radishes, red pepper, garlic and ginger
Kefir	*Lactobacillus kefir, Lactobacillus brevis*	Russia	Fermented milk product
Swiss cheese	*Lactobacillus lactis, Lactobacillus delbrueckii*	Switzerland	Dairy product
nham	*Weissella cibaria, Leuconostoc citreum*	Thailand	Fermented fresh Pork
Magou	*Leuconostoc mesenteroides, Pediococcus cerevisiae, Streptococcus lactis*	South africa	Fermented maize porridge
Balao balao	*Streptococcus sp., Leuconostoc sp., Pediococcus sp.*	Phillipines	Fermented rice and shrimp mixture
Gari	*Lactobacillus pentosus, Leuconostoc fallax*, *Weissella paramesenteroides Lactobacillus fermentum*	Nigeria	Fermented cassava
Kishk	*Lactobacillus sakei, Leuconostoc sp.*	Egypt	Fermented cereal and milk mixture
Laban rayeb	*Streptococcus feacalis*	Egypt	Fermented milks
Ras cheese	*Lactococcus sp., Lactobacillus sp., Enterococcus sp.,* and *Pediococcus sp.*	*Egypt*	Dairy product
Sauerkraut	*Leuconostoc mesenteriodes, Lactobacillus plantarum, Pediococcus acidilactici*	Western countries	Fermented cabbage
Sourdourgh	*Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus panis, Weissella cibaria*	Europe	Fermented cereals

1.3 Microbial diversity of India

Microbial diversity comprises the spectrum of variability among all types of microorganisms *viz*. bacteria, fungi, viruses and also mutated microorganisms that are altered at genomic level by human intervention. North eastern states of India is

blessed with a wide range of physiographic and eco-climatic conditions and the geographical 'gateway' for much of country's endemic flora as well as fauna and hence is considered as biodiversity hot spot of India (Myers *et al*., 2000; Singh *et al*., 2009). The eastern Himalayas biodiversity 'hotspot' was modified to the 'Indo-Burma hotspot' covering central Nepal to whole of north east India, Andaman and Nicobar Islands, Hainan island in southern China, Myanmar, Thailand and southern Malaysian peninsula and the second largest with an area 2,20,60,000 sq km among the 25 identified globally (Myers, 2000). Western ghats and the north eastern region of India are the biodiversity rich areas and are a genetic treasure houses of plant, animal and microbial resources (Singh *et al.,* 2009; Jeyaram *et al*., 2009). The north eastern region comprising eight states *viz.* Assam, Arunachal Pradesh, Sikkim, Manipur, Mizoram, Meghalaya, Tripura and Nagaland are rich in numerous endemic species of microorganisms (Singh *et al*., 2009). Diverse microorganisms ranging from filamentous fungi to enzyme and alcohol producing yeast, LAB, bacilli and micrococci are associated with fermentation and production of ethnic food alcoholic beverages in north eastern region of India (Tamang *et al*., 2012).

1.3.1 Importance of LAB in fermented food products of north east India

In the north east India the rural population use various traditional fermented food products and also use fermentation for preserving food. More than 250 different indigenous fermented foods (fermented soyabean, bamboo shoot, fish, milk, meat and leafy vegetables) contribute to a large proportion of the daily food intake of people of north eastern states of India (Tamang *et al*., 2005). In addition to preservation, fermented foods can also have additional benefits of enhanced flavour, increased

digestibility and improved nutritional and pharmaceutical values (Jeyaram *et al*., 2009). Each fermented product is associated with a unique group of microflora which increases the level of protein, vitamins, essential amino acids and fatty acids (Satish Kumar *et al*., 2012). Some of the non dairy fermented food products and their associated LAB are listed in Table 1.2.

Table 1.2 Non dairy fermented food products of north east India.

Product	State	Substrate	Nature and use	Reference
Marcha	Sikkim	Rice, Wild herbs, spices	Starter culture to ferment alcoholic beverages	Tamang *et al*., 2007
Kodo ko Jaanr	Sikkim	Finger Millet	Mild-alcoholic beverage	Thapa and Tamang, 2006
Ngari	Manipur and Assam	Fish	Side dish with Cooked rice	Jeyaram *et al*., 2009
Tungtap	Meghalaya	Fish	Pickle	Tamang *et al*., 2005
Uttongiri	Assam	Fish	Side dish	Jeyram et al., 2009
Kheuri	Sikkim	Yak/beef	Curry product	Rai *et al*., 2009
Kargyong	Sikkim	Goat/pigs	Sausage	Rai *et al*., 2009
Stachu	Sikkim	Beef	Side dish	Rai *et al*., 2009
Mesu	Manipur and Assam	Bamboo shoot	Pickle	Tamang *et al*., 2012
Hentak	Manipur	Fish	Curry product	Jeyaram *et al*., 2009
Tungrymbai	Meghalaya	Soyabean	Pickle	Thokchom and Joshi, 2012
Khalpi	Meghalaya	Cucumber	Pickle	Rai *et al*., 2009
Hamei	Manipur	Rice and wild herbs	Starter culture to ferment alcoholic beverages	Tamang *et al*., 2007
Geema	Sikkim	Red goat meat	Curry product	Rai *et al*., 2009
Chilu	Sikkim	Lamb meat	Edible oil	Rai *et al*., 2009
Phab	Manipur and Sikkim	Hebrs and spices	Alcoholic beverages	Tamang *et al*., 2012
Suka ka masu	Sikkim and Darjeeling Hills	Buffalo or goat meat	Curry product	Rai *et al*., 2009

1.3.2 Preparation of Marcha

Marcha is prepared from soaked glutinous rice mixed with various parts from locally grown plants, such as roots of ‘guliyo jara’ (*Plumbago zeylanica*), leaves of

'bheemsen paate' (*Buddleja asiatica*), flowers of 'sengreknna' (*Vernonia cinerea)* (Tamang *et al.,* 2007). The flow diagram for preparation of Marcha is shown in Fig. 1.2.

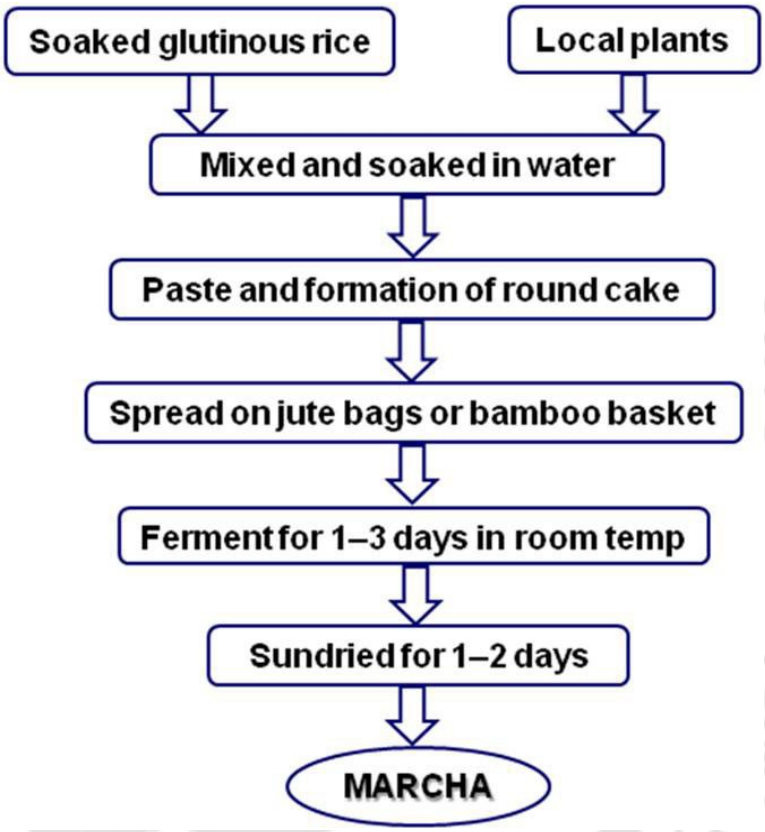

Fig. 1.2 Flow chart for Marcha preparation.

Marcha is used to prepare alcoholic beverages such as Kodo ko jaanr (fermented finger millet beverage), Bhaati jaanr (fermented rice beverages) and Raksi (distilled liquor) in Sikkim and the Darjeeling hills (Tamang *et al*., 2012). It is reported that the population of lactic acid bacteria (LAB) is 7.1 log CFU/g in Marcha. (Tamang *et al*., 2007).

Fig. 1.3 Sun dried Marcha cake after fermentation in Jute bags.

1.4 LAB as probiotic

The present day most active functional foods in world market are probiotic dairy products, in particular probiotic yogurts and milk. Traditionally, probiotic cultures are added to yogurt and other fermented dairy products, but lactose intolerance and the cholesterol content are the two major drawbacks related to their consumption (Schrezenmeir and de Vrese, 2001, Ouwehand *et al.,* 2002). As a result, the development of non dairy probiotic food products is a challenge to the food industry. A healthy digestive system is primarily made up of these beneficial bacteria or probiotics. Probiotics are one of the functional food supplements that have health promoting and beneficial effects on human. The name “probiotic” comes from the Greek word 'pro bios' which means 'for life' (Kollath, 1953) and now it is defined as 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host’ (Fuller, 1989; Lamari *et al*., 2013). Lactic acid bacteria (LAB) are the most common type of microorganisms used as probiotics and mainly *Lactobacilli,*

Streptococci, Enterococci and *Lactococci* can be used as probiotics (Schrezenmeir and de Vrese, 2001; Ouwehand *et al.,* 2002). They possess the property by which they can be considered as probiotic such as colonization or adhesion properties, good *in vitro* development, ability of cells to produce metabolites and enzymes, stability in bile and gastric juices, production of antimicrobial substances, antagonistic action against noted pathogenic bacteria and/or viruses and no adverse interactions with host especially, in terms of pathogenicity (Dunne *et al*., 2001; Ouwehand *et al.,* 2002; Jin *et al*., 2011). It has been recommend that the selection processes for new potential probiotic strains should mainly focus on the functional properties of the probiotic strains rather than its "origin" (Dunne *et al*., 2001; Forssten *et al*., 2011).

Probiotics are now used in a variety of food products, dietary supplements and in medicines (Schrezenmeir and de Vrese, 2001; William, 2010). LABs are also potentially used as starter cultures for the manufacture of dairy-based probiotic foods (Saarela *et al*., 2002). There are several examples of probiotic foods commercially available are shown in Table 1.3. Probiotic acidophilus capsule is a naturally occurring antibiotic that helps enhance digestion produces vitamin B and brings down the risk of vaginal infection and colorectal cancer (Friedlander *et al*., 1986; Osterlund *et al*., 2007). In addition, antimicrobial production by probiotic LAB might play a role during *in vivo* interactions occurring in the human gastrointestinal tract, hence contributing to gut health. Probiotics may regulate local and systemic immunity, thereby reducing allergic disease severity and susceptibilities of infants and children to allergies and atopic diseases (Hsieh and Versalovic, 2008; Forssten *et al*., 2011).

Table 1.3 Probiotic food and their applications (Das and Goyal, 2012).

Food	Microrganism	Manufactured by	Uses
Actimel	*Lb.bulgaricus, S. thermophilus* and *Lb. casei.*	Dannon Company	Used as delicious sweet milk
Align	*Bifidobacterium infantis* 35624		Found in clinical studies to help build and maintain a a healthy digestive system as well as benefitting those suffering from irritable bowel syndrome (IBS)
LC1	*Lb. johnsonii*	Nestle	Used as baby food to reduce the risk of infant diarrhea
Lifeway Kefir	*Lb. lactis, Lb. cremoris, Lb. diacetylactis, Lb. casei*	Lifeway and is available at Wild oat markets	Used in many food products in order to improve immune system.

1.4.1 Selection criteria of a potent probiotic strain

Several guidelines have been developed for considering a product to be probiotic such as strain specification, viable numbers, the shelf life and storage conditions, which would prevent misleading the consumer (Ouwehand *et al.,* 2002). The absence of pathogenicity and infectivity is an essential pre-requisite of probiotic safety (Williams, 2010; Lamari *et al*., 2013). Several other *in vitro* studies are recommended for screening putative probiotic strains such as resistance to gastrointestinal condition, antimicrobial activity against potentially pathogenic bacteria (acid and bacteriocin production), ability to reduce pathogen adhesion to surfaces and bile salt hydrolase activity (Dunne *et al*., 2001; Jin *et al*., 2011; Bao *et al*., 2012). The several *in vitro* tests used for selecting a potential probiotic strain are shown in Fig. 1.4.

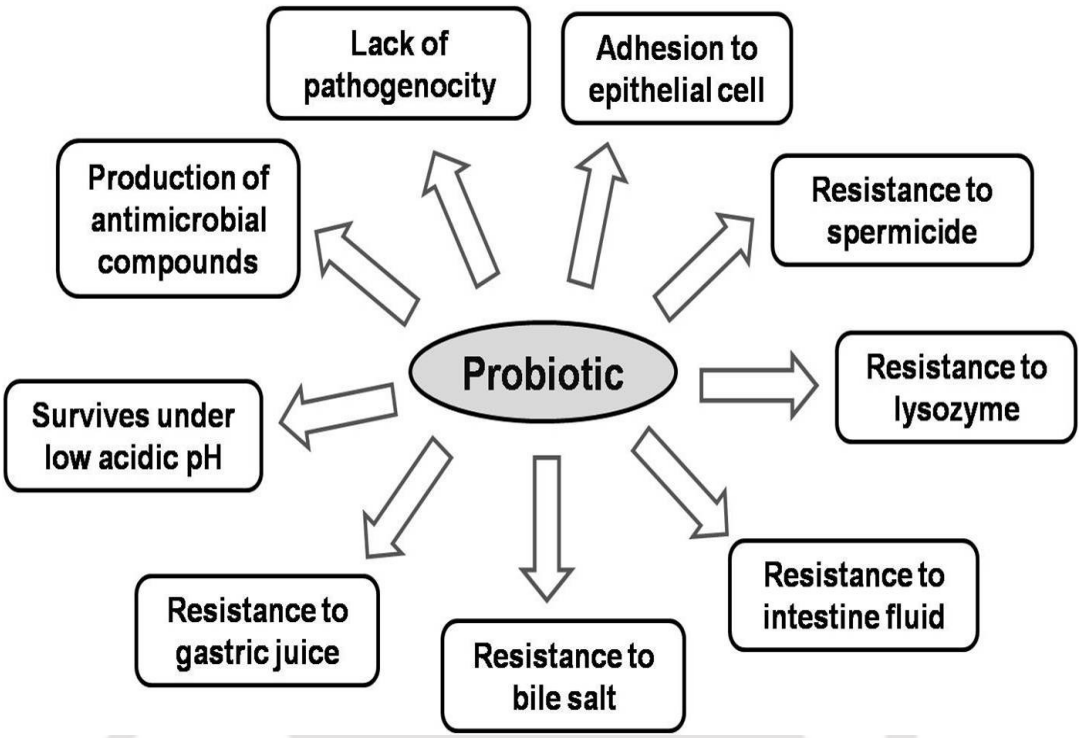

Fig. 1.4 *In vitro* selection criteria for a putative probiotic strain.

1.5 Antioxidants from LAB

Antioxidants are substances that delay or prevent the oxidation of cellular oxidisable substrates by scavenging free radicals and reactive oxygen species (ROS), preventing the generation of free radicals and ROS, and/or activating a battery of detoxifying proteins (Sies, 1997). Reactive oxygen species (ROS) are produced during passage of nutrients through the GI tract. The ROS mainly includes hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-), and free radicals, such as the hydroxyl radical (OH^-) (Hazra *et al*., 2008; Mao *et al*., 2013). Elevated levels of these highly active free radicals have been found to be associated with numerous human diseases, such as carcinogenesis, atherosclerosis, Alzheimer's disease, ageing and degenerative processes (Maxwell, 1995; Hazra *et al*., 2008). Both eukaryotic and aerobic

prokaryotic organisms have developed an overall antioxidative defence system to alleviate the damaging effects of ROS, by the help of antioxidative enzymes like superoxide dismutase (SOD), glutathione peroxidise (GSHPx) and catalase (CAT) (Miller and Britigan, 1997; Kullisar *et al*., 2002).

Antioxidants are considered important nutraceuticals on account of many health benefits (Droge, 2002; Valko *et al*., 2007). Various synthetic and natural antioxidants have been reported, however, there are doubts about the safety and long-term effects on health of synthetic antioxidants. Synthetic antioxidants, including BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and TBHQ (t-butyl hydroquinone), are commonly used in foodstuffs, however, the use of these products has been restricted because they are now suspected to be carcinogenic (Kumar *et al*., 2008). Naturally occurring antioxidants from LAB have received a great deal of attention during the past decade (Terahara *et al*., 2001). LAB also possessed several antioxidant enzymes, such as SOD, NADH-oxidase and NADH peroxide for protection against oxidative stress caused by ROS (Kullisaar *et al*., 2002; Lin and Yen, 1999). These intracellular enzymes could be obtained after breaking the bacterial cells as cell-free extracts that showed *in vitro* antioxidant activity, as demonstrated by several LAB strains (Lin and Yen, 1999; Li *et al*., 2012). The effect of anti-oxidative lactic acid bacteria on rats deficient in vitamin E has been studied by (Kaizu *et al*., 1993). Also, Sanders *et al*. (1995) reported that *Lactococcus* demonstrates anti-oxidative superoxide dismutase enzyme activity. *Lactobacillus acidophilus* ATCC 4356 inhibited linoleic acid peroxidation and scavenged free radicals (Lin and Chang, 2000). The anti-oxidative activities of some LAB are listed in Table 1.4. The anti-oxidative activity of lactic acid bacteria were assayed by

several methods, such as, inhibition of ascorbate auto-oxidation, superoxide scavenging activity, inhibition of lipid peroxidation, scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, Oxygen radical absorption capacity (ORAC), Scavenging of hydroxyl radical (Mishra and Korachich, 1984; Lin and Yen, 1999; Wang *et al*., 2009; Li *et al*., 2012).

Table 1.4 Intracellular cell free extract of LAB showing antioxidant activity.

Strain	**Inhibition of ascorbate autooxidation (%)**	**Hydrogen peroxide scavenging (%)**	**DPPH free radical scavenging (%)**	**Superoxide anion radicals scavenging (%)**
Lactobacillus acidophilus LA-1 (Lin and Yen, 1999)	9.8	3.7	-	32.44
Lactobacillus bulgaricus 448 (Lin and Yen, 1999)	9.3	3.1	-	31.09
Streptococcus thermophilus 821 (Lin and Yen, 1999)	12.0	5.3	-	46.04
Lactobacillus casei SY13 (Zhang *et al*., 2011)	-	31.2	27.5	33.5
Lactobacillus plantarum C88 (Li *et al*., 2012)	-	50.1	55.2	-

1.6 γ-Aminobutyric acid producing ability of LAB

Gamma-aminobutyric acid (GABA) is an ubiquitous non protein amino acid and act as major inhibitory neurotransmitter in mammalian brains (Schousboe and Waagepetersen, 2007). GABA is synthesized by glutamate decarboxylase (GAD; EC 4.1.1.15), a pyridoxal 5′-phosphate-dependent enzyme that catalyzes the irreversible α-decarboxylation of L-glutamate to GABA (Fig. 1.5) *via* GABA shunt metabolic pathway (Schousboe and Waagepetersen, 2007; Li and Cao, 2010). Apart from major inhibitory neurotransmitter in mammalian brains, GABA has several well-characterized physiological functions such as hypotensive, tranquilizing and diuretic effects, prevention of diabetes, improve the concentration of plasma growth hormone

and inhibit small airway-derived lung adenocarcinoma (Hayakawa *et al*., 2004; Schuller *et al*., 2008; Di cagno *et al*., 2010; Li *et al*., 2010).

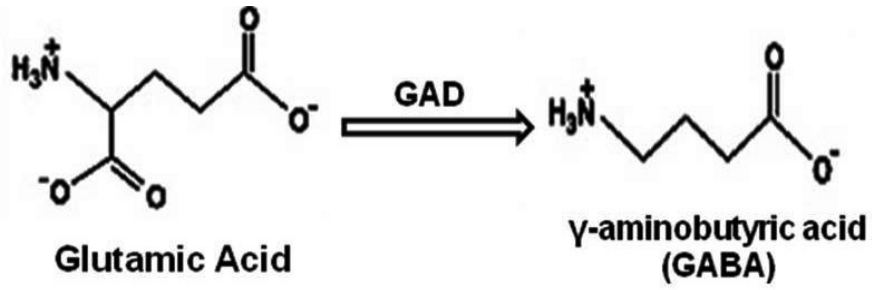

Fig. 1.5 Synthesis of GABA from glutamate by glutamate decarboxylase (GAD).

Some reports showed the presence of GAD activity in lactic acid bacteria (Komatsuzaki *et al.,* 2005; Cho *et al*., 2007). Several LAB such as, *Lactobacillus brevis, Lactobacillus paracasei, Lactobacillus delbrueckii, Lactobacillus buchneri, Lactobacillus plantarum, Lactobacillus helveticus, Lactococcus lactis* and *Streptococcus salivarius* subsp. *thermophilus* have been reported to show GABA producing ability with the help of intracellular GAD (Cho *et al*., 2007; Siragusa *et al*., 2007, Di Cagno *et al*., 2010). Among these LAB, *Lb. brevis* produced the maximum amount (346 mM) of GABA (Li *et al*., 2010). It has been observed that three lactobacillus strains isolated from Italian cheese could survive and synthesize GABA under simulated gastrointestinal conditions (Siragusa *et al*., 2007). Hence, it can be said that LABs with a high GAD activity have potential as probiotics. Several methods such as amino acid analyzer, thin layer chromatography, gas chromatography, high performance liquid chromatography, capillary liquid chromatographic tandem mass spectrometric method and flow-injection analysis

(FIA) are used for the detection of GABA (Komatsuzaki *et al*., 2005; Li and Cao, 2010; Di cagno *et al*., 2010).

1.7 Bacteriocin from LAB

A large number of ribosomally synthesized bacteriocins or bacteriocin-like substances are produced by lactic acid bacteria (LAB) have been identified and characterized in recent years due to their antimicrobial activity against food-borne pathogenic as well as spoilage bacteria. The bacteriocins are not antibiotics and the major difference between bacteriocins and antibiotics is that bacteriocins restrict their activity to genetically closely related species of the producing species and particularly to strains of the same species (Klaenhammer, 1993; Topisirovic *et al*., 2006). On the contrary, antibiotics have a wider activity spectrum and does not show any privileged effect on closely related strains (Savadogo *et al*., 2006; De vuyst and Leroy, 2007; Zacharof and Lovitt, 2012). The antibacterial spectrum of bacteriocin from LAB frequently includes spoilage organisms, oral pathogens and food-borne pathogens such as *Listeria monocytogenes, Escherichia coli, Streptococcus oralis* and *Staphylococcus aureus* (De vuyst and Leroy, 2007; Zoumpopoulou *et al*., 2013). For these reasons there has been increased interest in bacteriocins for their application in food preservation. This helps in reduction of use of chemical preservatives, heat and other physical treatments, thus satisfying the demands of consumers for better taste of foods. Bacteriocin can be added to foods in the form of concentrated preparations as food preservatives, shelf-life extenders, additives and ingredients (*ex-situ*) or they can be produced *in situ* by bacteriogenic starters, adjunct or protective cultures (Galvez *et al*., 2007; O'Shea *et al*., 2013). *In situ* bacteriocin production offers several

advantages compared to *ex situ* production regarding both legal aspects and costs. Bacteriocins are usually inactivated by low pH, heat and from digestive enzymes such as proteases (Todorov *et al*., 2011; O'Shea *et al*., 2013).

Bacteriocin production is often proposed as an advantageous characteristic of probiotic bacteria as it contribute to the colonisation resistance of the host and its protection against gastrointestinal pathogens (Bourlioux, 1997; Fooks and Gibson, 2002; Avonts *et al*., 2004). In addition bacteriocin production by probiotic lactic acid bacteria increases stability of the food product during its storage and shelf-life (Salminen *et al*., 1996; Avonts *et al*., 2004). Nisin, the bacteriocin from some strains of *Lactococcus lactis* subsp. *lactis*, which was accorded GRAS (Generally recognized as safe) status and approved for food use by the U.S. Food and Drug Administration, has already found a variety of applications in food preservation (Twomey *et al.,* 2002; Cotter, 2012). Till date only nisin and pediocins have been used as biopreservatives in food systems (Rodríguez *et al*., 2002; Cotter, 2012). Bacteriocins have been isolated from the commercial probiotic strains *Lactobacillus casei* and *Lactobacillus johnsonii* La1 (Avonts *et al*., 2004).

Immobilized bacteriocins can also find application for development of bioactive food packaging (Galvez *et al*., 2007). The effectiveness of bacteriocins requires careful testing in the food systems for which they are intended to be applied against the target bacteria. Application of bacteriocin-producing starter cultures in sourdough (to increase competitiveness), in fermented sausage (anti-listerial effect), and in cheese (anti-listerial and anti-clostridial effects), have been studied during in vitro laboratory fermentations as well as on pilot-scale level (De vuyst and Leroy, 2007). The broad industrial applications of bacteriocin from LAB are shown in Fig.

1.6. Although, the industrial application of bacteriocins becomes restricted by several limiting factors such as; little or inconsistent production levels, high production costs, a non-ideal antimicrobial spectrum and potency, the risk of the emergence of resistance and the poor/lack of growth of some producing strains in particular foods (O'Shea *et al*., 2013).

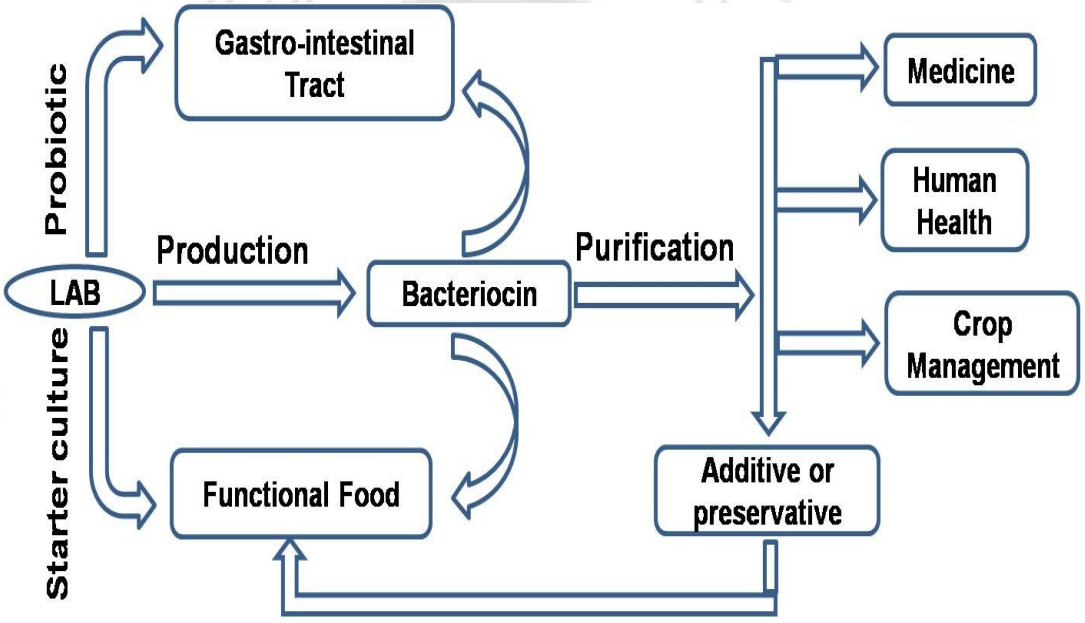

Fig. 1.6 Potential applications of bacteriocins from Lactic acid bacteria.

1.7.1 Characterization and nomenclature of bacteriocin

By definition, bacteriocins are small proteins with bactericidal or bacteriostatic activity against genetically closely related species (Topisirovic *et al*., 2006). Bacteriocin production in LAB is growth associated and the antimicrobial activity is found in the growth medium between the late exponential phase and early exponential phase of the growth. LAB-bacteriocins comprise a heterogeneous group of physic-chemically diverse ribosomally-synthesized peptides or proteins showing a narrow or

broad spectra of inhibition against both gram positive and gram negative bacteria. Several *in vitro* and *in vivo* experiments are carried out on antagonism effect of different LAB strains against *Helicobacter pylori, Clostridium difficile*, *Campylobacter jejuni*, *E. coli* and *Listeria monocytogenes*. Bacteriocins from LAB are small peptides, 3-10 kDa, in size (Nes *et al*., 1996), although there are exceptions (Jorger and Klaenhammer, 1990). The mode of action of bacteriocins from LAB is of two types bacteriostatic and bactericidal. In bacteriostatic mode of action, the bacteriocin retard the reproducibility of the pathogenic strain and after the exclusion of bacteriocin, the pathogenic strain starts to grow again. In bactericidal mode the bacteriocin completely destroyed the pathogenic cell by pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA and inhibition of peptidoglycan synthesis (Heu *et al*., 2006; Todorov and Dicks, 2009).

The classification of bacteriocin was first proposed by Klaenhammer, 1993 who classified into four main classes based on their chemical and genetic properties. Class I, the lantibiotics (<5 kDa), contain the characteristic polycyclic thioether amino acids lanthionine or methyllanthionine, as well as the unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid; Class II, the small heat stable non lanthionine containing membrane active peptides (<10 kDa); Class III, large heat labile bacteriocins (>30 kDa); and Class IV, bacteriocin composed of an undefined mixture proteins, lipids or carbohydrates. Class II bacteriocin is again subdivided into three groups, Class IIa or pediocin like bacteriocin, Class IIb or two component bacteriocin and Class IIc or thiol activated bacteriocin. The other classification system was proposed by Cotter *et al*., (2005) and it contained only two class; Class I (the lantibiotics) and Class II (the non-lantibiotic bacteriocins). Later the Heng and Tagg,

(2006) proposed a universal scheme for classification of bacteriocin as shown in Fig. 1.7. According to this classification the bacteriocin is divided into four groups and Group I and Group II are subdivided in to three individual groups and Group III is subdivided into two individual groups.

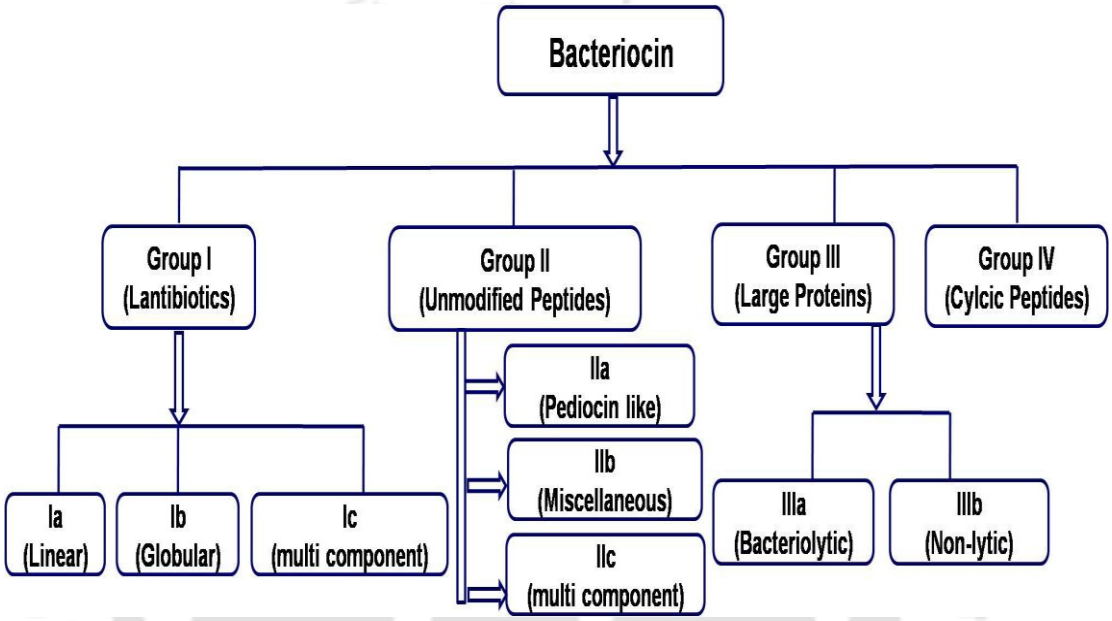

Fig. 1.7 Universal classification scheme of bacteriocin (Heng and Tagge, 2006).

1.7.2 Purification of Bacteriocin

Various strategies for purification of bacteriocin from LAB have been used as shown in Fig. 1.8. Most frequently applied techniques for purification of bacteriocin is salt precipitation followed by various combinations of chromatography (De vuyst and Leroy, 2007; Todorov and Dicks, 2009; Zacharof and Lovitt, 2012). Purification of bacteriocin from LAB can be achieved by adsorption-desorption method, in which bacteriocin is initially adsorbed to the producer cells at neutral pH and then released after being treated at low pH (between 2-2.5). Pediocin ACCEL from *P. pentosaceus*

ACCEL and pediocin NV5 from *P. acidilactici* LAB5 were purified by this method (Mandal *et al*., 2008). Bacteriocins can also be purified by organic solvent extraction method, in which bacteriocin is extracted using an organic solvent, butanol. Pediocin A from *P. pentosaceus* FBB61 was purified using this method (Piva and Headson, 1994).

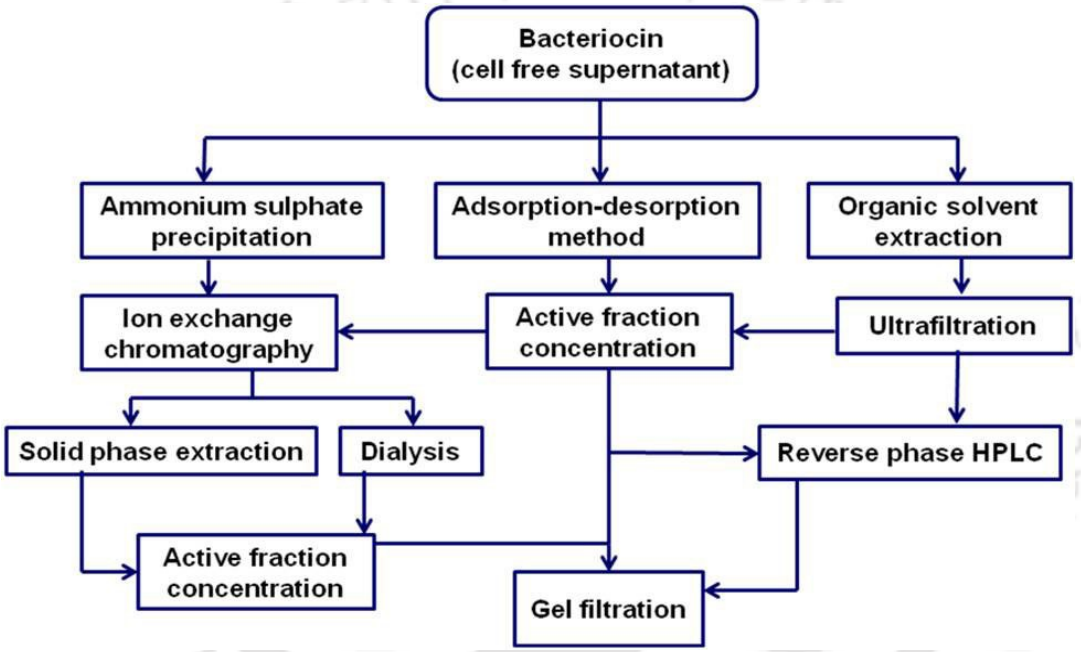

Fig. 1.8 Purification strategies of bacteriocin from Lactic acid bacteria.

1.8 Exopolysaccharides from LAB

Lactic acid bacteria (LAB) are known through ages for their wide applications in food, pharmaceutical and chemical industries. But recently LABs have aroused interest for their ability to secrete extracellular polysaccharides. These exopolysaccharides (EPS) have immense commercial applications because of their industrially useful physico-chemical properties (van Leeuwen *et al*., 2008). The exopolysaccharides from LAB are of two types;

A. Homopolysaccharides: polymers composed of glucose or fructose units, such as glucans which contain repetitive glucose units joined by α-(1→6) glycosidic linkages.

B. Heteropolysaccharides: polymers composed of a variety of sugar residues, mainly glucose, galactose, fructose and rhamnose, such as kefiran that contains equal proportion of galactose and glucose. In some Heteropolysaccharides charged groups such as acetate, phosphate or glycerol phosphate are also present (De Vuyst and Degeest, 1999).

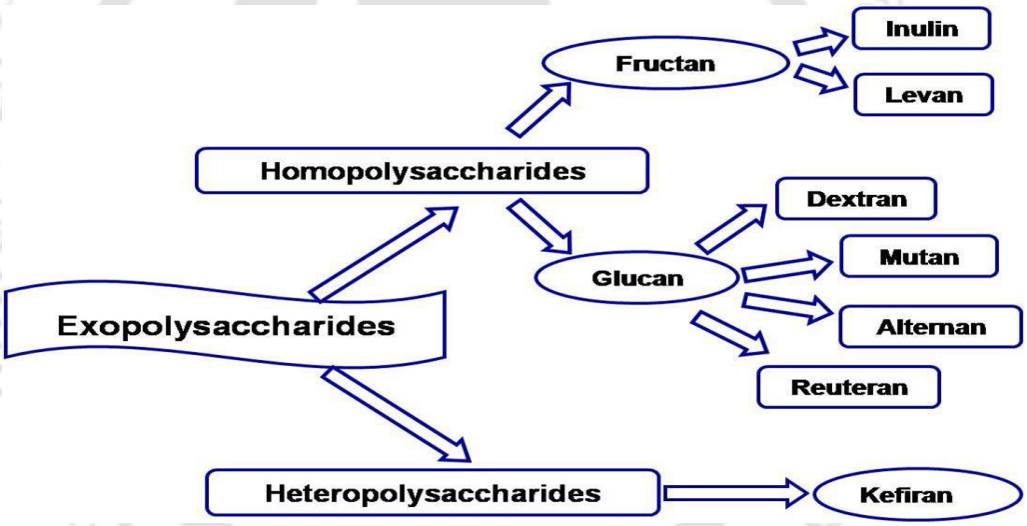

Fig. 1.9 Classification of microbial exopolysaccharides.

The function of exopolysaccharides in the microbial host is to provide protection against dehydration by retaining water (De Vuyst and Degeest, 1999; Tallon *et al*., 2003). The slimy and sticky nature of some EPS helps in adhering to surfaces, impeding the diffusion of toxic compounds to the host (De Vuyst and Degeest, 1999). The exopolysaccharides derived from LAB play crucial role in improving rheology, texture, mouth feel of fermented food formulations and conferring beneficial physiological effects on human health, such as antitumour

activity, immunomodulating bioactivity and anticarcinogenicity (Robyt, 1986; Talon *et al*., 2003; Puruma and Goyal, 2005; Patel *et al*., 2012; Shukla and Goyal., 2013)

1.8.1 Glucans from LAB

Four different genera of lactic acid bacteria, *Streptococcus, Leuconostoc, Pediococcus* and *Lactobacillus* are known to produce glucan. Although the glucan synthesis from *Leuconostoc* spp. has been extensively studied (Monsan *et al*., 2001; Majumder *et al*., 2009; Purama *et al*., 2009). The culture conditions of glucan and glucansucrase from *Leuconostoc* spp. has been optimized by statistical approach method. *Leuconostoc mesenteroides* NRRL B-640 is shown to produce glucansucrase that gives highly linear and soluble glucan (Uzochukwu *et al*., 2002, Puruma *et al*., 2008). A novel dextran produced by *Leuconostoc dextranicum* NRRL-B-18242 having a slushy, applesauce like appearance with a particulate gel-like structure is used in foods and other applications where texture is important (Pucci and Kunka, 1990).

1.8.2 Structure and function of glucan

1.8.2.1 α-D-Glucan

α-D-Glucan synthesized by glucansucrase in presence of sucrose is categorized mainly in four groups;

i) Dextran with α-(1→6) linkages, or with a majority of α-(1→6) linkages and α-(1→2), α-(1→3), and/or α-(1→4) branched linkages are mainly found in *Leuconostoc* species. The schematic representation of dextran is shown in Fig. 1.10.

ii) Mutan with a majority of α-(1→3) linkages generally found in *Streptococcus* species.

iii) Alternan with alternating α-(1→3) and α-(1→6) linkages, only reported in *L. mesenteroides.*

iv) Reuteran being a highly branched structure with mainly α-(1→4) linkages mainly found in *Lactobacillus reuteri.* (Monchois *et al*., 1999; van Leeuwen *et al*., 2009).

Other than these four distinct group of α-D-glucan, another group with containing large amounts of α-(1→2) linkages (predominantly α-(1→2,6) branching points), produced by *Leuconostoc mesenteroides* strain NRRL-B1299 and *Leuconostoc mesenteroides* NRRL B-1355 (mutant) was also reported (Smith *et al*., 1998; Bozonnet *et al*., 2002). The solubility of glucans depends upon the percentage of branched linkages. Presence of 95% linear linkages makes glucan water-soluble, and suitable for various applications (Leathers, 2002).

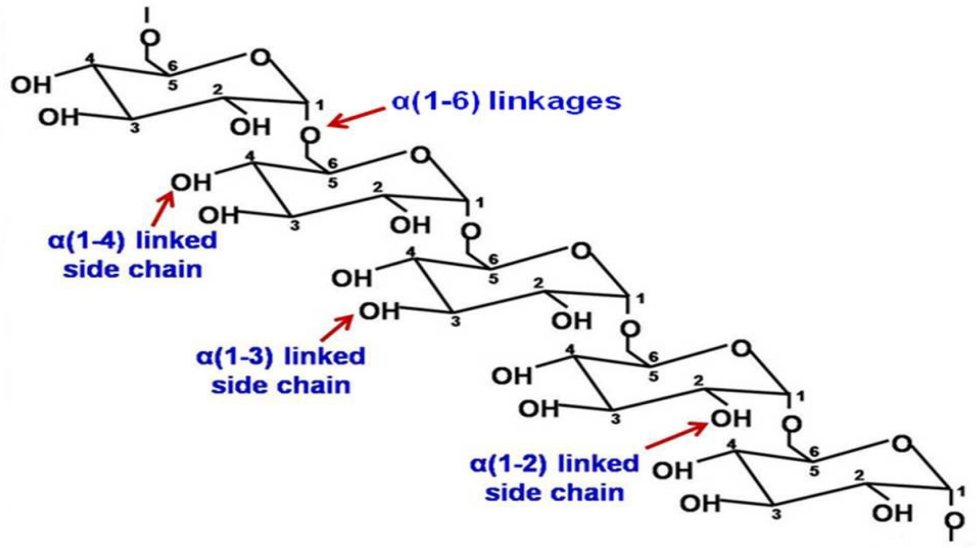

Fig. 1.10 Structure of α-D-glucan showing α(1→6) glycosidic bonds in main chain and possible branches of smaller chains with α(1→2), α(1→3) or α(1→4) links.

1.8.2.2 β-D-glucans

β-D-Glucan consists of linear un-branched polysaccharides of linked β-(1→3) and β-(1→4)-D-glucopyranose units, and it is a natural water-soluble polymer that cannot be digested by human enzymes, but is degraded by probiotic bacteria in the colon into short-chain fatty acids (SCFAs) in anaerobic condition (Dols-Lafargue *et al*., 2008). LAB strains belonging to the genera *Pediococcus* and *Lactobacillus* isolated from cider (Garai-Ibabe *et al*., 2010; Elizaquivel *et al*., 2011) and *Oenococcus* isolated from wine (Dols-Lafargue *et al*., 2008) synthesize β-glucan with the same primary structure: a trisaccharide repeating unit, with two (1,3)-β linked residues in the main chain, one of which is branched at position 2 by a terminal glucose residue (Dols-Lafargue *et al*., 2008; Garai-Ibabe *et al*., 2010; Elizaquivel *et al*., 2011).

1.8.3 Applications of glucan in foods and pharmaceuticals

Lactic acid bacteria produce a wide variety of food grade exopolysaccharides (EPS) with the help of glucosyltransferases that have nutritional and health applications. Certain exopolysaccharides are also potential therapeutic agents (Korakli and Vogel, 2006). They are also used as viscosifying, stabilizing, emulsifying, sweetening, flocculating, gelling, texturing or water-binding agents in the food as well as in the non-food industries owing to their non-ionic character and good stability under normal operating conditions (Sutherland, 1998; Welman and Maddox, 2000; Tallon *et al*., 2003; Dols-Lafargue *et al*., 2008; Bhavnani and Nisha, 2010).

Glucan also show,

A. *Cholesterol lowering effect*: it absorbs the cholesterol, helps in lowering the blood cholesterol level, thus reduces the risk of cardiovascular disease.

B. *Lowering of the glycaemic index*: Glucans are very viscous in nature which makes the gastric content thicker and help in slowing down the absorption rate of glucose. β-glucan spreads glucose absorption time (i.e. *reduction of glycaemic index*) and helps the body to fight against diabetes.

β-glucans are also used as edible film and as stabilizers in the manufacture of low-fat products such as salad dressings (Kontogiorgos *et al*., 2004), ice creams and yoghurts and cheese (Brennan *et al*., 2002). Two strains *Pediococccus parvulus* CUPV226 and *Lactobacillus suebicus* CUPV221 isolated from cider producing a 2-branched (1,3)-β-D-glucan have been reported, which decrease the serum cholesterol levels and affects the activation of human macrophages (Elizaquivel *et al*., 2011). The prebiotic properties of the 2-branched (1,3)-β-D-glucan produced by *Pediococcus parvulus* 2.6 has also been reported. This branched β-D-glucan also can resist the hydrolysis by the enzymes present in gastrointestinal tract and can induce the production of inflammation-related cytokines by polarized macrophages (Fernàndez de Palencia *et al*., 2007).

It has been reported that the α-D-glucan produced by *Leuconostoc dextranicum* NRRL B-1146, having α-(1→4) and α-(1→6) linkages, showed non-Newtonian pseudoplastic behaviour indicating its branched nature and also have unique rheological properties because of its potential of forming very viscous solution at low concentration and can be used as thickening or gelling agent in food

(Majumder and Goyal, 2009). Dextran from *Pediococcus pentosaceus* holds potential for usage as gelling agent in food formulations and as drug delivery carriers (Patel *et al*., 2010). The cytotoxicity test of dextran from *Pediococcus pentosaceus* on human cervical cancer (HeLa) cell line showed that there is no effect on the viability of HeLa cells for 72 h even at high concentration of 1000 mg/ml showing that it is non-toxic and biocompatible, rendering it safe for drug delivery, tissue engineering and various other biomedical applications (Patel *et al*., 2010). Recently, *in vitro* cytotoxicity analysis of novel dextran (2.93 x 10^5 Da) from *Pediococcus pentosaceus* CRAG3 displaying anti-cancer activity against cervical cancer (HeLa) and colon cancer (HT29) cell lines has been reported (Shukla and Goyal, 2013). The prebiotic effect of low molecular weight dextran with branched α-(1→2) linkages has also been reported (Sarbini *et al*., 2013). This particular type of dextran induces the growth of beneficiary bacteria such as *Bifidobacterium* sp. and *Lactobacillus* sp. (Sarbini *et al*., 2013). Major food and pharmaceutical applications of glucan are shown in Fig. 1.11.

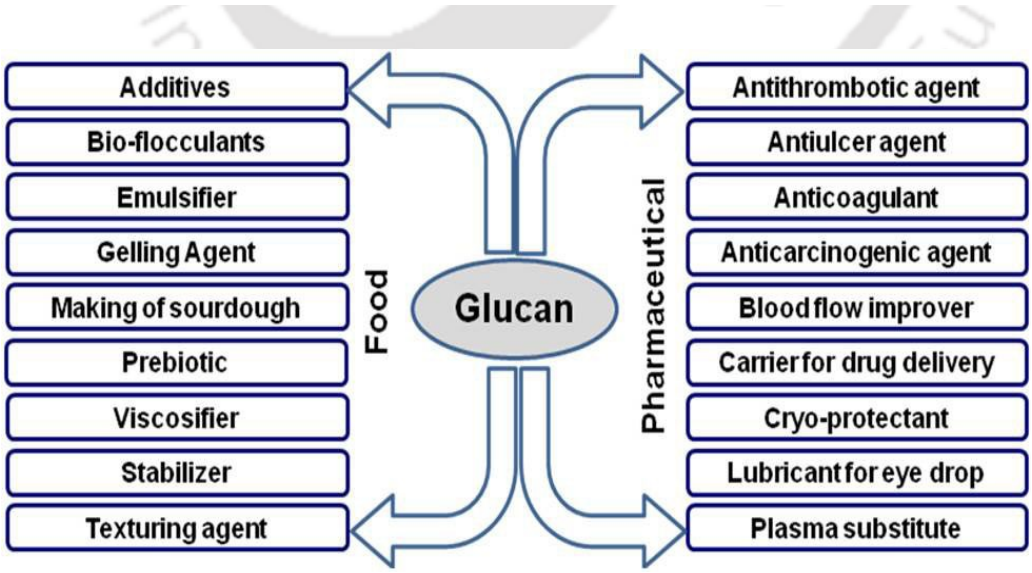

Fig. 1.11 Applications of glucan in foods and pharmaceuticals.

1.9 Glucansucrase from LAB

Glucansucrase (E.C. 2.4.1.5) catalyzes the polymerization of the glucopyranosyl moieties of sucrose to form glucan (Purama and Goyal, 2008). Glucansucrases have been listed within glycoside hydrolase (GH) family 70 in carbohydrate active enzyme database (http://www.cazy.org/Glycoside-Hydrolases.html) based on sequence similarity to GH13 α-amylases and GH77 amylomaltases (Cantarel *et al*., 2009; Vujicic Zagar *et al*., 2010). Together with the families GH13 and GH77 enzymes, they form the clan GH-H (Vujicic Zagar *et al*., 2010; Leemuhis *et al*., 2013). However, glucansucrases are much larger enzymes (~1600 - 1800 amino acid residues) than GH13 and GH77 (~500 - 600 amino acids), and they contain an N-terminal domain of variable region of unknown function (Vujicic Zagar *et al*., 2010).

Most of the enzymes classified in this family use sucrose as the D-glucopyranosyl donor to synthesize α-D-glucans of high molecular mass with the concomitant release of D-fructose. They are also referred to as glucosyltransferases (GTF) (Leemuhis *et al*., 2013). Glucansucrases usually display a $(\beta/\alpha)_8$ barrel shaped protein folding pattern and the acid-base-assisted substrate catalysis *via* a double displacement (retaining) mechanism. GH70 enzymes are transglucosylases produced by lactic acid bacteria from, e.g., *Streptococcus, Leuconostoc, Weisella* or *Lactobacillus* genera (Monchois *et al*., 1999; Ito *et al*., 2011; Leemuhis *et al*., 2013). The molecular architecture of glucansucrase of *Lb. reuteri* 121 from deduced amino acid sequence is shown in Fig. 1.12.

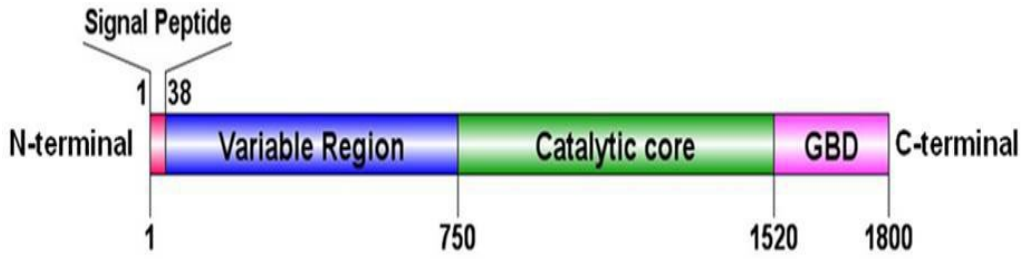

Fig. 1.12 Molecular architecture of glucansucrase.

The enzyme is composed of four distinct structural domains;

I. *Signal peptide N-terminal:* this region is responsible for protein secretion and is highly variable both in composition and in length.

II. *N-terminal variable region:* This region is composed of variable repeats although the function of this particular region is not identified. The removal of complete N-terminal variable domain in GTFI of *Streptococcus downei* MFe28 showed that it does not play any significant role in glucan structure determination but the enzyme activity was lost (Monchois *et al*., 1999; van Hijum *et al*., 2006).

III. *Catalytic core:* it is highly conserved region containing approximately, 1000 amino acids and also alternatively known as sucrose binding domain. Secondary structure prediction analysis of the catalytic domain showed that glucansucrases possess a $(\beta/\alpha)_8$ barrel structure similar to that of members of the GH13 family. This core structure is composed of 8 β-sheets alternated with 8 α-helices and involved in double displacement reaction mechanism (Kralj *et al*., 2004; Vujicic-Zagar *et al*., 2010; Leemuhis *et al*., 2013).

IV. *C-terminal domain or glucan binding domain:* It is composed of a series of tandem repeats which is thought to be involved in the determination of the structure of the synthesized glucan (Kralj *et al.*, 2011) and essential for glucansucrase activity (Van Hijum *et al*., 2006).

The production of glucansucrase of from *Leuconostoc* sp., *Pediodoccus* sp., and *Weisella* sp. is affected by factors like temperature, aeration and medium components (Tsuchiya *et al.* 1952; Lazic *et al.* 1993; Puruma and Goyal., 2005; Bejar *et al*., 2013). Among all *Lactobacillus* spp., *Lactobacillus reuteri* 121, *Lactobacillus sakei*, *Lactobacillus fermentum* and *Lactobacillus parabuchneri* are known to produce glucansucrase (van Hijum *et al.,* 2006; van Leeuwen *et al.,* 2009). Recently a novel strain *Lactobacillus satsumensis* isolated from a fermented beverage starter culture produced extracellular glucansucrase that synthesized dextran (Cote *et al*., 2012).

1.9.1 Reaction mechanism of glucansucrase

The enzyme glucansucrase synthesises glucan from sucrose with concomitant release of fructose (Fig. 1.13) by double displacement mechanism. In the first stage of double-displacement reaction α-(1→2) glycosidic linkage of sucrose is cleaved with the release of fructose and a glucosylenzyme intermediate is formed, in which the glucosyl unit is covalently attached to the catalytic nucleophile *via* a β-glycosidic linkage. In the second stage of reaction, the covalently bound glucosyl moiety is transferred to the accepting non-reducing end sugar of a growing glucan chain, with reformation of the α-glycosidic bond. Apart from the synthesis of glucan, glucansucrases also catalyze secondary transglycosylation reactions when

oligosaccharides (e.g. maltose, isomaltose) are used as acceptor molecules (Robyt *et al*., 2008).

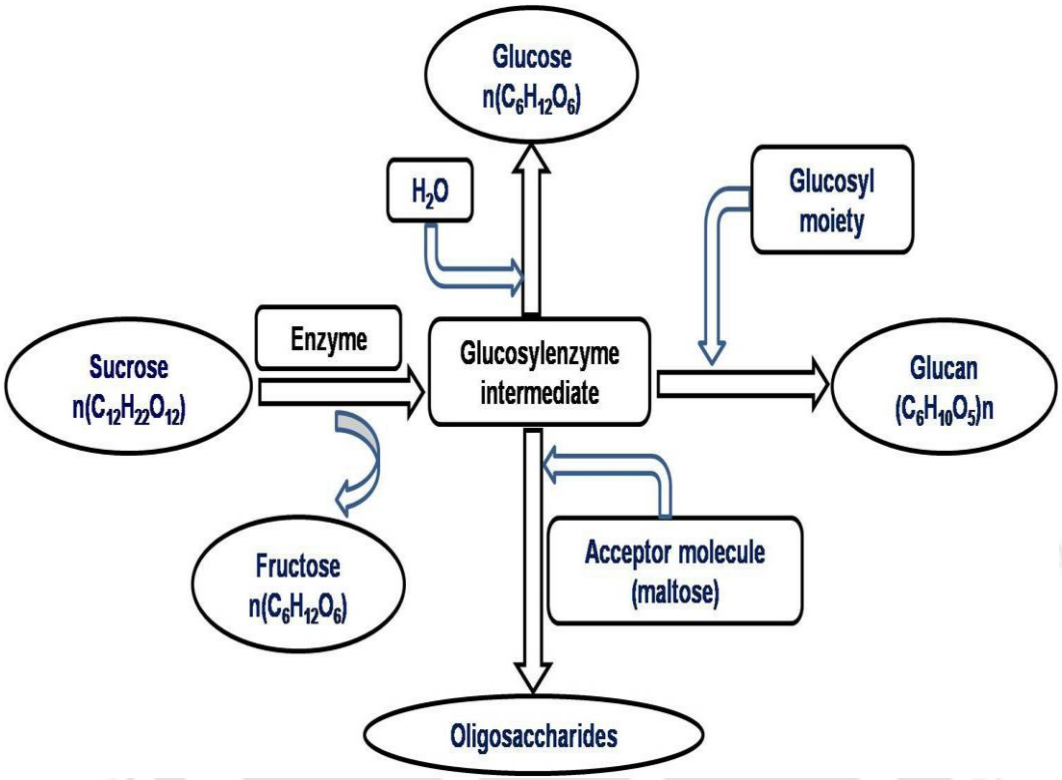

Fig. 1.13 Double displacement mechanism of glucansucrase reaction.

1.9.1 Purification and characterization of glucansucrase

Glucansucrases are generally high molecular mass proteins typically in the range of 120-200 kDa (Robyt *et al*., 2008; Leemuhis *et al*., 2013). Glucansucrase from LAB is produced in presence of sucrose in the culture medium which also leads to concomitant glucan synthesis during enzyme production. The presence of glucan results in aggregated forms of enzyme. In addition, glucans make the culture supernatants viscous, making enzyme purification troublesome. However, various methods such as precipitation by ammonium sulphate, ethanol or polyethylene glycol, phase partitioning, ultra-filtration and chromatography have been used to purify the

enzyme glucansucrase (Fig. 1.14) (Puruma and Goyal, 2005; Nigam *et al*., 2006; Majumder *et al*., 2007).

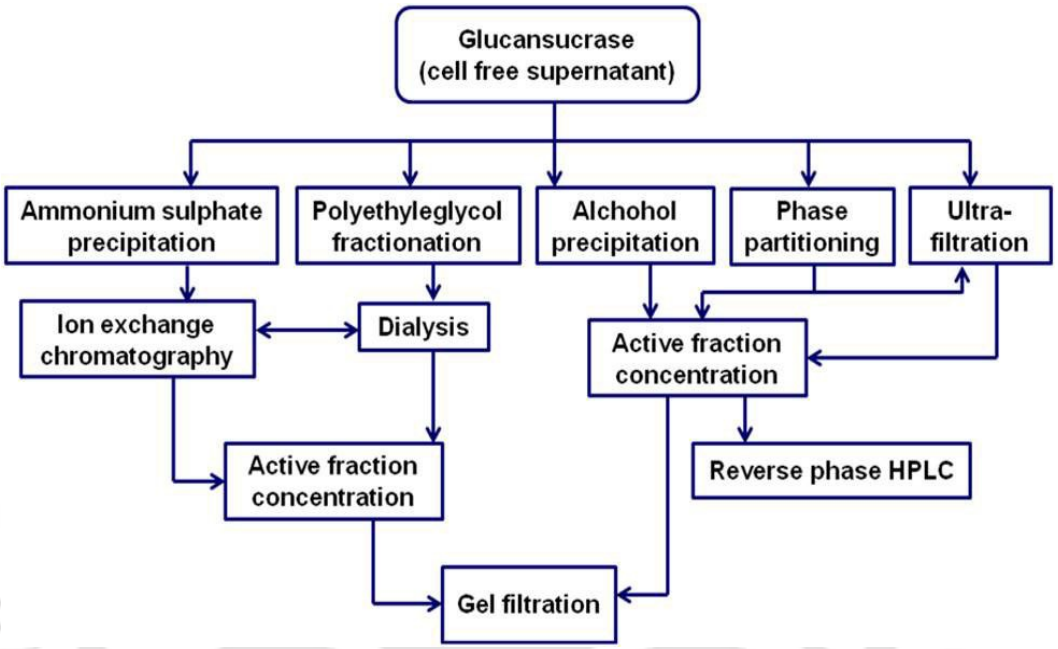

Fig. 1.14 Purification strategies of glucansucrase.

It has been reported that the polyethylene glycol (PEG) fractionation method is relatively easier and faster procedure to obtain purified form of glucansucrase (Goyal and Katiyar, 1994). PEG is known to selectively precipitate proteins, which have high molecular weights or exist in aggregated forms. The large size of glucansucrase, together with its tendency to form aggregates in solution has led to the use of non-ionic hydrophilic polymer PEG for precipitation of glucansucrase. However, the major disadvantage of this procedure is the contamination of enzyme by the glucan as it most often remains associated with enzyme. PEG is also present in the purified fractions but can be completely removed by extensive dialysis (Su and Robyt, 1994; Majumder *et al.*, 2008).

1.10 *Lactobacillus plantarum* as cell factories for nutraceuticals production

Lactobacillus plantarum represents a highly diverse group of Gram-positive, catalase negative, non-spore forming microaerophilic bacteria that microscopically appear as long to short rods. Originally, *Lb. plantarum* was designated as *Streptobacterium plantarum* by Orla-Jensen, (1999), but after biochemical identification it was placed in the *Lactobacillus* genus (Kandler and Weiss, 1986). The strain *Lb. plantarum* showed sequence similarity to *Lactobacillus pentosus*, *Lactobacillus arabinosus, Lactobacillus rudensis*, *Lb. paraplantarum* and *Lb. plantarum* var. *Mobilis* (Naser *et al*., 2007; Todorov and Franco, 2010). Among the lactic acid bacteria, *Lb. plantarum* has one of the largest genomes and the complete genome sequence (3,308,274 bp) of *Lb. plantarum* WCFS1 isolated from human saliva (Kleerebezem *et al*., 2003). *Lactobacillus plantarum* produces several organic acids such as lactic, malic, tartaric and acetic acid which in turn are used as acidulant, flavourings and pH buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods (Das and Goyal, 2012). *Lactobacillus plantarum* can enhance the intestinal integrity, metabolic activity of intestinal cells and stimulate immune response (Nissen *et al*., 2009). It can also induce a pro-inflammatory response to prevent an inflammatory outcome and to induce a higher immune alertness in intestinal epithelial cells (Cammarota *et al*., 2009). The intake of *Lb. plantarum* is shown to reduce certain gastrointestinal symptoms during treatment with antibiotics (Lonnermark *et al*., 2010). A functional beverage, grape must was prepared by fermentation of *Lb. plantarum* DSM19463 with enriched GABA and was reported to have potential anti-hypertensive effect (Di Cagno *et al.,* 2010). The strain *Lb. plantarum* C48 isolated from Italian cheese also possessed GABA (16 mg/kg)

producing ability (Sirgusa *et al*., 2008). Various studies have focused the addition of selected *Lb. plantarum* strains to dough, in order to improve the final quality of bakery products (Todorov and Franco, 2010). The antioxidant activity of *Lactobacillus plantarum* C88 isolated from traditional Chinese fermented food has been reported (Li *et al*., 2012). It was observed that on administration of *Lb. plantarum* C88 to senescent mice suffering from oxidative stress induced by D-galactose, the serum superoxide dismutase activity, the glutathione peroxidase activity and the total antioxidant capacity of the liver increased significantly, while the level of malondialdehyde in liver significantly decreased (Li *et al*., 2012). The occurrence of phytase enzyme in *Lb. plantarum* has also been reported which are usually used to upgrade the nutritional quality of food and feed (Khodaii *et al*., 2013). The concomitant production of glucansucrase and fructansucrase from *Lb. plantarum* PL9 during sourdough fermentation has been reported (Cagno *et al*., 2006). The production of two cell bound exopolysaccharide (EPS-b and EPS-r) from *Lb. plantarum* EP56 was reported (Tallon *et al*., 2003). The exopolysaccharide EPS-b (8.5×10^5 Da) was composed of glucose, galactose and *N*-acetylgalactosamine in a molar ratio of approximately 3:1:1 and traces of glycerol and phosphoglycerol, whereas exopolysaccharide EPS-r (4.0×10^4 Da) was composed of glucose, galactose and rhamnose in a molar ratio of 3:1:1 and traces of glycerol and phosphoglycerol. A heteropolysaccharide composed of galactose and glucose with a molar ratio of 1:2 from *Lb. plantarum* C88 (Zhang *et al*., 2013) has been reported, however, there are no reports on linear α-D-glucan with (1→6) linkages from *Lb. plantarum*. Several bacteriocins from *Lb. plantarum* have been reported and few of them are listed in Table 1.5. Recently a bacteriocin producing *Lb. plantarum* (ACA-DC-269) strain has

been isolated which showed activity against opportunistic pathogenic oral bacterium *Streptococcus oralis*. Most bacteriocins reported from *Lb. plantarum* appears as Class IIa bacteriocin (<10 kDa) (Carolissen-Mckay, 1997; De vuyst and Leory, 2007). The sequencing by Edman degradation of purified Plantaricin C (3.5 kDa) showed conserved N-terminal amino acid sequence (NH_2-Lys-Lys-Thr-Lys-Lys-Asn-Xaa-Ser-Gly-Asp-Ile) (Carolissen-Mckay, 1997). All aforementioned information suggest that *Lb. plantarum* may be useful in the design of novel probiotic functional foods.

Table 1.5 Bacteriocins from *Lactobacillus plantarum*.

Bacteria	Source	Bacteriocin	Mw (kDa)	Reference
Lb. plantarum C11	Cucumber	Plantaricin C11	>8.0	Daeschel *et al*., 1990
Lb. plantarum LPCO10	Green olive	i) Plantaricin S ii) Plantaricin T	2.5 <2.0	Jimenez-Diaz *et al*., 1993
Lb. plantarum LC74	Goat's milk	Plantaricin LC74	5.0	Rekhif *et al*., 1994
Lb. plantarum UG1	Dry sausage	Plantaricin UG1	3-10	Enan *et al*., 1996
Lb. plantarum NRIC 149	Pineapple	Plantaricin 149	2.2	Kato *et al*., 1994
Lb. plantarum 423	Sorghum beer	Plantaricin 423	3.5	vanReenen *et al*., 1998
Lb. plantarum ST31	Sourdough	Plantaricin ST31	2.75	Todorov *et al*., 1999
Lb. plantarum C19	Meat	Plantaricin C19	3.85	Atrith *et al*., 2001
Lb. plantarum 35d	Italian Sausage	Plantaricin 35d	4.5	Messi *et al*., 2001
Lb. plantarum NC8	Grass silage	i) Plantaricin NC8α ii) Plantaricin NC8β	3.6 4.0	Maldonado *et al*., 2003.
Lb. plantarum ST194BZ	Boza	i) Plantaricin ST194BZ(a) ii) Plantaricin ST194BZ(b)	3.3 14.0	Todorov and Dicks, 2005
Lb. plantarum TF711	Tenerife cheese	Plantaricin TF711	2.5	Hernandez *et al*., 2005
Lb. plantarum ST8KF	Kefir	Plantaricin ST8KF	3.5	Powell *et al*., 2006
Lb. plantarum AMA-K	Amasi (milk from Zimbabwe)	Plantaricin AMA-K	2.9	Todorov *et al*., 2007
Lb. plantarum L4/14	Rhizosperic isolate	Plantaricin L4/14	3.5	Tiwari and Srivastava, 2008
Lb. plantarum	Beloura and Chouriço	i) bacST202Ch ii) bacST216Ch	3.5 10.0	Todorov *et al*., 2010
Lb. plantarum KLDS1.0391	Jiaoke (Chinese cream)	Plantaricin MG	2.18	Gong *et al*., 2010
Lb. plantarum ST16Pa	*Carica papaya*	Plantaricin ST16Pa	6.5	Todorov *et al*., 2011
Lb. plantarum BM1	Sichuan pickle	Plantaricin BM1	4.7	Zhang *et al*., 2013
Lb. plantarum ST71KS	Goat feta cheese	Plantaricin ST71KS	5.0	Martinez *et al*., 2013

1.11 Objectives of the present study

Industrial interest in developing probiotics and probiotic functional foods is thriving, driven largely by the market potential for foods that target general health and well being. The main concern in the food industry these days is to select suitable microorganism that can be used in the food industry in order to improve the quality, taste and safety of the processed food. There is a huge potential for production of nutraceuticals; such as bacteriocin, exopolysaccharide, prebiotic oligosaccharides and antioxidants from probiotic lactic acid bacteria and not much work has been done on these aspects. The isolation of LAB strains as bioprotective cultures from different fermented food products could be useful as LAB inhibit other spoilage and pathogenic microorganisms. Various traditional fermented food sources of north eastern Himalyas like Marcha, Kodo ko jannr, Ngari, Suka ko masu will be used for isolation of probiotic LAB strains using selective MRS medium. Several factors such as the ability to survive in the acidic environment, adverse conditions in the gastrointestinal tract, resistance to bile salts, and production of bacteriocin, antioxidants and exopolysaccharide will be investigated for the selection of a probiotic. The selected probiotic strain will be identified by several biochemical tests and 16S rRNA gene sequence analysis. The 16S rRNA gene sequence will be used to carry out BLAST with the nr database of NCBI genbank and phylogenetic tree will be constructed. The isolate will be assayed for in its *in vitro* analysis for probiotic potentials, such as, resistance to gastric juices, resistance to bile salts, adhesion to the epithelial cells, auto-aggregation and co-aggregation ability. The cell free supernatant of the isolate will be assayed for bacteriocin activity against gram positive and gram negative bacteria. The bacteriocin will be purified by salt precipitation, ion exchange

chromatography and gel filtration. Bacteriocin bioassay will be performed for confirmation of antimicrobial activity. Purified bacteriocin will be analysed for its structure, and stability to temperature, pH and organic solvents. The intracellular cell free extract of the isolate will be investigated for antioxidative activity by several methods such as inhibition of ascorbate autooxidation and scavenging of DPPH free radical. LAB produces various types of exopolysaccharide with the help of extracellular glycoside hydrolases. The cell free extract of the isolate will be assayed for glucansucrase activity. The enzyme will be purified by polyethylene glycol fractionation and further by size exclusion chromatography. The enzyme will be biochemically characterized for temperature and pH optima, thermostability and pH stability. The influence of the physical factors such as temperature, shaking and pH on bacteriocin and exopolysachharide production will also be studied. The polysaccharide structure will be characterized by ^{1}H-NMR, ^{13}C-NMR, FTIR spectroscopic techniques and SEM and the biocompatibility of the isolate will also be evaluated using mammalian cell line studies.

Specific Objectives of the present study

1. Isolation, characterization and identification of bacteriocin, exopolysaccharide and antioxidant producing Lactic Acid Bacterium from fermented food.
2. *In vitro* analysis of probiotic properties and antioxidative activity of the isolate.
3. Production, purification and characterization of bacteriocin from the isolate.
4. Production, purification and functional characterization of glucansucrase from the isolate.
5. Production, purification and structural characterization of glucan from the isolate.

References

Atrih, A., Rekhif, N., Moir, A. J. G., Lebrihi, A. and Lefebvre, G. (2001) Mode of action, purification and amino acid sequence of plantaricin C19, an anti-Listeria bacteriocin produced by *Lactobacillus plantarum* C19. *Int. J. Food Microbiol*., 68, 93-109.

Avonts, L., Van Uytven, E. and De Vuyst, L. (2004) Cell growth and bacteriocin production of probiotic *Lactobacillus* strains in different media. *Int. Dairy J*., 14, 947-955.

Bao, Y., Zhang, Y., Li, H., Liu, Y., Wang, S., Dong, X., Su, F., Yao, G., Sun, T. and Zhang H. (2012) *In vitro* screen of *Lactobacillus plantarum* as probiotic bacteria and their fermented characteristics in soymilk. *Ann. Microbiol*., 62, 1311–1320.

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

Bhavani, A.L. and Nisha, J. (2010) Dextran: The polysaccharide with versatile uses. *Int. J. Pharma Biosci*., 1, 569-573.

Bourlioux, P. (1997) What is currently known about the molecular mechanisms of colonisation resistance? *Anaerobe*, 3, 179-184.

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.

Brennan, C.S., Tudorica, C.M. and Kuri, V. (2002) Soluble and insoluble dietary fibres (non-starch polysaccharides) and their effects on food structure and nutrition. *Food Ind. J*., 5, 261-272.

Cammarota, M., De Rosa, M., Stellavato, A., Lamberti, M., Marzaioli, I., Giuliano, M. (2009) *In vitro* evaluation of *Lactobacillus plantarum* DSMZ 12028 as a probiotic: emphasis on innate immunity. *Int. J. Food Microbiol*., 135, 90-98.

Cantarel, B.L., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V. and Henrissat, B. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res*., 37, D233-D238.

Cho, Y.R., Chang, J.Y. and Chang, H.C. (2007) Production of gamma-aminobutyric acid (GABA) by *Lactobacillus buchneri* isolated from kimchi and its neuroprotective effect on neuronal cells. *J. Microbiol. Biotechnol*., 17,104-109.

Cote, G.L., Skory, C.D., Unser, S.M. and Rich, J.O. (2012) The production of glucans via glucansucrases from *Lactobacillus satsumensis* isolated from a fermented beverage starter culture. *Appl. Microbiol. Biotechnol*., 97, 7265-7263.

Cotter, P.D. (2012) Bioengineering: a bacteriocin perspective. *Bioengineered.,* 3, 313-319.

Cotter, P.D., Hill, C. and Ross, R.P. (2005) Bacteriocins: developing innate immunity for food. *Nature Rev. Microbiol*., 3, 777-788.

Daeschel, M.A., McKeney, M.C. and McDonald, L.C. (1990) Bacteriocidal activity of *Lactobacillus plantarum* C-11. *Food Microbiol*., 7, 91-98.

Das, D. and Goyal, A. (2012) Lactic acid bacteria in food industry. *Microorganisms in Sustainable Agriculture and Biotechnology*. Springer, pp: 757-772.

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

De Vuyst, L. and Leroy, F. (2007) Bacteriocins from lactic acid bacteria: Production, purification and food applications. *J. Mol. Microbiol. Biotechnol*., 13, 194-199.

Di Cagno, R., Mazzacane, F., Rizzello, C.G., De Angelis, M., Giuliani, G., Meloni, M., De Servi, B. and Gobbetti, M. (2010) Synthesis of gamma-aminobutyric acid (GABA) by *Lactobacillus plantarum* DSM19463: functional grape must beverage and dermatological applications. *Appl. Microbiol. Biotechnol.*, 86, 731-741.

Dols-Lafargue, M., Lee, H.Y., Le Marrec, C., Heyraud, A., Chambat, G., Lonvaud-Funel, A. (2008) Characterization of *gtf* , a glucosyltransferase gene in the genomes of *Pediococcus parvulus* and *Oenococcus oeni*. *Appl. Environ. Microbiol*.. 74, 4079-4090.

Droge, W. (2002) Free radicals in the physiological control of cell function. *Physiol. Rev*., 82, 47-95.

Dunne, C., O'Mahony, L., Murphy, L., Thornton, G., Morrissey, D., O'Halloran, S., Feeney, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., O'Sullivan, G.C., Shanahan, F. and Collins, J.K. (2001) *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *Am. J. Clin. Nutr.*, 73, 386S-392S.

Elizaquivel, P., Sanchez, G., Salvador, A., Fiszman, S., Dueñas, M.T., Lopez, P., Fernandez de Palencia, P and Aznar, R. (2011) Evaluation of yogurt and various beverages as carriers of lactic acid bacteria producing 2-branched (1,3)-β-D-glucan. *J. Dairy Sci*., 94, 3271-3278.

Enan, G., Essawy, A.A., Uyttendaele, M. and Debevere, J. (1996) Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: Characterization, production and bactericidal action of plantaricin UG1. *Int. J. Microbiol.,* 30, 189-215.

Ercolini, D., Moschetti, G., Blaiotta, G. and Coppola, S. (2001) Behaviour of variable V3 region from 16S rDNA of lactic acid bacteria in denaturing gradient gel electrophoresis. *Curr. Microbiol.,* 42, 199-202.

Fooks, L. J. and Gibson, G. R. (2002) *In vitro* investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. *FEMS Microbiol. Ecol.*, 39, 67-75.

Forssten, S.D., Lahtinen, S.J., and Ouwehand, A.C. (2011) The Intestinal Microbiota and Probiotics. *Probiotic Bacteria and Enteric Infections: Cytoprotection by probiotic bacteria*. J. J. Malago et al., (eds.), Springer., 2, 41-63.

Friedlander, A., Druker, M.M. and Schachter, A. (1986) *Lactobacillus acidophillus* and vitamin B complex in the treatment of vaginal infection. *Panminerva. Med.,* 28, 51-53.

Fugelsang, K.C. and Edward, C.G. (2007) Wine microbiology, *Practical application and procedure*. 2nd edition, Springer, 2, 29-44.

Fuller, R. (1989) Probiotics in man and animals. *J. Appl. Bacteriol*., 66, 365-378.

Galvez, A., Abriouel, H., Lopez, R.L. and Omar, N.B. (2007) Bacteriocin-based strategies for food biopreservation. *Int. J. Food Microbiol*., 120, 51-70.

Garai-Ibabe, G., Areizaga, J., Aznar, R., Elizaquivel, P., Prieto, A., Irastorza, A. and Duenas M.T. (2010) Screening and selection of 2-Branched (1,3)-β-D-

Glucan producing lactic acid bacteria and exopolysaccharide characterization. *J. Agric. Food. Chem*., 58, 6149-6156.

Gong, H.S., Meng, X.C. and Wang, H. (2010) Plantaricin MG activity agaist Gram-negative bacteria produced by *Lactobacillus plantarum* KLDS1.0391 isolated from "Jiaoke", a traditional fermented cream from China. *Food Control*, 21, 89-96.

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

Hayakawa, K., Kimura, M., Kasaha, K., Matsumoto, K., Sansawa, H. and Yamori, Y. (2004) Effect of a γ-aminobutyric acid enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar–Kyoto rats. *Br. J. Nutr.,* 92, 411-417.

Hazra, B., Biswas, S. and Mandal, N. (2008) Antioxidant and free radical scavenging activity of *Spondias pinnata. BMC Complement. Altern. Med.,* 8, 63.

Heng, N.C.K. and Tagg, J.R. (2006) What's in a name? Class distinction for bacteriocins. *Nat Rev Microbiol* 4, doi: DOI:10.1038/nrmicro1273-c1.

Hernández, D., Cardell, E. and Zárate, V. (2005) Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. *J. Appl. Microbiol*., 99, 77-84.

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.

Hsieh, M.H. and Versalovic, J. (2008) The human microbiome and probiotics: Implications for pediatrics. *Curr. Probl. Pediatr. Adolesc. Health Care*., 38, 309-327.

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.

Jeyaram, K., Singh, T.H., Romi, W., Devi, A.R., Singh, W.M., Dayanidhi, H., Singh, N.R. and Tamang, J.P. (2009) Traditional fermented foods of Manipur. *Indian J. Traditional Knowl.,* 8, 115-121.

Jimenez-Diaz, R., Rios-Sanchez, R.M., Desmazeaud, M., Ruis-Barba, J.L. and Piard, J.C. (1993) Plantaricin S and T, 2 new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. *Appl. Environ. Microbiol*., 59, 1416-1424.

Jin, L., Hyun, S.Y., Kyu, W.C., Sejong, O., Sae, H.K., Taehoon, C., Bongjoon, K. and Kwang, Y.W. (2011) Evaluation of probiotic characteristics of newly isolated Lactobacillus spp.: Immune modulation and longevity. *Int. J. Food Microbiol*., 148, 80-86.

Joerger, M.C. and Klaenhammer, T.R (1990) Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. *J. Bacteriol*., 172, 6339-6347.

Kaizu, H., Sasaki, M., Nakajima, H. and Suzuki, Y. (1993) Effect of antioxidative lactic acid bacteria on rats fed a diet deficient in vitamin E. *J. Dairy. Sci.*, 76, 2493-2499.

Kandler, O. and Weiss, N. (1986) Genus *Lactobacillus* beijerinck 1901, 212$^{AL,"}$ In: (Sneath, P. H. A. Mair, N. S. Sharpe, M. E. and Holt, J. G. ed.) *Bergey's manual of systematic bacteriology*. Baltimore: Williams and Wilkins, 2, 209-1234.

Kato, T., Matsuda, T., Ogawa, E., Ogawa, H., Kato, H., Doi, U. and Nakamura, R. (1994) Plantaricin-149, a bacteriocin produced by *Lactobacillus plantarum* NRIC 149. *J. Ferment. Bioeng*., 77, 277-282.

Khodaii, Z., Natanzi, M.M., Naseri, M.H., Goudarzvand, M., Dodson, H. and Snelling, A.M. (2013) Phytase activity of lactic acid bacteria isolated from dairy and pharmaceutical probiotic products. *Int. J. Enterpathog*., 1, 12-16.

Klaenhammer, T.R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev*., 12, 39-86.

Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Fiers, M.W.E.J. et al. (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. *PNAS*., 100, 1990-1995.

Kollath, W. (1953) Ernahrung und Zahnsystem. *Deutsche Zahnärztliche Zeitschrift*., 8716.

Komatsuzaki, N., Shima, J., Kawamoto, S., Momose, H. and Kimura, T. (2005) Production of γ-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. *Food Microbiol.,* 22, 497-504.

Kontogiorgos, V., Biliaderis, C.G., Kiosseoglou, V. and Doxastakis, G. (2004) Stability and rheology of egg-yolk-stabilized concentrated emulsions containing cereal β-glucans of varying molecular size. *Food Hydrocolloids*, 18, 987-998.

Korakli, M. and Vogel, R.F. (2006) Structure/function relationship of homo-polysaccharide producing glycansucrases and therapeutic potential of their synthesized glycans. *Appl. Microbiol. Biotechnol*., 71, 790-803.

Kralj, S., Grijpstra, P., van Leeuwen, S.S., Leemhuis, H., Dobruchowska, J.M., van der Kaaij, R.M., Malik, A., Oetari, A., Kamerling, J.P. and Dijkhuizen, L. (2011) 4,6-α-Glucanotransferase, a novel enzyme that structurally and functionally provides an evolutionary link between glycoside hydrolase enzyme families 13 and 70. *Appl. Environ. Microbiol*., 77, 8154-8163.

Kralj, S., van Geel-Schutten, G. H., Dondorff, M.M, Kirsanovs, S., van der Maarel, M.M. and L. Dijkhuizen. (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.

Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C. and Kilk, A. (2002) Two antioxidative lactobacilli strains as promising probiotics. *Int. J. Food Microbiol.*, 72, 215-224.

Kumar, S.K., Ganesan, K. and Subba Rao, P.V. (2008) Antioxidant potential of solvent extracts of *Kappaphycus alvarezii* (Doty) Doty - An edible seaweed. *Food Chem*., 107, 289-295.

Lamari, F., Sadok, K., Bakhrouf, A. and Gatesoupe, F.J. (2013) Selection of lactic acid bacteria as candidate probiotics and *in vivo* test on *Artemia nauplii*. *Aquacult. Int*., DOI 10.1007/s10499-013-9699-5

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.

Leathers, T.D. (2002) Biopolymers, in *Polysaccharides I: Polysaccharides from Prokaryotes.,*, ed. By E.J. Vandamme, S. DeBaets, A. Steinbüchel (Wiley-VCH, Weinheim, 2002), pp. 229-321.

Lee, B., Kim, J., Kang, Y.M., Lim, J., Kim, Y., Lee, M., Jeong, M., Ahn, C. and Je, J. (2010) Antioxidant activity and γ-aminobutyric acid (GABA) content in sea tangle fermented by *Lactobacillus brevis* BJ20 isolated from traditional fermented foods. *Food Chem.*, 122, 271-276.

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

Leroy, F. and De vuyst, L. (2004) Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci. Technol.,* 15, 67-78.

Li, H. and Cao, Y. (2010) Lactic acid bacterial cell factories for γ-aminobutyric acid. *Amino Acids,* 39, 1107-1116.

Li, H., Qiu, T., Huang, G. and Cao, Y. (2010) Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. *Microb. Cell Fact*., 9, 85, 2-7.

Li, S., Zhao, Y., Zhang, L., Zhang, X., Huang, L., Li, D., Niu, C., Yang, Z. and Wang, Q. (2012) Antioxidant activity of *Lactobacillus plantarum* strains isolated from traditional Chinese fermented foods. *Food Chem.*, 135, 1914-1919.

Lin, M. and Yen, C. (1999) Antioxidative ability of lactic acid bacteria. *J. Agric. Food Chem.*, 47, 1460-1466.

Lin, M.Y. and Chang, F.J. (2000) Antioxidative effect of intestinal bacteria *Bifidobacterium longum* ATCC 15708 and *Lactobacillus acidophilus* ATCC 4356. *Dig. Dis. Sci*., 45, 1617-1622.

Lonnermark, E., Friman, V., Lappas, G., Sandberg, T., Berggren, A. and Adlerberth, I. (2010) Intake of *Lactobacillus plantarum* reduces certain gastrointestinal symptoms during treatment with antibiotics. *J. Clin. Gastroenterol.,* 44, 106-112.

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

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

Majumder, A., Singh, A. and Goyal, A. (2008) Application of response surface methodology for glucan production from *Leuconostoc dextranicum* and its structural characterization. *Carbohydr. Polym*., 75, 150-156.

Maldonado, A., Ruiz-Barba, J.L. and Jimenez-Diaz, R. (2003) Purification and genetic characterization of plantaricin NC8, a novel co-culture inducible two peptide bacteriocin from *Lactobacillus plantarum* NC8. *Appl. Environ. Microbiol*., 69, 383-389.

Mandal, V., Sen, S.K. and Mandal, N.C. (2008) Optimized culture conditions for bacteriocin production by *Pediococcus acidilactici* LAB5 and its characterization. *Indian J. Biochem. Biophys* ., 45, 106-110.

Martinez, R.C., Wachsman, M., Torres, N.I., LeBlanc, J.G, Todorov, S.D. and Franco, B.D. (2013) Biochemical, antimicrobial and molecular characterization of a non-cytotoxic bacteriocin produced by *Lactobacillus plantarum* ST71KS. *Food Microbiol*., 34, 376-381.

Mao, J.W., Yina, J., Gea, Q., Jianga, Z.L. and Gong J.Y. (2013) *In vitro* antioxidant activities of polysaccharides extracted from Moso Bamboo-Leaf. *Int. J. Biological. Macromol*., 55, 1-5.

Maxwell, S.R. (1995) Prospects for the use of antioxidant therapies. *Drugs*, 49, 345-361.

Messi, P., Bondi, M., Sabia, C., Battini, R. and Manicardi, G. (2001) Detection and preliminary characterization of a bacteriocin (plantaricin 35d) produced by a *Lactobacillus plantarum* strain. *Int. J. Food Microbiol*., 64, 193-198.

Miller, R.A. and Britigan, B.E. (1997) Role of oxidants in microbial pathophysiology. *Clin. Microbiol. Rev.* 10, 1-18.

Mishra, O.P. and Korachich, G.B. (1984) Inhibition of the autoxidation of ascorbate and norepinephrine by extracts of *Clostridium butyrich*, *Megasphaera elsdenii*, and *Escherichia coli*. *Life Sci*., 35, 849-854.

Mitropoulou, G., Nedovic, V., Goyal, A. and Yiannis Kourkoutas, Y. (2013) Immobilization technologies in probiotic food production. *J. Nutr. Metab*., ID 716861, doi.org/10.1155/2013/716861

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

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

Naser, S.M., Dawyndt, P., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Vancanneyt, M. and Swings, J. (2007) Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. *Int. J. Syst. Evol. Microbiol.,* 57, 2777–2789.

Nes, I.F., Diep, D.B., Havarstein, L.S., Brurberg, M.B., Eijsink, V. and Holo, H. (1996) Biosynthesis of bacteriocins of lactic acid bacteria. *Antonie van Leeuwenhoek* 70, 113-128.

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.

Nissen, L., Chingwaru, W., Sgorbati, B., Biavati, B. and Cencic A. (2009) Gut health promoting activity of new putative probiotic protective *Lactobacillus* spp. Strains: a functional study in the small intestinal cell model. *Int. J. Food Microbiol*., 135, 288-294.

O' Shea, E.F., Cotter, P.D., Ross, R.P. and Hill, C. (2013) Strategies to improve the bacteriocin protection provided by lactic acid bacteria. *Curr. Opin. Biotechnol*., 24, 130-134.

Orla-Jensen, S. (1919). The lactic acid bacteria, Host: *Copenhagen*, pp. 1-118.

Osterlund, P., Ruotsalainen, T., Korpela, R., Saxelin, M., Ollus, A., Valta, P., Kouri, M., Elomaa, I. and Joensuu, H. (2007) *Lactobacillus* supplementation for diarrhea related to chemotherapy of colorectal cancer: a randomised study. *Br. J. Cancer.,* 22; 1028-1034.

Ouwehand, A.C., Salminen, S. and Isolauri, E. (2002) Probiotics: an overview of beneficial effects. *Antonie van Leeuwenhoek,* 82, 279-289.

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. *Bioresour. Technol.,* 101, 6852-6855.

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

Pilar Fernàndez de Palencia, P., Werning, M.L., Elena Sierra-Filardi, E., Duenas, M.T., Ana Irastorza, A., Angel L. Corbi, A.L. and Lopez, P. (2009) Probiotic properties of the 2-Substituted (1,3)-β-D-Glucan producing bacterium *Pediococcus parvulus* 2.6. Appl. Environ. Microbiol., 75, 4887-4891.

Piva, A and Headon, D.R. (1994) Pediocin A, a bacteriocin produced by *Pediococcus pentosaceus* FBB61. *Microbiol.,* 140, 697-702.

Powell, J.E., Witthuhn, R.C., Todorov, S.D. and Dicks, L.M.T. (2007) Characterization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF. *Int. Dairy J*., 17, 190-198.

Pucci, M.J. and Kunka, B.S. (1990) Novel dextran produced by *Leuconostoc dextranicum* NRRL B-18242. United States Patent 4, 933,191.

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

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.

Rai, A.K., Palni, U. and Tamang, J.P. (2009) Traditional knowledge of the Himalayan people on production of indigenous meat products. *Indian J. Traditional Knowl.,* 8, 104-109.

Rekhif, N., Atrih, A. and Lefebvre, G. (1994) Characterization and partial purification of plantaricin LC74, a bacteriocin produced by *Lactobacillus plantarum* LC74. *Biotechnol. Lett.*, 16, 771-776.

Robyt, J.F. (1986) Dextran. In: *Encyclopedia of polymer science and technology* (Mark HF, Bikales NM, Overberger CG, Menges G eds). John Wiley and Sons, pp 752-767.

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

Rodríguez, J.M., Martínez, M.I., and Kok, J. (2002) Pediocin PA-1, a wide-spectrum bacteriocin from lactic acid bacteria. *Crit. Rev Food Sci. Nutr*., 42, 91-121.

Saarela, M., Lahteenaki, L., Crittenden, R., Salminen, S. and Mattila-Sandholm, T. (2002) Gut bacteria and health foods—the European perspective. *Int. J. Food Microbol*., 78, 99-117.

Salminen, S., Isolauri, E. and Salminen, E. (1996) Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol*., 70, 347-358.

Sanders, J. W., Leehouts, K. J., Haandrikmam, A. J., Venema, G. and Kok, J. (1995) Stress response in *Lactococcus lactis*: cloning, expression analysis, and mutation of the lactococcal superoxide dismutase gene. *J. Bacteriol*., 177; 5254-5260.

Sarbinia, S.R., Kolida, S., Naeye, S., Einerhand, A.W., Gibson, G.R. and Rastall, R.A. (2013)The prebiotic effect of α-1,2 branched, low molecular weight dextran in the batch and continuous faecal fermentation system. *J. Funct. Foods*, http://dx.doi.org/10.1016/j.jff.2013.09.015

Satish-Kumar, R., Kanmani, P., Yuvraj, N., Paari, K.A., Pattukumar, V. and Arul V. (2012) Traditional Indian fermented foods: a rich source of lactic acid bacteria. *Int. J. Food Sci. Nutr.,* 64, 1-14.

Savadogo, A., Ouattara, C.A., Bassole, I.H. and Traore, S.A. (2006) Bacteriocins and lactic acid bacteria. *Afr. J. Biotechnol*., 5, 678-683.

Schousboe, A. and Waagepetersen, H.S. (2007) GABA: homeostatic and pharmacological aspects. *Progress in Brain Res.*, 160, 9-19.

Schrezenmeir, J. and de Vrese, M. (2001) Probiotics, prebiotics, and synbiotics – approaching a definition. *Am. J. Clin. Nutr.,* 73, 361S-364S.

Schuller, H.M., Al-Wadei, H.A.N. and Majidi, M. (2008) Gamma-aminobutyric acid, a potential tumor suppressor for small airway-derived lung adenocarcinoma. *Carcinogenesis.,* 29;1979-1985.

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

Sies, H. (1997) Oxidative stress: oxidants and antioxidants. *Exp. Physiol*., 82; 291-295.

Singh, E.J., Singh, N.K.S. and Singh, N.R. (2009) Biodiversity conservation and natural resources in North East India with special reference to Manipur. *NeBio.,* 1, 42-47.

Siragusa, S., De Angelis, M., Di Cagno, R., Rizzello, C.G., Coda, R., and Gobbetti, M. (2007) Synthesis of γ-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Appl. Environ. Microbiol.*, 73, 7283-7290.

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.

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.

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

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

Tamang, J.P., Dewan, S., Tamang, B., Rai, A., Schillinger, U. and Holzapfel, W.H. (2007) Lactic acid bacteria in Hamei and Marcha of North East India. *Indian J. Microbiol*., 47, 119-125.

Tamang, J.P., Tamang, B., Schillinger, U., Franz, C.M.A.P., Gores, M. and Holzapfel, W.H. (2005) Identification of predominant lactic acid bacteria isolated from traditional fermented vegetable products of the Eastern Himalayas. *Int. J. Food Microbiol*., 105, 347-356.

Tamang, J.P., Tamang, N., Thapa, S., Dewan, S., Tamang, G., Yonzan, H., Rai, A.K., Chettri, R., Chakrabarty, J. and Kharel, N. (2012) Microorganism and nutritional values of ethnic fermented food and alcoholic beverages of north east India. *Indian J. Traditional Knowl*., 11, 7-25.

Terahara, M., Kurama, S. and Takemoto, N. (2001) Prevention by lactic acid bacteria of the oxidation of human LDL. *Biosci. Biotechnol. Biochem*., 65, 1864-1868.

Thapa, S. and Tamang, J.P. (2006) Microbiological and physicochemical changes during fermentation of kodo ko jaanr, a traditional alcoholic beverage of the Darjeeling hills and Sikkim. *Indian J. Microbiol*., 46, 333-341.

Thokchom, S. and Joshi, S.R. (2012) Antibiotic resistance and probiotic properties of dominant lactic microflora from *Tungrymbai*, an ethnic fermented soybean food of India. *J. Microbiol*., 50, 535-539.

Tiwari, S.K. and Srivastava, S. (2008) Characterization of a bacteriocin from *Lactobacillus plantarum* strain LR/14. *Food Biotechnol*., 22, 247-261.

Todorov, S., Onno, B., Sorokine, O., Chobert, J.M., Ivanova, I. and Dousset, X. (1999) Detection and characterization of a novel antibacterial substance produced by *Lactobacillus plantarum* ST31 isolated from sourdough. *Int. J. Food Microbiol*., 48, 167-177.

Todorov, S.D. and Dicks, L.M.T. (2005) Effect of growth medium on bacteriocin production by *Lactobacillus plantarum* ST194BZ, a strain isolated from boza. *Food Technol. Biotechnol*., 43, 165-173.

Todorov, S.D. and Dicks, L.M.T. (2009) Effect of modified MRS medium on production and purification of antimicrobial peptide ST4SA produced by *Enterococcus mundtii*. *Anaerobe*, 15, 65-73.

Todorov, S.D. and Franco, B.D.G.M. (2010) *Lactobacillus Plantarum*: Characterization of the species and application in food production. *Food Rev. Int.*, 26, 205-229.

Todorov, S.D., Ho, P., Vaz-Velho, M. and Dicks, L.M.T. (2010) Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. *Meat Sci*., 84, 334-343.

Todorov, S.D., Nyati, H., Meincken, M. and Dicks, L.M.T. (2007) Partial characterization of bacteriocin AMA-K, produced by Lactobacillus plantarum AMA-K isolated from naturally fermented milk from Zimbabwe. *Food Control*, 18, 656-664.

Todorov, S.D., Prevost, H., Lebois, M., Dousset, X., LeBlanc, J.G. and Franco, B.D.G.M. (2011) Bacteriocinogenic *Lactobacillus plantarum* ST16Pa isolated from papaya (Carica papaya) -From isolation to application: Characterization of a bacteriocin. *Food Res. Int*., 44, 1351-1363.

Todorov, S.D., Prevost, H., Lebois, M., Dousset, X., LeBlanc, J.G. and Franco, B.D.G.M. (2011) Bacteriocinogenic *Lactobacillus plantarum* ST16Pa isolated

from papaya (*Carica papaya*) -From isolation to application: Characterization of a bacteriocin. *Food Res. Int*., 44, 1351-1363.

Topisirovic, L., Kojic, M., Fira, D., Golic, N., Strahinic, I. and Lozo, J. (2006) Potential of lactic acid bacteria isolated from specific natural niches in food production and preservation. *Int. J. Food Microbiol*., 112, 230-235.

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 culture factors on production on dextransucrase by *Leuconostoc mesenteroides. J. Bacteriol*., 64, 521-526.

Twomey, D., Ross, R.P., Ryan, M., Meaney, B. and Hill, C. (2002) Lantibiotics produced by lactic acid bacteria: structure, function and applications. *Antonie van Leeuwenhoek*., 82, 165-185.

Uzochukwu, S., Balogh, E., Loefler, R.T. and Ngoddy, P.O. (2002) Structural analysis by13C nuclear magnetic resonance spectroscopy of glucan extracted from natural palm wine. *Food Chem*., 76, 287-291.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. and Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.,* 39, 44-84.

van Hijum, S.A.F.T., Kralj, S., Ozimek, L.K., Dijkhuizen, L. and van Geel-Schutten, I.G.H. (2006) Structure function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol. Mol. Biol. Rev*., 70, 157–176.

van Leeuwen, S.S., Kralj, S., van Geel-Schutten, I.H., Gerwig, G.J., Dijkhuizenb, L. and Kamerlinga, J.P. (2008) Structural analysis of the α-D-glucan (EPS35-5)

produced by the *Lactobacillus reuteri* strain 35-5 glucansucrase GTFA enzyme. *Carbohydr. Res*., 343, 1251-1265.

van Leeuwen, S.S., Kralj, S., Eeuwema, W., Gerwig, G.J., Dijkhuizen, L. and Kamerling, J.P. (2009) Structural characterization of bioengineered α-D-Glucans produced by mutant glucansucrase GTF180 enzymes of *Lactobacillus reuteri* strain 180. *Biomacromol.,* 10, 580-588.

van Reenen, C.A., Dicks, L.M.T. and Chikindas, M.L. (1998) Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *J. Appl. Microbiol*., 84, 1131-1137.

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. PNAS., 107, 21406-21411.

Wang, A.N., Yi, X.W., Yu, H.F., Dong, B. and Qiao, S.Y. (2009) Free radical scavenging activity of *Lactobacillus fermentum in vitro* and its antioxidative effect on growing-finishing pigs. *J. Appl. Microbiol.*, 107, 1140-1148.

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

Williams, N.T. (2010) Probiotics. *Am. J. Health Syst. Pharm.,* 67,449-458.

Zacharof , M.P. and Lovitt, R.W. (2012) Bacteriocins produced by lactic acid bacteria a review article. *APCBEE Procedia*., 2, 50-56.

Zhang, H., Liu, L., Hao, Y., Zhong, S., Liu, H., Tao Han, T. and Yuanhong Xie, Y. (2013) Isolation and partial characterization of a bacteriocin produced by

Lactobacillus plantarum BM-1 isolated from a traditionally fermented Chinese meat product. *Microbiol. Immunol*., doi: 10.1111/1348-0421.12091

Zhang, L., Liu, C, Li, D., Zhaoa, Y., Zhanga, X., Zenga, X., Yanga, Z. and Li, S. (2013) Antioxidant activity of an exopolysaccharide isolated from *Lactobacillus plantarum* C88. *Int. J. Biol. Macromol*. 54, 270-275.

Zhang, S., Liu, L., Su, Y., Li, H., Sun, Q., Liang, X. and Lv, J. (2011) Antioxidative activity of lactic acid bacteria in yogurt. *Afr. J. Microbiol. Res*., 5; 5194-5210.

Zoumpopoulou, G., Pepelassi, E., Papaioannou, W., Georgalaki, G., Maragkoudakis, P.A., Tarantilis, P.A., Polissiou, M., Tsakalidou, E. and Papadimitriou, K. (2013) Incidence of bacteriocins produced by food-related lactic acid bacteria active towards oral pathogens. *Int. J. Mol. Sci.*, 14, 4640-4654.

Chapter 2

Screening and isolation of new Lactic acid bacterium from Marcha: an ethnic fermented beverage of North eastern Himalayas

2.1 Introduction

The main concern in the food industry these days is to select suitable microorganism as a food additive in order to improve the quality, taste and safety of the processed food. During the past decade there has been increasing demand from consumers for the decreased or no use of chemical additives in food processing because many of their detrimental effects on health (Kumar *et al*., 2008). Lactic acid bacteria (LAB) are known through ages for their wide applications in food, pharmaceutical and chemical industries. Lactic acid bacteria play a very important role as starters in the production of fermented foods as they are generally regarded as safe (GRAS). The screening of bioprotective lactic acid bacteria from natural resources of the north eastern region of Himalayas has been in focus for its high biodiversity and profusion of natural flora and fauna (Singh *et al*., 2009). The fermented food products of north east India, such as koozh, rai, kanjika are wealthy source of lactic acid bacteria and are reported to have significant medicinal properties (Satishkumar *et al*., 2012).

LAB are well known for production of various antimicrobial compounds such as bacteriocin, organic acids, diacetly, acetoin, reuterin during fermentation (Holzapfel *et al*., 1995; Galvez *et al*., 2007). The bacteriocin from lactic acid bacteria may offer novel approach in food preservation as they inhibit food spoilage and pathogenic microorganisms. Several *in vitro* and *in vivo* experiments have revealed the antagonism effect of bacteriocin from lactic acid bacteria against major food borne pathogens such as *Helicobacter pylori, Camphylobacter jejuni, Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus* (Spelhaug and Harlander, 1989; Aymerich *et al*., 2000; Thokchom and Joshi, 2012). In addition to these, Lactic Acid Bacteria of the genera *Leuconostoc*, *Pediococcus*, *Lactobacillus*, *Weisella* also produce exopolysaccharides (EPSs), with inimitable rheological properties capable of improving the texture of fermented products (Cerning, 1990). The EPSs are used as viscosyfying, stabilizing, emulsifying, sweetening, gelling and water binding agents in the food as well as non food industries (Wellman and Maddox, 2003; Purama *et al*., 2009).

The classification of lactic acid bacteria into different genera is largely based on phenotypic methods such as morphology, mode of glucose fermentation, growth at different temperatures, ability to grow at high salt concentrations, acid or alkaline tolerance and configuration of the lactic acid produced (Corsetti *et al*., 2001). More recently, the advent of powerful analytical technique like SDS-PAGE of whole cell proteins, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and polymerase chain reaction (PCR) using the molecular marker like the 16S ribosomal DNA and housekeeping genes such as *rpoA, atpA* and *pheS* as species-specific identification tag have emerged as reliable tools for

identification of Lactic acid bacteria at genus and species level (Massi *et al*., 2004; Nasser *et al*., 2007; Buddhiman *et al.*, 2008).

Lactobacillus plantarum represents a highly diverse group of Gram-positive, microaerophilic bacteria that microscopically appear as long to short rods or even coccobacilli (Kandler and Weiss, 1986). *Lb. plantarum* is considered as probiotic (Nissen *et al*., 2009) and successfully used in the treatment of irritable bowel syndrome, abdominal distension and flatulence (Bixquert, 2009). Though the probiotic properties of *Lactobacillus plantarum* are known but, exocellular glucansucrase producing probiotic *Lactobacillus plantarum* has not been reported. In the present study, a novel *Lactobacillus* species was isolated from fermented beverage Marcha of Sikkim and was identified up to genus and species level based on morphological, biochemical, physiological and molecular characterization using 16S rRNA and *rpoA* gene sequence analysis.

2.2 Material and Methods

2.2.1 Chemicals and reagents

The components of MRS medium, enzyme production medium, Luria Bertani medium, Nutrient broth medium, antibiotic octadisc, carbohydrates, genomic DNA extraction kit, sodium carbonate, copper sulphate and ammonium molybdate were purchased from Hi-Media India Pvt., Ltd. Phenol and sulphuric acid were purchased from Merck India Pvt. Ltd. Agarose, glacial acetic acid (99.9 % pure), Trizma base (Tris free base), ethidium bromide were procured from Sigma Aldrich, USA.

2.2.2 Preparation of MRS medium

The MRS medium was prepared as described by DeMan *et al*., (1960) and comprised of (%, w/v): glucose, 2; yeast extract powder, 0.5; beef extract and peptone, 1; K_2HPO_4, 0.2; tri-ammonium citrate, 0.2; sodium acetate, 0.5; Tween 80, 0.1 (v/v); $MgSO_4.7H_2O$, 0.02; $MnSO_4.4H_2O$, 0.02. The pH of the medium was adjusted to 6.4 by 0.2 N HCl solution. After pH adjustment, the media was sterilized by autoclaving at a steam pressure of 10.3 kPa (15 lb/in^2) and a temperature of 121°C for 20 min.

2.2.3 Preparation of enzyme production medium

The enzyme production medium was prepared as described by Tsuchiya *et al.,* (1952). The medium contained (g/l) sucrose, 20; yeast extract, 20; K_2HPO_4, 20; $MgSO_4$.$7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.2; $FeSO_4.7H_2O$, 0.01; $CaCl_2.2H_2O$, 0.01; NaCl 0.01. The pH of the medium was adjusted to 6.9 with 0.2 N HCl solution. The medium was sterilized by autoclaving at a steam pressure of 10.3 kPa (15 lb/in^2) and at a temperature of 121°C for 20 min.

2.2.4 Sources and origins of the fermented food and beverage

Several ethnic fermented foods and beverage were selected as the sources for isolation of Lactic acid bacteria. The description of each source and their origin was mentioned in Table 2.2.1. The dry samples of 100g ngari, sukoko masu, marcha and hentak were collected aseptically in sterile falcon tubes from local markets of Assam and Sikkim in India. Tubes were kept in an ice-box and transported to the laboratory for analyses.

Table 2.2.1 Sources for isolation of lactic acid bacteria from north eastern Himalayas.

Product	Origin	Composition	Nature and uses
Ngari[1]	Manipur and Assam	Phoubu Fish (*Punitus sophore*)	Soft fish, curry or side dish
Sukoko masu[2]	Sikkim and Darjeeling hills	Buffalo/Goat/Chevon	Hard brownish, dried, curry or side dish.
Marcha[3]	Sikkim and Darjeeling hills	Rice with wild herb and spices	Dry, solid ball/fat, starter culture for preparation of alcoholic beverages
Hentak[4]	Manipur and Assam	Local fish (*Esomus danricus*)	ball-like thick paste, curry or side dish.

[1]Jeyaram *et al*., 2009; [2]Rai *et al*., 2009; [3]Tamang *et al*., 2007; [4]Thapa *et al*., 2004

2.2.5 Isolation and propagation of lactic acid bacteria

One gram of each Ngari, Sukoko masu, Marcha and Hentak were crushed separately using mortar pestle and homogeneously mixed with 9 ml 0.85% (w/v) sterile saline solution. The each suspension was then subjected to serial dilution up to 10^{-7} in the same diluents. One hundred microliter from all the dilutions of each source was spread on MRS agar (2%, w/v) plate and the plates were incubated at 30°C for 48 h or until the colonies appeared. Ten distinct colonies from 10^{-6} plates and ten distinct colonies from 10^{-7} plates were randomly picked by sterile inoculation loop and inoculated into 5 ml MRS medium in the test tubes. The test tubes were incubated at

30°C for 24 h. The purity of each single colony was checked by streaking again it into MRS agar (2%) plate and incubated at 30°C for 24 h and then sub-cultured in MRS medium.

2.2.6 Screening of new Lactic acid bacterium

The isolates from each source were screened on the basis of antagonistic activity and glucansucrase activity.

2.2.6.1 Screening of the isolates for antagonistic activity

The isolates were screened on the basis of antagonistic activity by agar well diffusion method using *Escherichia coli* DH5α as indicator strain (Todorov and Dicks, 2009). The indicator strain *Escherichia coli* DH5α was maintained in Luria broth medium (pH 6.8) containing 5 g/L (w/v) sodium chloride, 5 g/L (w/v) peptone, 1 g/L (w/v) beef extract and 2 g/L (w/v) yeast extract at 37°C. The isolates were grown in 5 ml MRS medium at 37°C for 18-20 h and then the broth was centrifuged at 10,000*g* at 4°C for 15 min. The pH of cell free supernatant was adjusted to 6.0 using sterile 2N NaOH and filtered through 0.2 µm filter membrane. The resultant filtrate was used for determining antimicrobial activity by agar well diffusion method using *E. coli* DH5α as indicator strain. For agar well diffusion method, an overnight culture of the indicator strain *E. coli* DH5α (1%, v/v) was inoculated in soft agar LB medium (0.7% agar) and 10 ml of this soft agar was plated over the LB agar base (1.5% agar). The wells were created with the help of cork borer and were inoculated with 50 µl of cell free supernatant of the isolates and the plates were then placed at 4°C for 6 h which allowed diffusing the supernatant into the agar. Finally, the plates were incubated at

37°C and examined after 24 h for zone of inhibition around the well against indicator strain *E. coli* DH5α (Das and Goyal, 2010).

2.2.6.2 Screening of isolates for glucansucrase activity

The isolates were also screened on the basis of glucansucrase activity and for that the isolates were grown in 5 ml enzyme production medium by Tsuchiya *et al*., (1952) as described in Section 2.2.3 and incubated at 30ºC for 24 h. 1% (v/v) of these 24 h grown cultures were again inoculated in 5 ml enzyme production medium and incubated at 27°C for 18 h under static condition. The culture broth was then centrifuged at 10,000*g* at 4°C for 10 min and the cell free supernatant was analyzed for enzyme activity by measuring the amount of reducing sugar released as described in Section 2.2.7.

The isolate showing the maximum zone of inhibition against indicator strain *E. coli* DH5α and glucansucrase activity was selected for further study and was designated as DM5 after author and source. The isolate DM5 was grown in MRS agar (2%) stabs at 30°C for 24 h and maintained at 4°C. The isolate was propagated by sub-culturing every 2 weeks. The culture was also preserved as glycerol stock frozen at -80°C in 22% (v/v) final concentration of sterile glycerol for long term storage.

2.2.7 Glucansucrase assay

The glucansucrase assay was carried out in 1 ml reaction mixture containing 5% (w/v) sucrose, 20 mM sodium acetate buffer (pH 5.4) and 20 µl cell free supernatant. The enzymatic reaction was performed at 30°C in water bath for 15 min.

The enzyme activity was calculated by measuring the released reducing sugar by Nelson and Somogyi method as described in the next Section 2.2.7.1. To 100 µl aliquot from the reaction mixture, 100 µl of reagent D (Section 2.2.7.1) was added for reducing sugar estimation. The solution was mixed and heated for 20 min in boiling water bath. It was cooled to room temperature and then 100 µl of reagent C (Section 2.2.7.1) was added. The colour developed rapidly with the evolution of CO_2. The mixture was diluted by adding 700 µl distilled water. The absorbance of colour developed was measured at 500 nm on a UV-visible spectrophotometer (Varian, Cary 100) using fructose (0.5 µg/ml to 500 µg/ml) as standard.

2.2.7.1 Preparation of reagents for reducing sugar estimation

The reagents for estimation of reducing sugar were prepared as described by Nelson, (1944) and Somogyi, (1945).

Reagent A: Sodium carbonate anhydrous (25 g), sodium potassium tartarate (25 g), sodium bicarbonate (20 g) and sodiumsulfate anhydrous (200 g) dissolved in distilled water and the volume made upto 1 ltr. Filtered and stored at a temperature between 30-37°C.

Reagent B: 15% copper sulphate containing one or two drops of concentrated sulphuric acid.

Reagent C: Ammonium molybdate (25 g) in 450 ml, added 21 ml of concentrated sulphuric acid and mixed. To this was added sodium arsenate (3 g)

dissolved in 25 ml of distilled water, mixed and stored at 37°C for 24 h before use.

Reagent D: Prepared fresh, by mixing 25 ml of reagent A and 1 ml of reagent B.

2.2.7.2 Calculation of enzyme activity

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

$$\text{Enzyme Activity (U/ml)} = \frac{\Delta A_{500} \times C \times V}{M \times t \times v} = (\mu\text{moles/min/ml})$$

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

2.2.8 Morphological, biochemical and physiological characterization of isolate DM5

2.2.8.1 Gram staining and scanning electron microscopic analysis of isolate DM5

The screened isolate DM5 was subjected to Gram staining for its morphological study (Gram, 1884; Bergey *et al.,* 1994). Bacterial smear was air-dried on a glass slide and then flooded with filtered crystal violet for 30 s. After washing briefly in water to remove excess crystal violet, the bacterial smear was flooded with Gram's iodine for 30 s. It was washed again briefly with water and decolourized with

alcohol and washed immediately in tap water. After washing, it was counterstained with safranin for 30 s, washed in tap water and dried. The smear was observed under compound microscope.

The cell shape, size and their arrangement of isolate DM5 was studied by Scanning Electron Microscopy (SEM). The sample was prepared by centrifuging 1 ml of 12 h grown culture at 10,000*g* at 25°C for 10 min. The cell pellet was re-suspended in 1 ml of NaCl solution (0.85%, w/v). The sample was fixed with equal volume of glutaraldehyde (2.5%, v/v) for 2-4 h. One drop of this bacterial smear was dehydrated using different percent of acetone and dried in a vacuum desiccator. This dried sample was attached to the SEM stub with double-sided tape and coated with 10 nm Au in a sputter coater (Leo, SCH 620). The surface of the sample was viewed at various magnifications in Scanning Electron Microscope (Leo, 1330 VP) operated at 10.0 kV.

2.2.8.2 Triple sugar iron and motility indole test of isolate DM5

Isolate DM5 was subjected to triple sugar iron test (Bergey *et al.,* 1994) by using Triple Sugar Iron Agar Slant procured from Hi-Media Pvt. Ltd. India. The slant was inoculated with a loopful of culture and incubated at 37°C for 24-48 h to check whether the isolate could ferment any of the three sugars, lactose, sucrose or glucose in the medium. The isolate DM5 was also checked for motility, tryptophanase production and production of enzymes for breakdown of lysine (Bergey *et al.,* 1994) by using Motility Indole Lysine Agar Slant procured from Hi-Media Pvt. Ltd. India. A loopful of culture of the isolate DM5 was stabbed into the semisolid agar medium and incubated at 37°C for 24-48 h. *E. coli* DH5α was used as a positive control for both the tests.

2.2.8.3 Detection of nitrate production and catalase activity

The isolate DM5 was subjected to nitrate reduction test by using a nitrate-agar slant from Hi-Media Pvt. Ltd. India. The slant was inoculated with a loopful of culture and incubated at 37°C over a period of 48 h. *E. coli* DH5α was used as a positive control. The catalase activity of the screened isolate was determined by adding few drops of 5% (v/v) H_2O_2 to 5 ml of overnight grown culture of isolate DM5. The *E. coli* DH5α culture was treated under identical conditions with H_2O_2 as positive control for catalase activity (Bergey *et al.,* 1994).

2.2.8.4 Temperature, pH and salt tolerance of isolate DM5

Temperature tolerance of isolate DM5 was determined by growing 1.0% of the isolate DM5 in MRS medium as described in Section 2.2.2 and incubated at different temperatures ranging from 10°C-50°C for 24-48 h. The salt tolerance of the isolate was determined by adding NaCl ranging from 4-9% (w/v) in MRS medium and growing at 37°C for 24-48 h. The growth of the isolate DM5 was measured by culturing the isolate DM5 in MRS medium at initial pH of 2.0-10.0 to determine its pH tolerance.

2.2.9 Antibiotic susceptibility profile of isolate DM5

The isolate DM5 was tested for its susceptibility to thirty-eight antibiotics using agar disc diffusion assay (Barry and Thornsberry, 1980) in Petri plates. The petri-plates were first laid with MRS medium containing (as described in Section 2.2.3) 1.8% (w/v) agar. An overnight culture of 100 µl of the isolate DM5 grown in MRS medium was mixed in MRS soft agar (0.8%, w/v agar) and overlaid on the MRS

medium containing 1.8% (w/v) agar. After 2 min, the antibiotic octadiscs were gently placed at the centre over the surface of the agar plates. The petri plates were incubated in inverted position at 37°C for 36-48 h and were observed for zone of inhibition around the discs (Purama *et al.*, 2008). The commercially available antibiotic octadiscs impregnated with amikacin, ampicillin, amoxyclav, amoxycillin, bacitracin, carbenicillin, cefaclor, cefexime, cephalexin, cephaloridine, cephalothin, cephatoxamine, chloramphenicol, ciproflaxacin, clindamycin, cloxacillin, co-trimaxazole, erythromycin, gentamicin, imipenam, kanamycin, lincomycin, methicillin, nalidixic acid, nitrofurantoin, novobiocin, norflaxacin, oleandomycin, oxacillin, oxytetracyclin, penicillin-G, piperacillin, sulphamethoxazole, tetracyclin, ticarcillin, tobramycin, trimethoprim and vancomycin were used for the analysis. Based on the measurement of the diameter of zone of inhibition around disks, the isolate was determined to be resistant, moderate or susceptible to the antibiotic (Das and Goyal, 2010).

2.2.10 Carbohydrate fermentation profile of isolate DM5

The isolate DM5 was tested for its ability to ferment various carbohydrates using the method of Kandler and Weiss, (1986). An overnight culture of 50 µl of the isolate DM5 grown in MRS medium (described earlier in section 2.2.3) was again inoculated in 5.0 ml MRS medium lacking glucose (the carbon source) but containing phenol red and 2% (w/v) other carbohydrates. The test media were incubated at 30°C for 48 h under static condition. The acid production was observed between 24-48 h, as indicated by a change in colour of the phenol red indicator dye from red to yellow. The carbohydrates used for this test were arabinose, cellibiose, fructose, galactose, glucose,

glycerol, inulin, lactose, maltose, mannose, mannitol, mellibiose, raffinose, rhamnose, sorbitol, sucrose, trehalose and xylose.

2.2.11 Molecular characterization of the isolate DM5

2.2.11.1 Plasmid DNA profile of isolate DM5

The isolate DM5 was screened for the presence of plasmid DNA using an alkaline lysis protocol (Birnboim and Doly, 1979) with some minor modifications (Purama *et al*., 2008). For plasmid DNA extraction, the isolate DM5 was grown in 5 ml liquid MRS medium at 37ºC for 20-24 h. After incubation, the cell pellet was collected by centrifugation at 12,000*g* at 25ºC for 5 min and re-suspended in 100 µl TGE buffer (25 mM Tris HCl, pH 8.0, 50 mM glucose and 10 mM EDTA). A lysozyme solution was added (40 µl of stock solution consisting of 50 mg/ml) and the mixture was incubated at 37°C for 60 min. Following incubation, lysis solution of 200 µl (1% sodium dodecyl sulphate in 0.2N NaOH) was added to the mixture and mixed three times by inversion and incubated on ice for 5 min. 150 µl of ice-cold 5M potassium acetate buffer (pH 4.8) was added to the lysis mixture, mixed by vortex action for 10 s and incubated on ice for 5 min. The mixture was centrifuged at 16,000*g* at 25ºC for 10 min in a microcentrifuge (Eppendorf, model 5424) and the supernatant was transferred to a fresh tube. Supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The plasmid DNA was precipitated from the aqueous supernatant with two volumes of cold 100% ethanol, and the DNA pellet was collected by centrifugation at 16,000*g* at 25ºC for 5 min in a microcentrifuge. The pellet was washed twice with 500 µl 70% (v/v) ethanol and

resuspended in 50 µl of 20 mM Tris-EDTA buffer (pH 8.0). The plasmid DNA samples were run on agarose gel (0.7%) electrophoresis (as described later in Section 2.2.11.3).

2.2.11.2 Extraction of genomic DNA from isolate DM5

The genomic DNA of the isolate DM5 was extracted by Hi-PurA Bacterial and Yeast Genomic DNA Purification Spin Kit (Hi-Media India Pvt. Ltd.). The isolate DM5 was grown in 2 ml liquid MRS medium (as described in Section 2.2.3) at 37°C for 20-24 h. The 2 ml of broth was centrifuged at 12,000*g* at 25°C for 2 min and the supernatant was completely discarded. The cell pellet was re-suspended in 200 µl Gram positive lysis solution containing lysozyme (2.0×10^5 U/ml, 2 mg/ml) and incubated at 37°C for 30 min. The 20 µl (20 mg/ml) of RNaseA solution was added to the above solution, mixed and incubated at 25°C for 2 min. Following the incubation, 20 µl of the proteinase K solution (20 mg/ml) and 200 µl of lysis solution was added to the above mixture and vortexed thoroughly for 15 s and incubated at 55°C for 10 min. The genomic DNA was then precipitated by adding 200 µl of absolute alcohol to the lysate and mixed thoroughly by vortexing for 15 s. The lysate was passed through Hi-Elute miniprep spin column provided with the kit by centrifugation at 12,000*g* at 25°C for 10 min. The flow through liquid was discarded and the spin column was placed in a new 2.0 ml collection tube. The 500 µl of prewash solution was added to the column and centrifuged at 6,500*g* at 25°C for 1 min and discarded the flow through liquid. After that 500 µl of wash solution was added to the column and centrifuged at 18,000*g* at 25°C for 3 min to dry the column. The flow through was

discarded. Elution buffer of 50 µl was added directly to the column without touching the sides and incubated at 25ºC for 5 min. The column was placed on new autoclaved 1.5 ml micro-centrifuge tube and was centrifuged at 8,000*g* for 1 min to elute the genomic DNA. The purity of DNA was evaluated on 0.7% agarose gel as described in Section 2.2.11.3.

2.2.11.3 Agarose gel electrophoresis of plasmid DNA and genomic DNA

The plasmid DNA and genomic DNA of isolate DM5 were run on 0.7% agarose gel prepared in 1x TAE buffer. A stock solution of TAE buffer was prepared by keeping the concentrations of components to 10x (400 mM Tris-acetate, 10 mM EDTA, pH 8.0) according to Sambrook and Russell, (2001). The gel (0.7%) was prepared by dissolving 0.35 g of agarose in 50 ml of 1x TAE buffer by heating in a microwave oven for few min to get a clear solution. Then 5 µl of ethidium bromide (5.0 mg/ml) was added when the solution temperature was around 50°C. The solution was mixed and poured on the casting apparatus, comb was placed and the gel was allowed to set at 25°C for 30 min. The DNA samples were mixed with DNA loading dye in 4:1 ratio and loaded in the gel. 1x TAE (Tris-Acetate-EDTA) buffer was used as an electrophoresis running buffer (Sambrook and Russel, 2001) and the gel was run at 70 Volts for 2 h. The bands were then visualized under UV illumination in a gel documentation system (Kodak, Gel Logic 1500).

2.2.11.4 Preparation of DNA loading dye

The 5x stock solution of DNA sample loading dye was prepared by mixing the components as mentioned below in Table 2.2.2. One volume of 5x stock solution of

DNA loading dye was mixed with 4 volumes of sample DNA to make the dye solution 1x, before loading on to agarose gel. The final pH of the DNA loading dye was adjusted to pH 8.0.

Table 2.2.2 Composition of 5x DNA loading dye.

Components	Final concentration (5x)
Tris-HCl	50 mM
Glycerol	25% (w/v)
EDTA	5.0 mM
Bromophenol blue	0.2% (w/v)
Xylene cyanol	0.2% (w/v)

2.2.11.5 Amplification and sequencing of 16S rRNA and rpoA gene of isolate DM5

The genomic DNA of isolate DM5 was used for amplification of 16S rRNA gene using the universal oligonucleotide primers 16S rRNA; forward primer 8F (5'-AGTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-ACCTTGTTACGACTT-3'). The RNA polymerase alpha subunit (*rpoA*) region of the isolate DM5 was amplified as described by Naser *et al.*, (2007) using two oligonucleotide primers, rpoA-21-F (5'-ATGATYGARTTTGAAAAACC-3') and rpoA-22-R (5'-ACYTTVATCATNTCWGVYTC-3'). The PCR amplifications and sequencing of PCR amplicons of 16S rRNA and rpoA gene were done by Xcelris Laboratory Ltd. (Ahmedabad, India). Forward and reverse DNA sequencing reaction of 16S rRNA and *rpoA* PCR amplicons were carried out with 27F/1492R and 21F/22R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The consensus gene sequences were generated from forward and reverse sequence data using aligner software. The 16S rRNA and *rpoA* consensus gene sequence were used to carry out BLAST with the non redundant database of NCBI genbank. Multiple

alignment of the sequences and calculations of percent of sequence similarity was performed by using the software CLUSTALW. Distance matrix was generated using RDP database and the evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and the phylogenetic tree was constructed using MEGA 4.

2.2.12 Antimicrobial spectrum of isolate DM5

The antimicrobial activity of screened isolate DM5 was also detected with several Gram positive and Gram negative indicator strains using agar well diffusion method as described in Section 2.2.6.1. The indicator strains *Alcaligenes feacalis, Enterobacter aerogenes, Klebsiella oxytoca, Listeria monocytogenes, Staphylococcous aureus,* and *Staphylococcus epidermis* were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, indicator strain *Bacillus cereus* was procured from National collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India and indicator strains *Leuconostoc citreum*, *Leuconostoc dextranicum, Leuconostoc mesenteroides, Pseudomonas fluorescens* were obtained from Agricultural Research Service Culture Collection (NRRL), U.S.A. The other two indicator strains *Pediococcus pentosaceous* SPA and *Weissella confusa* Cab3 were isolated from soil (Patel and Goyal, 2010) and fermented cabbage (Shukla and Goyal, 2011) in our laboratory. The 50 µl of filter sterilized (0.2 µm membrane) cell free supernatant of isolate DM5 (pH 6.0) was used for antimicrobial activity against different indicator stains and placed in the wells of soft agar inoculated with indicator strains over the base agar plate as described in

Section 2.2.6.1. The plates were incubated at the optimum growth temperature of the indicator strain used (Das and Goyal, 2011). The antimicrobial activity was determined by measuring the distance of the clear zone around the wells.

2.3 Results and Discussion

2.3.1 Selection of the isolate DM5

Twenty distinct colonies were randomly picked from each source *viz.* ngari, sukoko masu, marcha and hentak and screening of the isolates was done on the basis of antimicrobial activity and glucansucrase activity. Among the eighty isolates only 11 isolates showed antimicrobial activity against indicator strain *E. coli* DH5α at 37ºC as well as glucansucrase activity at 30ºC under static condition. Based on the zone of inhibition around the well against *E. coli* DH5α as indicator strain and glucansucrase activity, it was found that that among the 11 isolates, the isolate M5 from Marcha showed the maximum zone of inhibition (>15 mm) and highest glucansucrase activity of 1.8 U/ml (Table 2.3.1). The isolate M5 from Marcha was selected and was named as "DM5" and was used for further characterization and study.

Table 2.3.1 Screening of lactic acid bacteria from fermented food and beverage of North east India.

S. No.	Isolate[a]	Antimicrobial activity[b]	Enzyme Activity (U/ml)[c]	S. No.	Isolate[a]	Antimicrobial activity[b]	Enzyme Activity (U/ml)[c]
1.	N2	+	1.2	10.	S17	++	0.5
2.	N5	++	0.5	**11.**	**M5**	**+++**	**1.8**
3.	N6	-	0.9	12.	M15	++	1.1
4.	S2	++	0.6	13.	M18	-	1.9
5.	S3	+	0.8	14.	H1	+	0.8
6.	S4	+	0.5	15.	H3	++	-
7.	S8	+	-	16.	H4	-	0.6
8.	S10	++	0.4	17.	H9	+	-
9.	S14	+++	-	18.	H18	+	1.1

[a] *The isolates were named on the basis of the isolation source.* ***N****-isolates from ngari,* ***S****-isolates from sokako masu,* ***M****-isolates from marcha,* ***H****-isolates from hentak.*

[b] *Antimicrobial activity was measured by agar well diffusion assay against E. coli DH5α as indicator strain. Symbols:* ***(+++):*** *zone>15 mm,* ***(++):*** *10 mm<zone<15 mm,* ***(+):*** *5mm<zone<10mm,* ***(-):*** *no zone of inhibition.*

[c] *Enzyme activity was determined by estimating the released reducing sugar by Nelson and Somogyi method (Nelson, 1944; Somogyi, 1945)*

2.3.2 Morphological, biochemical and physiological characterization of the isolate DM5

2.3.2.1 Gram staining and scanning electron microscopic analysis of isolate DM5

The appearance of violet colour cells after Gram staining, confirmed the Gram positive nature of the isolate DM5, a characteristic of lactic acid bacteria (Fig. 2.3.1). It was also revealed through Gram staining that the cells were rod shaped.

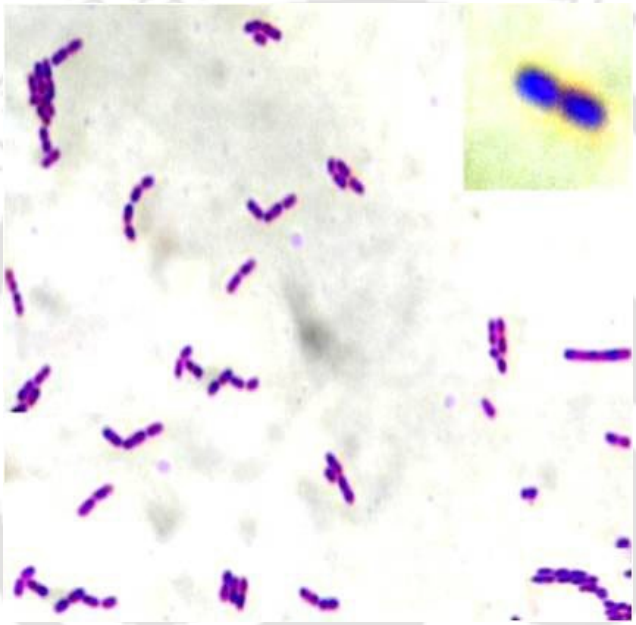

Fig. 2.3.1 Gram staining of the isolate DM5 showing violet colour cells, indicating the Gram positive nature.

Scanning electron microscopy of the isolate DM5 showed phenotypically homogeneous short rod cells, arranged singly or in pairs with a width and length of 0.5-0.6 μm and 1.2-1.4 μm, respectively (Fig. 2.3.2). Moreover, when the isolate DM5 was grown in MRS agar (2%, w/v) plate at 37°C for 24 h, it showed the white, smooth and small circular colonies (Fig. 2.3.3).

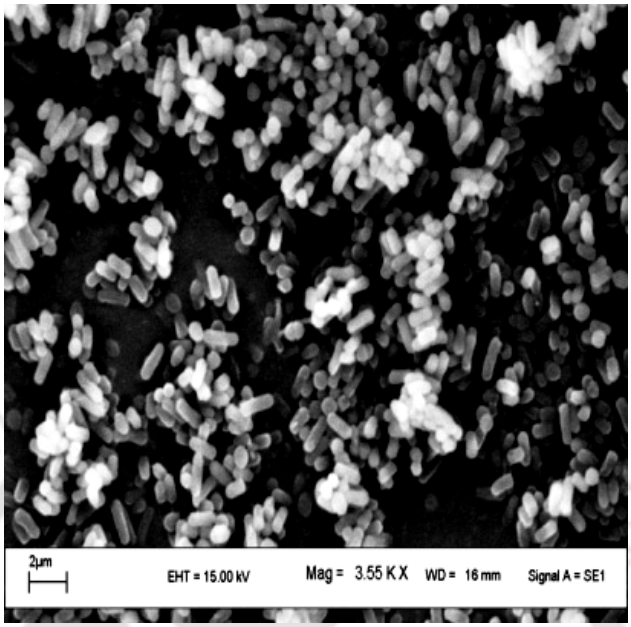

Fig. 2.3.2 Scanning Electron Microscopic analysis of the isolate DM5 showing rod shape and random arrangement of cells.

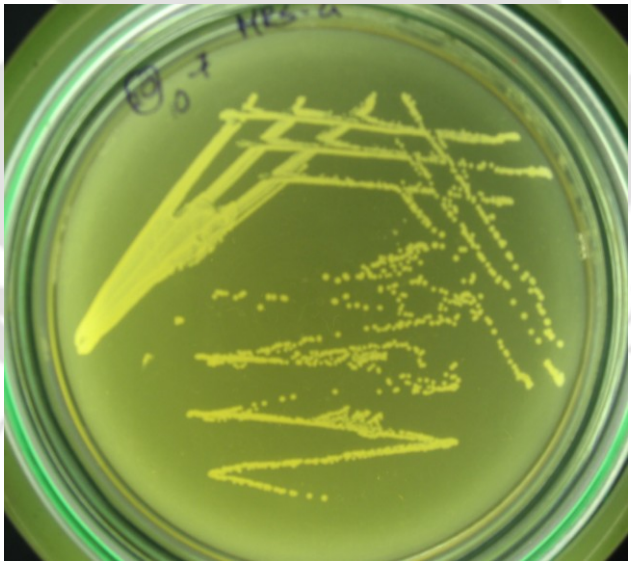

Fig. 2.3.3 Colony morphology of the isolate DM5 showing white, small circular colonies on MRS agar plate at 37ºC.

2.3.2.2 Triple sugar iron and motility indole test of isolate DM5

The triple sugar iron (TSI) slant contains agar, a pH-sensitive dye (phenol red), lactose, sucrose, glucose, as well as sodium thiosulfate and ferrous sulfate or ferrous

ammonium sulfate. If the test organism is able to ferment any of the three sugars in the medium and produce the by-products, which are usually acids, which will change the colour of the phenol red to yellow. If the test organism is able to utilize thiosulphate anion as a terminal electron acceptor, reducing it to sulphide, the newly-formed hydrogen sulfide reacts with ferrous sulfate in the medium to form ferrous sulfide, which is visible as a black precipitate. The isolate DM5 was able to ferment all the three sugars and change the red colour of the medium to yellow but it did not utilize thiosulphate anion and hence was unable to produce any black precipitate. On the other hand, *E. coli* DH5α used as a positive control was able to change the colour of the medium to yellow producing black precipitate as shown in Fig. 2.3.4.

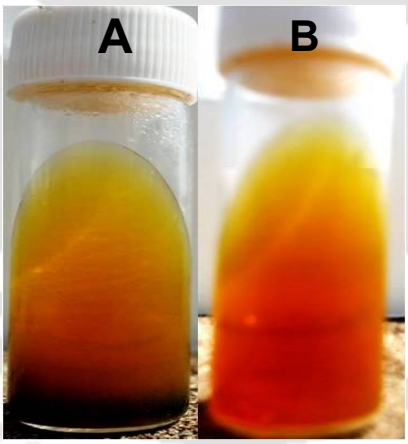

Fig. 2.3.4 Triple Sugar Iron Agar test of the isolate DM5. (A) Triple sugar iron agar slant inoculated with *E. coli* DH5α showing the black precipitate. (B) Triple sugar iron agar slant inoculated with isolate DM5.

The motility Indole Lysine Agar Slant is a semisolid agar used for the identification of the members of the family *Enterobacteriaceae* by detecting motility, indole and lysine decarboxylation or deamination. The isolate DM5 grew along the stab line keeping the surrounding medium clear which indicated that isolate DM5 was

non-motile and did not belong to the family *Enterobacteriaceae.* Whereas, *E. coli* DH5α which was used as a positive control when stabbed into the semi-solid agar migrated by means of its flagella and produced turbidity throughout the medium thus giving a positive result for the motility test (Fig. 2.3.5).

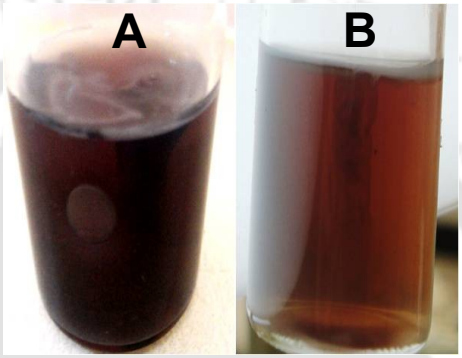

Fig. 2.3.5 Motility Indole Lysine test of the isolate DM5. (A) Motility indole lysine agar stab inoculated with *E. coli* DH5α showing turbidity throughout the medium. (B) Motility iron agar stab inoculated with isolate DM5 with no turbidity.

2.3.2.3 Detection of nitrate production and catalase activity

The nitrate agar slant is composed of a medium that contains large amount of nitrate along with alpha-napthylamine and sulphanilic acid. These two compounds react with nitrite and turn red in colour. If the test organism is able to reduce nitrate to nitrite or another nitrogenous compound such as ammonia or nitrogen gas, the medium turns red in colour due to production of nitrite, indicating a positive nitrate reduction test. The isolate DM5 was unable to grow or produce any colour change in the medium indicating a negative nitrate reduction test. *E. coli* DH5α was used as a control which showed nitrate reducing ability (Fig. 2.3.6).

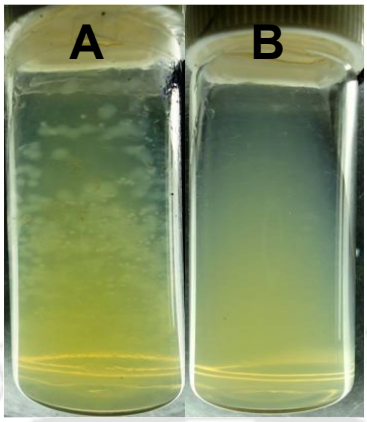

Fig. 2.3.6 Nitrate reduction test of bacteria. (A) Nitrate agar slant inoculated with *E. coli* DH5α showing red colour. (B) Nitrate agar slant inoculated with isolate DM5.

The catalase activity test proved the catalase negative nature of the isolate DM5 as it could not hydrolyse H_2O_2, whereas, *E. coli* DH5α was able to hydrolyse H_2O_2, which was evident from the bubbling that was observed when drops of H_2O_2 was added to *E. coli* DH5α culture (Fig. 2.3.7). The above all morphological and biochemical characteristic features of isolate DM5 are enlisted in Table 2.3.2. The results biochemical tests of isolate DM5 are compared with gram positive *Pediococcus pentosaceous* SPA and gram negative *Escherichia coli* DH5α.

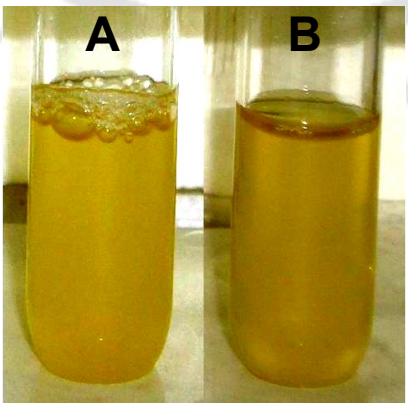

Fig. 2.3.7 Catalase activity test of bacteria. (A) *E. coli* DH5α showing bubble in presence of 3% H_2O_2. (B) Isolate DM5 showing no bubble formation.

Table 2.3.2 Morphological and Biochemical Characteristic features of isolate DM5.

Parameter	**Isolate DM5**	***P. pentosaceus* SPA**[a]	***E. coli* DH5α**
Gram staining	Gram positive	Gram positive	Gram negative
Cell morphology	Small rod shape	Cocci shape	Rod shape
Colony characteristic	White, smooth small colony	White smooth circular colony	Small circular colony
Catalase test	Negative	Negative	Positive
Motility test	Non motile	Non motile	Motile
Indole test	Negative	Negative	Positive
Nitrate test	Negative	Negative	Positive
H_2S gas production	Negative	Negative	Positive

[a]*Patel and Goyal, 2010.*

2.3.2.4 Temperature, pH and salt tolerance of isolate DM5

The isolate DM5 showed growth between 10 to 40°C, but not beyond 40°C, indicating the mesophilic nature of the isolate DM5. This result indicated that the isolate DM5 could be associated with widespread member of genus *Lactobacillus plantarum,* which was unable to grow at 45°C like *Lactobacillus helviticus* and *Lactobacillus delbrueckii* (Briggs, 1953). The isolate DM5 could grow in presence of 6.0% and 6.5% (w/v) NaCl; however, further increase in salt concentration inhibited the growth of the isolate DM5. The isolate DM5 could grow efficiently within pH range of 4-8 but, the pH of 2 and 10 did not support the growth of the isolate at 37°C. Among all the Lactobacillus species, *Lactobacillus plantarum* was able to grow in presence of 4% (w/v) NaCl and in low pH (Briggs, 1953), therefore the isolate DM5 could be cluster with *Lactobacillus plantarum*. The isolate DM5 also showed similar growth pattern with the isolate *Lb. plantarum* from Hamei (traditional alcoholic beverage in Manipur, India), which was able to grow in presence of 6.5% NaCl and at

pH 3.9 but was unable to grow in presence of 8% NaCl and pH 9.6 (Tamang *et al*., 2007).

2.3.3 Antibiotic susceptibility profile of isolate DM5

The susceptibility of isolate DM5 towards thirty-eight antibiotics was checked by agar disc diffusion assay using antibiotic octadisc. The susceptibility of microorganism to an antibiotic was measured by the size of the zone of inhibition around the disc. Based on this comparison, the test organism was classified as resistant, moderate or sensitive to the antibiotic and results were summarized in Table 2.3.3. The isolate DM5 was sensitive to almost all β-lactam antibiotics (ampicillin, amoxicillin, carbenicillin, cephalothin, penicillin-G, pipericillin, ticarcillin) like other *Lactobacillus* spp. (Zhou *et al*., 2005). The isolate DM5 showed moderate sensitivity towards oxacillin, resistant to cloxacillin and cefexime (third generation of cephalosporin) among β-lactam antibiotics. The data was in accordance with previous report where *Lb. plantarum* showed resistant towards antibiotic cloxacillin (Zhou *et al*., 2005) and third generation of cephalosporin (Danielsen and Wind, 2003). It has been reported that the *lactobacillus* spp. generally showed resistant towards aminoglycoside antibiotics (Zhou *et al*., 2005). The isolate DM5 was sensitive towards aminoglycoside antibiotics such as, gentamycin and novobiocin, but showed moderate resistance to kanamycin and resistant to amikacin and tobramycin. In addition, the isolate DM5 exhibited resistance towards norfloxacin, ciprofloxacillin, nalidixic acid and trimethoprim and susceptibility towards erythromycin, tetracycline, cholarmphenicol and methicillin. More frequently it has been observed that the Lactobacilli are susceptible to chloramphenicol, erythromycin and clindamycin and

resistance to trimethoprim and nalidixic acid (Danielsen and Wind, 2003; Klare *et al.*, 2007). The isolate DM5 also showed resistance towards glycopeptide type antibiotic vancomycin and was in harmony with the *Lactobacillus* species (Swenson *et al*., 1990). However, the resistance towards vancomycin and trimethoprim have been demonstrated as intrinsic by *Lactobacillus* species, which is chromosomally encoded and non transmissible (Swenson *et al*., 1990; Zhou *et al*., 2005).

Table 2.3.3 Antibiogram of isolate DM5 using antibiotic octodiscs on MRS agar at 37ºC.

S. No.	Antibiotic	Conc.	DM5	S. No.	Antibiotic	Conc.	DM5
1.	Amikacin (Ak)	10 µg	R	20.	Imipenam(I)	10 µg	S
2.	Ampicillin (A)	10 µg	S	21.	Kanamycin (K)	30 µg	M
3.	Amoxyclav(Ac)	10 µg	S	22.	Lincomycin (L)	2 µg	M
4.	Amoxycillin (Am)	10 µg	S	23.	Methicillin (M)	5 µg	S
5.	Bacitracin(B)	10 U	S	24.	Nalidixic acid (Na)	30 µg	R
6.	Carbenicillin (Cb)	100 µg	S	25.	Nitrofurantoin(Nf)	50 µg	S
7.	Cefaclor (Cj)	30 µg	S	26.	Novobiocin(Nv)	30 µg	S
8.	Cefexime (Cfx)	5 µg	R	27.	Norfloxacin (Nx)	10 µg	R
9.	Cephalexin (Cp)	10 µg	M	28.	Olaendomycin (OL)	15 µg	S
10.	Cephaloridine(Cr)	30 µg	S	29.	Oxacillin(Ox)	5 µg	M
11.	Cephalothin (Ch)	30 µg	S	30.	Oxytetracycline (O)	30 µg	S
12.	Cephotaxime(Ce)	30 µg	S	31.	Penicillin G (P)	10 U	S
13.	Chloramphenicol (C)	30 µg	S	32.	Piperacillin (Pc)	100 µg	S
14.	Ciprofloxacin(Cf)	10 µg	R	33.	Sulphamethoxazole (Sx)	50 µg	S
15.	Clindamycin(Cd)	2 µg	S	34.	Tetracycline(T)	30 µg	S
16.	Cloxacillin(Cx)	1 µg	R	35.	Ticarcillin (Ti)	75 µg	S
17.	Co-Trimaxazole (Co)	25 µg	S	36.	Tobramycin(Tb)	10 µg	R
18.	Erythromycin(E)	15 µg	S	37.	Trimethoprim(Tr)	2.5 µg	R
19.	Gentamicin(G)	10 µg	S	38.	Vancomycin (Va)	30 µg	R

R- Resistant (0-0.1 cm*); M- Moderate (0.2-0.8 cm*); S- Sensitive (0.9-2.5 cm*).

**Values in centimeters are the distances of zone of inhibition of growth of microorganism.*

2.3.4 Carbohydrate fermentation profile of isolate DM5

A carbohydrate fermentation reaction of isolate DM5 was measured by using the method of Kandler and Weiss, (1986). The nature of the fermentation reaction was

determined by observing the colour change of indicator dye due to the production of acid during fermentation. The extent of fermentation of carbohydrates by isolate DM5 was categorized and shown in Table 2.3.4.

Table 2.3.4: Carbohydrate fermentation profile of the isolate DM5 at 37ºC.

S. No.	Carbohydrate	DM5	S. No.	Carbohydrate	DM5
1.	Arabinose	++	10.	Mannitol	+++
2.	Cellobiose	++	11.	Mannose	+
3.	Fructose	++	12.	Mellibiose	++
4.	Galactose	+++	13.	Raffinose	++
5.	Glucose	+++	14.	Rhamnose	-
6.	Glycerol	-	15.	Sorbitol	-
7.	Inulin	++	16.	Sucrose	+++
8.	Lactose	+++	17.	Trehalose	+++
9.	Maltose	+++	18.	Xylose	+

Symbols: (+++) strongly positive; (++) fairly positive; (+) weakly positive; (-) negative.

The isolate DM5 could efficiently utilize arabinose, cellibiose, fructose, glucose, galactose, inulin, lactose, maltose, mellibiose, mannitol, raffinose, sucrose and trehalose (Table 2.3.4) like other *Lactobacillus plantarum* strains (Dicks and van Vuuren, 1987; Fugelsang and Edwards, 2007). The isolate DM5 showed weak fermentation towards mannose and xylose and inability to ferment sorbitol. However, it has been reported that the strain *Lactobacillus plantarum* displays variable response in case of mannose, sorbitol and xylose (Groenewald *et al*., 2006; Fugelsang and Edwards, 2007). Among the lactobacillus species *Lactobacillus plantarum* has the ability to utilize mannitol and trehalose (Kandler and Weiss, 1986; Fugelsang and Edwards, 2007). The isolate DM5 could not utilize glycerol and naturally occurring deoxy sugar rhamnose like *Lb. plantarum* ATCC 14917 (Kandler and Weiss, 1986).

2.3.5 Molecular characterization of the isolate DM5

2.3.5.1 Plasmid DNA Profile of isolate DM5

The plasmid DNA profile of isolate DM5 was determined by alkaline lysis method and is shown in Fig. 2.3.8. The isolate DM5 contained two plasmids of molecular sizes 19.6 kb and 2.3 kb. It has been reported that the *Lactobacillus plantarum* species often harbour one or more natural plasmids and the number of plasmids present in a single strain varied from 5 to 16 ranging between 68 kb and 2 kb in size, but in general low molecular weight plasmids predominated over the high molecular weight ones (Ruiz-Barba *et al*., 1991; Daming *et al*., 2003). *Lactobacillus plantarum* LTF154, isolated from a fermented sausage contained 14.4 kb plasmid involved in the production of plantaricin 154 (Kanatani and Oshimura, 1994). However, the strain *Lactobacillus plantarum* LR1 contained two cryptic plasmids pLR1 (2066 bp) and pLR2 (~7500 bp) and *Lactobacillus plantarum* 5057 strain showed single 10,877 bp tetracycline resistance plasmid pMD5057 (Li *et al*., 2009; Danielsen, 2002).

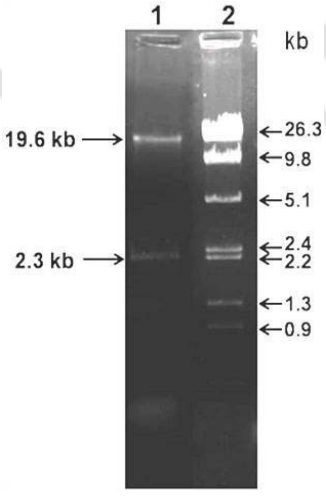

Fig. 2.3.8 Agarose gel (0.7%) showing two plasmid DNA of the isolate DM5 extracted by alkaline lysis method. Lane 1: Plasmid DNA of isolate DM5; Lane 2: DNA ladder.

2.3.5.2 Extraction of genomic DNA from isolate DM5

The genomic DNA of isolate DM5 was extracted by HiPurA Bacterial and Yeast Genomic DNA Purification Spin Kit (Hi-Media India Pvt. Ltd.). A single band of high molecular-weight DNA of about 23.8 kb was observed on 0.7% Agarose gel (Fig. 2.3.9). The genomic DNA profile of isolate DM5 was in accordance with earlier reports where *Lactobacillus* spp. showed genomic DNA of molecular size of approximately, 20 to 25 kb (Ulrich and Hughes, 2001; Sachinandan *et al*., 2010).

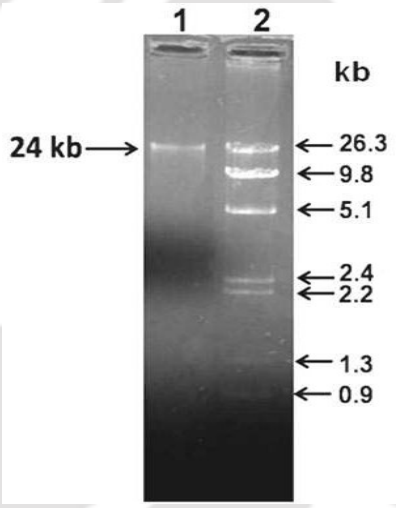

Fig. 2.3.9 Agarose gel (0.7%) showing genomic DNA of the isolate DM5 extracted using HiPurA bacterial and yeast genomic DNA purification Spin Kit. Lane 1: Genomic DNA of isolate DM5; Lane 2: DNA ladder.

2.3.5.3 Amplification and sequencing of 16S rRNA and rpoA gene of isolate DM5

The identification of the isolate DM5 was confirmed by 16S rRNA gene and *rpoA* gene sequence analysis. The amplified gene product of 1.5 kb was obtained after PCR amplification of genomic DNA of isolate DM5 by 16S rRNA specific primers (Fig. 2.3.10) and nucleotide sequence of the PCR amplified 16S rRNA gene was obtained from Xcelris Labs Limited, Ahmedabad, India.

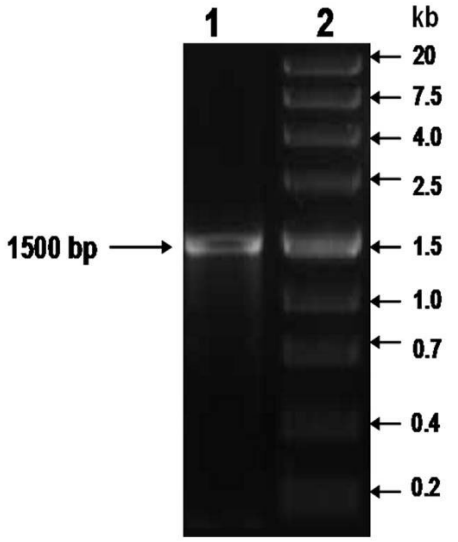

Fig. 2.3.10 Agarose gel (1.2%) showing 16S rRNA gene (1.5 kb) of the isolate DM5 amplified with universal primers 8F and 1492R. Lane 1: DNA ladder; Lane 2: Amplified product of full length 16S rRNA gene.

The consensus sequence of 1384 bp of 16S rRNA gene was generated from forward and reverse sequence data using aligner software (Fig. 2.3.11). The 16S rRNA consensus sequence of isolate DM5 was compared with reference bacteria from National Centre for Biotechnological Information (NCBI) Genbank. The BLAST analysis of the isolate DM5 using its 16S rRNA consensus sequence data showed 100% similarity with several *Lactobacillus plantarum* strains. Phylogenetic tree was constructed using neighbour joining method in MEGA4. The phylogenetic analysis revealed that the isolate DM5 has the highest homology with *Lactobacillus plantarum* Oh-3 as shown in Fig. 2.3.12. The16S rRNA gene sequence of the isolate *Lb. plantarum* DM5 was submitted to the NCBI Genbank database under the accession nos. KC020195.

CGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACA
CGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGA
CCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGA
TGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGAC
TGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGG
AGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAG
AGTAACTGTTCAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGA
TGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACTTGAGTGCAGAAGAGGACAGT
GGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTGG
TCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCG
TAAACGATGAATGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCG
CCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTAAGAGATTAGA
CGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGGCACTCTGGTGAGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT
GCTACAATGGATGGTACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTCAGTT
CGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAA

Fig. 2.3.11 Consensus sequence of 16S rRNA gene (1384 bp) of the isolate DM5.

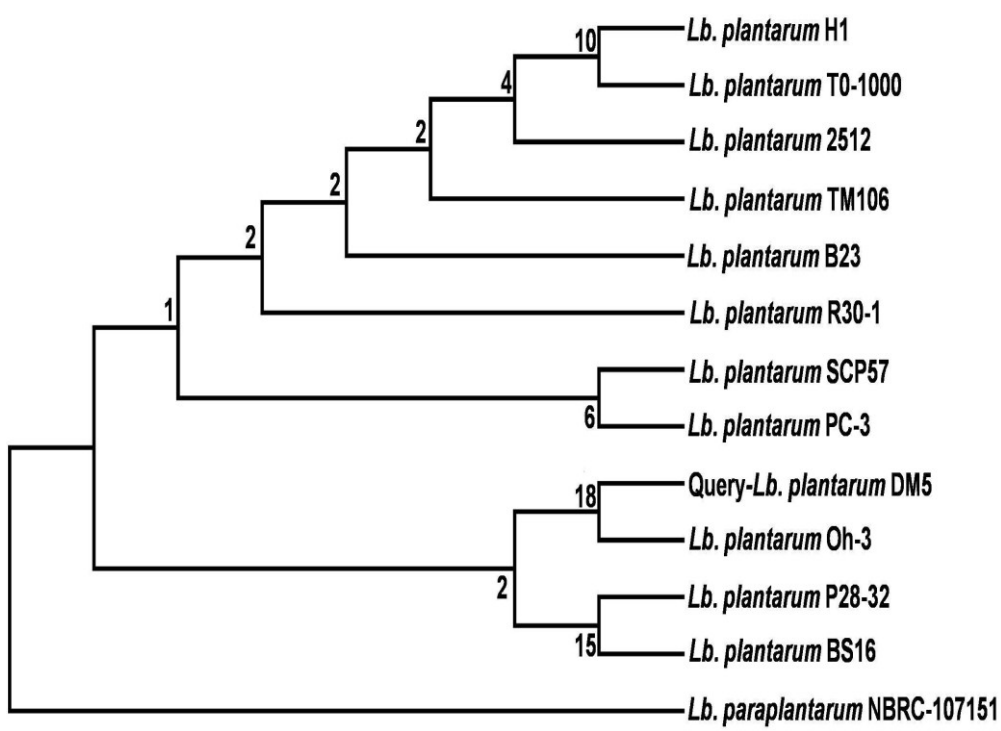

Fig. 2.3.12 Neighbour-joining tree based on 16S rRNA gene sequence of isolate *Lb. plantarum* DM5. Number at the nodes indicated the level of support based on a neighbour joining analysis after 500 simulations with bootstrap percentage (>50). *Lb. paraplantarum* NBRC-7 was showed as outgroup.

16S rRNA gene sequence analysis is the most common techniques currently used for bacterial species identification, however; 16S rRNA gene sequences have limited discriminating power for several closely related *Lactobacillus* species, such as *Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum*. The use of the housekeeping genes that code for the α-subunit of bacterial phenylalanyl-tRNA synthase (*pheS*) and the α-subunit of RNA polymerase (*rpoA*) has proven to be a robust system for the identification of all the closely related *Lactobacillus* and *Enterococcus* spp. as they provide sufficient variability to differentiate species of a particular genus and much more informative with an adequate degree of resolution (Zeigler, 2003). It has been reported that the *rpoA* gene sequence provides a higher discriminatory power for reliable identification of species of the genus *Lactobacillus* and can be used as alternative genomic markers to 16S rRNA gene sequences (Naser *et al*., 2007). Therefore, an alternative identification of the isolate DM5 was done by using the RNA polymerase alpha subunit (*rpoA*) partial gene sequence specific primers (21F rpoA/22R rpoA) to confirm the result obtained from 16S rRNA sequence analysis. The amplified gene product of 750 bp was obtained after PCR amplification of genomic DNA using *rpoA* gene specific primers (Fig. 2.3.13) and nucleotide sequence of the PCR amplified *rpoA* gene was obtained from Xcelris Labs Limited, Ahmedabad, India. The consensus sequence of 690 bp was generated from forward and reverse sequence data using aligner software (Fig. 2.3.14).

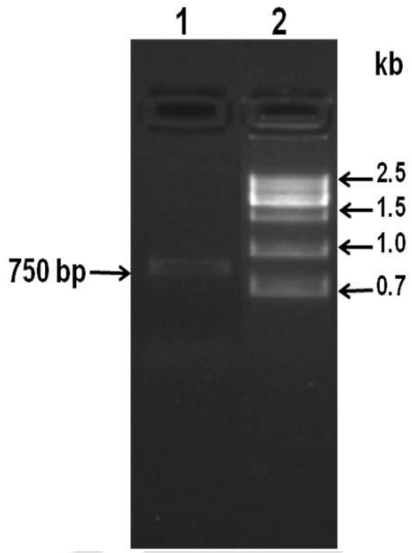

Fig. 2.3.13 Agarose gel (1.2%) showing *rpoA* gene (750 bp) of the isolate DM5 amplified with universal primers rpoA-21-F and rpoA-22-R. Lane 1: DNA ladder; Lane 2: Amplified full length *rpoA* gene.

ACCGCTTGAACGCGGTTATGGTACAACTTTAGGGAATTCACTTCGTCGGATTCTTCTTTCTTCTTTACC
TGGCGCTGCTGTTACTAGTATTCAAATTGATGGTGTTCTTCATGAATTTTCAACGATTGAGGGCGTAACG
GAAGACGTTACAGCAATTATCTTGAATGTTAAGAAGATTGCACTTAAGTTGGAATCAGACGAAACCAAGA
CGTTGGAAATCGACGTTAAGGGTCCTGCTAACGTTACTGCCGGTGATATCATTGGCGATGCGGACGTAGA
AGTCTTGAATCCAGACTTACCAATTTGTACCGTAGCAGACGGGGCACACTTCCATATGCGTATGACCGCA
AATACTGGTCGTGGTTATGTTTCCGCTGAGGATAACAAACATCGTGAAGATGACATGCCAATTGGCGTTT
TAGCTGTTGATTCATTGTATTCTCCAATCGAACGTGTCAACTATCAAGTTGAAAACACGCGGGTTGGTCA
ACGTGATGATTTCGATAAGTTAACCTTAGACGTTTGGACAAATGGTTCAATCACTCCAAGTGAAGCCATT
AGTCTATCAGCCAAAATCCTGACTGATCACCTTTCAATCTTCGTAAATCTCACCGATGAAGCTAAAAACA
CTGACGTGATGGTCGAGAAGGAAGAAACGCATAAGGAAAAGATGTTAGAAATGACGATTGA

Fig. 2.3.14 Consensus sequence of *rpoA* gene (690 bp) of the isolate DM5.

The partial sequence of *rpo*A gene of the isolate DM5 also showed highest homology with *Lb. plantarum* FHHMB120-8R-A3 (Genbank accession no. HQ379183) as shown in Fig. 2.3.15. Based on the evolution distance and the phylogenetic analysis resulting from partial sequencing of 16S rRNA and *rpoA* gene the isolate was identified as *Lb. plantarum* and the strain was designated as DM5 after author and source. The *rpo*A gene sequence of the isolate *Lb. plantarum* DM5 was submitted to the NCBI Genbank database under the accession no. KF286000.

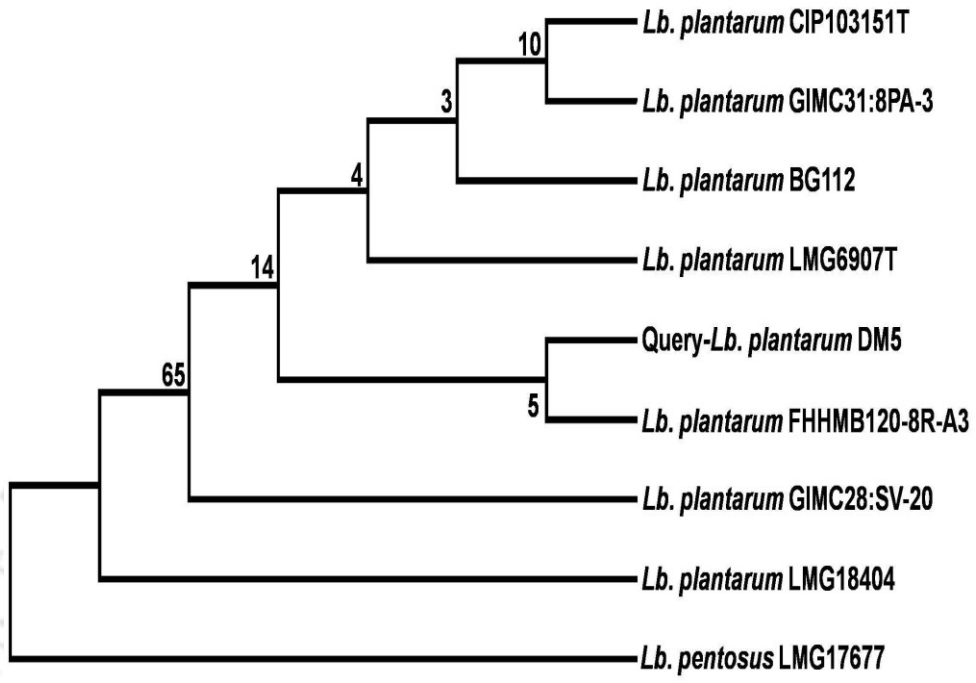

Fig. 2.3.15 Neighbour-joining tree based on *rpoA* gene sequence of isolate *Lb. plantarum* DM5. Number at the nodes indicated the level of support based on a neighbour joining analysis after 500 simulations with bootstrap percentage (>50). *Lb. pentosus* LMG 17677 was showed as outgroup.

2.3.6 Antimicrobial spectrum of isolate DM5

The antimicrobial activity of cell free supernatant of isolate DM5 was tested against Gram positive and Gram negative bacteria. The isolate DM5 showed broad range of antagonistic activity as shown in Table 2.3.5. The isolate DM5 inhibited the growth of Gram positive *Staphylococcous aureus* MTCC 737, *Staphylococcus epidermis* MTCC 6810, *Leuconostoc citreum* NRRL B-742, *Listeria monocytogenes* MTCC 1143, *Bacillus cereus* NCIM 2155 and *Pediococcus pentosaceus* SPA as well as Gram negative *Alcaligenes feacalis* MTCC 2952, *Enterobacter aerogens* MTCC 7016 and *Escherichia coli* DH5α. The isolate DM5 showed strong inhibitory effect

against Gram negative bacteria such as *E. coli* and *A. feacalis* whereas the other strains *Lb. plantarum* LPC010 (Jimenez-Diaz *et al*., 1993), *Lb. plantarum* 35d (Messi *et al*., 2001) and *P. pentosaceus* ACCEL (Wu *et al*., 2004) did not inhibit the growth of these bacteria.

Table 2.3.5 Antimicrobial spectrum of isolate *Lb. plantarum* DM5.

Test microorganism	Medium	Temp. (°C)	Sensitivity
Alcaligenes feacalis MTCC 2952	NB	30	+
Bacillus cereus NCIM 2155	NB	37	+
Enterobacter aerogenes MTCC 7016	NB	30	-
Escherichia coli DH5α	LB	37	+++
Klebsiella oxytoca MTCC 3030	NB	37	-
Leuconostoc citreum NRRL B-742	MRS	30	++
Leuconostoc dextranicum NRRL B-1146	MRS	30	-
Leuconostoc mesenteroides NRRL B-640	MRS	30	-
Leuconostoc mesenteroides NRRL B-1149	MRS	30	-
Leuconostoc mesenteroides NRRL B-512F	MRS	30	-
Listeria monocytogenes MTCC 1143	NB	37	++
Pediococcus pentoseous SPA	MRS	30	++
Pseudomonas fluorescens NRRL B-1612	NB	37	-
Staphylococcous aureus MTCC 737	NB	30	+++
Staphylococcus epidermis MTCC 6810	NB	30	++
Weisella confusa Cab3	MRS	30	-

Symbols: **(+++):** zone>15 mm*, **(++):** 10 mm<zone< 15 mm*, **(+):** 5mm<zone<10mm*, **(-):** no zone of inhibition.

**Values in millimeter are the distance of zone of inhibition of growth of microorganism*

The antimicrobial activity of the cell free supernatant of *Lb. plantarum* DM5 was compared with inhibitory activity of ampicillin (1 mg/ml) and kanamycin (1 mg/ml) against indicator strain *S. aureus* MTCC 737 as shown in Fig. 2.3.16. The isolate DM5 displayed clear zone of inhibition of around 16 mm against *S. aureus* MTCC 737 and the zone of inhibition by ampicillin (1 mg/ml) and kanamycin (1

mg/ml) was 25 mm and 23 mm, respectively, suggesting the strong antimicrobial activity of the isolate DM5 (Fig. 2.3.16).

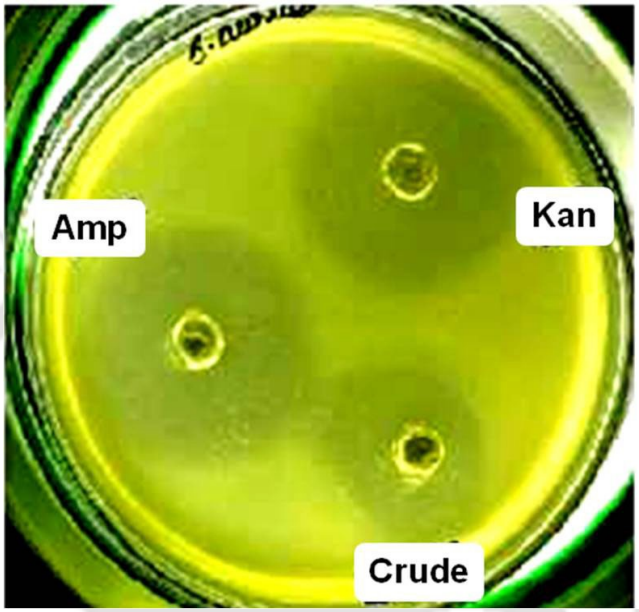

Fig. 2.3.16 Comparison of antimicrobial activity of isolate DM5 with standard antibiotics ampicillin and kanamycin (1 mg/ml) against indicator strain *Staphylococcus aureus* by agar well diffusion method. Amp: ampicillin; Kan: kanamycin; Crude: filter sterilized (0.22 µm) cell free supernatant of isolate DM5 (pH 6.0).

2.4 Conclusions

A novel lactic acid bacterium showing antagonistic activity and glucansucrase activity from non dairy fermented food products was isolated. Based on the finding of maximum zone of inhibition around the well against indicator strain *E. coli* DH5α and glucansucrase activity of 1.8 U/ml, the isolate DM5 was selected for further characterization. The isolate DM5 was Gram positive, rod shaped, non motile and did not possess catalase activity. The biochemical characterization of isolate DM5 was carried out to distinguish the isolate from other closely related lactic acid bacteria.

Based on biochemical and physiological studies the isolate showed its relation with other *Lactobacillus* spp. The antibiogram and carbohydrate fermentation profile study enabled the identification of isolated bacterium as it exhibited the typical characteristic features of *Lactobacillus plantarum*. The isolate DM5 was sensitive towards ampicillin, amoxicillin, bacitracin, carbenicillin, cholarmphenicol, erythromycin, methicillin, tetracycline and ticarcillin. The isolate DM5 was resistant to cloxacillin, cefexime, trimethoprim, nalidixic acid and vancomycin a recurrent attribute of *Lactobacillus plantarum* strain. The isolate DM5 could efficiently utilize arabinose, cellibiose, fructose, glucose, galactose, inulin, lactose, maltose, mellibiose, mannitol, raffinose, sucrose and trehalose like other *Lactobacillus plantarum* strains. The isolate DM5 could not ferment glycerol and rhamnose, a general trait of *Lactobacillus plantarum*.

The 16S rRNA gene sequence analysis revealed the identity of the isolate DM5 which was found to be *Lactobacillus plantarum* (Genbank Accession Number KC020195). As the 16S rRNA gene sequence data do not allow the identification of closely related species, an alternative identification of the isolate DM5 was done by

using the RNA polymerase alpha subunit (*rpoA*) partial gene sequence specific primers, which also revealed the isolate as *Lactobacillus plantarum* (Genbank Accession Number KF286000).

The isolate DM5 showed broad antimicrobial activity as it inhibited the growth of Gram positive as well as Gram negative bacteria. The isolate DM5 had shown clear zone of inhibition against several major food borne pathogens, such as *E. coli, S. epidermis, S. aureus, A. feacalis* and *L. monocyotogenes* in food stuffs, hence, it could be used as potential bio-preservative in foods. This is the first report of *Lactobacillus plantarum* showing antimicrobial activity as well as glucansucrase activity. The production of glucansucrase of isolate DM5 could be increased further by optimizing the fermentation process which could compensate the increasing demand of glucan as viscosifier and stabilizer in food industry. However, it is too early to claim the enzyme secreted by the isolate DM5 as glucansucrase. Further characterization of this enzyme is required to support this finding. This investigation also unravelled the abundance of industrially valuable microbial flora in fermented food products in North east India.

References

Aymerich, M.T., Garriga, M., Monfort, J.M., Nes, I. and Hugas, M. (2000) Bacteriocin-producing *Lactobacilli* in Spanish-style fermented sausages: characterization of bacteriocins. *Food Microbiol*., 17, 33-45.

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

Bergey, D.H., John, G. H., Noel, R. K. And Peter, H.A.S. (1994) Bergey's Manual of Determinative Bacteriology (9th ed.). Lippincott Williams & Wilkins. ISBN 0-683-00603-7.

Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.*, 7, 1513-1523.

Bixquert, J.M. (2009) Treatment of irritable bowel syndrome with probiotics: an etiopathogenic approach at last. *Rev. Esp. Enferm. Dig*., 101, 553-564.

Briggs, M. (1953) The classification of *lactobacilli* by means of physiological tests. *J. Gen. Microbiol.*, 9, 234-248.

Buddhiman, T., Jyoti, P.T., Ulrich, S., Charles, M.A.P.F., Michael, G. and Wilhelm, H.H. (2008) Phenotypic and genotypic identification of lactic acid bacteria isolated from ethnic fermented bamboo tender shoots of North East India. *Int. J. Food Microbiol*., 121, 35-40.

Cerning, J. (1990) Exocellular polysaccharides produced by lactic acid bacteria. *FEMS Microbiol. Rev.*, 87, 113-130.

Corsetti, A., Lavermicocca, P., Morea, M., Baruzzi, F., Tosti, N. and Gobbetti M. (2001) Phenotypic and molecular identification and clustering of lactic acid bacteria and yeasts from wheat (species *Triticum durum* and *Triticum aestivum*) sourdoughs of Southern Italy. *Int. J. Food Microbiol.*, 64, 95-104.

Daming, R., Yinyu, W., Zilai, W., Jun, C., Hekui, L. and Jingye, Z. (2003) Complete DNA sequence and analysis of two cryptic plasmids isolated from *Lactobacillus plantarum*. *Plasmid*, 50, 70-73.

Danielsen, M. (2002) Characterization of the tetracycline resistance plasmid pMD5057 from *Lactobacillus plantarum* 5057 reveals a composite structure. *Plasmid*, 48, 98-103.

Danielsen, M. and Wind, A. (2003) Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *Int. J. Food Microbiol*., 82, 1-11.

Das, D. and Goyal, A. (2010) Characterization and screening of antimicrobial activity of lactic acid bacterium isolated from a traditional beverage Marcha of Sikkim. *J Pharm. Chem.,* 4, 136-139.

Das, D. and Goyal, A. (2011) Expression of antagonism by lactic acid bacterium isolated from marcha: an ethnic fermented beverage of north-eastern Himalayas. *Int. J. Biotechnol. Biochem*., 7, 411-421.

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

De, S., Kaur, G., Roy, A., Dogra, G., Kaushik, R., Yadav, P., Singh, R., Datta, T.K. and Goswami, S.L. (2010) A Simple Method for the Efficient Isolation of Genomic DNA from *Lactobacilli* Isolated from Traditional Indian Fermented Milk (dahi). *Indian J. Microbiol*., 50, 412-418.

Dicks, L.M.T. and van Vuuren, H.I.I. (1987) A modification of the hot tube method for the detection of carbon dioxide produced by heterofermentative *Lactobacillus* strains. *J. Microbiol. Meth.,* 6, 273-275.

Fugelsang, KC, and Edward CG (2007). Wine microbiology, Practical application and procedure. 2nd edition, Springer, 2, 29-44.

Galvez, A., Abriouel, H., Lopez, R.L. and Omar, N.B. (2007) Bacteriocin-based strategies for food biopreservation. *Int. J. Food Microbiol.,* 120, 51–70.

Gram, C. (1884) Ueber die isolirte Farbung der Schizomyceten in Schnitt-und Trockenpraparaten. *Fortschritte der Medcin.*, 2, 185-189.

Groenewald, W.H., van Reenen C.A., and Dicks, L.M.T. (2006) Strains of *Lactobacillus plantarum* in Grape Must are also Present in the Intestinal Tract of Vinegar Flies. *S. Afr. J. Enol. Vitic.,* 27, 46-50.

Holzapfel, W.H., Geisen, R. and Schillinger, U. (1995) Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *Int. J. Food Microbiol.,* 24, 343–362.

Jeyaram, K., Singh, T.H., Romi, W., Devi, A.R., Singh, W.M., Dayanidhi, H., Singh, N.R. and Tamang, J.P. (2009) Traditional fermented foods of Manipur. *Indian J. Traditional Knowl.,* 8, 115-121.

Jimenez-Diaz, R., Rios-Sanchez, R.M. and Piard, J.C. (1993) Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPC010 isolated from a green olive fermentation. *Appl. Environ. Microbiol.*, 59, 1416-1424.

Kanatani, K. and Oshimura, M. (1994) Plasmid-associated bacteriocin production by a *Lactobacillus plantarum* strain. *Biosci. Biotechnol. Biochem*., 58, 2084-2086.

Kandler, O. and Weiss, N. (1986) Genus *Lactobacillus* beijerinck 1901, 212$^{AL,"}$ In: (Sneath, P. H. A. Mair, N. S. Sharpe, M. E. and Holt, J. G. ed.) *Bergey's manual of systematic bacteriology*. Baltimore: Williams and Wilkins, 2, 209-1234.

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.

Klare, I., Konstabe,l C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., Hildebrandt, B., Müller-Bertling, S., Witte, W. and Goossens, H. (2007) Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *J. Antimicrob. Chemother*., 59, 900-912.

Kumar, K.S., Ganesan, K. and Subba-Rao, P.V. (2008) Antioxidant potential of solvent extracts of *Kappaphycus alvarezii* (Doty) Doty –An edible seaweed. *Food Chem.,* 107, 289-295.

Li, R., Zhai, Z., Yin, S., Huang, Y., Wang, Q., Luo, Y. and Hao, Y. (2009) Characterization of a Rolling-Circle Replication Plasmid pLR1 from *Lactobacillus plantarum* LR1. *Curr. Microbiol*., 58, 106-110.

Massi, M., Vitali, B., Federici, F., Matteuzzi, D. and Brigidi, P. (2004) Identification method based on PCR combined with automated ribotyping for tracking probiotic *Lactobacillus* strains colonizing the human gut and vagina. *J. Appl. Microbiol*., 96, 777–786.

Messi, P., Bondi, M., Sabia, C., Battini, R. and Manicardi, G. (2001) Detection and preliminary characterization of a bacteriocin (plantaricin 35d) produced by a *Lactobacillus plantarum* strain. *Int. J. Food Microbiol*., 64, 193–198.

Naser, S.M., Dawyndt, P., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Vancanneyt, M. and Swings, J. (2007) Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. *Int. J. Syst. Evol. Microbiol.,* 57, 2777–2789.

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

Nissen, L., Chingwaru, W., Sgorbati, B., Biavati, B. and Cencic A. (2009) Gut health promoting activity of new putative probiotic protective *Lactobacillus* spp. Strains: a functional study in the small intestinal cell model. *Int. J. Food Microbiol*., 135, 288-294.

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

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.

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.

Rai, AK., Palni, U. and Tamang, J.P. (2009) Traditional knowledge of the Himalayan people on production of indigenous meat products. *Indian J. Traditional Knowl.,* 8, 104-109.

Ruiz-Barba, J.L., Piard, J.C. and Jimenez-Diaz, R. (1991) Plasmid profiles and curing of plasmids in *Lactobacillus plantarum* strains isolated from green olive fermentations. *J. Appl. Bacteriol*., 74, 417-421.

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

Sambrook, J. and Russell, D.W. (2001) Molecular Cloning: A Laboratory Manual, New York, Cold Spring Harbor Laboratory Press.

Satish-Kumar, R., Kanmani, P., Yuvraj, N., Paari, K.A., Pattukumar, V. and Arul V. (2012) Traditional Indian fermented foods: a rich source of lactic acid bacteria. *Int. J. Food Sci. Nutr.,* 64, 1-14.

Shukla, S. and Goyal, A. (2011) 16S rRNA based identification of a glucan hyper-producing *Weissella confusa*. *Enzym Res.,* 250842, doi:10.4061/2011/250842.

Singh, E.J., Singh, N.K.S. and Singh, N.R. (2009) Biodiversity conservation and natural resources in North East India with special reference to Manipur. *NeBio* 1, 42-47.

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

Spelhaug, S.R. and Harlander, S.K. (1989) Inhibition of foodborne bacterial pathogens by bacteriocins from *Lactobacillus lactis* and *Pediococcus pentosaceus*. *J. Food Prot.,* 52, 856-862.

Swenson, J.M., Facklamand, R.R. and Thornsberry, C. (1990) Antimicrobial susceptibility of vancomycin-resistant *Leuconostoc*, *Pediococcus*, and *Lactobacillus* species. *Antimicrob. Agents Chemother*., 34, 543-549.

Tamang, J.P., Dewan, S., Tamang, B., Rai, A., Schillinger, U. and Holzapfel, W.H. (2007) Lactic acid bacteria in Hamei and Marcha of North East India. *Indian J. Microbiol*., 47, 119-125.

Thapa, N., Pal, J. and Tamang, J.P. (2004) Microbial diversity in ngari, hentak and tungtap, fermented fish products of North-East India. *World J. Microbiol. Biotechnol.*, 20, 599-607.

Thokchom, S and Joshi, S.R. (2012) Antibiotic resistance and probiotic properties of dominant lactic microflora from *Tungrymbai*, an ethnic fermented soybean food of India. *J. Microbiol.,* 50, 535-539.

Todorov, S.D. and Dicks, L.M.T. (2009) Effect of modified MRS medium on production and purification of antimicrobial peptide ST4SA produced by *Enterococcus mundtii*. *Anaerobe*., 15, 65-73.

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 culture factors on production on dextransucrase by *Leuconostoc mesenteroides*. *J. Bacteriol*., 64, 521-526.

Ulrich, R.L. and Hughes, T.A. (2001) A rapid procedure for isolating chromosomal DNA from *Lactobacillus* species and other Gram-positive bacteria. *Lett. Appl. Microbiol*., 32, 52-56.

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

Wu, C.W., Yin, L.J. and Jiang, S.T. (2004) Purification and Characterization of Bacteriocin from *Pediococcus pentosaceus* ACCEL. *J. Agric. Food Chem*., 52, 1146-1151.

Zeigler, D.R. (2003) Gene sequences useful for predicting relatedness of whole genomes in bacteria. *Int. J. Syst. Evol. Microbiol*., 53, 1893-1900.

Zhou, J.S., Pillidge, C.J., Gopal, P.K. and Gill, H.S. (2005) Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *Int. J. Food Microbiol.,* 98, 211-217.

Chapter 3

Probiotic attributes and antioxidative activity of *Lactobacillus plantarum* DM5

3.1 Introduction

Lactic acid bacteria play a very important role as starters in the production of fermented health foods as they are food-grade organisms and generally regarded as safe (GRAS). The gut microbiota also includes lactic acid bacteria which have been implicated in a number of health promoting functions that affect general health and well-being of the host. Probiotics are defined as a live microbial feed supplement which beneficially affects the host by improving its intestinal microbial balance (Fuller, 1989). Lactic acid bacteria are widely used as probiotics as they are non pathogenic which is the most essential pre-requisite to select a potential probiotic bacterium. The other functional criteria used for selection of probiotics are, tolerance to low acidity and bile salt, bile salt hydrolase activity, production of antimicrobial substances, ability to reduce pathogen adhesion to epithelial cells and safety for food or clinical use (Ouwehand *et al.,* 2002). *Lactobacillus plantarum* is an abundant probiotic lactic acid bacterium and also being used as a starter culture in production of

various fermented food products (Bixquert, 2009; Lonnermark *et al.,* 2010). Probiotic *Lb. plantarum* enhances the intestinal integrity, metabolic activity of intestinal cells and stimulates the immune responses (Nissen *et al.,* 2009) and reduce certain gastrointestinal symptoms during the treatment with antibiotics (Lonnermark *et al.,* 2010). The use of *Lactobacillus plantarum* for the prevention and treatment of intestinal disorders has been reported but the antioxidative ability of probiotic *Lb. plantarum* is not well studied.

Environmental pollution, UV radiation and several normal metabolic processes have been associated with the induction of high level of reactive oxygen species (ROS) in mammalian cells. The ROS mainly includes highly reactive superoxide anion radical, hydroxyl radical and hydrogen peroxide. Elevated levels of these highly active free radicals have been found to be associated with numerous human diseases, such as carcinogenesis, atherosclerosis, Alzheimer's disease, ageing and degenerative processes (Maxwell, 1995). Although mammalian cells acquire efficient antioxidant defence and repair systems which have evolved to protect them against oxidative damage, these systems are not effective enough to completely prevent the damage (Simic, 1988; Li and Yen, 1999). Antioxidants are substances that can eliminate ROS, thus preventing or delaying the oxidation of cellular components. Various synthetic and natural antioxidants have been reported such as, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ); however, there are doubts about the safety and long-term effects of synthetic antioxidants on health (Kumar *et al.,* 2008). Therefore, antioxidants from natural sources are more desirable. It has been shown that some *Lactobacilli* possess antioxidative activity and are able to decrease the risk of accumulation of ROS (Kaizu *et al.,* 1993) and degrade the

superoxide anion and hydrogen peroxide (Kullisaar *et al.,* 2002). *Lactobacillus plantarum* 7FM10 isolated from the traditional Japanese food narezushi (Kanno *et al.,* 2012) and *Lactobacillus plantarum* isolated from Chinese fermented food (Li *et al.,* 2012) displayed DPPH, hydroxyl and superoxide radical scavenging capacities.

γ-Aminobutyric acid (GABA) is an ubiquitous non protein amino acid produced by α-decarboxylation of glutamate by glutamate decarboxylase and acts as a major inhibitory neurotransmitter in the mammalian central nervous system (Schousboe & Waagepetersen, 2007). In addition, GABA has hypotensive, tranquilizing and diuretic effects and can prevent diabetes (Hayakawa *et al.,* 2004; Siragusa *et al.,* 2007; Li and Cao, 2010). Several *Lactobacillus* spp have been reported to enhance the GABA content of fermented food products (Li and Cao, 2010; Lee *et al.,* 2010). The traditional fermented food sources enriched in glutamate are important sources for isolation of GABA-producing lactic acid bacteria (Di Cagno *et al.,* 2010; Lee *et al.,* 2010).

In the present study, a novel strain *Lactobacillus plantarum* DM5 (Genbank Accession No: KC020195) isolated from an ethnic fermented beverage Marcha of Sikkim, India (Das and Goyal, 2013) was explored for its prospective application in food industry as biopreservant probiotic. The isolate *Lb. plantarum* DM5 was also examined for its *in vitro* antioxidant activities including inhibition of ascorbate autooxidation and scavenging activity of DPPH radicals, superoxide anion radicals and hydroxyl radicals. GABA producing ability of *Lb. plantarum* DM5 was also explored for further establishing it as an exclusive probiotic anti-oxidative strain.

3.2 Material and Methods

3.2.1 Chemicals and reagents

The media components for maintenance of bacterial culture were purchased from Hi-Media Pvt. Ltd., India. All the chemicals required for probiotic analysis, buffer preparations and Fetal bovine serum (FBS) for maintaining the mammalian cell line were of high purity grade and purchased from Hi-Media Pvt. Ltd., India. The standard enzymes pepsin, trypsin, Bile salts, sodium turodeoxycholic acid (TDCA), γ aminnobutyric acid (GABA), ascorbate, cysteine, phenazine methosulphate, nitroblutetrazolium salt (NBT), Nicotinamide adenine dinucleotide (NADH), 1-diphenyl-2-picrylhydrazyl (DPPH), Bovine serum albumin (BSA) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma Aldrich, USA.

3.2.2 Microorganism and culture medium

The strain *Lb. plantarum* DM5 was isolated from an ethnic fermented beverage Marcha of Sikkim on the basis of antimicrobial activity (Das and Goyal, 2010) and glucansucrase activity (as described in Chapter 2, Section 2.2.6.1) and maintained in modified MRS agar medium (Goyal and Katiyar, 1996) as described in Chapter 2, Section 2.2.2 at 4ºC, sub cultured after every 2 weeks. The other bacterial cultures used in this study, *Lactobacillus plantarum* NRRL B-4495 and *Lactobacillus acidophilus* NRRL B-4496 were procured from Agricultural Research Service Culture Collection, USA. The strain *Staphylococcus aureus* MTCC 737 was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.

3.2.3 Mammalian cell line and maintenance

The human colon adenocarcenoma adherent cell line (HT-29) was purchased from National Centre for Cell Science (NCCS), Pune, India. The cell line was maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) containing 4.0 mM L-glutamine, 110 mg/L sodium pyruvate, 10% (v/v) fetal bovine serum (FBS) and 3.75 g/L sodium bicarbonate. The preparation of Dulbecco's Modified Eagle's Medium (DMEM) is described in Section 3.2.3.1. The cell line was grown in 75 cm^2 vent cap tissue culture flask containing 10 ml of complete DMEM medium and incubated at 37ºC±1ºC in 5% CO_2 atmosphere. The medium was changed at regular interval of 36 h and cell splitting was done after 90% confluency by 1x Trypsin EDTA solution (0.25% trypsin and 0.2% EDTA). The sub culturing of the cell line is described in the Section 3.2.3.2. The cell line was stored at -80ºC in cryopreservation medium comprised of 5 ml of 10% (v/v) FBS, 4 ml of complete DMEM medium and 1 ml DMSO for long term storage.

3.2.3.1 Preparation of DMEM medium

The incomplete serum free DMEM was prepared by adding 13.4 g dry powder of DMEM and 3.7 g sodium bicarbonate in pre autoclaved double distilled water. The DMEM medium is the modified version of Eagle's minimum essential medium (Darnell *et al*., 1970) that contains a fourfold higher concentration of amino acids and vitamins as well as supplementary components such as phenol red and iron (Delbecco, 1976). The pH of medium was adjusted to 7.4±0.2 by 1N HCl or 1N NaOH solution and the final volume of medium was adjusted to 1L. The medium was then supplemented with 50 µg/ml streptomycin and 50 IU/ml penicillin. The medium was

immediately filter-sterilized using a pre autoclaved 0.22 µm bottle filter (Tarson Products Pvt. Ltd. India). The complete serum added DMEM medium was used for maintaining and culturing the cell line. The fetal bovine serum was filter-sterilized using a pre autoclaved 0.22µm bottle filter and 50 ml (10%, v/v) of fetal bovine serum was added to 450 ml of incomplete DMEM medium and stored at 4°C. The complete DMEM medium should be pre-warmed before use by placing in a water bath at 37ºC for 15 min.

3.2.3.2 Sub-culturing of cell line

The attached HT-29 cells were sub cultured using 500 µl of 0.25% (w/v) trypsin-0.53 mM EDTA for detaching the cell from the bottom surface of the flask. After trypsinization, the enzymatic action of the trypsin-EDTA was stopped by adding 1 ml of complete DMEM medium to the detached cells and centrifuged at 300*g* at 4°C for 5 min. The supernatant was discarded and the cells were re-suspended in 5 ml of complete DMEM medium. A split ratio of 1:10 (1 ml of above cell suspension in 9 ml complete DMEM medium) or a seeding density of 4 x 10^4 viable cells/cm^2 was used for sub-culturing HT-29 cells.

3.2.4. *In vitro* analysis of probiotic properties of *Lb. plantarum* DM5

3.2.4.1 Acid tolerance of Lb. plantarum DM5

The tolerance of isolate *Lb. plantarum* DM5 to low pH was evaluated by method of Ehrmann *et al*., (2002). The 50 ml of MRS medium as described in Chapter 2, Section 2.2.2 was adjusted to pH 2, 3 and 4 by 2N HCl. The isolate DM5 was

initially grown in 5 ml of MRS medium (pH 6.4) at 37ºC for 18 h under static condition. The cell pellet was obtained by centrifugation at 8,000*g* at 4°C for 10 min and washed twice with deionised water. The cell pellet was re-suspended in sterile 0.85% NaCl to obtain a viable cell count of ~10^7 CFU/ml (Vinderola and Reinheimer, 2003). Cell suspension of 1 ml was then inoculated into 50 ml of MRS medium with different pH and incubated at 37ºC for 4 h. Viability of the isolate DM5 was quantified as CFU/ml, by counting colonies appeared by spreading 100 µl of culture on MRS agar plate (as described in Chapter 2, Section 2.2.2) at every 1 h interval up to 4 h. The plates were incubated at 37ºC for 18 h under static condition. The total number of viable cells was enumerated and the graph was plotted by taking log of CFU/ml (Oh *et al.,* 2000).

3.2.4.2 Gastric juice tolerance of Lb. plantarum DM5

The artificial gastric juice was prepared by supplementing MRS medium with pepsin (Sigma Aldrich, USA) solution of 1000 U/ml and pH of the medium was adjusted to 2.5 with 2N HCl (Oh *et al.,* 2000). The isolate DM5 was initially grown in 5 ml of MRS medium (pH 6.4) (as described in Chapter 2, Section 2.2.2) at 37ºC for 18 h under static condition. The cell pellet was obtained by centrifugation at 8,000*g* at 4°C for 10 min and washed twice with deionised water. The cell pellet was re-suspended in sterile 0.85% NaCl to obtain a viable cell count of ~10^7 CFU/ml (Vinderola and Reinheimer, 2003). Cell suspension of 1 ml was then inoculated into 50 ml of artificial gastric juice and incubated at 37ºC for 5 h. Viability of the isolate *Lb. plantarum* DM5 was quantified as CFU/ml and the graph was plotted by taking log of CFU/ml as described in Section 3.2.4.1.

3.2.4.3 Intestinal fluid tolerance of Lb. plantarum DM5

In order to prepare the intestinal fluid, the MRS medium was supplemented with 1% (w/v) Na_2CO_3 (Merck, India), 0.2% (w/v) NaCl (Merck, India), 0.5% (w/v) bile salt (Sigma Aldrich, USA) and 1000 U/ml of trypsin solution (Sigma Aldrich, USA) and the pH of the medium was adjusted to 8.0 with 1N NaOH (Farnadez *et al.,* 2003). The isolate *Lb. plantarum* DM5 was grown in 5 ml of MRS medium (pH 6.4) at 37ºC for 18 h and the cell pellet obtained by centrifugation at 8,000*g* at 4°C for 10 min. The cell pellet was washed twice with deionised water and re-suspended in sterile 0.85% NaCl to obtain a viable cell count of ~10^7 CFU/ml (Vinderola and Reinheimer, 2003). Cell suspension of 1 ml was then inoculated into 50 ml of intestinal fluid and incubated at 37ºC for 5 h. Viability of the isolate *Lb. plantarum* DM5 was quantified as CFU/ml and the graph was plotted by taking log of CFU/ml as described in Section 3.2.4.1.

3.2.4.4 Lysozyme tolerance of Lb. plantarum DM5

The MRS medium (pH 6.4) as described in Chapter 2, Section 2.2.2 was supplemented with different concentration of lysozyme (Hi-Media Pvt. Ltd., India) ranging from 50-300 µg/ml to determine the lysozyme sensitivity of the isolate *Lb. plantarum* DM5 following the method established by Suskovic *et al.,* (1997). The isolate *Lb. plantarum* DM5 initially was grown in 5 ml of MRS medium (pH 6.4) without lysozyme at 37ºC for 18 h under static condition and the cell pellet was obtained by centrifugation at 8,000*g* and 4°C for 10 min. The cell pellet of isolate *Lb. plantarum* DM5 was washed twice with deionised water and re-suspended in sterile 0.85% NaCl to obtain a viable cell count of ~10^7 CFU/ml. Cell suspension of 1 ml was

used for inoculating 50 ml of MRS medium containing 50, 100, 200 and 300 µg/ml of lysozyme separately. The cultures were then incubated at 37°C for 30 h under static condition and the total number of viable cells was analyzed by determination of CFU/ml at 5 h interval up to 30 h by plate count method as mentioned earlier in Section 3.2.4.1.

3.2.4.5 Bile salts tolerance of Lb. plantarum DM5

The isolate *Lb. plantarum* DM5 (1%, v/v) was inoculated in 5 ml MRS medium (pH 6.4) and incubated at 37ºC for 18 h under static condition. The cell pellet was collected by centrifugation (8,000*g* at 4ºC, for 10 min) and washed twice in deionised water. The cell pellet of isolate *Lb. plantarum* DM5 was re-suspended in sterile 0.85% NaCl to obtain a viable cell count of ~10^7 CFU/ml and 1 ml of cell suspension was used for inoculating 50 ml of MRS medium containing 0.3, 0.5 and 1% (w/v) of bile salt (Sigma Aldrich, USA), separately. The cultures were then incubated at 37°C for 30 h under static condition and the total number of viable cells was analyzed by determination of CFU/ml at 5 h interval up to 30 h by plate count method as mentioned earlier in Section 3.2.4.1.

3.2.4.6 Determination of cell surface hydrophobicity of Lb. plantarum DM5

3.2.4.6.1 Microbial adhesion to hydrocarbons

The *in vitro* cell surface hydrophobicity of the isolate *Lb. plantarum* DM5 was determined by the microbial adhesion to hydrocarbon (MATH) assay method as described by Kos *et al.,* (2003). The hydrocarbons used for this test were xylene, n-hexadecane, chloroform and ethyl acetate. Isolate DM5 was grown in 5 ml of MRS

medium at 37°C for 18-20 h and the cell pellet obtained by centrifugation at 8,000*g* at 4°C for 10 min. The cell pellet was then washed twice with 1x phosphate buffer saline (PBS) (pH 6.8) and re-suspended in the same buffer to an A_{600} of 0.5. Bacterial suspension of 3 ml was mixed with 0.6 ml of each solvent in a glass tube, vigorously shaken for 2 min and kept at 37°C for 30 min for phase separation (Kos *et al.,* 2003). The absorbance of the aqueous phase was measured at 600 nm. The percentage of bacterial adhesion to hydrocarbon was calculated as;

$$\textbf{MATH (\%)} = (1-A_1/A_0)\textbf{x}100$$

where, A_0= absorbance (A_{600}) of the bacterial suspension before extraction with hydrocarbons and A_1= absorbance (A_{600}) of the bacterial suspension after extraction.

3.2.4.6.2 Salt aggregation test

The cell surface hydrophobicity was analyzed by the Salt Aggregation Test (SAT) as described by Qiao *et al.,* (2012). The cell pellet obtained by centrifugation at 8,000*g* at 4°C for 10 min was washed twice with 1x PBS (pH 6.8) and re-suspended in PBS to give viable count of ~10^7 CFU/ml. Ammonium sulphate solution of 100 µl ranging from 0.01 M to 4 M was added to 500 µl of the bacterial cell suspension in PBS. The SAT value was measured as the lowest molarity of ammonium sulfate giving visible bacterial clumping (Qiao *et al.,* 2012).

3.2.4.7 Autoaggregation and coaggregation activity of Lb. plantarum DM5

Autoaggregation assay was performed according to the method established by Del Re *et al.,* (2000) with certain modifications. The isolate DM5 was grown in 10 ml

of MRS medium (pH 6.4) at 37°C for 18-20 h and cell pellet obtained by centrifugation at 8,000g at 4°C for 10 min. The cell pellet was washed twice in 1x PBS (pH 6.8) and re-suspended in 1x PBS to obtain viable counts of ~10^7 CFU/ml. Cell suspension of 4 ml was mixed by vortexing for 10 s and autoaggregation was determined at 37ºC during 6 h of incubation. Upper suspension of 0.1 ml was transferred to another tube with 3.9 ml of 1x PBS at every hour and the absorbance was measured at 600 nm (Kos *et al.,* 2003). The percentage of autoaggregation is expressed as:

$$\textbf{Autoaggregation (\%)} = (1-A_t/A_0)\textbf{x}100$$

where, A_t = represents the absorbance (A_{600}) at time t= 1, 2, 3, 4, 5 or 6 h and A_0 the absorbance at t=0.

The method for preparing the cell suspensions for coaggregation was the same as that for autoaggregation. Equal volume (2 ml) of each cell suspension was mixed together in pairs by vortexing for 10 s. Control tubes were set up at the same time, containing 4 ml of each bacterial suspension on its own. Absorbance of the bacterial suspension was measured at A_{600} after mixing and after 6 h of incubation at 37ºC. The samples were lifted in the same way as in the autoaggregation assay (Kos *et al.,* 2003). The percentage of coaggregation was calculated as;

$$\textbf{Coaggregation (\%)} = [\{((A_x+A_y)/2)-A_{(x+y)}\}/\ A_x+A_y/2]\textbf{x}100$$

where, A_x and A_y represent absorbance (A_{600}) of each of the two strains in the control tubes (t=0,1,2,3,4,5) and (x + y) represent absorbance (A_{600}) of the mixture (t=0,1,2,3,4,5,6).

3.2.4.8 In vitro cell adhesion assay of Lb. plantarum DM5

The *in vitro* adhesion ability of isolate *Lb. plantarum* DM5 was assayed as per the method described by Jacobsen *et al.,* (1999). The DMEM medium was prepared as described in Section 3.2.3.1 and the HT-29 cells were seeded in each well of six-well tissue culture plates at a density of $4x10^4$ cell/cm^2 and incubated at 37ºC for 24 h. After 24 h, the DMEM medium was removed and the cells were washed with 1x PBS (pH-7.4). The isolate *Lb. plantarum* DM5 was initially grown in MRS medium at 37ºC for 18 h under static condition and the cell pellet was obtained by centrifugation at 10,000*g* at 4ºC for 10 min. The cell pellet was washed twice with 1x PBS (pH-7.4) and re-suspended in DMEM medium without serum and antibiotics to obtain $1x10^8$ CFU/ml. Adhesion assay was done by adding 1 ml of bacterial cell suspension to each well of the six-well tissue culture plate containing monolayer of HT-29 cells. The tissue culture plate was then incubated at 37ºC for 2 h in the presence of 5% CO_2/95% air atmosphere. After incubation, the monolayer was washed with sterile 1x PBS (pH-7.4) and 2 ml of methanol was added to each well for fixing and incubated at 37ºC for 10 min. After that the methanol was completely removed and the fixed cells were stained with 1 ml of Giemsa solution (1x) at 37ºC for 20 min (Duary *et al.,* 2011). The wells were washed with absolute ethanol to remove the excess stain and air dried. The plate was then monitored under inverted microscope (TS100-F, Nikon International Inc.).

To determine the percent adhesion of isolate *Lb. plantarum* DM5 to HT-29 cells, bacterial cells were detached from monolayer by trypsinization using 500 µl of Trypsin-EDTA solution (as mentioned in Section 3.2.3.2). The tissue culture plate was then incubated at 37ºC for 15 min and the cell suspension was platted on MRS agar

plate (as described in Chapter 2, Section 2.2.2) by serial dilution for determining the adherent bacterial cells (Kaushik *et al*., 2009). The MRS agar plate was incubated at 37ºC for 24 h and colonies were counted. The results of the adhesion assay were expressed as adhesion percentage,

Adhesion (%)= (B_1/B_0)x100;

where, B_1 and B_0= No of viable cell (CFU/ml) of *Lb. plantarum* DM5 before and after adhesion.

3.2.4.9 Bile salt hydrolase activity (BSH) of Lb. plantarum DM5

The isolate *Lb. plantarum* DM5 was screened for bile salt hydrolase (BSH) activity by both qualitative direct plate assay method and quantitative ninhydrin assay method (Liong and Shah, 2005).

3.2.4.9.1 Qualitative determination of BSH activity by direct plate assay method

To determine the bile salt hydrolase activity of *Lb. plantarum* DM5, the isolate was initially grown in 5 ml of MRS medium (pH 6.4) at 37°C for 18 h under static condition. The isolate *Lb. plantarum* DM5 was streaked on MRS agar plate (as described in Chapter 2, Section 2.2.2) supplemented with 0.5% (w/v) sodium salt of taurodeoxycholic acid (TDCA; Sigma Aldrich, USA) and 0.04% (w/v) of $CaCl_2$ (Sieladie *et al.,* 2011). The plate was incubated at 37°C for 72 h in 2.5 L anaerobic jar (Sigma Aldrich, USA). The presence of white precipitation zone of bile acid or the formation of opaque granular white colonies due to deconjugation of bile salt around the colonies indicated the bile salt hydrolase activity of the isolate (Liong and Shah, 2005; Kumar *et al*., 2012).

3.2.4.9.2 Quantitative determination of BSH activity by ninhydrin assay method

The bile salt hydrolase activity was quantitatively measured by determining the amount of amino acids liberated from conjugated bile salts following the method described by Liong and Shah, (2005). The isolate *Lb. plantarum* DM5 was grown in MRS medium (as described in Chapter 2, Section 2.2.2) for 20 h under static condition and the cell pellet was obtained by centrifugation at 10,000*g* at 4ºC for 10 min. The cell pellet was washed twice with deionised water and re-suspended into 10 ml of 0.1M sodium phosphate buffer (pH 7.0). The cell concentration was adjusted to an absorbance (A_{600}) of 1 unit at 600 nm and 5 ml of the cell suspension was subjected to sonication (Sonics, Vibra cell) for three, 1 min intervals, by keeping cells in an ice bath. The cell debris was removed by centrifugation at 10,000*g* at 4ºC for 10 min. Cell free extract of 100 µl was mixed with 1.8 ml of 0.1 M sodium phosphate buffer (pH 7.0) and 0.1 ml of 6 mM conjugated bile salts. The conjugated bile salts used in the experiment was 6 mM sodium taurocholate or 6 mM conjugated bile salt mixture. The mixture was incubated at 37ºC for 30 min and the enzymatic reaction was terminated by adding 0.5 ml of trichloroacetic acid (15% w/v) to 0.5 ml of sample. The mixture was centrifuged (780*g* at 4ºC for 10 min) and 0.2 ml of supernatant obtained was added to 0.5 ml of distilled water and 0.5 ml of ninhydrin reagent (0.5ml of 1% ninhydrin in 0.5 M citrate buffer pH 5.5, 1.2 ml of 30% (v/v) glycerol, 0.2 ml of 0.5M citrate buffer pH 5.5). The preparation was mixed on a vortex and boiled for 15 min. After subsequent cooling, the absorbance at a 570 nm (A_{570}) was measured.

The glycine (in case of conjugated bile salt mixture) or taurine (in case of sodium taurocholate) was used as standards. One unit of BSH activity was defined as

the amount of enzyme that liberated 1 µmole of amino acid (glycine or taurine) from substrate per min at 37ºC.

$$\text{BSH Activity (U/ml)} = \frac{\Delta A_{570} \times C \times V}{M \times t \times v} = (\mu\text{moles/min/ml})$$

where,

ΔA_{570}	=	Optical Density (OD) change at 570 nm.
C	=	1 OD equivalent glycine or taurine concentration (mg/ml) from standard plot.
V	=	Volume of the reaction mixture (ml)
t	=	Time of reaction (min).
M	=	Molecular weight of glycine (75.06 g/mol) or taurine (125.15 g/mol).
v	=	Volume of the cell free extract (ml) for estimation of BSH activity.

3.2.5 Determination of protein concentration

The protein concentration of the cell free extract was estimated following the method of Lowry *et al*., (1951). The protein estimation was carried out by adding 0.2 ml of sample containing protein or bovine serum albumin (BSA) to 1 ml of reagent C (as described in Section 3.2.7.1) and incubated at 25ºC for 15 min. After the incubation, 0.1 ml of phenol reagent (as described in Section 3.2.7.1) was added to the reaction mixture and incubated at 25ºC for 30 min. The absorbance (A_{660}) of colour developed was measured at 660 nm on a UV-visible spectrophotometer (Varian, Cary 100). BSA in concentration range 0.5 µg/ml to 500 µg/ml was used as standard.

The concentration of protein was calculated as follows:

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

where,

ΔA_{660}	=	Optical Density (OD) change at 490 nm.
C	=	Amount (µg/ml) of BSA at OD=1 from standard plot.
V	=	Volume of reaction mixture (ml).
v	=	Volume of the protein sample (ml).

3.2.5.1. Preparation of reagents for protein estimation

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

Reagent B1 : 2% (w/v) sodium potassium tartarate.

Reagent B2 : 1% (w/v) copper sulfate.

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

Phenol reagent : 1 N phenol reagent.

3.2.5.2 Determination of specific activity

The specific activity (U/mg) of the cell free extract containing Bile salt hydrolase activity was calculated using the following equation;

$$\text{Specific activity (U/mg)} = \frac{\text{BSH Activity (U/ml)}}{\text{Protein Concentration (mg/ml)}} = (\text{µmoles/min/mg})$$

3.2.6 *In vitro* assessment of antioxidant activity of *Lactobacillus* strains

The *in vitro* antioxidant activity of *Lb. plantarum* DM5 was compared with the two standard *Lactobacillus* strains, *Lactobacillus acidophilus* NRRL B-4495 and *Lactobacillus plantarum* NRRL B-4496.

3.2.6.1 Resistance of Lactobacillus strains to hydrogen peroxide

Hydrogen peroxide resistance by *Lactobacillus* strains was assessed by the method of Wang *et al.,* (2009) with some modifications (Li *et al.,* 2012). The overnight grown cultures of the *Lactobacillus* strains were inoculated at 1% (v/v) into 100 ml of MRS medium (control) and 100 ml MRS medium containing 0.4, 0.8 or 1.0 mM hydrogen peroxide (50%, Merck) and incubated at 37ºC for 8 h. The cell growth was measured by taking absorbance at a 600 nm (A_{600}) by UV spectrophotometer (Cary 100, Varian) and the results were reported as change in absorbance of cells with varied concentration of hydrogen peroxide (Li *et al.,* 2012).

3.2.6.2 Preparation of cells and cell free extracts

The *Lactobacillus* strains were grown in MRS medium (as described in Chapter 2, Section 2.2.2) at 37°C for 20 h under static condition. The bacterial cells were harvested by centrifugation at 8,000*g* at 4°C for 10 min and washed twice with deionised water. The cells were re-suspended in deionised water to make the bacterial counts to 10^6, 10^8 and 10^{10} CFU/ml. The cell free extracts were prepared by the method described by Wang *et al.,* (2009) with minor modifications (Li *et al.,* 2012). The cells (10^6, 10^8 or 10^{10} CFU/ml) were incubated with 1 mg/ml lysozyme at 37°C for 30 min followed by ultrasonic disruption (Sonics, Vibra cell). Sonication was

performed for five, 1 min intervals, on an ice bath and the cell debris was removed by centrifugation at 8,000*g* at 4°C for 10 min. The resulting cell free extract was used for analyzing the antioxidative activity.

3.2.6.3 Assay of scavenging activity against DPPH radical

The DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging activity of *Lactobacillus* strains was measured by the method of Sun *et al.,* (2009) with certain modifications (Li *et al.,* 2012) and by using the intact cells (10^6, 10^8 and 10^{10} CFU/ml) re-suspended in deionised water. The 100 µl of ethanolic DPPH solution (0.4 mM) was mixed vigorously with 100 µl of 10^6, 10^8 or 10^{10} CFU/ml of *Lactobacillus* strains and the mixture was incubated at 37°C in the dark for 30 min. The absorbance of the mixture was then determined at 517 nm using a microplate reader (Tecan, Infinite 200 Pro). The blank included ethanol and the cells and the control contained deionised water and DPPH solution.

$$\textbf{Scavenging activity (\%)} = [1-(A_s - A_b)/A_c] \times 100,$$

where A_b is the absorbance of the blank, A_c is the absorbance of control and A_s is the absorbance of the sample.

3.2.6.4 Assay of inhibition of ascorbate autoxidation

The antioxidative activity of *Lactobacillus* strains was also assayed by the method of inhibition of ascorbate autoxidation (Lin and Yen, 1999) by using the cell free extracts of different cell concentrations (10^6, 10^8 or 10^{10} CFU/ml). A 0.1 ml of sample or distilled water (as control) was mixed with 9.8 ml of 0.2M sodium phosphate buffer (pH 7.0) and with 0.1 ml of 5 mM ascorbate solution (Sigma

Aldrich, USA). The 1 ml of this mixture was then quickly transferred to a cuvette and the decrease in the absorbance at a wavelength of 265 nm was measured with time for 10 min. The inhibition of ascorbate autoxidation rate of the sample was then calculated according to the following equation;

$$\textbf{Inhibition rate (\%)} = [(A_c - A_s/A_c)]\textbf{x}100$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample.

3.2.6.5 Assay of reducing activity

The reducing activity of the *Lactobacillus* stains was determined according to the method of Lin and Yen, (1999) with certain minor modifications (Sun *et al.,* 2009). A 0.2 ml sample of cell free extracts or distilled water (as control) was mixed with 0.2 ml of 1% potassium ferricyanide and 0.2 ml of 20 mM sodium phosphate buffer (pH 7.0). The mixture was incubated at 50°C for 20 min and then the reaction was stopped by adding 0.2 ml of 10% trichloroacetic acid. The mixture was centrifuged at 780*g* at 4ºC for 10 min. The supernatant (0.5 ml) obtained by the centrifugation was transferred to a new microcentrifuge tube and mixed with 0.1 ml of 0.1% ferrichloride and 0.4 ml of distilled water. The absorbance was measured at 700 nm. A higher absorbance of this mixture indicates a higher reducing activity and the reducing activity of cysteine was used as a standard.

3.2.6.6 Assay of scavenging activity against superoxide anion radical

This superoxide anion radical scavenging activity of cell free extracts of *Lactobacillus* strains at different cell concentrations (10^6, 10^8 or 10^{10} CFU/ml) was estimated by the reduction of nitrobluetetrazolium (NBT) according to a method

previously reported by Wang *et al.,* (2006). The 1.0 ml reaction mixture containing 20 mM sodium phosphate buffer (pH 7.4), 50 µM NBT (Sigma Aldrich, USA), 75 µM nicotinamide adenine dinucleotide (NADH), 15 µM phenazine methosulfate (PMS) (Sigma Aldrich, USA) and 50 µl of cell free extract sample or distilled water (control) was incubated at 37°C for 5 min. The absorbance at 560 nm was then measured by an automated microplate reader (Tecan, Infinite 200 Pro). The superoxide anion radical scavenging activity of the sample was calculated by the following equation;

$$\textbf{Scavenging activity (\%)} = [(A_c - A_s/A_c)] \times 100$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample.

3.2.6.7 Assay of scavenging activity against hydroxyl radical

The hydroxyl radical scavenging assay was conducted using the method of He *et al.,* 2004) with minor modification (Li *et al.,* 2012). The hydroxyl radical was generated by Fenton reaction method (He *et al.,* 2004). The 1 ml of reaction mixture containing 20 mM sodium phosphate buffer (pH 7.4), 0.435 mM brilliant green, 0.5 mM ferrous sulphate heptahydrate ($FeSO_4$, $7H_2O$), 3.0%, w/v hydrogen peroxide (H_2O_2) was mixed with 100 µl of cell free extract of *Lactobacillus* strain or distilled water (control). The reaction mixture was incubated at 37°C for 20 min and the absorbance was measured at 624 nm. The absorbance change of the reaction mixture indicated the scavenging ability of *Lactobacillus* strains for hydroxyl radicals and which was calculated as;

$$\text{Scavenging activity (\%)} = [(A_s - A_c/A_b - A_c)]x100$$

where A_s is the absorbance in the presence of the sample, A_c represents the absorbance of the control solution and A_b is the absorbance of blank without the sample and Fenton reaction system.

3.2.7 Identification of GABA producing ability of *Lb. plantarum* DM5 by thin layer chromatography

In order to evaluate the GABA-producing ability of *Lb. plantarum* DM5, it was grown in MRS medium containing 1% (w/v) monosodium glutamate (MSG) at 30ºC under static condition for 30 h. The cells were removed by centrifugation at 10,000*g* at 4°C for 10 min and then the cell free supernatants were filtered through 0.2 µm membrane filter. GABA was identified by TLC on activated silica gel plates (Silica gel 60 F254, Merck, India) following the method described by Lee *et al.,* (2010) using the solvent system containing n-butanol: acetic acid: water (5:2:2, v/v/v). The chromatogram was viewed after spraying 2% (w/v) ninhydrin solution and incubating at 80°C for 10 min.

3.3 Results and Discussion

3.3.1 In vitro probiotic assessment of Lb. plantarum DM5

3.3.1.1 Tolerance of Lb. plantarum DM5 to low pH

A probiotic bacterium should endure low acidic condition of upper gastrointestinal tract and high bile salt concentration of small intestine for their metabolic activity, multiplication or colonization. Probiotic bacteria mainly lactobacilli have been shown to exhibit a strain variation in their tolerance to low acidic pH (2-3) (Charteris *et al.,* 1998; Ramirez-Chavarin *et al.,* 2013). The sensitivity of the isolate *Lb. plantarum* DM5 towards the low acidic pH (2-4) was determined and shown in Fig. 3.3.1.

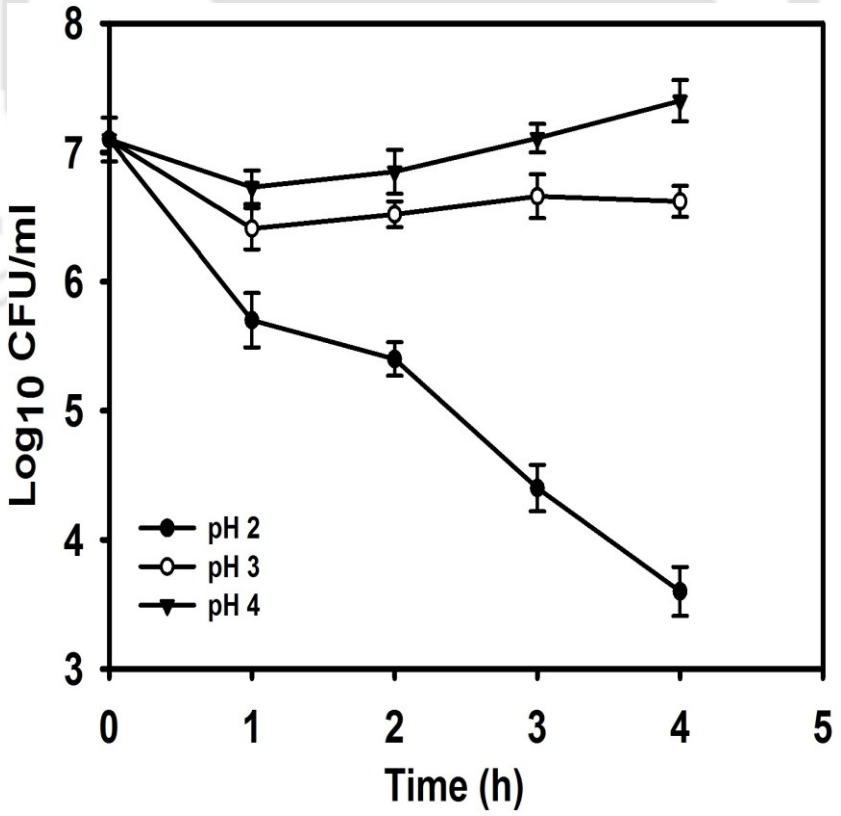

Fig. 3.3.1 Tolerance of *Lactobacillus plantarum* DM5 to low acidic pH ranging from 2 to 4 at 37ºC for 4 h. The data presented are mean value of three independent experiments with ± S.D.

The isolate *Lb. plantarum* DM5 showed 92% and 97% survival at pH 3 and pH 4, respectively, after 2 h of incubation at 37°C (Fig. 3.3.1). The highest fall of the cell viability of 38% was observed at pH 2.0 after 3 h and the cell population was further decreased by 51% after 4 h of incubation at 37°C. Compared to this, around 93% of the viable cells of the isolate *Lb. plantarum* DM5 were found at pH 3.0, after 4 h of incubation (Fig. 3.3.1). This was significantly higher than *Lb. plantarum* 17 isolated from cooked meat product which could resist pH 3.0 for only 1 h and did not revive even after 4 h (Ramirez-Chavarin *et al.,* 2013). High acid tolerance of *Lb. plantarum* DM5 was further supported by increase in the cell population by 4% at pH 4 after 4 h at 37°C (Fig. 3.3.1). The acid tolerance of isolate *Lb. plantarum* DM5 was comparable with other probiotic strain such as *Lb. plantarum* L4 (Suskovic *et al.,* 1997) and *Lb. plantarum* (Bao *et al.,* 2012).

3.3.1.2 Tolerance of Lb. plantarum DM5 to artificial gastric juice

Probiotic bacteria should be able to withstand the gastrointestinal stress conditions and colonize in the gastrointestinal tract for their metabolic activity. In human beings, the pH of gastric juice is generally around 2.0 (Huang and Adams, 2004) and therefore, the probiotic bacterium should be able to survive at pH range of 2.0-4.0 for 1-2 h, to exert the beneficial effects to the host (Ramirez-Chavarin *et al.,* 2013). The enzyme pepsin present in the stomach also had pH optimum at pH 2.0 to pH 3.0 (Nedelcheva *et al.,* 2010). The acid tolerance level of the isolate DM5 was significant at pH 2.5 in the presence of 1000 U/ml, as the decrease in viable cell count was only 7% after 5 h of incubation (Fig. 3.3.2). Similar trend of gastric juice tolerance (pH 2.5) was observed in case of *Lactobacillus* sp. isolated from

Tungrymbai, an ethnic fermented soybean food of India (Thokchom and Joshi, 2012), however; the gastric juice at pH 2.5 was inhibitory to *Lactobacillus plantarum* ZLP001 as the viable cell population reduced to 95% within 3 h (Wang *et al.,* 2011).

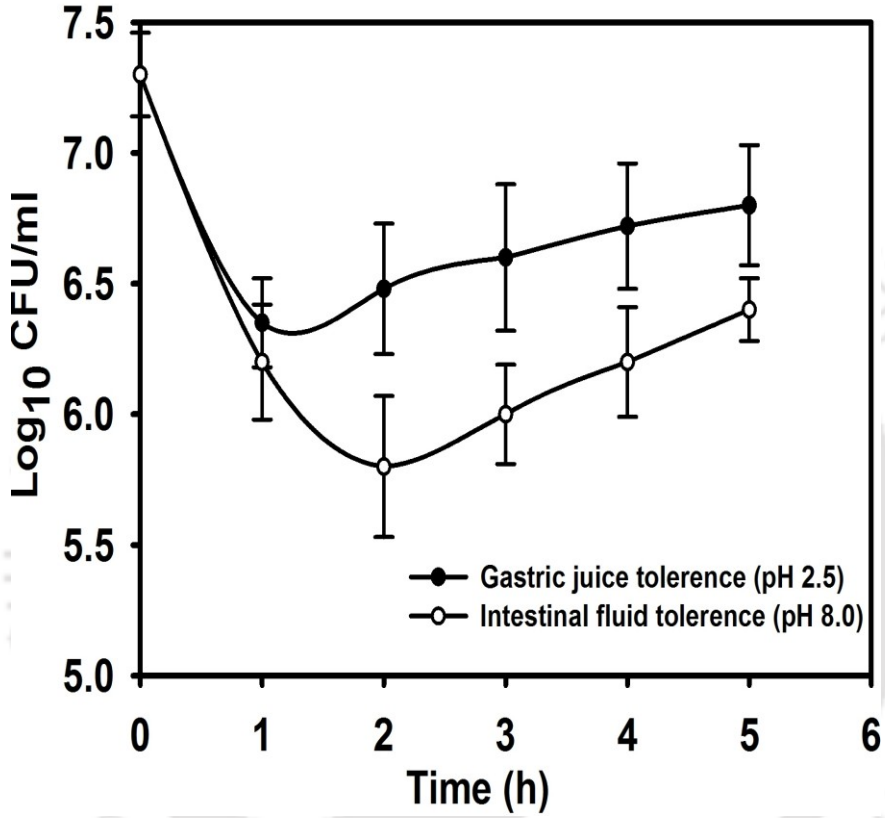

Fig. 3.3.2 Tolerance of *Lactobacillus plantarum* DM5 to artificial gastric juice and intestinal fluid for 5 h at 37ºC. The data presented are mean value of three independent experiments with ± S.D.

3.3.1.3 Tolerance of Lb. plantarum DM5 to intestinal fluid

After passing through the lower acidic condition of the upper gastrointestinal tract, the probiotic bacteria should survive the passage through small intestine. The pH of the small intestine varying from 7.2 to 8.0 and is ideal for serine proteases like trypsin and chymotrypsin (Huang and Adams, 2004). In presence of intestinal fluid (pH 8.0) although the total number of viable cells of the isolate *Lb. plantarum* DM5

initially decreased to 81% at 2 h of incubation but subsequently increased to 87% after 5 h of incubation (Fig. 3.3.2). The data indicated its high resistance towards the serine proteases and obnoxious environment of the small intestine. Similar trend of cell viability was observed in case of probiotic *Lactobacillus plantarum* ZLP001 (Wang *et al*., 2011).

3.3.1.4 Tolerance of Lb. plantarum DM5 to bile salt

In small intestine, bile salt tolerance is most crucial limiting factor for survival and proliferation of probiotic bacterium (Ouwehand *et al.,* 2002). The physiological concentration of human bile in the gastrointestinal tract varies from 0.3 to 0.5% (Vinderola and Reinheimer, 2003). It is necessary that efficient probiotic bacterium should be able to grow in bile salt with concentration ranging from 0.15-0.30% (Suskovic *et al.,* 1997). The isolate *Lb. plantarum* DM5 demonstrated excellent tolerance to bile salt. After 6 h of incubation the isolate *Lb. plantarum* DM5 showed 89%, 80% and 66% survival at 0.3%, 0.5% and 1% bile salt concentrations, respectively (Fig.3.3.3). The isolate *Lb. plantarum* DM5 not only exhibited excellent tolerance to 0.3% and 0.5% of bile salt (Fig. 3.3.3) but the viable cell count also increased by 25.3% and 21.4%, respectively, after 30 h of incubation. This scored over *Lactobacillus plantarum* ZLP001 where, 0.5% bile salt inhibited the growth by 9.4% (Wang *et al*., 2011). The isolate *Lb. plantarum* DM5 also showed survival in presence of 1% bile salt with sluggish growth response. This data indicated the survival potential of isolate DM5 in the presence of toxic bile salts of gastrointestinal tract. Similar result was also observed in case of *Lb. plantarum* Lp9, which could survive 5-6 fold higher concentration (1.5-2.0% bile) than the usual bile salt concentration

(0.3%) present in human stomach (Kaushik *et al*., 2009). Higher bile salt tolerance of isolate *Lb. plantarum* DM5 attains importance as most bile salt tolerant strains have been reported to reduce the symptoms of lactose intolerance (Vinderola and Reinheimer, 2003).

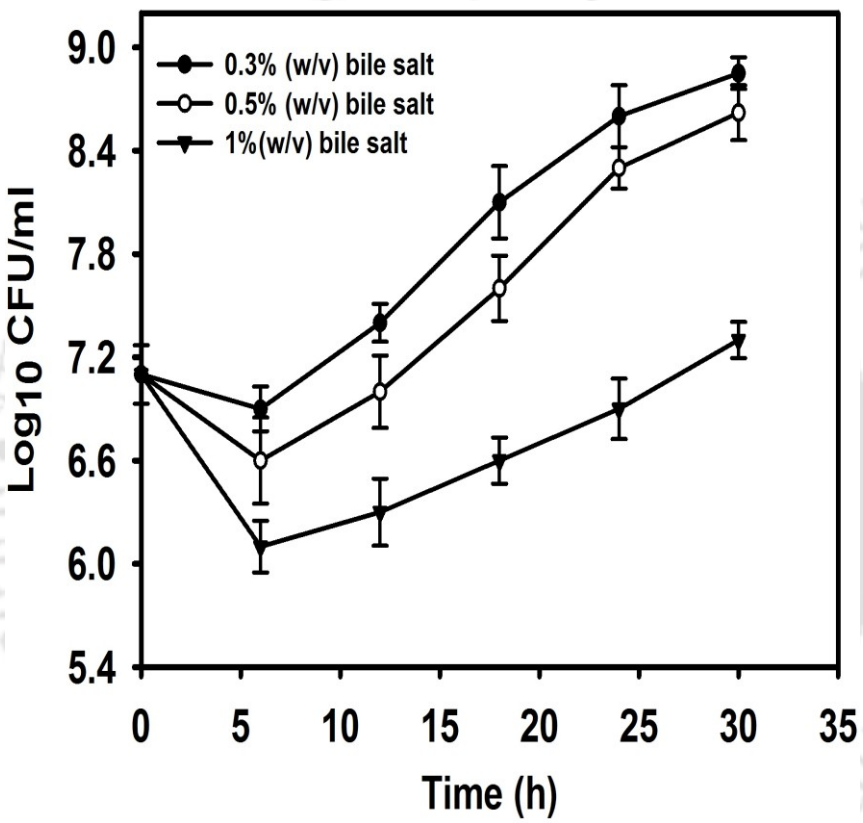

Fig. 3.3.3 Tolerance of *Lactobacillus plantarum* DM5 to bile salt ranging from 0.3% to 1% at 37ºC for 30 h. The data presented are mean value of three independent experiments with ± S.D.

3.3.1.5 Tolerance of Lb. plantarum DM5 to lysozyme

A probiotic bacterium should survive through the action of lysozyme present in the salivary glands and the gastrointestinal tract of human. The enzyme lysozyme cleaves the β-(1,4) bond between N-acetylmuramic acid and N-acetyl-D-glucosamineresidues in the peptidoglycan layer of the bacterial cell wall (Suskovic *et*

al., 1997). The sensitivity of *Lb. plantarum* DM5 to lysozyme was investigated in presence of 50, 100, 200 and 300 µg/ml lysozyme supplemented in MRS media, respectively. The isolate *Lb. plantarum* DM5 not only survived the deleterious effect of lysozme but also displayed enhanced growth of 17.4%, 16.5% and 12.3% at 50, 100 and 200 µg/ml of lysozyme supplemented in MRS medium at 37ºC after 30 h of incubation, respectively (Fig. 3.3.4). In presence of 200 µg/ml of lysozyme, the number of viable cells initially decreased by 4% at 6 h but after 6 h the number of viable cell increased with time (Fig. 3.3.4), however; in presence of 50 and 100 µg/ml of lysozyme there was no decrease in total number of viable cell was observed at any time of incubation.

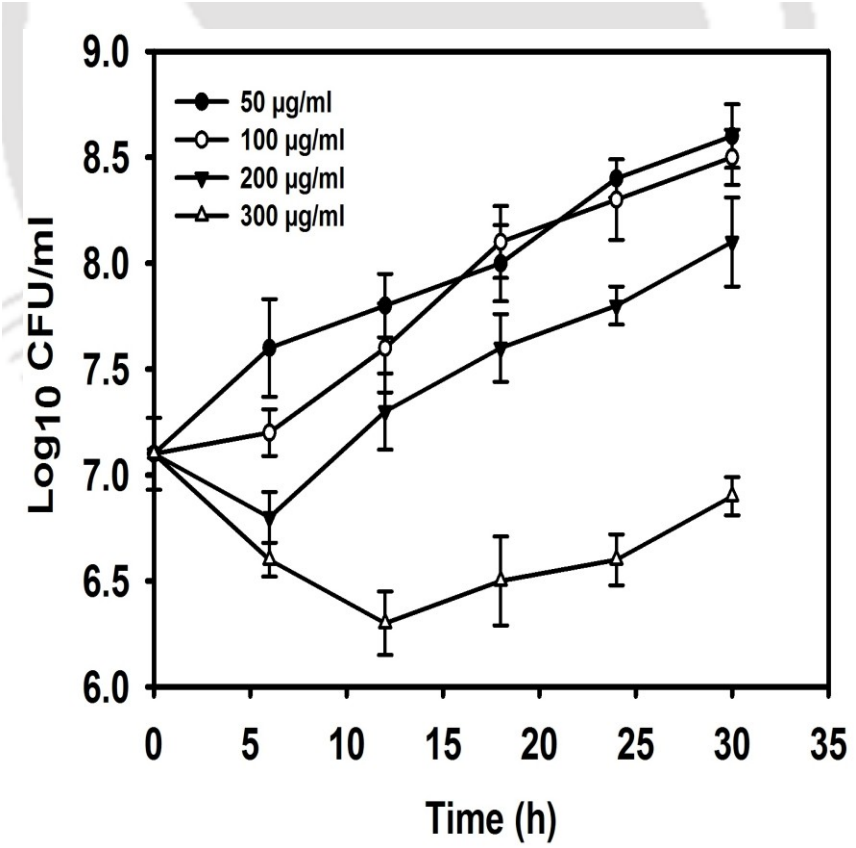

Fig. 3.3.4 Tolerance of *Lactobacillus plantarum* DM5 to lysozyme ranging from 50 µg/ ml to 300 µg/ml at 37ºC for 30 h. The data presented are mean value of three independent experiments with ± S.D.

In presence of 300 µg/ml of lysozyme, the delayed growth of *Lb. plantarum* DM5 was observed and number of viable cell decreased by 3% after 30 h of incubation. Similar result was observed in case of probiotic *Lactobacillus plantarum* L4 (Suskovic *et al.,* 1997). The lysozyme concentration used in this study was much higher than the concentration of lysozyme (50-100 µg/ml) found in saliva (Suskovic *et al.,* 1997), hence, it was clearly stated that the isolate could efficiently survive during the passage through salivary glands and the gastrointestinal tract of human.

3.3.1.6 Determination of cell surface hydrophobicity of Lb. plantarum DM5

The property of adherence to epithelial cells and mucosal surfaces is a key criterion for bacterial strains used as probiotics. The hydrophobic nature of the outermost surface of the microorganism has been implicated in the attachment of bacteria to host tissue (Kiely and Olson, 2000). The hydrophobicity of the isolate DM5 was measured by MATH and SAT test. The percent cell surface hydrophobicity of *Lb. plantarum* DM5 by MATH assay was carried out using two apolar solvents xylene and n-hexadecane. Determination of microbial adhesion to n-hexadecane and xylene is qualitative phenomenological approach to estimate the ability of a strain to adhere to epithelial cells (Kiely and Olson, 2000, *Kos et al.,* 2003). The cell surface hydrophobicity of *Lb. plantarum* DM5 was compared with *Lb. plantarum* NRRL B-4496, *Lb. acidophilus* NRRL B-4495 and *P. pentosaceus* SPA (Table 3.3.1). The percent hydrophobicity values obtained for *Lb. plantarum* DM5 with xylene and n-hexadecane were almost similar, 52.7 and 53.1%, respectively, within the experimental errors of 1.2% (Table 3.3.1). The hydrophobicity value of *Lb. plantarum* DM5 was much higher as compared with *Lb. plantarum* NRRL B-4496. According to

Del Re *et al.,* (2000), the minimum 40% cell surface hydrophobicity and autoaggregation ability is necessary for considering a strain as probiotic. The strains used as starter culture were of generally higher hydrophobicity value (> 40%) than probiotic lactic acid bacteria. The results suggested that the *Lb. plantarum* DM5 could be potentially used as probiotic as well as starter culture. The hydrophobic value less than 20% is negligible and the strains are not designated as probiotic. The cell surface hydrophobicity *P. pentosaceus* SPA was very low (<20%), indicating its hydrophilic nature (Table 3.3.1). The cell surface hydrophobicity of 46-48% of *Lb. acidophilus* NRRL B-4495 was in agreement with the reported value (38.1 to 67.8%) of probiotic *Lb. acidophilllus* (Vinderola and Reinheimer, 2003; Kos *et al.,* 2003). However, the cell surface hydrophobicity of *Lb. plantarum* DM5 was much higher than the reported probiotic strains such as *Lb. rhamnosus* (24.1%) and *Lb. plantarum* L4 (20.1%) (Vinderola and Reinheimer, 2003; Kos *et al.,* 2003).

Bacterial adhesion to chloroform and ethyl acetate was tested to assess the lewis acid-base characteristics of the bacterial cell surfaces (Kos *et al.,* 2003). All the strains showed stronger affinity towards chloroform, which is an acidic solvent and is strong electron pair acceptor than ethyl acetate, which is a basic solvent and is an electron pair donar (Table 3.3.1). The data suggested that the bacterial cell surface were electrophilic in nature. Similar results were also found in case of other probiotic strains (Kos *et al.,* 2003; Kaushik *et al.,* 2009).

Table 3.3.1 Percentage of microbial adhesion of lactic acid bacteria to hydrocarbons

Bacteria	(%) Adhesion[a]			
	Xylene	n-hexadecane	Chloroform	Ethyl acetate
Lb. plantarum DM5	52.7±1.12	53.1±1.21	19.6±1.4	12.7±0.93
Lb. plantarum NRRL B 4496	38.3±0.74	36.5±1.40	17.9±1.32	8.7±0.75
Lb. acidophilus NRRL B 4495	48.1±0.82	46.6±1.6	22.3±0.76	1.2±0.82
Pediococcus pentosaceus SPA	20.1±0.95	18.7±1.33	17.3±1.12	5.8±0.87

[a] *All experiments were carried out in triplicate (n =3) and results are expressed as mean ± S.D.*

The cell surface hydrophobicity of isolate DM5 was also determined by salt aggregation test and compared with MATH value of hydrophobicity. In salt aggregation test, a salting-out agent was used to induce aggregation of bacterial cells in order to determine the bacterial cell surface hydrophobicity. Ammonium sulphate solution with lowest molarity required by the bacterial strain for clumping displayed their SAT value. Strain with SAT values >4.0 M, 2.0-4.0 M, 1.0-2.0 M and 0.0-1.0 M of ammonium sulphate solution were designated as no, low, moderate and high hydrophobicity, respectively (Qiao *et al.,* 2012). The isolate *Lb. plantarum* DM5 showed visible clumping on glass slide after the addition of 1.5 M ammonium sulphate (SAT value) at 37ºC, exhibiting moderate cell surface hydrophobicity, whereas, the strain *Lb. planatrum* NRRL B-4496 showed clumping at 3.5 M (SAT value), displaying lower or no hydrophobicity. The stain *Lb. acidophilus* NRRL B-4495 showed clumping in 1.0 M (SAT value) ammonium sulphate solution and maximum hydrophobicity. The cell surface hydrophobicity of the strains by SAT assay is in good agreement with the value obtained by MATH assay.

3.3.1.7 Autoaggregation and coaggregation activity of Lb. plantarum DM5

Adhesion of probiotic bacterium to intestinal epithelial cells is an important prerequisite for colonization in the gastrointestinal tract and resisting their immediate elimination by peristalsis. Autoaggregation of probiotic strains appeared to be necessary for adhesion to intestinal epithelial cells (Del Re *et al.,* 2000). The isolate *Lb. plantarum* DM5 exhibited a high autoaggregation activity of 54.3% at 37ºC after 6 h of incubation and could be considered as strong auto aggregating phenotype (Fig. 3.3.5).

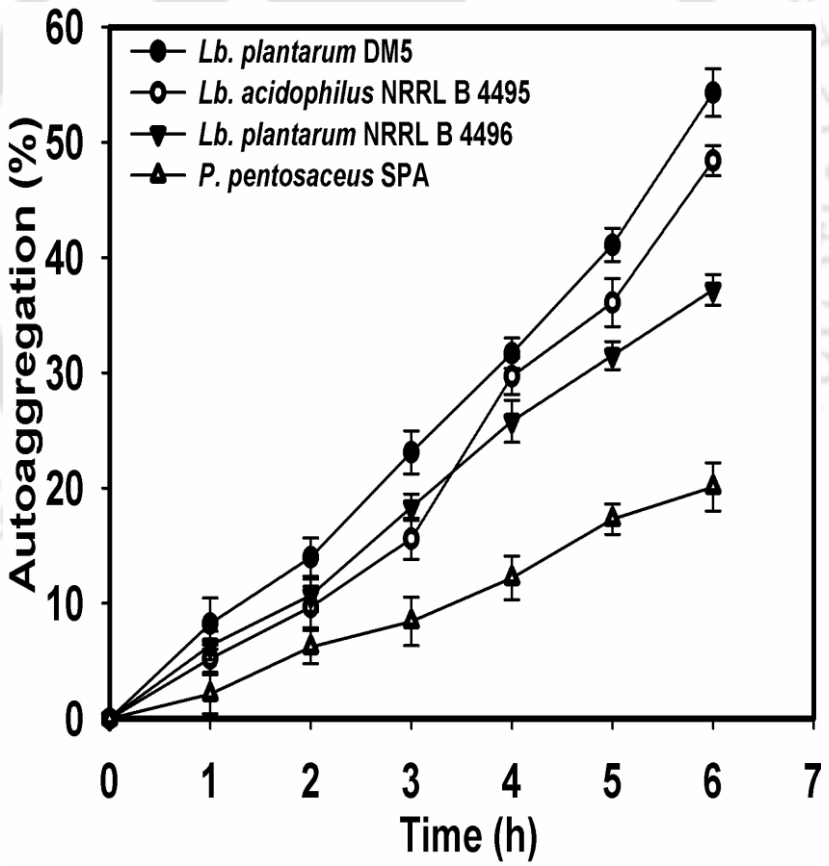

Fig. 3.3.5 Autoaggregation activity of the isolate *Lb. plantarum* DM5 at 37ºC for 6 h. Error bars represent the standard deviations of the mean values of results from three independent experiments.

The strains *Lb. acidophilus* NRRL B-4495, *Lb. plantarum* NRRL B-4496 and *P. pentosaceus* SPA showed lower autoaggregation activity of 48%, 37% and 20%, respectively, when compared with *Lb. plantarum* DM5 (Fig. 3.3.5). It has been reported that the two properties *viz* autoaggregation activity and cell surface hydrophobicity are strongly correlated and used for screening potentially adherent strains suitable for commercial application (Del Re *et al.,* 2000; Collado *et al.,* 2007).

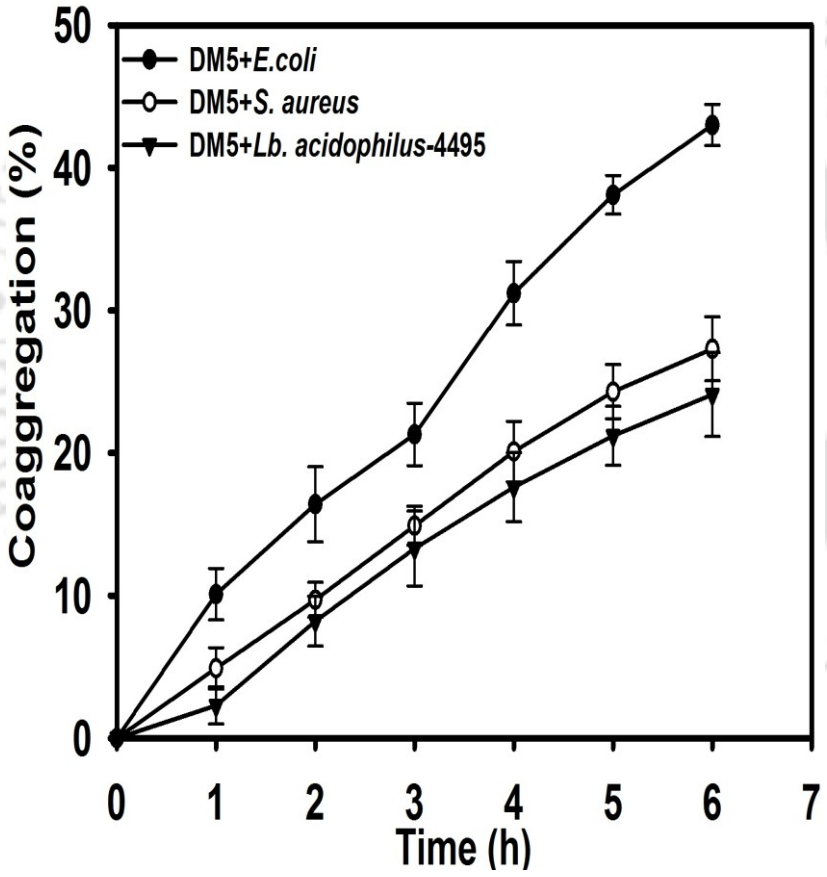

Fig. 3.3.6 Coaggregation activity of the isolate *Lb. plantarum* DM5 at 37ºC for 6 h. Error bars represent the standard deviations of the mean values of results from three independent experiments.

The coaggregation of a probiotic strain might enable it to form a barrier that prevents the colonization of pathogenic bacteria by creating a microenvironment around the pathogen with a higher concentration of inhibitory substances (Collado *et*

al., 2007). The coaggregation of *Lb. plantarum* DM5 was examined with *E. coli* DH5α*, S. aureus* MTCC 737 and *Lb. acidophilus* NRRL B-4495 and is shown in Fig. 3.3.6. The extent of coaggregation differed with probiotic *Lb. plantarum* strain and pathogen strain and with incubation time. It has been reported that the average coaggregation percentage of *Lb. plantarum* with *E. coli* and *S. aureus* was found to be 19 to 35% and 30 to 45%, respectively (Bao *et al.,* 2012). The isolate *Lb. plantarum* DM5 demonstrated maximum coaggregation of 43% with *E. coli* as compared to *S. aureus* (27%) and *Lb. acidophilus* NRRL B-4495 (24%) respectively (Fig. 3.3.6). These results suggested that the isolate DM5 could protect host epithelial cells from enteropathogenic *E.coli*-induced damage; an observation buttressed by the report of Bao *et al.,* (2012).

3.3.1.8 In vitro cell adherence of Lb. plantarum DM5

A probiotic bacterium should be able to adhere and colonize in the human gastrointestinal tract for sustaining health promoting effect. The adherence ability of putative probiotic is the most crucial criteria for its extended residence time in the host (Ramirez-Chavarin *et al.,* 2013). The good cell surface hydrophobicity of 53% and autoagrregasion of 43% ensured that the isolate *Lb. plantarum* DM5 possessed good adhesion ability, which was further confirmed by adhesion assay of isolate *Lb. plantarum* DM5 to HT-29 cell line. *Lb. plantarum* DM5 showed a good adhesion percentage of 8.63±3.03% with HT-29 cell line. The adhesion percentage of *Lb. plantarum* strains with HT-29 cell line were reported in between 5-13% (Duary *et al.,* 2011; Satish Kumar *et al.,* 2011). The strain *Lb. plantarum* Lp9 showed adhesion percentage of 7.4±1.3% with Caco-2 cell line (Kaushik *et al*., 2009).

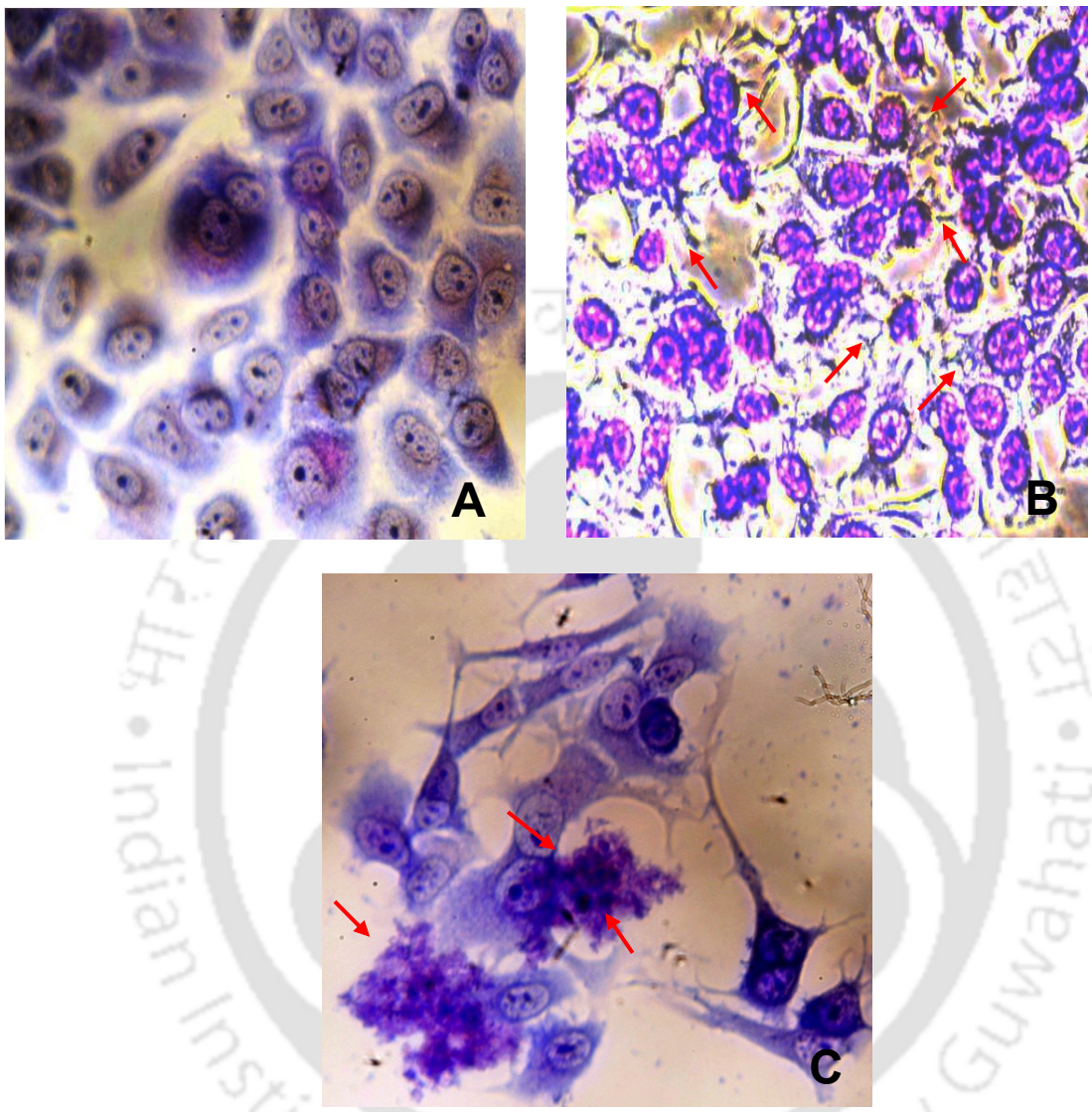

Fig. 3.3.7 Adhesion of *Lb. plantarum* DM5 on HT-29 cell line observed under inverted microscope (40x) after Giemsa staining. A) Control HT-29 cell line (B) and (C) treated HT-29 cells with *Lb. plantarum* DM5 for 2 h at 37ºC. The arrows indicate the attachment of bacterial cell to the mammalian HT-29 cells.

Cell adhesion is a complex process involving contact between the bacterial cell membrane and interacting cell surfaces of mammalian cell line (Chauviere *et al*., 1992; Duary *et al.,* 2011). It has been reported that *Lactobacillus plantarum* AS1 attached

efficiently to HT-29 cells *via* mechanisms that involve different combinations of carbohydrate and protein factors on the bacteria and eukaryotic cell surface (Satish Kumar *et al.,* 2011; Dhanani and Bagchi, 2013). The adhesion ability of *Lb. plantarum* DM5 with HT-29 cell line was also investigated by direct microscopic observation after Giemsa staining and is shown in Fig. 3.3.7. The microscopic image also confirmed the good adhesion ability of isolate *Lb. plantarum* DM5 with HT-29 cells. This result indicated the capability of *Lb. plantarum* DM5 to adhere in the gastrointestinal tract and resist their immediate elimination by peristalsis.

3.3.1.9 Bile salt hydrolase activity of Lb. plantarum DM5

Bile salt hydrolase activity provides several advantages to the probiotic bacterium for surviving in the competitive environment of the human gastrointestinal tract and therefore, it is a desirable property for selecting a potential probiotic bacterium. The isolate *Lb. plantarum* DM5 had high bile salt hydrolase activity indicated by the white precipitation zone of 12 mm around the colonies on MRS Thio-agar plate containing 0.5% (w/v) taurodeoxycholic acid and 0.04% (w/v) of $CaCl_2$ (Fig. 3.3.8). Similar results were observed for *Lactobacillus plantarum* Lp9 isolated from buffalo milk (Kaushik *et al.,* 2009) and *Lactobacillus plantarum* isolated from raw cow milk (Sieladie *et al.,* 2011). Several reports demonstrated that *Lactobacillus* species were able to reduce cholesterol *via* several mechanisms including bile salt hydrolase activity in case of hypercholesterolemia (Liong and Shah, 2005).

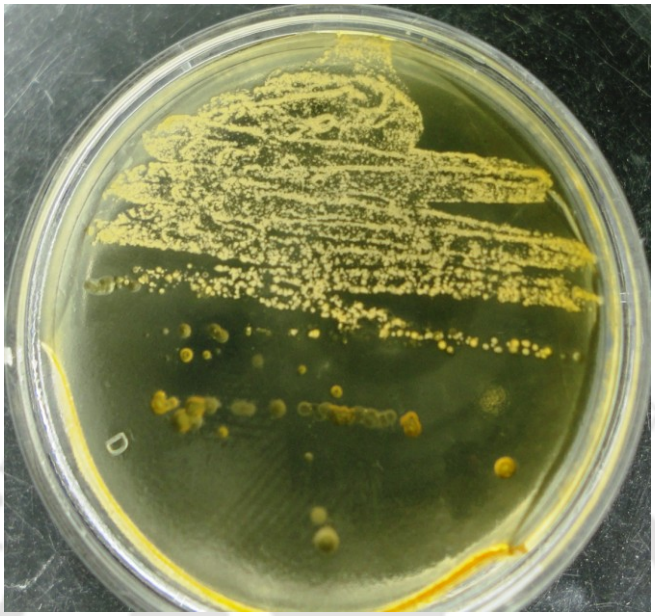

Fig. 3.3.8 Bile salt hydrolase activity of *Lb. plantarum* DM5 on MRS-Thio agar medium containing 0.5% (w/v) taurodeoxycholic acid and 0.04% (w/v) of $CaCl_2$. Plate was incubated anaerobically at 37º C for 72 h and formation of opaque granular shiny colonies on the medium indicated the BSH activity.

The bile salt hydrolase activity of *Lb. plantarum* DM5 was also quantitatively determined by ninhydrin assay method (Liong and Shah, 2005). The BSH activity obtained from cell free extract of isolate *Lb. plantarum* DM5 was measured for both glycine and taurine conjugated bile salt and is shown in Table 3.3.2. The isolate *Lb. plantarum* DM5 exhibited BSH activity of 0.63±0.02 U/mg towards sodium turocholate. However, when bile salt mixture containing glycocholic, glycochenodeoxycholic, taurocholic, taurochenodeoxycholic and taurodeoxycholic acid was used the isolate *Lb. plantarum* DM5 exhibited higher BSH activity of 1.18±0.03 U/mg. Similar results were also observed in case of *Lb. acidophilus* ATCC 4357 (0.64 U/mg) and *Lb. plantarum* Lp91 (0.12 U/mg) which showed lower activity towards sodium turocholate (Liong and Shah, 2005; Kumar *et al.,* 2012). However; the

strain *Lb. acidophilus* ATCC 4357 displayed higher BSH activity of 1.20 U/mg towards conjugated bile salt mixture. It has been reported that the strain *Lb. plantarum* Lp91 showed higher affinity towards bile salt glycodeoxycholic acid (GDCA) as compared to turodeoxycholic acid (TDCA) (Kumar *et al.,* 2012); however; *Lb. plantarum* BBE7 had a relatively high BSH activity toward both bile salts with a higher cholesterol-removing activity of 72.8% (Dong *et al*., 2012). The overall result suggested that the isolate *Lb. plantarum* DM5 is a promising probiotic candidate and could be potentially used for cholesterol assimilation *in vivo*.

Table 3.3.2 Bile salt hydrolase activity of *Lb. plantarum* DM5.

Bile salt[a]	BSH Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)
Sodium taurocholate	1.14±0.15	1.81±0.29	0.63±0.02
Conjugated bile mixture	2.11±0.36	1.81±0.29	1.18±0.03

[a] *All the experiments were carried out in triplicate (n =3) and the results are expressed as mean ± S.D. BSH activity was measured from cell free extracts of Lb. plantarum DM5 grown on MRS medium supplemented with 6 mM sodium taurocholate or 6 mM conjugated bile mixture.*

3.3.2 In vitro assessment of antioxidant activity of *Lb. plantarum DM5*

3.3.2.1 Survival efficacy of Lb. plantarum DM5 against hydrogen peroxide

The viability of *Lb. plantarum* DM5 in presence of 0.4 mM, 0.8 mM and 1 mM hydrogen peroxide was compared with *Lb. plantarum* NRRL B-4496 and *Lb. acidophilus* NRRL B-4495 (Table 3.3.3). All the three strains displayed tolerance to 0.4 mM and 0.8 mM hydrogen peroxide for 8 h at 37°C, with optical densities more than 1. However, *Lb. plantarum* DM5 exhibited maximum resistance in presence of 1.0 mM hydrogen peroxide with optical density of 0.70 as against 0.54 and 0.66 for

Lb. plantarum NRRL B-4496 and *Lb. acidophilus* NRRL B-4495, respectively. A similar trend of resistance up to 8 h was also observed in case *Lb. plantarum* C88 (Lee *et al.*, 2012), however; *Lb. fermentum* remained viable for up to 4 h (Wang *et al.,* 2009) in the presence of 1.0 mM hydrogen peroxide. It has been reported that the many *Lactobacillus* strains showed different extents of resistant to hydrogen peroxide, a weak antioxidant contributed in oxidative damage of cells either directly or as an oxidative precursor of hydroxyl radicals, but the mechanism behind the resistance is unclear. The intrinsic resistance of *Lb. plantarum* DM5 to hydrogen peroxide suggests that it may able to protect the oxidative damage caused by hydroxyl radical generated from hydrogen peroxide.

Table 3.3.3 Hydrogen peroxide (H_2O_2) tolerance of *Lactobacillus* strains.

Hydrogen peroxide (H_2O_2)	***Lb. plantarum* DM5***	***Lb. plantarum* NRRL B-4496***	***Lb. acidophilus* NRRL B-4495***
Control (0 mM)	2.57±0.07^{a}	2.39±0.03^{b}	2.71±0.03^{c}
0.4 mM	2.28±0.07^{a}	1.98±0.04^{b}	2.01±0.06^{b}
0.8 mM	1.29±0.02^{a}	1.01±0.02^{b}	1.21±0.04^{c}
1.0 mM	0.71±0.02^{a}	0.54±0.02^{a}	0.66±0.02^{b}

** Lactobacillus strains were grown in MRS medium supplemented with different concentrations of H_2O_2 and their growth was measured by taking the absorbance at a wavelength of 600 nm after 8 h incubation at 37 ºC. All experiments were carried out in triplicate (n =3) and the results are expressed as mean ± S.D.*

^{abc} *Different letters in the same rows are significantly different at P<0.01*

3.3.2.2 DPPH free radical scavenging activity

Scavenging of DPPH free radical is the basis of a common antioxidant assay and DPPH scavenging activities of antioxidants are attributed to their hydrogen donating abilities (Sharma and Bhatt, 2009). The purple colour of the DPPH solution

fade rapidly when it encounters proton radical scavenger. The DPPH free radical scavenging reaction of *Lb. plantarum* DM5 measured at 10^6, 10^8 or 10^{10} CFU/ml was compared with the two standard strains and is shown in Fig. 3.3.9.

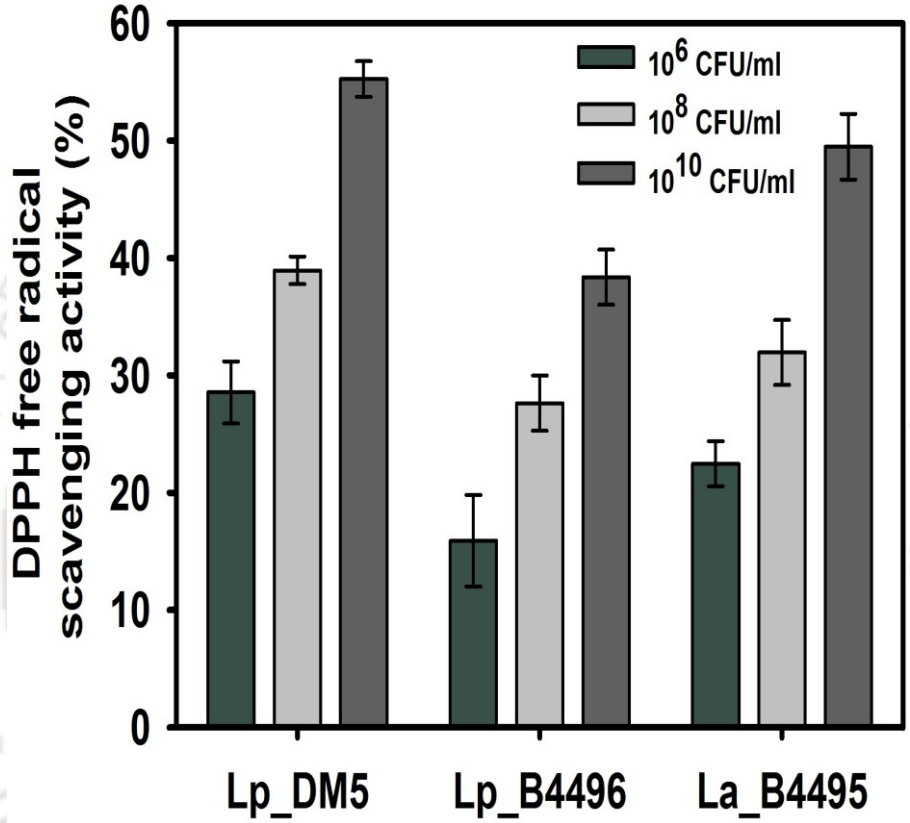

Fig. 3.3.9 DPPH free radical scavenging activity (%) of Lactobacillus strains. The DPPH activity of the tested strains was measured using different concentration of intact cell and each value is expressed as mean ± S.D (n=3). Lp_DM5= *Lb. plantarum* DM5, Lp_4496= *Lb. plantarum* NRRL B-4496 and La_4495= *Lb. acidophilus* NRRL B-4495.

Lb. plantarum DM5 exhibited maximum DPPH scavenging activity of 55.3% at 10^{10} CFU/ml and it was 31% and 10% higher than those of *Lb. plantarum* NRRL B-4496 (38.4%) and *Lb. acidophilus* NRRL B-4496 (49.5%), respectively. The data revealed that the DPPH free radical scavenging activity of *Lactobacillus* strains was dependent on the test concentration range of 10^6-10^{10} CFU/ml (Fig. 3.3.9) and

increased with increasing concentration of cells. These data was in accordance with earlier findings, where, *Lb. plantarum* C88 showed the DPPH scavenging activity of ~50% at 10^{10} CFU/ml (Lee *et al.,* 2012). The strong antioxidant activity of *Lb. plantarum* DM5 suggests that it can be used for production of ethanomedicine with probiotic activities without any cytotoxicity or adverse side effects.

3.3.2.3 Inhibition of ascorbate autoxidation

The cell free extract of several lactic acid bacteria containing antioxidant enzymes such as superoxide dismutase, ascorbate peroxidise, NADH oxidase, could be obtained only by breaking the bacterial cell wall (Kullisaar *et al.,* 2002; Lin and Yen, 1999) and the same observation was also found in the present study. The cell free extract of three tested *Lactobacillus* strain exhibited an ability to inhibit ascorbate autooxidation, which generates harmful superoxide anion radical. All three *Lactobacillus* strains demonstrated the antioxidative activity with inhibition in the range 11-20% at 10^{10} CFU/ml and their extent of inhibition depends on the concentration of cell free extract (Fig.3.3.10). However, *Lb. plantarum* DM5 exhibited a significantly higher extent of inhibition 20%, followed by *Lb. acidophilus* NRRL B-4495 (17.1%) and *Lb. plantarum* NRRL B-4496 (11.4%) at a concentration of 10^{10} CFU/ml. The Inhibition of ascorbate autooxidation and lipid peroxidation methods is generally used for searching antioxidants from natural sources (Lin and Yen, 1999). The benefit of ascorbate analysis over lipid peroxidation is that, it not only screens the antioxidants but also for the presence of adventitious catalytic metals ions (Lin and Yen, 1999).

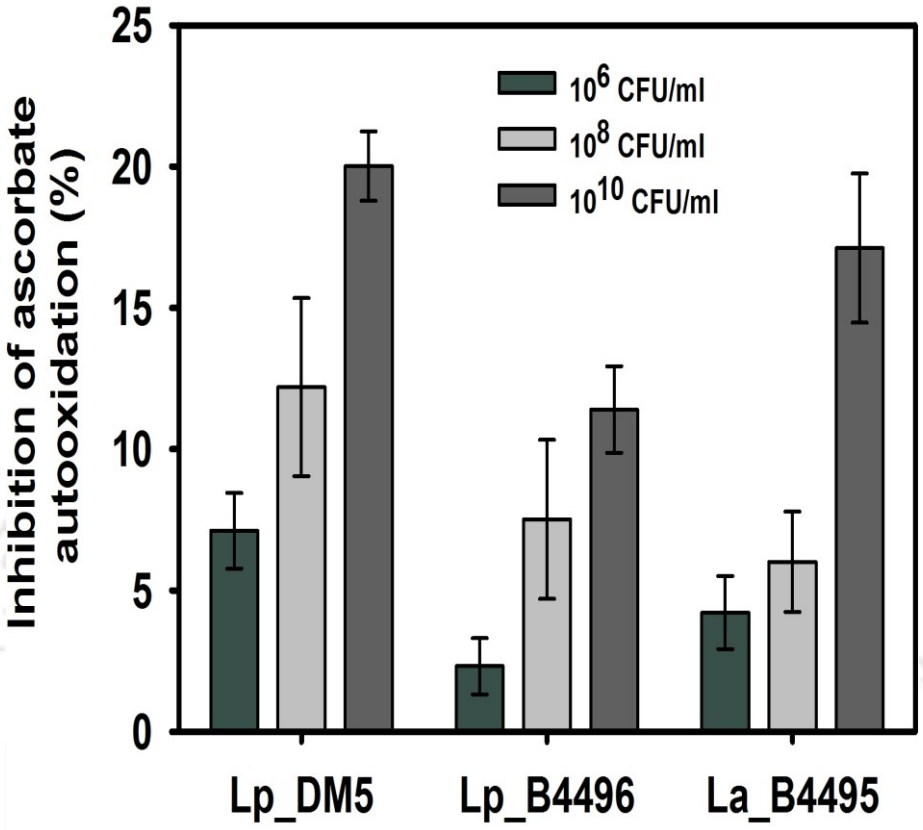

Fig. 3.3.10 Inhibition of ascorbate auto-oxidation (%) of Lactobacillus strains using different concentration of cell free extract. Each value is expressed as mean ± S.D (n=3). Lp_DM5= *Lb. plantarum* DM5, Lp_4496= *Lb. plantarum* NRRL B-4496 and La_4495= *Lb. acidophilus* NRRL B-4495.

3.3.2.4 Reducing activity

The reducing activity of cell free extract of *Lactobacillus* strains (10^6, 10^8 or 10^{10} CFU/ml) was measured and expressed as an equivalent amount of cysteine (Fig. 3.3.11). The three strains demonstrated various levels of reducing activity ranging from 30-140 µM cysteine equivalents and their reducing activity was dependent on the test concentration range of 10^6-10^{10} CFU/ml.

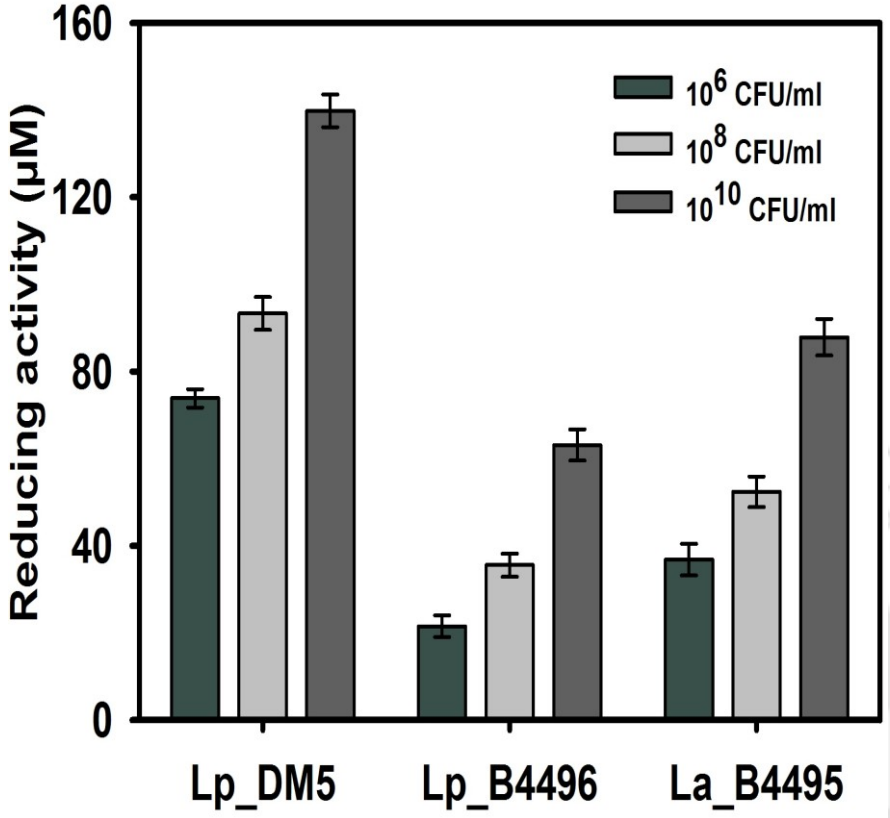

Fig. 3.3.11 Reducing activity of Lactobacillus strains expressed as equivalent to cysteine (µM). The each value is expressed as mean ± S.D (n=3). Lp_DM5= *Lb. plantarum* DM5, Lp_4496= *Lb. plantarum* NRRL B-4496 and La_4495= *Lb. acidophilus* NRRL B-4495.

It was observed that the reducing activity of *Lactobacillus* strains gradually increased with increasing concentration of cells. The isolate *Lb. plantarum* DM5 demonstrated maximum reducing activity of 140 µM at concentration of 10^{10} CFU/ml as compared with *Lb. plantarum* NRRL B-4496 (63 µM) and *Lb. acidophilus* NRRL B-4495 (88 µM). The reducing activity of isolate *Lb. plantarum* DM5 is comparable with other reported lactic acid bacteria at concentration of 10^{10} CFU/ml like *Lb. bulgaricus* 1006 (147 µM) and *S. thermophilus* MC (144.3 µM) (Lin and Yen, 1999). Most of the lactic acid bacteria possess high reducing activity due to intracellular

antioxidants and proteins presence in their cell free extracts (Lin and Yen, 1999, Wang *et al.,* 2006).

3.3.2.5 Superoxide anion radical scavenging activity

The non enzymatic PMS/NADH system generates superoxide radicals, which reduce NBT to a purple formazan (Hazra *et al.,* 2008). The decrease in absorbance at 560 nm in presence of cell free extract of three *Lactobacillus* strains indicates the consumption of superoxide anion in the reaction mixture. The superoxide anion radical scavenging activities of all three *Lactobacillus* strains varied in the range of 10-50% (Fig. 3.3.12). However, *Lb. plantarum* DM5 showed maximum scavenging activity of 48% at concentration of 10^{10} CFU/ml. The other two strains *Lb. plantarum* NRRL B-4496 and *Lb. acidophilus* NRRL B-4495 showed much lower superoxide anion radical scavenging activities of 33% and 37% at concentration of 10^{10} CFU/ml. The result indicates that the probiotic *Lactobacillus plantarum* DM5 is a more potent scavenger of superoxide radical than the other two standard *Lactobacillus* strain and can be used to alleviate oxidative stress caused by superoxide radicals. Antioxidant enzymes, such as Superoxide dismutase (SOD), NADH-oxidase and NADH peroxide, and heterologous non-haem catalase are regarded as important enzymatic defence systems against oxidative stress in lactic acid bacteria (Kullisaar *et al.,* 2008; Lee *et al.,* 2012) and the superoxide anion radical scavenging activity of this studied *Lactobacillus* strain might be due to the presence of SOD in their cell free extract.

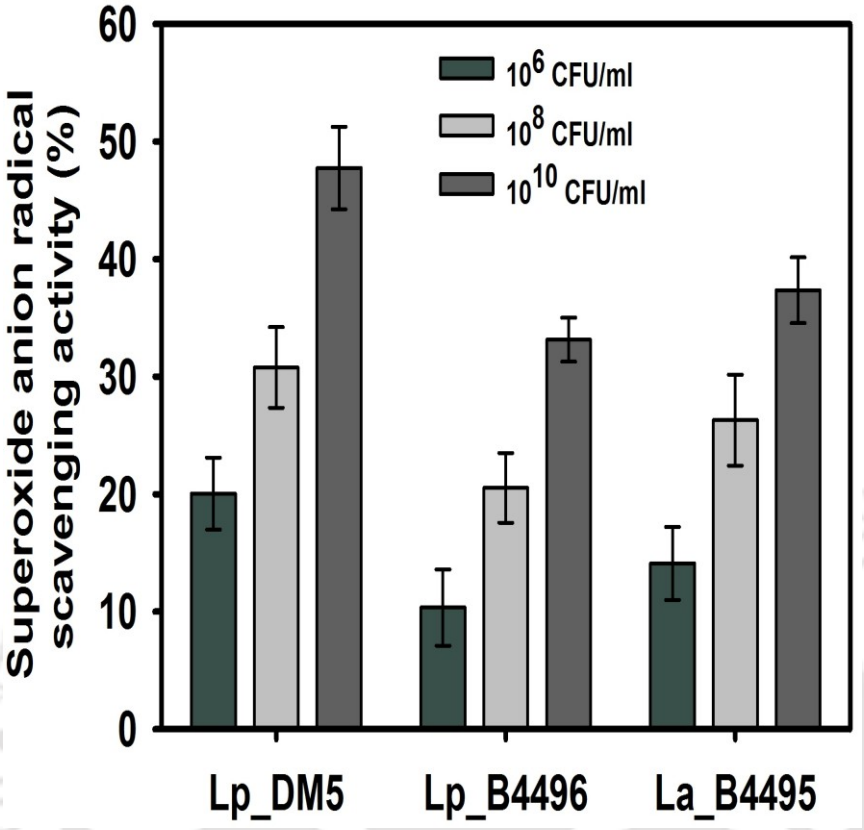

Fig. 3.3.12 Superoxide anion radical scavenging activity (%) of Lactobacillus strains. The superoxide anion radical scavenging activity of the tested strains was measured using different concentration of cell free extract and each value is expressed as mean ± S.D (n=3). Lp_DM5= *Lb. plantarum* DM5, Lp_4496= *Lb. plantarum* NRRL B-4496 and La_4495= *Lb. acidophilus* NRRL B-4495.

3.3.2.6 Hydroxyl radical scavenging activity

Hydroxyl radicals are the major reactive oxygen species causing lipid peroxidation and other massive biological damage to the human cells (Hazra *et al.,* 2008). In the present study hydroxyl radicals are generated by Fenton reaction and hydroxyl radical scavenging activity of *Lactobacillus* strains are shown in Fig. 3.3.13.

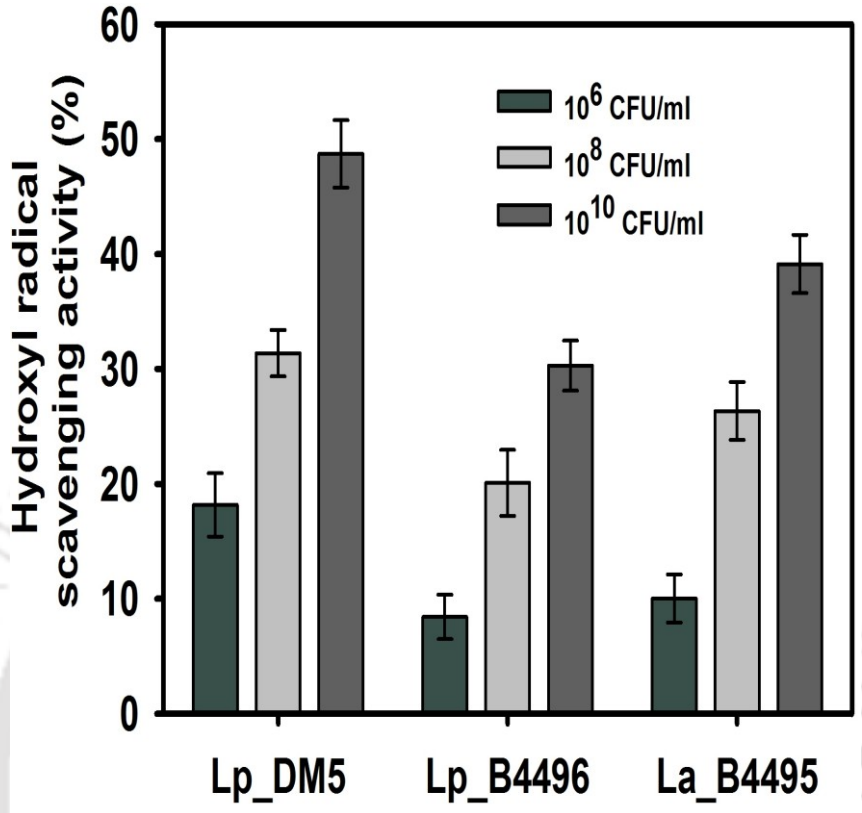

Fig. 3.3.13 Hydroxyl radical scavenging activity (%) of Lactobacillus strains using different concentration of cell free extract. The each value is expressed as mean ± S.D (n=3). Lp_DM5= *Lb. plantarum* DM5, Lp_4496= *Lb. plantarum* NRRL B-4496 and La_4495= *Lb. acidophilus* NRRL B-4495.

Lb. plantarum DM5 showed 18.2%, 31.4% and 48.7% scavenging activity at 10^6, 10^8 and 10^{10} CFU ml, respectively. The hydroxyl radical scavenging activity of *Lb. plantarum* DM5 was higher than those of the two lactobacillus strains using cell free extract at each cell concentration. The hydroxyl radical scavenging activity of *Lb. plantarum* NRRL B-4496 and *Lb. acidophilus* NRRL B-4496 was found to be 30.3% and 39.1% at 10^{10} CFU ml. The hydroxyl radical scavenging ability of these *Lactobacillus* strains could be due to the presence of natural physiological chelators of Cu^{2+} and Fe^{2+} in the intracellular cell-free extract of lactic acid bacteria (Lin and Yen, 1999; Kullisaar *et al.,* 2002). Evidently, the high total antioxidative activity of *Lb.*

plantarum DM5 as observed in DPPF free radical scavenging activity was one of the reasons for its increased resistance to reactive oxygen species like hydroxyl radicals and superoxide radicals. The present study has shown that ingestion of probiotic *Lb. plantarum* DM5 can serve as potent antioxidant and hence can be used as an alternative of synthetic antioxidants.

3.3.3 GABA producing ability of isolate *Lb. plantarum* DM5

Lactobacillus strains were cultivated in MRS medium (pH 6.4) containing 1% MSG and the production of GABA in the cell free supernatant was detected by TLC (Fig. 3.3.13).

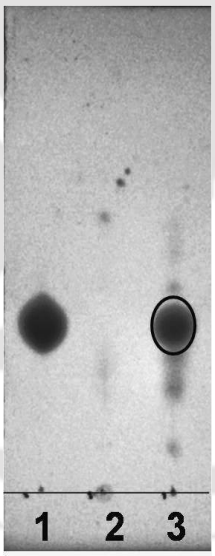

Fig. 3.3.13 TLC chromatogram showing GABA producing ability of *Lb. plantarum* DM5. The solvent system containing n-butanol: acetic acid: water (5:2:2, v/v/v) and the chromatogram was viewed after spraying 2% (w/v) ninhydrin solution followed by incubation at 80°C for 10 min. Lane 1: GABA standard (2 mg/ml), Lane 2: control MRS medium (pH 6.4) supplemented with 1% MSG. Lane 3: Filter sterilized (0.22 µm membrane) cell free supernatant of *Lb. plantarum* DM5 grown in MRS medium supplemented with 1% MSG at 37ºC for 30 h. The GABA standard (lane 1) and the intense spot obtained in lane 3 shows the same retention factor R_f=4.4 mm.

The chromatogram displayed a clear and strong spot for GABA from the culture supernatant of *Lb. plantarum* DM5 (Fig. 3.3.12, Lane 2). No spot was detected in fermented MRS broth by *Lb. plantarum* DM5 without 1% MSG (Fig. 3.3.12, Lane 3). The result ensured the GABA producing attribute of the isolate *Lb. plantarum* DM5 during fermentation. Several lactobacillus species such as *Lb. brevis, Lb. lactis, Lb. buchneri, Lb. plantarum* and *Lb. helveticus* have been reported to demonstrate GABA producing ability with vast difference in production level (Li and Cao, 2010). Previous studies showed that the doses of GABA (0.5 mg/kg) significantly lowered the systolic blood pressure of hypertensive rats after administration (Hayakaw *et al.,* 2004).

3.4 Conclusions

A novel isolate DM5 identified *as Lactobacillus plantarum* displayed *in vitro* probiotic properties as well as antioxidative activity. It showed adequate level of survival to the harsh conditions of the gastrointestinal tract and survived low acidic pH ranging from 2-4 for 5 h. Artificial gastric juice and intestinal fluidic environment decreased the initial viable cell population of isolate *Lb. plantarum* DM5 only by 7% and 13%, respectively. In presence of lysozyme (200 µg/ml) and bile salt (0.5%), the isolate *Lb. plantarum* DM5 displayed enhanced growth of 12.3% and 21.4% respectively after 30 h of incubation at 37ºC. Isolate *Lb. plantarum* DM5 demonstrated cell surface hydrophobicity of 53% and autoaggregation of 54% which are the prerequisite for adhesion to epithelial cells and colonization to host. Coaggregation of *Lb. plantarum* DM5 with two enteropathogens (*E. coli* and *S. aureus*) was also determined and it was found that the isolate *Lb. plantarum* DM5 demonstrated maximum coaggregation of 43% with *E. coli* as compared to *S. aureus* (27%). The adhesion ability of isolate DM5 was confirmed by a good adhesion ratio of 8.63% with HT-29 cell line. The isolate *Lb. plantarum* DM5 was found to deconjugate taurodeoxycholic acid, indicating its potential to cause hypercholesterolemia. In addition the isolate showed bile salt hydrolase activity of 0.63 U/mg and 1.18 U/mg towards sodium taurodeoxycholic acid and conjugated bile salt mixture, respectively.

The isolate *Lb. plantarum* DM5 also displayed antioxidant properties. A concentration of 10^{10} CFU/ml of *Lb. plantarum* DM5 demonstrated the hydroxyl radical, superoxide anion radical and DPPH scavenging activities of 49%, 48%, and 55%, respectively and reducing activity of 149 µM (cysteine equivalents). *Lactobacillus plantarum* DM5 showed 38% and 20% higher hydroxyl radical, 31%

and 22% higher superoxide anion radical and 43% and 33% higher DPPH scavenging activities than those displayed by *Lactobacillus plantarum* and *Lactobacillus acidophilus*, respectively. Probiotic *Lb. plantarum* DM5 has the ability to produce bioactive γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in mammalian brain. The cell free supernatant of the isolate *Lb. plantarum* grown in MRS medium supplemented with 1% MSG, showed intense GABA spot in TLC plates. It also possessed the ability to multiply in MRS medium containing abundant hydrogen peroxide (1 mM) and exhibited 20% inhibition rates of ascorbate autoxidation at 10^{10} CFU/ml. These findings overall suggest that *Lactobacillus plantarum* DM5 have the potential to protect the oxidative damage mediated by reactive oxygen species and can act as an antioxidative probiotic.

References

Bao, Y., Zhang, Y., Li, H., Liu, Y., Wang, S., Dong, X., Su, F., Yao, G., Sun, T. and Zhang H. (2012) *In vitro* screen of *Lactobacillus plantarum* as probiotic bacteria and their fermented characteristics in soymilk. *Ann. Microbiol*., 62,1311–1320.

Bixquert, J.M. (2009) Treatment of irritable bowel syndrome with probiotics: an etiopathogenic approach at last. *Rev. Esp. Enferm. Dig*., 101, 553-564.

Charteris, W.P., Kelly, P.M., Morelli, L. and Collins, J.K. (1998) Development and application of an *in vitro* methodology to determine 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, S. 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.

Collado, M.C., Surono, I., Meriluoto, J. and Salminen, S. (2007) Indigenous dadih lactic acid bacteria: cell-surface properties and interactions with pathogens. *J. Food Sci.,* 72, 89-93.

Darnell, J.E., Levintow, L. and Scharff, M.D. (1970) Harry Eagle. *J. Cell. Physiol.,* 76, 241-252.

Das, D. and Goyal, A. (2010) Characterization and screening of antimicrobial activity of lactic acid bacterium isolated from a traditional beverage Marcha of Sikkim. *J. Pharm. Chem.,* 4, 136-139.

Das, D. and Goyal, A. (2013) Potential probiotic attributes and antagonistic activity of an indigenous isolate *Lactobacillus plantarum* DM5 from an ethnic fermented beverage Marcha of north eastern Himalayas. *Int. J. Food Sci. Nutr.* (Accepted)

Del Re, B., Sgorbati, B., Miglioli, M. and Palenzola, D. (2000) Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Lett. Appl. Microbiol*., 31, 438-442.

Dhanani, A.S. and Bagchi, T. (2013) *Lactobacillus plantarum* CS24.2 prevents *E. coli* adhesion to HT-29 cells and also down-regulates enteropathogen induced TNF-α and IL-8 expression. *Microbiol. Immunol*., 57, 309-315.

Di Cagno, R., Mazzacane, F., Rizzello, C.G., De Angelis, M., Giuliani, G., Meloni, M., De Servi, B. and Gobbetti, M. (2010) Synthesis of gamma-aminobutyric acid (GABA) by *Lactobacillus plantarum* DSM19463: functional grape must beverage and dermatological applications. *Appl. Microbiol. Biotechnol.*, 86, 731-741.

Dong, Z., Zhang, J., Lee, B., Li, H., G. Du, G. and Chen, J. (2012) A bile salt hydrolase gene of *Lactobacillus plantarum* BBE7 with high cholesterol-removing activity. *Eur. Food Res. Technol*., 235:419-427.

Duary, R.K., Rajput, Y.S., Batish, V.K. and Grover, S. (2011) Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. *Indian J. Med. Res.,* 134, 664-671.

Dulbecco, R. (1976) From the molecular biology of oncogenic DNA viruses to cancer. *Science,* 192, 437-440.

Ehrmann, M.A., Kurzak, P., Bauer, J. and Vogel, R.F. (2002) Characterization of *lactobacilli* towards their use as probiotic adjuncts in poultry. *J. Appl. Microbiol.,* 92, 966-975.

Farnandez, M.F., Boris, S. and Barbes, C. (2003) Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. *J. Appl. Microbiol*., 94, 449-445.

Fuller, R. (1989) Probiotics in man and animals. *J. Appl. Bacteriol*., 66, 365–378.

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

Hayakawa, K., Kimura, M., Kasaha, K., Matsumoto, K., Sansawa, H. and Yamori, Y. (2004) Effect of a γ-aminobutyric acid enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar–Kyoto rats. *Br. J. Nutr.,* 92, 411-417.

Hazra, B., Biswas, S. and Mandal, N. (2008) Antioxidant and free radical scavenging activity of *Spondias pinnata. BMC Complement. Altern. Med.,* 8, 63.

He, Z.S., Luo, H., Cao, C.H. and Cui, Z.W. (2004) Photometric determination of hydroxyl free radical in Fenton system by brilliant green. *Am. J. Clin. Med.*, 6, 236-237.

Huang, J. and Adams, M.C. (2004) *In vitro* assessment of the upper gastrointestinal tolerance of potential probiotic dairy Propionibacteria. *Int. J. Food Microbiol*., 91, 253-260.

Jacobsen, C.N., Nielsen, V.R., Hayford, A.E., Moller, P.L., Michaelsen, K.F., Paerregaard, A., Sandstrom, B., Tvede, M. and Jakobsen, M. (1999) Screening

of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. *Appl. Environ. Microbiol.,* 65, 4949-4956.

Kaizu, H., Sasaki, M., Nakajima, H. and Suzuki, Y. (1993) Effect of antioxidative lactic acid bacteria on rats fed a diet deficient in vitamin E. *J. Dairy Sci.*, 76, 2493-2499.

Kanno, T., Kuda, T., An, C., Takahashi, H. and Kimura, B. (2012) Radical scavenging capacities of saba-narezushi, Japanese fermented chub mackerel, and its lactic acid bacteria. *LWT-Food Sci.Technol.*, 47, 25-30.

Kaushik, J.K., Kumar, A., Duary, R.K., Mohanty, A.K., Grover, S. and Batish, V.K. (2009) Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. *PloS One*., 4(12): e8099, doi:10.1371/journal.pone.0008099.

Kiely, L.J. and Olson, N.F. (2000) The physicochemical surface characteristics of *Lactobacillus casei*. *Food Microbiol*., 17:277-291.

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. Appl. Microbiol.,* 94, 981-987.

Kullisaar, T., Zilmer, M., Mikelsaar, M., Vihalemm, T., Annuk, H., Kairane, C. and Kilk, A. (2002) Two antioxidative lactobacilli strains as promising probiotics. *Int. J. Food Microbiol.*, 72, 215-224.

Kumar, R., Grover, S. and Batish, V.K. (2012) Bile salt hydrolase (Bsh) activity screening of *Lactobacilli*: *in vitro* selection of indigenous *Lactobacillus* strains

with Potential bile salt hydrolysing and cholesterol-lowering ability. *Probiotics Antimicrobial Prot*., 4,162-172.

Kumar, S.K., Ganesan, K. and Subba Rao, P.V. (2008) Antioxidant potential of solvent extracts of *Kappaphycus alvarezii* (Doty) Doty – An edible seaweed. *Food Chem*., 107, 289-295.

Lee, B., Kim, J., Kang, Y. M., Lim, J., Kim, Y., Lee, M., et al. (2010). Antioxidant activity and γ-aminobutyric acid (GABA) content in sea tangle fermented by *Lactobacillus brevis* BJ20 isolated from traditional fermented foods. *Food Chem.*, 122, 271-276.

Li, H. and Cao, Y. (2010) Lactic acid bacterial cell factories for γ-aminobutyric acid. *Amino Acids* 39, 1107-1116.

Li, S., Zhao, Y., Zhang, L., Zhang, X., Huang, L., Li, D., Niu, C., Yang, Z. and Wang, Q. (2012) Antioxidant activity of *Lactobacillus plantarum* strains isolated from traditional Chinese fermented foods. *Food Chem.*, 135, 1914-1919.

Lin, M. and Yen, C. (1999) Antioxidative ability of lactic acid bacteria. *J. Agric. Food Chem.*, 47, 1460-1466.

Liong, M.T. and Shah, N.P. (2005) Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. *J. Dairy Sci.,* 88, 55-66.

Lonnermark, E., Friman, V., Lappas, G., Sandberg, T., Berggren, A. and Adlerberth, I. (2010) Intake of *Lactobacillus plantarum* reduces certain gastrointestinal symptoms during treatment with antibiotics. *J. Clin. Gastroenterol.,* 44, 106-112.

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.

Maxwell, S.R. (1995) Prospects for the use of antioxidant therapies. *Drugs*, 49, 345-361.

Nedelcheva, P., Denkova, Z., Denev, P., Slavchev, A. and Krastanov, A. (2010) Probiotic strain *Lactobacillus plantarum* MBIMCC 2415 with antioxidant activity as starter cultures in the production of dried fermented meat products. *Biotechnol. Biotechnol. Eq.,* 24, 1624-1630.

Nissen, L., Chingwaru, W., Sgorbati, B., Biavati, B. and Cencic, A. (2009) Gut health promoting activity of new putative probiotic/protective *Lactobacillus* spp. Strains: a functional study in the small intestinal cell model. *Int. J. Food Microbiol*., 135, 288-294.

Oh, S., Kim, S.H. and Worobo, R.W. (2000) Characterization and purification of a bacteriocin produced by a potential probiotic culture, *Lactobacillus acidophilus* 30SC. *J. Dairy. Sci.,* 83, 2747-2752.

Ouwehand, A.C., Salminen, S. and Isolauri, E. (2002) Probiotics: an overview of beneficial effects. *Antonie van Leeuwenhoek,* 82, 279–289.

Qiao, G., Li, H., Xu, D.H. and Park, S. (2012) Modified a colony forming unit microbial adherence to hydrocarbons assay and evaluated cell surface hydrophobicity and biofilm production of *Vibrio scophthalmi*. *J. Bacteriol. Parasitol.,* 3:130, doi:10.4172/2155-9597.1000130.

Ramirez-Chavarin, M.L., Wacher, C., Eslava-Campos, C.A., Perez-Chabela, M.L. (2013) Probiotic potential of thermo tolerant lactic acid bacteria strains isolated from cooked meat products. *Int. Food Res. J*., 20, 991-1000.

Satish Kumar, R., Kanmani, P., Yuvaraj, N., Paari, K.A., Pattukumar, V. and Arul, V. (2011) *Lactobacillus plantarum* AS1 binds to cultured human intestinal cell line

HT-29 and inhibits cell attachment by enterovirulent bacterium *Vibrio parahaemolyticus*. *Lett. Appl. Microbiol*., 53, 481-487.

Schousboe, A. and Waagepetersen, H.S. (2007) GABA: homeostatic and pharmacological aspects. *Progress in Brain Res.*, 160, 9-19.

Sieladie, D.V., Zambou, N.F., Kaktcham, P.M., Cresci, A. and Fonteh, F. (2011) Probiotic properties of lactobacilli strains isolated from raw cow milk in the western highlands of Cameroon. *Innovative Romanian Food Biotechnol*., 9, 12-28.

Simic, M.G. (1988) Mechanisms of inhibition of free-radical processed in mutagenesis and carcinogensis. *Mutat. Res.*, 202, 377-386.

Siragusa, S., De Angelis, M., Di Cagno, R., Rizzello, C.G., Coda, R., and Gobbetti, M. (2007) Synthesis of γ-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Appl. Environ. Microbiol.*, 73, 7283-7290.

Sun, Y.P., Chou, C.C., and Yu, R.C. (2009) Antioxidant activity of lactic-fermented Chinese cabbage. *Food Chem.*, 115, 912-917.

Suskovic, J., Kos, B., Matosic, S. and Maric, V. (1997) Probiotic properties of *Lactobacillus plantarum* L4. *Food Technol. Biotechnol*., 35, 107-112.

Thokchom, S. and Joshi, S.R. (2012) Antibiotic resistance and probiotic properties of dominant lactic microflora from *Tungrymbai*, an ethnic fermented soybean food of India. *J. Microbiol*., 50, 535-539.

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.

Wang, A.N., Yi, X.W., Yu, H.F., Dong, B. and Qiao, S.Y. (2009) Free radical scavenging activity of *Lactobacillus fermentum in vitro* and its antioxidative effect on growing-finishing pigs. *J. Appl. Microbiol.*, 107, 1140-1148.

Wang, J., Ji, H., Zhang, D., Liu, H., Wang, S., Shan, D. and Wang, Y. (2011) Assessment of probiotic properties of *Lactobacillus plantarum* ZLP001 isolated from gastrointestinal tract of weaning pigs. *Afr. J. Biotechnol*., 10, 11303-11308.

Wang, Y.C., Yu, R.C. and Chou, C.C. (2006) Antioxidative activities of soymilk fermented with lactic acid bacteria and bifidobacteria. *Food Microbiol*., 23, 128-135.

Chapter 4

Production, purification and characterization of bacteriocin from *Lactobacillus plantarum* DM5

4.1 Introduction

Screening of autochthonous bio-protective lactic acid bacteria from conventional food preparation and other natural sources of the north eastern region of Himalayas has been in focus for its high biodiversity and profusion of natural flora and fauna (Singh *et al*., 2009). Lactic acid bacteria are considered as major group of probiotic bacteria (Klein *et al*., 1998). They possess an important bio-defence mechanism by which the colonization and subsequent proliferation of pathogenic bacteria in the intestine is prevented (Yoon *et al*., 2006). Lactic acid bacteria are well known for production of various antimicrobial compounds such as bacteriocin, organic acids, diacetyl, acetoin and reuterin (Holzapfel *et al*., 1995; Galvez *et al*., 2007). Bacteriocins are ribosomally synthesized small peptides with bactericidal or bacteriostatic activity against genetically closely related species (Klaenhammer, 1993). Bacteriocin from lactic acid bacteria inhibit the spoilage and pathogen microorganism and are being used as bio-preservative by enhancing the quality and the safety of the

processed food (Galvez *et al*., 2007; De Vuyst and Leroy, 2007). There is a renewed interest in antimicrobial peptides (bacteriocins and bacteriocin-like compounds) produced by lactic acid bacteria because of their potential use as antimicrobial agents for improving the quality and safety of the food product (Galvez *et al*., 2007). The empirical use of antimicrobial compounds for the preservation of food is encouraged by the commercial use of the bacteriocin nisin, produced by *Lactococcus lactis* (Delves-Broughton, 1990). Besides lactic acid bacteria, Bacillus spp. such as *Bacillus thuringiensis* B439 (Ahern *et al*., 2003) and *Bacillus subtillis* Ec1 (Nithya *et al*., 2012) have also displayed broad antagonistic activity against pathogenic bacteria. These antimicrobial proteins are generally purified by ammonium sulphate precipitation followed by gel filtration, ion-exchange chromatography and reverse-phase high-performance liquid chromatography (Carolissen-Mackay *et al*., 1997; Vera Pingitore *et al*., 2007). The production of antimicrobial peptides are often regulated by medium pH and growth temperature as observed in case of antimicrobial peptide ST4SA from *Enterococcus mundtii* isolated from soybean (Todorov and Dicks, 2009a). *Lactobacillus plantarum* has been extensively utilized as probiotic culture in dairy products (Klein *et al*., 1998; Nissen *et al*., 2009) and has ability to produce bacteriocins (Todorov and Dicks, 2005; Galvez *et al*., 2007, Tiwari and Srivastava, 2008; Todorov and Franco, 2010, Hurtado *et al*., 2011). Bacteriocins from *Lactobacillus plantarum* are generally classified as a small, heat stable, listeria active peptides (Messi *et al*., 2001; Todorov and Dicks, 2005; De Vuyst and Leroy, 2007) which belong to class IIa bacteriocin (Klaenhammer, 1993). The bacteriocin can be added to foods as food preservatives, additives and as shelf life extenders (Ross *et al*., 2002).

In recent years, the main concern of the food industry is to use such a preservative that that can selectively inhibit certain high risk bacteria such as *Listeria monocytogenes* in food without affecting the quality and taste of the product (Klaenhammer, 1993; Marth, 1998). This has prompted screening of potential lactic acid bacteria with strong antimicrobial activity to inhibit food borne pathogens. In this regard, *Lactobacillus plantarum* DM5 was isolated from fermented beverage Marcha of Sikkim (Das and Goyal, 2013a) and was explored for its bioprotective potential. The effect of various physiochemical factors on *Lactobacillus plantarum* DM5 for production of bacteriocin and its mode of action was evaluated. In addition, the bacteriocin was purified by ammonium sulphate precipitation, cation exchange chromatography and gel filtration. The bacteriocin was partially characterized and named as plantaricin DM5.

4.2 Material and Methods

4.2.1 Chemicals and reagents

The media components for maintenance of bacterial culture, protein estimation and buffer preparations were purchased from Hi-Media Pvt. Ltd., India. All the chemicals required for SDS-PAGE analysis were of high purity grade and purchased from Sigma Aldrich, USA. The enzymes (pepsin, trypsin, α-amylase and catalase), Carboxymethyl Sepharose (CM sepharose), Sephacryl S-200HR, propidium iodide and Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich, USA.

4.2.2 Microorganism and culture medium

The strain *Lb. plantarum* DM5 was isolated from an ethnic fermented beverage Marcha of Sikkim on the basis of antimicrobial activity and glucansucrase activity as described in Chapter 2, Section 2.2.5 and maintained in modified MRS agar medium (as described in Chapter 2, Section 2.2.2) at 4ºC, repeatedly sub cultured after every 2 weeks (Goyal and Katiyar, 1996). The indicator strain *Escherichia coli* DH5α was maintained in Luria Broth medium (pH 6.8) as described in Chapter 2, Section 2.2.6. The strains *Staphylococcus aureus* MTCC 737 and *Listeria monocytogenes* MTCC 1143 were used as indicator strains as described Chapter 2, Section 2.2.7 and were maintained in Nutrient medium (pH 6.8) containing 5 g/L (w/v) sodium chloride, 5 g/L (w/v), 1 g/L (w/v) beef extract and 2 g/L (w/v) yeast extract.

4.2.3 Production of bacteriocin from *Lb. plantarum* DM5

The bacteriocin was produced by inoculating the isolate *Lb. plantarum* DM5 (1%, v/v) in sterile 100 ml MRS medium as described by DeMan *et al*., (1960) and

also described in Chapter 2, Section 2.2.3. The culture was incubated at 37°C for 20 h under static condition. The cell free supernatant was obtained by centrifugation at 10,000*g* and at 4°C for 15 min and the pH of cell free supernatant was adjusted to 6.0 using sterile 2N NaOH and filtered through 0.22 µm membrane (Das and Goyal, 2013a). The resultant filtrate was analyzed for bacteriocin activity as described in the next Section 4.2.4. The cell free supernatant was used subsequently for the purification of bacteriocin as described in Section 4.2.10.1.

4.2.4 Antimicrobial activity assay of *Lb. plantarum* DM5

The antimicrobial activity of bacteriocin from *Lb. plantarum* DM5 was determined by agar well diffusion method using *S. aureus* MTCC 737 or *E. coli* DH5α as indicator strain as described in Chapter 2, Section 2.2.6.1. The antimicrobial activity was expressed as arbitrary units (AU) per ml as described by Todorov and Dicks, (2009b). One arbitrary unit was defined as the reciprocal of the highest dilution showing a clear zone of inhibition (~ 2 mm) of the indicator strain *S. aureus* MTCC 737 or *E. coli* DH5α (Das and Goyal, 2013a).

4.2.5 Estimation of protein concentration

The protein concentration of the cell free supernatant was estimated as described in Chapter 3, Section 3.2.5 following the method of Lowry *et al.,* (1951) using Bovine Serum Albumin (BSA) as standard and a concentration range from 25 to 500 µg/ml was used to plot a standard curve. The specific activity (AU/mg) of the cell free supernatant was calculated using the following equation;

$$\text{Specific activity (AU/mg)} = \frac{\text{Bacteriocin Activity (U/ml)}}{\text{Protein Concentration (mg/ml)}} = (\mu\text{moles/min/mg})$$

4.2.6 Effect of different physicochemical parameters on production of bacteriocin from *Lb. plantarum* DM5

4.2.6.1 Effect of temperature and aeration on bacteriocin production

The production of bacteriocin was studied under different incubation temperatures and shaking conditions. For this purpose 1% (v/v) inoculums of *Lb. plantarum* DM5 was inoculated in 100 ml MRS medium (pH 6.4) as described in Chapter 2, Section 2.2.3 and incubated at different temperatures ranging from 20°C to 47°C under static condition. Later the production of antimicrobial compound was compared at 37°C under static and shaking conditions at 100 rpm and 150 rpm. 1.0 ml of culture from MRS broth was withdrawn at every 4 h till 28 h and centrifuged at 10,000*g* at 4°C for 15 min. The pH of cell free supernatant was adjusted to 6.0 by 2N NaOH and used for determination of antimicrobial activity by measuring the clear zone of inhibition around the well of the indicator strain *S. aureus* MTCC 737 as described in Section 4.2.4.

4.2.6.2 Effect of initial medium pH on production of bacteriocin

The effect of initial pH of MRS medium on the production of bacteriocin from *Lb. plantarum* DM5 was tested by varying the pH 4 to 8. 100 ml MRS medium were prepared by adjusting the pH from 4 to 8 by 2N HCl or 2N NaOH and then autoclaved at 121ºC at 15 psi for 20 min. Each medium with respective pH was then inoculated with (1%, v/v) 18 h old culture of *Lb. plantarum* DM5 and incubated at 37°C under

static condition for 18 h. The change in medium pH of the culture and antimicrobial activity (AU/ml) were determined after 18 h by agar well diffusion method using *S. aureus* MTCC 737 as described earlier.

4.2.6.3 Effect of different carbon sources on production of bacteriocin

The production of antimicrobial compound from *Lb. plantarum* DM5 was studied at 37°C in presence of different carbon sources. The MRS medium (pH 6.4) of 100 ml was separately supplemented with 20 g/L of each glucose, sucrose, fructose, maltose, mannose or raffinose as sole carbon source. Each 100 ml medium was then inoculated with 1% (v/v) 18 h old culture of *Lb. plantarum* DM5 and incubated at 37°C under static condition for 18 h. The antimicrobial activity was determined by agar well diffusion method using *S. aureus* MTCC 737 as indicator strain as described previously in Section 4.2.4.

4.2.6.4 Effect of glucose and maltose concentration on production of bacteriocin

To study the effect of different glucose and maltose concentration on the production of antimicrobial compound, 100 ml MRS medium (pH 6.4) were prepared containing glucose or maltose ranging from 0.5 g/100 ml to 4.0 g/100ml as sole carbon source. The antimicrobial activity was determined as mentioned previously in Section 4.2.4.

4.2.7 Fermentation profile of *Lb. plantarum* DM5 in MRS medium

The isolate *Lb. plantarum* DM5 (1%, v/v) was inoculated in sterile 200 ml MRS medium as described by DeMan *et al*., (1960) and mentioned in Chapter 2,

Section 2.2.3. The growth parameters of *Lb. plantarum* DM5 such as pH, cell density, and antimicrobial activity were studied at every 4 h intervals up to 36 h at 37°C under static condition. The change in pH was measured by using pH meter (Sartorius, PP-15) in every 2 h intervals. The growth of culture was determined by measuring the absorbance of cells at 600 nm (A_{600}) on UV-Vis spectrophotometer (Varian, Cary 100) using the sterile MRS medium (DeMan *et al*., 1960) as blank at every 2 h intervals. The cell free supernatant was obtained by centrifugation of 500 µl culture broth at 10,000*g* at 4°C for 10 min and 50 µl was used for determining the antimicrobial activity using *E. coli* DH5α as described in Section 4.2.4.

4.2.8 Effect of temperature and pH on bacteriocin activity

The thermostability of the antimicrobial compound was determined by incubating the 5 ml of cell free supernatant (pH 6.0) of *Lb. plantarum* DM5 at different temperatures ranging from 30 to 100°C for 90 min. Sample of 500 µl was taken after 15, 30, 60 and 90 min of incubation at each of these temperatures and antimicrobial activity was determined as mentioned previously in Section 4.2.4 (Todorov and Dicks, 2009b). In order to determine the pH stability of the antimicrobial compound, 1 ml cell free supernatant of *Lb. plantarum* DM5 was taken and the pH were adjusted from pH 2 to 12 with sterile 2N HCl or 2N NaOH and incubated at 30°C for 2 h. After incubation, the pH of samples were readjusted to 6.0 (eliminating the effect of low acid and high base on indicator strain) with sterile 2N HCl or 2N NaOH (Todorov and Dicks, 2009b). The antimicrobial activity was checked by agar well diffusion method using *S. aureus* MTCC 737 as describer earlier

in Section 4.2.4. Measurements were carried out in triplicates for thermostability and pH stability experiments.

4.2.9 Effect of enzymes on bacteriocin activity

Biological nature of the antimicrobial compound produced by the isolate *Lb. plantarum* DM5 was determined by testing its sensitivity to the proteolytic enzymes. The 2 ml of cell free supernatant of *Lb. plantarum* DM5 was neutralized to pH 6.0 by 2N NaOH and incubated with each 1 mg/ml of pepsin, trypsin, α-amylase, catalase, lysozyme or proteinase K at 37ºC for 1 h. The residual antimicrobial activities of the cell free supernatant then tested by agar well diffusion method as described earlier in Section 4.2.4.

4.2.10 Effect of organic solvents, surfactants, salts and detergents on bacteriocin activity

The effect of organic solvents (ethanol, methanol, acetone, ethyl acetate, n-butanol, isopropanol), surfactants (Tween 20, Tween 80, Triton X-100), detergent sodium dodecyl sulphate (SDS), urea and sodium chloride (NaCl) on bacteriocin was determined. The above mentioned reagents were added to the 5 ml of cell free supernatant (pH 6.0) of *Lb. plantarum* DM5 at a final concentration of 1% (v/v or w/v as appropriate) and incubated at 37ºC for 2 h. After the incubation, 50 µl of the sample was taken from each mixture and analyzed for residual antimicrobial activity by agar well diffusion method using *S. aurues* MTCC 737 as indicator strain as described in Section 4.2.4.

4.2.11. Mode of action of bacteriocin from *Lb. plantarum* DM5

4.2.11.1. Growth of test organism in presence of Lb. plantarum DM5 bacteriocin

The isolate *Lb. plantarum* DM5 was grown in 100 ml MRS medium at 37ºC for 18 h. The cell free supernatant was obtained by centrifugation at 10,000*g* at 4ºC for 15 min and pH was adjusted to 6.0 with 2N NaOH. 25 ml of filter-sterilized (0.22 µm membrane) cell free supernatant (pH 6.0) was added to each of 100 ml culture of *E. coli* DH5α (A_{600}=0.4, no of viable cells $4.7x10^4$ CFU/ml) in LB medium (as described in Chapter 2, Section 2.2.6), *L. monocytogenes* (A_{600}=0.4, no of viable cells $2.9x10^4$ CFU/ml) and *S. aureus* MTCC 737, (A_{600}= 0.5, no of viable cell $5.3x10^4$ CFU/ml) in NB medium (as described in Section 4.2.2) in their early exponential phase. All the cultures were incubated at 37ºC for 16-18 h under shaking condition of 120 rpm and absorbance at 600 nm (A_{600}) were recorded at every 1 h intervals.

4.2.11.2 Visualization of test organism in presence of Lb. plantarum DM5 bacteriocin by scanning and transmission electron microscopy

In a separate experiment, the cell morphology of *E. coli* DH5α, *L. monocytogenes* MTCC 1143 and *S. aureus* MTCC 737 treated with the cell free supernatant of isolate *Lb. plantarum* DM5 was examined by scanning electron microscope (SEM), (Leo1330 VP, Leo Electron Microscopy Ltd., Cambridge, UK) operated at 10.0 kV. The 5 ml of filter-sterilized (0.22 µm memberane) cell free supernatant (pH 6.0) of *Lb. plantarum* DM5 was added separately to each of 20 ml culture of *E. coli* DH5α, *L. monocytogenes* MTCC 1143 and *S. aureus* MTCC 737 in their early exponential phase and incubated at 37ºC for 18 h. The cell pellets of treated *E. coli* DH5α, *L. monocytogenes* MTCC 1143 and *S. aureus* MTCC 737 obtained by centrifugation at 10,000*g* and 4ºC for 15 min were washed three times with 1x

phosphate buffer saline (PBS) (pH 6.8) and fixed with equal volume of glutaraldehyde (2.5%, v/v) for 2-4 h. One drop of this was used to prepare a smear, dehydrated using graded concentration of acetone and dried in a vacuum desiccator. The dried samples were attached to the SEM stub with double-sided tape and coated with 10 nm gold in a sputter coater (SCH 620, Leo) for scanning electron microscopy. The cell suspension of *E. coli* DH5α, *L. monocytogenes* MTCC 1143 and *S. aureus* MTCC 737 without the addition of cell free supernatant of *Lb. plantarum* DM5 served as control.

The cell morphology of *E. coli* DH5α treated with the cell free supernatant of isolate *Lb. plantarum* DM5 was also examined by transmission electron microscope (TEM) (JEOLJEM 2100). The preparation of sample for transmission electron microscopy was same as for scanning electron microscopy. The dried sample of *E. coli* DH5α treated with the cell free supernatant of isolate *Lb. plantarum* DM5 was fixed on a metallic grid for transmission electron microscopic analysis.

4.2.11.3 Analysis of cell lysis by Lb. plantarum DM5 bacteriocin by flow cytometry

The 5 ml filter sterilized (0.22 µm membrane) cell free supernatant (pH 6.0) of isolate *Lb. plantarum* DM5 was added to each 20 ml culture of *E. coli* DH5α and *S. aureus* MTCC 737 growing in nutrient broth medium at their early exponential phase and were allowed to incubate at 37ºC for 18 h. The cells of *E. coli* DH5α and *S. aureus* MTCC 737 were harvested by centrifugation at 10,000*g* at 4ºC for 10 min. The cell pellet was washed three times with 1x PBS (pH 6.8) and re-suspended in the same buffer. One microlitre of 2 mg/ml of propidium iodide (final conc. 2 µg/ml) was added to ~ 10^7 cells suspended in 1 ml of 1x PBS (pH 6.8) and incubated at 4ºC for 15 min in dark. The cell viability was analyzed using flow cytometer (FACS Calibur, BD

Biosciences) with excitation at 488 nm and emission at 530 nm (FL2-H filter in FACS Calibur).

4.2.12 Purification of bacteriocin

4.2.12.1 Purification of bacteriocin by ammonium sulphate precipitation

The cell free supernatant of *Lb. plantarum* DM5 was partially purified by ammonium sulphate precipitation. Ammonium sulphate was slowly added to the 100 ml cell free supernatant (pH 6.0) to 40% saturation and then centrifuged at 10,000*g* and 4°C for 20 min. The supernatant was collected and taken for carrying out further 80% ammonium sulphate precipitation. The solution was stirred at 4°C for 4-6 h and then centrifuged at 10,000*g* at 4°C for 30 min. The resulting pellet was re-suspended in 25 mM ammonium acetate buffer (pH 6.0) and extensively dialyzed (Molecular mass cut off 3.5 kDa) against the same buffer with regular change of buffer for a time period of 24 h. Serial dilutions of partially purified bacteriocin were carried out and its activity was checked by zone of inhibition of the indicator strain *S. aureus* MTCC 737 obtained by agar well diffusion method at 37ºC after 24 h incubation as described in Section 4.2.4.

4.2.12.2 Purification of bacteriocin by cation exchange chromatography

The partially purified bacteriocin from *Lb. plantarum* DM5 by ammonium sulphate was further purified by cation exchange chromatography using CM Sepharose as matrix. The column (1.5 cm x 5.0 cm) was pre-equilibrated with 25 mM ammonium acetate buffer (pH 5.4) and the 20 ml partially purified bacteriocin (specific activity 2196 AU/mg, protein conc. 5.83 mg/ml) obtained in the previous

step was loaded to the column. After sample loading, the column was washed with 20 ml of 25 mM ammonium acetate buffer (pH 5.4) at a flow rate of 1 ml/min. The bound protein was eluted with a linear gradient of NaCl (0.0 to 0.5 M) in 25 mM ammonium acetate buffer (pH 5.4) with a flow rate of 1.0 ml/min in FPLC (Akta Prime, GE Healthcare). Each fraction of 2 ml was collected for estimation of protein content (A_{280}) and bacteriocin activity (AU/ml) by agar well diffusion method using *S. aureus* MTCC 737 as mentioned in Section 4.2.4. The fractions having bacteriocin activity were pooled and dialyzed against 25 mM ammonium acetate buffer (pH 6.0) overnight at 4°C. The dialyzed bacteriocin was examined for protein concentration, specific activity and was further subjected to the next step of purification by gel filtration as in Section 4.2.12.1.

4.2.12.3 Purification of bacteriocin by gel filtration

The partially purified bacteriocin (3137 AU/mg, 1.02 mg/ml) obtained by cation exchange chromatography was further purified by gel filtration using a column (1.5 cm x 50 cm) containing Sephacryl S-200HR matrix, connected to FPLC (Akta Prime, GE Healthcare). The column was pre-equilibrated with 25 mM ammonium acetate buffer (pH 6.0) and 2 ml of partially purified bacteriocin (3137 AU/mg, 1.02 mg/ml) was loaded onto the column. The bacteriocin was eluted using 25 mM ammonium acetate buffer (pH 6.0) at a flow rate of 0.5 ml/min and fractions of 2 ml up to 60 fractions were collected. The purified fractions showing maximum bacteriocin activity against *S. aureus* MTCC 737 by agar well diffusion method were pooled and analysed for protein concentration and specific activity as described in Section 4.2.5.

4.2.13 SDS-PAGE analysis of purified bacteriocin

4.2.13.1 Preparation of reagents for SDS-PAGE

(A) Preparation of 30% (w/v) acrylamide

The stock solution of 100 ml acrylamide/ bis acrylamide solution (29.2%, w/v acrylamide and 0.8% w/v bisacrylamide) was prepared in an amber colour bottle. The solution was then filtered using Whatman No. 1 paper under dark and stored at 4°C.

(B) ***Tris-HCl (1.5 M, pH 8.8)***

54.45 g Tris base (121.14 g/mol) was first dissolved in 150.0 ml of distilled water. The pH of solution was adjusted to 8.8. Finally the volume of the solution was made up to 300 ml and stored at 4°C.

(C) ***Tris-HCl (0.5 M , pH 6.8)***

6.0 g Tris base (121.14 g/mol) was first dissolved in 60.0 ml of distilled water. The pH of solution was adjusted to 6.8. Finally the volume of the solution was made up to 100 ml and stored at 4°C.

(D) ***SDS (10%, w/v)***

10.0 g SDS was first dissolved in 60.0 ml of distilled water with gentle stirring. Finally the volume of the solution was made up to 100 ml.

(E) ***Sample buffer (5x)***

The protein sample buffer (5x) was prepared by mixing the components mentioned below in Table 4.2.1. A 5x stock solution of sample buffer was prepared and mixed with 4 volumes of protein sample to make it to 1x before loading on to the gel.

Table 4.2.1 Composition of 5x sample buffer.

Sample buffer Component	Final Concentration
Tris HCl (pH 6.8)	62.5 mM
Glycerol	20% (v/v)
SDS	2% (v/v)
Bromophenol Blue	0.025% (w/v)
β-mercaptoethanol	5% (v/v)

(F) ***Running buffer (5x)***

The SDS-PAGE gel was run using 1x Tris-glycine buffer (pH 8.3) prepared from the 5x stock solution. The compositions of 5x Tris glycine buffer (pH 8.3) is described below in Table 4.2.2. The 5x running buffer was filtered (Whatman, Filter No. 1) and stored at 4°C.

Table 4.2.2 Composition of 5x running buffer.

Components	Final concentration (5x buffer)
Tris base	0.125 M
Glycine	1.25 M
SDS	0.5% (w/v)

4.2.13.2 Preparation of SDS-PAGE gels

The SDS-polyacrylamide gel electrophoresis of bacteriocin sample purified by 80% ammonium sulphate precipitation, cation exchange chromatography and gel filtration was performed using a vertical slab mini gel unit (Mini-PROTEAN®Tetra cell, BioRad, USA) using 1.5 mm thick gels, following the method of Laemmli, (1970). The resolving gel containing 15% (w/v) acrylamide and stacking gel containing 4% (w/v) acrylamide was prepared as described in Table 4.2.3 and Table 4.2.4, respectively, and used for electrophoresis of bacteriocin samples.

Table 4.2.3 Composition for preparation of 15% resolving gel.

Component	Volume (ml)
Acrylamide-bisacrylamide solution (30%, w/v)	5.0
SDS solution (10%, w/v)	1.00
1.5 M Tris (pH 8.8)	3.30
APS solution (10%, w/v)	0.10
TEMED	0.01
Deionized water	0.60
Total volume	10.00

Table 4.2.4 Composition for preparation of 4% stacking gel.

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

The protein sample was mixed with 5x loading dye buffer in the ratio 4:1 and subjected to heat denaturation by putting the sample in boiling water bath for 5 min. The samples were loaded on 15% acrylamide gel along with protein molecular mass marker from Bangalore Genei Pvt. Ltd., India and the electrophoresis was carried out using 1x running buffer prepared from 5x running buffer as described in Section 4.2.13.1 with a current of 2 mA per lane. After the electrophoresis, the gel containing protein bands were stained with Coomassie Blue R250 staining solution as described in Section 4.2.13.3.

4.2.13.3 Staining and destaining of SDS-PAGE gels

The Coomassie staining solution was prepared by dissolving 250 mg of CBB R-250 dye in 50 ml of deionized water and the solution was filtered through Whatman, Filter No. 1. After filtration 40 ml of methanol and 10 ml of glacial acetic acid were

added and the solution was stored in amber colour bottle. For staining of the gel, the gel was immersed in 30 ml of staining solution and incubated at 25ºC for 30-45 min in a gel rocker. The destaining of the gel was carried out by several changes of destaining solution (40% methanol and 10% glacial acetic acid) until the background became transparent and blue colour protein bands were visible.

4.2.14 *In situ* assay of bacteriocin activity by agar gel overlay method

In situ bacteriocin activity was determined by agar gel overlay method as described by Bhunia *et al*., (1987). After electrophoresis the gel was divided into two vertical parts and one half of the gel was stained with Coomassie Blue R250 as described in Section 4.2.13.3 and the other part was used for detection of antimicrobial activity. For determining the *in situ* antimicrobial activity, the gel was fixed in 20% isopropanol and 10% acetic acid and washed in sterile double distilled water for 6 h with frequent water exchange. The gel was placed above the Nutrient base agar (1.8%) and after that it was overlaid with Nutrient soft agar (0.7%) medium inoculated with the indicator strain *S. aureus* MTCC 737 (~10^6 CFU/ml) and incubated at 37ºC for 24 h.

4.2.15 Assessment of cytotoxicity of bacteriocin

4.2.15.1 Culturing and maintenance of cell line

The human embryonic kidney (HEK-293) and the human cervical cancer (HeLa) cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India. The cell lines were maintained in Dulbecco's Modified Eagle's Medium

(DMEM) at 37°C in 5% carbon dioxide atmosphere as described in Chapter 3, Section 3.2.3.

4.2.15.2 In vitro cyotoxicity assay

The 5 ml purified bacteriocin with specific activity of 2196 AU/mg was lyophilized and used for cytotoxicity assay by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (van Meerloo *et al*., 2011). The human embryonic kidney cell line (HEK-293) and the human cervical cancer cell line (HeLa-293) were seeded separately in 250 ml, 75 cm^2 vent cap tissue culture flasks and after ~90% confluence, the cells were detached by 1x trypsin EDTA solution (Sigma Aldrich, USA) and counted by haemocytometer. For cytotoxicity test, the cells were re-suspended in Fetal Bovine Serum (FBS) containing DMEM medium (referred to as complete medium and is mentioned in Chapter 3, Section 3.2.3). 200 µl of this DMEM medium containing $2x10^4$ HEK 293 or HeLa cells per well were separately seeded in a 96 well plate. The plates were incubated at 37ºC for 12 h for cell adherence in CO_2 incubator (5%). After the incubation, the complete DMEM medium was completely removed and the adhered cells were exposed to different concentrations of bacteriocin (ranging between 10 µg/ml and 1000 µg/ml) dissolved in FBS free DMEM medium (referred to as incomplete medium and is described in Chapter 3, Section 3.2.3). The MTT assay was done at regular time interval by removing the whole 200 µl medium and washing the each well containing the adherent cells in the bottom of the well with 200 µl of 1x PBS (pH 7.1) to remove any bacterial contamination. Finally, 100 µl MTT (500 µg/ml) was added to each well and the plates

containing MTT solution was further incubated at 37ºC for 4 h. After the incubation, the 100 µl of MTT solution from each well was replaced with equal volume (100 µl) of DMSO. The absorbance at 570 nm, (A_{570}) was measured using a multi-mode microplate reader (Tecan, Infinite 200 Pro) and the viability (%) was calculated by an equation as described by Patel *et al.,* (2010) and as mention below.

$$\textbf{Cell viability (\%)} = (N_t/N_c) \times 100$$

where, N_t is absorbance (A_{570}) of cells treated with glucan and N_c is the absorbance (A_{570}) of untreated cells.

4.3 Results and Discussion

4.3.1 Antimicrobial activity assay of *Lb. plantarum* DM5

The cell free supernatant of *Lb. plantarum* DM5 showed antimicrobial activity of 6400 AU/ml (protein concentration 15.6 mg/ml) and specific activity of 410 AU/mg against gram positive *S. aureus* MTCC 737, *Listeria monocytogenes* MTCC 1143 and gram negative *E. coli* DH5α, respectively. The pH of cell free supernatant was adjusted to 6.0 by 2N NaOH to neutralize the effect of lactic acid on the indicator strains. Hence, it could be concluded that the zone of inhibition obtained around the well of the indicator strains by the cell free supernatant of *Lb. plantarum* DM5 was due to antimicrobial peptide (specific activity 410 AU/mg) and not due to lactic acid (Das and Goyal, 2013b). The cell free supernatant from *Lb plantarum* DM5 can be exploited as bio-perseverant in food industry as it possessed the ability to eliminate or retard the growth of *L. monocytogenes, E. coli* and *S. aureus,* well known virulent food borne pathogens responsible for listeriosis and food poisoning (Loir *et al*., 2003; Mook *et al*., 2011).

4.3.2 Effect of different physicochemical parameters on production of bacteriocin from *Lb. plantarum* DM5

4.3.2.1 Effect of temperature and aeration on bacteriocin production

The isolate *Lb. plantarum* DM5 was grown in MRS medium (pH 6.4) at temperatures ranging from 20°C to 47°C for production of bacteriocin. The maximum antimicrobial activity of 6400 AU/ml was observed at 37°C as determined by agar well diffusion method using *S. aureus* MTCC 737 as indicator strain (Fig. 4.3.1). At a temperature lower than 30ºC, the cell growth was slow which might be the cause for lower antimicrobial activity as shown in Fig. 4.3.1. It has been reported that the

incubation temperature of 30 to 37ºC under static condition is favourable for the production of bacteriocin from *Lb. plantarum* 35d (Messi *et al*., 2001) and *Lb. plantarum* ST194BZ (Todorov and Dicks, 2005).

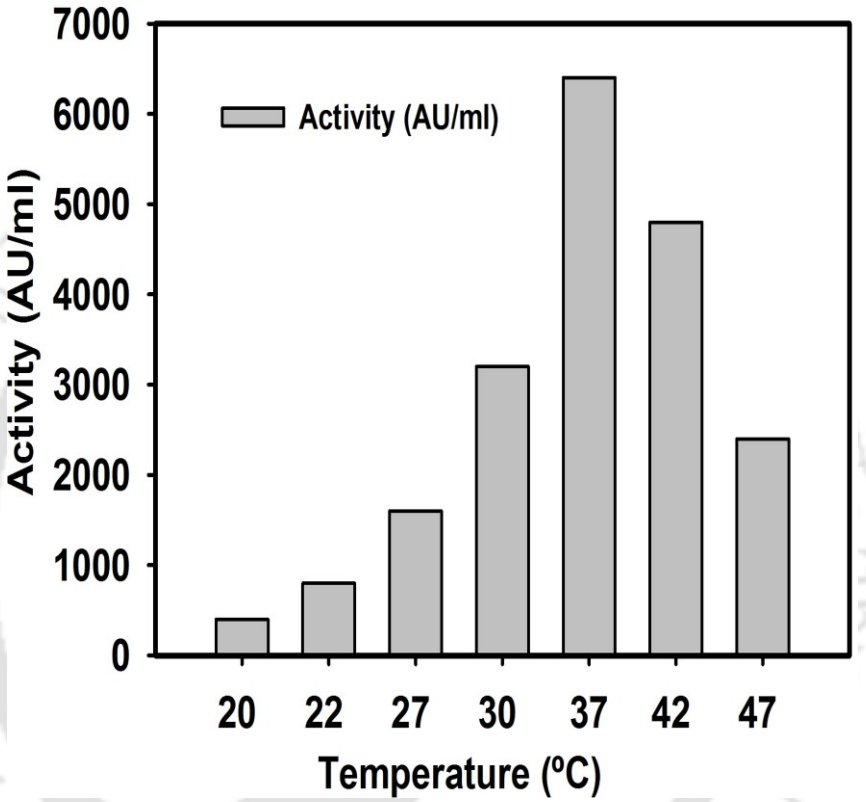

Fig. 4.3.1 Effect of temperature on bacteriocin production from *Lb. plamnatrum* DM5 under static condition. The incubation temperature was varied from 20ºC to 47ºC and changes in antimicrobial activity were recorded. All the experiments were carried out in triplicates and the results represent mean values with less than 2% of error and were not indicated.

The production of bacteriocin under static flask condition was compared with the shaking flask conditions at 100 and 150 rpm at 37°C (Fig. 4.3.2). The maximum antimicrobial activity of 4800 AU/ml and 3200 AU/ml was observed at 100 and 150 rpm after 20 h and 25 h of incubation, respectively (Fig. 4.3.2) at 37°C. It was observed that the antimicrobial activity was increased by ~ 25% at static condition

when compared with shaking at 100 rpm at 37ºC as shown in Fig. 4.3.2 (Das and Goyal, 2013b). The maximum activity in static condition was also achieved earlier (18 h) in static condition as compared with shaking condition of 100 rpm and 150 rpm (25 h).

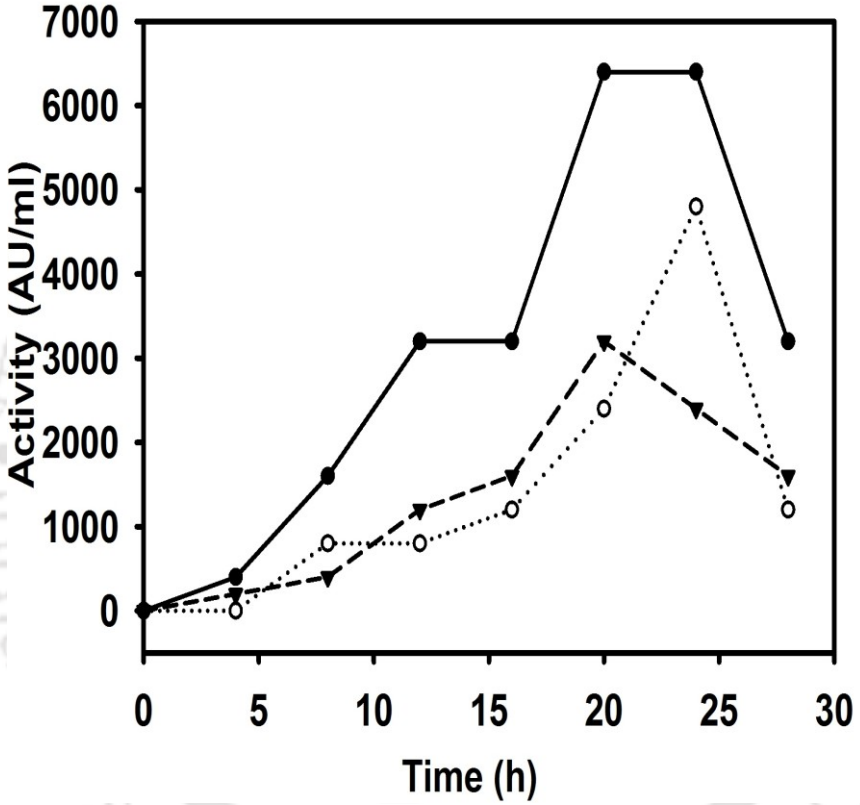

Fig. 4.3.2 Effect of shaking conditions on production of bacteriocin from *Lb. plantarum* DM5 at 37ºC. The shaking conditions of 100 rpm (--o--) and 150 rpm (--▼--) was compared with static condition (--●--). All the experiments were carried out in triplicates and the results represent mean values with less than 2% of error and were not indicated.

4.3.2.2 Effect of initial medium pH on production of bacteriocin

It was observed that the maximum production of bacteriocin occurred at pH between 6.0 and 6.5 (Table 4.3.1). The residual antimicrobial activity obtained at each pH was calculated by comparing with the highest activity (6400 AU/ml) as shown in Table 6.3.1. At pH lower than 6.0 the antimicrobial activity decreased sharply and

finally at pH 4.0 the activity was lost by 91%. The medium pH higher than 6.5, also did not support the production of antimicrobial compound as it showed 50% reduction in activity at pH 7.0. Similar results were also reported for other strains such as *Lb. plantarum* ST194BZ (Todorov and Dicks, 2005) and *Lb. plantarum* UG1 (Enan *et al*., 1996), which showed highest antimicrobial activity at pH 6.0 and 6.5, respectively. The final pH values of *Lb. plantarum* DM5 ranged between 3.8 and 4.2 (Table 4.3.1), irrespective of the initial medium pH of the MRS medium (Das and Goyal, 2013b).

Table 4.3.1 Influence of initial medium pH on production of bacteriocin from *Lb. plantarum* DM5 at 37°C under static condition.

Initial Medium pH	**4.0**	**4.5**	**5.0**	**5.5**	**6.0**	**6.5**	**7.0**	**7.5**
Final pH	3.8	3.8	3.9	3.9	4.0	3.9	4.0	4.2
OD at 600 nm	4.5	4.7	5.2	5.4	5.6	5.6	5.7	5.4
Antimicrobial Activity (AU/ml)	600	800	1600	3200	6400	6400	3200	1600
Specific Activity(AU/mg)	43	67	115	227	410	410	232	210
Residual Activity (%)[a]	9	13	25	50	100	100	50	25

[a]*compared with the highest antimicrobial activity (6400 AU/ml). The experiments were carried out in triplicates and the results represent mean values with less than 2% of error and were not indicated.*

4.3.2.3 Effect of different carbon sources on production of bacteriocin

The maximum production of bacteriocin (6400 AU/ml) was recorded in the presence of 20 g/L of maltose or 20 g/L of glucose as sole carbon source as shown in Table 4.3.2. Growth of *Lb. plantarum* DM5 in presence of 20 g/L glucose and 20 g/L maltose yielded the same bacteriocin activity of 6400 AU/ml however; in case of 20 g/L of maltose the specific activity was higher by 3% with ± 0.8% standard error (Das and Goyal, 2013b). The data was in accordance with other reported strain like *Lb. plantarum* ST194BZ (Todorov and Dicks, 2005) and *Lb. plantarum* SA6 (Rekhif *et al*., 1995), which also showed that glucose or maltose favoured the production of

bacteriocin. The lowest activity of 1600 AU/ml was observed in case of 20 g/L fructose or 20 g/L sucrose (Table 4.3.2). On the other hand 20 g/L raffinose and 20 g/L mannose showed the similar antimicrobial activity (3200 AU/ml) with a minor variation in specific activity.

Table 4.3.2 Influence of carbon source on production of bacteriocin from *Lb. plantarum* DM5 at 37°C.

Carbon Source (2%, w/v)	Activity (AU/ml)	Residual Activity[a]	Protein Conc. (mg/ml)	Specific Activity (AU/mg)
Glucose	6400	100	15.6	410
Maltose	6400	100	15.1	424
Mannose	3200	50	15.5	206
Raffinose	3200	50	15.2	210
Sucrose	1600	25	15.4	104
Fructose	1600	25	15.7	102

[a]*compared with the highest antimicrobial activity (6400 AU/ml). All the individual experiments were carried out in triplicates and the results represent mean values with less than 1% of error and were not indicated.*

4.3.2.4 Effect of glucose and maltose concentration on production of bacteriocin

The glucose and maltose (2% w/v) as sole carbon source showed same bacteriocin activity of 6400 AU/ml, therefore the effect of glucose and maltose concentration on production of bacteriocin was studied broadly. Lower concentration of glucose (0.25, 0.5, 0.75, 1, 1.25 and 1.5 g/100 ml) gave 400, 800, 800, 1600, 1600 and 3200 AU/ml, respectively (Fig. 4.3.3). Maximum bacteriocin activity of 6400 AU/ml was observed in presence 2 g/100 ml of glucose. However, maximum antimicrobial activity of 6400 AU/ml was achieved with lower concentration of maltose (1.5 g/100 ml) as compared with glucose (2 g/100 ml). Lower concentration of maltose (0.25, 0.5, 0.75, 1 and 1.25 g/100 ml) yielded 800, 800, 1600, 3200 and

3200 AU/ml bacteriocin activity, respectively (Fig. 4.3.3). Beyond 2% glucose and 1.5% maltose there was no increase was observed in the production of antimicrobial compound (Fig. 4.3.3).

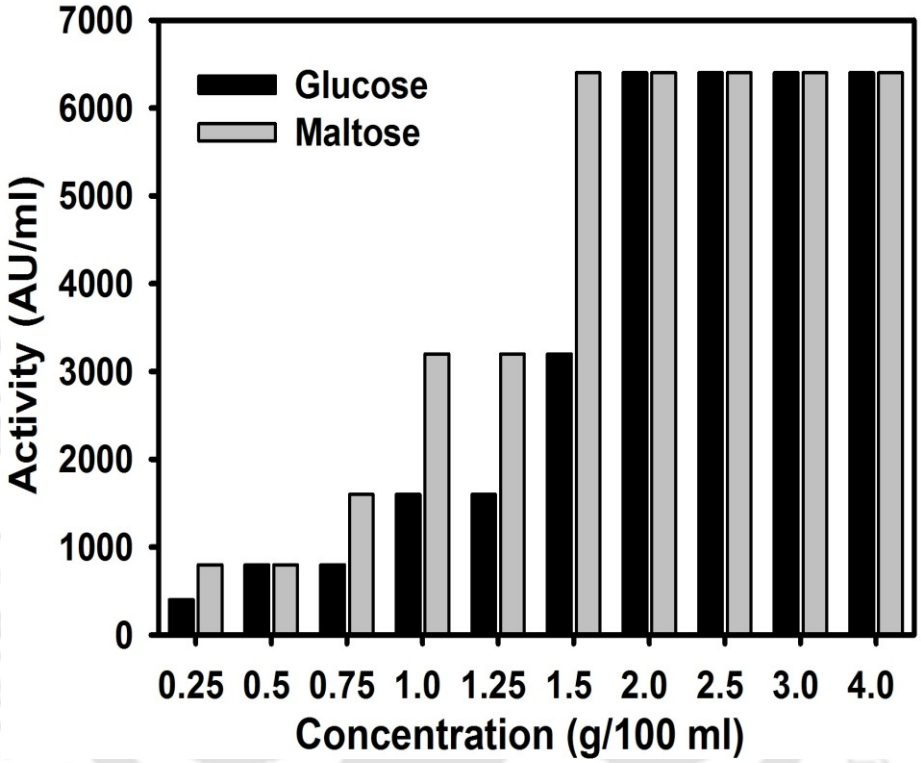

Fig. 4.3.3 Effect of glucose and maltose concentration on production of bacteriocin from *Lb. plantarum* DM5 at 37ºC. The concentration of glucose and maltose was varied from 0.25 to 4 g/100 ml in MRS medium (pH 6.4) and changes in antimicrobial activity were recorded. All the experiments were carried out in triplicates and the results represent mean values with less than 2% of error and were not indicated.

4.3.3 Fermentation profile of *Lb. plantarum* DM5 in MRS medium

The fermentation of *Lb. plantarum* DM5 was studied at 37ºC under static condition and the variation in cell absorbance, pH and the production of bacteriocin is shown in Fig. 4.3.4 Maximum antimicrobial activity of 6400 AU/ml of the cell free supernatant of *Lb. plantarum* DM5 against *E. coli* DH5α was observed during the

transition from late exponential phase to early stationary phase after 16 h of incubation at 37ºC and lasted for about 4 h. It decreased after 20 h at the end of the stationary phase when the cell growth was ceased, indicating that the production of extracellular antimicrobial compound primary metabolite was growth associated (Fig. 4.3.4) (Das and Goyal, 2013a).

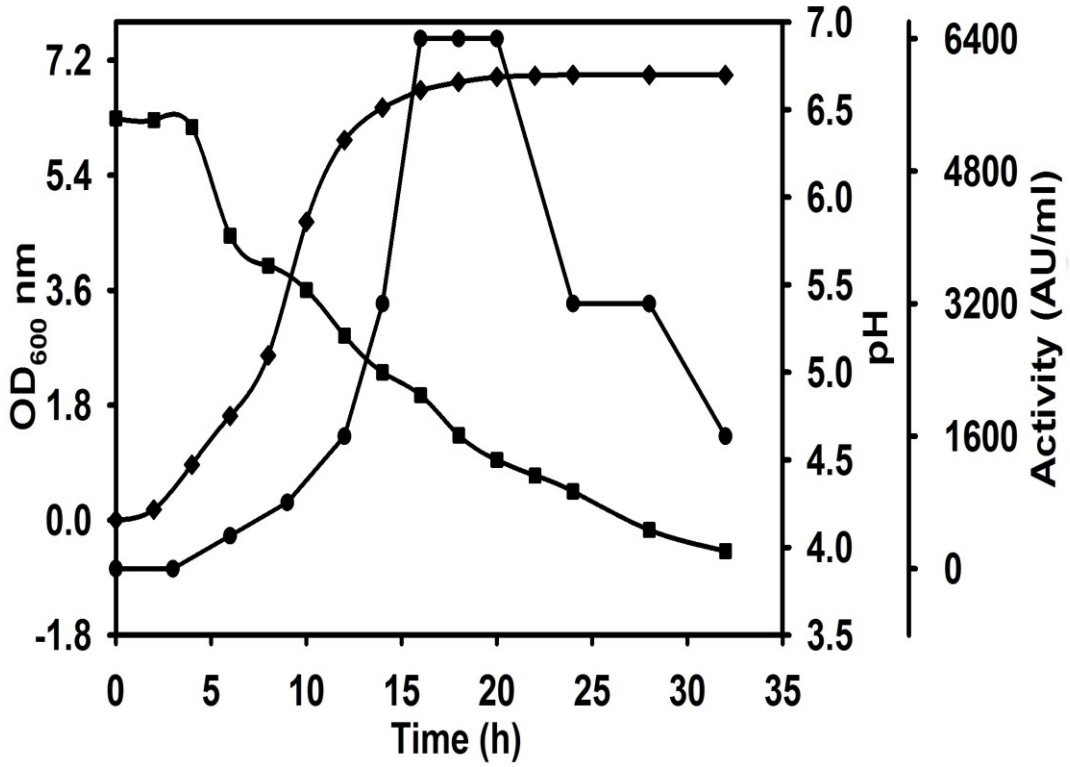

Fig. 4.3.4 Fermentation profile and production of bacteriocin in MRS medium (pH 6.4) at 37ºC by *Lb. plantarum* DM5. Antimicrobial activity (--●--) is represented as AU/ml against indicator strain *E. coli* DH5α. The change in optical density (--♦--) and pH (--■--) during the production of antimicrobial compound was monitored at 37 ºC for 35 h. Standard deviation recorded from three repeats was less than 2% and is not indicated.

The pH of the broth decreased from 6.4 to 4.0 and the absorbance (A_{600}) of cell increased from 0.02 to 4.93 after 32 h. The decrease in antimicrobial activity after 20 h

was due to low pH of the broth or degradation of antimicrobial compound by extracellular proteolytic enzymes. Similar results of decrease in antimicrobial activity was reported due to extracellular proteolytic enzyme and low medium pH (<4.5) for plantaricin ST194BZ (Todorov and Dicks, 2005) and pediocin ST44AM (Todorov and Dicks, 2009b). The total protein concentration of the cell free supernatant was 15.6 mg/ml at 16 h when antimicrobial activity was 6400 AU/ml. One of the most important adjuncts of a probiotics strain was to provide protection against pathogens in the intestinal tract of the host (Thokchom and Joshi, 2012) and in the present study, it was observed that the isolate *Lb. plantarum* DM5 could be considered as potential probiotic strain as the cell free supernatant of the isolate displayed highest antimicrobial activity of 6400 AU/ml against both gram positive *S. aureus* MTCC 737 and gram negative *E. coli* DH5α.

4.3.4 Effect of temperature and pH on bacteriocin activity

The thermostability of bacteriocin from *Lb. plantarum* DM5 was studied by incubating the 5 ml of cell free supernatant (pH 6.0) at different temperatures ranging from 30 to 100°C for 90 min. Antimicrobial activity of the cell free supernatant containing bacteriocin was not altered by heat treatment at 60, 70, 80 and 90°C after 30, 60 and 90 min of incubation (Fig. 4.3.5). The antimicrobial activity was unaffected by heating up to 100ºC for 60 min and 75% activity still remained after a heat treatment at 100ºC for 90 min (Fig. 4.3.5). These results suggested that the antimicrobial compound from *Lb. plantarum* DM5 is heat stable like other bacteriocins from *Lb. plantarum* 35d (Messi *et al*., 2001) and *Lb. plantarum* SA6 (Rekhif *et al*., 1995).

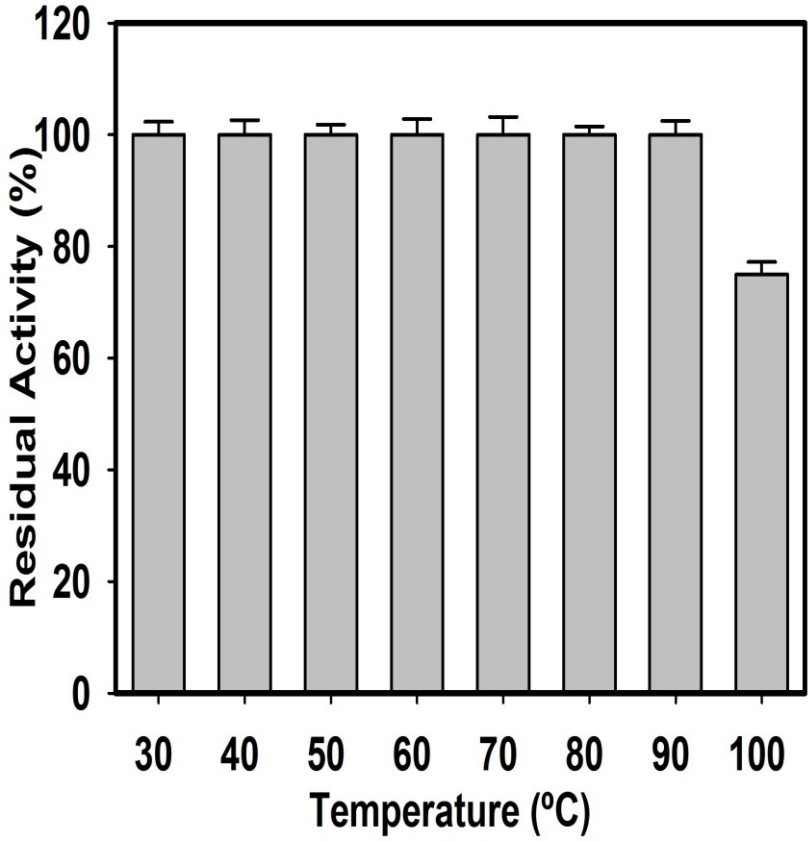

Fig. 4.3.5 Thermostability of bacteriocin from *Lb. plantarum* DM5. For thermostability the residual antimicrobial activity was studied after 90 min of heating at temperature ranging from 30 to 100ºC and compared with the highest activity of 6400 AU/ml at 37ºC. The mean value of three independent experiments of thermostability is presented with ± S.E.

The bacteriocin of *Lb. plantarum* DM5 was stable in a wide pH range from 4-8. It retained 100% activity between pH 5 to 7 (Fig. 4.3.6). The antimicrobial activity decreased by 20% and 75% at pH 2.0 and pH 8.0, respectively (Fig. 4.3.6 and Das and Goyal, 2013b). The antimicrobial activity was lost by 50% at pH 10, and no activity was found at pH 11 and pH 12 (Fig. 4.3.6). The thermostability and pH stability of bacteriocin from *Lb. plantarum* DM5 was similar to plantaricin C (Gonzalez *et al*., 1996), plantaricin 35d (Messi *et al*., 2001) and plantaricin ST16Pa (Todorov *et al*., 2011).

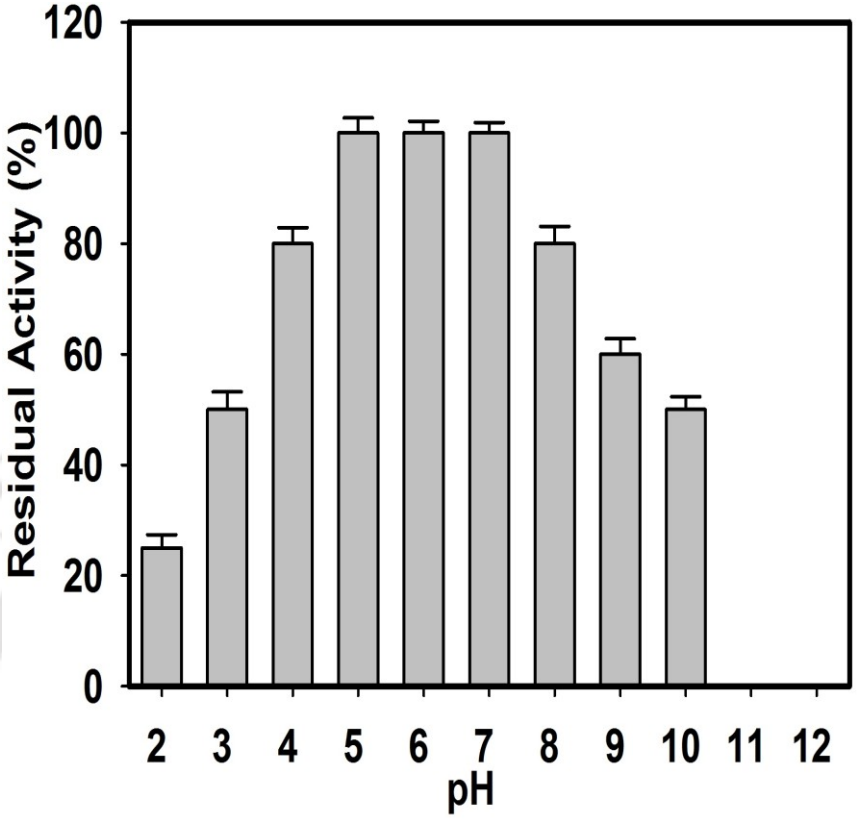

Fig. 4.3.6 pH stability of bacteriocin from *Lb. plantarum* DM5. For pH stability the residual activity was compared with the highest antimicrobial activity (6400 AU/ml) at pH 6.0.The mean value of three independent experiments of pH stability is presented with ± S.E.

4.3.5 Effect of enzymes on bacteriocin activity

Lactic acid bacteria are well known for production of antimicrobial compounds such as lactic acid, bacteriocin, reuterin, diacetyl and H_2O_2 (Galvez *et al*., 2007; De Vuyst and Leroy, 2007). The cell free supernatant of isolate *Lb. plantarum* DM5 was adjusted to pH 6.0 for eliminating the inhibitory effects of lactic acid in cell free extract. No change in antimicrobial activity of cell free supernatant was recorded when it was treated with catalase, indicating that H_2O_2 was not responsible for antagonistic activity present in cell free supernatant Fig. 4.3.7. The treatment of cell free

supernatant with proteolytic enzymes such as pepsin, trypsin or proteinase K at 37ºC for 1 h, completely destroyed the antimicrobial activity of the cell free supernatant as no zone of inhibition of *S. aureus* MTCC 737 was observed in NB agar plate at 37ºC after 24 h (Fig. 4.3.7).

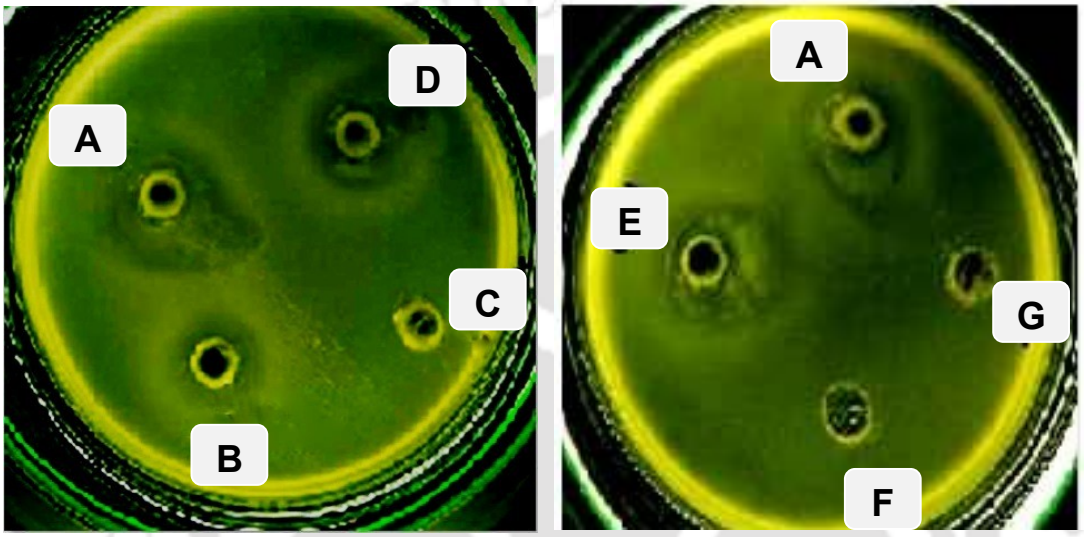

Fig. 4.3.7 Effect of enzymes (1 mg/ml) on antimicrobial activity of bacteriocin from *Lb. plantarum* DM5 at 37°C for 1 h. The enzyme treated bacteriocin was analyzed by agar well diffusion method against indicator strain *S. aureus* MTCC 737. A=bacteriocin without the treatment of enzymes, B=bacteriocin treated with catalase, C=bacteriocin treated with pepsin, D= bacteriocin treated with lysozyme, E=bacteriocin treated with α-amylase, F= bacteriocin treated with protinase-K, G= bacteriocin treated with trypsin.

The treatment with α-amylase and lysozyme did not affect the antimicrobial activity, suggesting that the activity was not due to any carbohydrate or lipid molecules present in the cell free supernatant (Fig. 4.3.7). Hence, the antimicrobial compound present in the cell free supernatant of isolate *Lactobacillus plantarum* DM5 was identified as bacteriocin due to its proteinaceous nature as the activity was completely destroyed by the proteolytic enzymes (Das and Goyal, 2013a). Similar

results were observed with plantaricin 35d (Messi *et al.*, 2001), plantaricin ST194BZ (Todorov and Dicks, 2005), plantaricin LR/14 (Tiwari and Srivastava, 2008), pediocin ST44AM (Todorov and Dicks, 2009b) and plantaricin ST16Pa (Todorov *et al*., 2011). Till date only two commercial probiotic strains *Lb. casei* Shirota and *Lb. johnsonii* La1 are reported to produce bacteriocins (Avonts *et al*., 2004).

4.3.6 Effect of organic solvents, surfactants, salts and detergents on bacteriocin activity

The antimicrobial activity of bacteriocin (plantaricin DM5) from *Lb. plantarum* DM5 was analyzed after treatment with organic solvents, surfactants, detergents, urea and NaCl by agar well diffusion method using *S. aureus* MTCC 737 as indicator strain (Table 4.3.3).

Table 4.3.3 Effect of organic solvents, surfactants, salts and detergents on plataricin DM5.

Reagents	Residual Activity[a]
Control[b]	100
Ethanol	100
Methanol	100
1-Butanol	100
Iso-propanol	100
Ethyl acetate	100
Acetone	100
SDS	100
Tween 80	100
Tween 20	100
Triton X-100	100
NaCl	100
Urea	100

[a]*compared with the highest antimicrobial activity (6400 AU/ml). All the individual experiments were carried out in triplicates and the results represent mean values with less than 1% of error and were not indicated.*

[b]*The cell free supernatant (pH 6.0) of Lb. plantarum DM5 without any organic solvents, salts, surfactants and detergents incubated at 37ºC for 2h. The antimicrobial activity of 6400 AU/ml was observed against indicator strain S. aureus by agar well diffusion method.*

It was observed that the bacteriocin activity of *Lb. plantarum* DM5 was not altered in presence of 1% (v/v) organic solvents and surfactants such as ethanol, methanol, 1-butanol, iso-propanol, ethyl acetate, acetone, sodium dodecyl sulphate (SDS), Tween 20, Tween 80, Triton X-100. The antimicrobial activity of bacteriocin also did not decrease when treated with 1% (w/v) urea and NaCl at 37ºC for 2 h. Similar observations were also reported for plantaricin 423 (Verellen *et al*., 1998), plantaricin C19 (Atrih *et al*., 2001), plantaricin L4/14 (Tiwari and Srivastava, 2008), pediocin ST44AM (Todorov and Dicks, 2009b) and plantaricin ST16Pa (Todorov *et al*., 2011). The high stability of planatricin DM5 suggests that it can be easily exploited in food industry as bio-perseverant and will not get affected during the food processing.

4.3.7 Growth of test organism in presence of plantaricin DM5

The effect of bacteriocin from *Lb. plantarum* DM5 on the growth of *E. coli* DH5α, *S. aureus* MTCC 737 and *L. monocytogenes* was studied at 37ºC for 18 h under shaking condition of 120 rpm and is shown in Fig. 4.3.8. Addition of cell free supernatant (bacteriocin activity of 6400 AU/ml; specific activity of 410 AU/mg) of isolate *Lb. plantarum* DM5 to early exponential phase (at 4h) of *E. coli* DH5α (Fig. 4.3.8A), *S. aureus* MTCC 737 (Fig. 4.3.8B) and *L. monocytogenes* (Fig. 4.3.8C) resulted in repression of growth for 18 h as observed by the reading cells absorbance (A_{600}). The absorbance of the cells of test cultures remained constant after the addition of cell free supernatant of *Lb. plantarum* DM5, however, the cell absorbance of test cultures without the addition of cell free supernatant increased progressively.

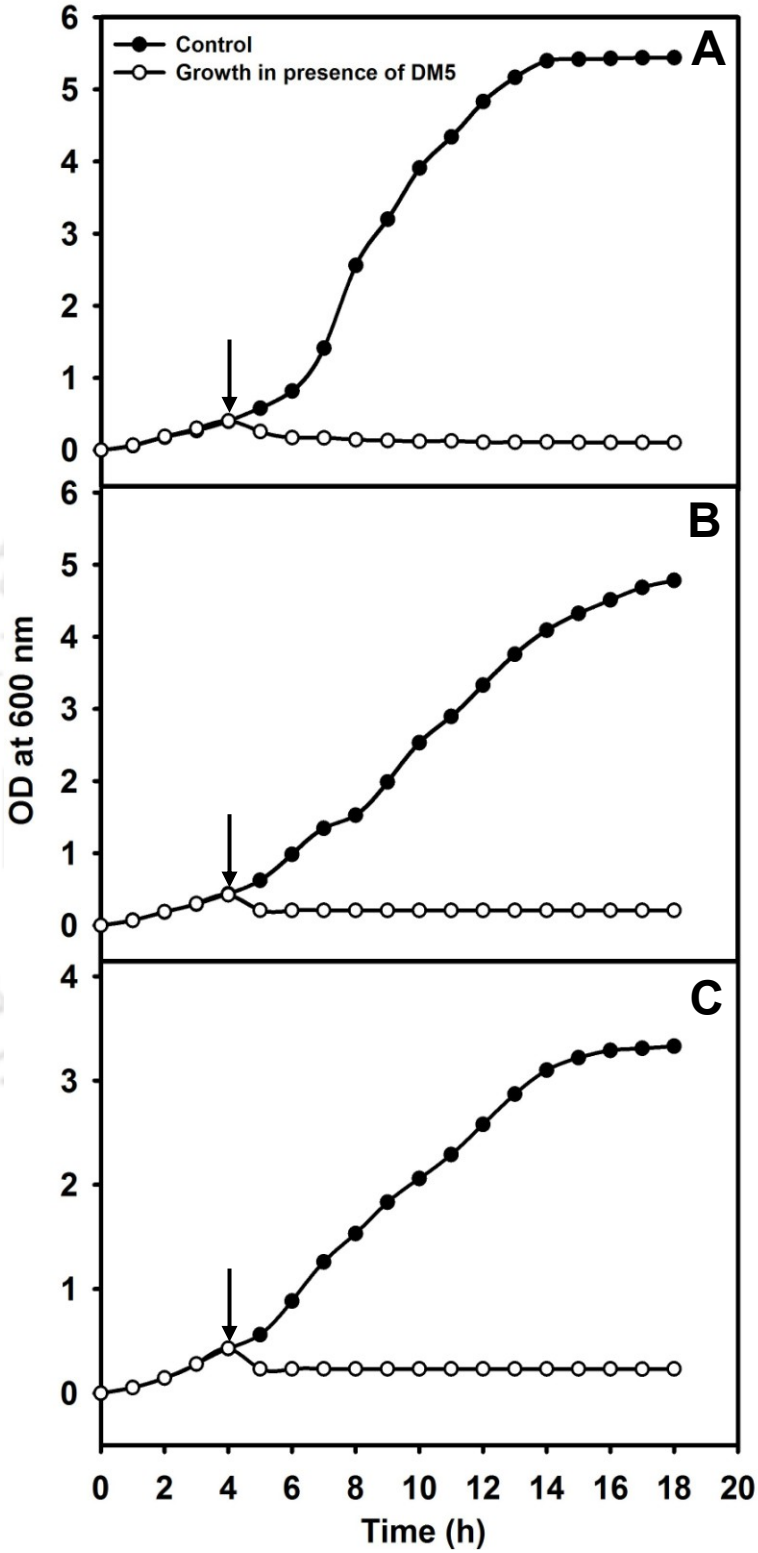

Fig. 4.3.8 Effect of bacteriocin from *Lb. plantarum* DM5 on the growth of (A) *E. coli* DH5α (B) *S. aureus* MTCC 737 and (C) *L. monocytogenes* at 37ºC for 18 h. The arrows indicate the addition of bacteriocin (specific activity 410 AU/mg). Standard deviation recorded from three repeats was less than 2% and is not indicated.

The number of viable cell of *E. coli* DH5α, *S. aureus* MTCC 737 and *L. monocyotgens* was reduced to $2.3x10^2$, $1.8x10^2$ and $0.6x10^2$ CFU/ml, respectively after bacteriocin treatment at 37ºC after 18 h. The bacteriocin untreated *E. coli* DH5α, *S. aureus* and *L. monocyotgens* showed $6.9x10^8$, $7.1x10^8$ and $8.3x10^7$ number of viable cell, respectively at 37ºC after 18 h. The drastic reduction of 99% of viable cells of *E. coli* DH5α, *S. aureus* and *L. monocyotogenes* indicated the potent bactericidal action of bacteriocin from *Lb. plantarum* DM5.

4.3.8 Visualization of test organism in presence of plantaricin DM5 by scanning and transmission electron microscopy

The cell morphology of *E. coli* DH5α, *S. aureus* MTCC 737 and *L. monocytogenes* MTCC 1143 treated with cell free supernatant (bacteriocin activity 6400 AU/ml; specific activity 410 AU/mg) of isolate *Lb. plantarum* DM5 for 18 h was observed by scanning electron microscopy (Fig. 4.3.9). The treated cells of *E. coli* DH5α (Fig. 4.3.9A2), *L. monocytogenes* (Fig. 4.3.9B2) and *S. aureus* (Fig. 4.3.9C2) showed the complete disruption of their cell wall. The cell wall of the treated cells was clearly deformed, vesiculated and the cell shape was disrupted as compared with respective untreated cells (Fig. 4.3.9A1, Fig. 4.3.9B1 and Fig. 4.3.9C1).

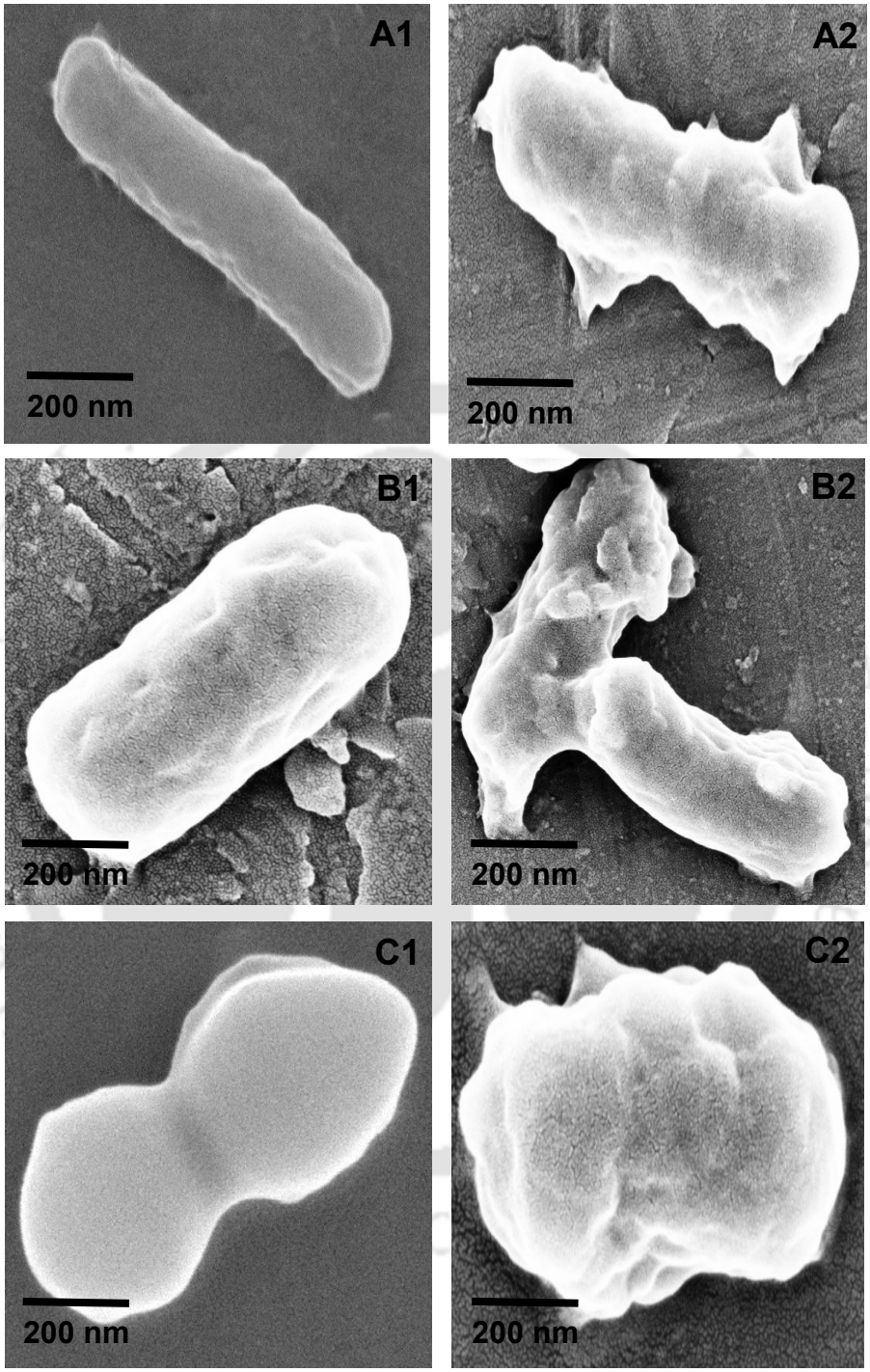

Fig. 4.3.9 Scanning electron microscopic image of treated (A) *E. coli* DH5α (B) *L. monocytogenes* and (C) *S. aureus* with cell free supernatant of *Lactobacillus plantarum* DM5 (6400 AU/ml). (1) untreated cell and (2) treated cell showing disruption in cell morphology.

The cell morphology of bacteriocin treated *E. coli* DH5α was also observed under transmission electron microscopy. It was found that the cell wall of treated *E. coli* DH5α was completely distorted and collapsed (Fig. 4.3.10). Similar observation was also found in case of *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 treated with pediocin PA-1 from *Pediococcus pentosaceus* (Todorov and Dicks, 2009). It has been reported that the bacteriocin from lactic acid bacteria show muralytic activity and disrupt the cell wall of test microorganism (Nilsen *et al*., 2003; Galvez et al. 2007).

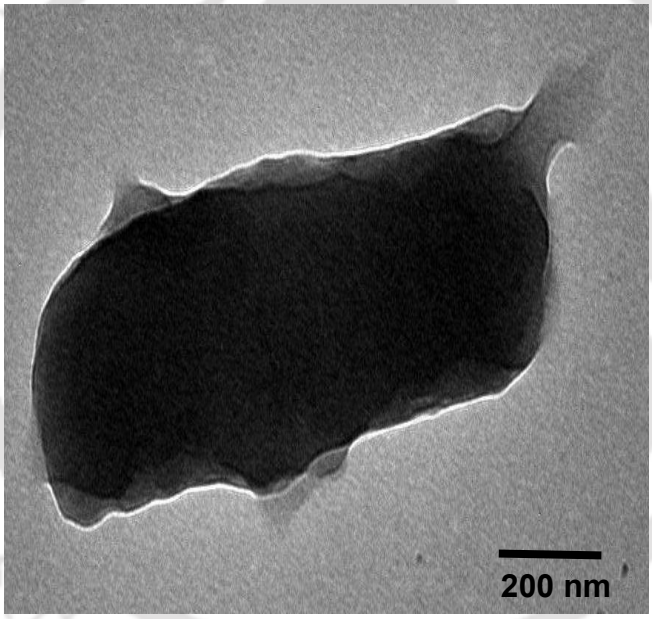

Fig. 4.3.10 Transmission electron microscopic image of bacteriocin treated *Escherichia coli* DH5α showing complete disruption of the cell wall.

4.3.9 Analysis of cell lysis of plantaricin DM5 by flow cytometry

The histogram plots obtained from the flow cytometer displayed a single measurement parameter (light scatter intensity) on the x-axis and the number of events (cell count) on the y-axis. In the histogram plots, two markers were used to specify a range of events for a single parameter. The marker M1 was placed around the negative

peak of the propidium iodide, indicating the proportion of live cells and the marker M2 was placed to designate the positive peak of the propidium iodide, indicating the proportion of dead cells. Propidium iodide (PI) binds to double stranded DNA by intercalating between base pairs and is excluded from the viable cells with intact plasma membranes. The histogram plot of the nonviable cells of *E. coli* DH5α was displayed in Fig. 4.3.11.

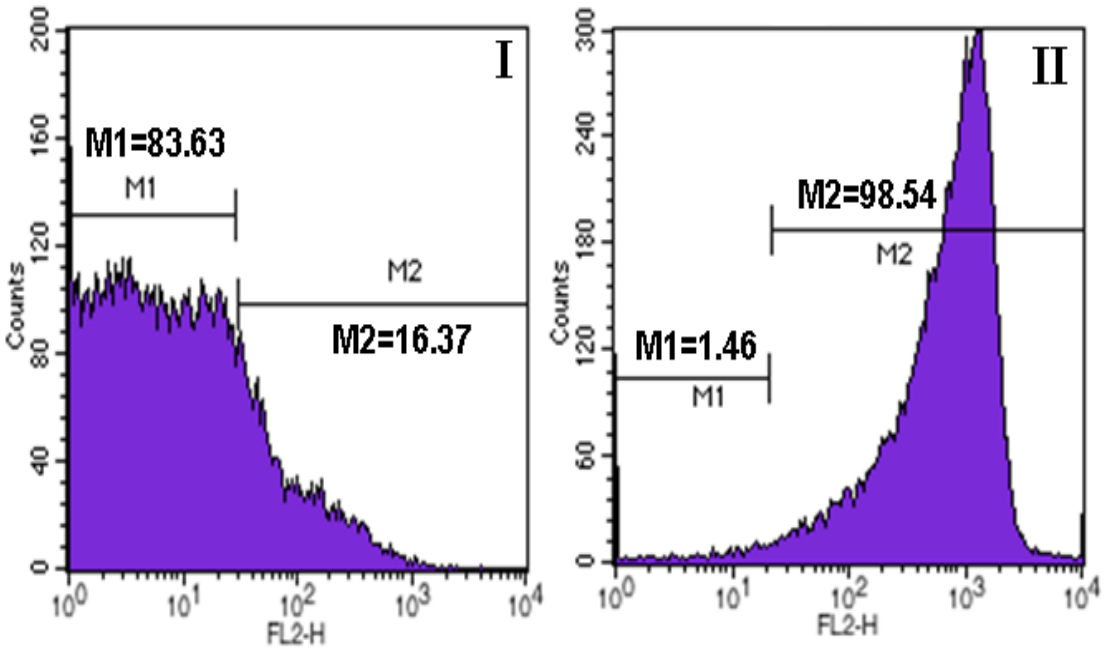

Fig. 4.3.11 Histogram plots of non viable cells of *E. coli* DH5α stained with propidium iodide. I) Untreated cell, II) Cell treated with bacteriocin from *Lb. plantarum* DM5 at 37ºC for 18 h. M1= PI unstained region (showing population of viable cell), M2= PI stained region (showing population of nonviable cell).

In the M1 region of the control *i.e.* without the treatment of plantaricin DM5 showed 83.63% of viable cells and 16.37% non viable cells present in the M2 region. The histogram peak was positively shifted towards the M2 region in bacteriocin treated cells of *E. coli* DH5α as shown in Fig. 4.3.11(II). The treated cells of *E. coli* DH5α showed 1.46% of the viable cells in the M1 region and 98.54% of the nonviable

cells in the M2 region. The mean fluorescence intensity also increased from 121 to 860 in case of treated cell as the treated cells were taking large amount of propidium iodide, as a result the increase in fluorescence intensity in terms of peak height and sharpness was observed (Fig. 4.3.11, II).

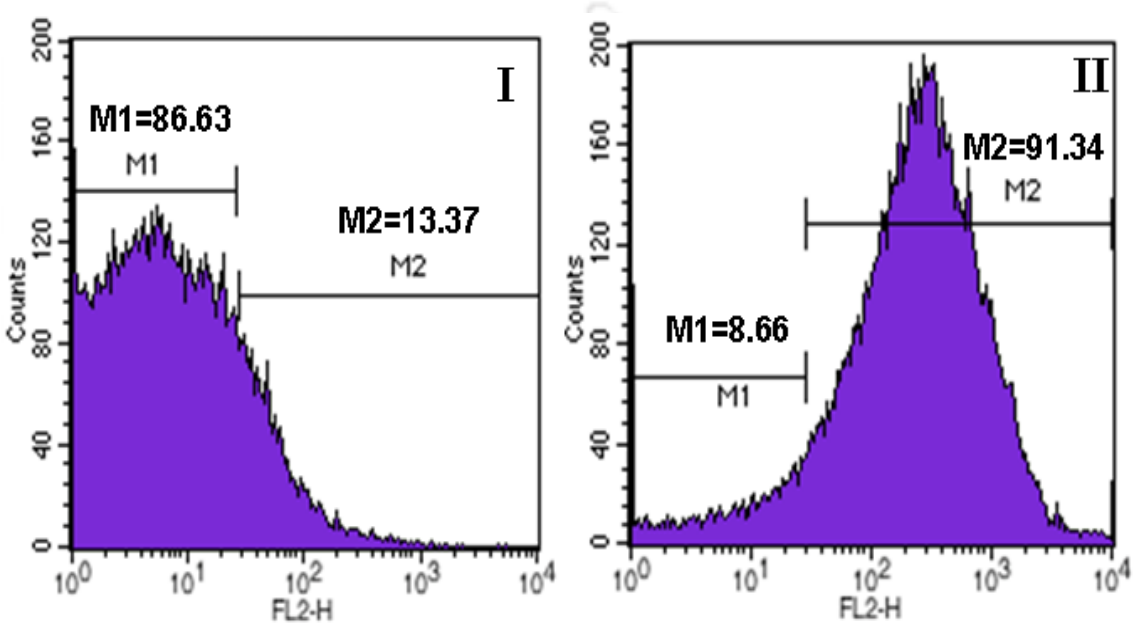

Fig. 4.3.12 Histogram plots of non viable cells of *S. aureus* stained with propidium iodide. I) Untreated cell, II) Treated cell with bacteriocin from *Lb. plantarum* DM5 at 37ºC for 18 h. M1= PI unstained region (showing population of viable cell), M2= PI stained region (showing population of nonviable cell).

The similar pattern of the cell viability was also observed in case of *S. aureus* MTCC 737 (Fig. 4.3.12). In the untreated sample, the number of viable cells (86.63%) in the M1 region was higher as compared with bacteriocin treated *S. aureus* MTCC 737 sample (8.66%). The M2 region of the treated cell showed positive peak shifting, containing 91.34% of non-viable cell (Das and Goyal, 2013a). The sharpness of the peak in the treated *S. aureus* MTCC 737 also indicated higher fluorescence intensity (443) than control (72). The cell viability assessment by flow cytometer indicated that

the bacteriocin from *Lb. plantarum* DM5 (plantaricin DM5) had bactericidal effect rather than bacteriostatic effect on the test organisms.

4.3.10 Purification of plantaricin DM5 by ammonium sulphate precipitation

The cell free supernatant of *Lb. plantarum* DM5 containing bacteriocin (6400 AU/ml) was purified by ammonium sulphate precipitation method. The precipitate obtained within 40-80% ammonium sulphate saturation showed specific activity of 2196 AU/mg with 5.4 fold increase in specific activity (Table 4.3.3).

Table 4.3.3 Purification of bacteriocin from *Lb. plantarum* DM5.

	Activity (AU/ml)	Total Units (AU)	Protein Conc. (mg/ml)	Total protein (mg)	Specific Activity (AU/mg)	Overall Activity Yield (%)	Fold Purifi-cation
Crude (cell free supernatant)	6400	640000	15.6	1560	410	100	–
Ammonium sulphate precipitation (80%)	12800	256000	5.83	116.6	2196	40	5.4
Ion exchange chromatography by CM sepharose	3200	32000	1.02	10.2	3137	5	7.7
Gel filtration by Sephacryl S-200HR	1600	6400	0.15	0.60	10667	1	26

No zone of inhibition was observed with 40% ammonium sulphate purified fraction of cell free supernatant against *S. aureus* (Fig. 4.3.13III), however, 80% ammonium sulphate precipitated fraction displayed significant growth inhibition against the indicator strain *S. aureus* as prominent clear zone was observed (Fig. 4.3.13, IV). The 50 µl of 25 mM ammonium acetate buffer was taken as negative control, which also showed no zone of inhibition. After 80% ammonium sulphate precipitation the partially purified bacteriocin from *Lb. plantarum* DM5 gave specific activity of 2196 AU/mg with 5.3 fold of purification and the overall activity yield was

40%. Similar results have been reported earlier where bacteriocins from lactic acid bacteria are precipitated within the range of 40-80% ammonium sulphate saturation (Nilsen *et al*., 2003; Todorov and Dicks, 2009b).

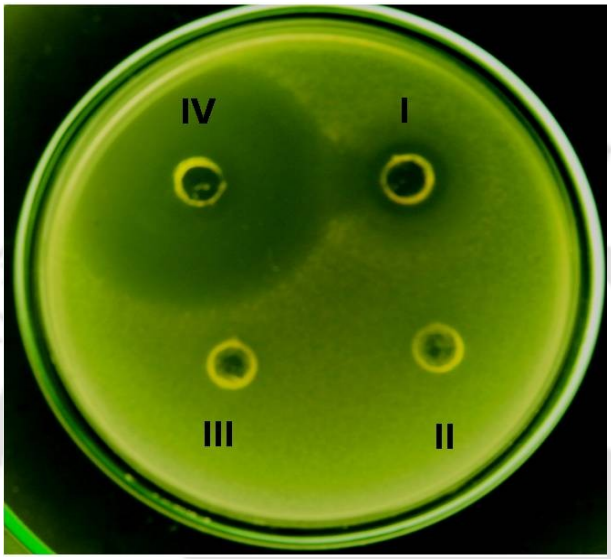

Fig. 4.3.13 *Lactobacillus plantarum* DM5 showed inhibitory activity by agar well diffusion method against indicator strain *S. aureus* MTCC 737. (I) Cell free extract (adjusted to pH 6.0), (II) 25 mM ammonium acetate buffer (pH 6.0), (III) 0-40% ammonium sulphate purified fraction and (IV) 40-80% ammonium sulphate purified fraction.

4.3.11 Purification of plantaricin DM5 by cation exchange chromatography

The partially purified bacteriocin by 80% ammonium sulphate precipitation with specific activity of 2196 AU/mg was loaded on CM Sepharose column pre-equilibrated with 25 mM ammonium acetate buffer (pH 5.4) and the bound proteins were eluted with a linear gradient of 0-0.5 M NaCl. The bacteriocin was eluted in form of single-symmetrical peak (Fig. 4.3.14) at about 0.24 M to 0.27 M sodium chloride (NaCl). The bacteriocin activity was confined within 23th to 27th fraction which also showed maximum protein content (A_{280}) as shown in Fig. 4.3.14. All the five fractions were pooled, which gave specific activity of 3137 AU/mg with 7.7 fold purification

(Table 4.3.3). The pooled fraction was dialyzed and used for gel filtration and SDS-PAGE analysis as described in Sections 4.3.16 and 4.3.17, respectively.

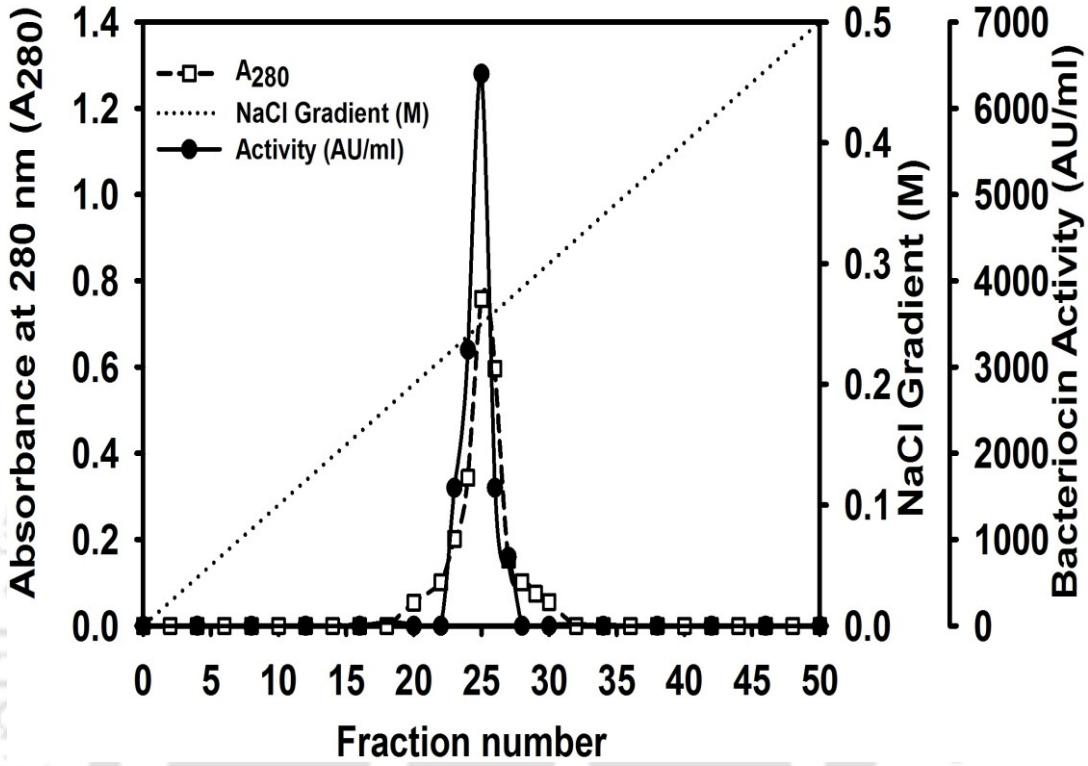

Fig. 4.3.14 Elution profile of 80% ammonium sulphate precipitated bacteriocin by cation exchange chromatography using CM sepharose. The bound proteins were eluted with linear salt gradient in range of 0.0-0.5M NaCl with a flow rate of 1 ml/min and fractions of 2 ml were collected. The fractions were assayed for bacteriocin activity (--▲--) and protein concentration (--□--).

4.3.12 Purification of plantaricin DM5 by gel filtration

The partially purified bacteriocin (3137 AU/mg) by cation exchange chromatography was subjected to next step of purification by gel filtration using Sephacryl S-200HR as matrix. The sample eluted in form of two consecutive peaks (Fig. 4.3.15) as observed by A_{280} measurements, however, the bacteriocin activity was found only to the first peak of A_{280}, within 13^{th} to 18^{th} fraction. Among these fractions, the fraction 14 and 15 were pooled as they showed maximum bacteriocin activity and

higher protein content (Fig. 4.3.15). The pooled fractions showed specific activity of 10667 AU/mg with 26 fold purification (Table 4.3.3). The pooled plantaricin DM5 sample was lyophilised and subjected to SDS-PAGE for analyzing its purity and molecular mass.

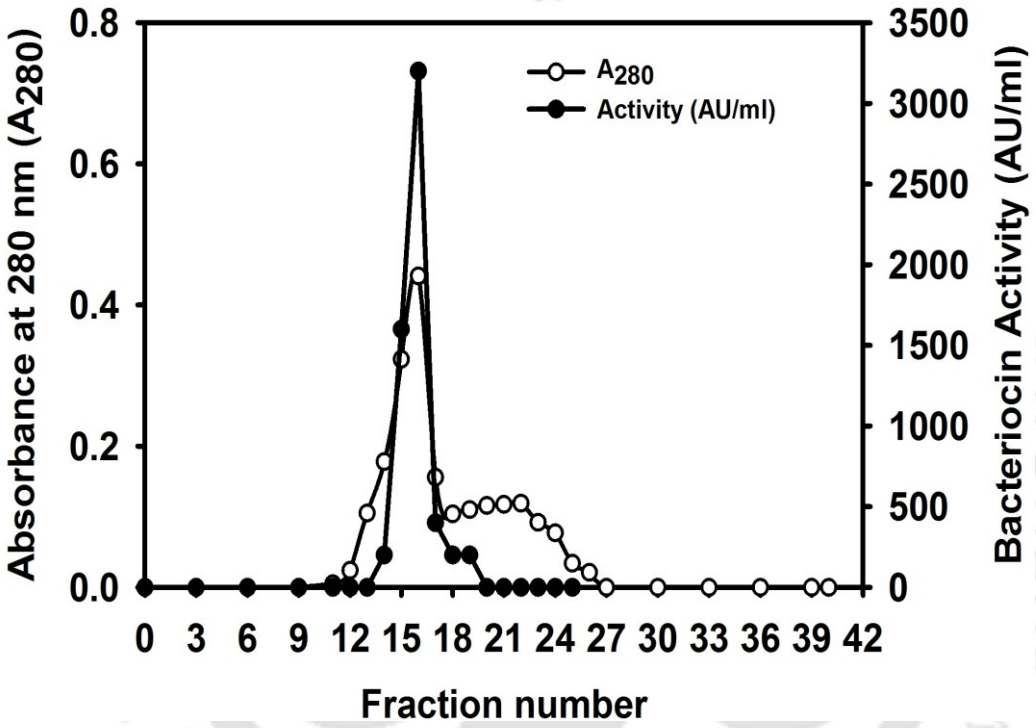

Fig. 4.3.15 Elution profile of bacteriocin purified by cation exchange chromatography from gel filtration column using Sephacryl S-200HR as matrix. The flow rate was 0.5 ml/min and fractions of 2 ml were collected. The fractions were assayed for enzyme activity (--■--) and protein concentration (--○--).

4.3.13 SDS-PAGE analysis and *in situ* assay of plantaricin DM5

The molecular mass of bacteriocin by 80% ammonium sulphate precipitation was found to be approximately, 15.2 kDa as determined by 15% SDS-PAGE (Fig. 4.3.16A, Lane 2). The result was also confirmed by an *in situ* agar gel overlay method using *S. aureus* MTCC 737 as indicator strain. A prominent inhibition zone (Fig. 4.3.16B) was observed corresponding to the protein band of molecular weight 15.2

kDa, in the activity gel against the indicator strain *S. aureus* MTCC 737. The molecular mass of bacteriocin was estimated to be 15.2 kDa from the graph of relative mobility (Rf) versus log molecular weight (Log MW) of the standard protein molecular weight markers (Fig. 4.3.17). The bacteriocin from *Lb. plantarum* was DM5 was larger than bacteriocin produced by *Lb. plantarum* 35d (Mol. mass 4.5 kDa) (Messi *et al*., 2001), *Lb. plantarum* ST194BZ (Mol. mass 14 kDa*)* and *Pediococcus acidilactici* LAB5 (Mol. mass 10.3 kDa) (Mandal *et al*., 2008) but smaller than bacteriocin produced by and *Pediococcus pentosaceus* FBB61 (Mol. mass 80 kDa) (Piva and Headon, 1994) and *Pediococcus pentosaceus* ACCEL (Mol. mass 17.5 kDa) (Wu *et al*., 2004).

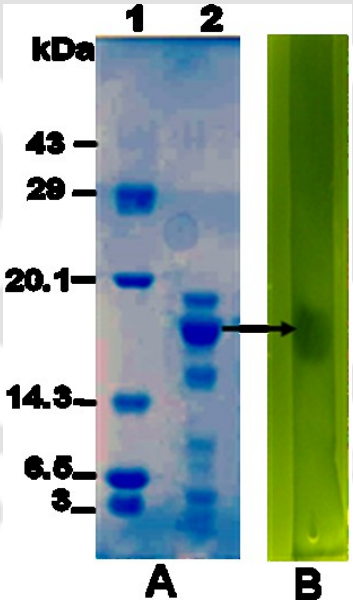

Fig. 4.3.16 SDS-PAGE (15% gel) analysis and *in situ* detection of antimicrobial activity of partially purified bacteriocin from *Lactobacillus plantarum* DM5 (A) Gel stained with Coomassie Brilliant Blue R250; Lane 1: Molecular mass marker (3.5 kDa - 43 kDa) from Bangalore Genei, India, Lane 2: 80% ammonium sulphate purified fraction of bacteriocin, (B) Clear zone of inhibition of indicator strain *S. aureus* corresponding to the position of the peptide band of 15.2 kDa. The gel was overlaid with indicator strain *S. aureus* (~10^6) embedded in NB soft agar (0.7%) and incubated at 37ºC for 24 h.

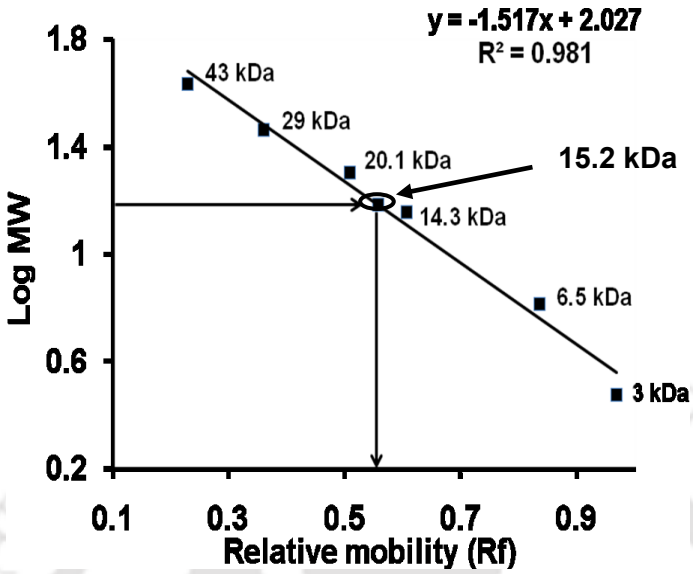

Fig. 4.3.17 Molecular mass determination of purified bacteriocin by relative mobility (Rf) versus log molecular weight (log Mw) plot. The standard markers used are Ovalbumin (43 kDa), Carbonic Anhydrase (29 kDa), Soyabean Trypsin Inhibitor (20.1 kDa), Lysozyme (14.3 kDa), Aprotinin (6.5 kDa) and Insulin (3kDa).

The partially purified bacteriocin by 80% ammonium sulphate precipitation was further purified by cation exchange chromatography and showed multiple band when it was run on SDS-PAGE (Fig. 4.3.18, Lane 2). In order to obtain the purified bacteriocin without any contaminating proteins, plantaracin DM5 containing sample was subjected to next stage of purification by gel filtration. The purified plantaricin DM5 by gel filtration showed single band when subjected to SDS-PAGE analysis (Fig. 4.3.18, Lane 3) confirming 15.2 kDa molecular size and the homogeneity of protein. The bacteriocin from *Lb. plantarum* DM5 was larger than most bacteriocin from *Lb. plantarum* which are generally grouped into class II bacteriocin (<10 kDa and heat stable) (Klaenhammer, 1993; Carolissen-Mackay *et al*., 1997). The thermostability and bactericidal nature of plantaracin DM5 with its large molecular

mass reveals some novel features of this bacteriocin which can be potentially utilized in food industry.

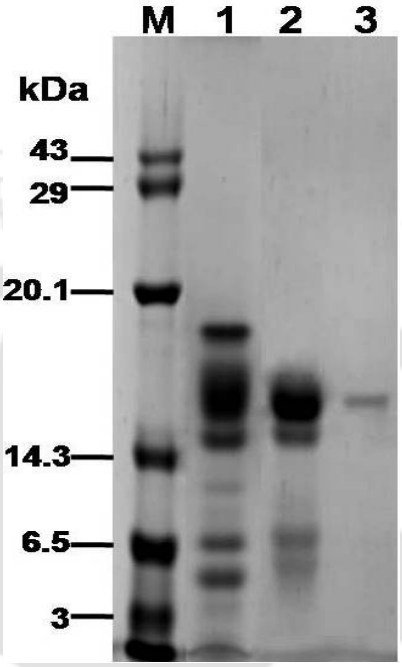

Fig. 4.3.18 SDS-PAGE (15% gel) analysis purified bacteriocin from *Lb. plantarum* DM5. Lane M: Molecular mass marker (3.5 kDa - 43 kDa) from Bangalore Genei, India, Lane 1: 80% ammonium sulphate purified fraction of bacteriocin, Lane 2: partially purified bacteriocin by cation exchange chromatography, Lane 3: purified bacteriocin by gel filtration showing single band. The gel was stained with Coomassie Brilliant Blue R250.

4.3.14 *In vitro* cyotoxicity assay of bacteriocin from *Lb. plantarum* DM5

The *in vitro* cytotoxic test of partially purified plantaricin DM5 (specific activity 2196 AU/mg) by 80% ammonium sulphate precipitation on HEK-293 and HeLa cells was carried out by MTT assay. The HEK-293 and HeLa cells were exposed to various concentrations of bacteriocin dissolved in serum free DMEM medium at 10, 20, 50, 100, 250, 500 and 1000 µg/ml for 3, 6, 12, 24 and 48 h. It was observed that the viability of both HEK-293 and HeLa cells remained constant at all concentrations of bacteriocin revealing its non-toxic nature (Fig. 6.3.19).

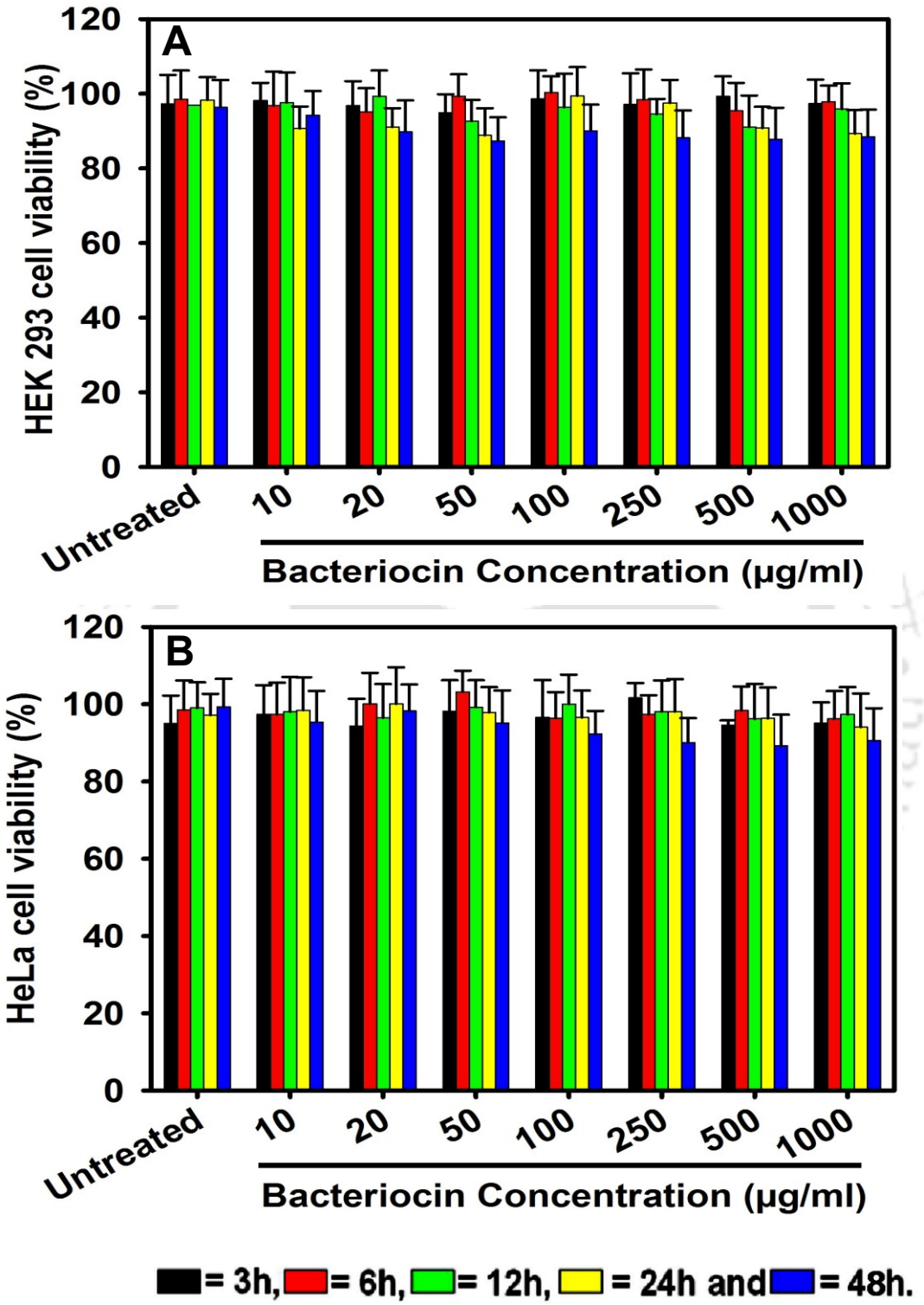

Fig. 6.3.13 The *in vitro* cytotoxicity assay showing the cell viability (%) of (A) HEK-293 and (B) HeLa cells treated with various concentrations of bacteriocin (10-1000 µg/ml) with a specific activity of 2196 AU/mg over a period of 3- 48 h of incubation.

In presence of 1000 µg/ml of bacteriocin, almost 85-90% of HEK-293 and HeLa cells remained viable over an incubation period of 48 h, however bacteriocin did not influence the cell proliferation. It was observed that the viability of treated HEK cells (Fig. 4.3.19A) and HeLa cells (Fig. 4.3.19B) was alike as compared with the respective untreated cells in serum free medium with no significant toxic effect at all concentrations. The data indicated that in presence of bacteriocin the mammalian cells were metabolically active without any toxic effect. All these results suggested that the bacteriocin from *Lb. plantarum* DM5 possessed the ability to eliminate or retard the growth of potent food borne pathogens *viz. S. aureus, E. coli* and *L. monocytogens* without any cytotoxic effect on mammalian cells and can be exploited for bio-preservation of various food, feed and beverages.

4.4 Conclusions

The antimicrobial activity of probiotic *Lb. plantarum* DM5 isolated from an ethnic fermented beverage Marcha of Sikkim was studied. The cell free supernatant of *Lb. plantarum* DM5 exhibited bacteriocin activity of 6400 AU/ml against gram positive indicator strain *S. aureus* 737 and gram negative indicator strain *E. coli* DH5α at 37ºC. The various physicochemical factors affected the production of antimicrobial compound as well as its activity. The highest antimicrobial activity (6400 AU/ml) was achieved at 37ºC under static condition when compared with shaking at 150 rpm at 37ºC, signifying the microaerophilic nature of *Lb. plantarum* DM5. The maximum bacteriocin activity was observed with an initial medium pH of 6.0 to 6.5 and the bacteriocin activity was lost by 91% and 87% at initial medium pH of 4.0 and 4.5, respectively. Growth of *Lb. plantarum* DM5 in presence of 20 g/L glucose and 20 g/L maltose yielded the same activity of 6400 AU/ml but in case of 20g/L of maltose the specific activity was higher by 3% with ± 0.8% standard error. The antimicrobial activity reduced by 50% in presence of 20 g/L of raffinose or 20g/L of mannose and 25% in presence of 20 g/L sucrose or 20 g/L fructose, respectively. The bacteriocin activity of *Lb. plantarum* DM5 was unaffected by heating upto 100ºC for 60 min and 75% activity still remained after a heat treatment at 100ºC for 90 min. The antimicrobial activity of *Lb. plantarum* DM5 was stable in a wide pH range from 4-8 while 100% activity was recorded between pH 5 to 7. The antimicrobial activity was lost by 50% at pH 10 and no activity was found in pH 11 and pH 12.

The bacteriocin from *Lb. plantarum* DM5 showed bactericidal action by repressing the growth of *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* well known virulent food borne pathogens responsible for listeriosis

and food poisoning. The cell morphology of the treated *E. coli*, *S. aureus* and *L. monocytogenes* was completely deformed as revealed by scanning electron microscopy, suggesting the high potential of *Lb. plantarum* DM5 as natural preservatives in food industry. The bactericidal action of bacteriocin from isolate was analyzed by flow cytometry. The bactericidal mode of action of bacteriocin gives a competitive advantage to *Lb. plantarum* DM5 used as probiotic and bio-preservative.

The bacteriocin produced by *Lb. plantarum* DM5 purified by 80% ammonium sulphate precipitation gave 2196 AU/mg with 5.3 fold purification. The bacteriocin from *Lb. plantarum* DM5 was further purified by cation exchange chromatography and gel filtration, which showed single distinct band of molecular mass of 15.2 kDa on 15% SDS PAGE, confirming the homogeneity of the protein. The result was also confirmed by an *in situ* agar gel overlay method which gave a prominent inhibition zone of *S. aureus* MTCC 737 in agar plate. The purified bacteriocin by gel filtration showed specific activity of 10667 AU/mg with 26 fold purification. The bactertiocin from *Lb. plantarum* DM5 was subjected to cytotoxicity assay using HEK-293 and HeLa cells in order to commercially exploit it as food additives and bio-preservative. The bacteriocin from *Lb. plantarum* DM5 exerted considerable non toxic effect on HEK-293 and HeLa cells revealing its biocompatible nature and can be exploited for bio-preservation of various food, feed and beverages.

References

Ahern, M., Verschueren, S. and van-Sinderen, D. (2003) Isolation and characterisation of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. *FEMS Microbiol. Lett*., 220, 127-131.

Atrih, A., Rekhif, N., Moir, A.J.G., Lebrihi, A. and Lefebvre, G. (2001) Mode of action, purification and amino acid sequence of plantaricin C19, an anti-Listeria bacteriocin produced by *Lactobacillus plantarum* C19. *Int. J. Food Microbiol*., 68, 93-109.

Avonts, L., Van Uytven, E. and De Vuyst, L. (2004) Cell growth and bacteriocin production of probiotic *Lactobacillus* strains in different media. *Int. Dairy J*., 14, 947-955.

Bhunia, A.K., Johnson, M.C. and Ray, B. (1987) Direct detection of an antiniicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Ind. Microbiol*., 2, 319-322.

Carolissen-Mackay, V., Arendse, G. and Hastings, J.W. (1997) Purification of bacteriocins of lactic acid bacteria: problems and pointers. *Int. J. Food Microbiol*., 34, 1-16.

Das, D. and Goyal, A. (2013a) Potential probiotic attributes and antagonistic activity of an indigenous isolate *Lactobacillus plantarum* DM5 from an ethnic fermented beverage Marcha of north eastern Himalayas. *Int. J. Food Sci. Nutr.* (accepted).

Das, D. and Goyal, A. (2013b) Anti-listerial bactericidal activity of *Lactobacillus plantarum* DM5 isolated from fermented beverage Marcha. *Probiotics Antimicrob. Prot*., 5, 206-215.

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

De Vuyst, L. and Leroy, F., (2007) Bacteriocins from lactic acid bacteria: production, purification and food applications. *J. Mol. Microbiol. Biotechnol.,* 13, 194-199.

Delves-Broughton, J. (1990) Nisin and its uses as a food preservative. *Food Technol*., 44:100-117.

Enan, G., Essawy, A.A., Uyttendaele, M. and Debevere, J. (1996) Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: Characterization, production and bactericidal action of plantaricin UG1. *Int. J. Microbiol.,* 30, 189-215.

Galvez, A., Abriouel, H., Lopez, R.L. and Omar, N.B. (2007) Bacteriocin-based strategies for food biopreservation. *Int. J. Food Microbiol*., 120, 51-70.

Gonzalez, B., Glaasker, E., Kunji, E.R.S., Driessen, A.J.M., Suarez, J.E. and Konings, W.N. (1996) Bactericidal mode of action of plantaricin C. *Appl. Environ, Micrbiol*., 62, 2701-2709.

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

Holzapfel, W.H., Geisen, R. and Schillinger, U. (1995) Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. *Int. J. Food Microbiol*., 24, 343-362.

Hurtado, A., Othman, N.B., Chammem, N., Hamdi, M., Ferrer, S., Reguant, C., Bordons, A. and Rozes, N. (2011) Characterization of *Lactobacillus* isolates

from fermented olives and their bacteriocin gene profiles. *Food Microbiol*., 28, 1514-1518.

Klaenhammer, T.R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol. Rev*., 12, 39-86.

Klein, G., Pack, A., Bonaparte, C. and Reuter, G. (1998) Taxonomy and physiology of probiotic lactic acid bacteria. *Int. J. Food Microbiol*., 41,103-125.

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

Loir, Y.L, Baron, F. And Gautier, M. (2003) *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res*., 2, 63-76.

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.

Mandal, V., Sen, S.K. and Mandal, N.C. (2008) Optimized culture conditions for bacteriocin production by *Pediococcus acidilactici* LAB5 and its characterization. *Indian J Biochem Biophys* 45, 106-110.

Marth, E.H. (1998) Extended shelf life refrigerated foods: microbiological quality and safety. *Food Technol*., 52, 57-62.

Messi, P., Bondi, M., Sabia, C., Battini, R. and Manicardi, G. (2001) Detection and preliminary characterization of a bacteriocin (plantaricin 35d) produced by a *Lactobacillus plantarum* strain. *Int. J. Food Microbiol*., 64, 193-198.

Mook, P., O'Brien, S.J. and Gillespie, I.A. (2011) Concurrent conditions and human listeriosis, England, 1999-2009. *Emerg. Infect. Dis*., 17, 38-43.

Nissen, L., Chingwaru, W., Sgorbati, B., Biavati, B. and Cencic, A. (2009) Gut health promoting activity of new putative probiotic protective *Lactobacillus* spp.

Strains: a functional study in the small intestinal cell model. *Int. J. Food Microbiol*., 135, 288-294.

Nithya, V. and Halami, P.M. (2012) Antibacterial peptides, probiotic properties and biopreservative efficacy of native *Bacillus* species isolated from different food sources. *Probiotics Antimicro. Prot*., 4, 279-290.

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. *Bioresour. Technol.,* 101, 6852-6855.

Piva, A and Headon, D.R. (1994) Pediocin A, a bacteriocin produced by *Pediococcus pentosaceus* FBB61. *Microbiol.,* 140,697-702.

Rekhif, N., Atrih, A., Michel, M. and Lefebvre, G. (1995) Activity of plantaricin SA6, a bacteriocin produced by *Lactobacillus plantarum* SA6 isolated from fermented sausages, *J. Appl. Bacteriol*., 78:349-358.

Ross, P.P., Morgan, S. and Hill, C. (2002) Preservation and fermentation: past, present and future. *Int. J. Food Microbiol*., 79, 3-16.

Singh, E.J., Singh, N.K.S. and Singh, N.R. (2009) Biodiversity conservation and natural resources in North East India with special reference to Manipur. *NeBio* 1, 42-47.

Thokchom, S. and Joshi, S.R. (2012) Antibiotic resistance and probiotic properties of dominant lactic microflora from *Tungrymbai*, an ethnic fermented soybean food of India. *J. Microbiol*., 50, 535-539.

Tiwari, S.K. and Srivastava, S. (2008) Characterization of a bacteriocin from *Lactobacillus plantarum* strain LR/14. *Food Biotechnol*., 22, 247-261.

Todorov, S.D. and Dicks, L.M.T (2005) Effect of growth medium on bacteriocin production by *Lactobacillus plantarum* ST194BZ, a Strain isolated from Boza. *Food Technol. Biotechnol*., 43, 165-173.

Todorov, S.D. and Dicks, L.M.T. (2009a) Effect of modified MRS medium on production and purification of antimicrobial peptide ST4SA produced by *Enterococcus mundtii*. *Anaerobe*., 15, 65-73.

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

Todorov, S.D. and Franco, B.D.G.M. (2010) *Lactobacillus Plantarum*: Characterization of the species and application in food production. *Food Rev. Int.*, 26, 205-229.

Todorov, S.D., Prevost, H., Lebois, M., Dousset, X., LeBlanc, J.G. and Franco, B.D.G.M. (2011) Bacteriocinogenic *Lactobacillus plantarum* ST16Pa isolated from papaya (Carica papaya) -From isolation to application: Characterization of a bacteriocin. *Food Res. Int*., 44, 1351-1363.

van Meerloo, J., Kaspers, G.J. and Cloos, J. (2011) Cell sensitivity assays: the MTT assay. *Methods Mol. Biol.,* 731, 237-245.

van Reenen, C.A., Dicks, L.M.T. and Chikindas, M.L. (1998) Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *J. Appl. Microbiol*., 84, 1131-1137.

Vera Pingitore, E., Salvucci, E., Sesma, F. and Nader, M.E. (2007) Different strategies for purification of antimicrobial peptides from lactic acid bacteria. In: Mendez-

Vilas A (ed) Communicating current research and educational topics and trends in applied microbiology. *Formatex*, Badajoz, pp 557-568.

Verellen, T.L.J., Bruggeman, G., Van Reenen, C.A., Dicks, L.M.T. and Vandamme, E.J. (1998) Fermentation optimisation of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423. *J. Ferment. Bioeng.,* 86, 174-179.

Wu, C.W., Yin, L.J. and Jiang, S.T. (2004) Purification and Characterization of Bacteriocin from *Pediococcus pentosaceus* ACCEL. *J. Agric. Food Chem*., 52, 1146-1151.

Yoon, K.Y., Woodams, E.E. and Hang, Y.D. (2006) Production of probiotic cabbage juice by lactic acid bacteria. *Biores. Technol*., 97, 1427-1430.

Chapter 5

Production, purification and functional characterization of glucansucrase from *Lactobacillus plantarum* DM5

5.1 Introduction

The glucansucrases, commonly referred to as glucosyltransferases (GTFs) (Leemuhis *et al*., 2013) from lactic acid bacteria are known to catalyze the transfer of glucosyl units from the cleavage of sucrose to a growing α-glucan chain (Purama and Goyal, 2008a; Robyt *et al*., 2008). Glucansucrase is grouped into glycoside hydrolase family 70 (GH70) according to the CAZy classification system (Cantarel *et al*., 2009) as it shows sequence similarity to GH13 α-amylases and GH77 amylomaltases (Majumder *et al*., 2008; Kralj *et al*., 2011). Glucansucrases are also often named according to the product they synthesize, *e.g.* dextransucrase that synthesize dextran with α-(1→6) linkages and alternansucrase that synthesize alternan with alternating α-(1→3) and α-(1→6) linkages (Leemuhis *et al*., 2013). So far, α-glucan formation by extracellular glucansucrase has been reported from the lactic acid bacteria of genera *Lactobacillus* (Pijning *et al.,* 2008; Leemuhis *et al*., 2013), *Leuconostoc* (Goyal and Katiyar, 1996; Purama and Goyal, 2008a) *Streptococcus* (Leemuhis *et al*., 2013),

Pediococcous (Patel *et al.,* 2010; Leemuhis *et al*., 2013) and *Weissella* (Shukla and Goyal, 2011a; Rao and Goyal, 2013). The glucan produced by glucansucrase from *Lactobacillus* spp. has found numerous potential applications such as viscosifying and water-binding agent in food and non-food industry (Purama and Goyal, 2008a; Badel *et al*., 2011).

Among all *Lactobacillus* spp., *Lactobacillus reuteri* 121, *Lactobacillus sakei*, *Lactobacillus fermentum* and *Lactobacillus parabuchneri* are known to produce glucansucrase (van Hijum *et al*., 2006; van Leeuwen *et al*., 2009). Recently a novel strain *Lactobacillus satsumensis* isolated from a fermented beverage starter culture produced extracellular glucansucrase that synthesized dextran (Cote *et al*., 2012). Several methods such as fractionation by polyethylene glycol, ultra-filtration, precipitation by salt, glycerol and alcohol, chromatography and phase-partitioning are used for purification of glucansucrase (Nigam *et al*., 2006; Purama and Goyal, 2008a). The presence of associated glucan in glucansucrase during the purification results in aggregated forms of enzyme, making the enzyme purification troublesome. However, the purification of glucansucrase by polyethylene glycol fractionation is a simple, effective and single step purification method as polyethylene glycol readily removed by dialysis (Purama and Goyal, 2008a; Majumder and Goyal, 2008). It has been reported that the enzyme glucansucrase can be stabilized by high-molecular-weight dextran, polyethylene glycol or nonionic detergents such as Tween 80 (Miller and Robyt, 1984; Majumder and Goyal, 2008). Glucansucrases are inactivated by EDTA and can be reactivated with Ca^{2+} (Miller and Robyt, 1986, Purama *et al.,* 2010). Various metal ions such as Ca^{2+}, Mg^{2+}, Co^{2+} enhanced the glucansucrase activity whereas metal ions like Cu^{2+}, Fe^{3+} and Mn^{2+} strongly inhibited the enzyme

activity (Robyt and Walseth, 1979; Purama and Goyal, 2008a; Majumder and Goyal, 2008).

Lb. plantarum is one of the most studied lactic acid bacteria due to their ability to reduce and eliminate potentially pathogenic micro-organisms by synthesis of antimicrobial agents and by competition with pathogens for receptor sites at the intestinal mucosa (Adlerberth *et al*., 1996). *Lb. plantarum* has been proven effective against diarrhoea, irritable bowel disorder and lactose intolerance (Lonnermark *et al*., 2010). However, the strain *Lb. plantarum* has not been explored for production of glucansucrase so far. It has been reported that *Lb. plantarum* PL916 concomitantly produce glucansucrase and fructansucrase during sourdough fermentation (Cagno *et al*., 2006), but no report is available on the purification of glucansucrase from *Lb. plantarum.* A probiotic lactic acid bacterium, *Lactobacillus plantarum* DM5 (Genbank Accession No: KC020195) was isolated from traditional fermented beverage Marcha of biodiversity hot spot region Sikkim, India (Das and Goyal, 2010; Das and Goyal, 2013) and its ability to produce extracellular glucansucrase was explored considering its enormous commercial applications in food industry. In the present study the culture and nutrient conditions for higher glucansucrase production from *Lactobacillus plantarum* DM5 were investigated. Glucansucrase produced by *Lactobacillus plantarum* DM5 was purified by polyethylene glycol fractionation followed by gel filtration. Biochemical properties of the glucansucrase were analysed. To the best of our knowledge this is the first report on purification of glucansucrase from *Lactobacillus plantarum*.

5.2 Material and Methods

5.2.1 Chemicals and reagents

The media components for maintenance of culture and enzyme production were purchased from Hi-Media Pvt. Ltd., India. All the chemicals required for reducing sugar estimation, protein estimation and buffer preparations were of high purity grade and purchased from Hi-Media Pvt. Ltd., India. PEG-400 was procured from Merck, India and PEG-1500 was obtained from BDH Chemicals Ltd. UK. Sephacryl S-300HR and Bovine Serum Albumin (BSA) was purchased from Sigma Aldrich, USA.

5.2.2 Microorganism and culture medium

The strain *Lb. plantarum* DM5 was isolated from an ethnic fermented beverage Marcha of Sikkim on the basis of antimicrobial activity and glucansucrase activity as described in Chapter 2, Section 2.2.5 and maintained in modified MRS agar medium (as described in Chapter 2, Section 2.2.2) at 4ºC, repeatedly sub cultured after every 2 weeks (Goyal and Katiyar, 1996).

5.2.3 Production of glucansucrase from *Lb. plantarum* DM5

The glucansucrase was produced by inoculating the isolate *Lb. plantarum* DM5 (1%, v/v) in sterile 100 ml enzyme production medium as described by Tsuchiya *et al*., (1952) and also described in Chapter 2, Section 2.2.3. The culture was incubated at 27°C for 18 h under static condition. The cell free supernatant was obtained by centrifugation at 10,000*g* and at 4°C for 10 min and analyzed for enzyme activity and protein concentration as described in the next Sections 5.2.4 and 5.2.5,

respectively. The cell free supernatant was used subsequently for the purification of glucansucrase as described in Section 5.2.10.1.

5.2.4 Enzyme activity assay

The enzyme assay was carried out in 1 ml reaction mixture containing 5% (w/v) sucrose as substrate, 20 mM sodium acetate buffer (pH 5.4) and 20 µl of cell free supernatant. The enzymatic reaction was performed at 30°C in water bath for 15 min. Aliquot of 100 µl from the reaction mixture was taken and the enzyme activity was determined by estimating the released reducing sugar by the method as described by Nelson, (1944) and Somogyi, (1945) using fructose as a standard as mentioned in Chapter 2, Section 2.2.7. The absorbance was measured at 500 nm using spectrophotometer (Varian, Cary 100). The enzyme activity was calculated as mentioned earlier in Chapter 2, Section 2.2.7.

5.2.5 Estimation of protein concentration

The protein concentration of the cell free supernatant was estimated as described in Chapter 3, Section 3.2.5 following the method of Lowry *et al.,* (1951) using Bovine Serum Albumin (BSA) as standard. The specific activity (U/mg) of the cell free supernatant was calculated using the following equation;

$$\text{Specific activity (U/mg)} = \frac{\text{Enzyme Activity (U/ml)}}{\text{Protein Concentration (mg/ml)}} = (\mu\text{moles/min/mg})$$

5.2.6 Effect of temperature and aeration on glucansucrase production from *Lb. plantarum* DM5

The production of enzyme by *Lb. plantarum* DM5 was studied under different physicochemical conditions, such as temperature and shaking. The effect of temperature on enzyme production was studied by varying the temperature from 20°C to 35°C under static condition, using 100 ml of Tsuchiya medium (as described in Chapter 2, Section 2.2.3) in 250 ml Erlenmeyer flask. The effect of shaking condition on enzyme production was analyzed under different shaking condition of 90, 120, 150 and 180 rpm at 27°C. The culture of 500 µl was withdrawn from each flask at different time intervals and centrifuged at 10,000*g* and 4°C for 10 min. The cell free supernatant (10-20 µl) was used for enzyme assay and the enzyme activity was calculated by measuring the released reducing sugar as described earlier in Chapter 2, Section 2.2.7.

5.2.7 Fermentation profile of *Lb. plantarum* DM5 in enzyme production medium

The isolate *Lb. plantarum* DM5 (1%, v/v) was inoculated in sterile 200 ml enzyme production medium as described by Tsuchiya *et al*., (1952) as also mentioned in Chapter 2, Section 2.2.3. The growth parameters of *Lb. plantarum* DM5 such as pH, cell density, enzyme activity and glucan production were studied at every 4 h intervals up to 24 h at 27°C under static condition. The change in pH was measured by using pH meter (Sartorius, PP-15) in every 2 h intervals. The growth of culture was determined by measuring the optical density of cells at 600 nm on UV-Vis spectrophotometer (Varian, Cary 100) using the sterile enzyme production medium (Tsuchiya *et al.,* 1952) as blank at every 2 h intervals. Cell free supernatant was

obtained by centrifugation of 500 µl culture broth at 10,000*g* at 4°C for 10 min and 20 µl was used for determining the enzyme activity as described in Chapter 2, Section 2.2.7. The amount of glucan produced by *Lb. plantarum* DM5 was analyzed by precipitation of 200 µl of cell free supernatant by the addition of 3 volume of pre-chilled 95% (v/v) ethanol at 4°C, following centrifugation at 10,000*g* for 30 min. The process of precipitation was repeated three times to remove any trace impurities or free reducing sugars. Finally, the precipitate was dissolved in 200 µl of deionised water. The concentration of glucan was estimated by phenol sulphuric acid method as described in Section 5.2.8.

5.2.8 Determination of glucan concentration

The glucan concentration was determined by phenol-sulfuric acid method described by Dubois *et al*., 1956 using a micro-titer plate (Kitaoka and Robyt, 1998). A 25 µl sample containing glucan was added to microtiter plate, along with 25 µl of 5% (w/v) phenol and the content were mixed by shaking the plate at slow speed on a vortex mixer for 30 s. The plate was then placed onto ice bath and 125 µl of concentrated sulphuric acid was added to each well containing sample and phenol. The content was mixed uniformly by gently shaking the plate for 30 s. The plate was wrapped with cling film followed by incubation at 80°C in water bath for 30 min. The plate was finally cooled and the absorbance was measured at 490 nm on a microtiter plate reader (Tecan, Infinite 200 Pro). The standard curve was prepared using dextran T-500 (500 kDa) from Sigma Aldrich, USA, in the concentration range 0.05-0.5 mg/ml.

Glucan (mg/ml) = ΔA_{490} x C

ΔA_{490} = Optical Density (OD) change at 490 nm.

C = 1 OD equivalent dextran T-500 concentration (mg/ml) from standard plot.

5.2.9 Effect of medium components on glucansucrase production

The effects of various medium components on glucansucrase production were studied by one variable at a time approach, by changing the concentration of one variable, while keeping other variables constant. The effects of various medium nutrients such as sucrose, yeast extract, beef extract, peptone, Tween 80 and K_2HPO_4 were studied by varying their concentrations in 100 ml Tsuchiya medium contained in 250 ml Erlenmeyer flask. The cultures were grown at 27°C under static condition. Broth samples of 5 ml were periodically withdrawn and analyzed for enzyme activity by measuring the released reducing sugar as described in Chapter 2, Section 2.2.7.

5.2.10 Purification of glucansucrase

5.2.10.1 Purification of glucansucrase by PEG fractionation

The purification of enzyme was carried out by fractionation with different concentrations of pre-chilled polyethylene glycol 400 (PEG-400) ranging from 25-40% (v/v, final concentration) and PEG-1500 ranging from 10-25% (w/v, final concentration) in 50 ml of cell free supernatant. Mixture was incubated overnight at 4°C to allow the enzyme to precipitate and then centrifuged at 10,000*g* at 4°C for 30 min to separate the fractionated enzyme (Dutta *et al*., 2012). Enzyme pellet was dissolved in 20 mM sodium acetate buffer (pH 5.4) and subjected to dialysis using 14

kDa cut-off membrane (Hi-Media Pvt. Ltd., India). The dialyzed enzyme was characterized further by both denaturing and non-denaturing SDS-PAGE using silver staining as described in Section 5.2.12 and Periodic acid schiff (PAS) staining as described in Section 5.2.13.

5.2.10.2 Purification of glucansucrase by gel filtration

The partially purified enzyme (10.1 U/mg, 0.38 mg/ml) by PEG-1500 (15%) was further purified by gel filtration using a column (1.5 cm x 50 cm) containing Sephacryl S-300HR matrix, connected to FPLC (Akta Prime, GE Healthcare). The column was pre-equilibrated with 20 mM sodium acetate buffer (pH 5.4) and 2 ml of enzyme partially purified (10.1 U/mg, 0.38 mg/ml) by 15% PEG-1500 was loaded onto the column. The enzyme was eluted using 20 mM sodium acetate buffer (pH 5.4) at a flow rate of 0.3 ml/min and fractions of 3 ml up to 30 fractions were collected. The purified fractions showing maximum enzyme activity were pooled and analysed for protein concentration and specific activity as described in Section 5.2.5.

5.2.11 SDS-PAGE analysis of purified enzyme

5.2.11.1 Preparation of reagents for SDS-PAGE analysis

Stock solution preparation of Acrylamide/ Bis acrylamide solution (29.2%, w/v acrylamide and 0.8% w/v bisacrylamide) has been described in Chapter 4, Section 4.2.13.1. The preparation of SDS (10%) solution, Tris-HCl (1.5 M, pH 8.8) and Tris-HCl (0.5 M, pH 6.8) buffers and 5x Tris-glycine-SDS running buffer (pH 8.3) have also been described in Chapter 4, Section 4.2.13.1.

5.2.11.2 Preparation of SDS-PAGE gels and sample buffer

The SDS-polyacrylamide gel electrophoresis was performed using a vertical slab mini gel unit (BioRad) using 1.5 mm thick gels, following the method of Laemmli, (1970). The resolving gel containing 7.5% (w/v) acrylamide was prepared as described in Table 5.2.1 and stacking gel containing 4% (w/v) acrylamide was prepared as mentioned in Table 4.2.4 in Chapter 4, Section 4.2.13.1.

Table 5.2.1 Composition for preparation of 7.5% resolving gel.

Component	**Volume (ml)**
Acrylamide-bisacrylamide solution (30%, w/v)	2.50
SDS solution (10%, w/v)	1.00
Glycerol (50%, v/v)	1.00
1.5 M Tris (pH 8.8)	3.30
APS solution (10%, w/v)	0.10
TEMED	0.01
Deionized water	2.09
Total volume	10.00

The samples of glucansucrase purified by 15% PEG-1500 fractionation and by gel filtration (Sephacryl S-300HR) were run on the gel along with protein molecular weight marker from Fermentas, Thermo Fisher Scientific, India. The sample loading buffer for denaturing condition was prepared as described in Chapter 4, Section 4.2.13.1. In case of non-denaturing conditions β-mercaptothanol was not added in the loading dye. The purified enzyme sample was mixed with 5x loading dye buffer in the ratio 4:1 and subjected to heat denaturation by putting the sample in boiling water bath for 5 min in case of denaturing condition, but, the sample was not subjected to heat denaturation in case of non-denaturing condition. The samples were loaded on 7.5% acrylamide gel and the electrophoresis was carried out using 1x running buffer (as described in Chapter 4, Section 4.2.13.1.) with a current of 2 mA per lane. After

the migration, protein bands were stained with silver solution (Rabilloud, 1992) as described in Section 5.2.12.

5.2.12 Silver staining analysis of purified protein

5.2.12.1 Preparation of reagents for silver staining

The use of silver staining technique for detecting proteins separated by gel electrophoresis provides a very sensitive tool for protein visualization with a detection level down to the 0.3-10 *n*g level (Switzer *et al*., 1979, Rabilloud, 1992). The solutions used in silver staining are listed in Table 5.2.3 and the staining procedure is described in Section 5.2.12.2.

Table 5.2.3 Composition for preparation of silver staining reagents.

Reagents	**Components**
Fixing solution	40 (%, v/v) ethanol, 10 (%, v/v) acetic acid in water
Sensitizing solution	0.2 (%, w/v) $Na_2S_2O_3$, 6.8 (%, w/v) Sodium acetate, 30 (%, v/v) ethanol and 0.025 (%, v/v) glutaraldehyde
Silver nitrate solution	0.1 (%, w/v) $AgNO_3$, 0.076 (%, w/v) formalin
Developing solution	2.5 (%, w/v) Na_2CO_3, 0.05 (%, w/v) formaldehyde
Stop solution	1.46 (%, w/v) EDTA

5.2.12.2 Silver staining procedure

Silver staining of the gel was carried out by following the method established by Rabilloud, (1992). After the electrophoresis, the gel was immersed in 50 ml of fixing solution at 25ºC for 1-2 h to fix the protein bands and placed on a shaker at a very gentle speed. After 1-2 h, the fixing solution was discarded and the gel was washed in 20% ethanol for 20 min to remove the remaining detergent ions as well as fixation acid from the gel. The gel was then incubated in 100 ml sensitizing solution

at 25°C for 20-30 min. After the incubation, the sensitizing solution was discarded and the gel was washed thrice in 50 ml deionised water at every 10 min intervals. The silver staining solution of 100 ml was added to the gel and the gel was incubated for 20 min in dark to allow the silver ions to bind with proteins. After staining, the gel was rinsed with 100 ml of deionised water for 2-3 min and 100 ml of developing solution was added to the gel. The reaction was stopped as soon as the protein bands appeared in desired intensity by adding the 50 ml of stop solution. The gel was stored in preserving solution comprising of 30% (v/v) absolute ethanol and 4% (v/v) glycerol prepared in deionised water.

5.2.13 Identification of *in situ* glucansucrase activity by Periodic Acid Schiff's (PAS) staining

The *in situ* glucansucrase activity was determined following the method of Holt *et al*., (2001) with minor modifications (Dutta *et al*., 2012). The enzyme samples purified by 15% (w/v) PEG-1500 fractionation and gel filtration were loaded on another 7.5% acrylamide gel and run under non-denaturing condition. In the non-denaturing condition, the samples were prepared without the addition of β-mercaptoethanol in loading dye and were not subjected to boiling. After the run, the gel was divided into two parts and both the parts were washed thrice by 20 mM sodium acetate buffer (pH 5.4) with 0.3 mM $CaCl_2$ and 0.1%, v/v Tween 80. The gels were incubated at 30ºC for 30 min to remove SDS. After the removal of SDS, one part of the gel was incubated in 20 mM sodium acetate buffer (pH 5.4) supplemented with 10% sucrose and 0.3 mM $CaCl_2$ at 30°C for 48 h and the other part was incubated under the same conditions with 10% raffinose instead of sucrose. After

incubation, the gels were washed with 75% ethanol for 40 min and incubated in periodic acid solution (periodic acid, 0.7% w/v and acetic acid 5% v/v) for 1 h at 25ºC. After the periodic acid treatment, the gels were washed with a solution containing 0.2% (w/v) sodium metabisulphite and 5% (v/v) acetic acid. Finally, the gels were stained with 15 ml Schiff reagent (0.5%, w/v Fuchsin basic, 1% sodium bisulphite and 0.1 N HCl) until the discrete magenta colour band within the gel matrix appeared. The gels were washed in distilled water to remove the excess stain and stored in 10% (v/v) acetic acid at 25ºC.

5.2.14 Optimum temperature and thermal stability of glucansucrase

The optimum temperature for assay of glucansucrase was studied by adding 20 µl of the purified enzyme (18.7 U/mg, 0.08 mg/ml) to 1 ml enzyme reaction mixture containing 150 mM sucrose in 20 mM sodium acetate buffer (pH 5.4). The reaction mixture was incubated at different temperatures varying from 15°C to 55°C for 15 min. The reaction mixture of 100 µl was taken for reducing sugar estimation following the method established by Nelson, (1944) and Somogyi. (1945) as mentioned in Chapter 2, Section 2.2.7. The thermostability of the enzyme was determined by incubating 0.5 ml of enzyme (18.7 U/mg, 0.08 mg/ml) at different temperatures ranging from 10-50°C for 30 min and the 20 µl aliquots of enzyme were assayed for residual enzyme activity in 1 ml reaction mixture containing 150 mM sucrose in 20 mM sodium acetate buffer (pH 5.4) and incubated at 30ºC. The assays were carried out in triplicates for temperature optima and for thermostability experiments.

5.2.15 Optimum pH and pH stability of glucansucrase

The optimum pH of purified enzyme was determined by incubating 20 µl (18.7 U/mg, 0.08 mg/ml) of purified enzyme in 1 ml reaction mixture containing 150 mM of sucrose in 20 mM sodium acetate buffer having different pH, ranging from 3 to 7. The reaction mixture was incubated at 30°C for 15 min and reducing sugar was estimated by taking 100 µl of reaction mixture as mentioned previously in Chapter 2, Section 2.2.7. In order to determine the pH stability, of purified enzyme (18.7 U/mg, 0.08 mg/ml) was incubated at different pH ranging from 3.0 to 6.0 in 20 mM sodium acetate buffer and pH 6.2 to pH 8.4 in 20 mM sodium phosphate buffer at 30°C for 30 min and then aliquots of 20 µl were assayed for residual enzyme activity as described earlier. Measurements were carried out in triplicates for pH optima and pH stability experiments.

5.2.16 Effect of ionic strength on glucansucrase activity

The effect of ionic strength on the enzyme activity was studied by varying the ionic strength of the sodium acetate buffer (pH 5.4) between 10 mM- 500 mM. Reaction mixture of 1 ml containing 150 mM sucrose in sodium acetate buffer (pH 5.4) of different ionic strengths and 20 µl of purified enzyme (18.7 U/mg, 0.08 mg/ml) was incubated in water bath at 30°C for 15 min. Aliquot of 100 µl from the 1 ml reaction mixture was taken after 15 min incubation for reducing sugar estimation as mentioned in Chapter 2, Section 2.2.7 and the enzyme activity was calculated.

5.2.17 Determination of kinetic parameters of glucansucrase

The purified glucansucrase (18.7 U/mg, 0.08 mg/ml) was used to study the effect of sucrose concentration on its activity. The reaction was carried out in 1 ml of 20 mM sodium acetate buffer (pH 5.4) containing 20 µl of enzyme and varying concentration of sucrose ranging from 0.05 mM to 400 mM at 30ºC for 15 min. The enzyme activity was determined by estimating the released reducing sugar, as described earlier in Chapter 2, Section 2.2.7. The data was used to generate Lineweaver Burk plot and the kinetic parameters were analyzed from the plot.

5.2.18 Effect of salts and denaturing agent on glucansucrase activity

The effects of $CaCl_2$, $MgCl_2$, $CoCl_2$, $MnSO_4$ and $HgCl_2$ between 0-12 mM and EDTA between 0-5 mM concentrations were studied on the activity of purified glucansucrase (18.7 U/mg, 0.08 mg/ml). The assays were carried out in 1.0 ml reaction mixture containing the salt or EDTA, the substrate sucrose of 5% in 20 mM sodium acetate buffer (pH 5.4) and 20 µl enzyme. The effect of urea was studied by prior incubation of enzyme with urea (0-5 M, final concentration) at 30°C for 30 min. The 20 µl was taken out from the reaction mixture and enzyme activity was measured as described earlier in Chapter 2, Section 2.2.7. The percent of residual activities were calculated with respect to the activity in absence of metal ion or denaturing compound. All the data expressed are the average of three independent experiments.

5.2.19 Effect of stabilizing agents on glucansucrase activity

To study the effect of different additives on the stability of glucansucrase, aqueous solutions of dextran T-40, PEG-6000, PEG-8000, glutaraldehyde, glycerol,

Tween-80 and acetonitrile were added to purified glucansucrase (18.7 U/mg, 0.08 mg/ml) in sodium acetate buffer (pH 5.4). The final concentrations of 2 µg/ml dextran T-40, 10 µg/ml PEG-6000, 10 µg/ml PEG-8000, 0.1% glutaraldehyde, 0.5% glycerol, 1% acetonitrile and 10 µl/ml Tween 80 in a final volume of 0.6 ml containing enzyme were incubated in water bath at 30ºC for 36 h. Samples of 20 µl were withdrawn at different time intervals and analysed for residual enzyme activity as mentioned earlier in Chapter 2, Section 2.2.7. All the experiments were carried out in triplicates and the data used is the average of three independent experiments.

5.2.20 Effect of storage temperature on stability of glucansucrase

The storage temperature of purified glucansucrase (18.7 U/mg, 0.08 mg/ml) was studied by incubating the enzyme at different temperatures (0°C, 4°C and -20°C) with or without additives. The additives used for long term storage of glucansucrase were dextran T-40 (2 µg/ml) and Tween 80 (10 µl/ml). Samples of 20 µl withdrawn at different time intervals and analysed for residual enzyme activity as described earlier in Chapter 2, Section 2.2.7.

5.3 Results and Discussion

5.3.1 Effect of temperature and shaking on glucansucrase production from *Lb. plantarum* DM5

The effect of incubation temperature on glucansucrase production from *Lb. plantarum* DM5 was studied by varying the temperature from 20 to 35°C and the maximum activity of 2.71±0.39 U/ml was observed at 27°C under static condition as shown in Fig. 5.3.1.

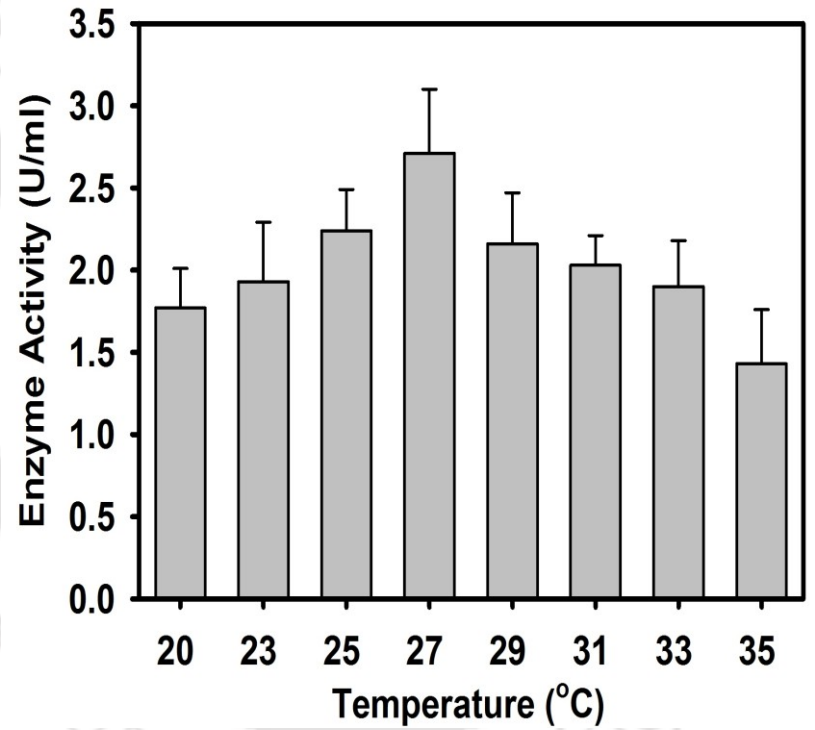

Fig. 5.3.1 Effect of temperature on glucansucrase production from *Lactobacillus plamnatrum* DM5 under static condition. The mean value of three independent experiments is presented with ± S.E.

The enzyme activity decreased as the temperature increased above 27°C, and it further decreased by 47% at 35°C due to the deactivation of the enzyme at higher temperatures. The enzyme activity reduced by 35% at 20°C, which might be due to the slower growth rate of cells consequently resulting in lower enzyme production. It

has been reported that the fermentation temperature ranging from 20 to 30°C have been used for glucansucrase production from *Leuconostoc* spp. (Cortezi *et al*., 2004; Majumder and Goyal, 2008) and *Weisella* spp. (Shukla and Goyal, 2011a; Rao and Goyal, 2013).

The production of enzyme was also studied at varying orbital shaking speed of 90, 120, 150 and 180 rpm at 27°C and results were compared with the static condition as displayed in Fig. 5.3.2. The maximum enzyme activity of 2.4± 0.23 U/ml observed at 90 rpm at 27°C, which was 11% less than that obtained in static condition (2.7 U/ml). The enzyme activity gradually decreased as the shaking speed increased and was found to be 2.16±0.26 U/ml at 180 rpm. The results indicated the microaerophilic nature of the bacterium.

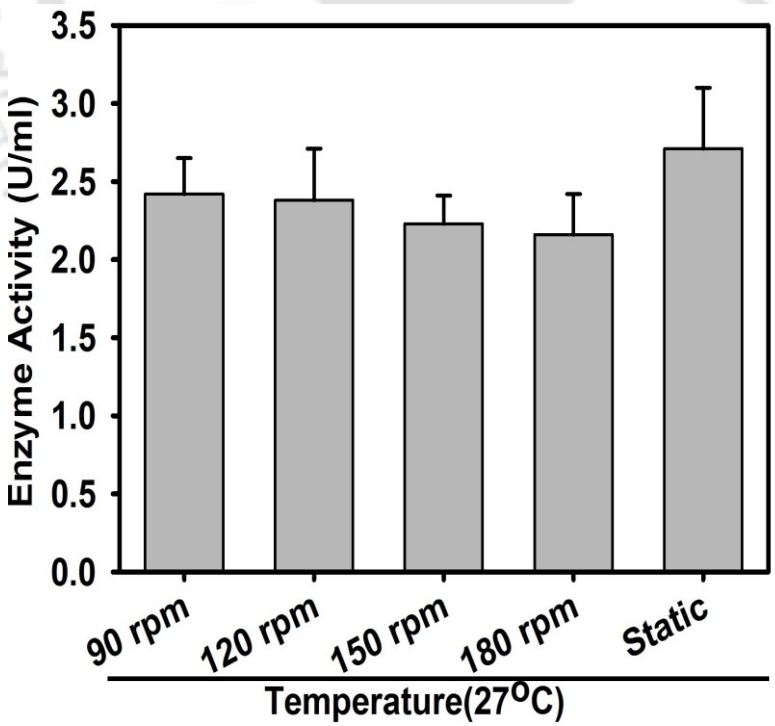

Fig. 5.3.2 Effect of different shaking conditions on glucansucrase production from *Lactobacillus plamnatrum* DM5 at 27ºC. The mean value of three independent experiments is presented with ± S.E.

5.3.2 Fermentation profile of *Lb. plantarum* DM5 in enzyme production medium

The variation in cell optical density and the pH profile during the fermentation of *Lb. plantarum* DM5 were studied at 27°C under static condition. The profiles of cell optical density, pH, enzyme activity and glucan concentration are shown in Fig.5.3.3.

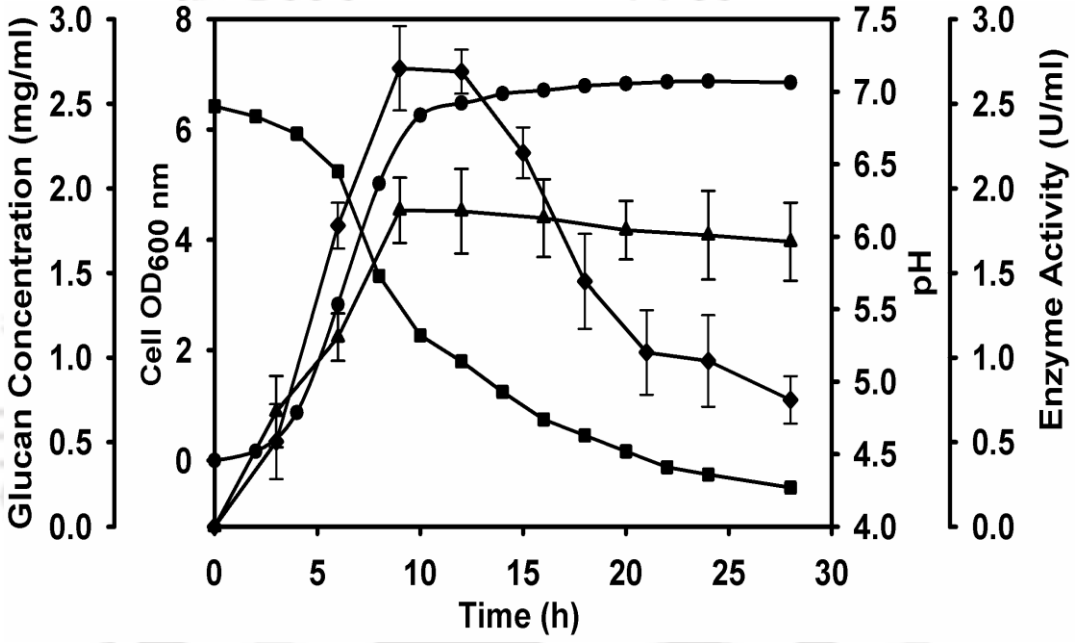

Fig. 5.3.3 Fermentation profile of *Lb. plantarum* DM5 at 27°C under static condition showing the cell optical density (--●--),changes in pH (--■--), enzyme activity (--♦--) and glucan concentration (--▲--). Standard error of cell optical density and changes in pH recorded from three repeats was less than 0.1% and is not indicated.

The probiotic *Lb. plantarum* DM5 gave the maximum glucansucrase activity of 2.71 U/ml and specific activity of 0.48 U/mg after 9 h incubation under static condition and remained constant up to 12 h and decreased thereafter. The lag phase extended up to 3 h followed by the exponential phase which reached maximum at 12 h and thereafter the stationary phase followed, and no further increase in cell growth was observed which might be due to the depletion of nutrients. The maximum enzyme

activity was observed at the onset of stationary phase when pH of the medium reached 5.3. It could be inferred from the data that the enzyme activity was growth associated (Fig. 5.3.3). The pH of the growth medium fell from pH 6.9 to pH 4.2 after 25 h of incubation. The cell free supernatant of *Lb. plantarum* DM5 gave crude glucan concentration of 1.87 mg/ml at 9 h when the enzyme activity and the specific activity were found to be maximal (Fig. 5.3.3). The slight decrease in glucan concentration was observed after 12 h, which was probably due to the presence of glycohydrolases which catalyzes the degradation of polysaccharide (Degeest *et al.*, 2002). The decrease in total exopolysaccaride amounts were also reported in case of *Streptococcus thermophilus* LY03 (De Vuyst *et al.*, 1998) and *Lactobacillus plantarum* C88 (Zhang *et al.*, 2013).

5.3.3 Effect of nutrients on glucansucrase production from *Lb. plantarum* DM5

5.3.3.1 Effect of sucrose

The effect of sucrose on glucansucrase production from *Lb. plantarum* DM5 was studied by varying its concentration from 1 to 7% (w/v) in the 100 ml enzyme production medium and compared with the control medium containing 2% (w/v) sucrose as described by Tuschiya *et al.*, (1952). The maximum enzyme activity of 6.23±0.11 U/ml was observed at 5% (w/v) sucrose concentration as shown in Fig. 5.3.4. It was observed that the enzyme activity increased by 2.3 fold with an increase in sucrose concentration from 2% (control) to 5% in the medium (Table 5.3.1). The production of enzyme decreased by 21% at 7% (w/v) sucrose concentration (Fig. 5.3.4), which might be due to the subsequent utilization of available sucrose for the formation of glucan by the released glucansucrase. The enhancement of glucansucrase

activity by 3 fold has also been reported in case of *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2008b) and *Weissella confusa* Cab3 (Shukla and Goyal, 2011b) in the presence of 7% and 5% (w/v) sucrose, respectively.

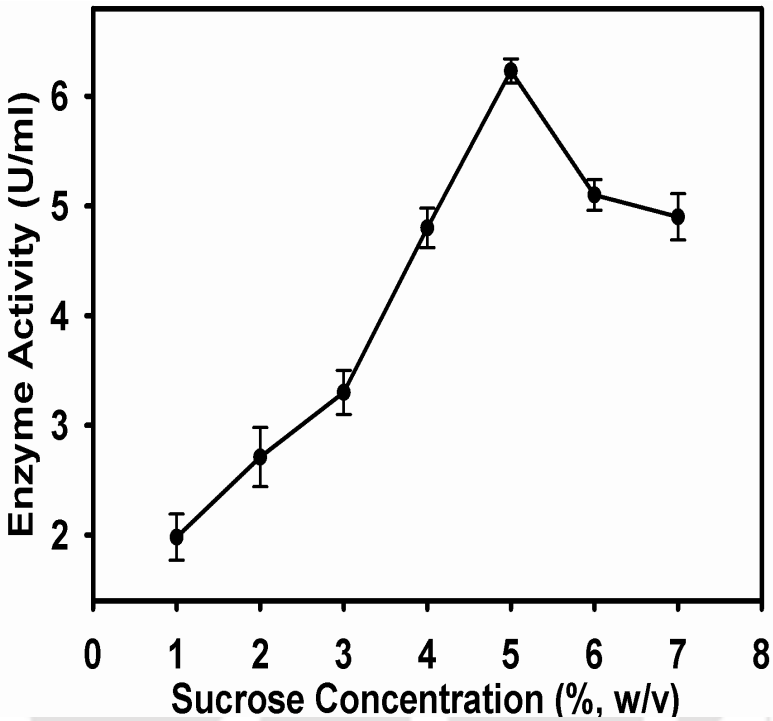

Fig. 5.3.4 Effect of sucrose on glucansucrase production from *Lb. plantarum* DM5. The mean value of three independent experiments is presented with ± S.E.

Table 5.3.1. Effect of nutrients on glucansucrase production by *Lb. plantarum* DM5.

Nutrient concentration (%, w/v)	Enzyme activity (%)
Control[a]	100
Sucrose (5%)	230
Yeast Extract (2.5%)	122
Beef Extract (2%)[b]	71
Peptone (1.5%)[b]	89
Yeast Extract (2%) + Beef extract (2%)	114
Yeast Extract (2%) + Peptone (1.5%)	129
K_2HPO_4 (2.5%)	160
Tween 80 (0.6%, v/v)	168

[a] *Control medium was prepared as described by Tsuchiya et al., (1952) and contained no beef extract, peptone or Tween 80.*

[b] *Medium contained only beef extract or peptone as sole nitrogen source, no yeast extract was used.*

5.3.3.2 Effect of nitrogen source

The effects of various nitrogen sources like yeast extract, peptone and beef extract on glucansucrase production by *Lb. plantarum* DM5 were studied. It was found that the yeast extract enhanced the production of glucansucrase and the maximum enzyme activity of 3.3±0.21 U/ml was achieved at 2.5% (w/v) yeast extract (Fig. 5.3.5), which was 22% higher than that obtained with control (Table 5.3.1). Beyond 2.5% yeast extract, the enzyme activity progressively decreased and enzyme activity of 1.31 U/ml was observed at 4% (w/v) yeast extract (Fig. 5.3.5), which was 52% less as compared to the control medium (2.71 U/ml) that contained 2% (w/v) yeast extract. Similar result was also observed in case of *Weissella confusa* Cab3 (Shukla and Goyal, 2011b), where higher concentration of yeast extract inhibited the enzyme production. The effects of peptone and beef extract as sole nitrogen source on glucansucrase production were studied by varying their concentration from 0.5% to 4% as shown in Fig. 5.3.5. The maximum enzyme activity of 2.4±0.20 U/ml and 1.9±0.20 U/ml was observed by the addition of 1.5% (w/v) peptone and 2% (w/v) beef extract, respectively (Fig. 5.3.5), with a reduction of enzyme activity by 11% and 30% as compared with control medium that contained (2%, w/v) yeast extract (Table 5.3.1). The results indicated that the yeast extract was the most effective nitrogen source for production of glucansucrase from *Lb. plantarum* DM5 and this was in good agreement with other reports where yeast extract served as a source of vitamin and amino acid supplement in production of glucansucrase (Majumder and Goyal, 2008; Purama and Goyal, 2008b). However, the addition of 2% (w/v) beef extract and 1.5% (w/v) peptone separately to the control medium (with 2%, w/v yeast extract) enhanced the enzyme production by 14% and 29%, respectively (Table 5.3.1). Similar results

were observed in case of *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2008b) and *L. mesenteroides* PCSIR-3 (Ul-Qader *et al*., 2001), where peptone and beef extract in addition to yeast extract resulted in enhanced enzyme activity.

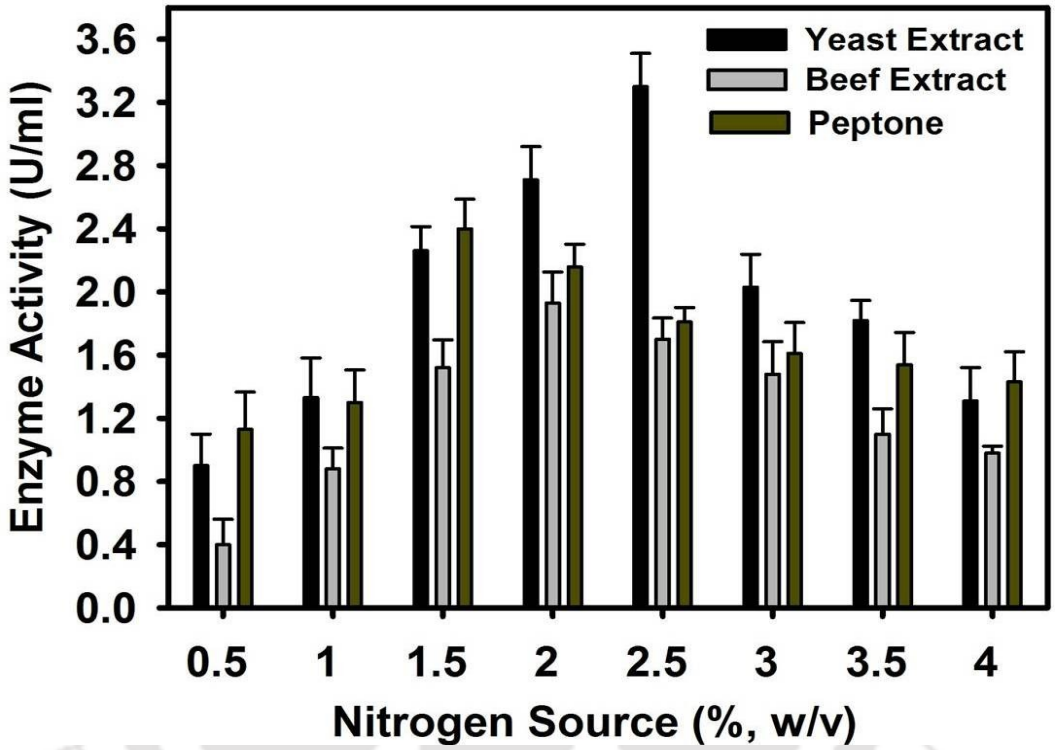

Fig. 5.3.5 Effect of nitrogen source on glucansucrase production from *Lb. plantarum* DM5. The yeast extract (black shaded box), beef extract (grey shaded box) and peptone (olive green shaded box) were used as sole nitrogen source for production of enzyme. The mean value of three independent experiments is presented with ± S.E.

5.3.3.3 Effect of Tween 80

The effect of Tween 80 on glucansucrase production was studied by varying its concentration from 0.1 to 1% (v/v) in the control medium that contained no Tween 80 as described by Tsuchiya *et al*., (1952) and mentioned in Chapter 2, Section 2.2.3. It was observed that with increasing concentration of Tween 80 the enzyme production increased, as displayed in Fig. 5.3.6. The maximum enzyme activity of

4.54±0.2 U/ml was observed at 0.6% (v/v) Tween 80 (Fig. 5.3.6), which was 68% higher as compared with the control medium containing no Tween 80 (Table 5.3.1). The enzyme activity remained constant in between 0.6% to 1% Tween 80 (Fig. 5.3.6). It has been reported that the use of the non ionic surfactant Tween 80, increases the glucansucrase secretion from the cells by altering the fatty acid composition of the cell membrane (Sato *et al*., 1989; Majumder and Goyal, 2008).

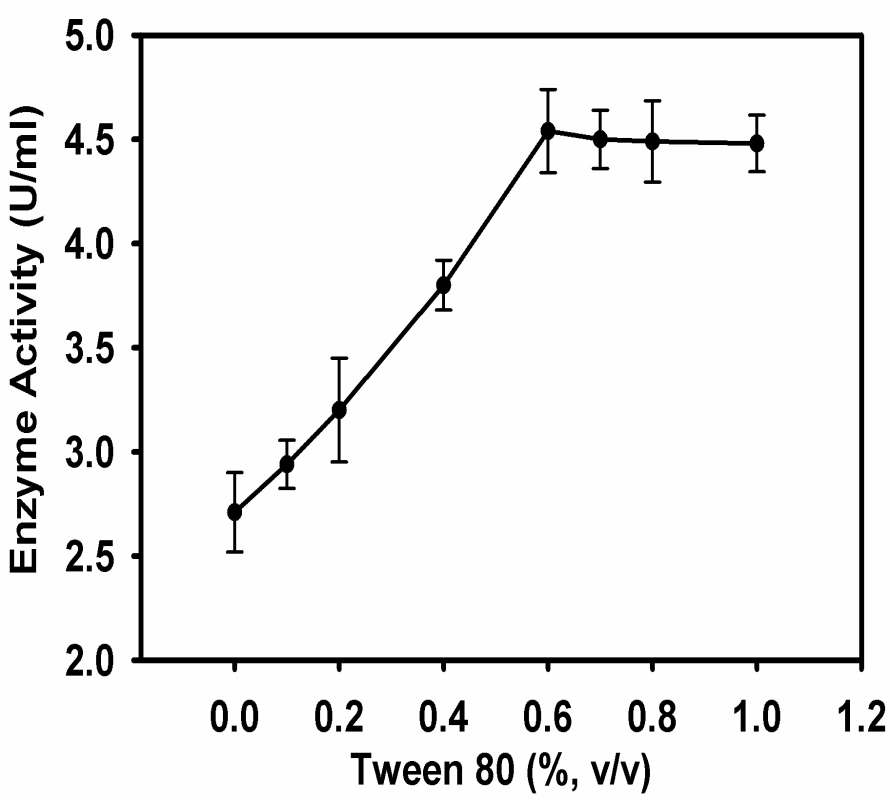

Fig. 5.3.6 Effect of Tween 80 on glucansucrase production from *Lb. plantarum* DM5. -The mean value of three independent experiments is presented with ± S.E.

5.3.3.4 Effect of K_2HPO_4

The effect of K_2HPO_4 on glucansucrase production was also studied by varying its concentration from 1 to 4% (w/v) in the control enzyme production

medium as mentioned in Chapter 2, Section 2.2.3. It was observed that the maximum enzyme activity of 3.2±0.48 U/ml was achieved at 2.5% (w/v) K_2HPO_4 concentration (Fig. 5.3.7). It was 18% higher than the control medium containing 2% K_2HPO_4 (Table 5.3.1). The higher enzyme activity in presence of 2.5% K_2HPO_4 might be due to buffering activity of K_2HPO_4 in the medium, which lowered the effect of lactic acid production during the fermentation (Shukla and Goyal, 2011b). However, the concentration beyond 2.5 % K_2HPO_4 did not support the enzyme activity and at 3.5% K_2HPO_4 it was 1.84±0.27 U/ml, resulting 44% decreases in the enzyme activity (Fig. 5.3.7).

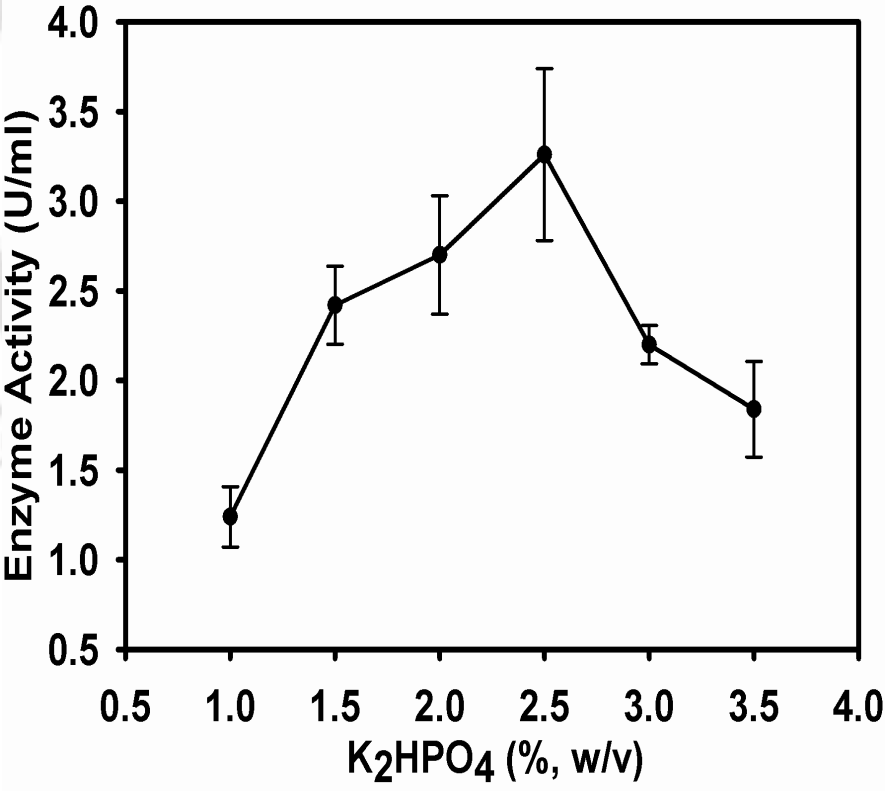

Fig. 5.3.7 Effect of di-potassiun hydrogen phosphate (K_2HPO_4) on glucansucrase production from *Lb. plantarum* DM5. The mean value of three independent experiments is presented with ± S.E.

5.3.4 Purification of enzyme by polyethylene glycol fractionation

The cell free supernatant containing extracellular glucansucrase (0.48 U/mg, 5.7 mg/ml) was subjected to fractionation with various concentrations of PEG-400 and PEG-1500. The specific activity profiles of glucansucrase with corresponding concentrations of PEG-400 and PEG-1500 are shown in Fig.5.3.8.

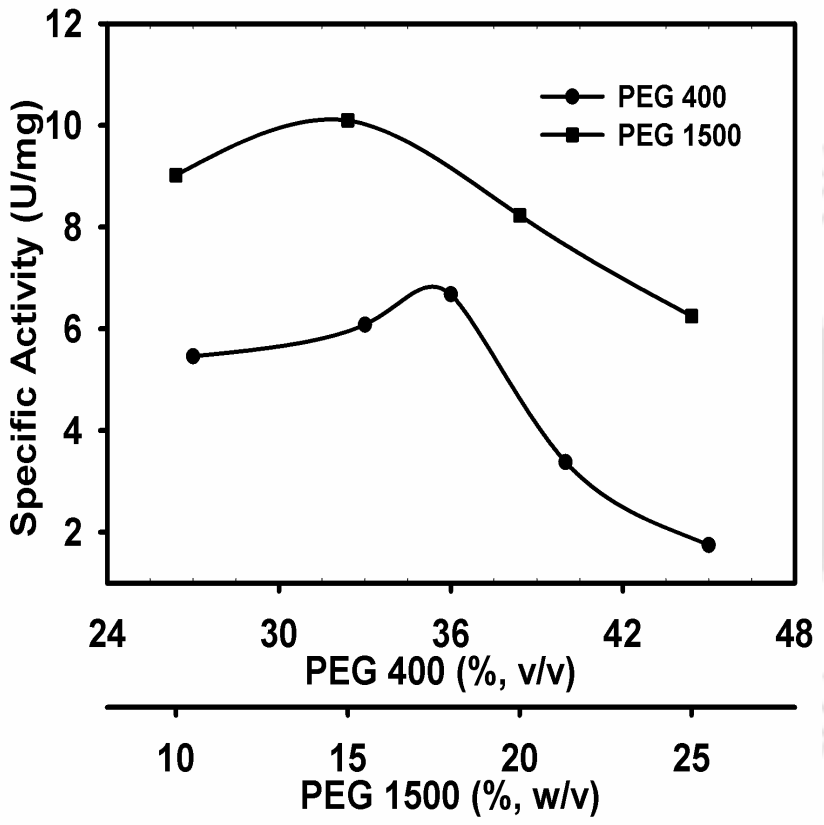

Fig. 5.3.8 Purification of glucansucrase from *Lb. plantarum* DM5 by PEG-400 and PEG-1500.

The maximum specific activity of 6.7 U/mg and 10.1 U/mg was achieved at 36%, (v/v) PEG-400 and 15%, (w/v) PEG-1500, respectively. The enzyme purified by 15%, (w/v) PEG-1500 fractionation exhibited 21-fold purification with 14% overall yield in single step (Table 5.3.2). The higher concentrations of PEG-400 (40%) and PEG-1500 (25%) were not effective as the enzyme activity decreased by 73% and

38%, respectively, as shown in Fig. 5.3.8. It has been reported that the higher concentration of PEG precipitated other non-glucansucrase proteins, while lower concentration of PEG did not fractionate most of the enzyme and therefore a decrease in specific activity was observed (Russell, 1979, Majumder et al. 2008). Similar results were also reported in case of *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2008a) and *L. dextranicum* NRRL B-1146 (Majumder *et al*., 2008).

Table 5.3.2. Purification of glucansucrase from *Lb. plantarum* DM5.

	Vol. (ml)	Enzyme Activity (U/ml)	Total Units	Overall Activity Yield (%)	Protein (mg/ml)	Total (mg)	Specific Activity (U/mg)	Fold Purifi-cation
Crude (cell free supernatant)	50	2.71	136	–	5.70	285	0.48	–
Fractionation by PEG-400 (36%)	5.4	2.54	14	10	0.38	2.1	6.7	14
Fractionation by PEG 1500 (15%)	5.0	3.84	19	14	0.38	2	10.1	21
Gel filtration by Sephacryl S-300HR	9	1.50	13	9	0.08	0.72	18.7	40

5.3.5 SDS-PAGE analysis and activity staining of purified glucansucrase by 15% PEG-1500

The molecular mass of the purified enzyme (15% w/v, PEG-1500) was determined by 7.5% (w/v) SDS PAGE which showed two molecular forms of approximately, 189 kDa and 148 kDa after silver staining as shown in Lane 1 and Lane 2 of Fig. 5.3.9, under both non-denaturing and denaturing condition, respectively. The purified enzyme (15% w/v, PEG-1500) was also run on SDS-PAGE gels under non-denaturing and denaturing condition for *in situ* activity detection of glucansucrase by PAS staining. The PAS staining of the gel showed three activity

bands of molecular weight of approximately 189 kDa, 150 kDa and 148 kDa when incubated in 10% sucrose solution (Fig. 5.3.9, Lane 3 and Lane 4).

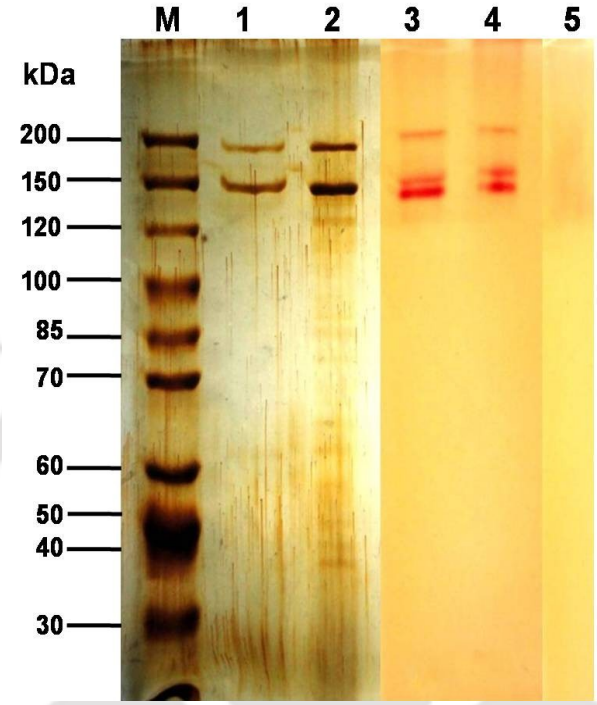

Fig. 5.3.9 SDS-PAGE (7.5%) analysis of 15% PEG-1500 purified enzyme. Lane M: Protein Molecular Mass marker (10 kDa to 200 kDa) from Fermentas Thermo Fisher Scientific, India (silver stained). Lane 1 and Lane 2: Purified glucansucrase by 15% PEG-1500 fractionation under non-denaturing and denaturing condition (silver stained), Lane 3 and Lane 4: Purified glucansucrase by 15% PEG-1500 under non-denaturing and denaturing condition (PAS stained) using sucrose as substrate, Lane 5: Absence of fructansucrase using raffinose as substrate by PAS staining method confirming the presence of glucansucrase.

The faint band of 150 kDa was observed only in PAS staining but not in silver staining which might be due to active form of enzyme was present in very low amounts (*P*g level) and therefore, could not be stained by the silver staining. As the active form of enzyme in *P*g levels also synthesized glucan in presence of sucrose, it was easily detected by PAS staining. It was observed that the most active form of glucansucrase was of 148 kDa, as it showed intense band on PAS staining (Fig. 5.3.9,

Lane 1 and Lane 2) and as well as silver staining (Fig. 5.3.9, Lane 3 and Lane 4). It has been reported that the molecular mass of the extracellular glucansucrases are in the range of 120-200 kDa (Leemhuis *et al*., 2013) and can exist in multiple molecular forms (Purama and Goyal, 2008a; Patel *et al.*, 2011). No band after activity staining of purified enzyme by 15% (w/v) PEG-1500 fractionation was observed upon the incubation of the gel with raffinose as shown in Fig. 5.3.9, Lane 5, which excluded the presence of fructosyltransferase.

5.3.6 Purification of enzyme by gel filtration

The partially purified glucansucrase by 15% (w/v) PEG-1500 with an enzyme activity of 10.1 U/mg was further purified by gel filtration using Sephacryl S-300HR.

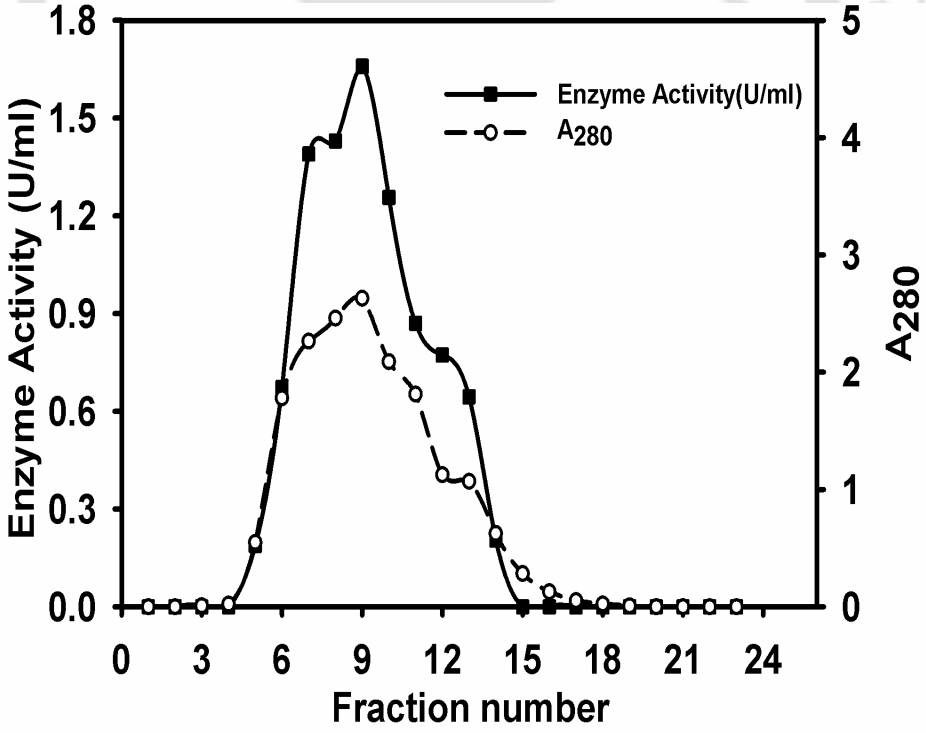

Fig. 5.3.10 Elution profile of glucansucrase by gel filtration using Sephacryl S-300HR. The flow rate was 0.3 ml/min and fractions of 3 ml were collected. The fractions were assayed for enzyme activity (--■--) and protein concentration (--○--).

The enzyme eluted as a single asymmetrical peak (Fig. 5.3.10) and the higher enzyme activity was confined between 7th to 11th fractions. Among these 5 eluted fractions, the fractions 7-10 showed single homogeneous bands on SDS-PAGE under denaturing condition after silver staining as shown in Fig 5.3.11 and the fractions 8, 9 and 10 showed maximum enzyme activity and higher protein content as observed by A_{280} (Fig. 5.3.10). Therefore, these three fractions were pooled and which showed specific activity of 18.7 U/mg with 40- fold purification (Table 5.3.2).

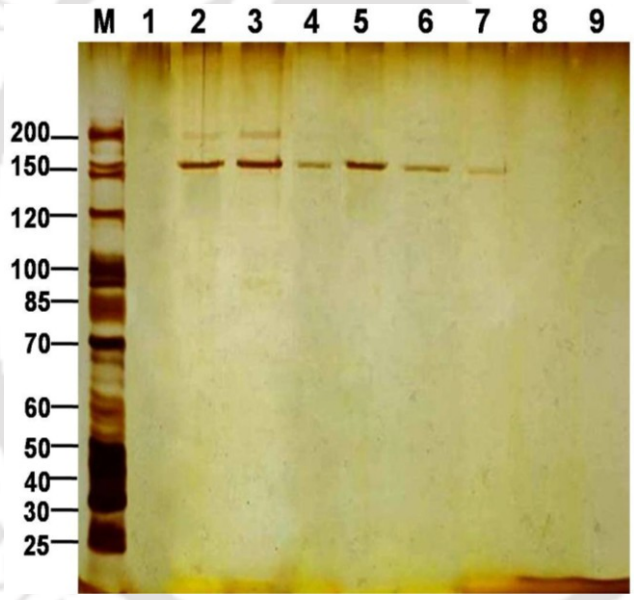

Fig. 5.3.11 Denaturing SDS-PAGE (7.5%) analysis of enzyme purified by gel filtration. Lane M: Protein Molecular Mass marker (10 kDa to 200 kDa) from Fermentas, Thermo Fisher Scientific, India (silver stained). Lane 1 to Lane 3: Fraction no. 4 to 6, Lane 4 to Lane 7: Purified fraction no. 7 to 10 showing single homogeneous band, Lane 8 and Lane 9: Fraction no. 11 and 13.

5.3.7 SDS-PAGE analysis and activity staining of purified glucansucrase by gel filtration

As mentioned in earlier Section 5.3.6, the fractions 8, 9 and 10 were selected on the basis of higher enzyme activity and protein content and showed specific activity of 18.7 U/mg. The pooled purified fractions was then run on 7.5% SDS-PAGE under denaturing condition which showed a single distinct band of molecular size of approximately, 148 kDa on silver staining (Fig. 5.3.12, Lane 1).

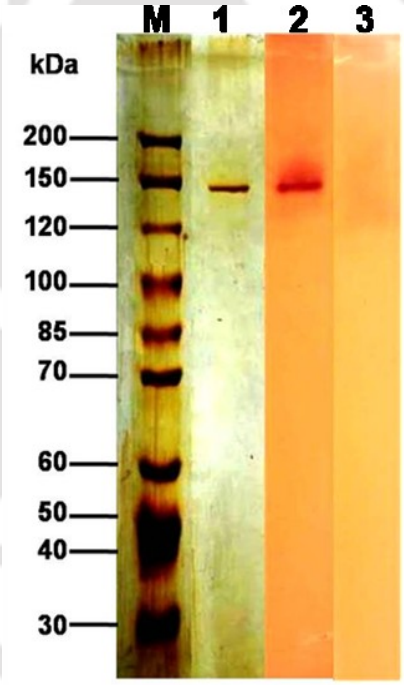

Fig. 5.3.12 SDS-PAGE (7.5%) analysis of purified glucansucrase from *Lb. plantarum* DM5 obtained from pooled fractions (8-10) of gel-filtration. Lane M: Protein ladder (10 kDa to 200 kDa) from Fermentas Thermo Fisher Scientific (silver stained). Lane 1: denatured enzyme (silver stained), Lane 2: Identification of purified glucansucrase (run under non-denaturing condition) by PAS staining of glucan formed using sucrose, Lane 3: purified glucanscuarse showing no glucan formation after PAS staining method using raffinose as substrate confirming absence of fructosyltransferase and presence of glucansucrase.

The pooled purified fraction was run on another 7.5% SDS-PAGE under non-denaturing condition for *in situ* activity detection of purified glucansucrase by PAS staining. The PAS staining of the gel incubated in 10% sucrose solution also showed the activity band that corresponded to the presence of active form of the purified glucansucrase of molecular size 148 kDa (Fig. 5.3.12, Lane 2). The result confirmed that the molecular size of the most active form of glucansucrase produced by *Lb. plantarum* DM5 is 148 kDa. No band appeared after activity staining of purified enzyme upon the incubation of the gel with raffinose (Fig. 5.3.12, Lane 3), excluding the presence of fructosyltransferase.

5.3.8 Optimum temperature and thermostability of glucansucrase

The purified glucansucrase showed maximum activity in the temperature range of 30-33°C with a specific activity of approximately 18.6 U/mg at pH 5.4 in 20 mM sodium acetate buffer (Fig. 5.3.13). The result was similar to those of earlier findings where the optimum temperature for glucansucrase activity reported was also within the same range of 30 to 35°C (Patel *et al*., 2011; Rao and Goyal, 2013). The loss of enzyme activity was observed at a temperature below and above of the range of 30-33°C. The enzyme activity rapidly decreased after 37°C and almost completely lost at 55°C. The thermostability results showed that glucansucrase was stable at lower temperatures (10-30°C) and rapidly lost activity at temperatures higher than 30°C (Fig. 5.3.13). The enzyme could retain only 6% of initial activity at 50°C.

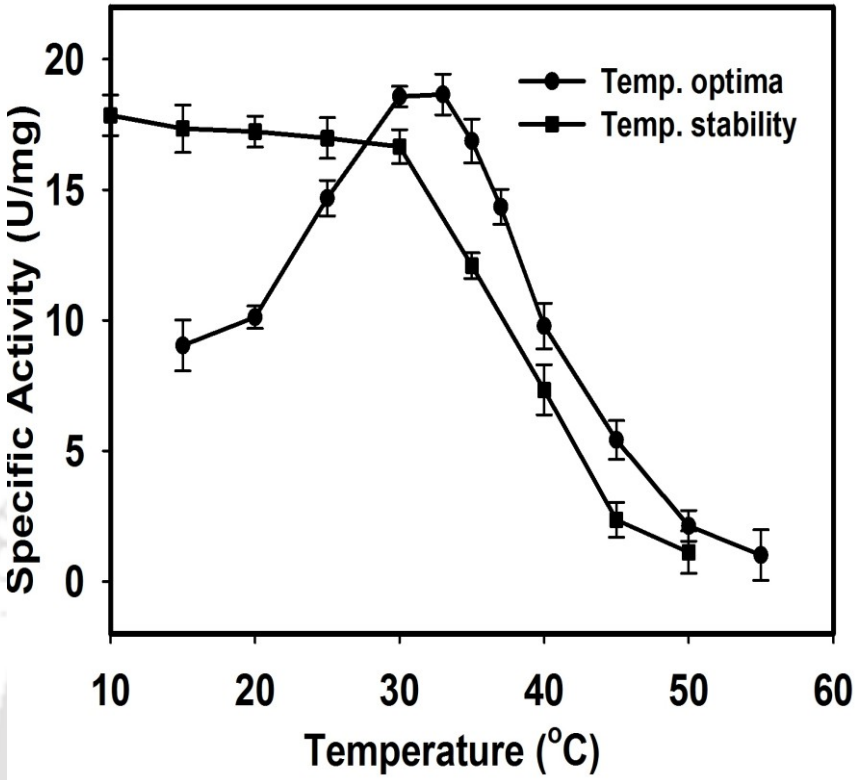

Fig. 5.3.13 Effect of temperature on activity (--●--) and stability (--■--) of glucansucrase. The temperature optimum was performed at different temperature ranging from 15-55°C ranges and for stability studies the enzyme was incubated in different temperatures (10-50°C). The assay was performed in optimum conditions and the data presented are mean value of three independent experiments with ± S.E.

5.3.9 Optimum pH and pH stability of glucansucrase

The maximum glucansucrase activity was observed at pH 5.4 with specific activity of 18.6 U/mg (Fig. 5.3.14). The optimum pH of 5.4 of glucansucrase from *Lb. plantarum* DM5 was similar to the other strains such as *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2008a), *W. confusa* Cab3 (Shukla and Goyal, 2011a) and *P. pentosaceus* SPA (Patel *et al*., 2011). The enzyme activity decreased sharply below pH 4.5 and above pH 5.5. Glucansucrase lost activity by 38% and 93% at pH 6.6 and

pH 3.4, respectively (Fig. 5.3.14). The enzyme was stable in acidic pH (4.6-5.8) range like other glucansucrases from *W. cibaria* JAG8 (Rao and Goyal, 2013) and *P. pentosaceus* SPA (Patel *et al*., 2011). The maximum stability of the glucansucrase observed at pH 5.4, was similar to the optimum pH required for enzyme assay (Fig. 5.3.14).

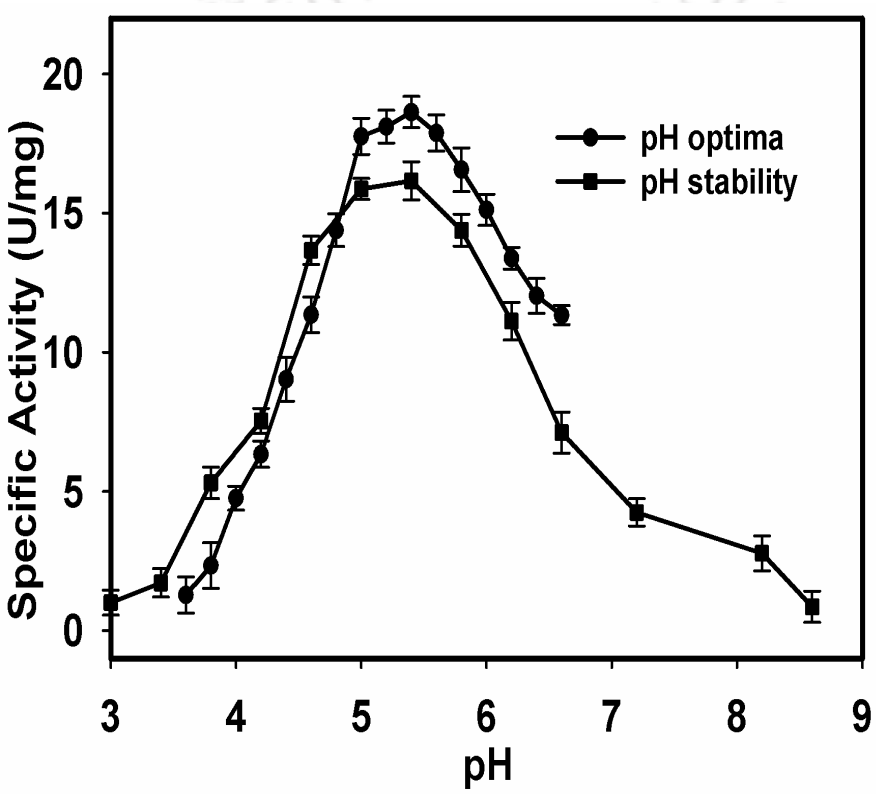

Fig. 5.3.14 Effect of pH on activity (--●--) and stability (--■--) of glucansucrase. The enzyme activity was measured in different pH ranging from 3.6-6.6 and for stability studies the enzyme was incubated for 30 min in different pH ranging from 3 to 9. The assay was performed in optimum conditions and the data presented are mean value of three independent experiments with ± S.E.

5.3.10 Effect of ionic strength on glucansucrase activity

The glucansucrase activity from *Lb. plantarum* DM5 was also determined with in a broad range of ionic strength between 10 mM-500 mM sodium acetate

buffer of pH 5.4. The results showed that the enzyme activity was stable with in experimental range of 10-200 mM with a minor variation in specific activity. The maximum activity of 18.64 U/mg was observed at 20 mM sodium acetate buffer (pH 5.4) as shown in Fig. 5.3.15. As the ionic strength of the buffer increased to 300 mM, the enzyme lost its 5% activity and further only 9% decrease in enzyme activity was observed at 500 mM sodium acetate buffer (pH 5.4). However, the decrease in the enzyme activity was not significant with increase of ionic strength. Similar trends of enzyme activity were also observed in case of *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2008a) and *P. pentosaceus* SPA (Patel *et al*., 2011).

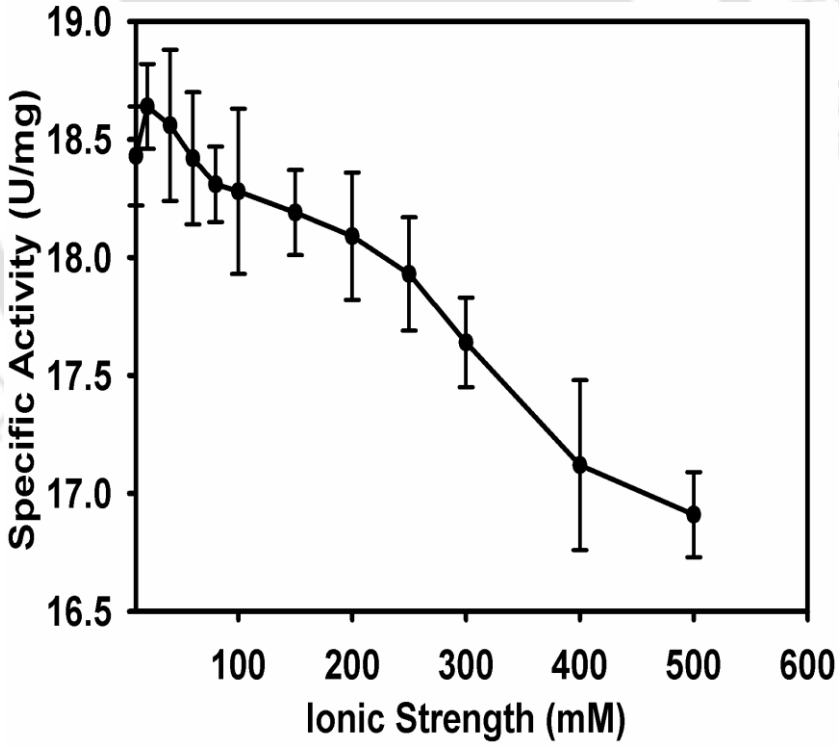

Fig. 5.3.15 Effect of ionic strength on activity (--●--) of glucansucrase. The enzyme activity was determined in sodium acetate buffer (pH 5.4) of different ionic strengths ranging from 10-500 mM. The assay was performed in optimum conditions and the data presented are mean value of three independent experiments with ± S.E.

5.3.11 Determination of kinetic parameters of glucansucrase

The effect of sucrose concentration on the enzyme activity was studied with varying sucrose concentration between 0.05 mM to 400 mM. The final sucrose concentration of 146 mM (5%, w/v) was found to be the optimum concentration for assay of the glucansucrase activity (Fig. 5.3.16).

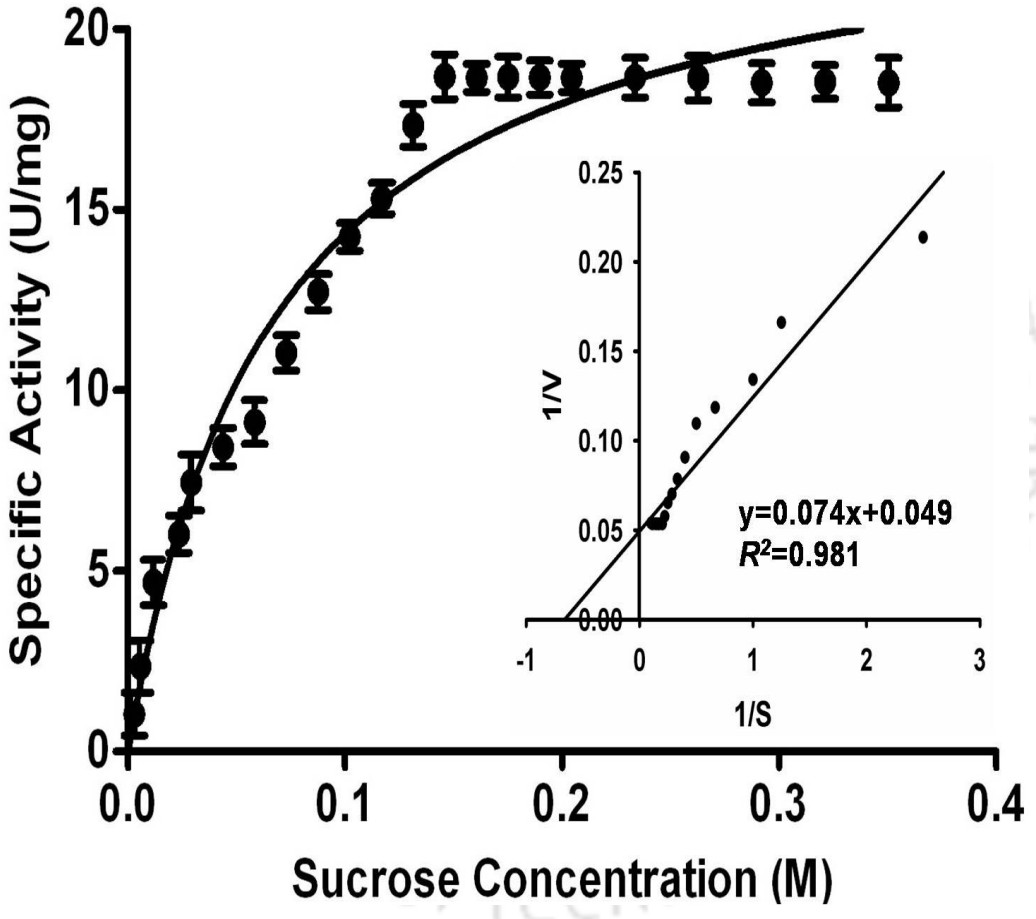

Fig. 5.3.16 Effect of sucrose concentration on glucansucrase activity. The assay was performed at 30°C in 20 mM sodium acetate buffer, pH 5.4. The value of K_m and V_m was calculated using Lineweaver Burk plot.

The results showed that it follows the classical Michaelis-Menten kinetics and the enzyme saturation was achieved at 5% sucrose concentration (Fig. 5.3.16). The V_m of 19.6 ± 0.83 µmole/mg/min and K_m of 4.5±0.58 mM was obtained from

Lineweaver Burk plot. The result indicates that the purified glucansucrase has high affinity and the specificity for the substrate and hence more effective for production of glucan. The value of K_m of glucansucrase from *Lb. plantarum* DM5 was comparable with K_m value (3.2±0.02 mM) of glucansucrase from *Lactobacillus reuteri* 180 (Pijning *et al*., 2008).

5.3.12 Effect of metal ions, salts and denaturing agent on glucansucrase activity

The Mg^{2+}, Ca^{2+} and Co^{2+} salts at lower concentrations exhibited a marginal increase in the enzyme activity of glucansucrase as shown in Fig. 5.3.17.

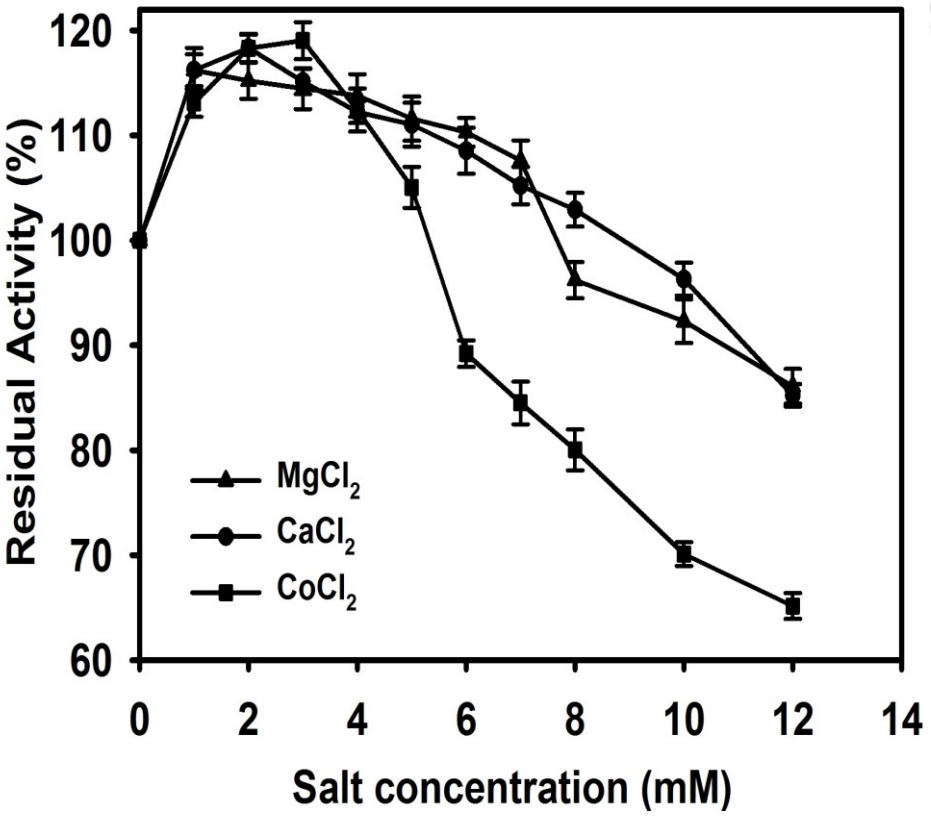

Fig. 5.3.17 Effect of metal ions on glucansucrase activity. The enzyme activity was measured in presence of varying concentrations (ranging from 1-12 mM) of $MgCl_2$ (--▲--), $CaCl_2$ (--●--) or $CoCl_2$ (--■--) and the assay was performed in optimum conditions. The data presented are mean value of three independent experiments with ± S.E.

The addition of 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 3 mM $CoCl_2$ to glucansucrase caused the enhancement of enzyme activity by 16%, 18% and 19%, respectively (Table 5.3.3).-Low concentrations of Mg^{2+}, Ca^{2+} and Co^{2+} showed similar enhancing effect on glucansucrase from *L. dextranicum* NRRL B-1146 (Majumder *et al*., 2008) and *W. cibaria* JAG8 (Rao and Goyal, 2013). The increase in the salt concentrations of Mg^{2+}, Ca^{2+} and Co^{2+} to 12 mM decreased the enzyme activity by 14%, 15% and 35%, respectively (Fig. 5.3.17 and Table 5.3.3). It has been reported that these salts stabilize the active site of enzyme by stabilizing the three dimensional protein structures (Miller and Robyt, 1984; Majumder *et al*., 2008).

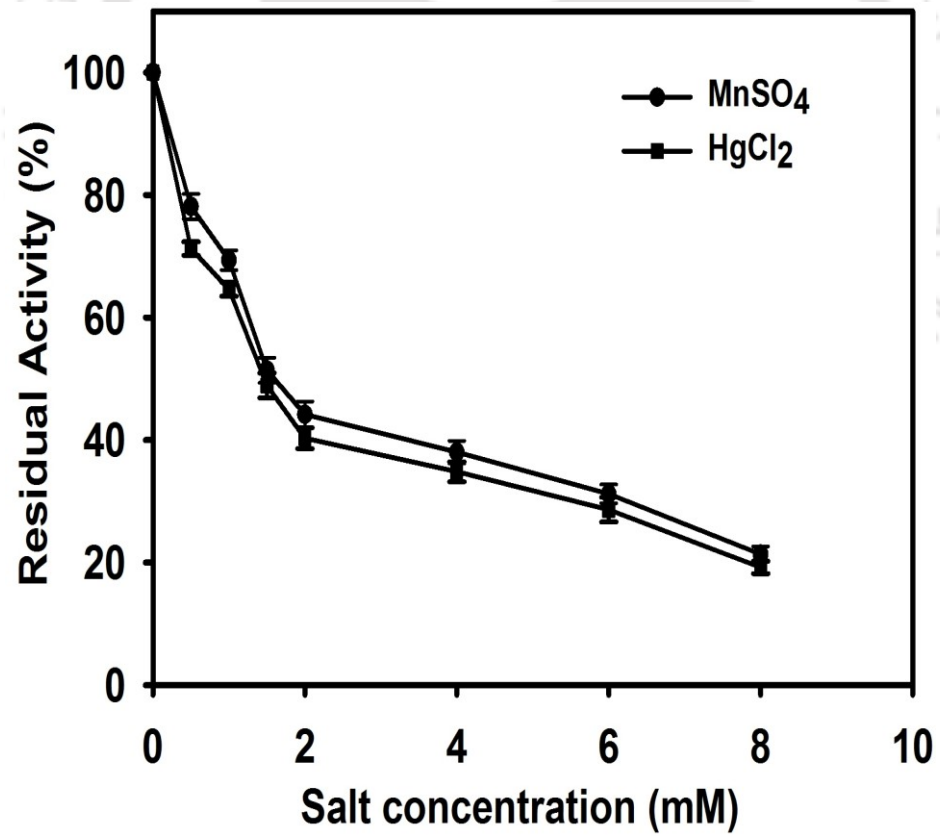

Fig. 5.3.18 Effect of metal ions on glucansucrase activity. The enzyme activity was measured in presence of varying concentrations (ranging from 0.5-8 mM) of $MnSO_4$ (--●--) and $HgCl_2$ (--■--). The assay was performed in optimum conditions. The data presented are mean value of three independent experiments with ± S.E.

The metal ions Mn^{2+} and Hg^{2+} exhibited negative effect on glucancusrase activity. The addition of 0.5 mM $MnSO_4$ and 0.5 mM $HgCl_2$ resulted in 78% and 71% residual enzyme activity. The enzyme activity further decreased to 21% and 19% in the presence of 8 mM $MnSO_4$ and 8 mM $HgCl_2$, respectively (Fig. 5.3.18 and Table 5.3.3).

Table 5.3.3 Effect of metal ions and chaotropic agent on the activity of glucansucrase.

Regents	Residual activity (%)	
	Maximum	Minimum
$MgCl_2$	116.2±2.1 (1 mM)	86.1±1.6 (12 mM)
$CaCl_2$	118.3±1.3 (2 mM)	85.2±1.1 (12 mM)
$CoCl_2$	119.0±1.8 (3 mM)	65.2±1.2 (12 mM)
$MnSO_4$	78.1±2.1 (0.5 mM)	21.4±1.3 (8 mM)
$HgCl_2$	71.3±1.1 (0.5 mM)	19.2±1.0 (8 mM)
Urea	58.4±1.4 (0.5 M)	3.2±1.3 (6 M)
EDTA	70.1±1.0 (1 mM)	31.3±1.2 (6 mM)

Urea at all concentrations displayed a denaturing effect on glucansucrase (Fig. 5.3.19) and 97% loss of enzyme activity was observed with 6M urea (Table 5.3.3). The treatment of enzyme with 1 mM EDTA inactivated the enzyme by 30% (Table 5.3.3). Further increase in the concentration of EDTA resulted in decrease of the enzyme activity and at 6 mM EDTA it decreased by 69% (Fig.5.3.19). Similar results were also obtained with glucansucrase from *W. cibaria* JAG8 (Rao and Goyal, 2013) and *P. pentosaceus* SPA (Patel *et al*., 2011) for effects of urea and EDTA.

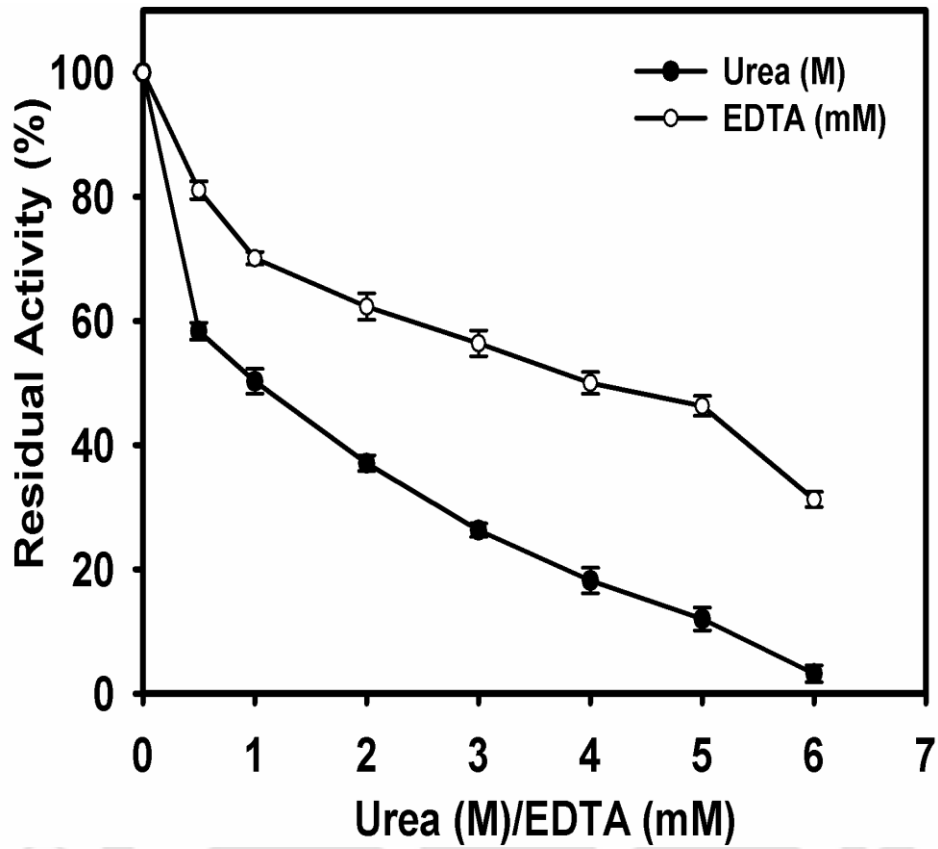

Fig. 5.3.19 Effect of chaotropic agents on glucansucrase activity. The enzyme activity was measured in presence of varying concentrations of urea ranging from 0.5-6.0 M (--●--) and EDTA ranging from 0.5 mM to 6.0 mM (--o--). The assay was performed in optimum conditions and the data presented are mean value of three independent experiments with ± S.E.

5.3.13 Effect of additives and stabilizer on glucansucrase activity

The residual enzyme activity of glucansucrase was measured at 30ºC with respect to time in presence as well as absence of the stabilizer for 30 h. The half life ($t_{1/2}$), mean life time and decay constant (λ) were calculated using the web available software, (http://www.calculator.net/half-life). The enzyme without any added stabilizer (control) lost activity rapidly ($t_{1/2}$= 10.3 h) at 30°C and only 13.3% residual activity remained after 30 h (Table 5.3.4). Glycerol, PEG-6000 and PEG-8000 acted

as stabilizers and displayed stabilising effects on glucansucrase as the residual activity was 42% ($t_{1/2}$= 24 h), 38% ($t_{1/2}$= 21.5 h) and 33% ($t_{1/2}$= 18.6 h), respectively after 30 h. Dextran T-40 also acted as good stabilizer with 56% residual activity ($t_{1/2}$= 41 h). Glutaraldehyde and acetonitrile ($t_{1/2}$= 5 h and $t_{1/2}$= 7 h, respectively) acted as inhibitor of enzyme as the enzyme activity was inhibited by 95% and 92%, respectively.

Table 5.3.4 Effect of stabilizers on glucansucrase activity at 30ºC.

Stabilizer[a]	[b]Residual Activity (%)	Half life ($t_{1/2}$) (h)	Mean life time (h)	Decay constant (λ)
[c]Control	13.3	10.3±0.1	14.8±1.2	1.6±1.2
Glycerol (0.5%, v/v)	42.1	24.0±1.0	34.6±0.8	0.8±1.1
PEG-6000 (10 µg/ml, w/v)	38.0	21.5±1.0	31.0±1.4	0.8±1.0
PEG-8000 (10 µg/ml, w/v)	33.0	18.6±0.9	26.8±1.2	0.9±1.5
Dextran T40 (2 µg/ml, w/v)	56.3	41.0±1.3	59.0±0.6	0.4±1.1
Glutaraldehyde (0.1%, v/v)	5.4	5.0±1.2	7.2±0.8	3.4±1.2
Acetonitrile (1%, v/v)	8.3	7.0±1.0	9.4±1.0	2.5±1.5
Tween 80 (10 µl/ml, v/v)	86.0	64.8±1.3	93.3±1.0	0.2±0.8

[a] *The enzyme with or without any stabilizer incubated at 30ºC for 30 h.*

[b] *Residual enzyme activity was calculated by considering the initial enzyme activity as 100%.*

[c] *The enzyme without any stabilizer incubated at 30ºC for 30 h and residual activity was calculated from initial activity as 100%.*

Among all the stabilizers, Tween 80 displayed the maximum stabilization effect on glucansucrase with 86% of residual activity ($t_{1/2}$ = 64.8 h) after 30 h and a decay rate of approximately 20% per h at 30ºC. Therefore, Tween 80 and dextran T-40 were used as stabilizers for investigation of long term storage stability at -20°C, 0°C and 4°C temperatures. The stabilization data of glucansucrase from *Lb. plantarum* DM5 was in agreement with previous report, where glucansucrase from *L. mesenteroides* NRRL B-512F showed stabilization in presence of high molecular

weight dextran (2 µg/ml) and non-ionic detergent Tween 80 (10 µg/ml) (Miller and Robyt, 1984; Purama *et al.,* 2010).

5.3.14 Effect of storage temperature on stability of glucansucrase

To study the suitable storage temperature, glucansucrase was incubated with and without Tween 80 or dextran T-40 at three different temperatures -20°C, 0°C and 4°C and their half lives were calculated. The enzyme lost 50% activity in 24 days when stored at 4°C, whereas, in the presence of dextran T-40 and Tween 80, the 50% loss of enzyme activity was observed in 32 days and 40 days, respectively (Fig. 5.3.20A). The enzyme stored at 0°C without any stabilizer retained 50% activity for 48 days of incubation, however, in presence of dextran T-40 and Tween 80; it retained 50% activity for 60 and 70 days, respectively (Fig. 5.3.20B). The enzyme when stored at -20°C without any stabilizer, 50% of its initial activity lost after 90 days, whereas in the presence of additive dextran T-40 and Tween 80, the 50% loss of enzyme activity was observed in 110 days and 120 days, respectively as shown in Fig. 5.3.20C.

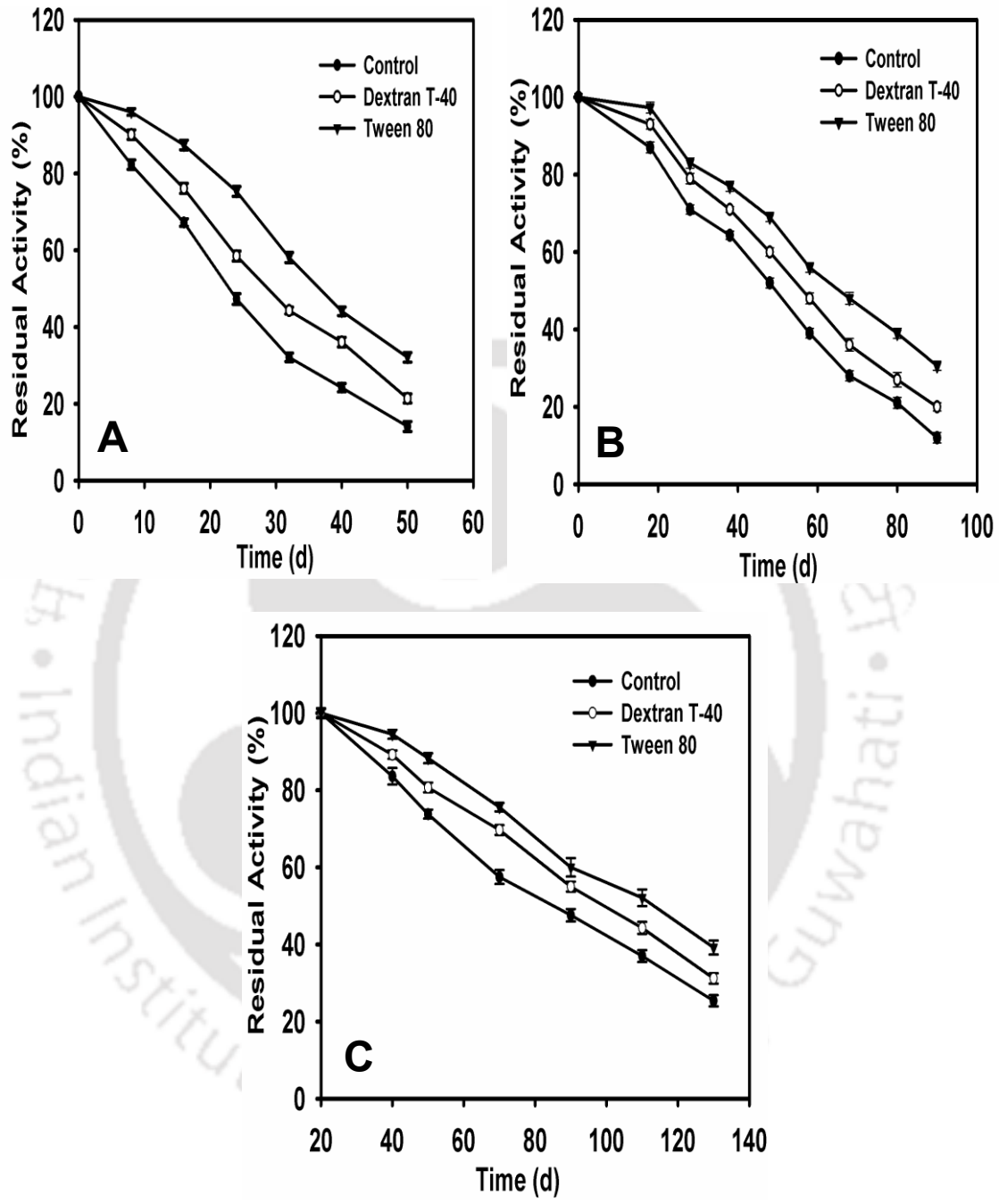

Fig. 5.3.20 Effect of storage temperature on glucansucrase from *Lactobacillus plantarum* DM5. The purified enzyme stored at (A) 4ºC (B) 0ºC (C)-20ºC with or without dextran T-40 and Tween 80 as stabilizer. The assay was performed in optimum conditions and the data presented are mean value of three independent experiments with ± S.E. The symbols (--●--), (--o--) and (--▼--) signifies enzyme stored with any stabilizer or control, enzyme stored with dextran T-40 and enzyme stored with Tween 80.

The enzyme in presence of dextran T-40 and Tween 80 also exhibited enhanced half life of 80 days and 86 days, respectively, as compared with the control ($t_{1/2}$= 65 d) at -20°C (Fig. 5.3.21). Tween 80 provided 33%, 35% and 16% higher stabilization than dextran T-40 at all the three temperatures 4°C, 0°C and -20°C, respectively. From all above data it can be summarized that Tween 80 as an additive ($t_{1/2}$=86 days, decay rate= 0.8% per day) proved to be best for the stabilization of glucansucrase from *Lb. plantarum* DM5 and could be used for long term storage.

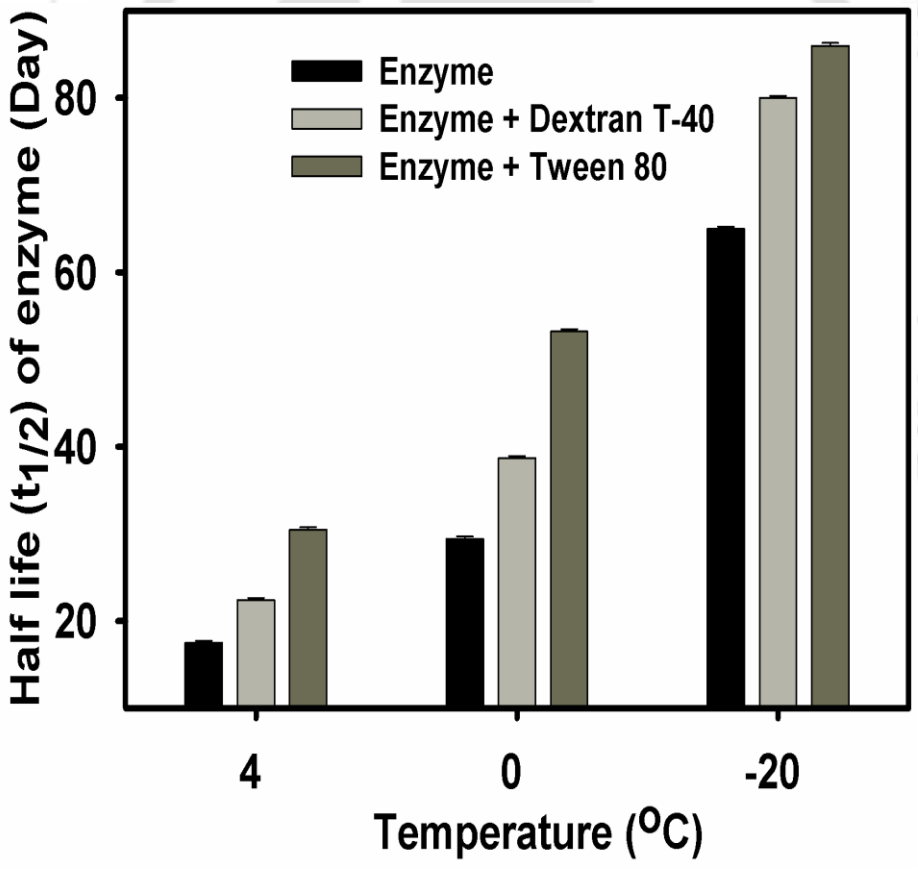

Fig. 5.3.21 The half lives of purified enzyme, enzyme with dextran T-40 and Tween 80 at all three above mentioned temperatures. The assay was performed in optimum conditions and the data presented are mean value of three independent experiments with ± S.E.

5.4 Conclusions

The probiotic *Lb. plantarum* DM5 isolated from fermented beverage Marcha of Sikkim was explored for its ability to produce extracellular glucansucrase during fermentation. The bacterium displayed glucansucrase activity of 2.7 U/ml (0.48 U/mg) at 27°C under static condition. It was observed that the production of glucansucrase from *Lb. plantarum* increased with increasing conc. of sucrose and the maximum activity of 6.23±0.11 U/ml was observed at 5% (w/v) sucrose concentration. The other medium compositions such as yeast extract (2.5%, w/v), K_2HPO_4 (2.5%, w/v) and Tween 80 (0.6%, v/v) were important nutrients for *Lb. plantarum* DM5 as they significantly enhanced the glucansucrase production by 20%, 22% and 68% respectively. The cell free supernatant of *Lb. plantarum* DM5 containing extracellular glucansucrase (0.48 U/mg, 5.7 mg/ml) was subjected to fractionation with various concentrations of PEG-400 and PEG-1500. The maximum specific activity of 6.7 U/mg and 10.1 U/mg was achieved at 36%, (v/v) PEG-400 and 15%, (w/v) PEG-1500, respectively. The glucansucrase purified by PEG-1500 followed by gel filtration using Sephacryl S-300HR gave enhanced specific activity of 18.7 U/mg with 40-fold purification and showed molecular mass of approximately, 148 kDa. The zymogram analysis of purified enzyme confirmed that it was glucansucrase as it showed magenta colour band only in presence of sucrose not in presence of raffinose. The purified glucansucrase was maximally active at 30ºC and pH 5.4 and was stable at acidic pH and low temperature. The V_m and K_m of purified glucansucrase for sucrose as substrate was 19.6 µmoles/mg/min and 4.5 mM. The metal ions, Ca^{2+}, Mg^{2+} and Co^{2+} enhanced the enzyme activity by 16%, 18% and 19%, respectively, whereas Mn^{2+} and Hg^{2+} decreased the enzyme activity by 78% and

71% respectively. The urea and EDTA even at lower concentrations denatured the glucansucrase which resulted in decrease of enzyme activity. The storage stability studies of the glucanscurase showed 25% higher stability in presence of Tween 80 at -20ºC ($t_{1/2}$= 86 days) as compared with the enzyme ($t_{1/2}$= 65 days) without any added stabilizer. The overall biochemical characterization reveals a promising novel glucansucrase which can compensate the increasing demand of glucan as viscosifier and stabilizer in food industry. Further studies are needed using the pure glucansucrase from *Lb. plantarum* DM5 in order to produce glucan to support its strong candidacy for application as a food supplement.

References

Adlerberth, J., Ahrne, S., Johansson, M.L., Molin, G., Hanson, L.A. and Wold, A.E. (1996) A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Appl. Environ. Microbiol*., 62, 2244-2251.

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

Cagno, R.D., Angelis, M.D., Limitone, A., Minervini, F., Carnevali, P., Corsetti, A., Gaenzle, M., Ciati, R. and Gobbetti, M. (2006) Glucan and Fructan production by sourdough *Weissella cibaria* and *Lactobacillus plantarum*. *J. Agric. Food Chem*., 54, 9873-9881.

Cantarel, B.L., Coutinho, P.M., Rancurel, C., Bernard, T., Lombard, V. and Henrissat, B. (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res*., 37, D233-D238.

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.

Cote, G.L., Skory, C.D., Unser, S.M. and Rich, J.O. (2012) The production of glucans via glucansucrases from *Lactobacillus satsumensis* isolated from a fermented beverage starter culture. *Appl. Microbiol. Biotechnol*., 97, 7265-7263.

Das, D. and Goyal, A. (2010) Characterization and screening of antimicrobial activity of lactic acid bacterium isolated from a traditional beverage marcha of Sikkim. *J. Pharm. Chem*., 4, 136-139.

Das, D., and Goyal, A. (2013) Potential probiotic attributes and antagonistic activity of an indigenous isolate *Lactobacillus plantarum* DM5 from an ethnic fermented beverage Marcha of north eastern Himalayas. *Int J Food Sci nutr.,* (Accepted)

De Vuyst, L., Vanderveken, F., de ven, S. and Degeest, B. (1998) Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in a milk medium and evidence for their growth-associated biosynthesis. *J. Appl. Microbiol*., 84, 1059-1068.

Degeest, B., Mozzi, F. and De Vuyst, L. (2002) Effect of medium composition and temperature and pH changes on exopolysaccharide yields and stability during *Streptococcus thermophilus* LY03 fermentations. *Int. J. Food Microbiol.,* 79**,** 161-174.

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

Dutta, A., Das, D. and Goyal, A. (2012) Purification and characterization of fructan and fructansucrase from *Lactobacillus fermentum* AKJ15 isolated from Kodo ka jaanr, a fermented beverage of north east Himalayas. *Int. J. Food Sci. Nutr*., 63, 216-224.

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

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

Kitaoka, M. and Robyt, J.F. (1998) Use of a microtiter plate screening method for obtaining *Leuconostoc mesenteroides* mutants constitutive for glucansucrase *Enzyme Microb. Technol*., 22, 527-531.

Kralj, S., Grijpstra, P., van Leeuwen, S.S., Leemhuis, H., Dobruchowska, J.M., van der Kaaij, R.M., Malik, A., Oetari, A., Kamerling, J.P. and Dijkhuizen, L. (2011) 4,6-α-Glucanotransferase, a novel enzyme that structurally and functionally provides an evolutionary link between glycoside hydrolase enzyme families 13 and 70. *Appl. Environ. Microbiol*., 77, 8154-8163.

Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophageT4. *Nature,* 227, 680-685.

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

Lonnermark, E., Friman, V., Lappas, G., Sandberg, T., Berggren, A. and Adlerberth, I. (2010) Intake of *Lactobacillus plantarum* reduces certain gastrointestinal symptoms during treatment with antibiotics. *J. Clin. Gastroenterol*., 44, 106-112.

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. and Goyal, A. (2008) Optimization of culture conditions of a novel glucan producing glucansucrase from *Leuconostoc dextranicum* NRRL B-1146. *Curr. Trends Biotechnol. Pharm.*, 2, 260-268

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

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

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

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

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

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

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

Purama, R.K. and Goyal, A. (2008b) Effect of nutrients by one variable at a time (OVAT) approach on the dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640. *Int. J. Microbiol*., 5:1, doi: 10.5580/1bc.

Rabilloud, T.A. (1992) Comparison between low background silver diammine and silver nitrate protein stains. *Electrophoresis,* 13, 429-439.

Rao, T.J. and Goyal, A. (2013) Purification, optimization of assay, and stability studies of dextransucrase isolated from *Weissella cibaria* JAG8. *Prep. Biochem. Biotechnol.,* 43, 329-41.

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

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

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

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. (2011a) 16S rRNA based identification of a glucan hyper-producing *Weissella confusa*. *Enzym. Res*., ID 250842, doi:10.4061/2011/250842.

Shukla, S. and Goyal, A. (2011b) 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.

Switzer, R.C., Merril, C.R. and Shifrin, S. (1979). A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal Biochem*., 98, 231-237.

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 culture factors on production on dextransucrase by *Leuconostoc mesenteroides. J. Bacteriol*., 64, 521-526.

Ul-Qader, S.A., Iqbal, L., Rizvi, H.A. and Zuberi, R. (2001) Production of dextran from sucrose by a newly isolated strain of *Leuconostoc mesenteroides* (PCSIR-3) with reference to *L. mesenteroides* NRRL B-512F. *Biotechnol. Appl. Biochem*., 34, 93-97.

van Hijum, S.A.F.T., Kralj, S., Ozimek, L.K., Dijkhuizen, L. and van Geel-Schutten, I.G.H. (2006) Structure function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol. Mol. Biol. Rev*., 70, 157–176.

van Leeuwen, S.S., Kralj, S., Eeuwema, W., Gerwig, G.J., Dijkhuizen, L. and Kamerling, J.P. (2009) Structural characterization of bioengineered α-D-Glucans produced by mutant glucansucrase GTF180 enzymes of *Lactobacillus reuteri* strain 180. *Biomacromol.,* 10, 580-588.

Zhang, L., Liub, C., Li, D., Zhaoa, Y., Zhanga, X., Zenga, X., Yanga, Z. and Li, S. (2013) Antioxidant activity of an exopolysaccharide isolated from *Lactobacillus plantarum* C88. *Int. J. Biol. Macromol*., 54**,** 270-275.

Chapter 6

Characterization and biocompatibility of glucan: a safe food additive from probiotic *Lactobacillus plantarum* DM5

6.1 Introduction

Over the past few decades, exopolysaccharides from microbial sources are being screened and have found numerous potential applications in food and pharmaceutical industry (Pan and Mei, 2010; Patel *et al*., 2010). The physiological roles of these exopolysaccharides in the microbial host are not yet completely understood, but they are involved in protection against dehydration, pathogenicity, biofilm formation and quorum sensing (Tallon *et al*., 2003). Lactic acid bacteria (LAB) of the genera *Leuconostoc*, *Lactobacillus*, *Pediococcus*, *Weisella* and *Streptococcus* species produces an abundant variety of exopolysaccharides synthesized by extracellular glucansucrases using sucrose as the glucosyl donor (Monsan *et al*., 2001; Purama and Goyal, 2008, Leemhuis *et al*., 2013). The exopolysaccharide synthesized by lactic acid bacteria are of two types, homopolysaccharides that consist of only one monosaccharide, such as glucose (glucans), fructose (fructans) or galactose (galactans) and heteropolysaccharides

formed of repeating units of different monosaccharides, including glucose, galactose, fructose and rhamnose (Monsan *et al*., 2001; Bejar *et al*., 2013). The α-D-Glucan synthesized by LAB are categorized mainly in four groups i) dextrans with α-(1→6) linkages, or with a majority of α-(1→6) linkages and α-(1→2), α-(1→3), and/or α-(1→4) branches (mainly found in *Leuconostoc*), ii) mutan with a majority of α-(1→3) linkages (found in *Streptococcus*), iii) alternan with alternating α-(1→3) and α-(1→6) linkages (only reported in *L. mesenteroides*) and iv) reuteran being a highly branched structure with mainly α-(1→4) linkages (found in *Lactobacillus reuteri*) (Monchois *et al*., 1999; van Leeuwen *et al*., 2009).

The α-D-glucan with unique physico-chemical properties produced by LAB has been reported that facilitate their applications in food industry as stabilizers and gelling agents (Monchois *et al*., 1999; Tallon *et al*., 2003). Among all the *Lactobacillus* spp., *Lactobacillus reuteri, Lactobacillus sakei*, *Lactobacillus fermentum* and *Lactobacillus parabuchneri* are known to produce glucansucrase and contain the same variety of glucansucrase and glucan products as *Leuconostoc* and *Streptococcus* spp. (van Leeuwen *et al*., 2009). However, the concomitant production of glucansucrase and fructansucrase from *Lb. plantarum* PL9 during sourdough fermentation has been reported (Cagno *et al*., 2006). *Lactobacillus plantarum* as probiotic has been proven effective against diarrhoea, irritable bowel disorder and lactose intolerance (Lonnermark *et al*., 2010). The production of cell bound exopolysaccharide from *Lb. plantarum* EP56 (Tallon *et al*., 2003) and a heteropolysaccharide composed of galactose and glucose with a molar ratio of 1:2 from *Lb. plantarum* C88 (Zhang *et al*., 2013) have been reported. However, the

production of linear α-D-glucan with (1→6) linkages from *Lb. plantarum* has not reported.

In the present study, an exopolysaccharide producing probiotic lactic acid bacterium, *Lactobacillus plantarum* DM5 was isolated from traditional fermented beverage Marcha of biodiversity hot spot region Sikkim, India (Das and Goyal, 2010) was studied. The production, purification and characterization of glucan from *Lactobacillus plantarum* DM5 was carried out. The effect of purified glucan was evaluated for cytotoxicity tests on Human embryonic kidney (HEK 293) and Human cervical cancer (HeLa) cell lines. To the best of our knowledge, production and purification of glucan (dextran) from probiotic *Lactobacillus plantarum* has been studied for the first time.

6.2 Material and Methods

6.2.1 Chemicals and reagents

The components for maintenance of bacterial culture, enzyme production medium were purchased from Hi-Media Pvt. Ltd., India. Absolute ethanol, phenol, sodium chloride and sulphuric acid were purchased from Merck India Pvt. Ltd. The chemicals required for buffer preparations, sodium alginate and Fetal bovine serum (FBS) for maintaining the mammalian cell line were of high purity grade and purchased from Hi-Media Pvt. Ltd., India. The hydrocarbon n-hexadecane was purchased from Spectrum Lab Pvt. Ltd. India. The standard dextrans (40 kDa, 100 kDa, 270 kDa, 410 kDa, 500 kDa and 2000 kDa), potassium bromide (KBr), guar gum and the matrix for gel filtration (Sepharose CL-6B and Sephacryl S-500HR) were procured from Sigma Aldrich, USA.

6.2.2 Microorganism and culture medium

The strain *Lb. plantarum* DM5 (Genbank Accession No: KC020195) was screened from an ethnic fermented beverage Marcha of Sikkim on the basis of antimicrobial activity (Das and Goyal, 2010) and glucansucrase activity (as described in Chapter 2, Section 2.2.6.1). The isolate was maintained in modified MRS agar medium (Goyal and Katiyar, 1996) as described in Chapter 2, Section 2.2.2, at 4ºC and sub cultured after every 2 weeks.

6.2.3 Production of glucan from *Lb. plantarum* DM5

The production of glucan from *Lb. plantarum* DM5 was carried out by inoculating 1 ml culture of *Lb. plantarum* DM5 in sterile 100 ml enzyme production

medium as reported by Tsuchiya *et al*., (1952) (as described in Chapter 2, Section 2.2.3). The culture was incubated at 27°C for 9 h under static condition and after 9 h of incubation; the cell free supernatant was collected by centrifugation at 10,000*g* and at 4°C for 10 min as mentioned in Chapter 5, Section 5.3.2. The cell free supernatant was used subsequently for the purification of glucan as described in Section 6.2.4.

6.2.4 Purification of glucan from *Lb. plantarum* DM5

6.2.4.1 Purification of glucan by ethanol precipitation

The cell free supernatant (50 ml) of *Lb. plantarum* DM5 obtained by centrifugation at 10,000*g* at 4°C for 10 min (as described in Section 6.2.3), was used for purification of glucan by ethanol precipitation. The chilled ethanol (95%) was added to the cell free supernatant in a ratio of 3:1 and centrifuged at 10,000*g* at 4°C for 30 min (Purama *et al*., 2009). The pellet was collected and dissolved in 50 ml of deionised water and the process was carried out thrice. The glucan content was determined by phenol-sulfuric acid method (Dubois *et al.,* 1956) in a micro titer plate (Fox and Robyt, 1991) as described in Chapter 5, Section 5.2.8.

6.2.4.2 Purification of glucan by gel filtration

Glucan solution (50 ml) after purification by ethanol was freeze dried by using lyophilizer (Christ GmbH, model ALPHA 1-4 LD) at -51°C and at a vacuum pressure of 3.5×10^{-2} mbar for 24 h and the dried powder of glucan was used for further purification by gel filtration using Sepharose CL-6B (Sigma Aldrich, USA). The dried powder of glucan (10 mg) was dissolved in 1 ml of 0.9% (w/v) sodium chloride

solution and loaded onto a gel filtration column (1.5 cm x 50 cm) containing Sepharose CL-6B and connected to Fast Performance Liquid Chromatography (FPLC) (Akta Prime, GE Healthcare). The glucan was eluted using 0.9% (w/v) sodium chloride solution at a flow rate of 0.3 ml/min and fractions of 3 ml were collected (Zhang *et al.,* 2013; Das and Goyal, 2013). The total carbohydrate content was determined by phenol sulphuric acid method (Dubois *et al.,* 1956) as described in Chapter 5, Section 5.2.8. The fractions showing higher absorbance at 490 nm were pooled and lyophilized for further physicochemical and structure analysis.

6.2.5 Structure analysis of glucan from *Lb. plantarum* DM5

6.2.5.1 Molecular mass determination of glucan

The average molecular mass of the glucan produced by *Lb. plantarum* DM5 was determined by gel permeation chromatography following the method as described by Zhang *et al.,* (2013). Standard dextrans (5 mg/ml) of molecular weight 40 kDa, 100 kDa, 270 kDa, 410 kDa, 500 kDa and 2000 kDa (Sigma Aldrich, USA) were passed through a Sephacryl S-500HR column (1.5 cm x 50 cm) pre-equilibrated with sodium chloride (0.9%, w/v) solution. The column was connected to FPLC (Akta Prime, GE Healthcare) and the 1 ml (5 mg/ml) of the each standard dextran was loaded on the column. The standard dextrans were eluted using 0.9% (w/v) sodium chloride solution at a flow rate of 0.3 ml/min and fractions of 2 ml were collected (Das and Goyal, 2013). The purified glucan (5 mg/ml) was also passed through the column and eluted under the same conditions with a flow rate of 0.3 ml/min and fractions of 2 ml were collected. The total carbohydrate content of all standard dextrans and glucan was determined by phenol sulfuric acid method (Dubois *et al.,*

1956) as described in as described in Chapter 5, Section 5.2.8. The logarithmic values of the molecular mass of the standard dextrans were plotted against their respective retention times (Zhang *et al*., 2013; Sajna *et al*., 2013). The average molecular mass of glucan from *Lb. plantarum* DM5 was determined from the standard graph which was plotted using standard dextrans and their respective retention time.

6.2.5.2 Monosaccharide composition analysis of glucan

Monosaccharide analysis of column purified glucan (2 mg/ml) was done by acid hydrolysis (100 mM H_2SO_4) at 100°C for 4 h. The hydrolysate was neutralized by 2N NaOH and the released monosaccharides were analyzed by high performance anion exchange chromatography (HPAEC, Dionex, ICS-3000 system) with Pulse Amperometric Detection (PAD) using Carbo-Pac P20 column (150 x 0.3 cm). The isocratic elution was carried out using 0.1N NaOH at a constant flow rate of 0.5 ml/min at 30°C and the detection of monosaccharide was done with an electrochemical detector (ED 50).

6.2.5.3 Optical rotation

The optical rotation of column purified glucan (50 mg/ml) was measured in a polarimeter (Perkin-Elmer Instruments, Model 343 Polarimeter) using a sodium D-line (589 nm) at 25ºC. The glucose (50 mg/ml) and fructose (50 mg/ml) were used as reference.

6.2.5.4 Fourier Transform Infrared Spectroscopic analysis of glucan

The FTIR spectrum was recorded for column purified glucan in a KBR pellet using a spectrophotometer (Perkin Elmer Instruments, Spectrum Two). The purified glucan (2 mg) was finely grinded with 400 mg of potassium bromide (KBr) powder and pressed into pellets of thickness 0.5 to 1 mm using hydraulic press. For a solid sample preparation in FTIR analysis, the concentration of the sample in KBr should be in the range of 0.2% to 1% and the pellet thickness should be in the range of 0.5 mm to 1 mm for avoiding the noisy spectra (Davarcioglu, 2010). The pellet was then placed in a transmission holder and scanned in the region of 4000 cm^{-1} to 500 cm^{-1} with 20 scans per min.

6.2.5.5 ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectral analysis of glucan

The structure of glucan purified from gel filtration was elucidated using ^{1}H NMR and ^{13}C NMR techniques using NMR spectrophotometer (Varian, Model AS400) equipped with VnmrX for Sun Microsystems Ver. 6.1 software. The ^{1}H NMR spectra of glucan (10 mg/ml) dissolved in D_2O (Merck India Pvt. Ltd.) was recorded on at a temperature of 293K with a base frequency of 400 MHz. The ^{13}C NMR spectra of glucan (50 mg/ml) dissolved in D_2O was recorded with a base frequency of 100 MHz.

6.2.5.6 Rheological properties of glucan

The rheological property of the glucan (0.5%, w/v) purified from gel filtration dissolved in deionised water was determined at 25ºC by using a rheometer (Thermo

Electron model, Haake rheostress RSI) interfaced with HAAKE RheoWin 323 software. The shear rate was in the range of 0.1–1000 s^{-1}.

6.2.5.7 Thermo-gravimetric analysis of glucan

The thermal property of glucan was studied by thermo-gravimetric analysis (TGA) using Thermo-gravimetric analyzer (DSC 821, Mettler Toledo India Pvt. Ltd.). The compound (5 mg) was subjected to a temperature range of 30-600ºC under nitrogen atmosphere with a linear heating at rate of 5ºC/min and the corresponding weight loss was determined. The graph was plotted by taking the percentage of weight loss and derivative of weight against the temperature. The change in mass of glucan sample, expressed as percentage with respect to original mass was plotted on the vertical axis as a function of temperature on the horizontal axis.

6.2.6 Physicochemical characterization of glucan from *Lb. plantarum* DM5

6.2.6.1 Scanning Electron Microscopic analysis of glucan

The glucan (1 mg) purified from gel filtration sample was coated with ~10 nm Au in a sputter coater (SCH 620, Leo) and viewed in Scanning Electron Microscope (SEM) (Leo1330 VP) at magnification of 1.35 Kx operated at 14.0 kV.

6.2.6.2 Solubility properties of glucan

Solubility of glucan from *Lb. plantarum* DM5 in water was determined by the method established by Chang and Cho, (1997). The glucan (30 mg) was dissolved in 1 ml deionised water with continuous agitation at 25ºC for 24 h. The glucan suspension was then centrifuged at 5,000*g* at 25ºC for 15 min and the upper layer (0.2 ml) of

supernatant was collected. The 0.6 ml of chilled ethanol (95%) was added to the 0.2 ml of the supernatant in a ratio of 3:1 and centrifuged at 10,000*g* at 4°C for 20 min. The resulting precipitate was vacuum dried at 80ºC and difference in weight loss was recorded.

The solubility of glucan was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{Total glucan concentration in supernatant}}{\text{Initial dry weight of the glucan}} \times 100$$

6.2.6.3 Water holding capacity of glucan

The glucan polymer was also characterized for water holding capacity (WHC) by following the method established by Ahmed *et al.,* (2013). The glucan (0.2 g) was dissolved in 10 ml of deionised water and was centrifuged at 13,000*g* at 4ºC for 30 min. Unbound water that was not held by glucan was discarded and the precipitated glucan was placed on pre weighed filter paper to remove the moisture of the pellet. The wet weight of precipitated glucan in form of pellet was determined. The percentage of WHC was calculated as;

$$\text{WHC (\%)} = \frac{\text{Wet weight of glucan after water absorption}}{\text{Initial dry weight of the glucan}} \times 100$$

6.2.6.4 Emulsion stability of glucan

The emulsifying activity of glucan from *Lb. plantarum* DM5 was assayed by the method described by Bramhachari *et al.,* 2007. Lyophilized glucan (0.5 mg) was

dissolved in 0.5 ml deionised water by heating at 100ºC for 15 min and allowed to cool at 25ºC. The volume was then made up to 2 ml using 1x phosphate-buffered saline (PBS), pH 7.4. The sample was mixed on a vortex for 1 min after the addition of 0.5 ml n-hexadecane. The absorbance at 540 nm (A_0) was immediately measured after mixing (A_0). The sample was then incubated at 25ºC and decrease in absorbance was recorded at 30 and 60 min (A_t). A control was run simultaneously with only 2 ml of 1x PBS (pH 7.4) and 0.5 ml n-hexadecane. The emulsification activity was expressed as the percentage retention of emulsion during incubation for 30 or 60 min;

$$\textbf{Emulsion (\%)} = (A_t/A_0) \times 100$$

where, A_0= absorbance (A_{540}) of the suspension at time t=0 and A_t= absorbance (A_{540}) of the suspension at time t=30 or 60 min.

6.2.6.5 Flocculating activity of glucan

The flocculating activity of glucan from *Lb. plantarum* DM5 was determined by following the method of Lim *et al*., (2007) using charcoal activated carbon. In a test tube, 50 mg of charcoal activated carbon was mixed in 10 ml of deionised water and mixed with 0.1 ml of 6.5 mM $CaCl_2$ solution. The glucan with various concentrations ranging from 0.1 to 0.8 mg/ml was added to the suspension and mixed on a vortex for 30 s. The reaction mixture was allowed to stand at 30ºC for 10 min and the absorbance (A_{550}) at 550 nm of the upper phase (1 ml) was measured. The absorbance of the control experiment (A_c) without the addition of glucan or guar gum was also measured. The flocculating activity (%) was measured as;

$$\textbf{Flocculating activity (\%)} = [(A_c-A_s)/A_c] \times 100$$

where, A_s= absorbance of glucan or guar gum containing suspension; A_c= absorbance of control.

6.2.7 Assessment of cytotoxicity and biocompatibility of glucan

6.2.7.1 Culturing and maintenance of cell line

The human embryonic kidney (HEK-293) and the human cervical cancer (HeLa) cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India. The cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C in 5% carbon dioxide atmosphere as described in Chapter 3, Section 3.2.3.

6.2.7.2 In vitro cyotoxicity assay

The cytotoxic effect of glucan (ranging between 10 µg/ml and 1000 µg/ml) on the cell lines was studied by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (van Meerloo *et al*., 2011). The human embryonic kidney cell line (HEK-293) and the human cervical cancer cell line (HeLa-293) were seeded separately in 250 ml, 75 cm^2 vent cap tissue culture flasks and after ~90% confluence the cells were detached by 1x trypsin EDTA solution (Sigma Aldrich, USA) and counted by haemocytometer. For cytotoxicity test, the cells were re-suspended in Fetal Bovine Serum (FBS) containing DMEM medium (referred to as complete medium and is mentioned in Chapter 3, Section 3.2.3). 200 µl of this DMEM medium containing $2x10^4$ HEK 293 and HeLa cells/ well were separately seeded in a 96 well plate. The plates were incubated at 37ºC for 12 h for cell

adherence in CO_2 incubator (5%). After the incubation, the complete DMEM medium was completely removed and the adhered cells were exposed to different concentrations of glucan dissolved in FBS free DMEM medium (referred to as incomplete medium and is described in Chapter 3, Section 3.2.3). In order to evaluate the cyototoxic effect of any test compound, it is dissolved in serum free media (Haubler *et al.,* 1998; Hirsch *et al.,* 2012). The MTT assay was done at regular time interval by removing the whole 200 µl medium and washing the each well containing the adherent cells in the bottom of the well with 200 µl of PBS (1x) to remove any bacterial contamination. Finally 100 µl MTT (500 µg/ml) was added to each well and the plates containing MTT solution was further incubated at 37ºC for 4 h. After the incubation, the 100 µl of MTT solution from each well was replaced with equal volume (100 µl) of DMSO. The absorbance was measured at 570 nm using a multi-mode microplate reader (Tecan, Infinite 200 Pro) and the viability (%) was calculated as described by Patel *et al.,* (2010).

$$\textbf{Cell viability (\%)} = (N_t/N_c)\textbf{x100}$$

where, N_t is absorbance (A_{570}) of cells treated with glucan and N_c is the absorbance (A_{570}) of untreated cells.

6.3 Results and Discussion

6.3.1 Purification of glucan from *Lb. plantarum* DM5

The cell free supernatant of *Lb. plantarum* DM5 obtained by centrifugation at 10,000*g* at 4°C for 10 min, was subjected to ethanol precipitation as described in Section 6.2.4.1. The crude glucan with slushy applesauce like appearance was achieved after ethanol precipitation and gave glucan concentration of 1.87 mg/ml as determined by phenol sulphuric acid method (as described in Chapter 5, Section 5.2.8). The crude glucan was lyophilized to give white powder with gel like structure and was further purified by gel filtration using Sepharose CL-6B as matrix.

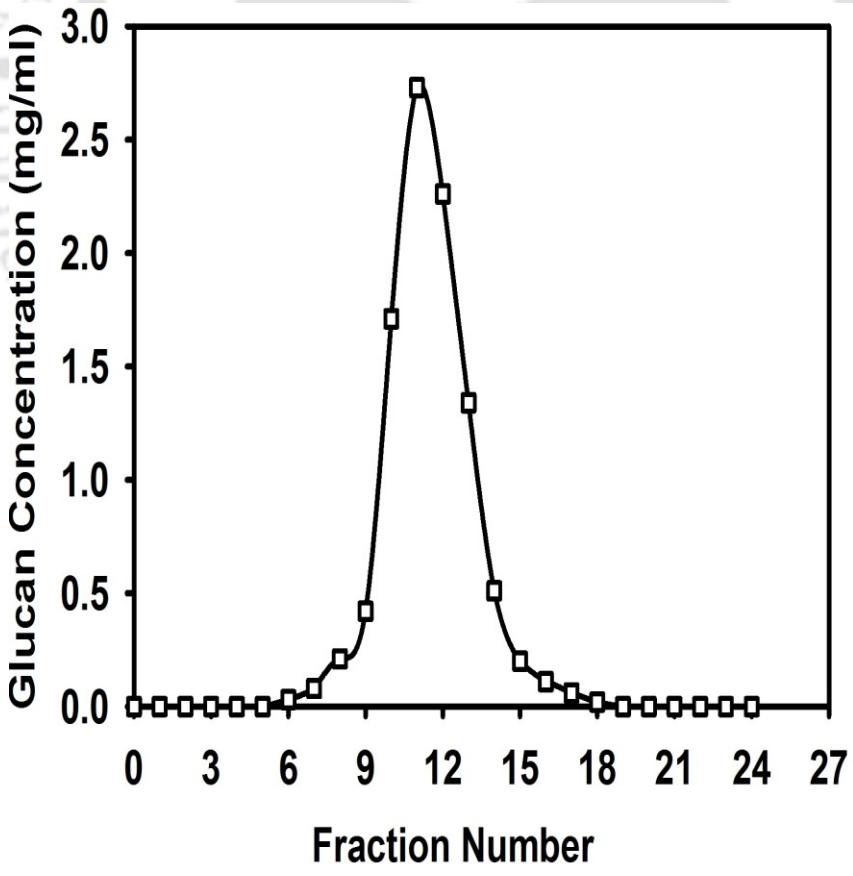

Fig. 6.3.1 Elution profile of glucan of *Lb. plantarum* DM5 from Sepharose CL-6B using 0.9% (w/v) sodium chloride solution at a flow rate of 0.3 ml/min and fractions of 3 ml were collected.

The glucan was eluted by 0.9% (w/v) sodium chloride solution from gel filtration column using Sepharose CL-6B as a matrix. The glucan was eluted as a single major peak (Fig. 6.3.1) as detected by phenol sulfuric acid method (described in Chapter 5, Section 5.2.8). The purified fractions from 10 to 13 were collected as they showed maximum glucan concentration. The fractions were pooled; lyophilized and resulting glucan was structurally characterized using FTIR, ^{1}H NMR and ^{13}C NMR spectroscopy.

6.3.2 Structural analysis of glucan from *Lb. plantarum* DM5

6.3.2.1 Molecular mass estimation of glucan

The purified glucan was subjected to by gel permeation chromatography using Sephacryl S-500HR as matrix to determine the molecular mass. The standard dextrans (as described in Section 6.2.3) and purified glucan were eluted from the column at a flow rate of 0.3 ml/min (Fig.6.3.2). The retention time of each standard dextran and glucan from *Lb. plantarum* DM5 was calculated from their fraction number and elution volume and is shown in Table 6.3.1. Molecular weight of purified glucan was estimated from the standard graph which was plotted by taking logarithmic values of standard dextrans and their retention time (Fig.6.3.2). The retention time of the purified glucan from *Lb. plantarum* DM5 from the column was 159.84 min as shown in Table 6.3.1.

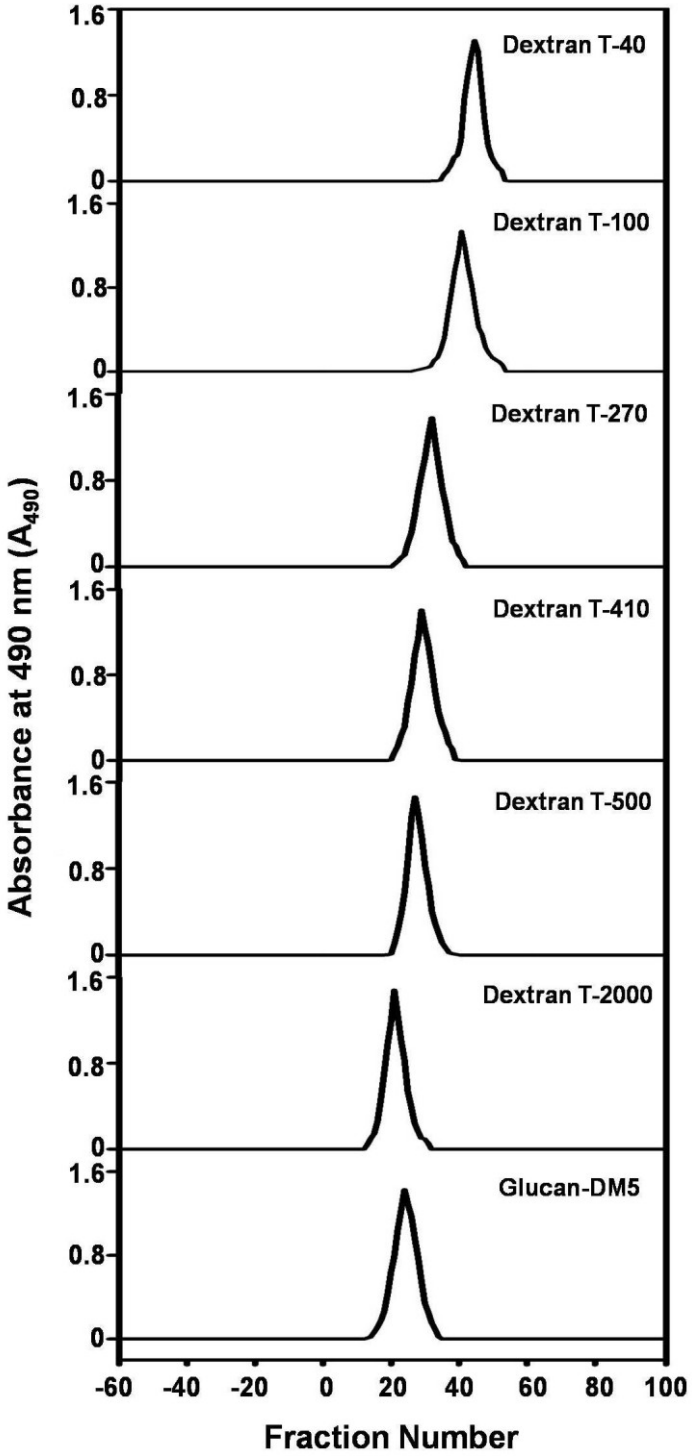

Fig. 6.3.2 Elution profile of standard dextran and glucan of *Lb. plantarum* DM5 from Sephacryl S-500HR column using 0.9% (w/v) NaCl. Standard molecular mass dextrans used were 40 kDa (T-40), 100 kDa (T100), 270 kDa (T-270), 410 kDa (T-410), 500 kDa (T-500) and 2000 kDa (T-2000).

Table 6.3.1 Elution profile of standard dextrans and glucan from *Lb. plantarum* DM5 from Sephacryl S-500HR column.

Sample	[a]Fraction No.	Elution Vol. (ml)	[b]Retention Time (min)
Dextran T-40	44	88	293.04
Dextran T-100	40	80	266.40
Dextran T-270	32	64	213.12
Dextran T-410	29	58	193.14
Dextran T-500	27	54	179.82
Dextran T-2000	21	42	139.86
[c]Glucan-DM5	24	48	159.84

[a] *The individual fraction showed highest absorbance at 490 nm. The standard dextrans were eluted using 0.9% (w/v) sodium chloride solution at a flow rate of 0.3 ml/min and fractions of 2 ml were collected.*

[b] *The retention time was calculated from the flow rate of 0.3 ml/min.*

[c] *The purified glucan was eluted following the same condition as the standard dextrans.*

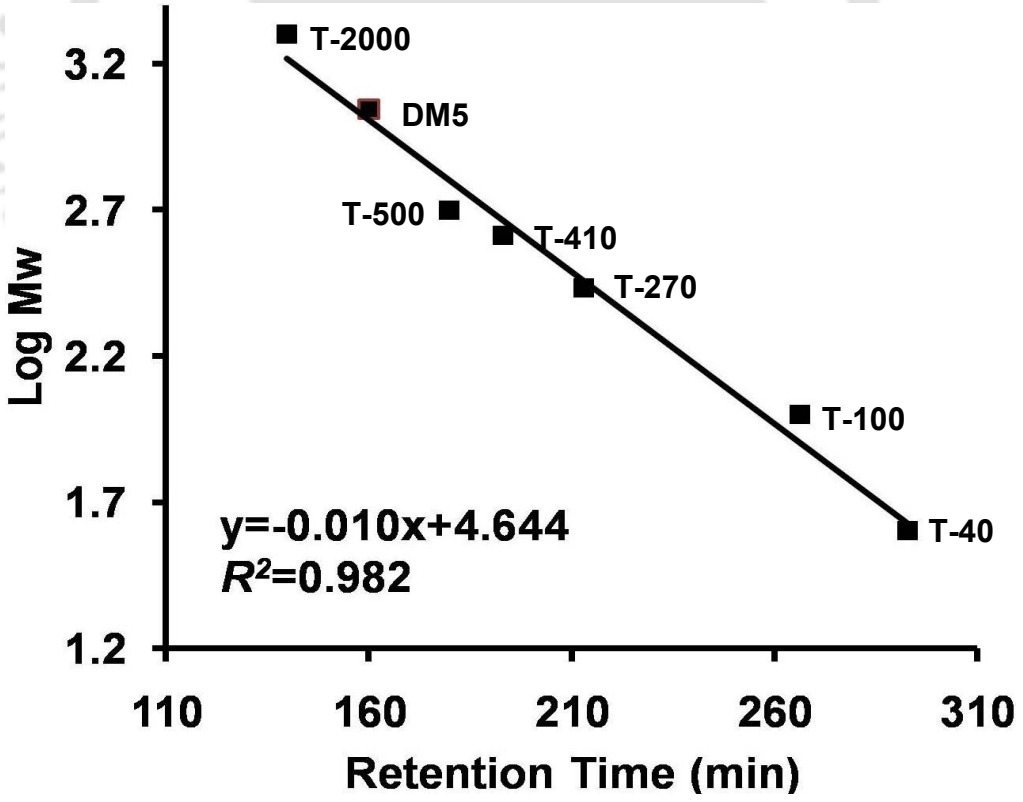

Fig. 6.3.3 Determination of molecular mass of purified glucan by gel permeation chromatography using Sephacryl S-500HR as matrix.

Based on the calibration curve as shown in Fig. 6.3.3 (y= -0.010x+4.644, R^2= 0.982, where y= log molecular weight of the standard dextrans and x= retention time), the average molecular mass of the glucan was 1.11×10^6 Da. High molecular mass of glucan from *Lb. plantarum* DM5 might be the reason for its easy precipitation by ethanol and viscous nature. Similar molecular mass of exopolysaccharide (1.7×10^6 Da) was reported from *Pseudozyma sp.*NII 08165 (Sajna *et al*., 2013). The exopolysaccharide LPC1 from *Lb. plantarum* C88 also showed high molecular mass of 1.15×10^6 Da (Zhang *et al*., 2013).

6.3.2.2 Monosaccharide composition analysis of glucan

The identification of released monosaccharide from hydrolyzed glucan by 100 mM H_2SO_4, was analyzed by HPAEC by comparing the retention time with the reference standard glucose and fructose (Fig. 6.3.4). The hydrolysate showed a single peak at 4.15 min (Fig. 6.3.4C) corresponding to that of standard glucose peak at 4.18 min (Fig. 6.3.4B) whereas the retention time of fructose was 5.15 min (Fig. 6.3.4A). Thus, the exopolysaccharide synthesized by glucansucrase from *Lb. plantarum* DM5 contained only glucose as building block, confirming its glucan nature.

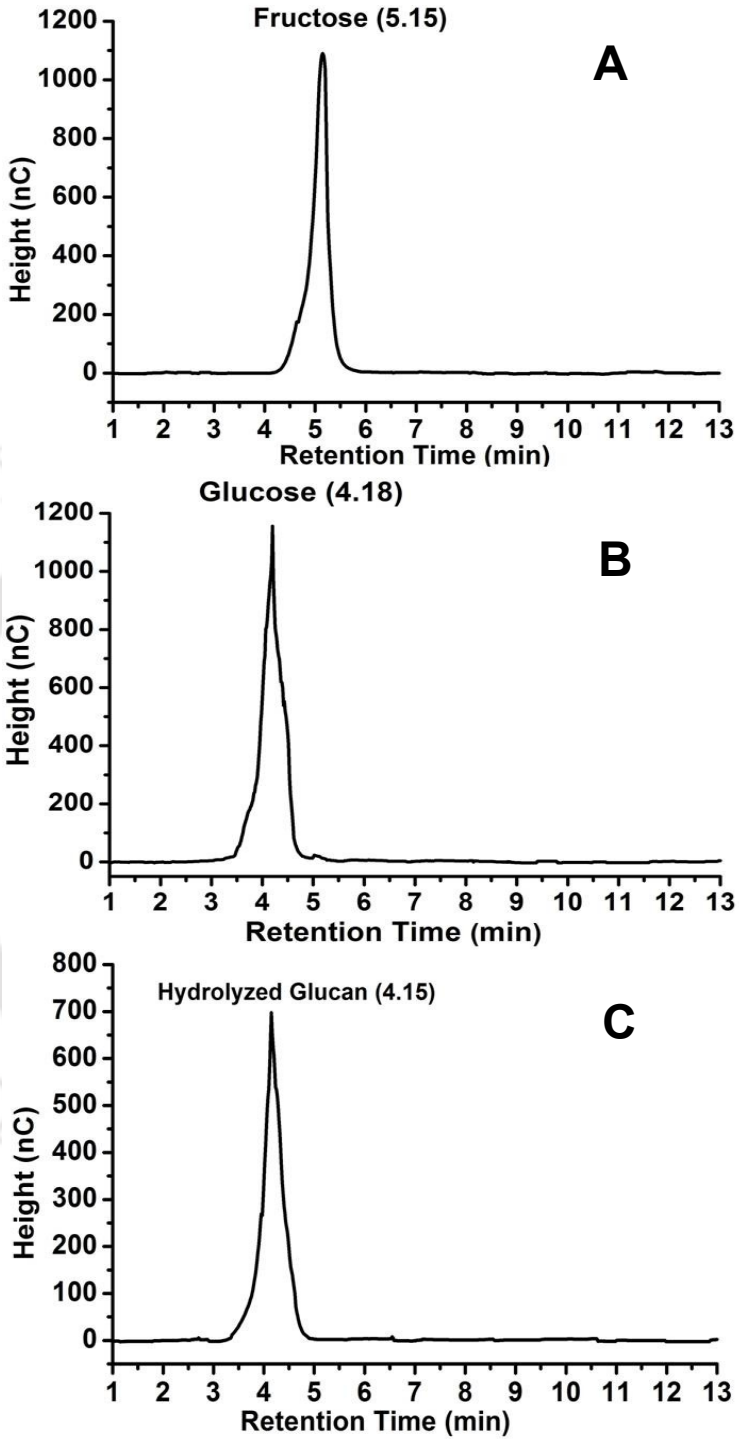

Fig. 6.3.4 Monosaccharide analysis of glucan from *Lb. plantarum* DM5 by high performance anion exchange chromatography (HPAEC), using a Carbo-Pac P20 column by isocratic elution using 0.1 N NaOH at a constant flow rate of 0.5 ml/min at 30°C. A) Fructose, B) Glucose and C) Hydrolysed glucan.

6.3.2.3 Optical rotation

The glucan synthesized by glucansucrase from *Lb. plantarum* DM5 showed optical rotation $[\alpha]_D^{25^oC}$ +147º (c = 5% in dH_2O, 25°C), indicating that it rotates the plane-polarized light to the right like glucose molecule which also showed optical rotation $[\alpha]_D^{25^oC}$ +121º (c = 5% in dH_2O, 25°C). Hence it was confirmed that the glucan synthesized by the glucansucrase has dextrorotatory property unlike fructose which showed laevorotation of $[\alpha]_D^{25^oC}$ is -131º (c = 5% in dH_2O, 25°C).

6.3.2.4 FTIR Spectrometric analysis of glucan

The FTIR spectrum of purified glucan from *Lb. plantarum* DM5 was analyzed and is shown in Fig. 6.3.5. A broad stretching in the region 3420 cm^{-1} was observed due to the hydroxyl stretching vibration of the polysaccharide. This is the characteristic absorption band of carbohydrate ring and is responsible for the water solubility of the polymer (Liu *et al*., 2007). The band in the region of 2927 cm^{-1} was due to CH stretching vibration of the methyl group and the band in the region of 1638 cm^{-1} corresponded to the stretching vibrations of the CHO and C=O bonds of the carboxyl group present in exopolysaccharide (Liu *et al*., 2007, Purama *et al*., 2008; Sajna *et al*., 2013). The wave number region from 1200 cm^{-1} to 800 cm^{-1} was considered as the fingerprint region and can be used to characterize different exopolysaccharides (Copikova *et al*., 2006). The intensity of band at 1152 cm^{-1} was due to the valent vibrations of the C-O-C bond and glycosidic bridge and the band at 1102 cm^{-1} corresponded to the polysaccharide with α-(1→3) branched linkages (Shingel, 2002; Liu *et al*., 2007). The intense peaks at 919 cm^{-1} and 1024 cm^{-1}

indicated the existence of α-(1→6) glycosidic bond and were also reported for glucan from *L. mesenteroides* NRRL B-640 (Purama *et al*., 2009) and *P. pentosaceus* SPA (Patel *et al*., 2010). The absence of band at 870-890 cm^{-1} indicated that there could be no β-glycosidic linkage present in the polymer, suggesting its α-configuration (Majumder *et al*., 2008; Sajna *et al*., 2013).

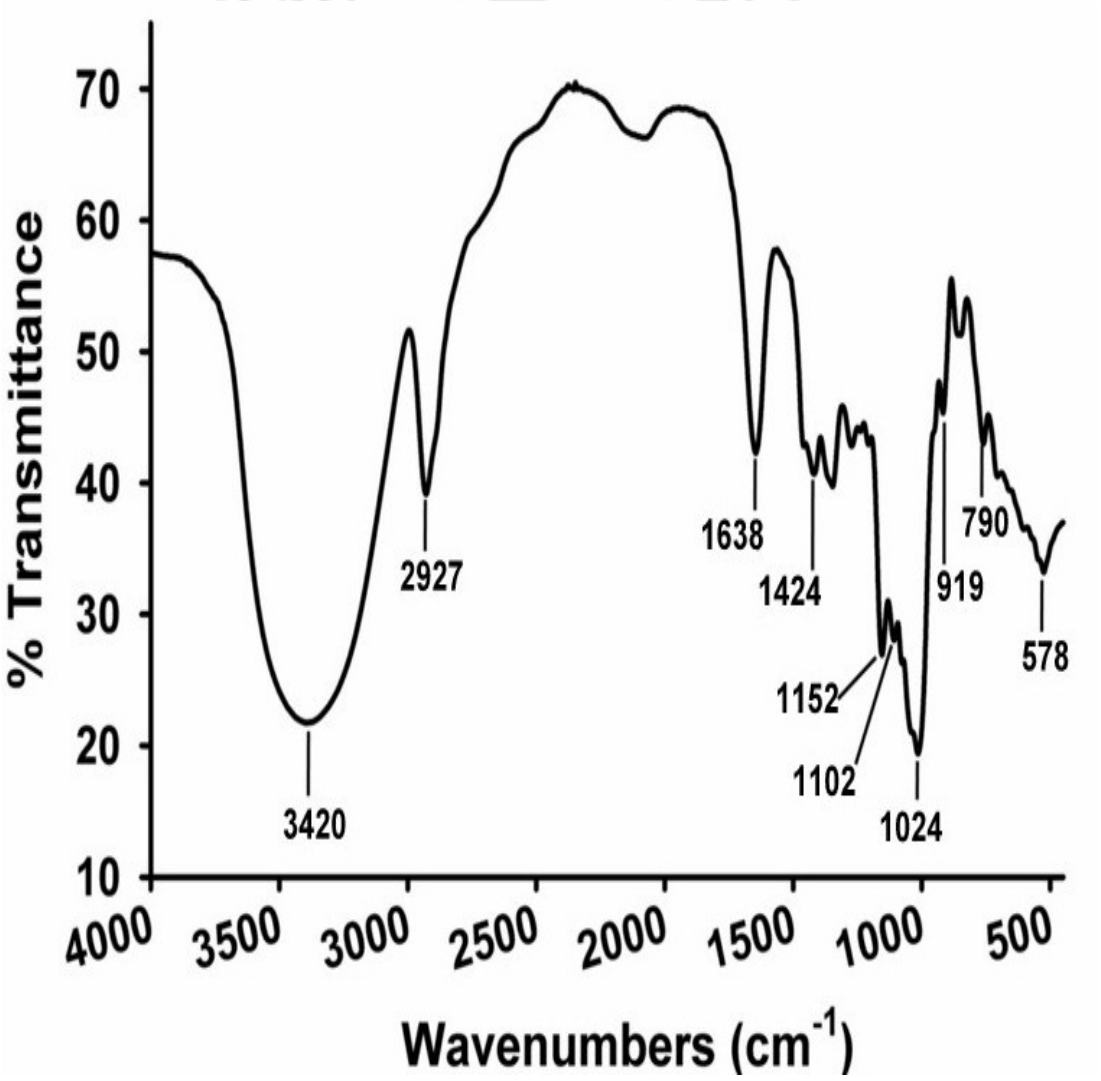

Fig. 6.3.5 FTIR (KBr) spectrum of *Lb. plantarum* DM5 glucan showing the presence of α-(1→6) glycosidic linkages and other functional groups.

6.3.2.5 ^{1}H NMR and ^{13}C NMR Spectroscopic analysis of glucan

The ^{1}H NMR (400 MHz, D_2O) spectrum of glucan from *Lb. plantarum* DM5 showed anomeric proton signals at 4.96 ppm assigned to α-(1→6) glycosidic linkage and at 5.31 ppm assigned to α-(1→3) branching (Table 6.3.1; Fig. 6.3.6). It has been reported that various glucans have ^{1}H NMR spectral resonances (C-2, C-3, C-4, C-5 and C-6) in the 3- to 4-ppm region and the hemiacetal C-1 resonance in 4-6 ppm region (Sidebotham, 1974; Bejar *et al*., 2013). Integration analysis of ^{1}H NMR revealed 86.5% of α-(1→6) and 13.5% α-(1→3) branching with average chain length of 7 glucose units between branch linkages (Fig. 6.3.6).

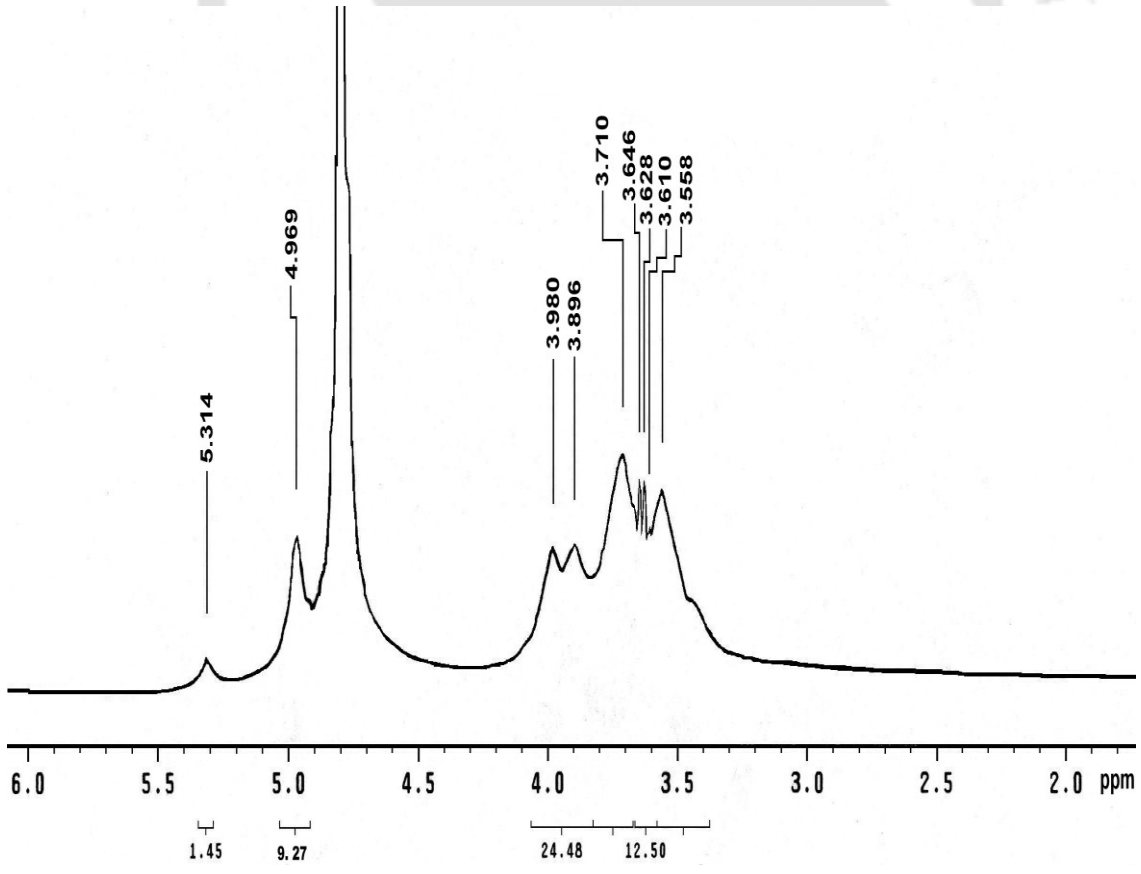

Fig. 6.3.6 ^{1}H NMR (400 MHz, D_2O) spectrum of glucan from *Lb. plantarum* DM5.

The ^{13}C NMR (400 MHz, D_2O) spectrum of glucan also displayed all the characteristic chemical shifts for α-glucose moiety as given in Table 6.3.2. The major resonance in the anomeric region occurs at 98.09 (rather than 90 ppm) ppm showing that the C-1 is linked and an equally intense signal at 65.9 ppm (rather than 60 ppm), indicated that most of the C-6 are also linked, corresponded to C1 α-(1→6) linkages. The up field signal at 99.7 ppm in ^{13}C NMR was assigned to α-(1→3) anomeric carbon (Table 6.3.2; Fig. 6.3.7). The signal at 60.9 ppm, assigned to C6 atom on the non-reducing glucose units and corresponds to the branching. Similar result was observed in case of α-glucan synthesized by glucosyltransferase I (GTF-I) from *Streptococcus downei* (Monchois *et al*., 2000). The intensities of characteristic peaks at 5.31 ppm (^1H NMR) and 99.7 ppm (^{13}C NMR) of glucan from *Lb. plantarum* DM5 as shown in Fig. 6.3.6 and Fig. 6.3.7, respectively indicated the presence of α-(1→3) linkages along with α-(1→6) linkages.

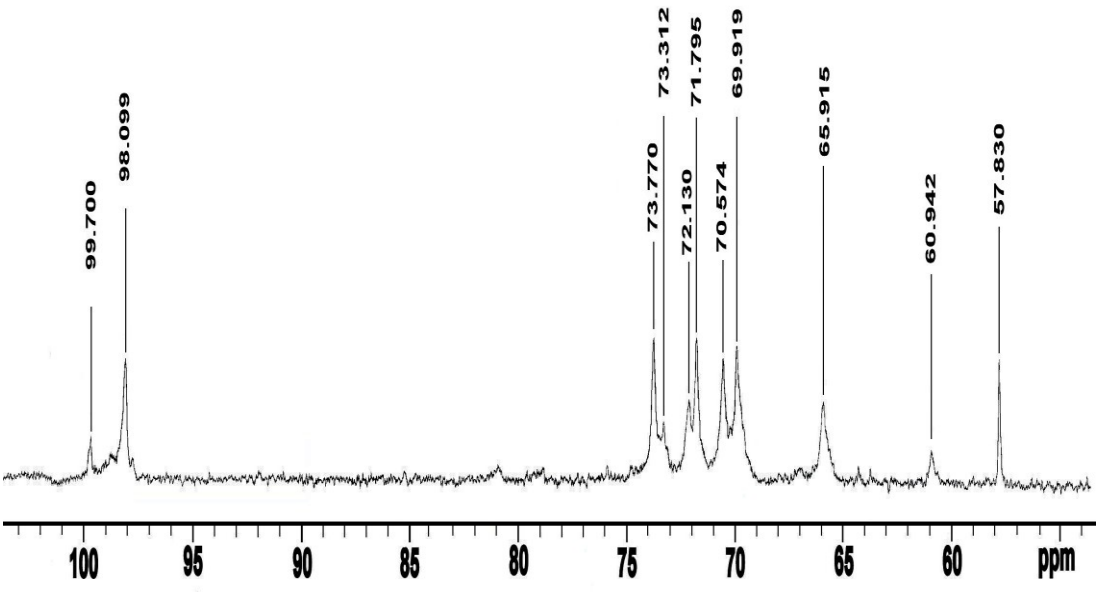

Fig. 6.3.7 ^{13}C NMR (400 MHz, D_2O) spectrum of glucan from *Lb. plantarum* DM5.

Table 6.3.2 Comparison of ^{1}H NMR and ^{13}C NMR chemical shifts (δ) of glucan from *Lb. plantarum* DM5 with *L. mesenteroides* NRRL B-640 and *Weissella sp.* TN610.

^{1}H NMR	**Chemical shift (δ) of ^{1}H (ppm)**		
Proton[a]	**Glucan from *Lb. plantarum* DM5**	**Glucan from *L. mesenteroides* NRRL B-640[b]**	**Glucan from *Weissella sp.* TN610[c]**
AH-1	4.96	4.98	4.99
H-2	3.61	3.58	4.04
H-3	3.71	3.73	3.72
H-4	3.55	3.54	3.53
H-5	3.89	3.92	3.87
H-6	3.98	3.99	3.99
BH-1	5.31	-	5.27
^{13}C NMR	**Chemical shift (δ) of ^{13}C (ppm)**		
Carbon Atom	**Glucan from *Lb. plantarum* DM5**	**Glucan from *L. mesenteroides* NRRL B-640[b]**	**Glucan from *Weissella sp.* TN610[c]**
C-1	98.0	97.8	98.5
C-2	72.1	71.5	74.2
C-3	73.7	73.5	72.2
C-4	71.7	69.7	70.98
C-5	70.5	70.3	70.3
C-6	65.9	65.7	66.35
C-6’	60.9	-	-

[a] *^{1}H NMR chemical shifts are referenced to an internal deuterium oxide (D_2O). Peaks AH-1 (4.92 ppm) and BH-1 (5.27 ppm) were assigned to anomeric protons representing 86.5% α-(1→6) and 13.5% α-(1→3) of glucosidic linkages, respectively.*

[b] *Puruma and Goyal, 2008.*

[c] *Bejar et al., 2013.*

The comparison of individual peak assignments of glucan in ^{1}H NMR and ^{13}C NMR of *Lb. plantarum* DM5 with glucan from *L. mesenteroides* NRRL B-640 (Purama *et al*., 2009) and *Weissella sp.* TN610 (Bejar *et al*., 2013) are shown in Table 6.3.2. The structure of glucan from *Lb. plantarum* DM5 was in accordance with the α-D-glucan produced from sucrose by glucansucrase GTF180 from *Lb. reuteri* strain 180 which also contains α-(1→6) and α-(1→3) linkages (van Leeuwen *et al*., 2009). The predominance of α-(1→6) linkages in the main chain of the glucan from *Lb.*

plantarum DM5 facilitate high water absorption capacity and could be used as texturing agent in food industry.

6.3.2.6 Rheological analysis of aqueous glucan solution

The rheological study of glucan from *Lb. plantarum* DM5 exhibited shear rate thinning effect as the apparent viscosity (η) decreases as shear rate increases (Fig. 6.3.8) and revealed classical non-Newtonian pseudoplastic behaviour.

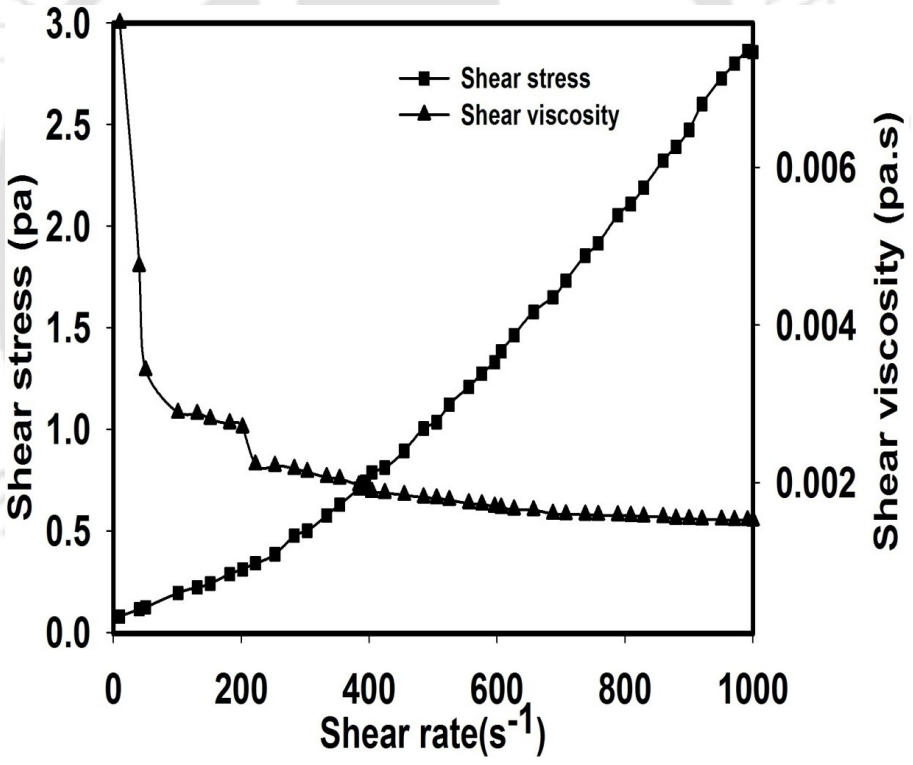

Fig. 6.3.8 Viscosity shear rate profile of *Lb. plantarum* DM5 glucan (0.5%) at 25ºC.

A similar pseudoplastic behaviour of glucan was also observed in case of *L. mesenteroides* NRRL B-640 (Purama *et al*., 2009) and *P. pentosaceus* SPA (Patel *et al.,* 2010). Moreover, the glucan from *Lb. plantarum* DM5 did not demonstrate time

dependent change in viscosity and showed nonthixotropic behaviour in water solution like glucan polymer from *L. mesenteroides* NRRL B-512F (Irague *et al*., 2012). The non-Newtonian pseudoplastic and nonthixotropic behaviour of glucan from *Lb. plantarum* DM5 suggested that it belongs to a class of very flexible and extended polymer and can be used as thickening, stabilizing and gelling agent in the food industry.

6.3.2.7 Thermo gravimetric analysis (TGA) of glucan

The thermo gravimetric analysis (TGA) of glucan from *Lb. plantarum* DM5 showed an initial weight loss of approximately, 14% at the temperature in between 65ºC to 95ºC, which corresponded to the elimination of surface bound water molecule (Fig. 6.3.9). EPS with high carboxyl groups is always rich in moisture contents (Ahmed *et al*., 2013; Sajna *et al*., 2013) and initial weight loss in glucan from *Lb. plantarum* DM5 suggests that the polymer was rich in carboxyl contents. The data was supported by FTIR analysis, in which glucan revealed an intense peak in the region 1638 cm^{-1} due to the presence of carboxyl group (Das and Goyal, 2013). Similar observation was also found in case of exopolysaccharide from *Pseudozyma sp.* NII 08165 (Sajna *et al*., 2013). The decrease in weight loss above 95ºC is attributed to the degradation of the glucan polymer and the onset of decomposition occurred at 270ºC with an observed weight loss of 21%. The degradation temperature (T_d) of glucan was found to be 292.2ºC. The weight loss of glucan decreased drastically around 300ºC and completely decomposed at 530ºC.

The degradation temperature of glucan from *Lb. plantarum* DM5 was in accordance with degradation temperature (T_d=292.2ºC) of exopolysaccharide from *Lactobacillus kefiranofaciens* ZW3 (Ahmed *et al*., 2013). Along with other physiochemical characteristics; industrial applicability of exopolsaccharide is largely dependent on its rheological and thermal behaviour (Marinho-Soriano and Bourret, 2005, Ahmed *et al*., 2013). The high thermo-stability of glucan from *Lb. plantarum* DM5 indicates that it can be potentially used in the food industry for manufacturing and processing of several food products which are generally carried out at high temperatures.

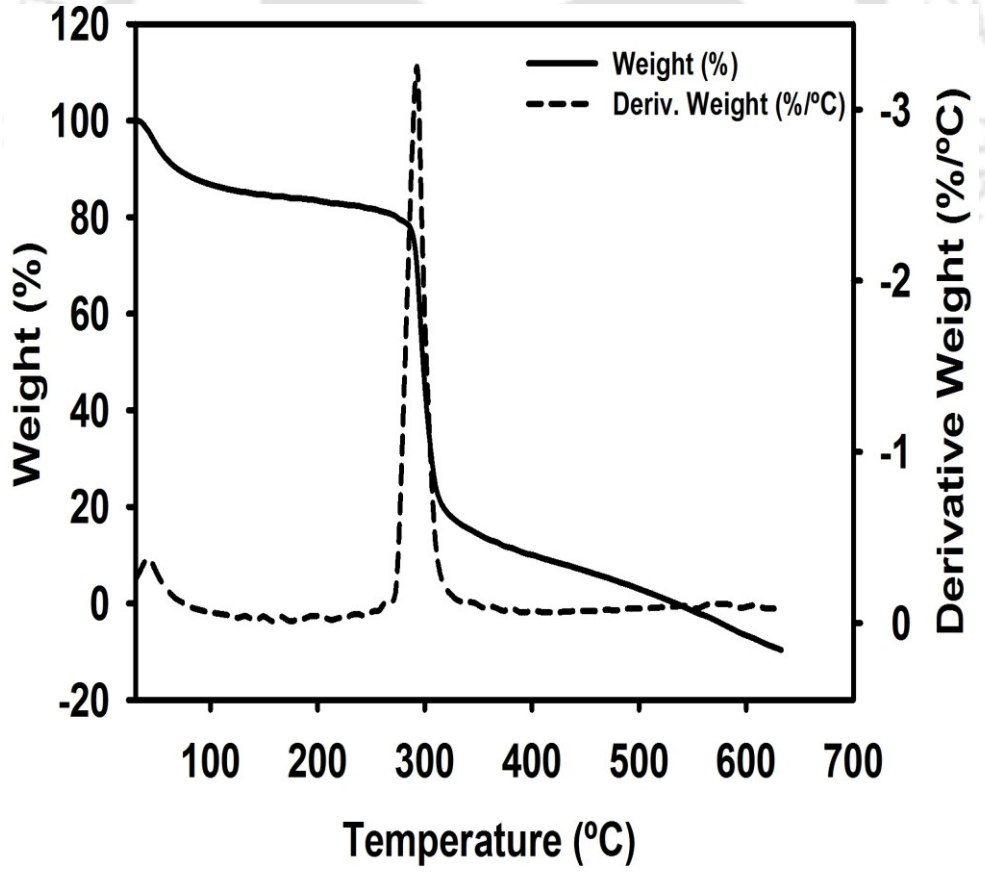

Fig. 6.3.9 Thermogravimetric analysis (TGA) of glucan from *Lb. plantarum* DM5 showing the thermal stability up to 270ºC and degradation temperature (T_d) of 292.2ºC.

6.3.3 Physicochemical characterization of glucan from *Lb. plantarum* DM5

6.3.3.1 Scanning electron microscopic analysis of glucan

The glucan produced by *Lb. plantarum* DM5 displayed slushy applesauce like appearance with gel like structure was further confirmed by SEM analysis. The SEM image of glucan also showed smooth porous or web like structure (Fig. 6.3.10) prerequisite for using as emulsifier or stabilizer in food industry. The hydroxyl groups present in the glucan also increases the water holding capacity and the polymer can be used as a texturing agent in food industry.

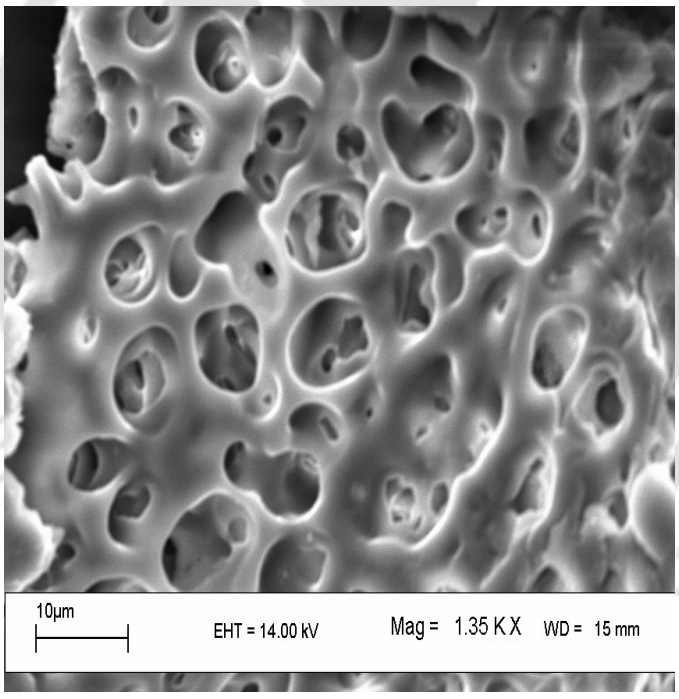

Fig. 6.3.10 Scanning Electron Micrograph (SEM) of *Lb. plantarum* DM5 glucan at magnification 1.35 Kx showing surface morphology.

6.3.3.2 Solubility and water holding capacity of glucan

The glucan polymer from *Lb. plantarum* DM5 displayed 21.6% solubility and 317% water holding capacity as shown in Table 6.3.3. These properties are associated

to the porous matrix structure formed by polysaccharide chains which can hold large amounts of water through hydrogen bonds (Zhou *et al*., 2010). This result was in good agreement with the scanning electron microscopic analysis of glucan from *Lb. plantarum* DM5, which also showed smooth porous and gel like structure (Das and Goyal, 2013). The solubility of 14.2% and water holding capacity of 496% was observed in case of exopolysaccharide from *Lb. kefiranofaciens* ZW3 (Ahmed *et al*., 2013). It has been reported that the solubility of glucan polymer largely depends on its molecular weight and the percentage of branching (Cote and Robyt, 1995; Tususaki *et al*., 2009). The good solubility and water holding abilities of glucan are attributed to its use as an emulsifier or stabilizer in the food industry.

Table 6.3.3 Solubility and water holding capacity (WHC) of glucan.

Sample	Solubility (%)	WHC (%)
Lb. plantarum DM5 Glucan [a]	21.6±0.35	316.9±0.38
Lb. kefiranofaciens ZW3 Exopolysaccharide [b]	14.2	496.0

[a] *The mean value of three independent experiments is presented with ± S.E.*

[b] *The exopolysaccharide produced by Lactobacillus kefiranofaciens ZW3, isolated from Tibet kefir (Ahmed et al., 2013).*

6.3.3.3 Emulsion stability of glucan

The emulsifying activity of EPS is determined by its strength in retaining the emulsion of the hydrocarbon in water. The emulsion stabilities of glucan from *Lb. plantarum* DM5 was compared with guar gum, a natural polysaccharide and sodium alginate, a synthetic hydrocolloid, both commercially used as emulsifiers. The glucan retained 80.6% and 71.4% of the emulsification activity after 30 and 60 min, respectively however; the emulsion stability of guar gum was found to be 73.4% and

39.2% after 30 and 60 min, respectively (Fig. 6.3.11) against n-hexadecane. The emulsifying activity of sodium alginate was 67.9% and 37.6% after 30 and 60 min, respectively (Fig. 6.3.11) against n-hexadecane. To determine the emulsion-stabilizing capacity of an emulsifier, it should be able to retain at least 50% of the emulsion after formation (Kanmani *et al*., 2013). For this a control experiment was run with 2 ml PBS (1x) mixed with 0.5 ml n-hexadecane without any added glucan or standard polymers. The control was unable to retain its 50% emulsion activity after 30 min of incubation, suggesting the emulsion activity of glucan, guar gum and sodium alginate.

The glucan polymer displayed superior emulsifying activities of 32.2% and 33.8% as compared with guar gum and sodium alginate, respectively after 60 min, suggesting that it could be potentially used as emulsifier in food industry. The emulsifying activity of glucan was in accordance with exopolysaccharide (88.9%) from *Lb. kefiranofaciens* ZW3 (Wang *et al*., 2008) and exopolysaccharide (84.5%) from *Enterococcus faecium* MC13 (Kanmani *et al*., 2013) after 30 min of incubation. In earlier Section 6.3.2.5, it was shown that the glucan from *Lb. plantarum* DM5 possessed a shear rate thinning effect and revealed classical non-Newtonian pseudoplastic behaviour. The overall results suggest that the glucan from *Lb. plantarum* DM5 is a prospective candidate for use as an emulsifier and viscosifier in the food industry.

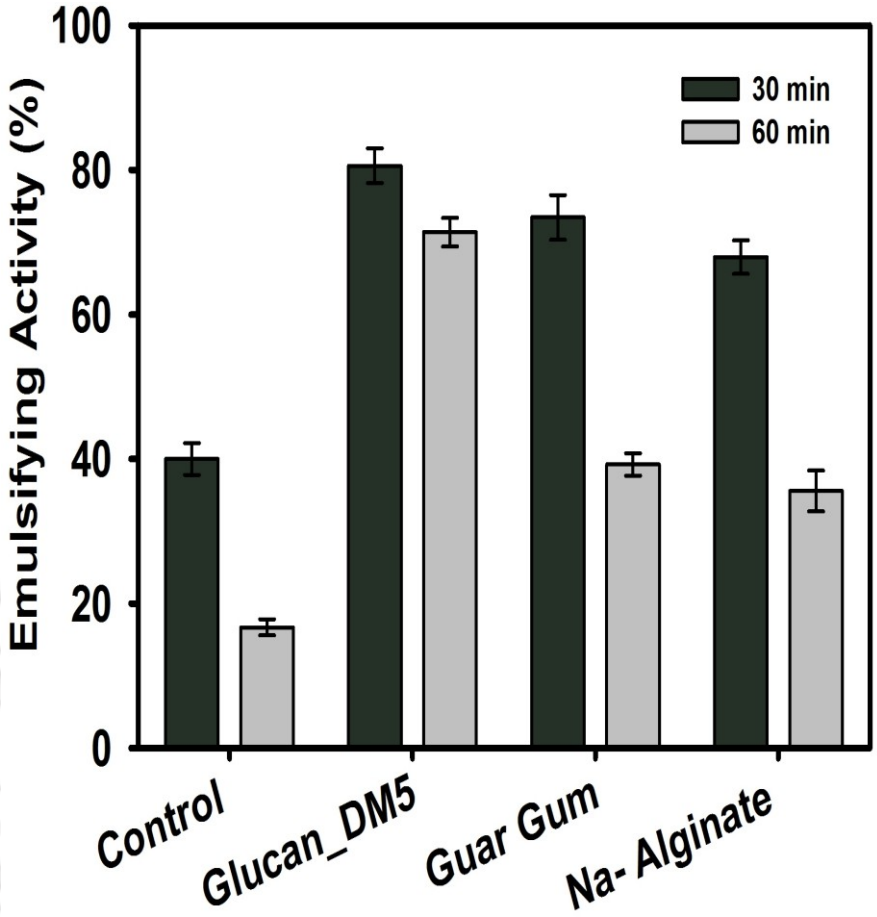

Fig. 6.3.11 Emulsion stability of glucan from *Lb. plantarum* DM5 after 30 or 60 min at 25ºC. A control was run with 2 ml PBS (1x) mixed with 0.5 ml n-hexadecane without any added glucan or standard polymers and absorbance was measured at 540 nm. The mean value of three independent experiments is presented with ± S.D.

6.3.3.4 Flocculating activity of glucan

Flocculants are categorized into two groups, chemically synthesized flocculants (organic and inorganic flocculants) and natural flocculants (chitosan, algin and microbial flocculants). Presently the use of chemical synthesized flocculants is restricted as they are hard to degrade and harmful to human, therefore, the screening of natural flocculants from microbial sources has been in focus as they are nontoxic,

benign and biodegradable polymers (Shih *et al*., 2001; Liu *et al*., 2013). The flocculating activity of *Lb. plantarum* DM5 glucan ranging from 0.05 to 0.8 mg/ml in 5 mg/ml dispersion of activated charcoal containing 6.8 mM $CaCl_2$ solution was compared with guar gum and is shown in Fig. 6.3.12. It was observed that the flocculation activity of glucan initially increased with increasing the concentration of glucan and the maximum flocculating activity of 86.2% was achieved at concentration of 0.1 mg/ml. Above 0.1 mg/ml concentration of glucan, the flocculating activity decreased as the concentration increased. The exopolysaccharide from *Lb. kefiranofaciens* ZW3 showed similar trend of flocculating activity (Wang *et al*., 2008), however; flocculating activity of the exopolysaccaride from *Enterococcus faecium* MC13 increased with increasing concentration of EPS (Kanmani *et al*., 2013). In case of guar gum, the maximum flocculating activity of 78.6% was observed at concentration of 0.2 mg/ml. The flocculating activity of guar gum gradually increased up to 0.4 mg/ml concentrations and subsequently decreased up to the concentration of 0.8 m/ml (Fig. 6.3.12). The glucan polymer from *Lb. plantarum* DM5 showed higher flocculating activity of 8.8% and at half concentration as compared to commercial hydrocolloid guar gum against activated charcoal. The aforementioned data imply that the glucan from *Lb. plantarum* DM5 can be used as bio-flocculants in dairy industry for making cheese from curd. Apart from food industry, the glucan from *Lb. plantarum* DM5 as a bio- flocculants can be used widely in a variety of industrial processes, such as wastewater treatment, drinking water purification and for harvesting microbial cells from culture broth in industrial downstream process.

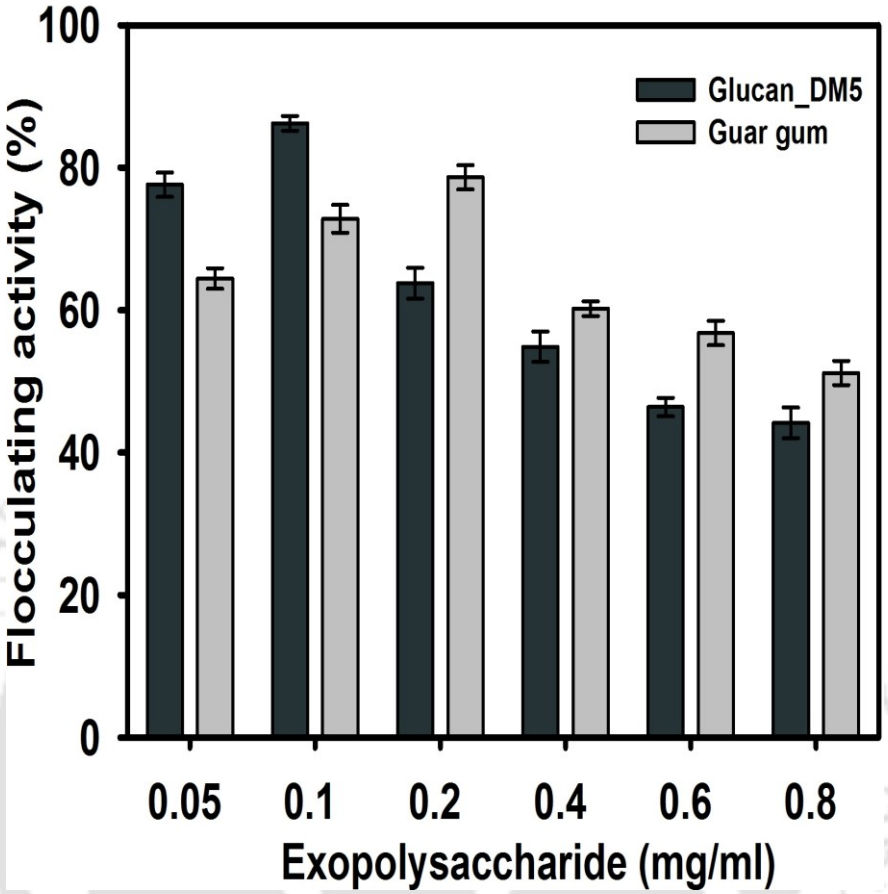

Fig. 6.3.12 Flocculating activity of glucan from *Lb. plantarum* DM5 and guar gum against activated charcoal containing 6.8 mM $CaCl_2$ at room temperature (25°C). The mean value of three independent experiments is presented with ± S.D.

6.3.4 Assessment of cytotoxicity and biocompatibility of glucan

The *in vitro* cytotoxic test of glucan from *Lb. plantarum* DM5 was done by MTT assay. The MTT assay is an established method of determining viable cell, as the yellow colour of MTT dye was reduced to purple colour formazan by oxidoreductase enzymes present in the cytosolic compartment of the living cells (van Meerloo *et al*., 2011). The HEK-293 and HeLa cells were exposed to glucan from *Lb. plantarum* DM5 dissolved in serum free DMEM medium at 10, 20, 50, 100, 250, 500 and 1000 µg/ml for 3, 6, 12, 24 and 48 h and it was observed that the cell viability

remained constant as a function of both time and the dose (Fig. 6.3.13). The presence of serum in the complete medium (*i.e.* DMEM + 10% FBS) also contributes to the cell growth along with the presence of test compounds or drugs and interferes with cell viability assessment, thus serum free media are preferably used to study the interactions of hormones or drugs for cell cytotoxicity (Barnes and Sato, 1980; Haubler *et al*., 1998; Hirsch *et al*., 2012). The cyto-compatibility test of glucan using HEK-293 cells and HeLa cells revealed favourable cell viability, but it did not influence the cell proliferation over an incubation period of 48 h. It was observed that the viability of treated HEK cells (Fig. 6.3.13A) and HeLa cells (Fig. 6.3.13B) was alike as compared with the respective untreated cells in serum free medium with no significant toxic effect at all concentrations. The higher concentration of 1000 µg/ml of glucan did not show significant cytotoxicity in both normal HEK-293 and cancerous HeLa cell lines and almost 85-90% of cell viability was observed in both the cell lines, like the glucan untreated normal HEK-293 cells and cancer HeLa cells in serum free DMEM medium after 48 h of incubation. The data indicated that even at higher glucan concentrations the cells were metabolically active without any toxic effect. The results of cytotoxicity assay of glucan was in accordance with previous report where the α-D-glucan from *P. pentosaceous* SPA was found to be non-toxic and biocompatible (Patel *et al*., 2010). Therefore, the glucan from *Lb. plantarum* DM5 can be considered as biocompatible and can be used for drug delivery, tissue engineering and other biomedical applications.

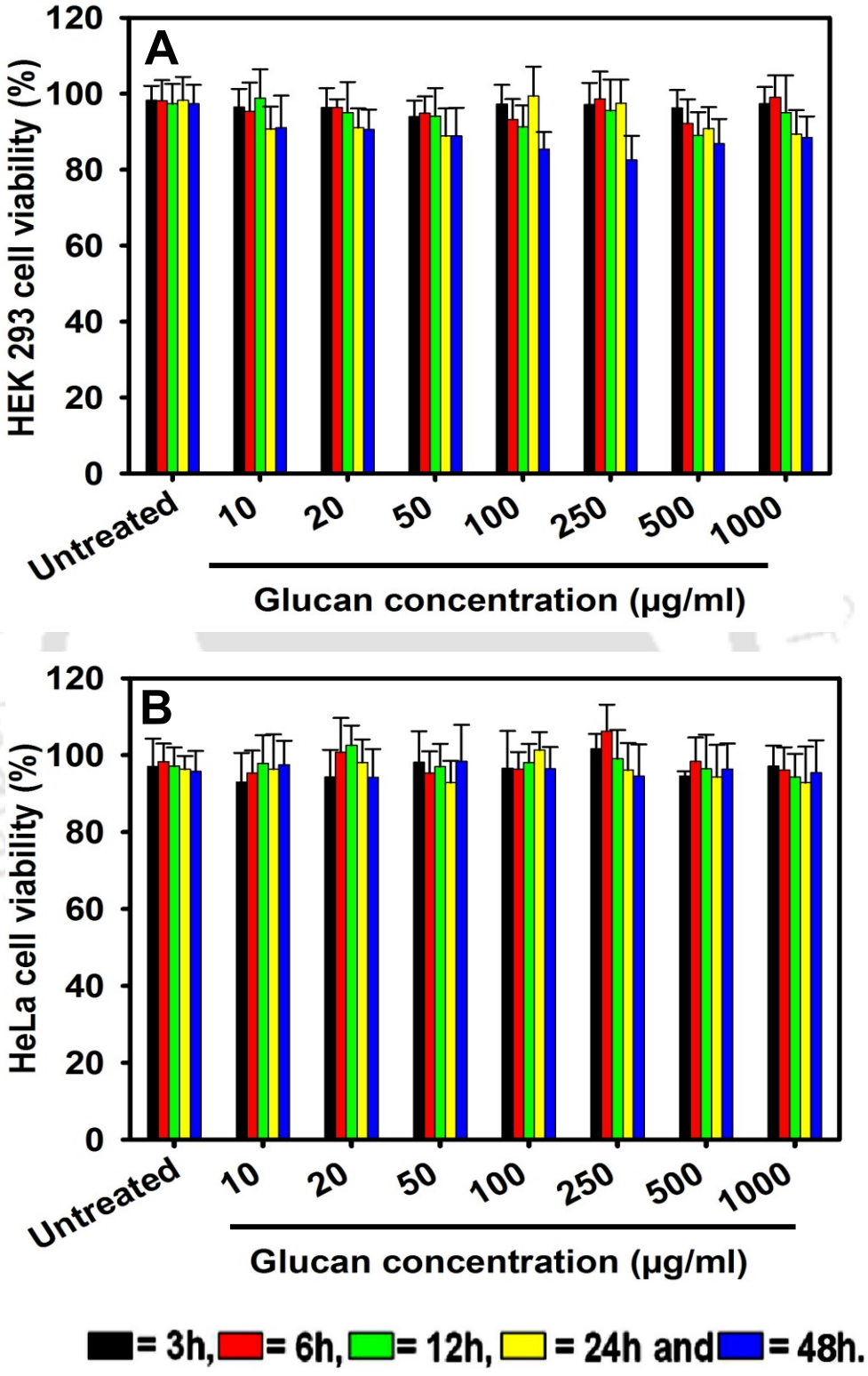

Fig. 6.3.13 The *in vitro* cytotoxicity assay showing the cell viability (%) of (A) HEK-293 and (B) HeLa cells treated with various concentrations of glucan (10-1000 µg/ml) over a period of 3 h- 48 h of incubation.

6.4 Conclusions

An exopolysaccharide producing probiotic lactic acid bacterium (*Lb. plantarum* DM5) was isolated from fermented beverage Marcha of north eastern Himalayas. The exopolysaccharide synthesized by *Lb. plantarum* DM5 was purified by ethanol precipitation and gel filtration using Sepharose CL-6B and characterised. Monosaccharide analysis of purified exopolysaccharide showed only glucose residues as building blocks and hence confirmed its glucan nature. The gel permeation chromatography of glucan revealed an average molecular mass of 1.11×10^6 Da.

The structural characterization of purified glucan was carried out using FTIR, ^{1}H-NMR and ^{13}C-NMR techniques. The peak at 1024 cm^{-1} in the FTIR spectrum indicated the presence of α(1→6) linkages and the peak at 1102 cm^{-1} corresponded to the α-(1→3) branched linkages. ^{1}H-NMR and ^{13}C-NMR data confirmed the abundance of α-(1→6) glycosidic bonds in the backbone of the glucan, with α-(1→3) linkage. The integration analysis of ^{1}H NMR revealed 86.5% of α-(1→6) and 13.5% α-(1→3) branching with average chain length of 7 glucose units between branch linkages.

The rheological study of glucan revealed that the viscosity was decreased with the increase in shear rate exhibiting a typical non-Newtonian pseudoplastic behaviour. The surface morphology showed that it has porous structure and may be used as thickening and gelling agent in dairy and bakery industry. The glucan polymer displayed 21.6% solubility and 317% water holding capacity. It also showed flocculation activity and emulsification activity of 80.6% and 86.2% respectively and can be potentially used as bio-flocculent and emulsifier in food as well as non-food industry. The glucan polymer revealed excellent thermal stability with degradation

temperature (T_d) of 292.2ºC, which indicates that the polymer can able to withstand high temperature during food processing and manufacturing.

This is the first report on the structure and biocompatibility of homopolysaccharide α-D-glucan (dextran) from probiotic *Lactobacillus plantarum* strain with its unique physical and rheological properties that facilitate its application in food industry as viscosyfying and gelling agent. The glucan exerted considerable nontoxic effect on HEK 293 and HeLa cell lines, displaying its biocompatible nature. The glucan can be potentially used to manufacture glucan-based nanofibers for bio-sensing and biomedical applications. The overall data suggest that the glucan from *Lb. plantarum* DM5 can be explored for its potential applications as stabilizing, emulsifying, texturing, bio-thickening, biocompatible and biodegradable agents in food and pharmaceutical industry.

References

Ahmed, Z., Wang,Y., Anjum, N., Ahmad, A. and Khan, S.T. (2013) Characterization of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir-Part II. *Food Hydrocolloids*, 30, 343-350.

Barnes, D. and Sato, G. (1980) Methods for growth of cultured cells in serum-free medium. *Anal. Biochem.,* 102, 255-270.

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

Bramhachari, P.V., Kavi kishor, P.B., Ramadevi, R., Kumar, R., Rao, B.R. and Dubey, S.K. (2007). Isolation and characterization of mucous exopolysacchride (EPS) produced by *Vibrio furnissii* strain VBOS3. *J. Microbiol. Biotechnol*., 17, 14-51.

Cagno, R.D., Angelis, M.D., Limitone, A., Minervini, F., Carnevali, P., Corsetti, A., Gaenzle, M., Ciati, R. and Gobbetti, M. (2006) Glucan and Fructan production by sourdough *Weissella cibaria* and *Lactobacillus plantarum. J. Agric. Food Chem*., 54, 9873-9881.

Chang, P. S. and Cho, G. B. (1997) Oxidation of primary alcohol groups of polysaccharides with 2,2,6,6-tetramethyl-1-piperidine oxoammoumion. *Korean J. Food Sci.Technol.,* 29, 446-451.

Copikova, J., Barros, A.S., Smidova, I., Cerna, M., Teixeira, D.H., Delgadillo, I., Synytsya, A. and Coimbra, M.A. (2006) Influence of hydration of food additive polysaccharides on FT-IR spectra distinction. *Carbohydr. Polym.,* 63, 355-359.

Cote, L.G. and Robyt, J.F. (1984) The formation of α-D-(1→3) branch linkages by a D-glucansucrase from *Streptococcus mutans* 6715 producing a soluble D-glucan. *Carbohydr. Res*., 127, 95-107.

Das, D. and Goyal, A. (2010) Characterization and screening of antimicrobial activity of lactic acid bacterium isolated from a traditional beverage marcha of Sikkim. *J. Pharm. Chem.* 4, 136-139.

Das, D. and Goyal, A. (2013) Characterization and biocompatibility of glucan: a safe food additive from probiotic *Lactobacillus plantarum* DM5. *J. Food Sci. Agric*., doi: 10.1002/jsfa.6305.

Davarcioglu, B. (2010) FTIR Spectroscopic techniques for quantitative characterization by abrasion with potassium bromide. *Int. J. Nat. Eng. Sci.,* 4, 35-40.

De Vuyst, L., Vanderveken, F., de Ven, S. and Degeest, B. (1998) Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in a milk medium and evidence for their growth-associated biosynthesis. *J. Appl. Microbiol*., 84, 1059-1068.

Degeest, B., Mozzi, F. and De Vuyst, L. (2002) Effect of medium composition and temperature and pH changes on exopolysaccharide yields and stability during *Streptococcus thermophilus* LY03 fermentations. *Int. J. Food Microbiol.,* 79, 161-174.

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.

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

Haubler, S., Nimtz, M., Domke, T., Wray, V. and Steinmetz, I. (1998) Purification and Characterization of a Cytotoxic Exolipid of *Burkholderia pseudomallei. IAI.,* 66, 1588-1593.

Hirsch, A., Hahn, D., Kempna, P., Hofer, G., Mullis, P.E., Nuoffer, J.M. and Fluck, C.E. (2012) Role of AMP-activated protein kinase on steroid hormone biosynthesis in adrenal NCI-H295R Cells. *PLoSone* 7, e30956. doi:10.1371/journal.pone.0030956.

Irague, R., Rolland-Sabate, A.,Tarquis, L., Doublier, J.L., Moulis, C., Monsan, P., Remaud-Simeon, M., Potocki-Veronese, G. and Buleon, A. (2012) Structure and property engineering of α-D-Glucans synthesized by dextransucrase mutants. *Biomacromol.,* 13, 187-195.

Kanmani, P., Suganya, K., Satish kumar, R., Yuvaraj, N., Pattukumar, V., Paari, K.A. and Arul, V. (2013) Synthesis and Functional Characterization of Antibiofilm Exopolysaccharide Produced by *Enterococcus faecium* MC13 Isolated from the Gut of Fish. *Appl. Biochem. Biotechnol*., 169, 1001-1015.

Kralj, S., Grijpstra, P., van Leeuwen, S.S., Leemhuis, H., Dobruchowska, J.M., van der Kaaij, R.M., Malik, A., Oetari, A., Kamerling, J.P. and Dijkhuizen L. (2011) 4,6-α-Glucanotransferase, a novel enzyme that structurally and functionally provides an evolutionary link between glycoside hydrolase enzyme families 13 and 70. *Appl. Environ. Microbiol*., 77, 8154-8163.

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

Lim, D.J., Kim, J.D., Kim, M.Y., Yoo, S. H. and Kong, J.U. (2007) Physicochemical properties of the exopolysaccharides produced by marine bacterium *Zoogloea* sp. KCCM10036. *J. Microbiol. Biotechnol*., 17, 979-984.

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, Z.Y., Hu, Z.Q., Wang, T., Chen, Y., Zhang, J., Yu, J., Zhang, T., Zhang, Y.F. and Li, Y. (2013) Production of novel microbial flocculants by Klebsiella sp. TG-1 using waste residue from the food industry and its use in defecating the trona suspension. *Bioresour. Technol*., 139, 265-271.

Lonnermark, E., Friman, V., Lappas, G., Sandberg, T., Berggren, A. and Adlerberth, I. (2010) Intake of *Lactobacillus plantarum* reduces certain gastrointestinal symptoms during treatment with antibiotics. *J. Clin. Gastroenterol.* 44, 106-112.

Marinho-Soriano, E. and Bourret, E. (2005) Polysaccharides from the red seaweed *Gracilaria dura* (Gracilariales, Rhodophyta). *Bioresour.Technol*., 96, 379-382.

Monchois, V., Vignon, M. and Russell, R.R.B. (2000) Mutagenesis of Asp-569 of glucosyltransferase I glucansucrase modulates glucan and oligosaccharide synthesis. *Appl. Environ. Microbiol.,* 66, 1923-1927.

Monchois, V., Willemot, R.M. and Monsan, P. (1999) Glucansucrases: mechanism of action and structure-function relationships. *FEMS Microbiol. Rev*., 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.

Pan, D. and Mei, X. (2010) Antioxidant activity of an exopolysaccharide purified from *Lactococcus lactis* subsp. *lactis* 12. *Carbohydr. Polym*., 80, 908-914.

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. *Bioresour. Technol.,* 101, 6852-6855.

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.

Sajna, K.V., Sukumaran, R.K., Gottumukkala, L.D., Jayamurthy, H., Dhar, K.S. and Pandey, A. (2013) Studies on structural and physical characteristics of a novel exopolysaccharide from *Pseudozyma* sp. NII 08165. *Int. J. Biol. Macromol.,* 59, 84-89.

Shih, I.L., Van, Y.T., Yeh, L.C., Lin, H.G. and Chang, Y.N. (2001) Production of a biopolymer flocculant from Bacillus licheniformis and its flocculation properties. *Bioresour. Technol*., 78, 267-272.

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.

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

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 culture factors on production on dextransucrase by *Leuconostoc mesenteroides. J. Bacteriol*., 64, 521-526.

Tsusaki, K., Watanabe, H., Nishimoto, T., Yamamoto, Y., Kubota, M., Chaen, H., Fukuda, S. (2009) Structure of a novel highly branched a-glucan enzymatically produced from maltodextrin. *Carbohydr. Res*., 344, 2151-2156.

van Leeuwen, S.S., Kralj, S., Eeuwema, W., Gerwig, G.J., Dijkhuizen, L. and Kamerling, J.P. (2009) Structural characterization of bioengineered α-D-Glucans produced by mutant glucansucrase GTF180 enzymes of *Lactobacillus reuteri* strain 180 *Biomacromol.,* 10, 580-588.

van Meerloo, J., Kaspers, G.J. and Cloos, J. (2011) Cell sensitivity assays: the MTT assay. *Methods Mol Biol.,*731, 237-245.

Wang, Y., Ahmed, Z., Feng, W., Li, C. and Song, S. (2008) Physicochemical properties of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir. *Int. J. Biol. Macromol.,* 43,283-288.

Zhang, L., Liub, C, Li, D., Zhaoa, Y., Zhanga, X., Zenga, X., Yanga, Z. and Li, S. (2013) Antioxidant activity of an exopolysaccharide isolated from *Lactobacillus plantarum* C88. *Int. J. Biol. Macromol*. 54, 270-275.

LIST OF PUBLICATIONS

Published/accepted

1. **Deeplina Das** and Arun Goyal **(2013)** Potential probiotic attributes and antagonistic activity of an indigenous isolate *Lactobacillus plantarum* DM5 from an ethnic fermented beverage "Marcha" of North Eastern Himalayas. *International Journal of Food Science and Nutrition*. DOI: 10.3109/09637486.2013.869792.
2. **Deeplina Das** and Arun Goyal **(2013)** Characterization and biocompatibility of glucan: a safe food additive from probiotic *Lactobacillus plantarum* DM5. *Journal of Food Science and Agriculture*. DOI 10.1002/jsfa.6305.
3. **Deeplina Das** and Arun Goyal **(2013)** Anti-listerial Bactericidal Activity of Lactobacillus plantarum DM5 Isolated from Fermented Beverage Marcha. *Probiotics & Antimicrobial Protein*. 5:206–215.
4. Arijita Dutta, **Deeplina Das** and Arun Goyal **(2012)** Purification and characterization of fructan and fructansucrase from *Lactobacillus fermentum* AKJ15 isolated from Kodo ko jaanr, a fermented beverage from north-eastern Himalayas. *International Journal of Food science and Nutrition,* 63, 216-224.
5. **Deeplina Das** and Arun Goyal **(2011)** Expression of antagonism by Lactic acid bacterium isolated from Marcha: An ethnic fermented beverage of North-Eastern Himalayas. *International Journal of Biotechnology and Biochemistry*, 7, 411-421.
6. Seema Patel, **Deeplina Das** and Arun Goyal **(2011)** Structural characterization of the exopolysaccharide produced by a new strain of *Pediococcus pentosaceus* (SPO) isolated from Orissa. *Proceedings of National Academy of Sciences India,* Section B, 81, 291-298.
7. **Deeplina Das** and Arun Goyal (**2010**) Characterization and screening of antimicrobial activity of Lactic acid bacterium isolated from a traditional beverage Marcha of Sikkim. *Journal of Pharmacy and Chemistry* 4 (4) 136-139.

Submitted

8. **Deeplina Das** and Arun Goyal **(2013)** Isolation, purification and functional characterization of glucansucrase from probiotic *Lactobacillus plantarum* DM5. *Annals of Microbiology*.
9. **Deeplina Das** and Arun Goyal **(2013)** Antioxidant activity and γ-aminobutyric acid (GABA) producing ability of probiotic *Lactobacillus plantarum* DM5 isolated from Marcha of Sikkim. *LWT-Food Science and Technology*.
10. **Deeplina Das**, Rwivoo Baruah and Arun Goyal (2013) Potential food additive and prebiotic properties of a novel glucan from *Lactobacillus plantarum* DM5. *International Journal of Biological Macromolecules*.

Book Chapters

1. **Deeplina Das** and Arun Goyal (**2012)** Lactic Acid Bacteria in Food Industry. *Microorganisms in Sustainable Agriculture and Biotechnology*. Springer, pp: 757-772.
2. **Deeplina Das** and Arun Goyal (**2013)** Pharmaceutical and personal care products. *Biotransformation of Waste Biomass into High Value Biochemicals.* Springer, 367-387.

LIST OF CONFERENCES

International

1. **Deeplina Das** and Arun Goyal (**2014**). Emulsifying, flocculating and prebiotic properties of novel a glucan from *Lactobacillus plantarum* DM5 isolated from Marcha. 27th International Carbohydrate Symposium (ICS). January 12-17, Indian Institute of Science, Bangalore, India *(Abstract accepted)*.
2. **Deeplina Das** and Arun Goyal **(2012)**. *In vitro* analysis of probiotic properties of *Lactobacillus plantarum* DM5 isolated from fermented beverage Marcha of Sikkim. 5th International Conference on Industrial Bioprocesses (International Forum on Industrial Bioprocesses). October 7-11, NTUST, Taipei, Taiwan.
3. **Deeplina Das** and Arun Goyal **(2012)**. Purification and Characterization of glucansucrase produced by *Lactobacillus plantarum* DM5 isolated from a fermented beverage Marcha of Sikkim. Prospective and Challenges in Chemical and Biological Sciences: Innovation Cross Roads (18th International conference Post ISCBC), IASST, Guwahati, Assam, India.
4. **Deeplina Das** and Arun Goyal **(2011)**. Antagonistic activity of bacteriocin produced by a potential probiotic *Lactobacillus plantarum* DM5 isolated from a fermented beverage of Sikkim. International Conference on Microbial Biotechnology for Sustainable Development (52nd Annual Conference of Association of Microbiologists of India), November 3-6, Panjab University, Chandigarh, Punjab, India.
5. **Deeplina Das**, Arijita Dutta and Arun Goyal **(2010)**. Antibiotic sensitivity, Carbohydrate Fermentation Characteristics, Purification and Characterization of Glucansucrase produced by natural isolates of lactic acid bacteria from fermented beverages. International symposium on recent advances in cross disciplinary microbiology: Advances and challenges (51st Annual Conference of Association of Microbiologists of India), December 14-17, Ranchi, India.
6. **Deeplina Das** and Arun Goyal **(2010).** Production and characterization of bacteriocin from natural isolate of lactic acid bacteria from traditional fermented food of Sikkim; International Conference on Genomic Sciences; Nov 12-14, Madurai Kamraj University, Madurai, India.

National

7. Seema Patel, Damini Kothari, S. Krishna Bindu, **Deeplina Das** and Arun Goyal **(2009)**. Statistical optimization of medium as a strategy to enhance the dextransucrase activity of mutant of a new isolate of lactic acid bacteria. 50th Annual Conference of Association of Microbiologists of India, December 15-18, National Chemical Laboratory, Pune, India.
8. **Deeplina Das** and Arun Goyal **(2012)**. Structural analysis and effective purification of glucan produced by *Lactobacillus plantarum* DM5 isolated from fermented beverage Marcha of Sikkim. 81st Annual Meeting of the Society of Biological Chemists (India) & Symposium on Chemistry and Biology: Two Weapons against Diseases. November 8-11, Science city, Kolkata, India

VITAE

The author was born on December 6, 1983 in Kolkata, West Bengal, India. She passed the Secondary School Examination conducted by West Bengal Board of Secondary Education, Kolkata, in 2000, and Higher Secondary School Examination conducted by West Bengal Council of Higher Secondary Education, Kolkata, in 2002. She completed B. Tech in Biotechnology from West Bengal University of Technology, Kolkata, India, in 2006. She did her M. Tech in Biotechnology from West Bengal University of Technology, Kolkata in 2009.

Deeplina Das joined Ph.D. programme in July 2009 at Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, Assam, India. She received Senior Research Fellowship from Institute under the scheme run by the Ministry of Human Resource and Development (MHRD), India. She also received Senior Research Fellowship Council of Scientific and Industrial Research (CSIR), New Delhi, India. She successfully completed the course work with 8.5/10 Cumulative Point Index (CPI). She gave the Open (PhD Synopsis) Seminar on January 03, 2014 and presented her thesis work before the Doctoral Committee and her performance was satisfactory. She submitted the PhD thesis in January, 2014.