ISOLATION AND CHARACTERIZATION OF ANTIFUNGAL COMPOUNDS FROM LACTIC ACID BACTERIA

THESIS SUBMITTED TO
THE UNIVERSITY OF KERALA
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

UNDER THE FACULTY OF APPLIED SCIENCE

BY

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JULY 2015

Dedicated to my parents and brother

DECLARATION

I hereby declare that the PhD thesis entitled **"Isolation and characterization of antifungal compounds from lactic acid bacteria"** is an independent work carried out by me under the supervision of Dr K Madhavan Nampoothiri, Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Thiruvananthapuram-695 019, India, and it has not been submitted anywhere else for any other degree, diploma or title.

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CERTIFICATE

This is to certify that the work embodied in the thesis entitled **"Isolation and characterization of antifungal compounds from lactic acid bacteria"** is based on the original research work carried out by Ms.Varsha KK under my supervision in the Biotechnology Division of CSIR-NIIST, Thiruvananthapuram and that no part of this work has been submitted previously anywhere for the award of any degree.

Place: Trivandrum **Dr. K. Madhavan Nampoothiri**

Date : Research Supervisor

ACKNOWLEDGMENTS

This thesis would not have been possible without the kind support, help, criticism and encouragement of many individuals and organization. I would like to extend my sincere thanks to all of them.

First of all I thank Council of Scientific and Industrial Research, New Delhi for providing me the research fellowship to carry out this study.

With gratitude and pleasure I thank my research supervisor Dr. K. Madhavan Nampoothiri, for giving me the opportunity to work with his research group and for allowing me to choose this topic for my research. He always provided the freedom to work independently and showed support and confidence during my entire study for which I will always remain grateful to him.

I am deeply indebted to Prof. Ashok Pandey, HOD of our division for always being supportive and encouraging during my work. His suggestions helped me to overcome the hard times of my research for which I will stay thankful to him.

I place on record, my thanks to Director, CSIR-NIIST for providing me the necessary facilities and infrastructure of the institute for carrying out this work.

My heartfelt thanks for the helps received for bioactive structure analysis from Dr. Leena Devendra, without whose support I would never have completed this work. I gratefully acknowledge her for the encouragement and fruitful discussions which enabled me to achieve my target.

I deeply appreciate Dr. Priya S and Ms. Shilpa G from Agroprocessing Division, CSIR-NIIST for the help and guidance provided for cell-line studies that added much significance to my work.

I thank Dr. N. Ramesh Kumar for carrying out DNA sequencing and also for his valuable advices and suggestions which inspired me to explore more domains of my work.

I extend my thanks to Dr. Binod Parameswaran, Dr. Rajeev K Sukumaran, Dr. Muthu Arumugam, Dr. Sindhu R and Mr. Kiran Kumar for their helps, support and encouragement.

My completion of this work could not have been accomplished without the support and help of Anusree, Nishant, Kiran, Rajasree and Kuttiraja. I am deeply indebted to each one of you for sparing your valuable time to find solutions for the troubles I encountered during my work. The suggestions and advices provided by them helped me focus on various possibilities to reach my goal. My sincere thanks to Sneha for the helps received for aminopeptidase inhibition studies.

I deeply acknowledge the helps received from Dr. Neetha Joseph and Dr. Venkata Ramana Vemuluri, Microbial culture collection, National Centre for Cell Science for performing the FAME analysis and DDH experiment.

I thank Mr. Saravana Kumar and Mr. Arun Kumar from Mass spectrometry and Proteomic core facility of Rajiv Gandhi Centre for Biotechnology for performing MALDI-TOF and Peptide Mass Fingerprinting analyses.

I am grateful to the faculty of Analytical facility unit especially Mass and NMR spectrometry Units, CSIR-NIIST for performing my analyses. I extend my thanks to Dr. Sinu C R, Chemical Science and Technology for performing the FTIR analysis.

I sincerely thank my respectful seniors and colleagues Dr. Vipin Gopinath , Dr. Arya Nandan , Dr. Dhanya Gangadharan, Dr. Shyam Krishna, Dr. Raji, Dr. Nisha, Dr. Bindumol, Dr. Sai shyam, Dr. Vidya J, Dr. Ushasree M V, Deepthi Alex, Deepa and Dr. Abraham Mathew for giving support and motivation during my stay in lab.

I extend my thanks to my colleagues Aravind, Ramya, Divya, Nimisha, Sabeela, Sajna, Preeti and Lalitha for their help and support at various stages of my work along with my gratitude to my lab mates Dilna, Vini, Surya, Ram

Kumar, Niyas, Karthik, Soya, Koyna, Vani, Leya, Anju, Dhanya, Sowmya, Seeta, Rahul and Vivek.

I deeply acknowledge the technical support provided by the technical staff of our division. I am thankful to Mrs Radhamony, Mrs Sasikala and Mrs Rajalekshmi for all their support and would like to extend my gratitude to the administration, library and all other non-scientific staff members of CSIR-NIIST for the technical guidance.

Finally, to my loving, caring and encouraging family: my deepest gratitude. It's their faith and support that give me the strength and inspiration to pursue my dreams.

Varsha K K

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List of Abbreviations

2, 4 DTBP	2, 4 di- tertiary butyl phenol
α- AL	α-acetolactate
%	Percentage
°C	Degree Celsius
µ	Micro
µg	Microgram
µg/µL	Microgram per microlitre
µL	Microlitre
µM	Micro molar
AGE	Agarose gel electrophoresis
AMP	Antimicrobial peptide
APS	Ammonium persulphate
ATCC	American Type Culture Collection
A_{600}	Absorbance at 600 nm
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCF	Concentrated culture filtrate
CCUG	Culture collection University of Goteborg
CF	Culture filtrate
CFS	Cell free supernatant
CFU/mL	Colony forming units per millilitre
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribo Nucleic acid
dNTPs	Deoxynucleotide phosphates
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylene diamine tetraacetic acid
ESI	Electron spray ionization
Fe3+	Trivalent iron
Fe2+	Divalent iron
FBS	Fetal bovine serum
FTIR	Fourier transform infrared
g	Gram
GC	Gas chromatography
GRAS	Generally recognized as safe
h	Hour
HCl	Hydrochloric acid
HPLC	High pressure liquid chromatography
IC_{50}	The half maximal inhibitory concentration
IR	Infrared
IU	International Units
KACC	Korean agricultural culture collection
kDa	Kilo Dalton
L	Liter
LAB	Lactic acid bacteria

LAP	Leucine aminopeptidase
LC	Liquid chromatography
Leu- *p*NA	Leucine paranitroanilide
M	Molar
MALDI	Matrix-assisted laser desorption/ionization
MEM	Eagles minimum essential medium
mg	Milligram
mg/mL	Milligram per milliliter
min	Minute
mL	Milliliter
mM	Millimolar
MS	Mass spectrometry
MTCC	Microbial type culture collection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium Chloride
NCBI	National center for biotechnology information
nM	Nanomolar
NMR	Nuclear magnetic resonance
OD	Optical density
PAP	Proline aminopeptidase
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
pH	Hydrogen ion concentration
PMF	Peptide mass fingerprinting

*p*NA	Para-nitroaniline
Pro- *p*NA	Proline paranitroanilide
QPS	Qualified presumption of safety
RP	Reverse phase
Rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec	Second
SEM	Scanning Electron Microscopy
sp.	Species
SPE	Solid phase extraction
subsp.	Subspecies
TAC	Total antioxidant capacity
TEMED	N, N, N′, N′-Tetramethylethylenediamine
TFA	Trifluro acetic acid
TLC	Thin layer Chromatography
UFLC	Ultra fast liquid chromatography
UPR	Unfolded protein response
U/mL	Units per milliliter
UV	Ultraviolet
v/v	Volume per volume
VOC	Volatile organic compound
w/v	Weight per volume
w/w	Weight per weight

CHAPTER 1
Introduction

1. 1. Introduction

Biological preservation of food has gained major attention during recent years where antagonistic microorganisms or their antimicrobial metabolites can prevent the growth of pathogenic bacteria and fungus in food. Fungal growth is one of the causes of spoilage in vegetables and baked foods causing significant reduction in their quality and quantity. In addition, allergenic fungal spores and mycotoxins such as aflatoxins, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone can cause serious health problems. These mycotoxins are reported to be carcinogenic, immunotoxic, teratogenic, neurotoxic, nephrotoxic and hepatotoxic and hence the occurrence of these moulds in food is a major health concern and economic problem (Kabak et al., 2006; Sweeny and Dobson, 1999). Bacterial pathogens that account for food borne illness include *Salmonella*, *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium botulinum. Penicillium* and *Aspergillus* species have been reported as spoilage fungi during storage of wide range of food and feed. *Fusarium* species often contaminate cereal grains (Buzby et al., 1996).

Physical and chemical methods have been developed to control the occurrence of these microorganisms and their toxins but several molds have acquired resistance against some chemical treatments and preservatives. For example, various strains of the *Penicillium*, *Saccharomyces* and *Zygosaccharomyces* can grow in the presence of potassium sorbate (Davidson, 2001). Frequent use of antibiotics and preservatives may increase the resistance phenomena in the future. Instead of chemical preservatives or additives in food or feed, consumers want high quality, preservative free, safe and mildly processed food with extended shelf life.

Lactic acid bacteria (LAB) are found in nutrient rich surroundings and are normal inhabitants of gut microbiota. They naturally occur in various food products such as vegetables, dairy and meat products. LAB have a long tradition of being used in food fermentations since they greatly influence the nutritional, sensory and shelf-life characteristics of fermented food products and secured the position of food grade bacteria due to their "generally recognized as safe" (GRAS) status. LAB are established as probiotics and good candidate for biological food preservation due to their wide spectrum antimicrobial activities. The metabolic processes behind the biopreservative properties of LAB have begun to unveil during last century. As food additives, LAB can perform as protective culture for improving microbiological safety of the product without changing the sensory characteristics of the food.

This thesis discusses identification and characterization of antifungal and antibacterial compounds from a new isolate of LAB and explores the possibility of using the same to prevent food spoiling fungi. In order to achieve this, the following objectives were made

1. 2. Objectives of the study

- Isolation of lactic acid bacteria (LAB) and evaluation of its antifungal activity
- Identification and characterization of selected LAB strains including probiotic properties
- Production optimization of the selected antifungal metabolites from LAB
- Purification and characterization of the antifungal metabolites
- Wide spectrum studies of the metabolite on the antifungal effect
- Application studies related to food spoilage

The thesis is framed into 10 chapters. Chapter 1 provides a brief introduction and an outline of the work and chapter 2 reviews the research developments in the area of LAB probiotics and antifungal compounds produced by LAB. Chapter 3 deals with the general materials and methods used in this study. Chapter 4

describes isolation and probiotic characterization of antifungal LAB strains and chapter 5 checks the role of organic acids on antifungal activity of LAB and identify them as a group of possible antifungal compounds from three different genera of LAB. Chapter 6 covers the phylogenetic characterization and determination of taxonomic position of an antifungal *Lactococcus* strain (BSN307) based on biochemical and molecular studies and chapter 7 describes the purification, characterization and identification of a phenolic compound and a phenazine compound produced by this strain. Chapter 8 explains the production, purification and characterization of an antifungal biopeptide produced by the strain BSN307 and chapter 9 demonstrates the application of BSN307 and the antifungal compounds in preventing food spoilage caused by fungi. Chapter 10 summarizes the entire work and conclusions derived from the study followed by a bibliographic section of all the chapters. Three annexures are attached. Annexure I contains the media composition, annexure II contains the list of major instruments used and finally annexure III contains the list of publications.

CHAPTER 2

Review of Literature

2. 1. Introduction

This chapter reviews the recent advances in the field of antifungal lactic acid bacteria (LAB) and their use as protective cultures against fungal contamination. The probiotic potential of LAB, different antifungal compounds produced, purification and characterization of compounds as well as the possible application studies are also covered.

2. 2. Lactic acid bacteria (LAB)

LAB are a group of Gram-positive, catalase negative, microaerophilic, non sporing cocci, coccobacilli or rods with lactic acid as the main product of carbohydrate fermentation. LAB comprise of the genera *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, and Weissella.* Lactobacilli, Carnobacteria and some *Weissella* are rods while the rest of the genera are cocci (Axelsson 2004; Stiles and Holzapfel 1997). The natural habitat of LAB include different environment niches that comprise vegetables and other plant materials, fruits, cereals, meat, pickles, milk and other dairy products as well as the gastrointestinal tract of animals contributing to the maintenance of a healthy gut microbiota.

LAB have two different metabolic pathways for carbohydrate fermentation, the homo and heterofermentative pathway. Homofermentative pathway follows glycolysis (Embden- Meyerhof-Parnas pathway) and occurs in *Lactococcus*, *Pediococcus* and *Streptococcus*. In the homofermentative pathway, a hexose is processed and split into two lactate molecules. Heterofermenters use pentose phosphate pathway to generate carbon dioxide, lactate and ethanol or

acetate by utilizing hexoses. *Leuconostoc* and *Weissella* follow heterofermentative pathway (Axelsson, 2004).

Humans have consumed LAB fermented foods for centuries although the role of these microbes was not established at the time. Because of this long history of safe use in food they are considered non-toxic and food-grade microorganisms with a wide acceptance in food products by the society for varying applications. LAB play crucial roles in the fermentation of milk products, vegetables and meat in addition to processing of wine along with application in biopreservation of food products as protective culture. LAB have been studied meticulously in their genetic, physiological and application aspects in order to manipulate and modify them so as to adapt more to diverse applications in food industry. Fig.2.1 summarizes the application of LAB in industry

Fig. 2.1 Application of LAB in industry

2.2.1. Phylogenetic characterization of LAB

The use of 16S rRNA gene sequences has been by far the most common housekeeping genetic marker to study bacterial phylogeny and taxonomy for a number of reasons such as (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed; and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Janda and Abbott, 2007). Apart from this, additional data are required to propose a new bacterial taxon that include source of isolation; phenotype variations such as optimum growth conditions, gram staining value, colony morphology, motility, spore-forming capacity; biochemical profiles including oxidase and catalase testing and carbohydrate metabolizing ability. Antibiotic susceptibility of the isolates is also importatant in some cases. Apart from 16S rRNA gene, sequences of 23S rRNA gene, 16S-23S rRNA gene internal transcribed sequences (ITS), housekeeping genes such as rpoB gene encoding the β- subunit of DNA gyrase and groELgene encoding the heat shock protein are also important when characterizing a new bacterium (Drancourt and Raoult, 2005).

DNA-DNA hybridization (DDH) is considered as the "gold standard" for bacterial classification. Organisms that show more than 70% DDH values and less than 5% difference in their melting temperature (Δ*Tm*) are considered to belong to the same species (Wayne et al., 1987). Genomic fingerprinting methods such as rep-PCR (ERIC-, REP-, BOX-PCR), RAPD and AFLP also provide more differentiation power during bacterial classification (Rajendhran and Gunasekaran, 2010).

The genus *Lactococcus* currently contains 11 species including *L. lactis*, *L. garvieae*, *L. piscium*, *L. plantarum*, *L. raffinolactis*, *L. fujiensis*, *L. chungangensis*, *L. taiwanensis* and *L. formonensis*, *L. hircilactis*, *L. laudensis* (Cai et al., 2011; Chen et al., 2014; Cho et al., 2008; Meucci et al. 2015) and the subspecies- *L. lactis* subsp. *cremoris*, *L. lactis* subsp.*hordniae*, *L. lactis* subsp.*lactis*, *L. lactis* subsp. *biovar. Diacetylactis*, *L. lactis* subsp.*tructae* are

established within *L. lactis* (Kim et al., 1999; Schleifer and Kilpper-Balz, 1987; Schleifer et al., 1985). The genus *Lactobacillus* contains 214 species along with 29 subspecies and *Pediococcus* contains 15 species (LPSN- List of prokaryotic names with standing in nomenclature). Fig.2.2 shows rooted phylogenetic tree of LAB constructed on the basis of alignments of the DNA-dependent RNA polymerase (Price et al., 2012). The minimum-evolution tree was built using MEGA 6.0 software.

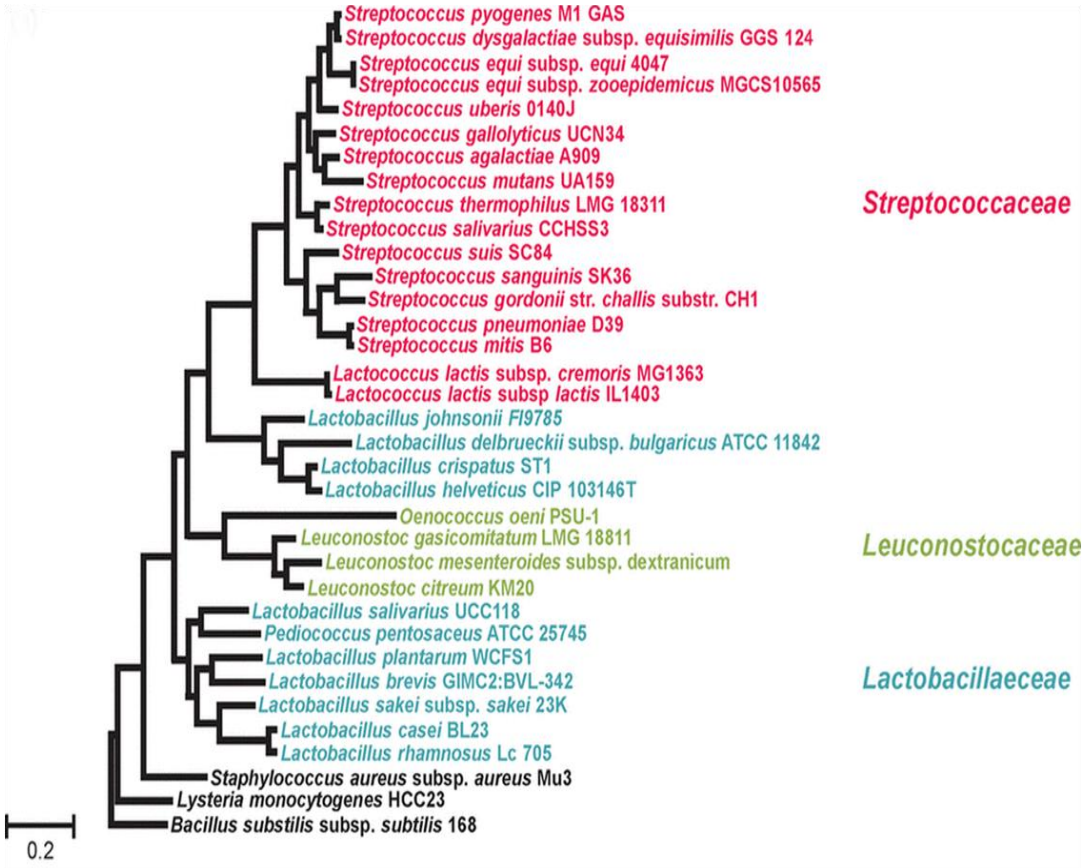


Fig.2.2 Rooted phylogenetic tree of LAB constructed on the basis of alignments of the DNA-dependent RNA polymerase.

2. 3. LAB as probiotics

The word probiotic means “for life” and the world health organization defines probiotics as, “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. In the last two decades, research in the probiotic area has achieved major progresses in the selection and characterization of specific probiotic cultures and confirmed the health benefits associated with them. Traditionally, fermented foods are the main source of probiotics and hence one of the major dietary supplements of modern world.

LAB are the most important group of microorganisms commercially used as starter cultures for the manufacture of dairy based probiotic foods (Heenan et al., 2002) and have been established as a natural consumer. Strains of the genera *Lactobacillus*, *Bifidobacterium* and *Propionibacterium* are the most widely used and commonly studied probiotic bacteria.

Nowadays probiotics are consumed as fermented dairy products or as freeze dried cultures. Probiotics, upon reaching the lower part of small intestine and colon, colonize and multiply to exert their beneficial effects. Table.2.1 shows some of the probiotics present in the market, manufacturer, the LAB strain used in each preparation and the health benefit that it provides.

The mechanisms by which LAB differentially modulate the host cell functions depend upon the cell wall constituents of bacteria and corresponding host cell receptors that modulate downstream processes. High tolerance to acid and bile helps them survive the harsh physico-chemical conditions of gastro-intestinal (GI) tract. The ability of the bacteria to colonize the mucosal surfaces by adhering to the host GI epithelial cells and extracellular matrix proteins depends on cell surface hydrophobicity (Zareba et al., 1997). This prevents pathogen access by steric interactions or specific blockage on cell receptors. Probiotic interaction with the mucin layer of the host epithelial cells increases their retention time in the host (Otero et al., 2004).

The antimicrobial activity of LAB has been attributed to the production of organic acids (Cabo et al., 2002; Lind et al., 2007), reuterin (Talarico et al., 1988), proteinaceous compounds (Magnusson and Schnurer, 2001) and cyclic dipeptides

(Strom et al., 2002). Bacteriocins <20 kDa cause depolarization of the target cell membrane and/or inhibit cell wall synthesis and those with >20 kDa degrade the murein layer (Settanni and Corsetti, 2008).

Table.2.1 Probiotics on Market

Trade name	**Manufacturer**	**Organism**	**Health benefit**
Ultimata flora critical care 50 billion	Renew life	Bifidobacteria Lactobacilli	Promotes digestive health, regularity and immune health
Yakult	Yakult	*L. casei* shirota	Improve bowel movement, aid digestion, maintain gut flora
Fem-Dophilus	Jarrow formulas	*L. reuteri*, *L. rhamnosus*	Vagina/urinary tract health
Culturelle	Amerfit brands	*L. rhamnosus* GG	Restores natural balance of intestinal microbiota, boost immune system
Activia yogurt	Dannon Inc.	*B. lactis* DN-173 010	Digestive well-being
Align capsules	Align	*B. infantis* 35624	Maintain natural balance of digestive system
Attune nutrition bar	Attune foods	*L. acodophilus* NCFM, *L. casei* LC-11, *B. lactis* HN019	Helps digestion
Probulin	Probulin	Bifidobacteria,	Supports digestive

original probiotic		Lactobacilli	health and balance
DanActive	Danone	*S. thermophilus, L. bulgaricus, L. casei* DN-114001	Contributes to healthy gut flora
Yo-plus yogurt		*B. animalis* subsp *lactis* Bb-12, *S. thermophilus, L. bulgaricus*	Replenish gut bacterial flora, supports natural defence
RepHresh Pro-B		*L. rhamnosus, L. reuteri*	Maintain the microbiota important to overall vaginal health in females

2. 3. 1. Probiotic market

The probiotic market has been one of the prime beneficiaries of the recent fad over functional foods. Europe and Asia-Pacific are the dominating regions for these products. The most active area within the functional foods market in Europe is probiotic dairy products, in particular, probiotic yogurts and milks. In 1997 these products accounted for 65% of the European functional foods market (Stanton et al., 2001). Indian probiotic market, which is comparatively nascent now, valued at USD 12 million in 2011, is expected to witness a compound annual growth rate (CAGR) of 11% by 2016, Frost & Sullivan (2012) said. According to the report published by Markets and Markets, probiotic products market was valued at $26,125.9 million in 2012. (http://www.marketsandmarkets.com/PressReleases/probiotics.asp;http://articles.economictimes.indiatimes.com/2012-08-22/news/33322348_1_probiotic-ice-creams-health-and-wellness).

2. 3. 2. Health benefits of probiotics

Probiotic LAB bring about health benefits. Studies show probiotic intake can be an important means to reduce infectious diarrhea. Research groups working under various countries reported that consumption of probiotic fermented dairy products have shortened the episodes or reduced the risk of rotavirus induced diarrhea in humans. Many strains of LAB are reported to reduce the risk of acute diarrhea.

In a study, one hundred and twelve newborn infants in rural India were randomized to receive a daily oral dose of 10^8 CFU/g *L.sporogenes* or a placebo for one year. Results has shown that feeding *L. sporogenes* on a prophylactic basis in the first year of life has a significant preventive impact on the incidence and duration of diarrhea as well as the total number of days of illness (Chandra, 2002). A combination of nitazoxanide and *Lactobacillus* was found to be efficient in the treatment of acute rotavirus diarrhea in children (Teran et al., 2009). The efficacy of *Lactobacillus* GG strain isolated from healthy humans in decreasing the incidence of traveller's diarrhea in Finnish travellers (Hilton et al., 1997) has been studied. In a recent study the inefficiency of non-viable *L. acidophilus* in prevention of traveller's diarrhea has been found by a randomized, double-blind, controlled study (Briand et al., 2006).

Excessive use of antibiotics may lead to colitis caused by *Clostridium difficile.* This is a common inhabitant of intestine but an imbalance in the indigenous microbiota leads to an elevation in their number and production of toxin. In such cases probiotics can be administered to restore the lost intestinal microbiota. Intake of *Lactobacillus* GG is reported to be very effective in alleviating the signs and symptoms of *C. difficile* infection (Biller et al., 1995). A daily supplementation with two strains of *L. acidophilus* (CUL60 and CUL21) and two strains of *Bifidobacterium* sp. during and post antibiotic therapy reduced the extent of disruption to the intestinal microbiota as well as the incidence and total numbers of antibiotic -resistant strains in the re-growth population(Madden et al., 2005; Plummer et al., 2005).

Helicobacter pylori is a gram negative pathogen that causes peptic ulcers and type B gastritis. *L. johnsonii* La1, when administered as a whey-based culture supernatant is reported to have attenuated the colonization of *H. pylori* in the gut (Michetti et al., 1999). Administration of *L. rhamnosus, P. freudenreichii* and *B. breve* after an anti *H. pylori* treatment resulted in minor changes in intestinal microbiota and slightly diminished the microbial disturbances (Myllyluoma et al., 2007). In a mouse study, the strain *L. casei* Shirota administered for nine months reduced colonization of *H. pylori* in the antrum and corpus regions of the stomach, associated with a reduced intensity of mucosal inflammation (Sgouras et al., 2004).

Alterations in the gut biota including infection may lead to inflammatory bowel disease (IBD) like Crohn's disease and ulcerative colitis (Shanahan, 2000). Intestinal microbiota play a critical role in inflammatory conditions in the gut and hence the therapeutic restoration of the altered microbiota with appropriate probiotics can be an ideal treatment for IBD. Several studies show interesting effects of probiotics on IBD. Children with mildly to moderately active Crohn's disease were given enterocoated *L. rhamnosus* GG twice a day for six months and had a notably reduced inflammation after four weeks (Gupta et al., 2000). Another proof that demonstrates the potential role of probiotics in prevention or treatment of inflammatory bowel disease came from a clinical study in which an IL-10 deficient mouse was pretreated with *L. reuteri* and *L. paracasei* and then infected with IBD causing *H. hepaticus*. Intestinal inflammation was reduced and the levels of proinflammatory colonic cytokines were lowered after the pretreatment (Pena et al., 2005). Deconjugation and absorbtion of bile acids can be performed by lactobacilli and bifidobacteria, possibly reducing the colonic mucosal secretion of mucin and fluids that may donate to functional diarrhea or IBS with diarrhea (Camilleri, 2006).

Among the many health promoting functions of probiotics much interest has given to their immune modulatory activity. The immunomodulation roles may involve the activation of both specific and nonspecific immune responses. The

cell wall components of probiotics include peptidoglycans, polysaccharides, and teichoic acid which are previously reported to have immunostimulatory effects. Probiotics boost the mucosal barrier function by innate immune molecules, including goblet cell–derived mucins and trefoil factors and defensins produced by intestinal paneth cells or by promoting adaptive immune responses (Sherman et al., 2009). In a mouse study, 7 days of *L. casei* CRL 431 administration decreased the severity of infection with *Salmonella enteric*a serovar Typhimurium. This continuous administration diminished the counts of the pathogens in the intestine as well as their spreads outside this organ (de Moreno de LeBlanc et al., 2010). In a human study the effect of yogurt containing *L. casei* as the main probiotic component on immune system function was checked. Administration of LAB strains resulted in an increase of NK activity and proliferative response to *Candida albicans* in adults and secretory-IgA in children saliva (Zanini et al., 2007).

Experiments show that probiotic microorganisms can preclude or delay the start of certain cancers. The six day old extracts of fermented milk product kefir depressed the growth of human mammary cancer cells (MCF-7) in a dose-dependent manner, showing 29% inhibition of proliferation at a concentration as low as 0.63% without any antiproliferative effect against normal human mammary epithelial cells (HMECs) (Chen et al., 2007). Ornithine decarboxylase (ODC) and spermidine/spermine N1-acetyltransferase (SSAT), the key enzymes involved in polyamine biosynthesis and catabolism respectively are associated with cancer risk and are the specific markers for neoplastic proliferation. Administration of *L. rhamnosus* GG homogenate significantly reduced ODC mRNA and activity as well as polyamine content and neoplastic proliferation. In addition, an increase in both SSAT mRNA and activity was observed after *L. rhamnosus* GG administration in HGC-27 human gastric cell lines (Linsalata et al., 2010).

L. acidophilus VM 20 caused a decrease of the mycotoxins ochratoxin A and *B. animalis* VM 12 reduced patulin level from a liquid medium (Fuchs et al.,

2008). In another study antimutagenic effect of soymilk fermented with *S. thermophilus*, *L. acidophilus*, *B. infantis* and *B. longum* was checked against mutagenesis induced by 4-nitroquinoline-*N*-oxide (4-NQO), a direct-acting mutagen, and 3,2-dimethyl-4-amino-biphenyl (DMAB), an indirect-acting mutagen, on *S. typhimurium* TA 100 and concluded with lack of mutagenic activity of the fermented soymilk (Hsieh and Chou, 2006).

Preliminary evidences show lactobacilli, bifidobacteria and their metabolic products lower serum cholesterol level and thus reduce the risk of cardiovascular diseases due to hypercholestremia. The LAB strains *B. longum*, *L. plantarum*, *L. paracasei*, *S. thermophilus* and *L. delbreuckii* brought about significant lowering of the serum concentrations of total cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides in rats and hamsters. In humans a decrease in serum total cholesterol was observed as a result of the activity of probiotics (Wang et al., 2010; Xiao et al., 2003). The hypocholesterolemic effects exerted may be because of removal of cholesterol through incorporation into the cellular membranes of live or dead lactobacilli or by deconjugation of bile via bile salt hydrolase and co-precipitation of cholesterol with the deconjugated bile (Liong and Shah, 2005; Lye et al., 2009). Fig.2.3 summarizes the health benefits provided by LAB.

2. 4. Synbiotics, nutraceuticals and functional foods

The concept of synbiotics has been proposed by combining the rationale of pro and prebiotics to characterize nutritional food with some health enhancing activity called functional food. A prebiotic, according to (Gibson and Roberfroid, 1995) is, "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health". Synbiotics could beneficially affect the host by improving survival and implantation of live microbial dietary supplements in the GI biota. Roberfroid (2000) suggested that synbiotics may improve survival of the bacteria crossing the upper part of the gastrointestinal tract, thereby enhancing their effects in the large bowel. A mixture of 0.5% inulin and *Weissella cibaria* in the diet of *Pseudoplatystoma*

hybrid surubins trimmed down the number of pathogenic bacteria and stimulated the beneficial intestinal microbiota (Mourino et al., 2012). Recent studies show the significance of prebiotics in reducing the risk of diseases. A specific mixture of neutral oligosaccharides and pectin derived acidic oligosaccharides reduced the occurrence of early atopic dermatitis among low atopy risk-infants (Gruber et al., 2010). Modulation of cholesterol metabolism is another important function of prebiotics. A combination of *L. acidophilus* CHO-220 and inulin reduced plasma total cholesterol and LDL cholesterol by 7.84 and 9.27%, respectively thus reduced the risk of artherosclerosis (Ooi et al., 2010) is another example of the health advantage brought about by a symbiotic product.

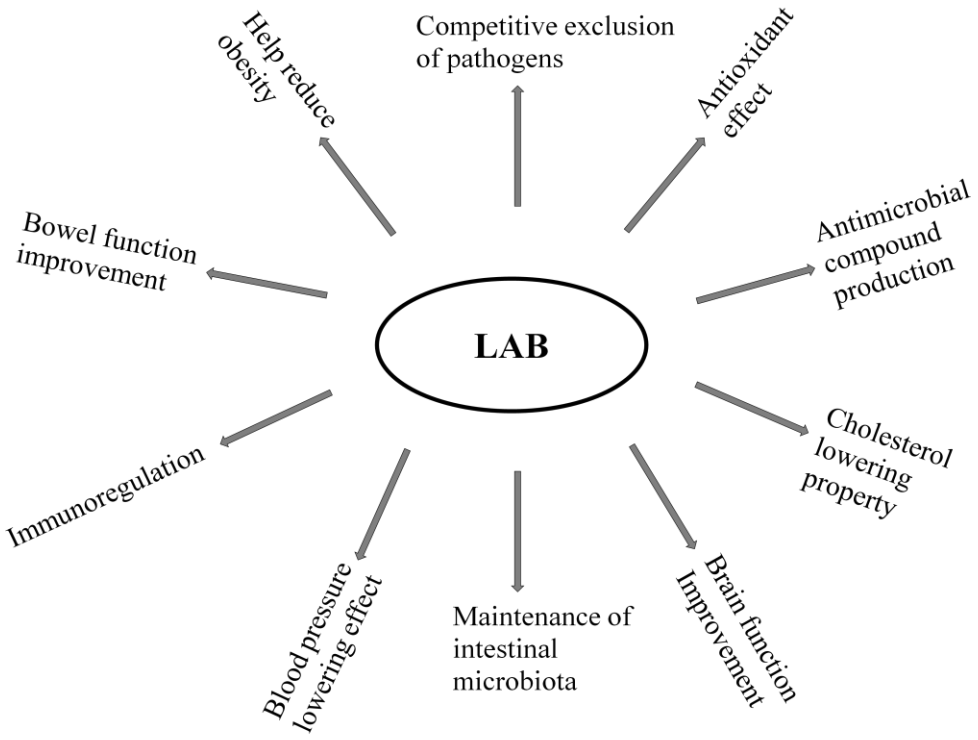


Fig.2.3. Health benefits of LAB

Dr Stephen De Felice, founder and chairman of the Foundation for Innovation in Medicine coined the term "Nutraceutical"from "Nutrition" and "Pharmaceutical" in 1989 and according to him nutraceutical can be defined as, "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease (Brower, 1998). Nutraceuticals are hugely popular among consumers and American sales for 2003 were an estimated $31 billion. Consumer interest in the relationship between diet and health has increased the demand for information on nutraceuticals. Traditional nutraceuticals -fruits, vegetables, grains, fish, dairy and meat products contain several natural components that deliver benefits beyond basic nutrition, such as lycopene intomatoes, omega-3 fatty acids in salmon or saponinsin soy. Nontraditional nutraceuticals, on the other hand, are foods resulting from agricultural breeding or added nutrients and/or ingredients.

The health benefits conveyed by nutraceuticals comprise areas including cancer, atherosclerosis, cardiovascular disease (CVD), aging process and immune response-enhancing effect, diabetes and mental health (Shahidi and Zhong, 2004). They also involved in prevention or reducing hypertension, hypercholesterolemia, obesity, arthritis, osteoporosis, macular degeneration, cataracts, menopausal symptoms, insomnia, diminished memory and lack of concentration, digestive upsets and constipation and headaches (Stauffer, 1999). LAB produce various nutraceuticals like B vitamins (mainly folate, riboflavin and cobalamin), low calorie sugars (mannitol, sorbitol, tagatose), L-alanine, exopolysaccharides (EPS) and antioxidants (Hugenholtz et al., 2002, Lu et al., 2008). Table 2.2 shows the important nutraceuticals reported from LAB and their health benefits.

Genetic engineering techniques focus on manipulating the biosynthetic pathway of LAB and make them suitable to produce nutraceuticals. The food grade and nisin induced controlled expression system (NICE) allows the gradual over expression of genes in LAB. Different metabolic engineering strategies resulted in the overproduction of B vitamins- folate, riboflavin and cobalamin as

well as the production of low calorie sugars – mannitol and sorbitol in different LAB strains (Hugenholtz et al., 2002; Ladero et al., 2007).

Table.2.2 Nutraceuticals from LAB

Neutraceutical	Health benefit
B vitamins	
Folate (Vitamin B9)	Prevents neural tube defects in newborns, take parts in nucleotide biosynthesis, anticarcinogen
Riboflavin (Vitamin B2)	Required for the proper development and function of skin, lining of digestive tract, blood cells
Cobalamin (Vitamin B12)	Necessary for the production of blood cells, helps in maintaining the health of CNS
Low-calorie sugars	
Sorbitol	Sugar free dietary sweetener, protects against tooth decay, possesses moisture-stabilizing properties
Mannitol	Sugar free dietary sweetener, protects against tooth decay
Tagatose	Antidiabetic, antioxidant, prebiotic, low calorie sugar substrate
Antioxidants	
Glutathione	Involved in tissue building and repair, helps eliminate toxins
Exopolysaccharides	Used as food additive, gives flavor and texture to food
Fatty acids	May reduce risk of coronary heart diseases

2. 5. Lactic acid bacteria and biopreservation

Biopreservation is the use of natural or added microbiota and/or their antimicrobial products for extending the shelf life and enhanced food safety. LAB as bio-preservation organisms are of particular interest since they greatly

influence the nutritional, sensory and shelf-life characteristics of fermented food products. As food additives, LAB can perform as protective culture for improving microbiological safety of the product without changing the sensory characteristics of the food.

2. 5. 1. Fungal spoilage of food and feed

Food, by its very nature is a rich nutrient source and attracts microbial colonization as a suitable habitat. Nutritional properties of the food alter successful colonization of the microbes and when the nutritional value, structure, and taste of the product are negatively influenced, it is called food spoilage. Many strains of food spoilage fungi including *Aspergillus*, *Penicillium* and *Fusarium* species are capable of producing mycotoxins which cause serious health problems once consumed. These are often produced when the fungi are under stress, like, when the temperature, water activity or amount of oxygen becomes less. Mycotoxins of greatest public health and agro-economic significance include aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins, and ergot alkaloids. Aflatoxin B_1, the most potent hepatocarcinogenic substance known, has been recently proven to be genotoxic as well. Ochratoxin A is nephrotoxic and nephrocarcinogenic and zearalenone, produced by various species of *Fusarium* has an osteogenous action and is significantly toxic to the reproductive system of animals (Milicevic et al., 2010; Zain, 2011). Since filamentous moulds and yeasts are common spoilage organisms of food products like fermented milk products, cheese, bread as well as stored crops and feed such as hay and silage (Filtenborg et al., 1996; Schnurer and Magnusson, 2005) the risk of mycotoxins incorporate into food and feed are very high. Cereals, before being consumed as food, go through the processes of cultivation, harvesting, drying, preparation and marketing (including storage) all under natural conditions, and therefore, often involve microbiological contamination and infection. Between 5 and 10% of the world's food production is estimated to be lost due to fungal attack (Oerke and Dehne, 2004; Pitt and Hocking, 2009).

2. 5. 2. Control of food spoilage fungi

Control of spoilage fungi has been achieved through techniques like dehydration (freeze and heat drying), cold storage, heat and microwave treatment as well as ultrasound and irradiation which come under physical methods of food preservation (Farkas et al., 2007). The organic acids such as acetic, lactic, propionic, sorbic and benzoic acid, sodium benzoate and the antibiotic natamycin produced by *Streptomyces natalensis* are used as chemical food preservatives to manage fungal growth (Brul and Coote, 1999; Davidson, 2001).

Despite of the application of above methods to control fungal growth on food, different species of fungi prevail over those conditions leading to colonization and deterioration of food. A number of *Penicillium*, *Saccharomyces* and *Zygosaccharomyces* species can grow in the presence of potassium sorbate. Furthermore, *P. roqueforti* isolates have been found to be resistant to benzoate and some other moulds possess the ability to degrade sorbate (Davidson, 2001; Nielsen and De Boer, 2000). Some strains of the mould *P. discolor*, a species that causes spoilage of hard cheese were found to be natamycin resistant (Filtenborg et al., 1996; Schnurer and Magnusson, 2005). Yeasts like *Debaryomyces hansenii*, *Candida versatilis* and *Torulaspora delbrueckii*, have also shown strong resistance to chemical sanitizers and cleaning compounds in dairy environments (Bennie C, 2001). Frequent use of antibiotics and preservatives may increase the resistance phenomena in the future. Instead of chemical preservatives or additives in food or feed, consumers want high-quality, preservative-free, safe, and mildly processed food with extended shelf life.

LAB occur naturally in different food sources and have been used for centuries in food fermentation and became a part of human diet without any adverse health effects which procured them the 'GRAS' status. The different fermentation end products of LAB like organic acids, prteinaceous compounds and other low molecular weight compounds are capable of inhibiting spoilage fungal growth (Dalie et al., 2009). The long tradition of using LAB in food and feed along with recent scientific knowledge on encouraging health effects caused

by probiotic LAB ingestion in addition to the antifungal potential suggest them as perfect alternatives to chemical preservatives.

2. 5. 3. Antifungal LAB

LAB produce a variety of antifungal compounds, the synergistic action of which inhibit the growth of a broad range of fungi. Most of the published reports explain the antifungal activity of *Lactobacillus* species and studies on the antagonistic activities of *Lactococcus* species are very few. In a study, where *L. lactis* CLFP 101, *L. plantarum* CLFP 238, and *L. fermentum* CLFP 242 were studied for their efficiency to prevent adhesion of fish pathogens *in vitro*, it was found that only *Lactococcus* could inhibit all the tested pathogens (Balcazar et al., 2008). Oranusi et al. (2013) reported the antifungal activity of *L. Lactis* against various *Aspergillus* spp., *Penicillium*, *Mucor* and *Rhizopus*. Growth inhibitory action of *Lactobacillus*, *Enterococcus* and *Leuconostoc* cultures were reported against varying fungal groups such as *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Penicillium* sp. (Voulgari et al., 2010). Application of *L. plantarum* IMAU10014 against *Botrytis cinerea*, *Glomerella cingulate*, *Phytophthora drechsleri Tucker*, *P. citrinum* and *Fusarium oxysporum* (Wang et al., 2012) as well as the use of *Lactobacillus* and *Weisella* against *A. niger* MUCL 28699, *Candida albicans* MUCL 30112, *A. tubingensis* MP1 and *P. crustosum* MY1 (Ndagano et al., 2011) have been reported. The culture filtrate (CF) of *L. fermentum*, *P. pentosaceus*, *L. pentosus* and *L. paracasei* delayed the growth of fungi for 23 to 40 days at 4 ^{o}C and 5 to 6 days at 20 and 30 ^{o}C in tomato puree, 19 to 29 days at 4 ^{o}C and 6 to 12 days at 20 and 30 ^{o}C in processed cheese and 27 to 30 days at 4 ^{o}C and 12 to 24 days at 20 and 30 ^{o}C in commercial bread (Muhialdin et al., 2011). *Lactobacillus* also reported to have strong inhibitory activity against the human pathogenic fungi *Microsporum canis*, *Microsporum gypseum* and *Epidermophyton floccosum* (Guo et al., 2013).

Several LAB species are reported to be able to bind aflatoxin B_1 which is classified by the International Agency for Research in Cancer as a class 1 human carcinogen and reduced the bioavailability in the medium (Gratz et al., 2005).

Studies conducted by El-Nezami et al. (El-Nezami et al., 2002a; El-Nezami et al., 2002b) showed the ability of *Lactobacillus* and *Propionibacterium* to remove *Fusarium* toxins, trichothecenes. The percentage of bound toxins varied between 18 - 93% and the removal of zearalenone and α-zearalenol by 38% and 46% respectively. The removal of mycotoxins by LAB pellet was assumed to be because of the binding of toxin to the bacterial cell wall components, most probably peptidoglycans (Lahtinen et al., 2004; Niderkorn et al., 2009).

2. 5. 4. Antifungal compounds produced by LAB

LAB produce antagonistic compounds that are able to control the growth of pathogenic bacteria as well as unwanted spoilage fungi and offer effective means for food preservation and safety. Antimicrobial activities of LAB have been ascribed to the production of antagonistic compounds like organic acids (Cabo et al., 2002; Lind et al., 2007) reuterin (Talarico et al., 1988), proteinaceous compounds (Magnusson and Schnurer, 2001) and cyclic dipeptides (Strom et al., 2002) and also competition for nutrients. Table.2.3 describes the antifungal compounds reported so far from various LAB strains.

2. 5. 4. 1. Organic acids

The major fermentative end product of LAB is lactic acid which reduces pH of the medium and has antibacterial activity. LAB have been reported to produce wide range of fungal growth inhibiting organic acids including acetic, caproic, formic, propionic, butyric, n-valeric acids and benzeneacetic acid (Corsetti et al., 1998, Wang et al., 2012) in addition to phenyllactic acid, 4-hydroxyphenyllactic acid (Lavermicocca et al., 2000). Palmitic acid, benzoic acid, salicylic acid and stearic acid were identified in our study as potential antifungal carboxylic acids (Varsha et al., 2014). Linoleic acid, oleic acid and pyroglutamic acid have been reported to have antifungal activity against *Aspergillus* by Sangmanee and Hongpattarakere (2014).

Table.2.3 Antifungal compounds reported from LAB

LAB isolate	**Compound**
L. reuteri	3HPA (reuterin)
L. sanfrancisensis CB1	Caproic acid, propionic acid, butyric acid, valeric acid, formic acid
*L. plantarum*VTT E78076	Benzoic acid, methylhydantoin, mevalonolactone, cyclo(Gly-L-Leu)
L. plantarum	Phenyllactic acid, 4-hydroxy-phenyllactic acid
*L. plantarum*MiLAB 393	3-Phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro), Hydroxy fatty acids
L. coryniformis Si3	peptide, phenyllactic acid, cyclo(Phe-Pro), cyclo(Phe-OH-Pro), reuterin
L. plantarum	Linoleic acid, oleic acid, pyroglutamic acid
Lactococcus BSN307	Palmitic acid, benzoic acid, salicylic acid, 3-PLA, stearic acid
L. plantarum HD1	5-oxododecanoic acid, 3-hydroxy decanoic acid, 3-hydroxy-5-dodecenoic acid
Weissella	2- hydroxy fatty acid, 2-hydroxy-4-methylpentanoic acid
L. plantarum strain LR/14	Antifungal peptides AMPs LR14
Lactobacillus	antifungal 3, 6-bis (2-methylpropyl)-2, 5-piperazinedione
Propionibacteria	cyclo(L-Phe-L-Pro), cyclo(L-Ile-L-Pro), antifungal peptides
L. casei	2,6-diphenyl-piperidine, and 5,10-diethoxy-2,3,7,8-tetrahydro-1*H*,6*H*-dipyrrolo[1,2-a;1′,2′-d]pyrazine
L. plantarum	2 propenyl ester

Lactobacillus	Hydrogen peroxide
Lactobacillus	Diacetyl

The hydroxyl fatty acids, 3-(R)-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecenoic acid, 3-(R)-hydroxydodecanoic acid and 3-(R)-hydroxytetradecanoic acid, from *L. plantarum* MiLAB 14 showed a very broad spectrum of antifungal activity against *Aspergillus*, *Penicillium*, *Kluyveromyces*, *Pichia* and *Rhodotorula* and their minimum inhibitory concentration (MIC) ranges between 10 and 100 µg/mL (Sjogren et al., 2003). Ryu et al. (2014) reported antifungal compounds from *L. plantarum* HD1 which were active against food and feed borne filamentous fungi and yeasts. These compounds were identified as 5-oxododecanoic acid, 3-hydroxy decanoic acid and 3-hydroxy-5-dodecenoic acid. Apart from 3- hydroxy fatty acids, 2- hydroxy fatty acid, 2-hydroxy-4-methylpentanoic acid produced by *Weissella* was reported by Ndagano et al. (2011). Propionic and sorbic acids are used for the preservation of different food products including bread preservation and benzoic acid for fruit preservation. Propionic and sorbic acids are allowed in bakery products up to concentrations of 3000 and 2000 ppm respectively and benzoic acid up to 1500 ppm (Suhr and Nielsen, 2004). Acetic acid is used in the food industry and has a food additive code E260 (UK food standards industry). Organic acids when included in animal feed have profound benefit in weanling piglets and have been observed to benefit poultry performance (Dibner and Buttin, 2002).

2. 5. 4. 2. Proteinaceous compounds

Antimicrobial peptides known as bacteriocins, are produced ribosomally by LAB include nisin, pediocin PA-1/AcH, lacticin 3147 and enterocin AS-48 or variacin, have considerable antibacterial effects and used in different food preservation strategies where the growth of bacteria have to be controlled (Galvez et al., 2007). Only a few reports are available about the production of antifungal proteinaceous compounds by LAB. A recent report explains the production and

purification of the antifungal peptides AMPs LR14 from *L. plantarum* strain LR/14 inhibited the growth of *Aspergillus niger*, *Rhizopus stolonifer*, *Mucor racemosus* and *Penicillium chrysogenum* (Gupta and Srivastava, 2014). Gourama and Bullerman (1997) reported that the antifungal activity of crude bacterial culture filtrate of *Lactobacillus casei pseudoplantarum* 371 against *A. flavus* was reduced after treatment with trypsin and α-chymotrypsin and concluded that the activity was due to a small peptide of less than 1 kDa. Another report explains growth inhibitory effect of a small peptide of about 3 kDa from *Lactobacillus coryniformis subsp.coryniformis* strain Si3 against moulds and yeast. The peptide was heat stable and completely inactivated by treatment with proteinase k (Magnusson and Schnurer, 2001). Short peptides with antifungal activity have been purified from bacteria, fungi, insects, mollusks, crabs, spiders, plants and mammals. These have evolved to act via a number of different mechanisms, and a single peptide is often capable of more than one mode of action, depending on the target cell type (van der Weerden et al., 2013). Peptide- WH produced by *Bacillus amyloliquefaciens* has been reported to achieve antifungal property through inhibition of glucan synthesis, apoptosis, caspase like activity and cytochrome release and showed growth inhibiting activity against *Rhizoctonia solani*, *Candida albicans* and *Fusarium oxysporum*. The peptide- PAF from *P. chrysogenum* causes hyperpolarization of plasma membrane, activation of ion channels, ROS and apoptosis and is active against *A. nidulans*, *A. fumigatus*, *A. niger* and *B. cinerea* (De Brucker et al., 2011).

Cyclic peptides are another type of proteinaceous compounds with antifungal activity produced by LAB. They include cyclo (Phe-Pro), cyclo (Phe-OH-Pro) by *L. plantarum* MiLAB 393, *P. pentosaceus*, *L. sakei* and *L. coryniformis* (Magnusson et al., 2003; Strom et al., 2002) and cyclo (Gly-L-Leu) from *L. plantarum* VTTE-78076 (Niku-Paavola et al., 1999). Several antifungal peptides, His-Pro-Leu-Pro-Leu, Phe-Leu-Pro-Tyr-Pro, Gly-Pro-Phe-Pro-Ile, Gly-Pro-Phe-Pro-Leu, Gly-Pro-Phe-Pro-Leu-Val, Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile and Val-Ala-Pro-Phe-Gly-Val-Ala-Val-Phe-Gly along with several antifungal

diketopiperazines (DKPs); cyclo (L-Leu-cis-4-OH-D-Pro), cyclo (L-Leu-trans-4-OH-L-Pro), cyclo (L-Phe-cis-4-OH-D-Pro), cyclo (L-Phe-trans-4-OH-L-Pro) and cyclo (L-Phe-L-Pro) isolated from different LAB strains and found to be antifungal against *Aspergillus, Rhodotorula* and *Ganoderma boninense* (Kwak et al., 2014; Lind et al., 2007; Sjogren, 2005). (Niku-Paavola et al., 1999) and Yang and Chang (2010) reported the production of antifungal 3, 6-bis (2-methylpropyl)-2, 5-piperazinedione from *Lactobacillus*.

Diketopiperazines or piperazinedione are cyclodipeptides, in which the two nitrogen atoms of a piperazine 6-membered ring are part of amide linkages and obtained by the condensation of two α-amino acids. They are commonly biosynthesized from amino acids and are considered to be secondary functional metabolites. They are not only bound in nature but are often produced as degradation products of polypeptides (Borthwick, 2012). The mode of action by which they exert biological effects varies greatly depending on the amino acid they possess. The cyclic peptide, PAI-1 is the main inhibitor of the serine proteases, urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA). Diketopiperazines cause microtubule depolymerisation which results in inhibition of cell cycle progression and can also act as structural analogues of enzyme substrates (Martins and Carvalho, 2007). In a study conducted by Musetti et al. (2007), infection of grapewine downy mildew was controlled by *Plasmapara viticola* by three diketopiperazines-cyclo (L-alanine-trans-4-hydroxy-L-proline), cyclo (L-leucine-trans-4-hydroxy-L-proline) and cyclo (L-phenylalanine-trans-4-hydroxy-L-proline). Here *P. viticola* exhibited marked structural changes such as abnormal vacuolization, accumulation of electron-dense material in the vacuoles and necrotic, collapsed or incompletely developed haustoria. The cyclic proline-containing dipeptide- cyclo (L-Arg-D-Pro) or CI -4, isolated from broth of a marine bacterium which is growth inhibitory in action against *C. albicans* is reported to be a chitinase inhibitor (Izumida et al., 1996) by structurally mimicking a reaction intermediate (Houston et al., 2002).

2. 5. 4. 3. Low molecular weight compounds

Fungal cell wall is rigid and the presence of chitin along with polysaccharides and sterols such as ergosterol make the penetration of drugs into the cell a difficult process. A number of low molecular weight compounds (< 1 kDa) produced by different LAB species are reported to possess considerable fungal growth inhibitory activity. Methylhydantoine (5-methyl-2, 4-imidazolidine dione) and Mevalonolactone (tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one) from *L. plantarum* were identified as antifungal against *Fusarium avenaceum* and *Pantoea agglomerans* by Niku-Paavola et al. (1999). Li et al. (2012) reported the production of 2,6-diphenyl-piperidine, and 5,10-diethoxy-2,3,7,8-tetrahydro-1*H*,6*H*-dipyrrolo[1,2-a;1′,2′-d]pyrazine by *L. casei*. Another compound, 2 propenyl ester from *L. plantarum* showed antifungal activity against various fungi (Wang et al., 2012).

Hydantoins are oxidized derivatives of imidazolidine and the antifungal activity of imidazole derivatives has been reported before (Kanagarajan et al., 2011; Tang et al., 2013). The imidazolidine derivatives act by inhibiting fatty acid amide hydrolase (FAAH) which is essential for hydrolyzing endogenous bioactive fatty acid derivatives (Muccioli et al., 2005) A study by Dos Santos Gomes et al. (2012), where imidazolidine derivatives checked against *Trypanosoma cruzi* revealed severe damage to the parasite's mitochondrial complex along with myelin bodies, enlargement of cytoplasm vacuole, fragmentation of endoplasmic reticulum, and some treated samples clearly showed signs of necrosis. The metabolic pathway that lead to the production of this compound is not elucidated in LAB. Lactones are esters of hydroxycarboxylic acids. Mevalonolactone exists in equilibrium with mevalonic acid, where, later is the main intermediate in mevalonate pathway which serves as the basis biosynthetic pathway for the production of sterols and isoprenoid. Exogenous mevalonolactone acts by reducing HMG CoA reductase enzyme activity and thus impairs cholesterol synthesis pathway (Houten et al., 2003). It is reported to have antibacterial effect against *Staphylococcus epidermidis* (Scopel et al., 2014).

Reuterin or 3-hydroxypropionaldehyde is one of the mostly studied antimicrobial compounds produced by *L. reuteri* when glycerol was added into the growth medium. It is produced from glycerol by starving cells under anaerobic conditions and exists as an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-hydroxy propionaldehyde (Talarico et al., 1988). This antifungal compound which is active against *Aspergillus* and *Fusarium* is postulated to be able to suppress ribonuclease activity in the target organism and block DNA synthesis but not scientifically proved (Chung et al., 1989; Dalie et al., 2009). Schaefer et al. (2010) described that reuterin induces oxidative stress in cells most likely by modifying thiol groups in proteins and small molecules which indicates that reuterin negatively affects a large number of cellular targets.

Diacetyl is another antifungal compound reported from LAB. It is produced by oxidative decarboxylation of the metabolic intermediate α-acetolactate (α- AL). α- AL is mainly produced by LAB as a result of the metabolism of citric acid (Hugenholtz et al., 2000; Oberman et al., 1982). Diacetyl is more effective in growth inhibition against gram-negative bacteria, yeasts, and molds than against gram-positive bacteria (Jay, 1982). It inhibits the growth of gram negative bacteria by antagonizing arginine utilization by reacting with arginine binding proteins. Gram positive bacteria are more resistant due to their lack of similar periplasmic binding proteins and their possession of larger amino acid pool (Jay, 1986). Combined antimicrobial activity of reuterin and diacetyl against foodborne pathogens were studied and found to be effective against *E.coli* O157:H7, *Salmonella* Enteritidis, and *Listeria monocytogenes* (Langa et al., 2014). 2- propenyl ester purified from *L. plantarum* showed antifungal activity against *Glomerella, Botrytis*, *Penicillium* and *Fusarium* species (Wang et al., 2012). Hydrogen peroxide is another antimicrobial compound produced by many strains of LAB in the presence of oxygen (Boateng et al., 2011; Ito et al., 2003). Since LAB do not produce catalase it accumulates in the

environment and cause chemical oxidation of cellular components of bacteria (Finnegan et al., 2010).

2-butyl-4-hexyloctahydro-1H-indene and 2,4-di-*tert*-butylphenol have been identified in the culture filtrate of *L. plantarum* which inhibited the growth of *A. niger* and *A. parasiticus* (Sangmanee and Hongpattarakere, 2014). In our study, the volatile organic compound (VOC), 2,4- di-*tert*- butyl phenol (2,4 DTBP) was purified from the culture supernatant of *Lactococcus* sp. This compound has shown complete growth inhibition of *Aspergillus*, *Penicillium* and *Fusarium* and possesses antioxidant properties along with anti-cancer potential (Varsha et al., 2015). 2,4 DTBP is present in fruits as well as seeds and contributes to their antioxidant properties (Choi et al., 2013). *In vitro* antimalarial activity of this compound was reported by Kusch et al. (2011). A summary of different group of antifungal compounds produced by LAB and their mode of action is provided in Fig.2.4.

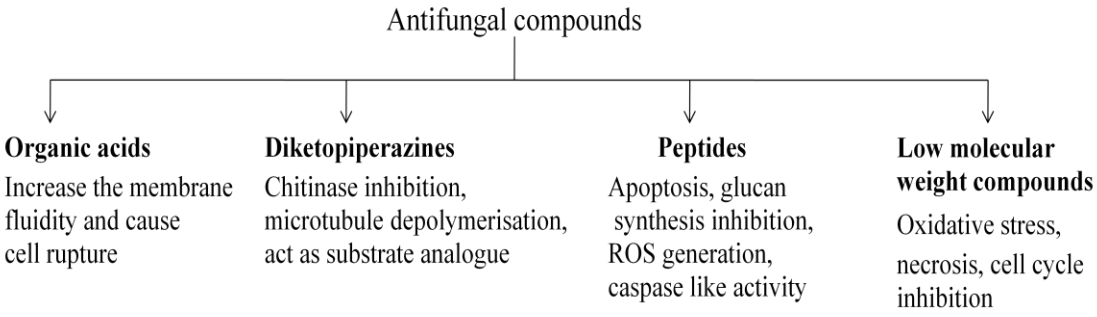


Fig.2.4. Antifungal compounds and the mode of action

2. 6. Purification and identification of antifungal compounds

Solid phase extraction (SPE) and high pressure liquid chromatography (HPLC) are the most commonly used methods for purification of hydrophobic low molecular weight antifungal compounds from LAB. For purification of cyclo(L-Phe–L-Pro), cyclo(L-Phe–trans-4-OH-L-Pro) and phenyllactic acid from *L. plantarum* strain MiLAB 393 and *L. coryniformis* strain Si3, (Magnusson et al.,

2003). Strom et al. (2002) fractionated the supernatant on a C18 SPE column and the 95% aqueous acetonitrile fraction was further separated on a preparative HPLC C18 column. The fractions collected were subjected to antifungal activity assay and antifungal compounds were identified by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). This bioassay guided fractionation method was followed for fractionation of 3- hydroxyfatty acids by Sjogren et al. (2003). 3-hydroxydodecanoic acid and 3-hydroxytetradecanoic acid were detected in cell-free supernatant by GC-MS and 3-hydroxydecanoic acid and 3-hy-droxy-5-cis-dodecenoic acid were identified by NMR, EI-MS and HPLC. Ryu et al. (2014) identified 3-hydroxy-5-dodecenoic acid from *L. plantarum* by this two step purification method. Niku-Paavola et al. (1999) fractionated the filtered culture supernatant of *L. plantarum* by gel chromatography. The fractionation was carried out in a Sephadex G- 10 column with distilled water and antimicrobial activity was checked by turbidometry. The fractions were subjected to GC-MS and also monitored at 280 nm for organic acids by HPLC. Identity of the compounds was confirmed by procuring commercially available standards and comparing with the identified compounds by HPLC and GC-MS.

Lavermicocca et al. (2000) extracted the cell free supernatant (CFS) four times with ethyl acetate and concentrated under reduced pressure to get the crude residue. The crude residue (100 mg) was partially purified by preparative TLC (Merck; 60 F_{254}, 0.5mm) and the different fractions were dissolved in chloroform-methanol. GC- MS analysis and comparison with the commercially available standards by HPLC were employed to identify the compounds as phenyllactic and 4-hydroxy-phenyllactic acids. Wang et al. (2012) followed a different strategy for purification of antifungal compounds from LAB. Here, the supernatant was concentrated through freeze- drying and made up to 100 mL with distilled water. Then 20 mL portions were extracted three times using petroleum ether, cyclohexane, dichloromethane, ethyl acetate or n-butanol as extraction solvent (1:3 v/v) and checked for antifungal activity. The active fraction was then subjected to silica gel column chromatography and eluted using a mixture of

petroleum ether and anhydrous alcohol (9:1 and 4:1, v/v) in a stepwise manner. In the third stage of purification, the active fraction was purified by preparative RP-HPLC with a gradient of acetonitrile and water with 0.1% (v/v) trifluro acetic acid (TFA). The antifungal compounds were characterized by LC-MS, GC-MS and NMR analysis. The compounds identified by this method include 3- phenyllactic acid, benzeneacetic acid and 2-propenyl ester. Another approach by Li et al. (2012) include ultrafiltration of the freeze dried, 15 fold concentrated culture filtrate (CCF) of *L. casei* by 10, 3 and 1 kDa membrane gradually with an ultrafiltration system. The ultrafiltered CCF was further separated using semi preparative HPLC (Rs-5u-C18 column) with a linear gradient of methanol/ 0.05% (v/v) TFA (solvent A) and water/0.05% TFA (solvent B) as mobile phase. Identification of the compounds were performed by GC-MS and the main antifungal compounds identified include ctclo-(Leu-Pro); 5,10-Diethoxy-2,3,7,8-tetrahydro-1*H*, 6*H*-dipyrrolo[1,2-a;1`,2`-d]pyrazine; and 2,6-diphenyl-piperidine. In our study we purified phenolic compounds and organic acids from *Lactococcus* BSN307 CFS by solvent extraction and Prep-HPLC methods.

Only two reports explain the purification of antifungal proteins from LAB. A proteinaceous compound of about 3 kDa from the CFS of *L. coryniformis* subsp. *coryniformis* Si3 was partially purified by Magnusson and Schnurer (2001). Freeze dried, 15 fold concentrated CFS was first purified by ion-exchange chromatography using Q-Sepharose with 20 mM citrate-phosphate buffer (pH 5.0) followed by precipitation with 60, 80 and 100% (w/v) ammonium sulfate saturations. The precipitates were dialyzed against 20 mM citrate-phosphate buffer (pH 5.0) and antifungal activity was evaluated with microtiter plate assay. The dissolved pellets from the 80 and 100% (w/v) $(NH_4)_2SO_4$ saturations were pooled and run on a gel filtration column (Superdex Peptide PC 3.2/30) using the SMART chromatography system (Pharmacia, Uppsala, Sweden) with 10 mM acetic acid as buffer and different fractions were checked for antifungal activity. Although they could determine the size, amino acid sequencing of this small protein failed. Antifungal peptides, AMPs LR14 from *L. plantarum* were purified

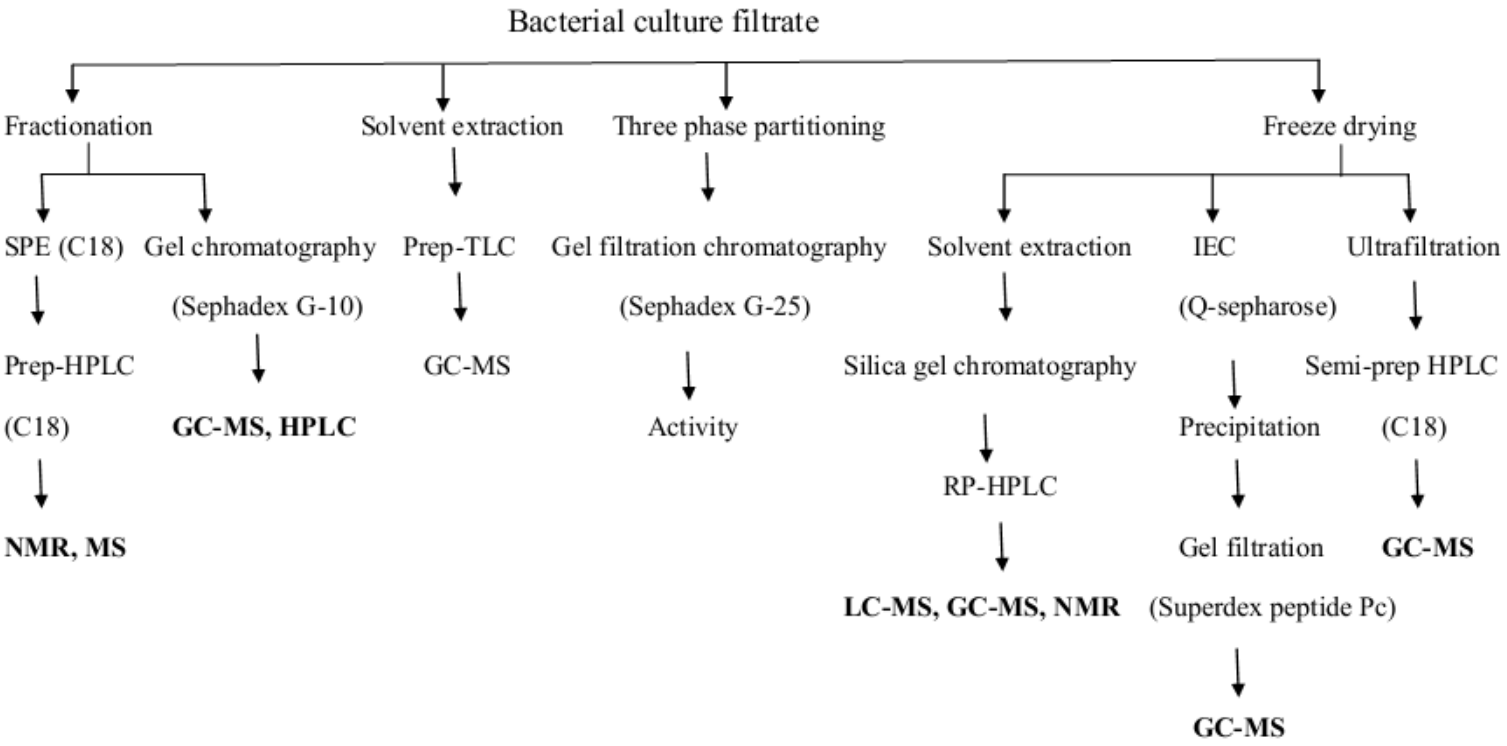


Fig.2.5. Purification methods of antifungal compounds from LAB

by Gupta and Srivastava (2014). Here proteins were precipitated by three-phase partitioning using ammonium sulfate and tertiary butanol and the proteins on the interfacial layer was separated and washed using sterile distilled water and further subjected to gel-filtration chromatography using Sephadex G-25 desalting columns (GE-Healthcare Bio-Sciences, USA). A summary of different methods used for purification of antifungal compounds from LAB is given in Fig.2.5.

2. 7. Application of LAB as protective culture

Fermentation is a long age process used to improve the quality and shelf life of food products and the secret of their extended shelf life is the presence of antimicrobial LAB, which are natural inhabitants of fermented foods. The presence and function of different LAB strains in fermented foods like rice- wine/ beer- takju, tapuy; acid leavened bread/ noodle- idli, puto, khanomjeen; fermented vegetable- kimchi, dhamuoi, burong; fermented fish and meat- sikhae, narezushi, nham are well described (Rhee et al., 2011). The biologically active compounds in kimchi include benzylisothiocyanate, indol compounds, thiocyanate and sistosterol which are antibiotic, anticarcinogenic, immunestimulant and cholesterol reducing in activity respectively (Rhee et al., 2011).The viability of LAB and *Bifidobacterium* in fermented soymilk after drying, subsequent rehydration and storage was studied by Wang et al. (2004) and found that in the freeze-dried fermented soymilk after 4 months of storage at 4 ^{o}C in laminated pouch, the survival of *S. thermophilus* and *B. longum* were 51.1% and 68.8%, respectively. Meanwhile, *S. thermophilus* and *B. infantis* in the spray-dried fermented soymilk showed a survival percent of 29.5% and 57.7%, respectively and after freeze-drying, lactic acid bacteria and bifidobacteria exhibited a survival percent of 46.2–75.1% and 43.2–51.9% respectively.

Studies have been conducted regarding the application of LAB as biopreservative to control mold/fungal spoilage of bread. Ryan et al. (2008) used *L. plantarum* for dough fermentation and the resulted bread significantly reduced the outgrowth of *A. niger*, *F. culmorum* and *P. expansum* spores. In another study they used antifungal *L. amylovorus* which has amylase activity, for sourdough

fermentation and found a positive effect in decreasing the staling rate during storage (Ryan et al., 2011). The use of 20% (w/w) sourdough fermented with *L. hammesii* in breadmaking increased the mold-free shelf life by 2 to 3 days and it was concluded that the antifungal activity of bread is due to the conversion of linoleic acid to monohydroxy octadecenoic acid by *L. hammesii* (Black et al., 2013). A study by Gerez et al. (2009) showed that the inclusion of antifungal LAB strains during bread manufacturing permitted 50% reduction in the concentration of calcium propionate (CP) while still attaining a shelf life equal to traditional bread containing 0.4% (w/v) CP. Bread produced through sourdough fermentation with *L. plantarum* also extended the shelf life under common storage conditions (Coda et al., 2011; Lavermicocca et al., 2000). LAB was effectively used to control the growth of *Listeria monocytogenes* and *salmonella enteritidis* in chicken meat (Maragkoudakis et al., 2009) and growth of *S. typhimurium*, *E. coli* and *L. monocytogenes* in iceberg lettuce and golden delicious apples (Randazzo et al., 2009; Trias et al., 2008). Citrus fungal pathogens *Penicillium digitatum* and *Geotrichum citriaurantii* were efficiently inhibited by LAB (Gerez et al., 2010). Production of the antagonistic compounds by *L. plantarum* in grass silage was investigated, that led to the possibility of using LAB for silage preservation efficiently (Broberg et al., 2007).

Biopreservation ability of different LAB strains on rice cake, fruits, vegetable and in malting of barley were showed by different research groups (Baek et al., 2012; Crowley et al., 2013; Laitila et al., 2002; Sathe et al., 2007). LAB strains inoculated into cottage cheese prevented the growth of *P. commune* by between 14 - 25 days longer than cottage cheese that contained either no LAB or LAB that did not have antifungal activity (Cheong et al., 2014). The *L. reuteri* R29 CFS, produced with barley based malt extract (wort) as substrate represents a practical and inexpensive antifungal alternative to the malt industry. *L. reuteri* R29 CFS using a 3 ^{0}P wort substrate inhibited the germination of *F. culmorum* spores and reduced the mycotoxin accumulation in the final malt grains (Oliveira et al., 2014a; Oliveira et al., 2014b; Oliveira et al., 2014c). In a study, Abdel-

Wahhab et al. (2011) found that *L. reuteri* and *L. casei* protected against oxidative stress in rats fed aflatoxins-contaminated diet.

So far, nisin is the only bacteriocin licensed as a food preservative (E234). Many preliminary studies on the activity of bacteriocins *in vitro* or in food systems are carried out with partially-purified preparations obtained from cultured broths (Galvez et al., 2007). Bacteriocin producing strain of *Lactobacillus* has been effectively used for the preservation of refrigerated and vacuum-packed *Dicentrarchus labrax* which prevented total volatile basic nitrogen contents and trimethylamine to some extent (El Bassi et al., 2009). *Enterococcus* and *Pediococcus* were used for preservation of vaccum packaged cold-smoked salmon (Tome et al., 2008). Enterocin AS-48 was found to reduce the number of *L. monocytogens* in artificially contaminated alfalfa and soybean sprouts as well as active against *Bacillus* contamination in rice, canned fruits and vegetables (Grande et al., 2006; Lucas et al., 2006; Molinos et al., 2005). *E. raffinosus* PS99 and *L. reuteri* TA43 could lower the counts of coliforms, *Pseudomanas* spp., proteolytic and haemolytic bacteria in blood while preservation (Davilla et al., 2006). In a study conducted by Einarsson and Lauzon (1995), use of bavaricin A and nisin Z extended the shelf life of brined shrimp by 16 and 31 days respectively. If LAB, in case cause spoilage of food, it can be controlled by the application of other strains of antimicrobial LAB. *Leuconostoc gelidum* UAL187 was successfully applied to control the growth of beef spoiling, sulfide producing *L. sakei* strain during anaerobic storage at 2 ^{o}C (Leisner et al., 1996). Gupta and Srivastava (2014) used partially purified AMPs LR14 produced by *L. plantarum* strain LR/14 for whole wheat grain preservation against *A. niger*, *R. stolonifer*, *Mucor racemosus* and *P. chrysogenum*. Treatment of wheat seeds with AMPs LR14 prevented fungal growth for a period of~ 2.5 years under laboratory conditions. Table.2.4 provides names of some commercially available LAB protective cultures.

Table.2.4 Commercially available LAB protective cultures

Protective culture	**Field of application**	**Producer**
FreshQ	Fermented dairy products	Chr.Hansen (Denmark)
SafePro ImPorous	Meat products	Chr.Hansen (Denmark)
Bactoferm	Meat starter culture	Chr.Hansen (Denmark)
HOLDBAC	Fermented foods and cheese	Dupont (USA)
Lyofast	Fermented milk products and cheese	Sacco (Italy)
Dairy Safe	Cheese manufacture	CSK (Netherlands)

The commercially available Feedtech Silage F3000 is a combination of four silage bacteria including antifungal *L. plantarum* Milab 393 along with *P. acidilactici*, *E. faecium* and *L. lactis*. When added to the forage it promotes the growth of useful bacteria and controls the growth of yeast, fungi and clostridia. Microgard, a commercial food additive extracted from *Propionibacterium freudenreichii* can be termed as an example of protective culture. It is marketed as a food preservative for use in cottage cheese and fruit flavored yogurt where the shelf life of cottage cheese was extended 6 to 9 days (Lund et al., 2000).

2. 8. Conclusion

A thorough review of present topic shows the possibility of developing new antifungal LAB strains and the antifungal compounds produced by them as potential biopreservatives. Use of probiotic LAB as protective culture increases the nutritional value of food and brings about health benefits. A wide range of antifungal compounds are detected in cell free supernatants of various LAB strains and many of them possess antioxidant and anticancer properties. Some of the low molecular compounds identified are reported to play major roles in

various metabolic activities. More reports are coming out on such bioactives detected in LAB fermentation medium. One of the obstacles about the use of such compounds in food systems is the production of very low amounts of such compounds and their separation from the culture medium. Screening of more potential strains and modification of the culture medium along with efficient purification techniques are expected to solve the problem to an extent.

CHAPTER 3

Materials and Methods

3. 1. Materials

3. 1. 1. Chemicals and reagents

Chemicals like potassium ferricyanide, DPPH (1,1-diphenyl-2-picryl-hydrazyl), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), Dulbecco's Modified Eagle's Medium (DMEM), Eagles Minimum Essential Medium (MEM), trichloroacetic acid, streptomycin, penicillin, amphotericin B, ammonium molybdate and ascorbic acid for antioxidant and cytotoxicity activity assays were procured from Sigma Chemicals (India). Molecular biology grade chemicals including lysozyme, primers, agarose and ethidium bromide for DNA extraction, PCR reaction and gel electrophoresis were purchased from Sigma Chemicals (India). Enzymes and markers for molecular biology were obtained from Fermentas Inc., (USA) and Bangalore Genei Pvt Ltd (India). Organic acid standards like lactic acid, acetic acid, propionic acid, butyric acid, phenyllactic acid, caproic acid, palmitic acid, benzoic acid and chemicals for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis such as SDS, acrylamide, bis-acrylamide, urea, tricine, ammonium per sulfate, TEMED (N, N, N′, N′-Tetramethylethylenediamine) were procured from Sigma Chemicals (India). XAD-16 and SP-sepharose used for protein purification were obtained from Sigma Chemicals (India). Bicinchoninic acid (BCA) assay kit for protein estimation and red dye PCR master mix were procured from Merck (USA).

The HPLC columns including prep-C18, prep-silica and C18 analytical columns used for purification and analysis purpose were obtained from Phenomenex (USA). Tissue culture plates and other plastic wares were from Costar (Corning, New York, USA), Nunc (Thermo Fisher scientific, USA) and Himedia (India).

Strips and reagents for antibiotic sensitivity and biochemical characterization were obtained from Hi-media Laboratories. Antibiotics gentamycin, penicillin and streptomycin were also from Hi-media Laboratories. Chemicals like ammonium sulphate, ammonium nitrate, ammonium chloride, ammonium citrate, sodium nitrite, potassium nitrate, sodium sulfate, sodium chloride,sodium acetate, di-potassium hydrogen phosphate, potassium di hydrogen phosphate, di-sodium hydrogen phosphate, sodium di hydrogen phosphate magnesium sulphate, ferric chloride, manganese sulphate, tween 80, glycerol, NaOH, mucin, n-hexadecane, HCl, H_2SO_4, TCA and silica gel for column chromatography were purchased from Hi-media Laboratories (India), Merck (India) and SRL Pvt. Ltd. Analytical and HPLC gradient solvents like chloroform, ethyl acetate, acetonitrile, ethanol, methanol and chloroform-D1 for Nuclear magnetic Resonance (NMR) spectroscopy were obtained from Merck (India). The stains used for microscopy such as crystal violet and safranin were procured from Fischer scientific chemicals (Mumbai, India).

3. 1. 2. Microorganisms, cell lines and culture media

The fungal strains, *Aspergillus niger* (KACC 42589), *Penicillium chrysogenum* (NII 08137), *Fusarium oxysporum* (KACC 42109), *Fusarium moniliforme* (KACC 08141), *Fusarium graminearum* (MTCC 1893), *Fusarium chlamydosporum* (MTCC 2399), *Listeria monocytogenes* (MTCC) and *Lactococcus garvieae* (CCUG 32208) were obtained from different culture collections. Strains of *Escherischia coli*, *Serratia marcescens* and *Staphylococcus aureus* (isolates from NIIST) were maintained on nutrient agar at 30 ^{o}C. LAB isolates *Pediococcus pentosaceus* (TG2), *Lactobacillus casei* (DY2) and *Lactococcus garvieae* subsp. *gaurensis* (BSN307) were used for this study.

Human colorectal adenocarcinoma cells HT-29 was obtained from NCCS, Pune. Mammalian cell lines, H9c2 (myoblast cell line), HeLa (cervical adenocarcinoma cell line) and MCF-7 (breast carcinoma cell line) were obtained from ATCC.

Culture media like MRS (de Man Rogosa Sharpe) broth, MRS agar, nutrient broth, tryptic soy broth, brain heart infusion broth, arginine dehydrolase media, YEME (yeast extract malt extract) medium, agar, PDB (potato dextrose broth), PDA and media components like glucose, lactose, sucrose, galactose, yeast extract, beef extract, tryptone were purchased from Hi-media Laboratories.

3. 2. General instruments

The major Instruments used for experiments are listed in **Annexure I1**

3. 3. General methods

3. 3. 1. Methods in general microbiology

3. 3. 1. 1. Gram staining

The bacterial cells were heat fixed on a slide and flooded with crystal violet and allowed to remain for one minute. The crystal violet was rinsed off in running tap water followed by addition of iodine solution and incubation for one minute. After washing off the iodine in running tap water, absolute alcohol was added on the smear and kept for two seconds. The smear was again washed in tap water and flooded with safranin. After thirty seconds, safranin was cleared off in running tap water and the smear was dried followed by microscopical examination.

3.3.1. 2. Microorganisms and cell lines maintenance

All the lactic acid bacteria (LAB) isolates were grown on MRS (de Man Rogosa Sharpe) medium at 30 ^{o}C and the fungal cultures were grown in potato dextrose medium at 30 ^{o}C. They were stored at 4 ^{o}C for immediate use and sub-cultured every two weeks. For long time preservation they were maintained in 20% (w/v) glycerol stock and stored at -80 ^{o}C. Bacterial isolates used for antimicrobial study were maintained on nutrient agar at the above mentioned conditions. HT-29 cells were maintained in Eagles Minimum Essential Medium (MEM), supplemented with 10% (w/v) fetal bovine serum (FBS), 100 mg/L streptomycin, 100 U/L penicillin and 50 µg/mL gentamycin. H9c2, HeLa and MCF-7 were maintained in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% (w/v) FBS, 10 mg/L streptomycin, 100 U/L penicillin

and 25 µg/mL amphotericin B. Compositions of media used are given in **Annexure I**.

3. 3. 1. 3. Inoculum preparation

To prepare the inoculum of LAB, a single colony of bacterium from 24h old plate was inoculated into 50 mL MRS broth in 250 mL Erlenmeyer flask and incubated at 30 ^{o}C for 24 h under static condition. The culture absorbance was noted at 600 nm and unless specified, 10^9 CFU/mL culture was used as inoculum.

3. 3. 4. Analytical methods

3. 3. 4. 1. Bicinchoninic acid (BCA) assay for protein estimation

The BCA protein assay (Smith et al., 1985) is based on a biuret reaction, the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline solution and a concentration-dependent detection of the monovalent copper ions produced. Bicinchoninic acid is a chromogenic reagent that chelates with the reduced copper, producing a purple reaction complex with strong absorbance at 562 nm.

BCA kit was purchased from Merck (USA). Components used for the assay are BCA solution (BCA, sodium carbonate, sodium tatrate and sodium bicarbonate in 0.1 M NaOH, pH 11.25), 4% (w/v) cupric sulfate and bovine serum albumin (BSA) as standard. Working reagent was prepared by mixing 50 parts BCA solution with 1 part of 4% (w/v) cupric sulfate. Standard graph is presented in Fig.3.1.

3. 3. 4. 2. Thin layer chromataography

The sample was added as spot or band on the TLC plate and the plate was placed in the TLC chamber (CAMAG, Switzerland) which was previously saturated with the solvent (chloroform: methanol: acetic acid in 9: 1: 0.1 ratio). The plate was developed in the solvent and dried. The dried plates were visualized under TLC scanner (CAMAG, Switzerland).

3. 3. 5. Molecular methods

3. 3. 5. 1. Extraction of genomic DNA

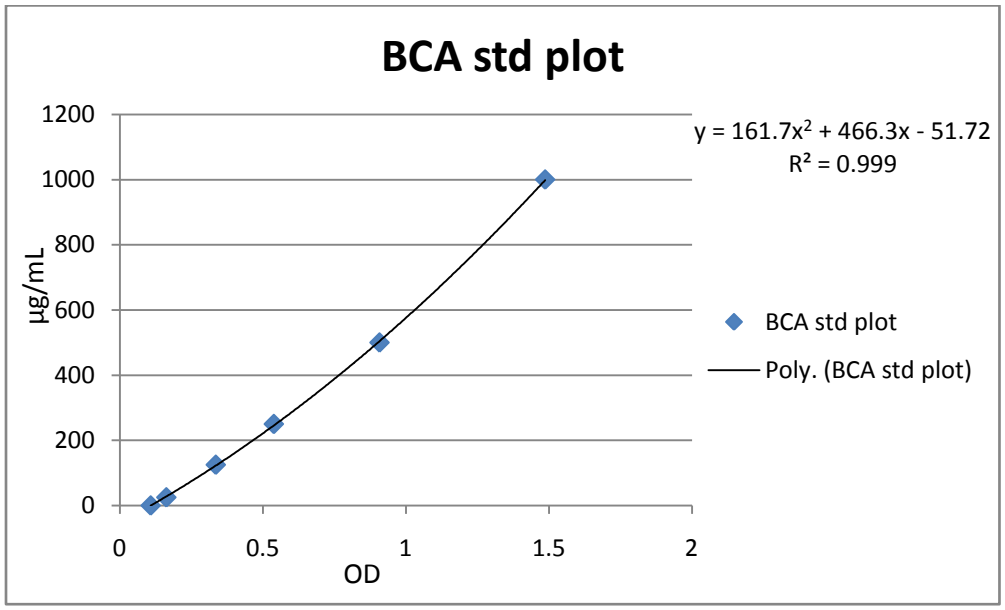


Fig.3.1. Standard graph of BSA for protein estimation

Total DNA was extracted from culture (2 mL) harvested in the mid-log phase (OD 600 of 0.5-1), employing a modified method of Savadogo et al. (2004). Cells were collected by centrifugation (3000×g, 10 min) and frozen for at least 1 h at -20 °C. The thawed pellet was washed in 500 µL TES buffer (6.7% (w/v) sucrose, 50 mM Tris-HCl, 1mM EDTA, pH 8.0) and re-suspended in 150 µL STET buffer (8% (w/v) sucrose, 5% (w/v) Triton X-100, 50 mM Tris-HCl, 50 mM EDTA, pH 8.0). 20 µL lysozyme (TES containing 40 mg/mL lysosyme) was added and the suspension was incubated at 37 °C for 1 h. After addition of 20 µL preheated (37 °C) 20 % (w/v)SDS in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0), cells were vortexed for 60 sec and incubated at 37 °C for 10 min, followed by 10 min incubation at 65 °C. 20 µL 5 M NaCl and 100 µL TE buffer were added and the lysate was extracted with 1 volume phenol/chloroform/isoamyl alcohol (49:49:1). Aqueous phase was separated by centrifugation (18000×g, 5 min) using phase lock gel tubes (Eppendorf, Germany). Double volume ethanol (70%, v/v) was added to the aqueous phase and kept at -20 ^{o}C for 1 h.The DNA was collected by centrifugation (20000×g, 30 min) and the pellet washed in ice-cold 70% (v/v) ethanol. Finally, DNA was dried

and re-suspended in 20 µL TE. Genomic DNA was analyzed by electrophoresis in 1 % (w/v) agarose gels in 1X Tris-acetate- EDTA buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA)

3. 3. 5. 2. Determination of 16S rRNA gene sequence

Polymerase Chain Reaction (PCR) was performed in mixtures (50 µL) containing 50 ng of DNA, 1.5 mM $MgCl_2$, the four deoxynucleoside triphosphates at 150 µM each, each primer (27 F 5′ AGAGTTTGATCCTGGCTCAG 3′; 1492 R ′ TACGGTTACCTTGTTACACTT 3′) at 10 pM in Taq buffer, and 0.5 U of Taq polymerase. The PCR reactions were performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) by following the programs as described. It consisted of 94 °C for 4 min, then 35 cycles at 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min and finally, one 72 °C cycle for 7 min. Amplicons were analyzed by electrophoresis in 1% (w/v) agarose gels in 1X Tris-acetate- EDTA buffer. 1 kb DNA ladder (Fermentas) was used to identify the molecular sizes of the bands. Sequencing of 16S rRNA gene was performed by ABI 3500 (Applied Biosystems, USA) genetic analyzer according to the instructions of manufacturer.

3. 3. 5. 3. Electrophoretic techniques

3. 3. 5. 3. 1. Agarose gel electrophoresis

DNA are separated by applying an electric field to move the negatively charged molecules through an agarose matrix in which shorter molecules move faster and migrate farther than longer ones through the pores of the gel. The yield and purity of genomic DNA, estimation of size of DNA molecules, analysis of PCR products etc were done using agarose (0.8-1.6%) gel electrophoresis with ethidium bromide to view DNA under UV light. Tris-acetate-EDTA (TAE) buffer (1X, 40 mM Tris-acetate and 1 mM EDTA, pH 8.3) was used as running buffer for agarose gel electrophoresis.

3. 3. 5. 3. 2. Tricine-SDS-PAGE

The protocol of Schagger (2006) was used for SDS-PAGE analysis of peptide. Tricine –SDS-PAGE is the preferred electrophoretic system for the

resolution of proteins smaller than 30 kDa. The concentration of acrylamide in the gel is lower than in other electrophoretic systems and this facilitates electroblotting, which is particularly important for hydrophobic proteins.

Reagent setup

Acrylamide-bisacrylamide stock solution (AB-6): 46.5 g acrylamide and 3 g bisacrylamide in 100 mL distilled water.

Sample buffer: 2% SDS (w/v), 30% glycerol (w/v), 0.05% Coomassie blue G-250, 150 mM Tris/HCL (pH 7), 8 M urea.

Compositions of anode buffer, cathode buffer and gel buffer are given in Table.3.1.

Table.3.1 Electrode and gel buffers for Tricine-SDS-PAGE

	Anode buffer (10X)	Cathode buffer (10X)	Gel buffer (3X)
Tris (M)	1	1	3
Tricine (M)	-	1	-
HCL (M)	0.225	-	1
SDS (%)	-	1	0.3
pH	8.9	~8.25	8.45

The sample was treated with non-reducing buffer for 20 min at 37 ^{o}C and was analyzed by Tris-tricine SDS-PAGE using 4% stacking gel and 16% separating gel with 6 M urea (Table.3.2). Initial voltage used was 30 V (for 30 min) and then 90 V constant. After run the gel was fixed in fixing solution (50% (v/v) methanol and 10% (v/v) acetic acid).

3. 3. 5. 3. 2a. Coomassie staining

The gel was stained with 0.05% (w/v) Coomassie dye in 10% acetic acid for 1 h and destained the gel twice in 10% acetic acid and transferred to water.

Table.3.2 Running gel composition

	4% sample gel	16%/ 6M urea
AB-6 (mL)	1	10
3X gel buffer (mL)	3	10
Urea (g)	-	10.8
Water to final volume (mL)	12	30
Polymerize by adding		
10% APS (µL)	90	100
TEMED (µL)	9	10

3. 3. 5. 3. 2b. Silver staining

Silver staining was performed by modifying the protocol of Schagger (2006). The gel after run was fixed in acetic acid: methanol: water (5: 45: 50) for 30 min and in methanol for 15 min. After washing five times (each run for two min) in water, the gel was sensitized with 0.02% (w/v) sodium thiosulphate for 6 min followed by incubating the gel in 0.2% (w/v) silver nitrate for 30 min. Following washing, the gel was developed in 3% (w/v) sodium carbonate with 0.036% (v/v) formaldehyde and the activity was stopped by treating with 1.4% (w/v) EDTA for 10 min.

3. 4. Conclusion

The chapter explains the general materials and methods used in this study. References are provided for all the standard protocols. Details of specific methods are discussed in respective chapters.

CHAPTER 4

Isolation and probiotic characterization of antifungal lactic acid bacteria

4. 1. Introduction

Lactic acid bacteria (LAB) ferment glucose primarily into lactic acid or lactic acid, CO_2 and ethanol. Although they lack catalase enzyme they possess superoxide dismutase and peroxidase enzymes to detoxify the peroxide radicals (Axelsson, 2004). They found in many habitats like dairy products, milk, fruits, vegetables and decaying substances. LAB are free living or found in association with animals as normal flora of oral cavity, intestinal tract and vagina where they impart beneficial effects. As probiotics, the health benefits provided by them are gaining huge attention from public and an important area of research. The health advantages they bring include lowering cholesterol and obesity, immunoregulation and improved bowel function. Because of their ability to produce different vitamins, antioxidants and sugar alcohols, they are used as nutraceuticals in functional food industry (Hugenholtz et al., 2002). Commercially, probiotic LAB strains are available as capsule or in the form of fermented food.

They require nutrient rich medium to grow and are the most important group of organisms used in fermentation industry. The antimicrobial compounds produced by them help to prevent bacterial and yeast infections and also used for food preservation because of their antifungal potential (Schnurer and Magnusson, 2005). Due to their ability to add flavor and texture to food along with the ability to control food spoilage microbes they are used in making cultured butter, sour cream, yogurt, cheese, sausage, cucumber pickles, olives and sauerkraut.

LAB fulfill the criteria that have to be met by the organisms to be selected as probiotics like resistance to the enzymes in the oral cavity, survival through the GI tract, arrival at the site of action in a viable physiological state and adherence

to the host cell surface (Otero et al., 2004). The possible mechanisms of action of probiotics include production of antimicrobial compounds, competitive exclusion of pathogen binding, competition for nutrients and immunomodulation (Magnusson et al., 2003). Health claims provided by LAB range from the regulation of intestinal microbial homeostasis to the modulation of immune responses. Alleviation of lactose intolerance, a reduction in the risk of diarrhea caused by bacteria and virus as well as lowering serum cholesterol are some other positive effects obtained with the consumption of probiotic (Roberfroid, 2007).

4. 2. Materials and methods

4. 2. 1. Microorganisms and growth conditions

Maintenance of selected LAB isolates for probiotic characterization and inoculum preparation are described in chapter 3 (sections **3. 1. 2** and **3. 3. 1**)

4. 2. 2. Isolation of lactic acid bacteria (LAB)

Ninety six LAB strains were isolated from different sources like rotten jackfruit, guava, deer and Indian gaur dung and tiger puke. The samples were collected from Silent Valley National Park which is a part of the Western Ghat's natural world heritage site of India and one of the biodiversity hot spots in the Kerala belt. The sources of isolation also included curd, butter, pickle, fish intestine and Ceylon spinach (*Talinum triangulare*). All samples (1 g) were added into MRS broth and kept for LAB growth for 24 h at 30 ^{o}C. Decimal dilutions of these samples were spread plated and cultivated on MRS agar plates at the same conditions for 48 h. Isolated pure colonies were picked and sub-cultured on MRS-$CaCO_3$ plates, where acid producing bacteria will produce clear zone around the colony. Preliminary identification was done by gram staining and catalase test.

4. 2. 3. Antifungal activity assay

Antifungal assay used was a modified method of Schillinger and Villarreal (2010). The LAB isolates were spot inoculated onto MRS agar plates and allowed to grow at 30 ^{o} C for 48 h. The plates were then overlaid with 10 mL of soft potato dextrose agar (PDA, 0.7%, w/v) containing about 10^4 spores per mL *F.*

oxysporum (KACC 42109). The isolates which prevented the growth of test fungus were selected and inoculated into MRS broth and incubated at 30 ^{o}C under static condition for 48 h. After incubation, the cells were removed by centrifugation at 8590 × g for 15 min and the culture supernatants were concentrated 10 fold in a vacuum concentrator at 30 ^{o}C with respect to their initial volume and used for agar well diffusion assay. For this, 100 µL of the above samples were added to the wells (5 mm diameter) cut on potato dextrose agar which was previously spread plated with 1×10^{4} spores of *F. oxysporum* and kept at 30 ^{o}C for four days. An inhibition of *F. oxysporum* growth more than 8 mm diameter was looked further for growth inhibition against other fungal cultures like *A. niger* (KACC 42589), *F. moniliforme* (KACC 08141), *P. chrysogenum* (NII 08137) and the yeast *C. albicans* (MTCC 3017).

4. 2. 4. Identification of selected strains by 16S rDNA sequencing

DNA extraction and PCR amplifications of 16S rRNA gene were carried out as described in section **3. 3. 5. 2** and **3. 3. 5. 3**. The sequences obtained were blasted in NCBI (National center for biotechnology information, www.ncbi.nlm.nih.gov) to identify the most related lactic acid bacteria

4. 2. 5. Probiotic characterization of selected antifungal LAB isolates

Out of 44 antifungal isolates, 11 [B6 (from butter), G1 (from Ceylon spinach), C9 (from curd), TGI (from tiger feces), TG2 (from tiger puke), RJF4 (from rotten jack fruit), SVG3 (from guava), DY1, DY2 (from jack fruit), DR2 (from deer feces), BSN307 (from Indian gaur dung)] which showed maximum antifungal activity were selected for probiotic characterization studies.

4. 2. 5. 1. Evaluation of tolerance to gastric/intestinal inhibitory substances

Ability of LAB isolates to tolerate gastric and intestinal conditions were primarily checked by survival through adverse growth conditions such as presence of phenol (0.3, 0.4, and 0.5 v/v %), bile salt (0.3, 0.5 and 0.8 w/v %) and low pH (2 and 2.5). MRS medium was used for experiments. Cells were incubated for 24 h under these varying conditions at 30 ^{o}C in static condition and the growth was monitored. An A_{600} above 0.1 was taken for further evaluation.

4. 2. 5. 2. Survival of LAB in simulated gastro intestinal environment

Simulated gastric and intestinal juices were prepared as previously described (Corcoran et al., 2005) with some modifications. It was prepared by dissolving 13.3 mg/L pepsin from porcine gastric mucosa (Sigma, USA) in 0.5% (w/v) sterile saline and adjusting the pH to 2.5. Simulated small intestinal juice was prepared by dissolving 250 mg/L pancreatin from porcine pancreas (Sigma) and 500 mg/L porcine bile (Himedia, India) in 0.5% (w/v) sterile saline and adjusting the pH to 7.5. Freshly prepared solutions were used for the experiments. Cultures of BSN307, TG2 and DY2 were grown overnight (18 h) in 5 mL MRS medium and centrifuged at 8590 × g for 15 min to obtain the pellets. The pellets were then re suspended and washed twice in sterile saline. After centrifugation the pellets were re suspended in equal volume of simulated gastric juice at 37 ^{o}C and incubated for 90 min with constant shaking. The initial number of viable cells re suspended in gastric juice was 1.4×10^{8} cells of BSN307, 6×10^{7} cells of TG2 and 4×10^{7} cells of DY2 per mL. Samples were retrieved after 90 min; serially diluted and appropriate dilutions were plated on MRS medium and incubated at 37 ^{o}C for 48 h. Cells after simulated gastric environment treatment were collected by centrifugation at 8590 × g for 15 min and re suspended in same amount of simulated intestinal juice and kept for 6 h incubation at 37 ^{o}C. Samples retrieved at 0 and 6 h and the appropriate dilutions were plated on MRS medium to check the viable number of bacteria.

4. 2. 5. 3. In vitro bacterial adhesion capacity to intestinal epithelial cell line

A modified method (Klingberg et al., 2005) was adapted for checking the intestinal adhesion ability of selected LAB isolates. Adhesion capacity of BSN307, TG2 and DY2 to the human colon adenocarcinoma cells HT-29 was investigated. The strain *Escherischia coli* E1T was kept as negative control. HT-29 cell line was obtained from NCCS (Pune) and maintained in Eagles Minimum Essential Medium (MEM), supplemented with 10% fetal bovine serum (FBS), 100 mg/L streptomycin, 100 U/L penicillin and 50 µg/mL gentamicin. The cells were grown at 37 ^{o}C in a humidified atmosphere of 5% CO_2 and 95% air until a

confluent monolayer was obtained. Monolayers of HT-29 cells were seeded at a concentration of 2×10^{5}cells/mL and dispensed into each 200 mm^2 well of a 24-well tissue culture plate. The 24 h old bacterial cells washed twice in PBS and re suspended in the HT-29 growth medium without gentamicin to a final concentration of approximately 3.5×10^{8} CFU/mL of BSN307, 3.3×10^{6} CFU/mL of DY2 and 4.5×10^{8} CFU/mL of TG2. Viable number of *E. coli* cells used was 9×10^{8} cfu/mL. This cell suspension (1mL) was added to each well of the tissue culture plate. After 90 min of incubation with the isolates, the monolayers were washed two times with phosphate-buffered saline (PBS, pH 7.4) in order to remove non-adherent bacteria. The HT-29 cells were lysed by addition of 0.1% (v/v) Triton-X100 and the number of viable adherent bacteria was determined by plating serial dilutions onto MRS medium. Colony-forming units were enumerated after incubation for 48 h at 37 ^{o}C and the adhesion capacity was described as the percentage of bacteria adhered to HT-29 cells in relation to the total number of bacteria added. Each adhesion assay was conducted two times with duplicates.

4. 2. 5. 4. Co-aggregation assay

Overnight cultures of BSN307 and TG2 were pelleted and washed twice in PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5) and resuspended in the same to get an OD of 0.5 at 600 nm. Four ml of this cell suspension was taken and vortexed for 10 sec and incubated at room temperature for 5 h. 0.1 mL of the upper suspension was taken and mixed with 900 µL of PBS and the absorbance at 600 nm was measured. Co-aggregation ability of the selected LAB isolates with *Staphylococcus aureus* and *Pseudomonas aeruginosa* (NIIST isolates) was studied. Equal volumes (2 mL) of each cell suspension were mixed together in pairs and vortexed for 10 sec. After 5 h, absorbance at 600 nm was measured. Percentage of coaggregation was calculated according to Handley et al. (1987) as:

$$\text{Coaggregation (\%)} = \frac{(Ax + Ay)/2 - A(x+y)}{Ax + Ay/2} \times 100$$

Where x and y represent each of the 2 strains in the control tubes and (x+y) in the mixture.

All experiments have been performed in triplicates and the results represented by their mean ± SD (standard deviation).

4. 3. Results and discussion

4. 3. 1. Isolation of LAB

A total of 96 LAB were isolated from various samples on MRS plate. All the isolates produced clear zone around the culture on MRS-$CaCO_3$ plates (Fig.4.1), were Gram positive and included rods, cocci and cocci in chain. All the isolates were catalase negative which showed no bubbling when hydrogen peroxide was added.

Fig.4.1 Lactic acid bacteria on MRS-$CaCO_3$ agar plates

4. 3. 2. Antifungal activity assay

From the 96 LAB isolates 44 showed antifungal activity by overlay method and were selected for agar well diffusion method. Out of 44 antifungal isolates, 11 showed maximum antifungal activity and were identified by 16S rDNA sequencing and also selected for preliminary probiotic characterization. These 11 isolates showed more than 8 mm diameter zone of growth inhibition

against *F. oxysporum* (Fig.4.2, Table.4.1). Out of these, three isolates; BSN307, TG2 and DY2 were selected and spectrum of activity was checked against three major plant pathogenic and mycotoxin producing genera of fungi that include *A. niger* (KACC 42589), *F. moniliforme* (KACC 08141) and *P. chrysogenum* (NII 08137) associated with the growth in and damage to food even at refrigeration temperatures. Anti yeast activity was confirmed against *C. albicans* which is a reference strain for antifungal susceptibility checking. Isolates TG2 and DY2 showed a wide spectrum of activity (+++) against *A. niger*, *F. moniliforme* and *P. chrysogenum* where BSN307 showed (+++) activity against *F. moniliforme* and *C. albicans* along with (++) activity against *P. chrysogenum* (Fig.4.3 and Fig.4.4)

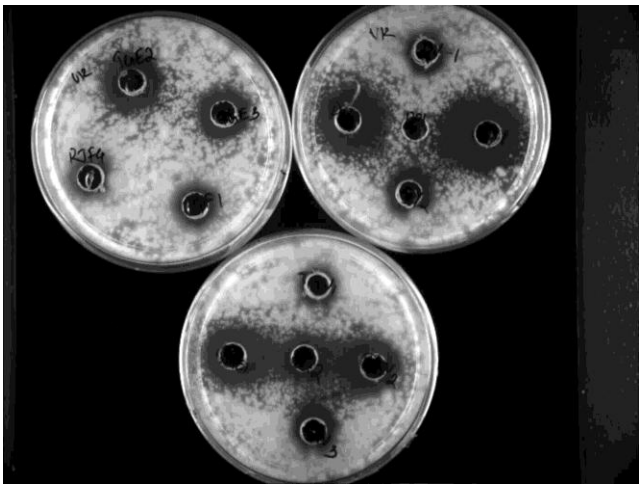


Fig.4.2 Antifungal activity of LAB isolates against *F. oxysporum* by agar well diffusion assay

4. 3. 3. Identification of selected strains by 16S rDNA sequencing

The 16S rDNA amplification of 11 isolates was performed by PCR reaction. A band of~1508 bp specific for bacterial 16S rDNA was obtained. The obtained gene sequence was compared with other 16S rDNA sequences in the GenBank by using the NCBI Basic Local Alignment Search Tool BLASTn program. All the isolates belonged to four LAB genera namely *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Enterococcus*. Three isolates from three different

genera were selected based on their activity against the test organism. The isolates include BSN307 (NCBI accession number KM261818), TG2 (NCBI accession number KM261819) and DY2 (NCBI accession number KM261820).The results are shown in Table.4.1.

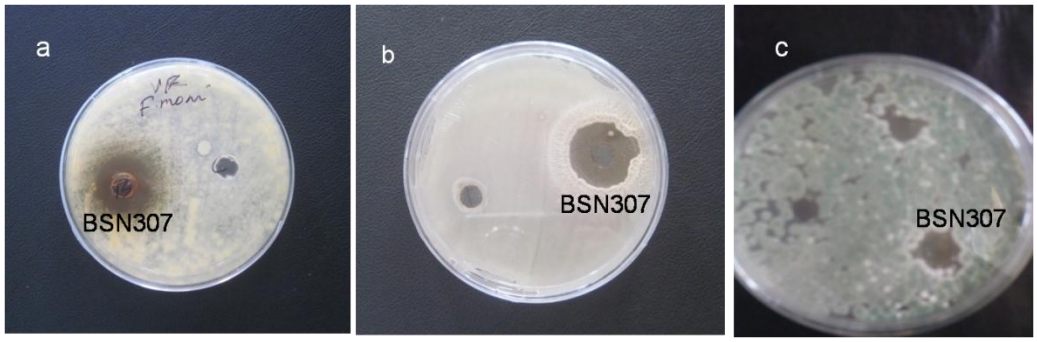


Fig.4.3 Antifungal activity of BSN307 against *F. moniliforme* (a), *C. albicans* (b) and *P. chrysogenum* (c)

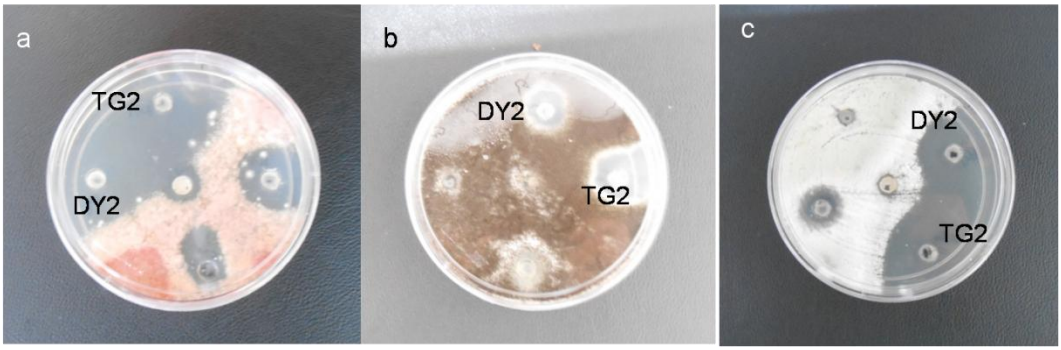


Fig.4.4 Antifungal activity of TG2 and DY2 against *F. moniliforme* (a), *A. niger* (b) and *P. chrysogenum* (c)

4. 3. 4. Probiotic characterization of selected antifungal LAB isolates

4. 3. 4. 1. Evaluation of tolerance to gastric/intestinal inhibitory substances

Endure through the gastrointestinal conditions are important characteristics of LAB strains since they are a very important group of intestinal

Table.4.1 Culture Identification and tolerance to growth inhibiting conditions by antifungal LAB

Strain	Source	pH		Bile (%)		Phenol (%)		Antifungal activity [a]	Identification 16S rDNA
		2	2.5	0.5	0.8	0.4	0.5		
BSN307	Indian gaur dung	+	+	+	+	+	-	++	*Lactococcus*
TG1	Tiger feces	-	+	+	+	+	-	++	*L. plantarum*
TG2	Tiger puke	+	+	+	+	+	-	+++	*P. pentosaceus*
DY1	Jackfruit	-	+	+	+	+	+	++	*L. plantarum*
DY2	Jackfruit	-	+	+	+	+	-	+++	*L. casei*
DR2	Deer feces	-	+	+	+	+	-	+	*E. faecalis*
SVG3	Guava	+	+	+	+	+	+	++	*L. plantarum*
RJF4	Rotten Jackfruit	-	+	+	+	+	-	++	*L. plantarum*
B6	Butter	+	+	+	+	+	+	+	*L. plantarum*
C9	Curd	+	+	+	+	+	+	+	*L. plantarum*
G1	Amaranthus	+	+	+	+	+	+	+	*L. plantarum*

+, positive (A_{600} above 0.1); -, negative

[a] Calculation of antifungal activity: +, 8 mm diameter clearing zone; ++, 10 mm diameter clearing zone; +++, more than 10 mm diameter clearing zone (against *F. oxysporum*)

microbiota and colonization of which are possible only if they subsist the acidic gastric acid environment as well as exposure to bile and pancreatic juice in the upper small intestine to exert its beneficial effects in the gut. All the isolates could survive 0.4% phenol and 0.8% bile in the medium. Phenol resistance is considered as an advantage of probiotics owing to the fact that it can be released to the body by deamination of aromatic amino acids (Suskovic et al., 1997). Survival at 0.5% bile is regarded as very good (Charteris et al., 1998) and our isolates could survive 0.8% bile in the medium. Both *Lactococcus* (BSN307) and *Pediococcus* (TG2) could survive pH 2 for 24 h which substantiates their origin and establishment in animal intestine and the *Lactobacillus* strain could tolerate a pH of 2.5. Survival at low pH is an important probiotic character since the bacteria have to tolerate the depleted pH of gastric juice whiles the journey from mouth to intestine. One of the indispensable qualities of probiotic is its ability to reach, survive and persist in the environment where it is proposed to act and to affect its environment favorably. The number of probiotic bacteria should be preferably between 10^5 to 10^8 CFU/g of intestinal content (Marteau et al., 1993), so the number of bacteria selected for this study was confined to the above range. The results are summarized in Table.4.1.

4. 3. 4. 2. Survival of LAB in simulated gastro intestinal environment

To confirm the initial results from low pH and bile tolerance as well as to check the ability of these isolates to survive gastro intestinal environment, survival in simulated gastric and intestinal juice was verified. After 90 min of incubation in the simulated gastric juice, approximately 3 log reduction of cell viability was occurred for BSN307, less than 2 log reduction for TG2 and below 1 log reduction for DY2 (Fig.4.1). It was revealed that *Lactobacillus* was more competent in artificial gastric conditions when compared to the survival of *Lactococcus* and *Pediococcus* in the same. After 6 h simulated intestinal juice treatment, about 2.5 log reduction of cell viability was noticed for both BSN307 and DY2 and less than 1 log decrease for TG2 when compared to the initial number of cells inoculated to simulated intestinal juice. TG2 was capable of

survival at pH 2 and in the presence of 0.8% bile with very less decrease in viability after treatment in the artificial gastro-intestinal environment.

In this model digestive system *Lactobacillus* showed highest survival rate which is in accordance with earlier reports (Faye et al., 2012). Only a few investigations showed that lactococcal strains can tolerate artificial gastric juice (pH 2.5) and artificial bile (Lee et al., 2007). The lactococcal strain under study could tolerate the artificial gastro-intestinal conditions and can be considered as a candidate to be used in functional foods. Ability of *Pediococcus* as a probiotic candidate is well studied and reported up to 0.5% bile (0.3% is the mean concentration in the intestine) tolerance along with low pH tolerance at 3 and 58% simulated gastric juice survival (Immerstrand et al., 2010; Ramirez-Chavarin et al., 2013; Shukla and Goyal, 2014).

4. 3. 4. 3. In vitro bacterial adhesion capacity to intestinal epithelial cell line

Three isolates were examined for their ability to adhere to HT-29 cell line along with *E. coli* as negative control. All the three isolates showed higher adhesion capacity than the *E. coli* strain tested which was 0.005% with a 4 log reduction of viable cells after binding. The isolate TG2 hold high adhesion capacity of 15.2% with around 1.5 log reduction from the initial cell number. DY2 possessed adhesion capability of about 1% and the lower adhesion ability was exhibited by BSN307 which was 0.3% with around 2 log reduction in the viable cell number for both BSN307 and DY2 after binding (Fig.4.1).

TG2 revealed a higher amount of adhesion (15.2%) than the previously reported *P. pentosaceus* Q3 which showed 6.2% (Jensen et al., 2012) and *P. pentosaceus* OZF strain which has 14.4% adhesion capacity on Caco-2 cells (Osmanagaoglu et al., 2010). The new *L. casei* strain hold a binding ability of 1% and both these values are higher when considering binding on HT-29 cells. Cell binding capability of BSN307 is lower (0.3%) but still higher than the adhesion shown by *E. coli*. The *in vitro* studies followed to explore the probiotic potential of new LAB isolates revealed that they can survive and adapt to the gastro intestinal conditions and are able to bind to the intestinal cell line. The *in vitro*

probiotic characterization experiments performed revealed that TG2 hold the maximum of probiotic features in all the isolates checked and can be a potential probiotic strain.

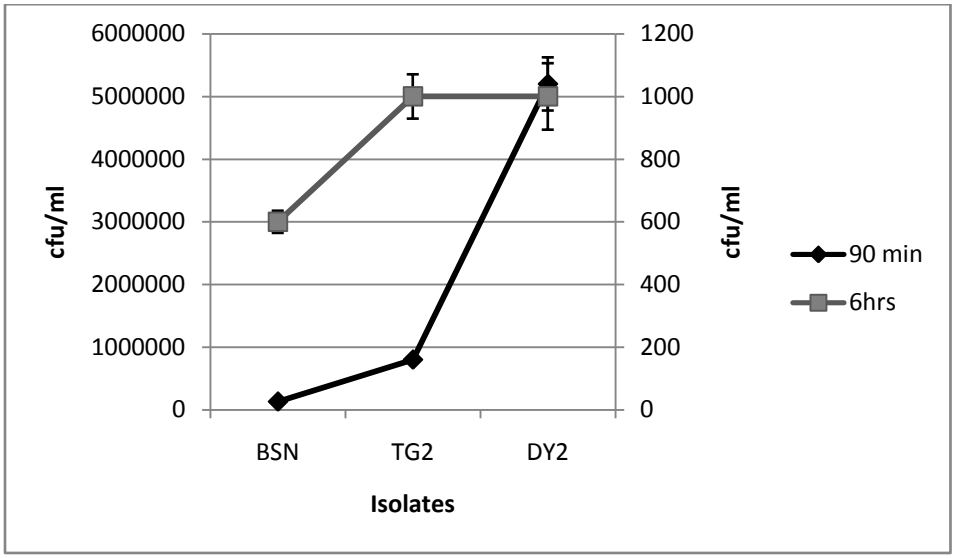


Fig.4.5 Survival of LAB isolates in simulated gastric juice (◆) along with efficiency to survive simulated intestinal juice on the secondary Y axis (▬). The initial number of viable cells re suspended in gastric juice was 1.4×10^8 cells of BSN, 6×10^7 cells of TG2 and 4×10^7 cells of DY2 ($p < 0.05$)

It is difficult to conclude the *in vivo* intestinal binding potential of probiotics from *in vitro* cell adhesion experiments. The situation in gastro-intestinal tract, where the host defense systems, competition with the resident microbiota, mucosal shedding, and peristaltic flow are likely to modify the bacterial adhesion (Lebeer et al., 2008). Though ability of bacteria to adhere to the intestinal cell lines is important to understand the mechanisms of adhesion and provide important information about differences among LAB species. Cell adhesion property of diverse LAB strains to different cell lines varies from 1-25% (Jensen et al., 2012; Klingberg et al., 2005). Adhesive properties of different bacteria depend on the origin and the initial doses provided and also when applied

as a combination of probiotic bacteria, stronger adhesive characteristics were reported (W et al., 2000). Investigations by Jankowska et al. (2008) showed that pathogenic bacteria adhered better to well-differentiated Caco-2 cells whereas lactobacilli and lactococci displayed better adhesion to non-differentiated Caco-2 cells which may give support to the competitive exclusion of pathogenic bacteria by LAB. Earlier studies (Gu et al., 2008) reported adhesion abilities below 5% and 14.4% for *L. casei* and very little binding abilities of *L. casei rhamnosu*s and *P. pentosaceus* (Ramirez-Chavarin et al., 2013) on Caco-2 cells.

4. 3. 4. 4. Co-aggregation assay

Bacterial aggregation between different species and strains (coaggregation) is of considerable importance in several ecological niches, especially in the human gut where probiotics are to be active (Gu et al., 2008) to exert their beneficial effects. With *S. aureus* BSN307 showed 50% co-aggregation ability and TG2 showed 20% co-aggregation ability. BSN307 had 74% co-aggregation ability and TG2 possessed 55% with *P. aeruginosa*. Co-aggregation ability determines the ability of the probiotic strain to adhere to the oral cavity, gastrointestinal tract and urogenital tract by competing with other bacteria and forms a barrier that prevents colonization by pathogens.

4. 4. Conclusion

Three LAB isolates; BSN307 (*Lactococcus*), TG2 (*Pediococcus pentosaceus*) and DY2 (*Lactobacillus casei*) with remarkable antifungal and probiotic potential were selected for initial screening of antifungal compounds production. Antifungal activity spectrum of the compounds produced by these strains includes different genera of food spoiling fungi such as *Fusarium*, *Aspergillus* and *Penicillium*. Simulated gastrointestinal juice survival and the *in vitro* HT-29 cell adhesion assay revealed their ability to survive and colonize the intestinal habitat.

CHAPTER 5

Organic acids from lactic acid bacteria as antifungal compounds

5. 1. Introduction

Fungal contamination is one of the major causes of spoilage in food and feed. Besides, mycotoxins cause serious health problems in humans ranging from immune suppression to death in severe cases. Contamination of feedstuff with mycotoxins also creates a serious threat to the health and productivity of animals (Kabak et al., 2006). Aflatoxins are hepatocarcinogenic and fumonisins may cause neural tube defects in maize consuming communities through disruption of ceramide synthase and sphingolipid biosynthesis (Wild and Gong, 2010). Thus, food safety remains a major concern when addressing current health problems in developing countries and pursuing natural methods for food protection is gaining huge attention now a day because of the consumers demand on chemical free, safe, and mildly processed food with extended shelf life. Previous studies report organic acids as a major group of antifungal compounds produced by LAB that include acetic, caproic, formic, propionic, butyric, n-valeric acids, phenyllactic acid, 4-hydroxyphenyllactic acid and benzeneacetic acid along with the hydroxyl fatty acids such as 3-(R)-hydroxydecanoic acid, 3-hydroxy-5-cis-dodecenoic acid, 3-(R)-hydroxydodecanoic acid and 3-(R)-hydroxytetradecanoic acid (Corsetti et al., 1998; Lavermicocca et al., 2000; Sjogren et al., 2003; Wang et al., 2012). Selected strains of LAB with potent antibacterial and antifungal properties can be used as "green preservatives" apart from their contribution to texture, flavor and nutritional value to the food or feed.

Organic acids in their undissociated, hydrophobic form diffuse through the membrane of the target organisms, dissociate inside the cell, releasing H^+ ions that acidify and reduce cytoplasmic pH and stop metabolic activities. Medium-sized acids (C4-C7) at near neutral pH act primarily at the cell membrane. For large fatty acids and small and medium acids, at increased concentrations,

membrane rupture and cell lysis occur as primary actions. It is hypothesized that organic acids neutralize the electrochemical potential of plasmic membrane and increase its permeability, leading to the death of susceptible organisms (Schnurer and Magnusson, 2005; Stratford and Eklund, 2003). The inhibitory effect of undissociated organic acids is l0-600 times stronger than that of their dissociated forms and the extent of dissociation is directly determined by pH (Helander et al., 1997). This chapter explains the identification of organic acids as possible antifungal agents from LAB.

5. 2. Materials and methods

5. 2. 1. Microorganisms and growth conditions

Newly isolated LAB strains BSN307 (*Lactococcus garvieae*), TG2 (*Pediococcus pentosaceus*) and DY2 (*Lactobacillus casei*) were used for the experiments. General maintenance and inoculum preparation had been described in chapter 3 (section **3. 3. 1**).

5. 2. 2. Preparation and purification of bioactive from culture supernatant

Ten litre of MRS medium was inoculated with cultures of BSN307, TG2 and DY2 independently and incubated at 37 ^{o}C for 48 h at static condition. After incubation, the supernatant was collected by centrifugation at 8590 × g for 15 min and extracted with double volume of hexane, chloroform and ethyl acetate in a sequential order. The solvent was evaporated under reduced pressure in a rotavapour (Buchi, Switzerland) and the semi viscous concentrated sample obtained was loaded onto a solid-phase extraction (SPE) silica column (600×40 mm). The active compounds were eluted using chloroform: methanol gradient. Growth inhibitory activity of all the fractions from three isolates was checked against *F. oxysporum* by agar well diffusion method. Active fractions of each isolate were purified further by preparative thin layer chromatography. Fractions of BSN307, TG2 and DY2 were separated by loading 200 µL of each sample on silica plate (Merck; 60 F254, 0.5 mm). Compounds adsorbed on silica were scraped out and extracted by treating with chloroform: methanol (1:1 v/v) and subjected to ESI-MS and HPLC analysis.

5. 2. 3. Identification of organic acids by ESI-MS and HPLC

Compounds separated by preparative TLC were identified by comparing data from ESI-MS and high-performance liquid chromatography with that of commercially available compounds including acetic acid, stearic acid, palmitic acid, 3- phenyllactic acid, caproic acid, benzoic acid, salicylic acid and propionic acid (Sigma, USA). Liquid chromatography–mass spectrometry (Thermo Orbitrap LC/MS) of separated compounds was performed using electrospray ionization method. Samples were prepared in acetonitrile and 0.1N formic acid was used as the mobile phase. For chromatographic analysis Luna silica column (Phenomenex- 250×4.60 mm) connected to PDA detector was used with chloroform as mobile phase at 254 nm.

5. 2. 4. HPLC analysis of active fractions

To compare the various compounds produced by the three strains based on the difference in retention times, the active fractions of BSN307, TG2 and DY2 were analyzed by analytical HPLC (Shimadzu, Japan). Rezex ROA column (Phenomenex, USA) was used with 0.01 N H_2SO_4 as mobile phase and PDA as detector. Compound diversity was compared based on the number of peaks at various retention times obtained for each sample at 210 nm.

5. 2. 5. Effect of temperature, pH and enzymes (lipase and proteolytic) on compound stability and antifungal activity

Antifungal activity of the culture filtrate of BSN307 (around pH 4.3) after exposure to high temperature (at 121 ^{o}C for 15 min) and varying pH (6.0, 6.5 and 7 adjusted with 0.1 M NaOH) was determined by agar well diffusion assay. The enzymes: lipase, pepsin, proteinase k, trypsin and α-chymotrypsin (Sigma-Aldrich, USA) were prepared in appropriate buffers and treated with the crude bioactive fractions at specific pH and temperature. Final concentrations of the enzymes in culture filtrates were 1mg/mL and samples without enzymes were kept as control. Antifungal activity was detected by the well diffusion method against *F. oxysporum*.

5. 2. 6. Effect of incubation on antifungal activity

Effect of incubation time on the antifungal activity was checked for the selected isolate. MRS broth was used as the growth medium and samples after 24, 48 and 72 h incubation were collected. Antifungal activity was checked against *F. oxysporum* by agar well diffusion assay as explained in section **4. 2. 3** with equal amount of samples from each experiment.

5. 2. 7. Effect of carbon sources and nitrogen sources on antifungal activity

Effect of different carbon sources on growth and antifungal activity was verified against *F. oxysporum* by agar well diffusion assay. Growth was checked by measuring A_{600} after 24 h incubation of the isolate in broth and samples for antifungal activity were collected after 48 h incubation. Composition of MRS broth was used where only the particular carbon or nitrogen source was included or replaced in the corresponding experiments along with other media components.

All experiments have been performed in triplicates and the results represented by their mean ± SD (standard deviation).

5. 3. Results and discussion

5. 3. 1. Extraction and identification of antifungal compounds

After sequential extraction with the solvents, it was found that ethyl acetate extract of BSN307, TG2 and DY2 culture supernatants retained major portion of antifungal activity against *F. oxysporum* leaving chloroform fraction with limited activity. Silica column purified fractions were checked for growth inhibiting activity against *F. oxysporum* and the active fractions were further separated on preparative TLC and the activity was confirmed against the same pathogen (Fig.5.1).

From the visible zone of inhibition, it was obvious that, the fractions from BSN307 (b) and TG2 (t) are more effective in fungal growth inhibition. These partially purified compounds were analyzed by ESI-MS (Fig.5.2) along with analytical HPLC and noticed that BSN307 produced a range of carboxylic acids including benzoic acid, caproic acid, salicylic acid, 3-phenyllactic acid (3PLA),

stearic acid and palmitic acid (Table.5.1). At the same time, TG2 and DY2 produced stearic acid and salicylic acid.

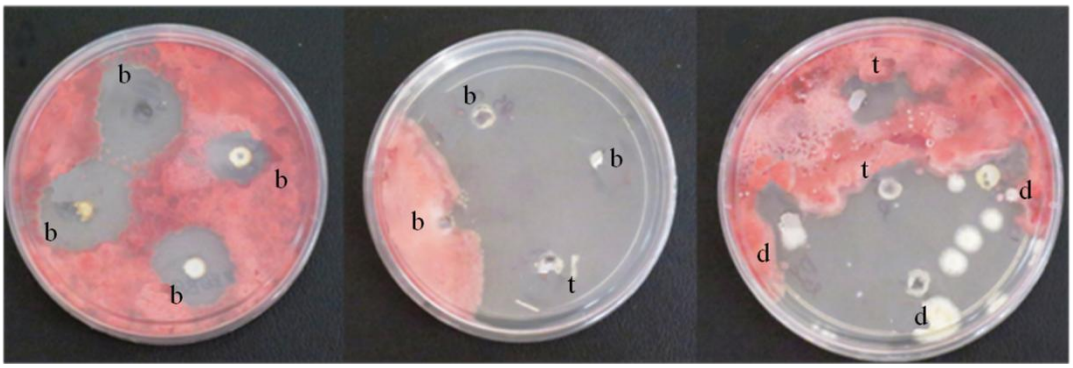


Fig.5.1Antifungal activity of preparatory TLC purified fractions against *F. oxysporum*.Antifungal activity of different fractions from BSN307 (b), TG2 (t) and DY2 (d).

P. pentosaceus as a biopreservative in bakery products and tomato puree by delaying the growth of *Aspergillus oryzae* and *Aspergillus niger* was described by Muhialdin et al. (2011). *Lactococcus*, *P. pentosaceus* and *L. casei* are reported to produce various carboxylic acids as antimicrobial compounds (Schnurer and Magnusson, 2005). In this study an array of carboxylic acids were detected in some of the fractions from BSN307 as stated above. Stearic acid and salicylic acid were also produced by TG2 and DY2. Role of these acids towards fungal growth inhibition can be considered vital based on previous studies by various research groups (Niku-Paavola et al., 1999; Schnurer and Magnusson, 2005).

Apart from these recognized compounds, presence of certain unidentified compounds directs to think about the existence and involvement of a few unknown antifungal compounds contributing to the antifungal activity. When considering the role of carboxylic acids as precursors to a variety of compounds including esters, amides and carboxylate salts the possibility of formation of various compounds are not to be kept aside.

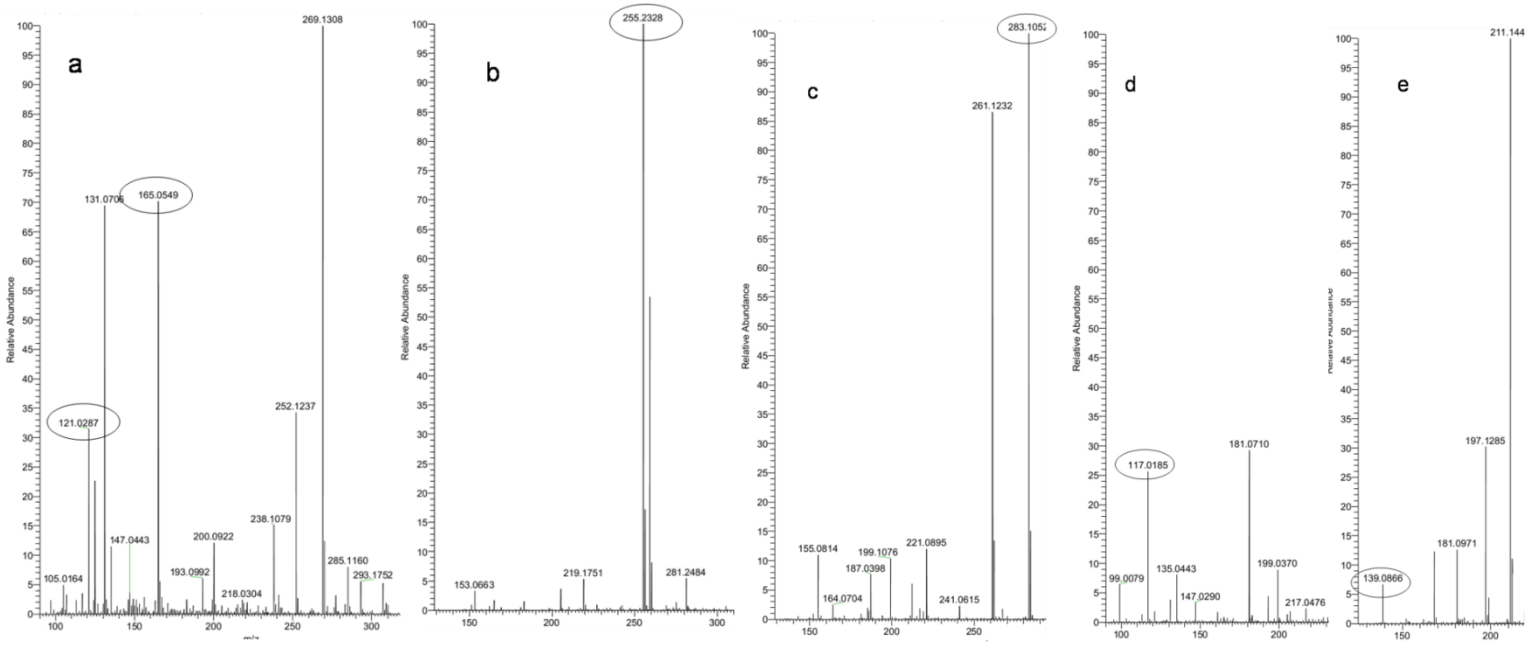


Fig.5.2 ESI-mass spectrum of BSN307 fraction with benzoic acid and 3-phenyllactic acid (a) palmitic acid (b), stearic acid (c) in the $[M-H]^-$ mode and caproic acid (d) and salicylic acid (e) in $[M+H]^+$ mode.

Table.5.1 ESI-MS and HPLC analysis results of the antifungal fractions of LAB isolates

Acid	Mass	Molecular formula	Isolate
Benzoic	122.12	$C_7H_6O_2$	BSN307
Palmitic	256.42	$C_{16}H_{32}O_2$	BSN307
Salicylic	138.12	$C_7H_6O_3$	BSN307, TG2, DY2
Stearic	284.47	$C_{18}H_{36}O_2$	BSN307, TG2, DY2
Caproic acid	116.15	$C_6H_{12}O_2$	BSN307
3-PLA	166.17	$C_9H_{10}O_3$	BSN307

5. 3. 2. HPLC analysis of active fractions

HPLC analysis of active fractions from the three isolates revealed that, the fractions from BSN307 had more number of compounds based on retention times of various compounds eluted out from the column (Fig.5.3). Table.5.2 shows the compiled result where retention times of compounds from BSN307, TG2 and DY2 are given.

From this analytical HPLC analysis results, it was clear that active fractions of BSN307 contained more compounds when comparing with the fractions from TG2 or DY2. All the compounds detected from TG2 were present in BSN307 and DY2 had a few compounds which were not present in BSN307 and TG2. Overall, BSN307 fractions had more number of compounds which are not present in the fractions of other two isolates and based on this analysis it was decided to proceed with the purification of antifungal compounds produced by BSN307.

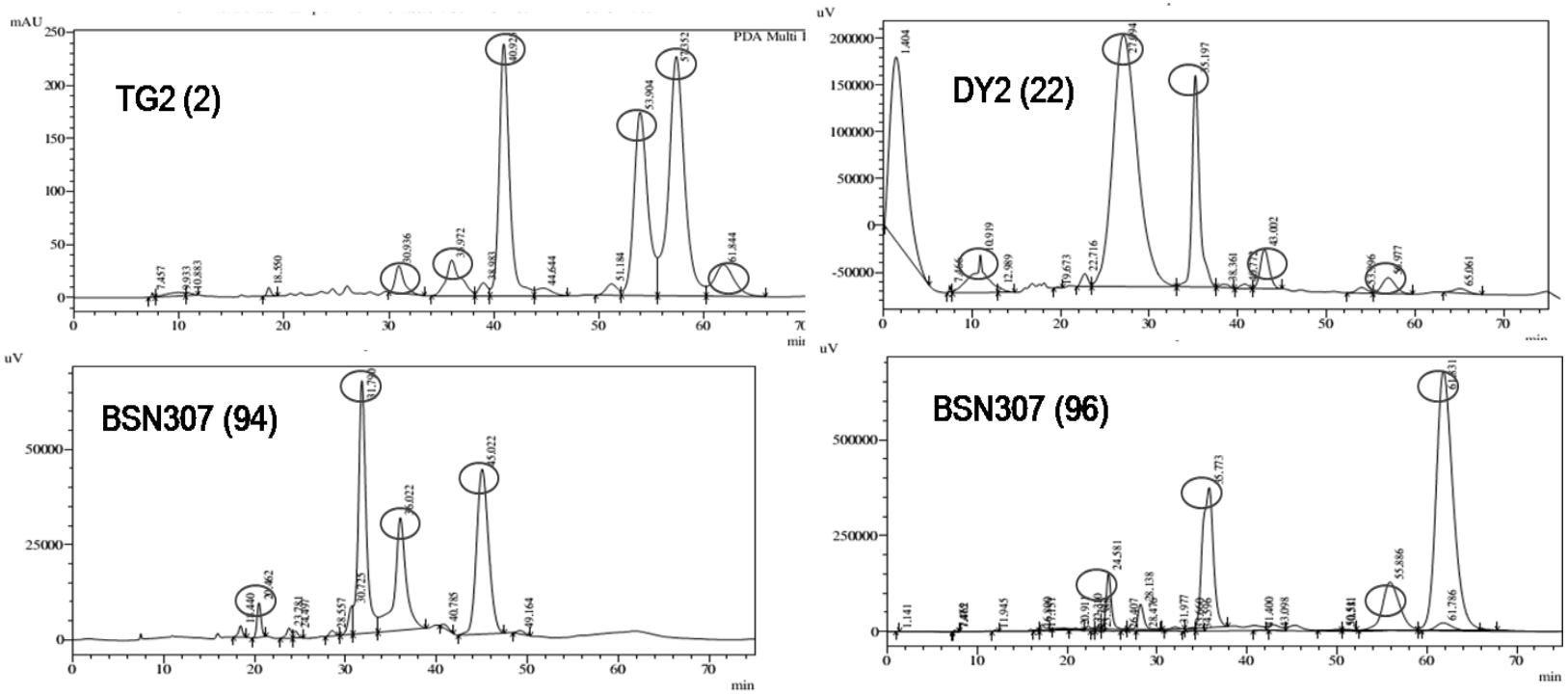


Fig.5.3. HPLC chromatograms of fractions TG2 (2), DY2 (22), BSN307 (94) and BSN307 (96)

Table.5.2 HPLC comparison of compounds from LAB isolates

Isolate (fractions)	Retention times * (min)
BSN307 (100)	7.4, 42.8
BS N307 (99)	61.8, 35.8
BSN307 (98 B)	35.2, 40.9, 53.8, 57.3
BSN307 (98 A)	35.2, 26
BSN307 (96)	61.8, 35.7, 24.5, 55.8, 28.1
BSN307 (94)	31.7, 36, 45, 20.4
TG2 (3)	26.7
TG2 (2)	40.9, 53.9, 57.3, 61.8
DY2 (22)	1.4, 27, 35.1, 43
DY2 (20)	7.4, 10.9

* Same retention times are marked in same color

5. 3. 3. Effect of temperature, pH and enzymes on antifungal activity of BSN307

Antifungal activity of the cell free supernatant (CFS) from BSN307 was retained after exposure to 121 ^{o}C for 15 min (Fig.5.4). Antifungal activity decreased with an increase in pH, holding 40% of the activity at pH 6.5 and 20% activity at pH 7 in comparison with the control which was an untreated sample of pH 4.3 (Fig.5.5). Treatment with proteolytic enzymes and lipase showed no effect on the growth inhibiting activity of the fractions (Fig.5.6).

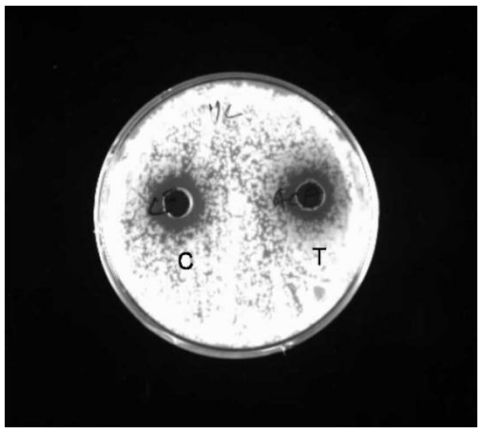


Fig.5.4 Effect of temperature on antifungal activity of BSN307 CFS. Control (C) which was untreated sample and test (T) which was treated at 121 ^{o}C for 15 min

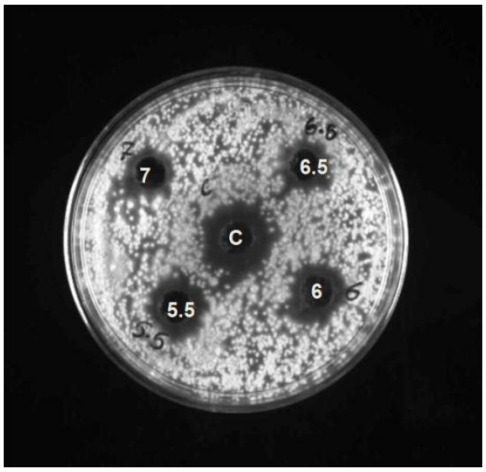


Fig.5.5 Effect of pH on antifungal activity of BSN307 CFS

5. 3. 4. Effect of incubation time on antifungal activity

The maximum antifungal activity was observed after 48 h of incubation (Fig.5.7) and for antifungal compound production 48 h was selected as the incubation period. The 24 h sample showed invasion of fungal mycelia into the zone of inhibition showing a diminished potential of antifungal compounds produced till that time. 48 h incubation was selected for antifungal compound production considering that there was not any increase in antifungal activity with

the 72 h sample and also to reduce the degradation of different compounds produced till 48 h.

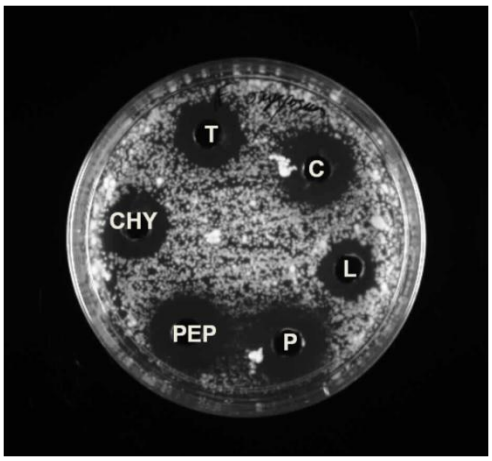


Fig.5.6 Effect of lipase (L), proteinase k (P), pepsin (PEP), chymotrypsin (CHY) and trypsin (T) on antifungal activity of BSN307 CFS along with control (C)

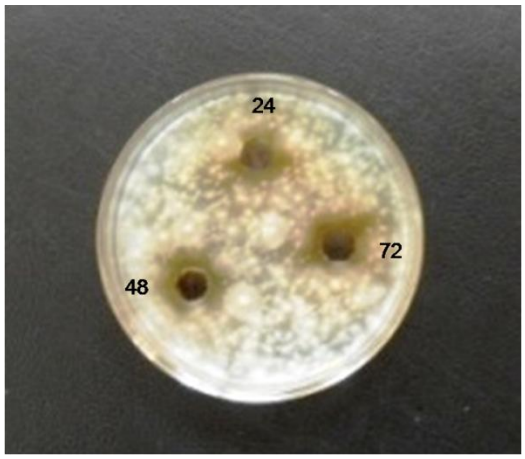


Fig.5.7 Effect of incubation time on antifungal activity

5. 3. 5. Effect of carbohydrate and nitrogen on growth and antifungal activity

Effect of different carbon sources (Table.5.3) and nitrogen sources (Table.5.4) after 48 h were checked and found that dextrose, yeast extract and

Table.5.3 Effect of carbohydrates on growth and antifungal activity

Sugar (2%, w/v)	Growth (A_{600}) 24 h	Inhibition zone (cm) 48 h
Dextrose	**2.034**	**1.7**
Fructose	2.01	1.5
Lactose	0.980	1.2
Sucrose	0.878	1.2

($p < 0.05$)

Table.5.4 Effect of nitrogen sources on growth and antifungal activity

Nitrogen (1%, w/v)	Growth (A_{600}) 24 h	Inhibition zone (cm) 48 h
Yeast extract	**2.334**	**1.3**
Ammonium citrate	**0.738**	**1.3**
Beef extract	2.378	1.1
Ammonium bicarbonate	0.685	1.1
Peptone	2.293	-
Ammonium chloride	0.561	-
Ammonium sulfate	0.567	-
Malt extract	1.119	-
Sodium nitrate	0.368	-

($p < 0.05$)

ammonium citrate had major effects on both growth and antifungal activity of BSN307. When different nitrogen sources were checked, carbon source used was dextrose. All the components that caused an increase in antifungal activity are part of the MRS broth and hence MRS broth was chosen as the production medium for antifungal compounds. Though good growth was observed with peptone and malt extract no antifungal activity was observed, showing the importance of media components on determination of LAB antifungal activity.

5. 4. Conclusion

This chapter concludes that organic acids produced by BSN307, TG2 and DY2 had a major role in the antifungal activity. Comparison of the active fractions of three strains by HPLC analysis revealed that the active fraction from BSN307 contains more number of compounds and hence it was selected for further purification studies. The effect of pH and different enzymes on the activity of BSN307 revealed that 20% of the activity retains at pH 7 indicating to the existence of compounds other than organic acids. Effect of various carbon and nitrogen sources on growth and antifungal activity of BSN307 showed that dextrose, yeast extract, ammonium citrate and beef extract are the major components contributing to both growth and antifungal compound production. Since all these ingredients are part of MRS medium, the same was selected as the production medium for large scale production of antifungal compounds.

CHAPTER 6

Phylogenetic characterization of the new isolate BSN307

6. 1. Introduction

The precise and definitive classification and identification of microorganisms is very important in taxonomic studies and most importantly when they are involved with health and diseases of humans and animals. Comparison of 16S rRNA gene sequences is the most common method followed for preliminary identification of bacteria. Since it is possible that the same bacterial isolate contains multiple copies of the 16S rRNA gene, sequencing of this gene alone is not sufficient for classification of a bacterial isolate. It was proposed that two bacterial isolates would belong to different species if the similarity in the 16S rRNA gene sequence between them were less than 97%. In contrast it was also proposed that two bacterial isolates do not necessarily belong to the same species if the similarity in 16S rRNA gene sequence between them is <3%. The ribosomal 23S rRNA gene, the 16S-23S rRNA gene ITS (internal transcribed sequences) as well as the house keeping genes such as homologous recombination encoding recA and the heat shock protein coding groEL are also shared by majority of bacterial species and used for identification purposes (Drancourt and Raoult, 2005; Janda and Abbott, 2002). Genomic fingerprinting methods such as rep-PCR (ERIC-, REP-, BOX-PCR), RAPD and AFLP also provide more differentiation power during bacterial classification (Rajendhran and Gunasekaran, 2010) along with phenotype differences and fatty acid composition of bacterial cells. Above all this, DNA-DNA hybridization (DDH) is considered as the "gold standard" for bacterial classification.Organisms that show more than 70% DDH values are considered to belong to the same species (Wayne et al., 1987).

In this chapter the strain BSN307 isolated from the dung of wild gaur (Indian bison, *Bos gaurus*) was studied. Phylogenetic analysis by 16S rRNA gene

sequencing revealed this novel strain is closely related to *L. garvieae* ATCC 49156. *L. garvieae* strains are found to be distributed in a range of environmental niches and are reported from vegetables, meat, and also have been isolated from dairy environments such as traditional Italian cheese, raw cow and goat milk (Fortina et al., 2003; Foschino et al., 2006; Ricci et al., 2013). They are considered as an important part of the microbial population and associated with the natural fermentation of artisanal Italian cheeses (Fortina et al., 2007). Many strains are reported to be isolated from disease conditions such as mastitis of cow and lactococcosis of fish (Ricci et al., 2013) and the pathogenecity is reported to be strain dependent (Kawanishi et al., 2006). Strains isolated from fish reported to have antibiotic resistance against erythromycin, lincomycin and tetracycline and harbour transferable R plasmids that carry *erm*B and *tet*S genes (Maki et al., 2009) but the real mechanism of how it is involved in the diseases is not yet very clear.

Further experiments including phenotypic characteristics, rep-PCR, fatty acid methyl ester (FAME) analysis and DNA-DNA hybridization (DDH) techniques were performed to establish the taxonomic position of this novel isolate and found to carry considerable difference from the type strain. Based on the results from above experiments, strain BSN307^T represents the type strain of a new subspecies within the species *L. garvieae*, for which the name *L. garvieae* subsp. *gaurensis* subsp. nov. is proposed.

6. 2. Materials and methods

6. 2. 1. Strains and culture conditions

The newly isolated strain BSN307 and type strain of *Lactococcus garvieae*^{T} (CCUG 32208) were used for phylogenetic studies. Culture conditions were explained in section **3. 3. 1. 2**.

6. 2. 2. Determination of 16S rRNA gene sequence and phylogenetic analysis

All the isolated colonies of lactic acid bacteria (LAB) were checked for cell morphology by phase contrast microscope (Leica DMLS, Germany), Gram stain, catalase test and growth pattern in MRS broth. All the isolates showed same

characteristics and out of these, strain BSN307^T was selected to carry out further studies. Chromosomal DNA of BSN307^T was extracted and polymerase chain reaction (PCR) for 16S rRNA gene was performed as described in section **3. 3. 5.1**.

Sequencing of 16S rRNA gene was performed by ABI 3500 (Applied Biosystems, USA) genetic analyzer according to the instructions of manufacturer. Sequence similarity searches were performed using the EzTaxon-e server (Kim et al., 2012) and sequences for phylogenetic analysis were obtained from the same. All sequences were aligned by using CLUSTAL W software (Thompson et al., 1997). Distances were calculated according to Kimuras two-parameter model (Kimura, 1980). Phylogenetic trees were constructed using neighbour-joining (NJ) method (Saitou and Nei, 1987), with bootstrap analysis based on 1,000 replications. MEGA version 6.0 (Tamura et al., 2013) was used for all the analysis.

6. 2. 3. Phenotypic characterization

Cells and spores were examined by phase contrast microscopy. Phenotypic tests including growth at pH 9.6 in brain heart infusion (BHI) medium, growth in the presence of 4% (w/v) NaCl in tryptic soy medium and growth at 42 ^oC in MRS agar (Eldar et al., 1999) were performed. Growth in nutrient agar with 0.5% (w/v) glucose, 0.5% (w/v) yeast extract and 6.5% (w/v) NaCl was also tested. Arginine dihydrolysis was checked in arginine dehydrolase media at 30 ^oC. Acid production from carbohydrates with API 50 CHL strips (Biomerieux, France) and antibiotic susceptibility of the strain on nutrient agar plates provided with 0.5% glucose and 0.5% yeast extract was checked by using HiComb strips (Himedia, India) according to the instructions of manufacturer. Antibiotics (µg/disc) checked include cephalothin (30), clindamycin (2), co-trimoxazole (25), erythromycin (15), gentamycin (10), ofloxacin (5), penicillin (10), vancomycin (30), ampicillin (10), chloramphenicol (30), oxacillin (1), linezolid (30), azithromycin (15), amikacin (30), clarithromycin (15), teicoplanin (10), methicillin (5), amoxyclav (30), novobiocin (5) and tetracycline (30).

6. 2. 4. Genetic and chemotaxonomic characterization

The genomic fingerprinting method, repetitive sequence based PCR (rep-PCR) pattern analysis has been reported to be useful in bacterial characterization (Perez et al., 2010; Rajendhran and Gunasekaran, 2010). In this study we analyzed the rep-PCR patterns of BSN307^T and CCUG 32208^T using the primers of $(GTG)_5$, ERIC (ERIC1 and ERIC2) and SERE which were previously proved to be effective in characterization of *Lactococcus* spp. by Cho et al. (2008). For PCR amplification red dye PCR master mix (Merck, USA) was used. Primers used are ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′),
ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′),
SERE (5'-GTGAGTATATTAGCATCCGCA -3′) and
$(GTG)_5$ (5′ -GTGGTGGTGGTGGTG-3′). The program for ERIC primers consisted of 95 ^oC for 7 min, then 40 cycles at 94 ^oC for 1 min, 52 ^oC for 1 min, and 65 ^oC for 8 min and finally, one 68 °C cycle for 16 min. For $(GTG)_5$, 95 ^oC for 5 min, then 35 cycles at 94 ^oC for 1 min, 42^oC for 1 min, and 65 ^oC for 8 min and finally, one 65 °C cycle for 15 min was used. The program, 95^oC for 5 min, 40 cycles at 94 ^oC for 1 min, 50 ^oC for 1 min, and 72 ^oC for 2 min and finally, one 72 °C cycle for 15 min was used for SERE primer. Amplicons were analyzed by electrophoresis in 1.6 % (w/v) agarose gels in 1X Tris-acetate- EDTA buffer

For DNA–DNA hybridization experiments, genomic DNA was extracted and purified according to the method of (Marmur, 1961) with additional *RNase* treatment. DNA suspended in the 2X SSC is used for the analysis and resulted values are calculated from the mean of triplicates. The analysis was done by fluorimetry using a Step One Plus Real-Time PCR system (Applied Biosystems, USA) fitted with 96 well thermal cycling block in 96 well plate as described and evaluated before (Ley et al., 1970; Loveland-Curtze et al., 2011; Gillis et al., 1970).

For cellular fatty acid analysis, cells were grown in MRS medium at 30 ^oC according to Chen et al. (2013). Analysis was performed by using the Sherlock

Microbial Identification System (version 6.1), according to the instructions of the Microbial Identification System (MIDI, Newark, DE, USA).

6. 3. Results and discussion

6. 3. 1. Phylogenetic analysis based on 16S rRNA gene sequence

A nearly complete 16S rRNA gene sequence (approximately 1404 nucleotides) (Fig.6.1a) was obtained. The BLAST results of 16S rRNA gene indicated 99.64% similarity with *L. garvieae* ATCC 49156^T and 99% similarity with *L. formosensis* 516^T. The next strains came close to BSN307^T are *L. lactis* subsp. *tructae* L105^T and *L. lactis* subsp. *cremoris* NCDO 607^T with 93.43% and 93.36% similarities respectively (Fig.6.1b). The GenBank accession number for 16S rRNA gene of BSN307^T is KM261818. Phylogenetic analysis based on 16S rRNA gene similarities revealed that the novel strain BSN307^T comes under the species *L. garvieae* (Fig.6.2), belongs to the genus *Lactococcus*.

6. 3. 2. Phenotypic characteristics

The colonies were cream, opaque, round and convex on MRS plate (Fig.6.3). The cells were catalase negative and microscopic analysis revealed Gram- positive, coccoid or ovoid-shaped cells present singly or in short chains (Fig.6.4a). Short ovoid chains were observed by scanning electron microscope (Carl Zeiss EVO 18, Germany) too (Fig.6.4b). No spores and mycelium were observed. A different growth pattern in MRS broth for BSN307^T from type strain was observed, where the novel strain completely sedimented to the bottom after 24 h incubation (Fig.6.5). BSN307^T failed to grow on TSA with 4% NaCl (Fig.6.6a) and pH 9.6 BHI medium (Fig.6.6b). Growth was not observed for BSN307^T in nutrient agar with 0.5% glucose, 0.5% yeast extract and 6.5% NaCl and at 42 ^oC (Fig.6.6c). At the same time type strain of *L. garvieae* CCUG 32208^T could grow on these conditions. These experiments which are specific for differentiation of *L. garvieae* (Eldar et al. 1999) clearly differentiate BSN307^T from the type strain. Another difference is the delayed arginine dihydrolysis reaction of this strain when compared to the reaction of type strain.

5′-3′ a

GATTGAAGATAGCTTGCTATTTTCATGAAGAGCGGCGAACGGGTGAGTAACGCGTGGGAAATCTGCCGAG
TAGCGGGGGACAACGTTTGGAAACGAACGCTAATACCGCATAACAATGAGAATCGCATGATTCTTATTTG
AAAGAAGCAATTGCTTCACTACTTGATGATCCCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACC
AAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAA
GAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACGTTAAGTAGAGTGGAAAATTACTTAAGTG
ACGGTATCTAACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCAAGCGT
TGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTCTTAAGTCTGATGTAAAAGGCAGTGGCTCA
ACCATTGTGTGCATTGGAAACTGGGAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGT
GAAATGCGTAGATATATGGAGGAACACCGGAGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGC
TCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGAGTGCTAGCTGT
AGGGAGCTATAAGTTCTCTGTAGCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGG
TTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTTCGAAGCAACGC
GAAGAACCCTTACCAGGTCTTGACATACTCGTGCTATCCTTAGAGATAAGGAGTTCCTTCGGGACACGGG
ATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATTACTAGTTGCCATCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGATAAACCGGAGGAAGG
TGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAC
GAGTCGCCAACCCGCGAGGGTGCGCTAATCTCTTAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTC
GCCTGCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCACGGAAGTTGGGAGTACCCAAAGTAGGTTGCCTAACCGCAAGGAGGGCGC

b

	Name	Strain	Accession	Pairwise Similarity(%)	Diff/Total nt	Completeness(%)
1	Lactococcus garvieae	ATCC 49156(T)	AP009332	99.64	5/1402	100
2	Lactococcus formosensis	516(T)	AB775178	99	14/1402	100
3	Lactococcus lactis subsp. tructae	L105(T)	EU770697	93.43	92/1401	100
4	Lactococcus lactis subsp. cremoris	NCDO 607(T)	AB100802	93.36	93/1401	100
5	Lactococcus taiwanensis	0905C15(T)	AB699722	93.07	97/1399	98.12
6	Lactococcus lactis subsp. lactis	JCM 5805(T)	BALX01000047	92.8	101/1402	100
7	Lactococcus lactis subsp. hordniae	NCDO 2181(T)	AB100804	92.72	102/1402	100
8	Lactococcus fujiensis	NJ317(T)	AB485959	92.44	106/1402	100
9	Lactococcus piscium	CCUG 32732(T)	DQ343754	91.53	118/1393	96.18
10	Lactococcus chungangensis	CAU 28(T)	EF694028	91.23	123/1402	98.65
11	Lactococcus raffinolactis	DSM 20443(T)	EF694030	90.72	130/1401	97.36
12	Lactococcus plantarum	DSM 20686(T)	EF694029	90.66	131/1402	97.43

Fig.6.1 The 16S rRNA gene sequence (a) and BLAST results of BSN307^T (b)

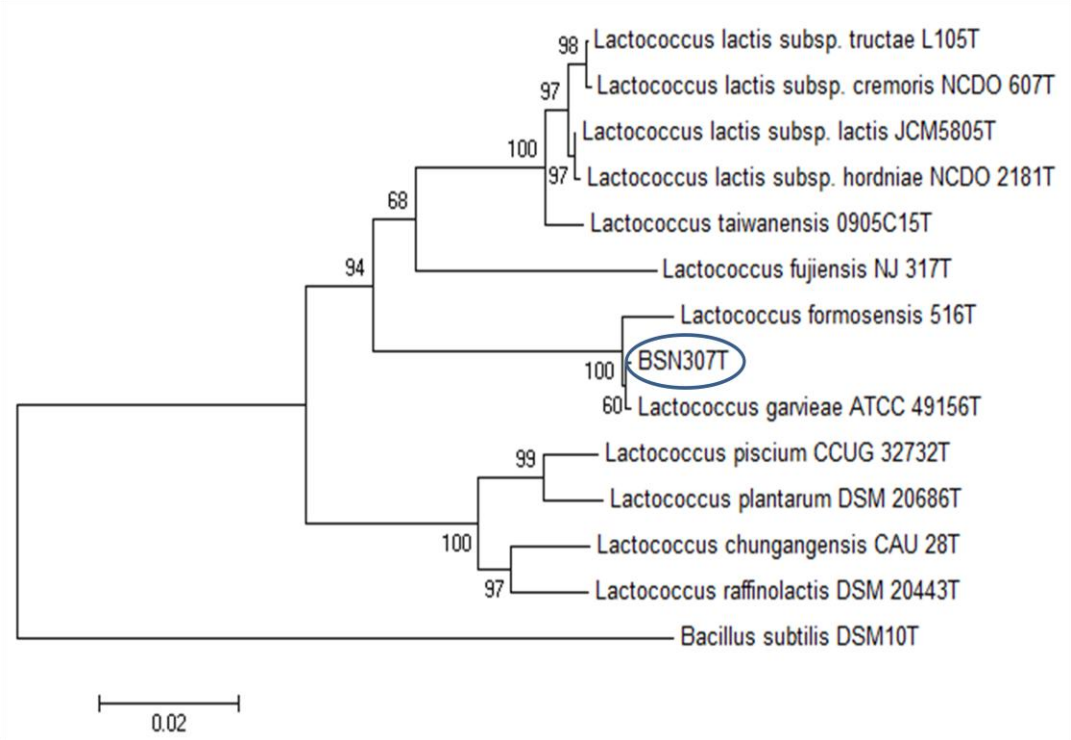


Fig.6.2 Neighbour-joining tree based on 16S rRNA gene sequences of strain BSN307^T and other related taxa. Bootstrap values > 50%, based on 1000 subsets, are given at branch points. Bar, 0.02

Fig.6.3 Colonies of BSN307^T on MRS agar

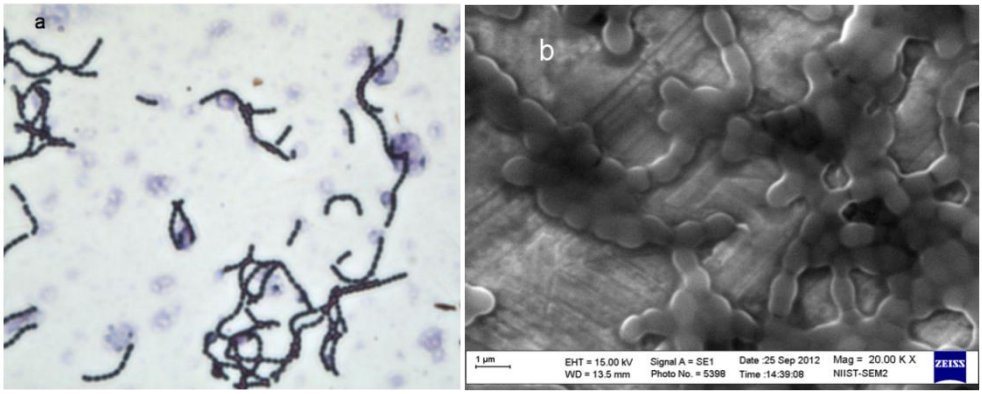


Fig.6.4 Cell morphology of BSN307^T revealed through phase contrast microscope (a) and scanning electron microscope (b)

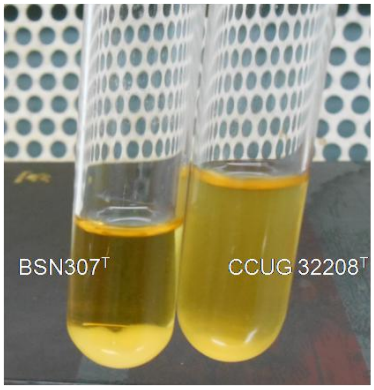


Fig.6.5 Growth (24 h) of BSN307^T and *L. garvieae* CCUG 32208^T in MRS broth

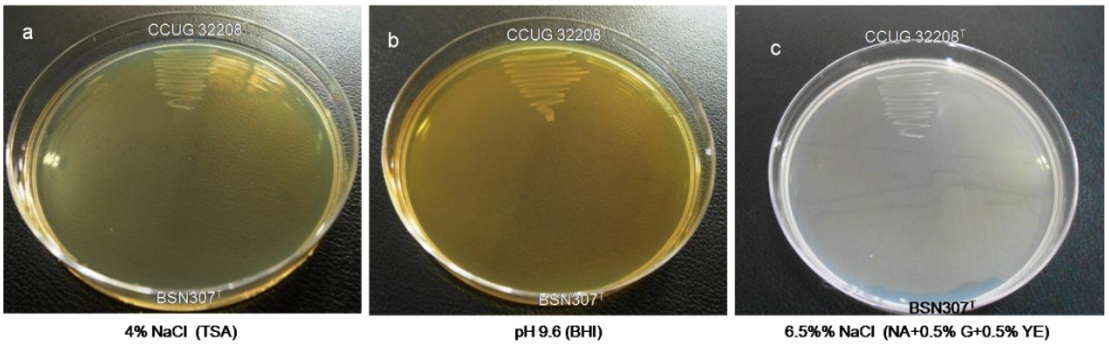


Fig.6.6 Growth of BSN307^T and *L. garvieae* CCUG 32208^T on 4% NaCl (a) and pH 9.6 (b) and 6.5% NaCl (c) [(Nutrient agar with 0.5% glucose (G) and 0.5% yeast extract (YE)]

Table.6.1 Characteristics of strain BSN307^T and other related taxa of the genus *Lactococcus*

Characteristics	1[a]	2[a]	3[b]	4[c]	5[c]
Growth in 4% NaCl	-	+	+	+	-
Growth in 6.5% NaCl	-	+	NA	NA	NA
Growth in pH 9.6	-	+	NA	NA	NA
Growth at 42 ^oC	-	+**	NA	NA	NA
Acid production from					
Ribose	+	+	+	+	-
Mannitol	+	+	+	+	-
Amygdalin	+	+	+	+	-
Melezitose	-	-	-	-	-
D-Xylose	-	-	-	-	-
Cellular fatty acid composition					
C12:0	0.80	0.57	-	-	-
C16:0	34.99	44.08	22.7	37.6	40.3
C17:0 cyclo	-	1.8	0.15	-	-
C18:0	5.38	3.27	-	0.98	0.7
C19:0 cyclo w8c	5.17	28.88	17.9	26.57	31.5
20:1 w9c	0.77	-	-	-	-
Summed Feature 8[*]	34.51	4.05	-	-	-

Strains: 1, BSN307^T; 2, *L. garvieae* CCUG 32208^T; 3, *L. formosensis* 516^T; 4, *L. lactis* subsp. *tructae* L105^T; 5, *L. lactis* subsp. *cremoris* DSM 20069^T. +, positive; -, negative; NA, Not available

[a] Data obtained from this study; [b] Data obtained from the study of Chen et al. (2014); [c] Data obtained from the study of Perez et al. (2010); ** Data from the study of Eldar et al. (1999)

[*]Summed Feature 8; C18:1 w7c/C18:1 w6c

Acid production from carbohydrates was determined using API 50 CHL fermentation kit after 48 h of incubation and an identical result to *L. garvieae* CCUG 32208^T was obtained. Acid was produced from D-glucose, ribose, galactose, D-fructose, D-mannose, N-acetyl glucosamine, mannitol, amygdalin, arbutine, esculine, salicine, cellobiose, maltose, trehalose, gluconate and β gentiobiose. Acid was not produced from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, β-methyl-xyloside, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, lactose, melibiose, sucrose, inulin, melezitose, D-raffinose, amidon, glycogene, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-keto-gluconate and 5-keto-gluconate. BSN307^T was susceptible to all the antibiotics checked. Phenotypic characters of BSN307^T are summarized in Table.6.1.

6. 3. 3. Genetic and chemotaxonomic characterization

DNA-DNA hybridization between BSN307^T and CCUG 32208^T showed a similarity of 75.8% which confirmed that BSN307^T comes under the species of *L. garvieae*. The rep-PCR pattern analysis showed clear difference between BSN307^T and CCUG32208^T with all the primers checked in both number of bands and also the molecular weight of bands present (Fig.6.7). The rep-PCR pattern analysis which previously used for characterization of *L. lactis* subsp *tructae* L105^T proved that the pattern will be similar for strains belonging to same subspecies but will be different among subspecies (Perez et al., 2010).

Some differences in the composition and amount of cellular fatty acid were obtained. The major fatty acids of strain BSN307^T are C:16 (34.99%) and summed feature 8 (C18:1 w7c/C18:1 w6c; 34.51%) and for CCUG 32208^T are C:16 (44.08%) and C19:0 cyclo w8c (28.88%). Absence of C16:1w5c, C17:0 cyclo, C18:1w7c 11-methyl, 20:2 w6,9c, presence of 20:1 w9c and substantial difference in summed feature 8, C19:0 cyclo w8c and C:16 differentiate strain BSN307^T from *L. garvieae* CCUG 32208^T. Complete fatty acid profile of both strains is given in Table.6.2.

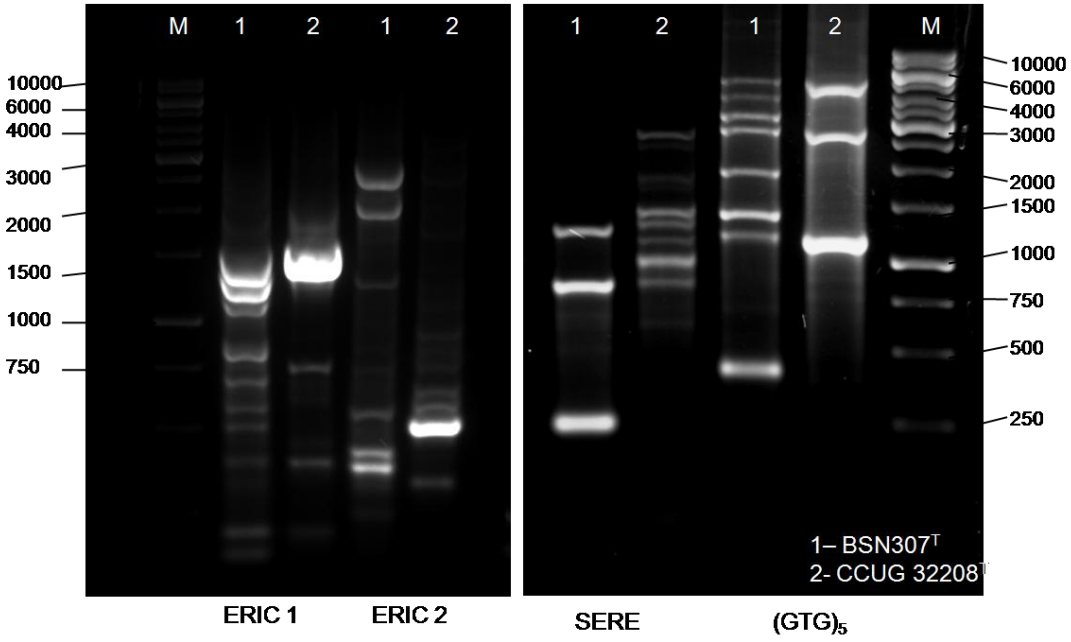


Fig.6.7 rep-PCR patterns (Primers; ERIC 1, ERIC 2, SERE and $(GTG)_5$) of BSN307^T (lane 1) and *L. garvieae* CCUG 32208^T (lane 2) along with 1kb DNA ladder (M).

Table.6.2 Cellular fatty acid content of BSN307^T and *L. garvieae* CCUG 32208^T

Fatty acid	BSN307^T	CCUG 32208^T
C12:0	0.80	0.57
C14:0	6.99	7.54
C16:1w5c	-	0.34
C16:0	**34.99**	**44.08**
C17:0 cyclo	-	**1.8**
C17:0	0.56	0.62
C18:1w9c	7.71	3.57
C18:0	5.38	3.27
C18:1w7c 11-methyl	-	**0.54**
C19:0 cyclo w8c	5.17	**28.88**

20:2 w6,9c	-	0.43
20:1 w9c	**0.77**	-
20:1 w7c	1.13	0.94
Summed Feature 3	2.20	3.41
Summed Feature 8	**34.51**	**4.05**

Summed Feature 3; C16:1w7c/C16:1 w6c; Summed Feature 8; C18:1 w7c/C18:1 w6c (p<0.05)

6. 4. Description of *L. garvieae* subsp. *gaurensis* subsp. nov.

L. garvieae subsp. *gaurensis* subsp. nov. (gau.ren'sis. N. L. masc. gaurensis of *Bos gaurus*)

The colonies are cream, opaque, round and convex on MRS plate. Cells are Gram positive, catalase negative, non-spore-forming, non-mycelium-forming, coccoid or ovoid-shaped and present as singly or in short chains. Strain cannot grow on BHI medium with pH 9.6, TSA with 4% NaCl and nutrient agar with 0.5% glucose, 0.5% yeast extract and 6.5% NaCl. Optimal growth occurs at 30 ^{o}C. Growth does not occur at 42 ^{o}C. Major cellular fatty acids are C:16 (34.99%) and summed feature 8 (C18:1 w7c/C18:1 w6c; 34.51%). Presence of 20:1 w9c in very small amount is another difference this novel strain carries from type strain.

6. 5. Conclusion

This study showed that the new isolate BSN307^{T} carries phenotypic and genotypic differences from the type strain *L. gravieae* CCUG 32208^{T} despite having a close 16S rRNA gene sequence similarity. At the same time comes under the reference strain based on DNA-DNA hybridization study. Based on the evidences provided in this study , the strain BSN307^{T} isolated from Indian gaur dung represent a novel subspecies of *L. garvieae* for which the name *L. garvieae* subsp. *gaurensis* subsp. nov. is proposed.

CHAPTER 7

Purification and characterization of antifungal compounds from *Lactococcus garvieae* subsp. *gaurensis* BSN307

7. 1. Introduction

Food industry is in continuous search for new bio-preservatives where antifungal Lactic acid bacteria (LAB) and their metabolites can be used effectively. LAB have received the GRAS (generally recognized as safe) and the QPS (Qualified presumption of safety) status and represent the microbial group most commonly used as protective cultures (Gaggia et al., 2011). It is estimated that over 3400 tonnes of pure LAB cells are consumed every year in Europe alone (Franz et al., 2010). An array of antifungal compounds produced by LAB are identified and characterized which include organic acids, reuterin, proteinaceous compounds, cyclic dipeptides (Oliveira et al., 2014; Schnurer & Magnusson, 2005), methylhydantoine, mevalonolactone (Niku-Paavola et al., 1999) and 2-propenyl ester (Wang et al., 2012).

Phenolic compounds as food additives are of particular interest when considering health and diet due to their antioxidant properties. LAB are capable of producing volatile phenolic compounds according to the availability of suitable substrates. It is reported that supplementation of caffeic acid, p- coumaric acid, malic acid and ferulic acid along with 5% v/v ethanol in MRS led to the production of volatile phenolic compounds, 4 vinyl phenol (4VP) and 4 ethyl phenol (4EP) by *Lactobacillus* through p- coumaric acid metabolic pathway (Silva et al., 2011a; Silva et al., 2011b). Production of 4VP and 4EP by *Lactobacillus* in wine conditions was detected by Fras et al. (2014).

Phenazines which are nitrogen containing heterocyclic compounds that differ in their chemical and physical properties based on the type and position of functional groups are extensively studied because of their broad spectrum antibiotic properties (Pierson Iii and Pierson, 2010).

The advantage of LAB metabolites having both antifungal and antioxidant properties is that they can be developed as food preservatives with dietary antioxidant properties once incorporated into food. Production of phenolic and phenazine compounds by LAB and their antifungal activity is not meticulously researched and remains to be explored.

This chapter discusses the purification and characterization of the phenolic compound 2,4 di- tertiary butyl phenol (2,4 DTBP) and a phenazine compound of 210 Da from the cell free supernatant (CFS) of BSN307. Both these compounds possess wide spectrum of biological activities such as antifungal, antioxidant and anti tumor properties.

7. 2. Materials and methods

7. 2. 1. Microorganisms and growth conditions

The LAB strain *Lactococcus garvieae* subsp. *gaurensis* BSN307, its maintenance and inoculum preparation had been described in chapter 3 (sections **3. 1. 2, 3. 3.1. 2** and **3. 3. 1. 3**).

7. 2. 2. Purification and characterization of the bioactives

For purification, 10 L of MRS medium was inoculated (5 % (v/v)) with 24 h old culture and incubated at 30 °C for 48 h at static condition. After incubation, the supernatant was collected by centrifugation at 8,590×g for 15 min and extracted with double volume of chloroform. The solvent was evaporated under reduced pressure in a rotavapor (Buchi, Switzerland) and the concentrated sample was further dissolved in acetonitrile (B1 fraction). The B1 fraction was purified by high pressure liquid chromatography (Shimadzu UFLC, Japan) using Prep-C18 column (Phenomenex, USA) and acetonitrile: water (90:10) as mobile phase with a flow rate of 4 mL/min. Two fractions named as compound 1 and compound 2 with antifungal activity were obtained which were collected and purified further by analytical HPLC (C18 column, Phenomenex, USA) using acetonitrile as mobile phase with a flow rate of 1 mL/min. Antifungal activity of the purified products (400 µg/disc) was confirmed against *F. oxysporum* (KACC 42109) by disc diffusion assay as described below in section **7. 2. 3**. Apart from antifungal

activity, these compounds purified from BSN307 CFS were also checked for other therapeutic activities.

7. 2. 2. 1. Structure analysis of compound 1

In order to analyze the structure, the pure compound (based on HPLC analysis observation) was subjected to ^{1}H nuclear magnetic resonance (NMR) spectroscopy using Bruker Avance II 500 spectrometer (USA). Mass spectrometry (Thermo Orbitrap, USA) employing electrospray (ESI) ionization method was used to determine molecular mass of the compound and Fourier transform infrared spectroscopy (FTIR) was performed with PerkinElmer model Spectrum 100 (USA) to obtain IR spectrum of the compound.

7. 2. 2. 2. Structure analysis of compound 2

Structure of the antifungal compound was determined by ^{1}H nuclear magnetic resonance (NMR) spectroscopy using Bruker Avance II 500 spectrometer (USA). Liquid chromatography–mass spectrometry (Thermo Orbitrap LC/MS, USA) employing electrospray (ESI) ionization method was used to determine molecular mass of the compound. The sample was prepared in acetonitrile with 0.1N formic acid and acetonitrile was used as mobile phase. Fourier transform infrared spectroscopy (FTIR) was performed with PerkinElmer model Spectrum 100 (USA) to obtain IR spectrum of the compound.

7. 2. 3. Antifungal activity assay of the pure compounds

Apart from *F. oxysporum* (KACC 42109), the wide spectrum antifungal activity of the individual pure compounds (400 µg/disc) dissolved in 50% acetonitrile was checked against *A. niger* (KACC 42589), *F. moniliforme* (KACC 08141) and *P. chrysogenum* (NII 08137) by disc diffusion method on PDA plates which were already swabbed with individual fungal suspension (1×10^{4} spores/mL). 50% acetonitrile was kept as control and the plates were incubated at 30 ^{o}C for four days to check the inhibition zones.

7. 2. 4. Total antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity (TAC) of the B1 fraction was determined according to the method of Prieto et al. (1999). The B1 fraction (1 mg/mL) was

mixed with 2.5 mL of phosphomolybdenum reagent (0.6 M sulphuric acid, 28 mM sodium sulphate and 4 mM ammonium molybdate were mixed together in 250 mL distilled water). The tubes were capped and incubated in boiling water bath at 95 ^{o}C for 90 min. After the samples had cooled to room temperature, absorbance of the reaction mixture was measured after 15 min at 695 nm against blank in spectrophotometer. A blank is prepared with 2.5 mL reagent solution and distilled water and incubated under same conditions. For samples, antioxidant capacity is expressed as equivalents of ascorbic acid.

7. 2. 5. Ferric ion (Fe^{3+}) reducing activity assay

Ferric cyanide (Fe^{3+}) reducing power of B1 fraction (1 mg/mL) was determined by Oyaizu method (Oyaizu et al., 1986). Sample was mixed with 1 mL of 0.2 M Na_2HPO_4 buffer (pH 6.6) and 1 mL of 1% (w/v) $K_3Fe(CN)_6$. The mixture was incubated at 50 ºC for 20 min, 1 mL of 10% (v/v) trichloroacetic acid was added, and the mixture was centrifuged at 3,500×g for 10 min. The supernatant (2.5 mL) was recovered, mixed with distilled water (2.5 mL) and freshly prepared 0.1% (w/v) $FeCl_3$ (0.5 mL) and placed immediately into the spectrophotometer. The absorbance at 700 nm was measured. Ascorbic acid was used as the positive control, and an increased absorbance reading indicated increased reducing power. For samples antioxidant capacity is expressed as equivalents of ascorbic acid.

7. 2. 6. Assay of free radical scavenging

Free radical scavenging activity of B1 fraction as well as the purified antifungal compounds (≥ 90% purity) were determined by the method of Shimada et al. (1992) which is based on the principle of scavenging the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical. DPPH was prepared in 95% (v/v) ethanol to a final concentration of 0.2 mg/5 mL. Samples were prepared in methanol in varying concentrations and the radical-scavenging activities of samples were expressed as percentage scavenging of DPPH. The reaction mixture was kept in the dark for 30 min and the absorbance was measured at 517 nm (Tecan NanoQuant, Switzerland) against a blank. The scavenging ability was defined as:

Scavenging activity (%) = ($A_{Control} - A_{Sample}$)/$A_{Control}$ × 100

7. 2.7. Cytotoxicity to mammalian cell lines

Cytotoxicity of pure compounds against mammalian cells were determined by MTT assay as described previously by Mossman (1983), where the conversion of tetrazolium salt MTT into blue colored formazan by the mitochondrial enzyme succinate dehydrogenase active in living cells was measured for quantifying cell survival and proliferation. In brief, mammalian cell lines, H9c2, HeLa and MCF-7 were maintained in DMEM supplemented with 10 % (w/v) FBS, 10 mg/L streptomycin, 100 U/L penicillin and 25 µg/mL amphotericin B. The cells were maintained at 37 ^{o}C in a humidified atmosphere of 5% CO_2 (SANYO CO_2 Incubator, Japan) until a confluent monolayer was obtained. Cells were seeded at a concentration of 1× 10^4cells into each well of a 96-well tissue culture plate followed by incubation at the same conditions for 24 h. The medium was removed and the cells were treated with varying concentrations of purified 2, 4 DTBP prepared in DMEM without FBS and incubated in the CO_2 incubator for 24 h. After incubation, medium was removed from each well by pipetting and 200 µL of MTT (0.5 mg/mL) dissolved in DMEM was added followed by incubation in dark for 3 h. The reaction was stopped by addition of 100 µL DMSO into the wells. Plates were further incubated for 30 min in a shaker and the absorbance was read at 570 nm (Synergy^{TM} 4 multimode Microplate Reader, USA). The experiments were performed in triplicates. Cytotoxicity (%) and IC_{50} (effective concentration of drug resulting in 50% of maximal toxicity) were calculated as follows;

Cytotoxicity (%) = [1- (A_{Test}/$A_{Control}$)] ×100

7. 2. 8. Aminopeptidase inhibitory activity

7. 2. 8. 1. Aminopeptidase production

Leucine aminopeptidase (LAP) was produced by *Streptomyces gedanensis* (IFO 13427) and proline aminopeptidase (PAP) was produced by *S. lavendulae* (ATCC 14162). The production medium for PAP was YEME (yeast extract malt extract) and for LAP was AP3 medium [(g/L): sucrose; 10, KH_2PO4; 2, $MgSO_4$.

$7H_2O$; 1, NaCl; 15, Na_2CO_3; 0.5, soybean powder; 5 and tween 80; 1.5, pH 7] (Raji et al., 2009). Individual 250 mL Erlenmeyer flasks containing 50 mL of the media were inoculated with 1% (v/v) of 24 h old inoculums of *S. gedanensis* and *S. lavendulae* separately and incubated for 5 days at 30 ^{o}C and 200 rpm. The cell free supernatants were collected after centrifugation at 8000×g and used as enzyme.

7. 2. 8. 2. Aminopeptidase assay

Aminopeptidase assay was done as per the protocol described by Arya et al. (2013). The reaction mixture contained 2.5 mM leucine *p*-nitroanilide (*p*NA) or proline *p*-nitroanilide as substrate, 50 mM Tris HCl buffer (pH 8.5), 50 µL enzyme sample (leucine or proline aminopeptidase) and 50 µL of either B1 fraction (250 mg/mL) or purified 2, 4 DTBP or phenazine compound (10 mg/mL). The assay mixture was incubated at 37 °C for 10 min. The bright yellow color produced due to the release of *p*NA by aminopeptidase enzyme activity was estimated spectrophotometrically by absorbance at 405 nm (Tecan NanoQuant, Switzerland). Enzyme activity (One IU) was defined as the amount of enzyme that hydrolyses 1µM of paranitroanilide substrate. When the inhibitor is added, a decrease in color due to inhibition or decrease in activity of enzyme happens which was estimated as explained before.

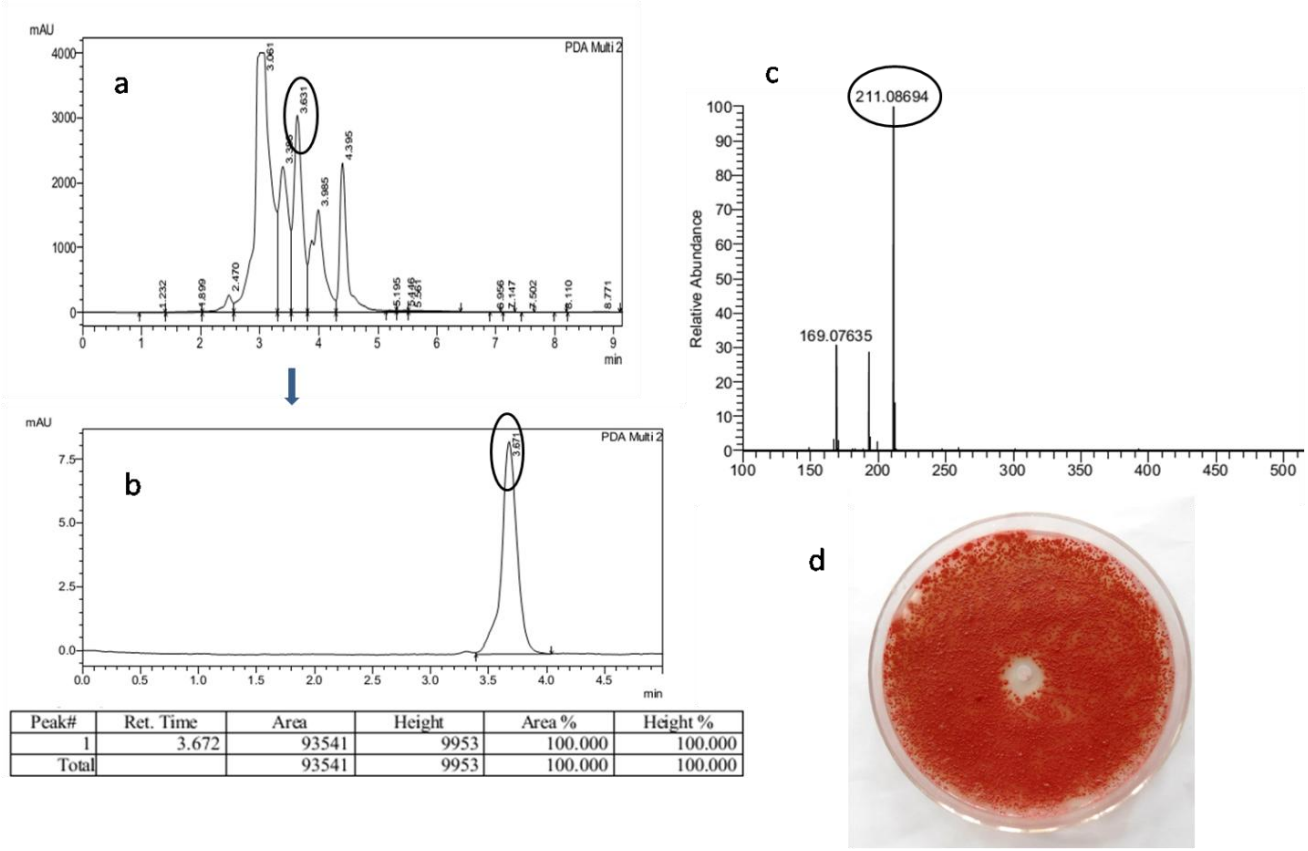
7. 2. 9. Statistical analysis

All the experiments were performed in triplicates and the results were expressed as mean values ± standard deviation. One-way ANOVA and Dunnet's test were performed to test the differences between test and control groups ($p < 0.05$). Statistical analyses were performed using the Minitab statistical package v. 17 (Minitab Inc., USA).

7. 3. Results and discussion

7. 3. 1. Purification and characterization of phenazine compound (compound 1)

After the final purification by analytical HPLC using C18 column (Fig.7.1a) and acetonitrile as mobile phase a single peak of the target compound was obtained (Fig.7.1b) at 270 nm. MS analysis of the pure compound indicated



Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.672	93541	9953	100.000	100.000
Total		93541	9953	100.000	100.000

Fig.7.1 Purification and antifungal activity of phenazine compound. HPLC chromatogram of B1 fraction (a), purified phenazine compound (b), MS chromatogram of phenazine (c) and antifungal activity of pure compound against *F. oxysporum* (d)

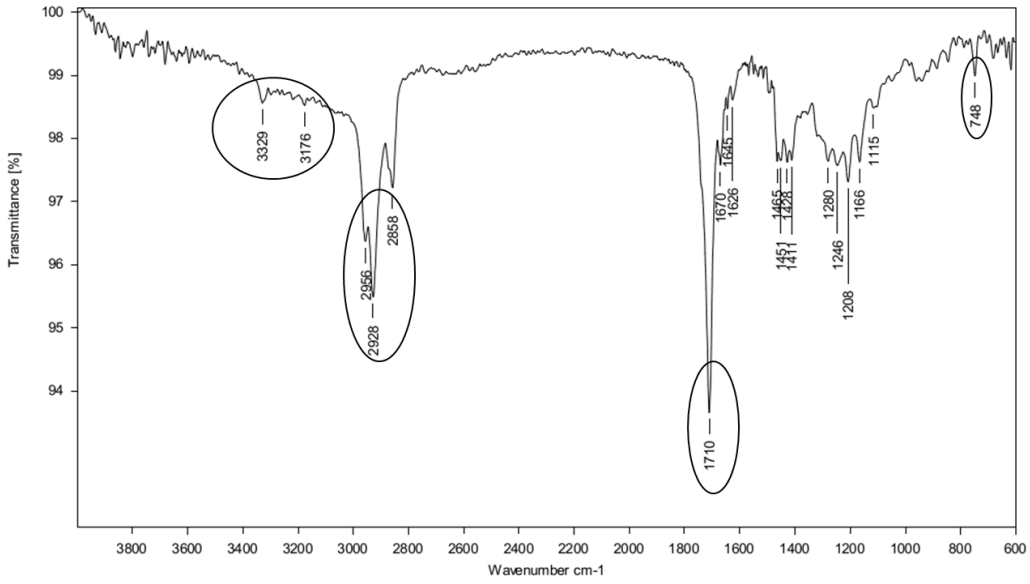


Fig.7.2 FTIR spectrum of phenazine compound

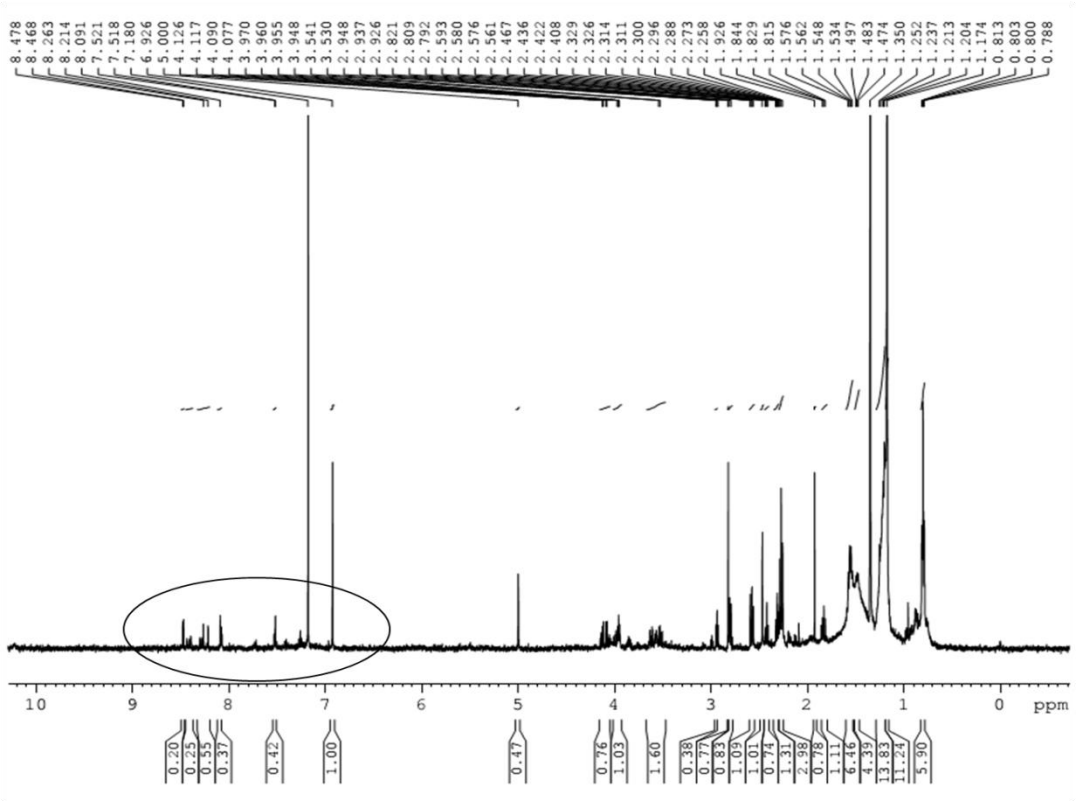


Fig.7.3 NMR spectrum of phenazine compound with the phenazine ring encircled

molecular ion at *m/z* of 211 $[M+H]^+$ (Fig.7.1c) and the mass was calculated to be 210 Da. FTIR analysis showed peaks at 2858 -2959 cm^{-1} indicated C-C stretching of alkyl group, C-H aromatic at 3059 cm^{-1} and 748 cm^{-1}, C=N at 1626 cm^{-1}, C-N at 1280 cm^{-1}, O-H at 3329 cm^{-1} and 1208 cm^{-1}, aromatic C=C stretchings in the region of 1400-1500 cm^{-1} and C=O at 1710 cm^{-1} (Fig.7.2).

^1H NMR analysis showed strong signals in the region 7-8.5 ppm indicating the presence of condensed nitrogen aromatic ring (Fig.7.3). Both IR and NMR data revealed the presence of a phenazine ring with C=O in the compound when compared with the already available data about phenazines.

Moreover, while extraction of cell free supernatant with chloroform presence of a blue coloured pyocyanin like pigment was detected (Fig.7.4) in small amount which is a phenazine compound with molecular weight of 210 Da. However, after HPLC purification of the B1 fraction the purified compound obtained was dark yellow in color and was fluorescent in nature (Fig.7.5) which leads to think about the possibility of this compound being some other phenazine derivative and may not be pyocyanin.

From the details of more than 6000 available phenazine derivatives (Mavrodi et al., 2006) we could not find one that matches with the mass and IR frequencies. Considering that phenazine compounds are involved in quorum sensing and they are produced in a strain specific manner, the production of a new phenazine derivative by this novel strain cannot be kept aside. Apart from the IR and NMR data, this compound showed an absorbance in the region of 370 nm apart from the maximum absorption at 260-280 nm during HPLC analysis which is another characteristic of phenazine compounds (Mavrodi et al., 2006) that further confirms the possibility of the compound being a phenazine derivative. The pure compound produced a clear zone of 1.5 cm against *F. oxysporum* (Fig.7.1d).

Phenazine production is based on *phz* gene expressions. Enzymes, PhzE, PhzD, PhzF, PhzB, and PhzG convert chorismic acid via 2-amino-2-desoxyisochorismic acid (ADIC), trans-2,3-dihydro-3-hydroxyanthranilic acid

(DHHA), 6-amino-5-oxocyclohex-2-ene-1-carboxylic acid (AOCHC), hexahydro-phenazine-1,6-dicarboxylate (HHPDC) and tetrahydro-phenazine-1-carboxylate (THPCA) to phenazine-1,6-dicarboxylic acid (PDC) and phenazine-1-carboxylic acid (PCA). PDC and PCA then act as‘‘core’’ phenazines that strain-specific enzymes convert to other phenazine derivatives (Wulf Blankenfeldt, 2013).

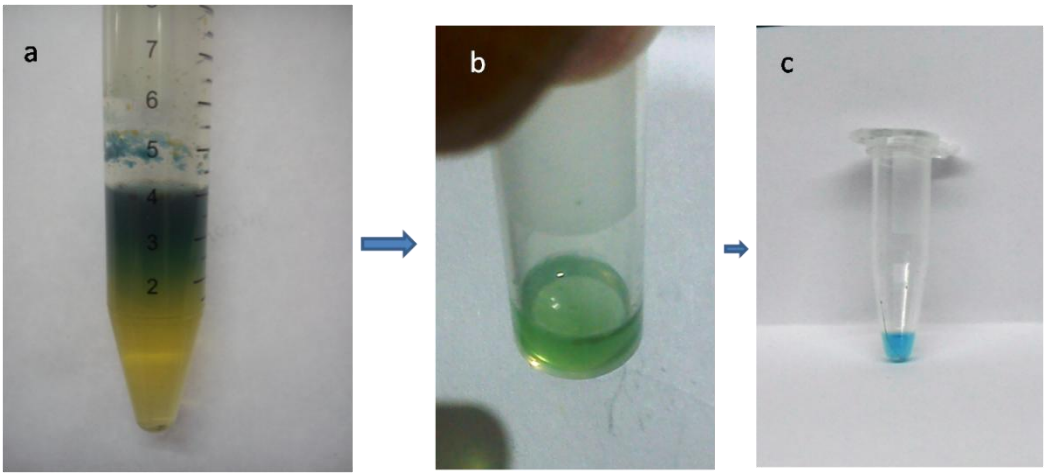


Fig.7.4 Different stages of recrystallization of chloroform extract (a and b) and purified pyocyanin like pigment (c)

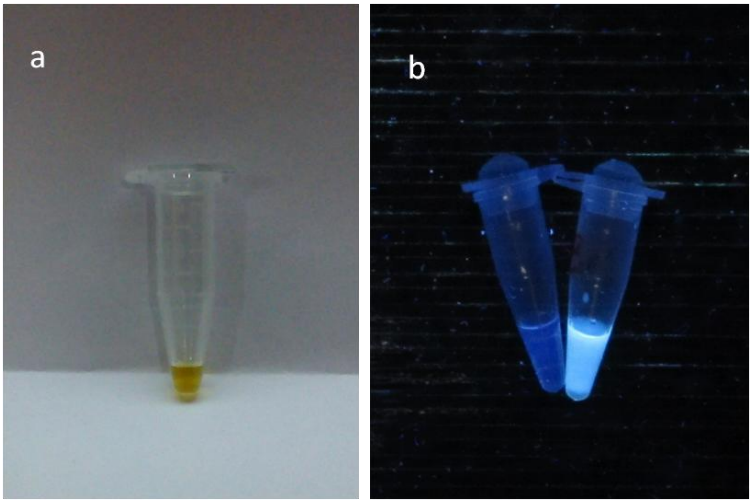


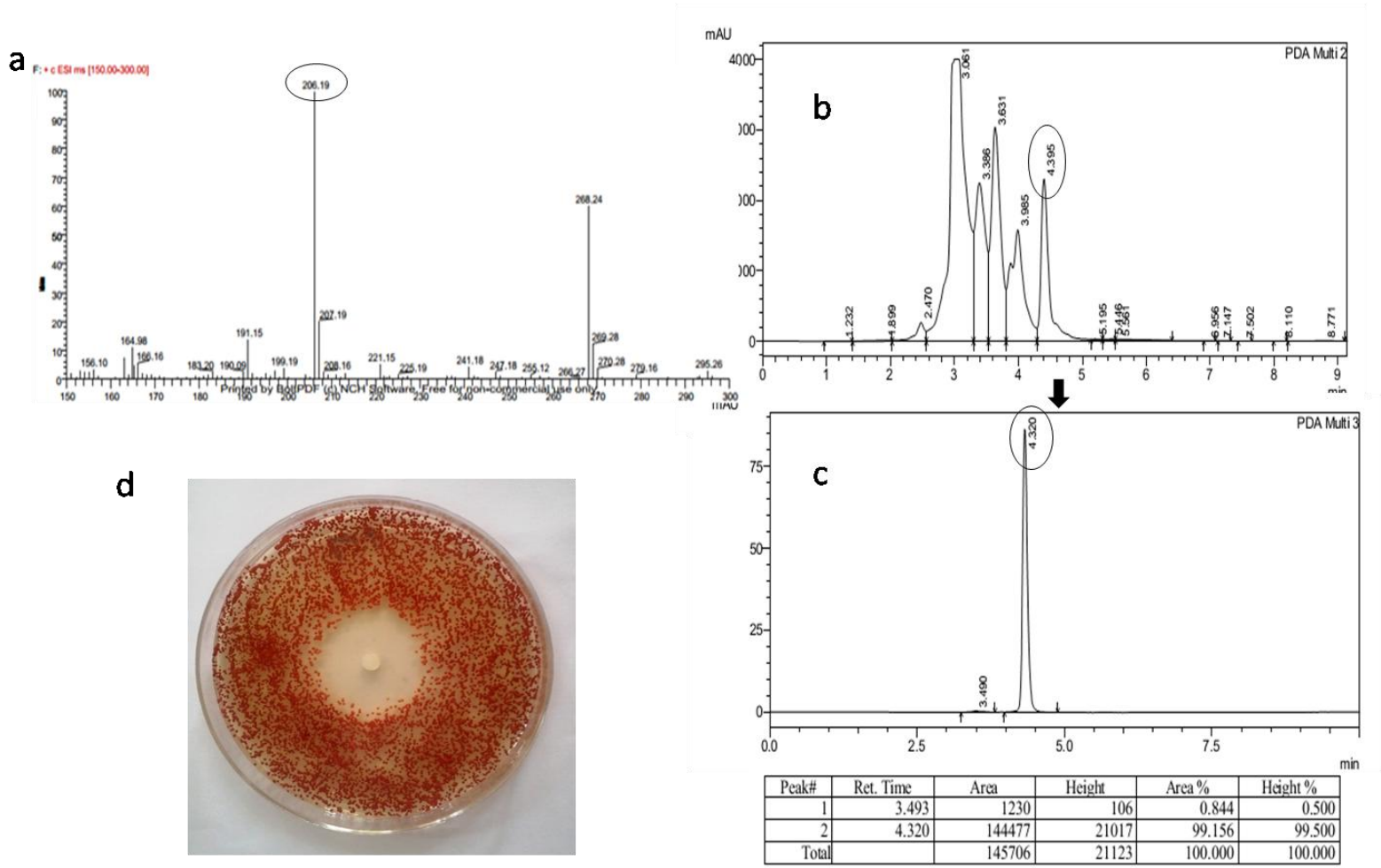
Fig.7.5 Pure phenazine compound (a) showing fluorescence under UV (b)

7. 3. 2. Purification and characterization of phenolic compound (compound 2)

HPLC analysis of prep- C18 purified sample revealed a major component of 90% and few minor impurities contributing to rest 10%. The sample was then subjected to LC-MS analysis which indicated a molecular weight of 206 at positive mode with corresponding to the major peak in the sample (Fig.7.6a). The sample was then further purified to 99.5% by analytical HPLC using C18 column. Fig.7.6b shows the HPLC chromatogram of crude B1 fraction and the purified compound (Fig.7.6c) at 270 nm. NMR and IR were performed. IR spectra of a purified sample showed a peak at 3533 cm^{-1} indicates stretching of O-H phenolic group. Peaks at 2871 -2959 cm^{-1} indicated C-C stretching of alkyl group. C-O stretching of phenols was seen at 1252 cm^{-1}. Presence of aromatic C=C stretching were identified by peaks at 1506-1600 cm^{-1} (Fig.7.7). Thus the presence of this functional group provided suggestive evidence that the compound is phenolic in nature.

NMR data of the purified sample revealed the presence of aromatic hydrogen corresponding to 7 to 7.3 ppm, a doublet at 7.07 ppm indicate aromatic meta hydrogen. Singlets of 9 hydrogen atoms at 1.309 and 1.431 suggested presence of di substituted tertiary butyl group. A downfield singlet at 4.662 indicates the presence of phenolic hydrogen (Fig.7.8a). Thus, after careful interpretation and co-relation of IR, NMR and LC-MS data, the compound was structurally elucidated as $C_{14}H_{22}O$ (Fig.7.8b) known as 2,4 di-*tert* butyl phenol (2,4 DTBP), a volatile organic compound (VOC) and discovered to play a major role in the antifungal activity of chloroform fraction. The final yield of 2,4 DTBP (≥90% purity) was 2 mg/ L. Using purified 2,4 DTBP a clearing zone of 3 cm against *F. oxysporum* (Fig.7.6d) was obtained.

2,4 DTBP is used as an intermediate in for the preparation of UV stabilizers and antioxidants as well as in the manufacture of pharmaceuticals and fragrances (Choi et al., 2013). The possible precursor and the pathway that leads to the production of this compound is not yet elucidated clearly although the gene



Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.493	1230	106	0.844	0.500
2	4.320	144477	21017	99.156	99.500
Total		145706	21123	100.000	100.000

Fig.7.6 Purification and antifungal activity of 2,4 di- *tert*- butyl phenol. LC-MS chromatogram of 2,4 DTBP (a), HPLC chromatogram of B1 fraction (b), purified 2,4 DTBP (c) and antifungal activity of 2,4 DTBP against *F. oxysporum* (d)

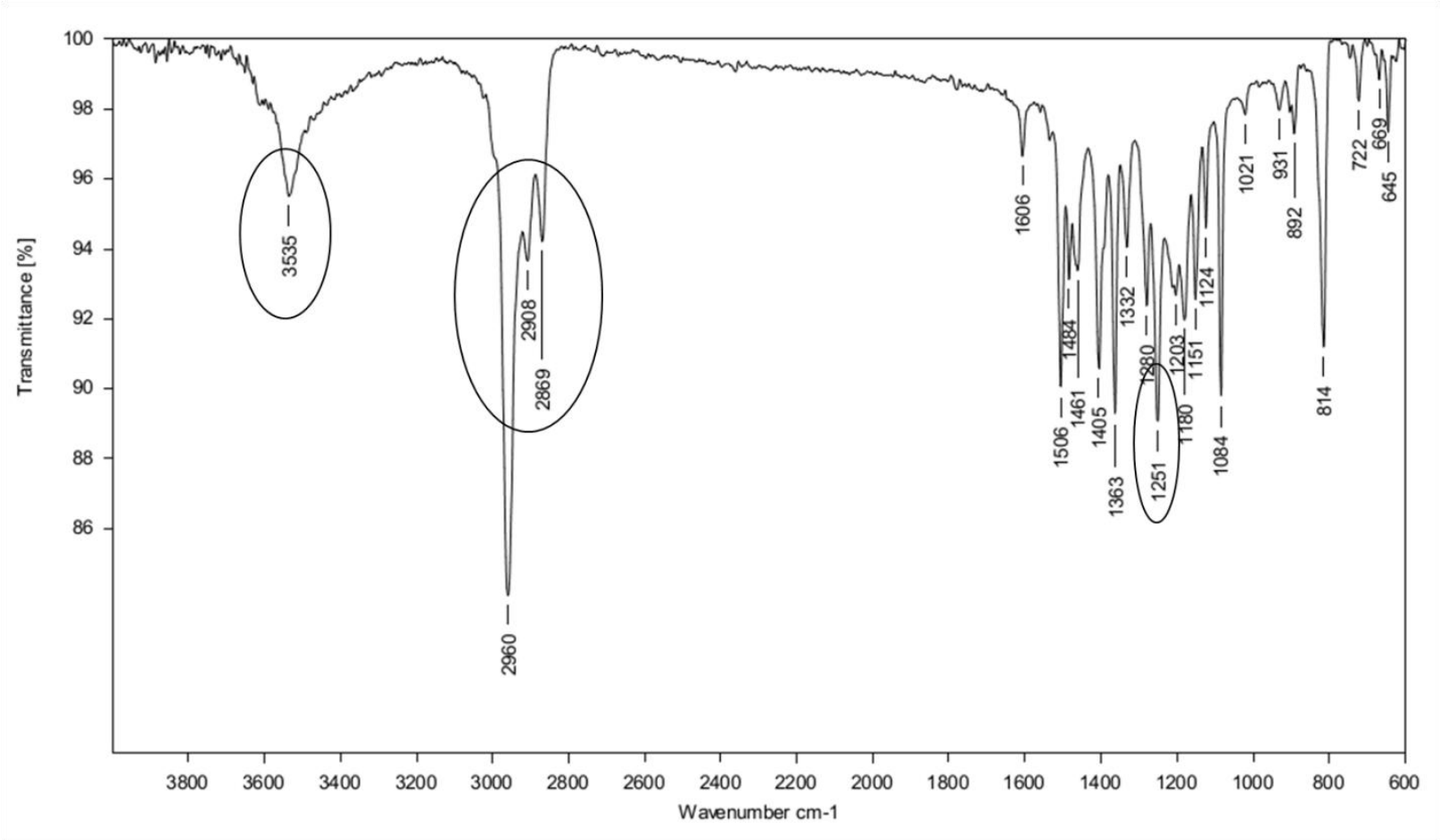


Fig.7.7 FTIR spectrum of 2,4 di- *tert*- butyl phenol

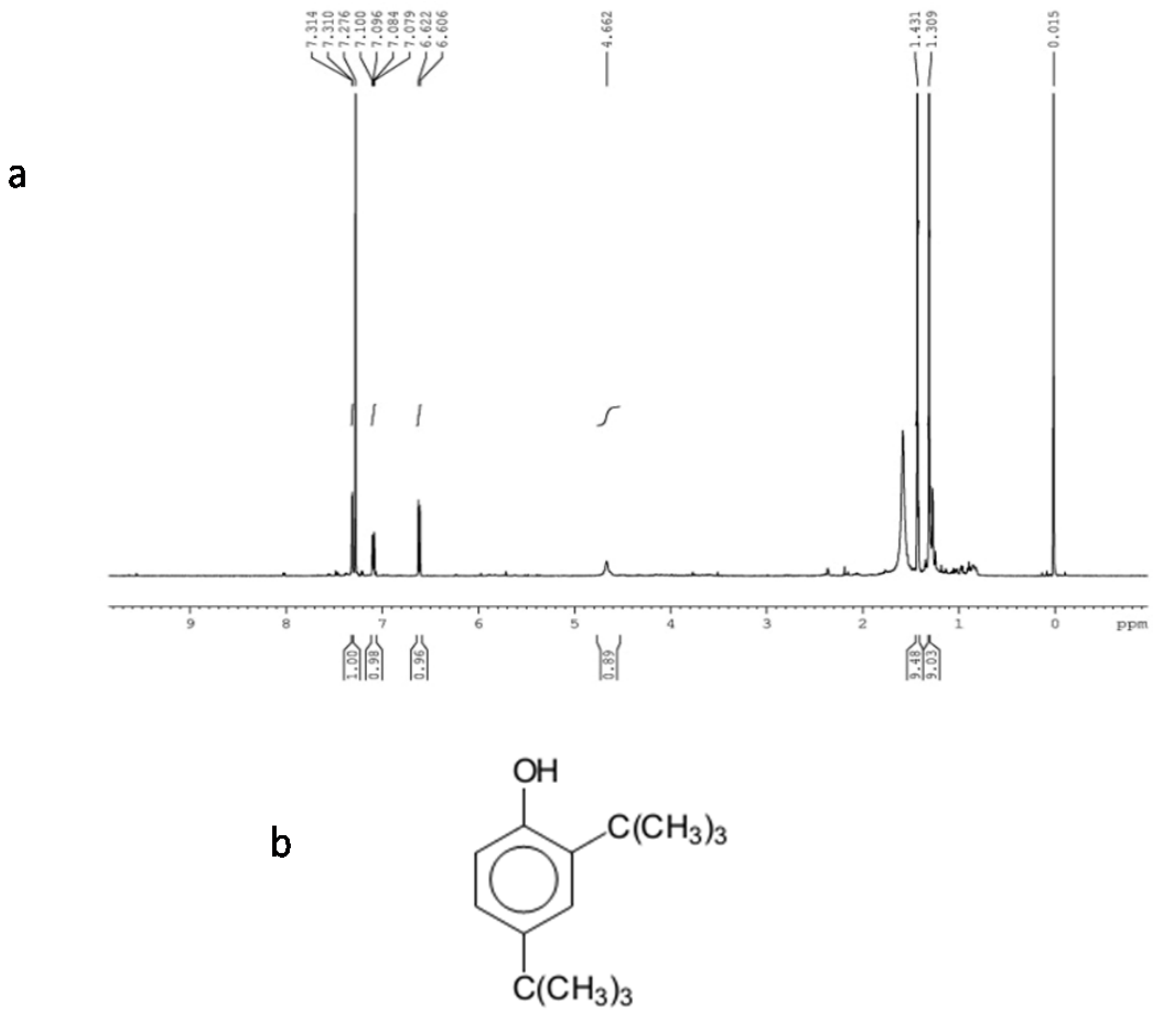


Fig.7.8 Structure elucidation of 2,4 di- *tert*- butyl phenol. ^{1}H NMR spectrum (a) structure of 2,4 DTBP (b)

clusters and enzymes required for shikimate and mevalonate pathway have been identified in *Lactobacillus* (Bolotin et al., 2001; Smeds et al., 2001).

7. 3. 3. Antifungal activity assay of the pure compounds

Apart from *F. oxysporum* (Fig.7.6d), 2,4 DTBP was fungicidal in activity against other fungal strains tested. 400 µg/disc produced a clearing zone of 0.8 cm against *A. niger*, 1.5 cm against *P. chrysogenum* and 1.6 cm against *F. moniliforme* (Fig.7.9). Similarly, the phenazine compound (400 µg/disc) also

produced a clear zone of 1.7 cm against *F. moniliforme* and 1.4 cm against *P. chrysogenum*. Growth inhibitory activity against *A. niger* was negligible (Fig.7.10). Three different genera of fungi often cause contamination in various food products were selected to study the antifungal activity and found that all of them are sensitive to the compounds.

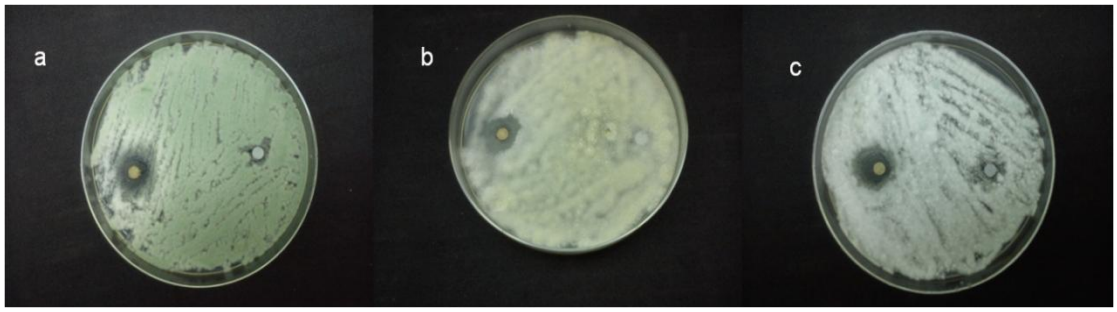


Fig.7.9 Antifungal activity of 2,4 di- *tert*- butyl phenol against *P. chrysogenum* (a), *A. niger* (b) and *F. moniliforme* (c)

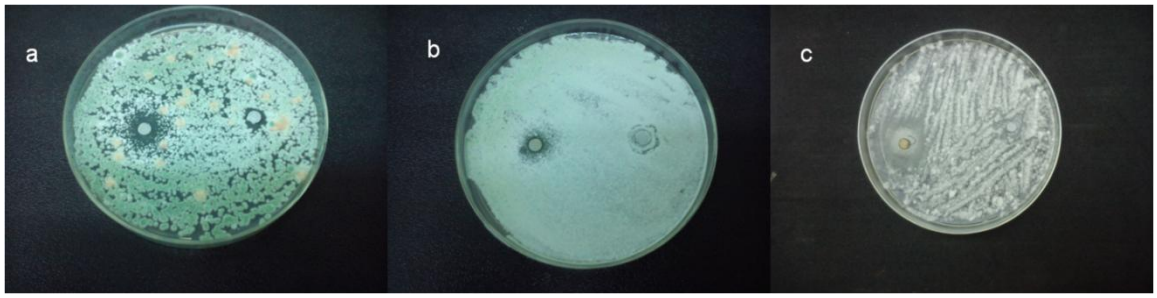


Fig.7.10 Antifungal activity of phenazine compound against *P. chrysogenum* (a), *A. niger* (b) and *F. moniliforme* (c)

This result supports and confirms previous researches on the antifungal activity of volatile organic compound (VOC) produced by bacteria (Yuan et al., 2012; Weisskopf, 2014). This volatile phenolic compound has been reported to be present in fruits as well as seeds and imparts antioxidant properties (Choi et al., 2013). 2,4 DTBP is reported to have *in vitro* antimalarial activity where the plasmodial growth was arrested with an amount of 100 mM compound (Kusch et al., 2011). One recent report indicates the presence of 2,4 DTBP in the culture

filtrate (CF) of an antifungal *Lactobacillus* strain where the compound was detected by GC-MS analysis (Sangmanee and Hongpattarakere, 2014). Dharni et al. (2014) reported prevention of spore germination of *F. oxysporum* with 100 µg/mL of 2,4 DTBP. The proposed mechanism of antifungal activity as described by this group was the inhibition of spore germination by preventing the emergence of normal germ tube and leads to abnormal swelling and branching of hyphae. It is demonstrated that, 2,4 DTBP inhibits the assembly of spindle microtubules and disturbs the chromosomal alignment at the metaphase plate and microtubule- kinetochore interactions, causing chromatids loss, which may reduce the mycelia growth and the germination of spores. Our study confirms the earlier reports as well as describes the efficiency of this compound in prevention of *A. niger* and *P. chrysogenum* growth along with growth inhibition of *F. oxysporum* and directs towards the possibility of production of other VOC with various biological activities from LAB.

The capacity of phenazine compounds to inhibit the growth of pathogens has been attributed to the ability to generate reactive oxygen species and oxidative stress in other organisms (Price-Whelan et al., 2006). Almost all phenazines are inhibitory to the growth of bacteria and fungi due to their ability to undergo cellular redox cycling in the presence of oxygen and reducing agents and cause accumulation of toxic superoxide and hydrogen peroxide (Mavrodi et al., 2001). Phenazine production by soil-borne bacteria was shown to control the growth of plant pathogenic fungi (Bolwerk et al., 2003). Phenazine-1-carboxamide (PCN) was shown to control *F. oxysporum* f. sp. *radicis lycopersici*, the causative agent of tomato foot and root rot and both PCA and PCN control *Pythium myriotylum*, the causative agent of root rot of cocoyam (Hofte and Altier, 2010; Tambong and Hofte, 2001).

7. 3. 4. Antioxidant activity and free radical scavenging assay

Evaluation of total antioxidant capacity (TAC) by phosphomolybdenum method showed that 40 µg of B1 fraction had 80% antioxidant activity where the same amount of ascorbic acid had 83% activity. Ferric ion reducing power assay

revealed that 1 mg of this fraction hold 33% of reducing power and the same amount of ascorbic acid owned 50% activity.

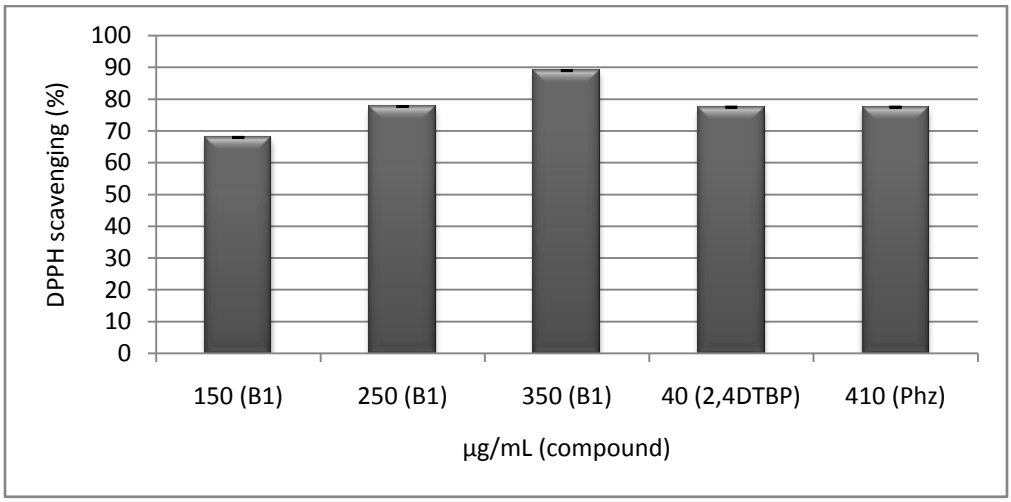


Fig.7.11 DPPH scavenging activity of different concentrations of B1 fraction (150-350 µg/mL) and pure 2,4 di- *tert*- butyl phenol (40 µg/mL 2,4 DTBP) and phenazine compound (410 µg/mL Phz) ($p < 0.05$)

The percentage of free radical scavenging activity of B1 fraction, phenazine compound and 2,4 DTBP is shown in Fig.7.11. 40 µg of purified 2,4 DTBP had 77.5% free radical scavenging ability and 410 µg of phenazine compound caused the same amount of free radical scavenging.

2,4 DTBP was reported to be associated with the antioxidant effect of methanolic extract from dried *Scolopendra subspinipes mutilans* (Scolopendridae), a centipede that has been utilized as a traditional Chinese and Korean medicine for a variety of diseases, such as spasm, seizure, poisonous nodules, childhood convulsions, diphtheria and tetanus. The antioxidant effects displayed by this compound include prevention of LDL oxidation and DPPH scavenging ability (Yoon et al., 2006). Choi et al. (2011) showed in a study that the ethanol extract of pomegranate had antioxidant and neuronal protective effects and diminished hydrogen peroxide induced oxidative stress in PC-12 cells. The protective effect against learning and memory impairment induced by $A\beta_{1-42}$ was

due to the antioxidant effect of 2,4 DTBP where ABTS radical assay was followed to confirm the antioxidant activity of 2,4 DTBP. The disease prevention roles of fruits, vegetables and red wine have been attributed, in part, to the antioxidant properties of their constituent polyphenols (vitamins E and C, and the carotenoids) and the antioxidant property of plant materials found to increase with an increase in the amount of total phenols (Rice-Evans et al., 1997, Velioglu 1998).

Benthocyanin B and its congener benthocyanin C, phenazine derivatives isolated from *Psueodomonas punicolar* are free radical scavengers that inhibit lipid peroxidation induced by free radicals in rat liver microsomes (Shinya et al., 1993). Apart from the excellent antifungal activity, the antioxidant activity assays revealed the potential of this fraction to act as reducing agent and thus inhibiting the oxidation of molecules as well as the production of free radicals. Based on this study and also the previous studies 2,4 DTBP and phenazine prove as natural antioxidants with protective effect against oxidative damage.

7. 3. 5. Assay of cytotoxicity to cancer cell lines

2,4 DTBP displayed remarkable cytotoxic activity against HeLa cell lines with an IC_{50} value of 10 µg/mL and IC_{50} against MCF-7 was achieved with 16 µg/mL. Against the normal cell line H9c2, a higher amount of 19 µg/mL was required to bring about IC_{50}. The values are represented in Fig.7.12. In the case of phenazine compound, 20 µg/mL and 24 µg/mL were required to bring abount IC_{50} in HeLa and MCF-7 cell lines respectively. At the same time no growth inhibition was observed against normal H9c2 cell lines up to an amount of 55 µg/mL (Fig.7.13). Cytotoxic property of 2,4 DTBP was reported before by different research groups. Malek et al. (2009) purified 2,4 DTBP from the leaves of *Pereskia bleo* (Kunth) where it represents a major component and found to have cytotoxic effect on the human cancer cell lines; KB, MCF-7, CasKi, HCT 116, A549 and in normal human cell line MRC-5. In their study, IC_{50} against MCF-7 was obtained with 5.75 µg/mL and in MRC-5 with 20 µg/mL.

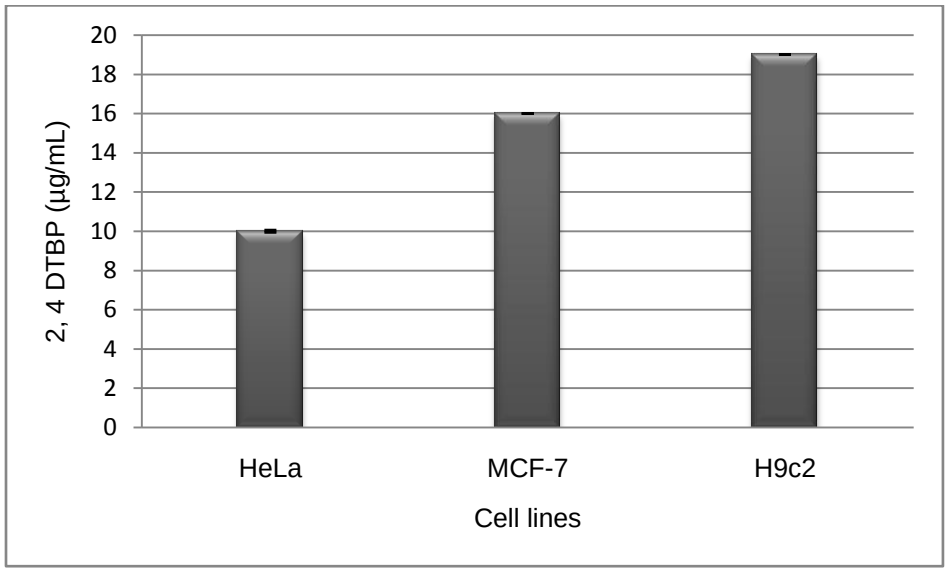


Fig.7.12 IC_{50} of 2,4 di- *tert*- butyl phenol in µg/mL against different cell lines ($p < 0.05$)

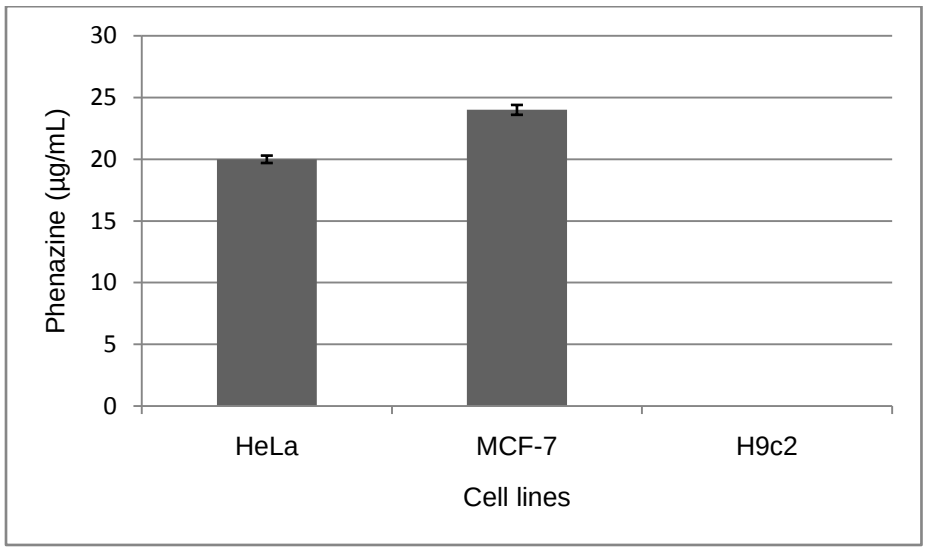


Fig.7.13 IC_{50} of phenazine compound in µg/mL against different cell lines ($p < 0.05$)

At the same time 29 µg/mL was required in HCT 116 cell line and below 6 µg/mL was enough to bring about IC_{50} in other cell lines used. Leaves of Kunth are used traditionally in Malaysia for the treatment of cancer, diabetes, high blood

pressure and diseases associated with rheumatism and inflammation as well as remedy for the relief of gastric pain, ulcers and for revitalizing the body. Presence of this compound in plants is not yet explained biogenetically but the same authors (Malek et al., 2009) reported the presence of 2,4 DTBP in other plants like *Termitomyces heimi* and *Hericium erinaceus.*

In a study, sweet potato extract is reported to have protective effect against hydrogen peroxide-induced oxidative stress and cytotoxicity on the pheochromocytoma cell line (PC12). The active component in this extract was purified and identified as 2, 4 DTBP and administered to check the effects on amyloid-beta peptide ($A\beta_{1-42}$) induced learning and memory impairment in mice and shown to have a protective effect against $A\beta_{1-42}$ by decreasing neuronal cell damage and increased spontaneous alteration behavior in mice when provided diet supplemented with 2,4 DTBP (Choi et al., 2013). A previous study (Chen et al., 2007), reported the effect of six day old extracts of fermented milk product kefir reduced the growth of human mammary cancer cells (MCF-7) in a dose-dependent manner, showing 29% inhibition of proliferation at a concentration of 0.63% without any anti-proliferative effect against normal human mammary epithelial cells (HMECs). Even though it has certain degree of cytotoxicity in normal cell lines, studies show that 2,4 DTBP is a common ingredient in many medicinal preparations and can be helpful during disease conditions.

Phenazines are known to be associated with anti-tumor activities. Actively respiring cells, like cancer cells appear to be more susceptible to respiratory interference and reactive oxygen species (ROS) generation by phenazines (Laursen and Nielsen, 2004). Phenazine compounds also interfere with topoisomerase I and II activities in eukaryotic cells and cancer cells, having high levels of both topoisomerases are more susceptible to this interference (Pierson Iii and Pierson, 2010). Development of anti-cancer drugs based on phenazine derivatives is an ongoing area of research. Lavanducyanin and two new brominated, terpenoid phenazines from marine *Streptomyces* sp.inhibited TNF-α-induced NFκB activity (IC_{50} values of 4.1, 24.2 and 16.3 µM respectively) in

mammalian cell culture studies and treatment of cultured HL-60 cells led to dose-dependent accumulation in the subG1 compartment of the cell cycle, as a result of apoptosis (Kondratyuk et al., 2012). Saphenamycin isolated from *S. canaries* and *S. antibioticus*, showed IC_{50} values of 0.15 µg/mL in L5178Y and of 0.6 µg/mL in L1210 mouse leukaemia cell lines (Kitahara et al., 1982) and IC_{50} of 0.6 µg/mL in CCRF/CEM T-cell leukaemia cells (Geiger et al., 1988; Laursen and Nielsen, 2004). Apart from this, saphenamycin displays life-prolonging effect on mice with leukaemia cell implants. 250 µg/mL mouse/day ×10 days slightly ($p>0.05$) prolonged by 19 and 20 % the survival of mice intra-peritoneally implanted with the L1210 leukaemia and the Ehrlich ascite carcinoma, respectively (Cimmino et al., 2012; Kitahara et al., 1982).

Presence of 2,4 DTBP and phenazine in the cell free supernatant (CFS) of LAB explains one of the reasons behind many of the health benefits including anticancer properties provided by them which represent the major human microbiota and the common starter culture in food fermentation. Since LAB are normal part of our daily diet, their intake can bring along therapeutic effects considering they are source of natural antioxidants and may be particularly useful when long term treatment is required.

7. 3. 6. Aminopeptidase inhibitory activity

The enzyme activity calculated for LAP was 4.1±0.04 IU/mL and 1±0.08 IU/mL for PAP. B1 fraction inhibited 65% activity of LAP and 91% activity of PAP. 2,4 DTBP inhibited 66% of LAP activity and 70% of PAP activity whereas, phenazine compound prevented 67.7% activity of LAP and 94% activity of PAP (Fig.7.14). The mechanisms of how these particular compounds cause aminopeptidase inhibition is not studied thoroughly and much data are not available about the same.

Analogues that stimulate the immune system are potent inhibitors of aminopeptidases (Rich et al., 1984). Natural phenolic compounds are reported to have aminopeptidase inhibitory activity. Psammaplin A (PsA), a phenolic natural product isolated from a marine sponge, showed potent cytotoxicity against several

cancer cell lines and also was found to inhibit mammalian aminopeptidase N (APN) that plays a key role in tumor cell invasion and angiogenesis. PsA inhibited the APN activity with an IC_{50} of 18 µM in a non-competitive manner (Shim et al., 2004). Curcumin, another phenolic natural product, binds CD13/aminopeptidase N (APN) and irreversibly inhibits its activity. CD13/aminopeptidase N (APN) is a membrane-bound, zinc-dependent metalloproteinase that plays a key role in tumor invasion and angiogenesis. Accordingly, APN is considered an important therapeutic target for tumor angiogenesis and metastasis (Shim et al., 2003).

Gilpin et al. (1995) reported that phenazine compounds produced by *Streptomyces* are reported to possess metalloenzyme inhibitory activity. Enzyme-inhibitor binding studies revealed that hydroxyl group of bestatin plays a major role in the initial collision complex between the inhibitor and the enzyme and thus in aminopeptidase inhibitory activity (Burley et al., 1991). This may explain the reason behind the inhibitory activity of 2,4 DTBP and the phenazine compound.

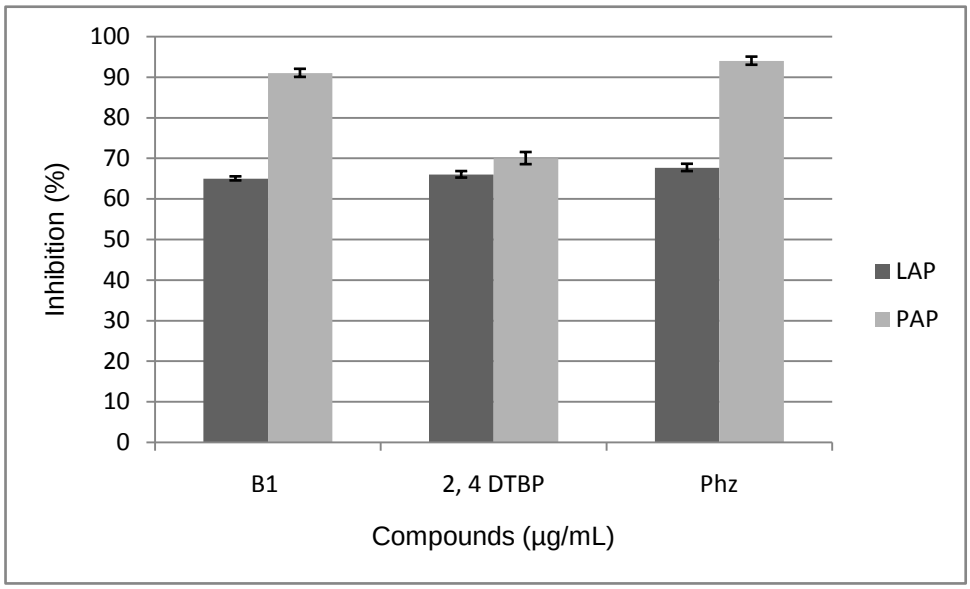


Fig.7.14 Inhibition of B1 fraction (12.5 mg), 2,4 DTBP (500 µg) and phenazine compound- phz (500 µg) against LAP and PAP ($p < 0.05$)

Moore et al. (2009) showed that myeloma cells depend on unfolded protein response (UPR) to assemble folded immunoglobulins correctly and targeting this protein handling within myeloma cells by aminopeptidase enzyme inhibition resulted in marked inhibition of myeloma cell growth and survival, and thus is a novel cancer treatment strategy. For cancer cells, the supply of cellular free amino acids, regulated by aminopeptidases, is of utmost importance for their survival and proliferation (Hitzerd et al., 2013). Amino peptidase inhibition disrupts protein turnover leading to an accumulation of peptides and a reduction in the cellular free amino acid content, which has profound effect on cell survival and proliferation especially in myeloma cells which are more reliant on protein production and UPR (Davenport et al., 2007). Clinical trials of cancer therapies combined with aminopeptidase inhibitors are in progress. Bestatin is an example of aminopeptidase inhibitor used to treat acute and chronic acute myeloid leukemia under the trademark Ubenimex in Japan (Ichinose et al., 2003). It is possible that a bioactive that is inhibitory to *Streptomyces* aminopeptidase can be inhibitory to eukaryotic aminopeptidases too. Both human and *Streptomyces* LAP belong to the M28 family that have two zinc atoms in their active site. The double -zinc coordinated active centre carries structural similarity among LAP from different sources (Arima et al., 2006). Amastatin which mimics the LAP catalytic transition state is a strong inhibitor of eukaryotic aminopeptidases and is also inhibitory to LAP of *Streptomyces hygroscopicus* (Liew et al., 2013).

7. 4. Conclusion

Antifungal and antioxidant property of 2,4 DTBP (206 Da) and the phenazine compound (210 Da) purified from BSN307 demonstrate the potential of these compounds to develop as food additive so as to improve the food safety as well as its health promoting characteristics. Cytotoxicity against cancer cell lines compared to normal cell lines and aminopeptidase inhibitor property revealed the diverse biological properties of these bioactives and their potential in development of new therapeutic drugs.

CHAPTER 8

Purification and characterization of a bioactive peptide from *Lactococcus garvieae* subsp. *gaurensis* BSN307

8. 1. Introduction

The majority of studies with peptides of lactic acid bacteria (LAB) are focused on the aspect of food preservation with a few concentrating on the area of therapeutic applications. Antimicrobial peptides (AMPs) or proteins produced by LAB are called bacteriocins and are more likely to meet the regulatory approval due to their origin because of the generally recognized as safe (GRAS) status of LAB and can be readily introduced into food products without further processing like purification. Since they are antimicrobial, their use as anti infectious agents in diseases caused by bacteria can also be envisaged. This is of particular interest when considering the fact that bacteriocins are easily degraded after exerting their action on pathogens by the proteolytic enzyme system of the host minimizing the opportunity for the pathogen to interact with and develop resistance.

In a non-antibiotic approach, lacticin 3147 produced by *Lactococcus lactis* was used to treat mastitis in non-lactating dairy cows effectively (Ryan et al., 1999). The primary metabolite nature and relatively simple biosynthetic mechanism make them acquiescent to bioengineering techniques to enhance their activity or specificity towards microorganisms (Perez et al., 2014). Study by Luders et al. (2003) showed that a combination of LAB bacteriocins, pediocin PA-1, sakacin P, and curvacin A with the eukaryotic AMP pleurocidin (from fish) increased the growth inhibitory potency of pleurocidin against *Escherichia coli* by about fourfold. The cationic, amphipathic nature of peptides can make them potential anti cancer agents. Cytotoxic AMPs are placed into two categories, AMPs that are highly potent against bacteria and cancer cells but not against normal mammalian cells and AMPs that are cytotoxic for bacteria, cancer cells, and normal mammalian cells (Hoskin and Ramamoorthy, 2008). The antioxidant

and anticancer potential of LAB peptides are not characterized well and no reports are available about the same.

Antimicrobial peptides possessing antifungal activity and of bacterial origin are very poorly addressed though a number of such peptides from plants, fungi, invertebrates and mammals are thoroughly studied and characterized. LAB bacteriocins are generally active against closely related species and only a few reports are available about the production of antifungal proteinaceous compounds by LAB. A recent report explains the AMPs LR14 from *L. plantarum* strain LR/14 inhibited the growth of *Aspergillus niger*, *Rhizopus stolonifer*, *Mucor racemosus* and *Penicillium chrysogenum* (Gupta and Srivastava, 2014). Antifungal peptides of less than 3 kDa from LAB are reported to have inhibitory activity against molds and yeast (Gourama and Bullerman, 1997; Magnusson and schnurer, 2001) though structural characterization and mechanism of action are remains to be unraveled.

This chapter discusses the purification and characterization of a novel AMP of 8595 Da size named niistin from the cell free supernatant (CFS) of the novel strain BSN307. Molecular weight of niistin is different from other peptides reported from *L. garvieae* and shows a wide range of biological activities unlike other bacteriocins from LAB. Bioactivities of this peptide include antibacterial, antifungal, free radical scavenging and anticancer potential. The preliminary assay for antifungal activity of niistin by neutralizing pH was explained in section **5. 3. 3**. This is the first study explaining the purification and potential of an AMP from LAB possessing antioxidant and anticancer property that can induce apoptosis selectively in cancerous mammalian cells.

8. 2. Materials and methods

8. 2. 1. Microorganisms and culture conditions

Lactococcus garvieae subsp. *gaurensis* BSN307 and *Enterococcus faecalis* were cultivated on de Man Rogosa Sharpe (MRS) medium (Himedia, India) at 30 ^{o}C for 24 h and maintained for longer storage at -20 ^{o}C in MRS broth with 20% (w/v) glycerol. The fungal test strain, *Fusarium oxysporum* (KACC

42109) was maintained on potato dextrose agar (Himedia, India) at 30 ^{o}C. Strains of *Escherischia coli*, *Serratia marcescens, Staphylococcus aureus* (isolates from NIIST) and *Listeria monocytogenes* (MTCC 657) were maintained on nutrient agar at 30 ^{o}C.

8. 2. 2. Production and purification of niistin

For production and purification, the method previously described by Maldonado-Barragan et al. (2013) was followed with some modifications. 5-liter of MRS broth was inoculated with 24 h old BSN307 cells [5 % (v/v)] and incubated at 30 °C for 24 h. After incubation, the cell free supernatant (CFS) was collected by centrifugation at 8,590×g for 15 min at 4 ^{o}C and subjected to hydrophobic interaction chromatography (HIC). The CFS was mixed with 30 g per liter of Amberlite XAD-16 by stirring in a conical flask at room temperature for 2 h. The resin was washed with 50% (v/v) ethanol in distilled water (1L) and niistin was eluted with 70% (v/v) 2-propanol in distilled water supplemented with 10 mM acetic acid (1L). After evaporation under reduced pressure in a rotavapour (Buchi, Switzerland), the precipitate was resuspended in 50 mM phosphate buffer (pH 7) and further purified by cation exchange chromatography (CEC) using SP sepharose according to Villani et al. (2001). The column was washed with 50 mM phosphate buffer (pH 7) and niistin was eluted with the same buffer supplemented with 1M NaCl. Purified niistin was desalted further by reverse-phase column (RPC) chromatography using HPLC (UFLC, Shimadzu, Japan). Column was equilibrated with 5% (v/v) acetonitrile containing 0.1% (v/v) trifluroacetic acid (TFA) and niistin was eluted with a linear gradient of 5-25% of acetonitrile containing 0.1% TFA for 15 min at 220 nm. Fractions obtained were checked for antimicrobial activity by microtiter plate method as described before. Amount of protein was determined by Bicinchoninic acid (BCA assay kit, Merck) assay (Smith et al., 1985). Purity of niistin was checked by HPLC analysis as explained above and by SDS tricine PAGE analysis as described below.

8. 2. 3. SDS- PAGE analysis

Fractions after CEC was analyzed by 16% Tris-tricine-SDS-PAGE gel with 6M urea as explained in section **3. 3. 5. 3. 2**

8. 2. 4. Mass spectrometry analysis and peptide mass fingerprinting

Molecular mass of pure niistin was determined by matrix assisted laser desorption ionization using BRUKER Ultraflex TOF/TOF (USA) in the linear positive ion mode using sinapinic acid as matrix. For PMF niistin was cut eluted from the gel, digested with trypsin and analyzed in reflectron positive ion mode using 19 KV acceleration voltage. The eluted peptides were co-crystallized with α-cyano-4-hydroxycinnamic acid matrix on the target plate (384-well ground steel plate; Bruker Daltonics, Bremen, Germany) and external peptide mass calibration was applied (Peptide mixture 1) as per the manufacturer's instructions. The mass spectra was acquired in the mass range of 700-3500m/z and MALDI-TOF/TOF fragment ion analysis of selected peptides was carried out in the LIFT mode of the instrument. In order to confirm the identification, all MS/MS data from LIFT TOF/TOF spectra were combined with the corresponding MS peptide mass and mascot search was done with following parameters. However Peak list was generated using flex analysis software 2.2. Human keratin and trypsin autodigest peptide ions were excluded prior mass spectra were imported into the database search engine (BioTools v2.2 connected to Mascot, version 2.2.04; Matrix Science).

Mascot searches were done using the NCBI and Swissprot with the following settings: number of miss cleavages permitted was 1; fixed modifications such as carbamidomethyl on cysteine, variable modification of oxidation on methionine residue; peptide tolerance of 100 ppm, enzyme used as trypsin and a peptide charge setting as +1.

8. 2. 5. Antimicrobial activity spectrum of niistin

Antimicrobial activity of niistin after HIC purification was determined against *E. coli*, *S. marcescens* and *S. aureus.* Pure niistin after CEC was checked for activity against *L. monocytogenes, E. faecalis* and *F. oxysporum.* Experiment was performed by microtiter plate (Nunc, Thermoscietific, USA) method based

on a decrease in absorbance at 600 nm using Tecan NanoQuant Plate Reader (Switzerland) in the presence of niistin. 200 µg of niistin was present in each reaction along with the corresponding growth medium to a final volume of 300 µL. 20 µL of 24 h old bacterial cells (A_{600} 0.1) and 20 µL of 24 h old fungal broth were inoculated into each well. The absorbance of *E. coli*, *S. marcescens*, *S. aureus* and *E. faecalis* were checked after 6 h of incubation and *L. monocytogenes* and *F. oxysporum* after 24 h incubation.

8. 2. 6. Effect of lipase and proteolytic enzymes on antimicrobial activity

The enzymes; lipase (1 mg), proteinase k (25 µg), trypsin (50 µg) and α-chymotrypsin (100 µg) from Sigma-Aldrich, USA were prepared in appropriate buffers and treated with niistin at specific pH and temperature required for the enzyme. The effects of different enzymes on antimicrobial activity were checked against *E. coli* by microtiter plate method as explained above.

8. 2. 7. Assay of free radical scavenging

Free radical scavenging activity of the peptide was determined by the method of Shimada et al. (1992) which is based on the principle of scavenging DPPH (1,1-diphenyl-2-picrylhydrazyl) radical. DPPH was prepared in 95% (v/v) ethanol to a final concentration of 0.2 mg/5 mL. Radical-scavenging activity of 500 µg of pure niistin was checked and expressed as percentage scavenging of DPPH. Each mixture was kept in the dark for 30 min and the absorbance was measured at 517 nm against a blank using Tecan NanoQuant (Switzerland). The scavenging activity was defined as:

$$\text{Scavenging activity (\%)} = (A_{\text{Control}} - A_{\text{Sample}})/A_{\text{Control}} \times 100$$

8. 2. 8. Assay of cytotoxicity to cancer cell lines

Cytotoxicity against mammalian cells was determined by MTT assay following the protocol of Mossman (1983), where the tetrazolium salt MTT is converted into blue colored formazan by the mitochondrial enzyme succinate dehydrogenase in living cells and measured for quantifying cell survival and proliferation. Briefly, mammalian cell lines, HeLa, H9c2, and MCF-7 were maintained in DMEM supplemented with 10% (w/v) FBS, 10 mg/L streptomycin,

100 U/L penicillin and 25 µg/mL amphotericin B. The cells were maintained at 37 ^{o}C in a humidified atmosphere of 5% CO_2 until a confluent monolayer was obtained. Cells were seeded at a concentration of 1×10^{4} cells into each well of a 96-well tissue culture plate followed by incubation at the same conditions for 24 h. After removing the medium, cells were treated with varying concentrations of purified niistin prepared in DMEM without FBS and incubated in the CO_2 incubator (SANYO CO_2 Incubator, Japan) for 24 h after which medium was removed and 200 µL of MTT (0.5 mg/mL) dissolved in DMEM was added followed by incubation in dark for 3 h. The reaction was stopped by addition of 100 µL DMSO into the wells. Plates were further incubated for 30 min in a shaker and the absorbance was read at 570 nm using Synergy^{TM} 4 multimode Microplate Reader (USA). The experiments were performed in triplicates. Cytotoxicity (%) and IC_{50} (effective concentration of drug resulting in 50% of maximal toxicity) were calculated as follows;

Cytotoxicity (%) = $[1- (A_{Test}/A_{Control})] \times 100$

8. 2. 9. Fluorescent microscope analyses

To understand the nature of cytotoxicity, IC_{50} concentration of niistin was added to the H9c2 and HeLa cell lines and after incubation (as explained in section **8.2.8**) were visualized for live cell imaging under spinning disc fluorescent microscope (BD Pathway TM Bioimager system, USA). DAPI (4', 6-diamidino-2-phenylindol) staining has been done as a qualitative probe for DNA molecules because of its high sensitivity where DNA-DAPI complexes emit strong bluish white fluorescence when excited by ultraviolet light. For this, H9c2 and HeLa cell lines after treating with IC_{50} concentration of niistin as explained before were stained with 50 µL of DAPI (300 nM in PBS) for 5 min followed by 3-4 times washing with PBS to remove unbound DAPI. The cells were then observed under spinning disc fluorescent microscope. Excitation maximum for DAPI bound to ds DNA is 358 nm, with 461 nm as emission maximum and was excited with a xenon arc lamp.

8. 2. 10. Caspase-3 activation assay

To confirm induction of apoptosis, activation of caspase-3 was checked by Caspase-3 fluorescence assay kit (Cayman chemicals, USA). The kit employs a specific caspase-3 substrate, N-Ac-DEVD-N'-MC-R110 which upon cleavage by active caspase generates a highly fluorescent product that can be measured using excitation and emission wavelengths of 485 and 535 nm. After treatment of H9c2 and HeLa cell lines with IC_{50} concentration of niistin as explained in section **8. 2. 8** the plates were centrifuged (Beckman Coulter, USA) at 800 × g for 5 min to remove the culture medium and the cells were collected and performed the assay according to the protocol provided by manufacturer. Synergy^{TM} 4 multimode Microplate Reader (USA) was used to take readings.

8. 2. 11. Statistical analysis

All the experiments were performed in triplicates and the results were expressed as mean values ± standard deviation. One-way ANOVA and Dunnet's test were performed to test the differences between test and control groups ($p < 0.05$). Statistical analyses were performed using the Minitab statistical package v. 17 (Minitab Inc., USA).

8. 3. Results and discussion

8. 3. 1. Purification, molecular weight determination and peptide mass fingerprinting of niistin

Niistin was purified from 5L cell free supernatant (CFS) of BSN307 after 24 h of incubation by the three step purification that includes HIC, CEC and reverse-phase HPLC. Throughout the purification process niistin showed the characteristics of highly hydrophobic and cationic peptide. Antimicrobial activity of the CFS was completely recovered in the 70% isopropanol fraction of HIC purification and in the 1M NaCl fraction after CEC. HPLC analysis showed a major peak of protein at 220 nm apart from the buffer components (Fig.8.1a) which were separated to get the pure protein (Fig.8.1b) and that fraction was used for Tris Tricine SDS-PAGE and molecular weight determination bt MALDI-TOF analysis.

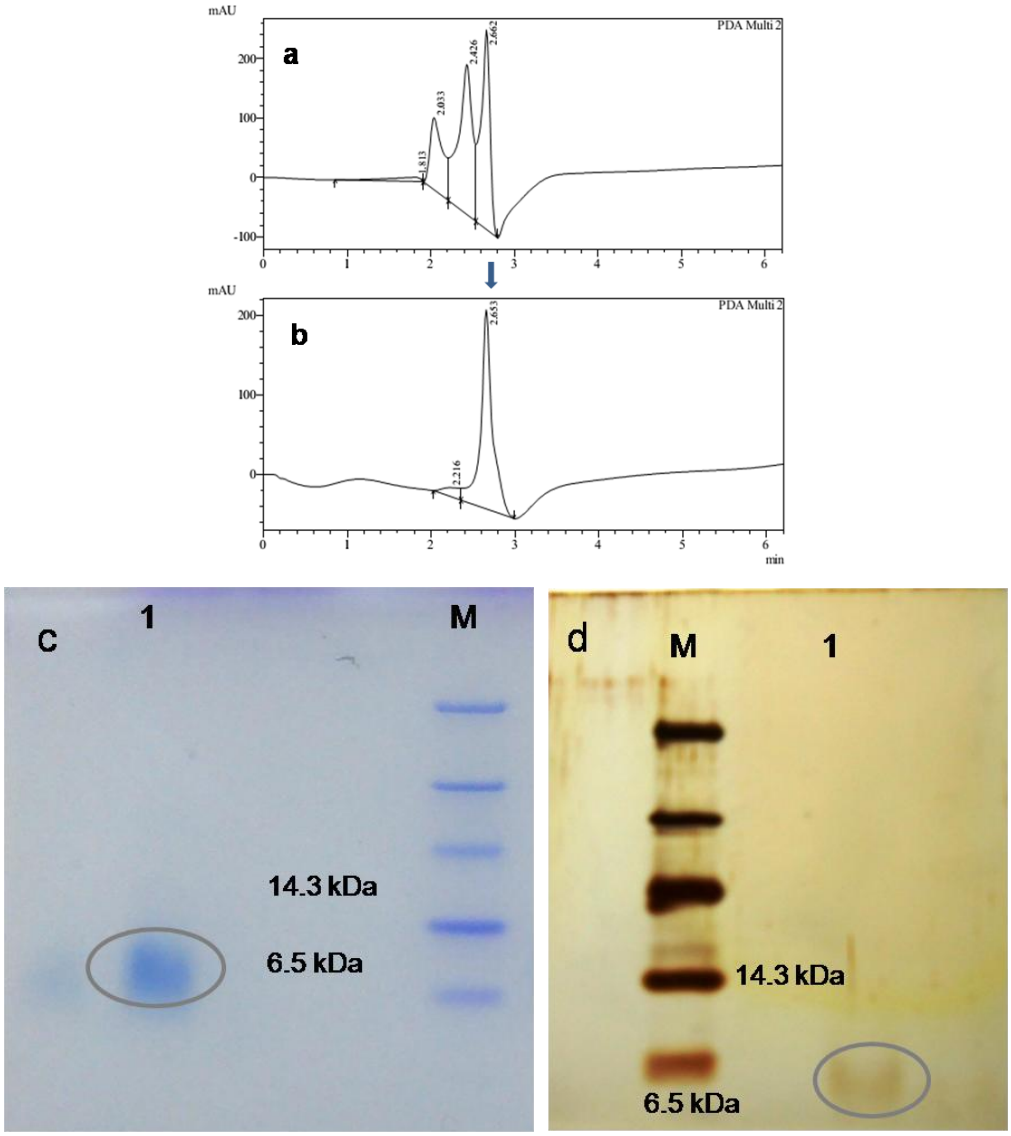


Fig.8.1 HPLC analysis of the CEC purified niistin fraction (a) and pure niistin after desalting (b) along with 16% tricine coomassie stained gel (c) and silver stained gel (d) with niistin in circles

Because of the highly hydrophobic nature, niistin appeared as a smear instead of a distinct band in the tricine gel and was later rectified to an extent with incorporation of 6 M urea into the separating gel and 8 M urea into the sample buffer according to Schagger (2006). The experiment was set in a cool

environment. The band obtained by both coomassie (Fig.8.1c, lane 1) and silver stain (Fig.8.1d, lane 1) were not very sharp and was near to 6.5-8.5 kDa. MALDI-TOF analysis of the pure protein indicated a monoisotopic peak $[M+H]^+$ of the AMP, suggesting that the molecular mass of niistin is 8595 Da (Fig.8.2). Four peptides from *L. garvieae* are reported so far, garviecin L1-5 (2.5 kDa), garvieacin Q (5339 Da), garvicin ML (6022 Da) and garvicin A (4678 Da) (Maldonado-Barragan et al. 2013), and the molecular weight of niistin is different from them.

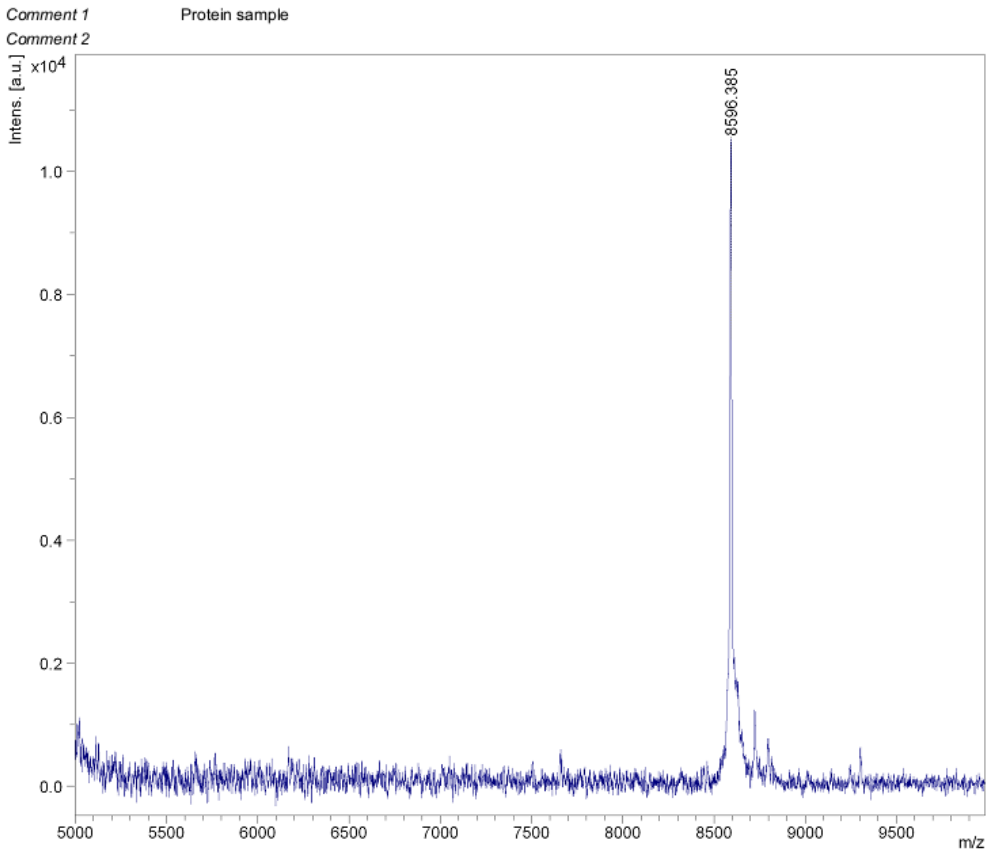


Fig.8.2 MALDI-TOF analysis of niistin indicating a monoisotopic peak $[M+H]^+$, suggesting that the molecular mass is 8595 Da

The peptides generated by PMF produced no significant search results. Four peptide fragments were selected after confirmation by MS/MS and

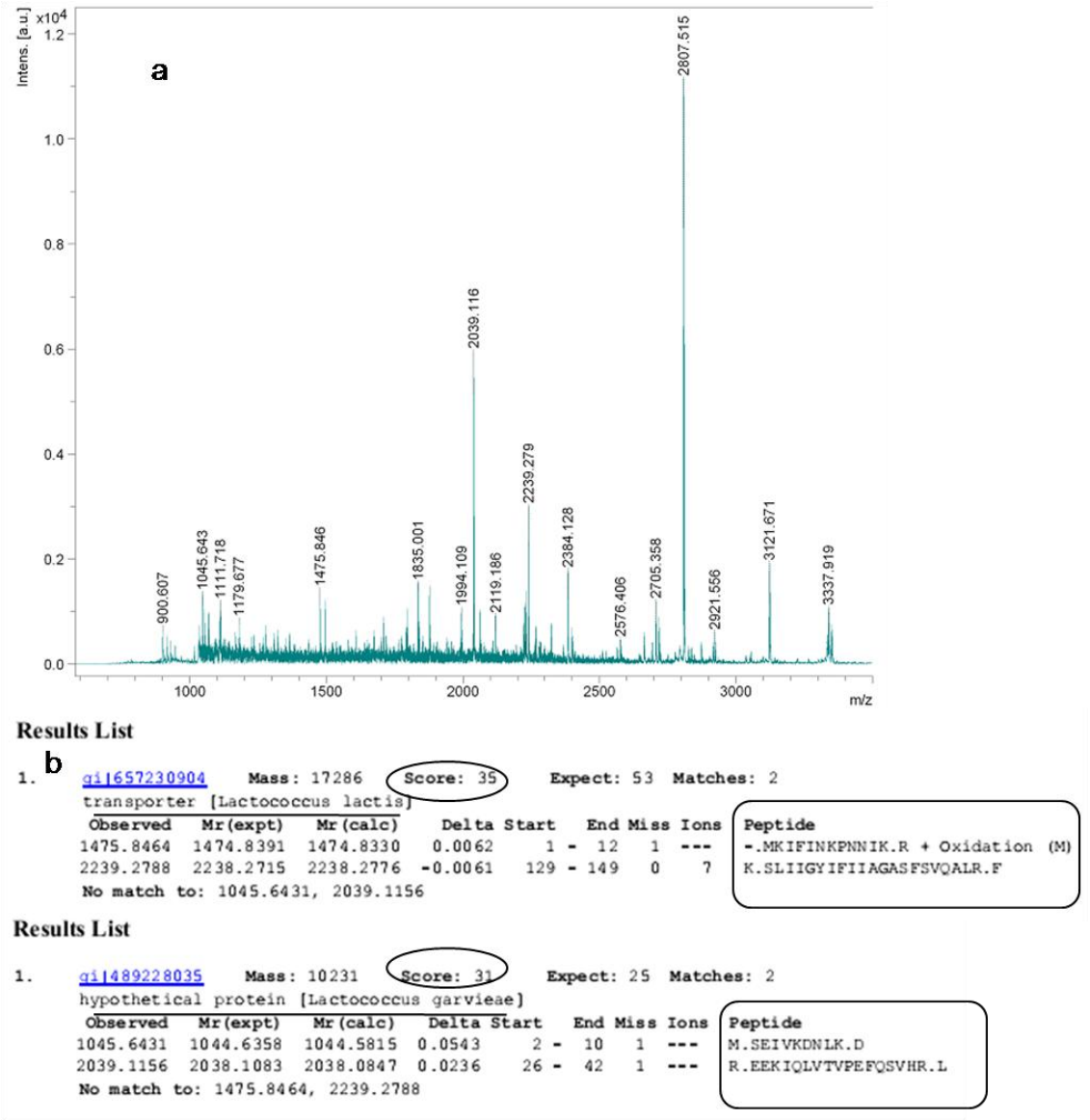


Fig.8.3 Peptide mass fingerprinting spectrum (a) and similarity search results of PMF generated peptides (b)

performed *de novo* MS-MS peptide mapping using Mascot search engine. The amino acid sequences obtained by *de novo* MS-MS peptide mapping of all the peptides are shown in Fig.8.3a. Niistin showed no significant similarity with any

of the AMPs previously reported. The MS/MS ion search produced similarity of two fragments, 1474 and 2238 to a transporter protein (17 kDa) of *L. lactis* with a very low score of 35 (>65 is significant). The fragments 1044 and 2038 produced even lesser homologies (score 31) to a hypothetical protein (10 kDa) of *L. garvieae* (Fig.8.3b). The PMF results clearly indicate that niistin is a novel peptide that is well separated from the known AMPs of bacterial origin.

8. 3. 2. Antimicrobial activity spectrum

Niistin had a broad spectrum of inhibitory activity against bacteria and fungus. The susceptible microbes include Gram positive bacteria (*S. aureus*, *E. faecalis*, *L. monocytogenes*), Gram negative bacteria (*E. coli*, *S. marcescens*) and the fungus *F. oxysporum* showing difference in activity from other LAB bacteriocins which are active against closely related species only. A fixed amount (200 µg) of niistin was used for the antimicrobial assays and found that in all cases the microbial growth was inhibited more than 70%. *S. aureus* was the most affected and *E. faecalis* was the least affected among the checked microorganisms. Inhibition (%) against all the checked microbes is given in Fig.8.4.

Growth inhibitory activity against both Gram positive and Gram negative bacteria can be explained on the basis of net positive charge of AMPs. Bacterial membranes are negatively charged due to peptides like cardiolipin (CL), phosphatidylglycerol (PG) or phosphatidylserine (PS). Electrostatic interaction between the positively charged peptides and negatively charged lipids allow the cationic peptides bind to the bacterial membranes. In Gram negative bacteria, the AMPs displace divalent cations like Ca^{2+} and Mg^{2+} which stabilize the negatively charged lipopolysaccharides and interact with the outer membrane (Hoskin and Ramamoorthy, 2009).

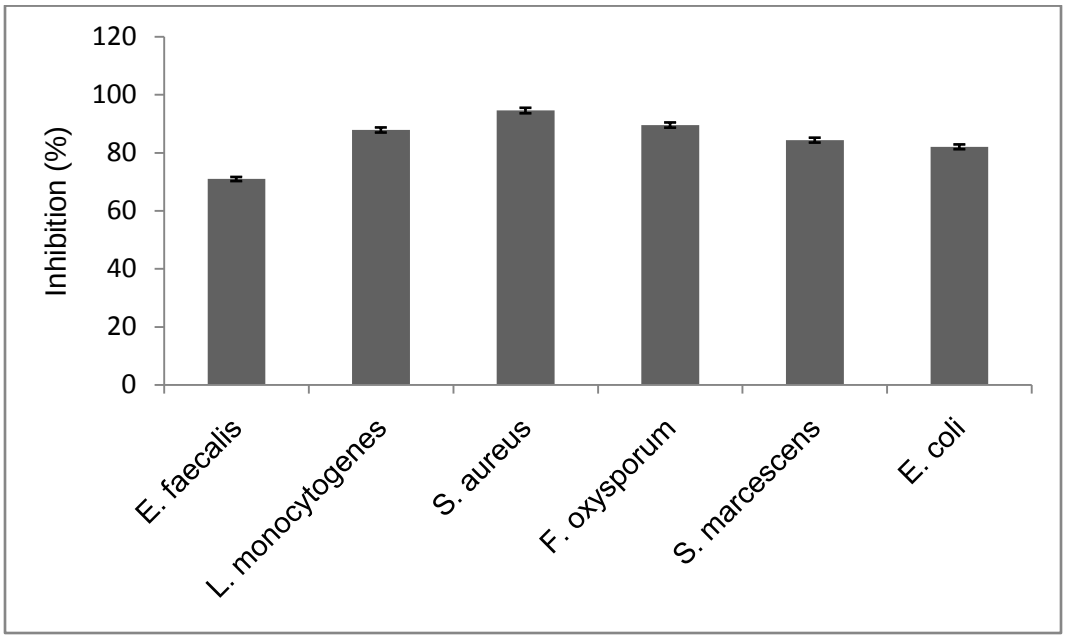


Fig.8.4 Antimicrobial activity spectrum of niistin obtained from the culture medium of BSN307 ($p < 0.05$)

Generally antimicrobial peptides are proposed to act through membrane permeabilization but mechanisms of antifungal peptides are more complex and little is known about the peptides of bacterial origin. Various mechanisms of action are proposed for the antifungal activity of AMPs include membrane permeabilization which results in loss of membrane potential and interaction with cellular targets or induction of signaling cascades that result in reactive oxygen species (ROS)/ programmed cell death (De Brucker et al., 2011; van der weerden et al., 2012). The net peptide charge and cationicity play an important role in the membranolytic function of antifungal peptides whereas the amphipathic structures and those containing more hydrophobic residues tend to have less favorable toxicity profiles (Duncan and O'Neil, 2013; Lopez-Abarrategui et al., 2012). Mechanism of activity of a single peptide may vary against different pathogens and the way of action by which niistin acts remains to be elucidated.

8. 3. 3. Effect of lipase and proteolytic enzymes on antimicrobial activity

Proteolytic enzymes trypsin and chymotrypsin treated samples lost considerable antimicrobial activity due to inactivation of niistin under the reaction conditions. When the control sample with active niistin and without any of the enzymes showed 80% growth inhibition, the enzyme treated ones, such as with 50 µg of trypsin and 100 µg of chymotrypsin the growth inhibition considerably reduced to 11% and 23.6% respectively. However, Proteinase k treated samples retained 80 % inhibition as that of control sample. This confirmed that no lipid moiety is involved with the antimicrobial activity and the reason of propteinase k having no effect on activity is not clear and may be revealed once the amino acid sequence of niistin is available.

8. 3. 4. Assay of free radical scavenging and cytotoxicity to cancer cell lines

Antioxidant potential of niistin was determined based on the ability to scavenge DDPH radicals. 500 µg of niistin could scavenge 50% of the free radicals generated. Remarkable cytotoxic activity against HeLa and MCF-7 cell lines was observed where the IC_{50} was achieved with 17 µg/mL of niistin. No cytotoxicity was observed in normal H9c2 cell line with this amount of niistin and showed negligible cytotoxicity (1%) when niistin concentration was increased to 30 µg/mL. Reports on antifungal peptides with anticancer property in mammalian cells of bacterial origin are not available. Niistin possesses this remarkable property and showed high selectivity towards cancer cells which is very important in developing anticancer drugs. The antifungal lipopeptide WH1 fungin from *Bacillus amyloliquefaciens* is an example of antifungal peptide that can bring about apoptosis in fungal cells by caspase-like activity with cytochrome c release (Qi et al., 2010).

The significantly selective cytotoxic activity of niistin towards cancer cell lines can be explained based on its highly cationic and hydrophobic nature and the membrane differences between normal and cancer cell. Positively charged AMPs have more affinity to cancer cells because the outer membranes of cancer cells over express negatively charged phosphatidylserine and O-glycosylated mucins

and thus carry a greater negative charge in comparison to the normal eukaryotic cells which consist largely of zwitterionic phospholipids such as phosphatidylethanolamine (PE), phosphatidylcholine (PC) or sphingomyelin (SM) which are neutral in charge (Bhutia and Maiti, 2008; Liu et al., 2015). Cholesterol, the major component of eukaryotic cell membranes protect the cells from cytolytic effect of AMPs by altering membrane fluidity and hence interfering with the membrane insertion of peptides (Leuschner and Hansel, 2004; Li et al., 2006). Moreover, the larger surface area of cancer cells due to the presence of higher number of microvilli allows them to bind more numbers of peptides (Chaudhary and Munshi, 1995). Multi-drug-resistant (MDR) proteins give a cancerous cell the ability to resist treatment by pumping out the neoplastic drugs out of the cell. The AMPs will kill the cell simply by disrupting the membrane and thus avoid such resistance mechanism (Hoskin and Ramamoorthy, 2008) and degraded by the proteolytic enzymes without accumulating in the host.

8. 3. 5. Fluorescent microscope analyses

The apoptotic effect of niistin on HeLa cell lines is visible in Fig.8.5a along with the normal H9c2 cell lines treated with same amount of compound (17 µg/mL). Cell shrinkage and blebbing which are characteristics of apoptosis were visible in the cancer cells whereas normal H9c2 cells remained intact without any change. Nuclear fragmentation, another feature of apoptosis was visible in the cancer cells by observation of the fluorescent DNA-DAPI complexes by DAPI staining (Fig.8.5b). These microscopic analyses of peptide treated cell lines confirms that cytotoxicity of niistin is restricted to cancer cells when normal cells are spared from their action and is caused by apoptosis.

8. 3. 6. Caspase-3 activation assay

Caspase-3 fluorescence assay showed the activation of caspase-3 in niistin treated HeLa cells where the fluorescent cleavage product of caspase-3 substrate, N-Ac-DEVD-N'-MC-R110 was detected. The increase in caspase-3 activity has been expressed in terms of relative fluorescence units. The HeLa cells treated with niistin showed activation of caspase-3 at the same time no activation was

observed in the control HeLa cells which was not treated with niistin (Fig.8.6). From this experiment, it can be concluded that niistin induces apoptosis through activation of caspase-3. Activation of caspase-3 has been used as a biomarker in assessment of apoptosis and in understanding mechanisms of apoptosis induction and the regulation of caspase-3 activation is one of the focuses in discovery for anti-cancer drugs.

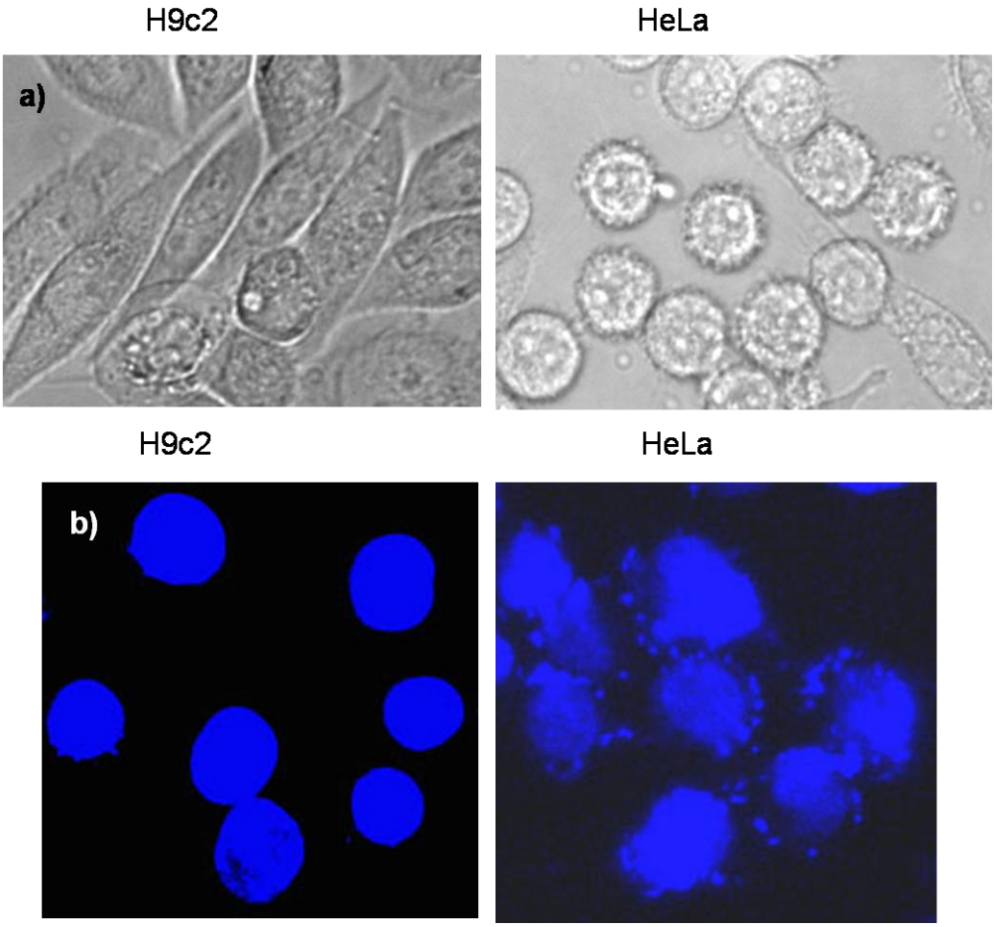


Fig.8.5 Live cell microscope image (a) and DAPI staining (b) of niistin treated normal H9c2 and HeLa cell lines

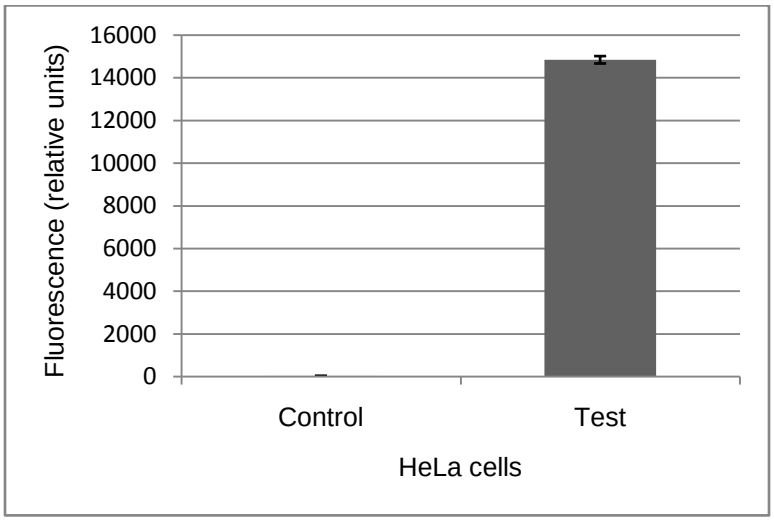


Fig.8.6 Caspase-3 activation in niistin treated (Test) and untreated (control) HeLa cells ($p < 0.05$)

Caspase activation can occur through two pathways, the cell surface death receptor pathway and the mitochondrial initiated pathway. Mitochondrial release of proapoptotic factors like cytochrome c in combination with apoptosis-activating factor 1 (Apaf-1), ATP and caspase-9 leads to activation of caspase-3 (Parrish et al., 2013; Zou et al., 1997). Caspase-3 normally exists in the cytosol as a 32 kDa precursor that is activated proteolytically into a 20 kDa and a 10 kDa heterodimer when cells are signaled to undergo apoptosis and activate other members of the caspase family leading to rapid and irreversible apoptosis (Wang et al., 1996). Activated caspase-3 will cleave and activate the 45 kDa subunit of DNA fragmentation factor, DNA fragmentation factor (DFF) which leads to degradation of DNA into nucleosomal fragments, a hallmark of apoptosis. This in turn confirms the results of DAPI staining where nuclear fragmentation was detected in cancer cells.

8. 4. Conclusion

The bioactive peptide, niistin purified from the cell free supernatant of *Lactococcus garvieae* subsp. *gaurensis* BSN307 showed no similarity to any of the peptides produced by lactic acid bacteria and has a broad range of biological activities. Niistin showed wide spectrum antimicrobial activity against both

bacteria and fungi and this could be exploited further to develop anti-infectives against bacterial and fungal infections. The selective cytotoxicity in cancer cells without affecting the normal cells as revealed from *in vitro* assays proves it can be an excellent and safe candidate to develop anti-cancer therapeutic drugs. Moreover, the antioxidant activity confirmed by free radical scavenging gives an additional advantage when niistin is consumed as a drug. These *in vitro* studies are further to be checked and confirmed by *in vivo* studies.

CHAPTER 9

Biocontrol of fungi on baked sourdough and wheat grains by *Lactococcus garvieae* subsp. *gaurensis* BSN307

9. 1. Introduction

Biological preservation of food has gained major attention during recent years where antagonistic microorganisms or their antimicrobial metabolites can prevent the growth of pathogenic bacteria and fungus in food. Lactic acid bacteria (LAB) have a long tradition of being used in food fermentations and secured the position of food grade bacteria due to their generally regarded as safe (GRAS) status. LAB are established as probiotics and good candidate for biological food preservation due to their wide spectrum antimicrobial activities. As food additives, LAB can perform as protective culture for improving microbiological safety of the product without changing the sensory characteristics of the food

Cereals comprise a major food source and contribute more than 60% to the world food production providing dietary fibre, proteins, minerals and vitamins required for human health. Beneficial effects of cereals can be exploited in different ways leading to the design of novel functional foods based on cereals or cereal ingredients that can target specific populations (Charalampopoulos et al., 2002). One of the major cause of cereal spoilage during storage is contamination by fungal pathogens where apart from diminishing the quality and nutritive value, mycotoxins produced by them cause severe health problems. In response to consumer demands, biopreservation technologies are being favoured to improve the safety, nutrition value and the organoleptic properties of cereals (Oliveira et al., 2014c). Previous investigations show that LAB can be used for cereal preservation through fermentation techniques (Blandino et al., 2003)

9. 2. Materials and methods

9. 2. 1. Materials

Wheat grains, all-purpose flour and Baker's yeast obtained locally. Microorganims used in this study include *Lactococcus garvieae* subsp. *gaurensis* BSN307, *A. niger*, *F. moniliforme*, *F. chlamydosporum*, *F. graminearum* and *F. oxysporum*.

9. 2. 2. Prevention of fungal growth on baked sourdough fermented with BSN307

The sourdough was baked to check the efficiency of BSN307 on prevention of fungal growth. BSN307 cells were collected after 24 h of incubation in MRS broth, washed twice with sterile distilled water and re suspended in the same. This cell suspension (4×10^9 CFU/ mL) was used along with commercially available yeast cells in sourdough manufacture. Two controls were kept during the study, the dough without BSN307 and the dough without yeast to test how the absence or presence of each would affect the fungal growth inhibiting activity of sourdough. The dough formulations (Table.9.1) were mixed thoroughly and were placed in separate aluminum pans and fermented at 30 ^oC for 36 h. After fermentation, the sourdoughs were baked in a batch oven at 180 ^oC for 30 min and the bread loaves were cooled at room temperature for 90 min.

Table.9.1 Dough formulations

	I [a]	II [b]	III [c]
Flour (g)	200	200	200
NaCl (g)	2	2	2
Glucose (g)	4	4	4
Yeast (g)	1	1	-
BSN (CFU)	-	$4x10^9$	$4x10^9$
Water (ml)	150	150	150

[a] Yeast alone; [b] Yeast and BSN307 together; [c] BSN307 alone

Antifungal potential of baked sourdough was checked against *A. niger*, *F. oxysporum* and *F. moniliforme.* 50 µL suspension of each fungus containing approximately 10^4 spores along with some mycelia were sprayed on both sides of the loaves and kept for incubation at 30 ^oC for five days under sterile conditions. The loaves were checked daily for visible fungal outgrowth and the shelf life was defined as the time for fungus to become visible on bread loaves.

9. 2. 3. Biocontrol of fungi on wheat grains

The wheat grains were washed twice with distilled water and surface sterilized with 4% (v/v) sodium hypochlorite. The surface sterilized grains were thoroughly washed with sterilized distilled water and air dried. The active fraction (B1) was extracted from 1L of BSN307 culture filtrate using double volume of chloroform, concentrated in a rota vapour and dissolved in 20% acetonitrile. Grains were treated in this active fraction for 15 min and air dried along with those treated in 20% (v/v) acetonitrile as control. The dried grains were distributed in to sterile glass vials and inoculated with 2-3 day old mycelia plugs (5mm) of *A. niger*, *F. moniliforme*, *F. graminearum*, *F. chlamydosporum*, *F. oxysporum* and kept for incubation at room temperature. Fungal growth on the grains was monitored every day.

9.3. Results and discussion

***9. 3. 1. Prevention of fungal growth on baked sourdough fermented with* BSN307**

The baked sourdough treated with BSN307 alone or without yeast lacked the normal texture and was brittle in nature whereas, the yeast cells alone or yeast cells together with BSN307 brought about a more porous and soft bread.

Comparison with the spoilage rate in the control sourdough revealed that the dough treated with both BSN307 and yeast could delay the growth of *A. niger*, *F. oxysporum* and prevented the attack of *F. moniliforme* (Fig.9.1). Sporulation of the respective fungi occurred in the bread treated with yeast alone in three days. BSN307 and yeast treated sourdough loaves delayed the growth of *A. niger* and *F.*

oxysporum and prevented the growth of *F. moniliforme*. Sporulation of *A. niger* and visible mycelia growth of *F. oxysporum* occurred on fifth day of incubation.

This experiment shows the potential of this new isolate to extend the shelf life of sourdough comparing to the sourdough treated with yeast alone. Ryan et al. (2008) reported that the antifungal activity of sourdough where the dough was treated with antibiotics to make sure that no viable bacteria remained in the dough. In this study, we baked the sourdough and checked the antifungal activity on the baked loaves. Ability of *Lactobacillus* to prevent fungal growth when present along with yeast is reported earlier by (Lavermicocca et al., 2000). Inability of the sourdough loaves treated only with BSN307, to prevent fungal growth demonstrates the synergistic action of yeast and BSN307 that led to extended shelf life of sourdough (Varsha et al., 2014). Much report are not available where lactococci have been used for preservation of baked foods and this experiment demonstrates the possibility of using the sourdough treated with BSN307 for manufacturing bread.

9. 3. 2. Biocontrol of fungi on wheat grains

All the vials with B1 fraction treated wheat grains completely prevented the fungal mycelia growth, where the inoculated mycelia plug failed to start growing and dried off. Fungal mycelia started growing on the third day of inoculation in the control vials and infested by seven days leading to grain spoilage. *F. chlamydosporum* and *F. oxysporum* formed a mat on the grain surface (Fig.9.2). Since microbial contaminants are found mostly on the grain surface (Oliveira, et al., 2014c), it is important to control their attack by natural ways.

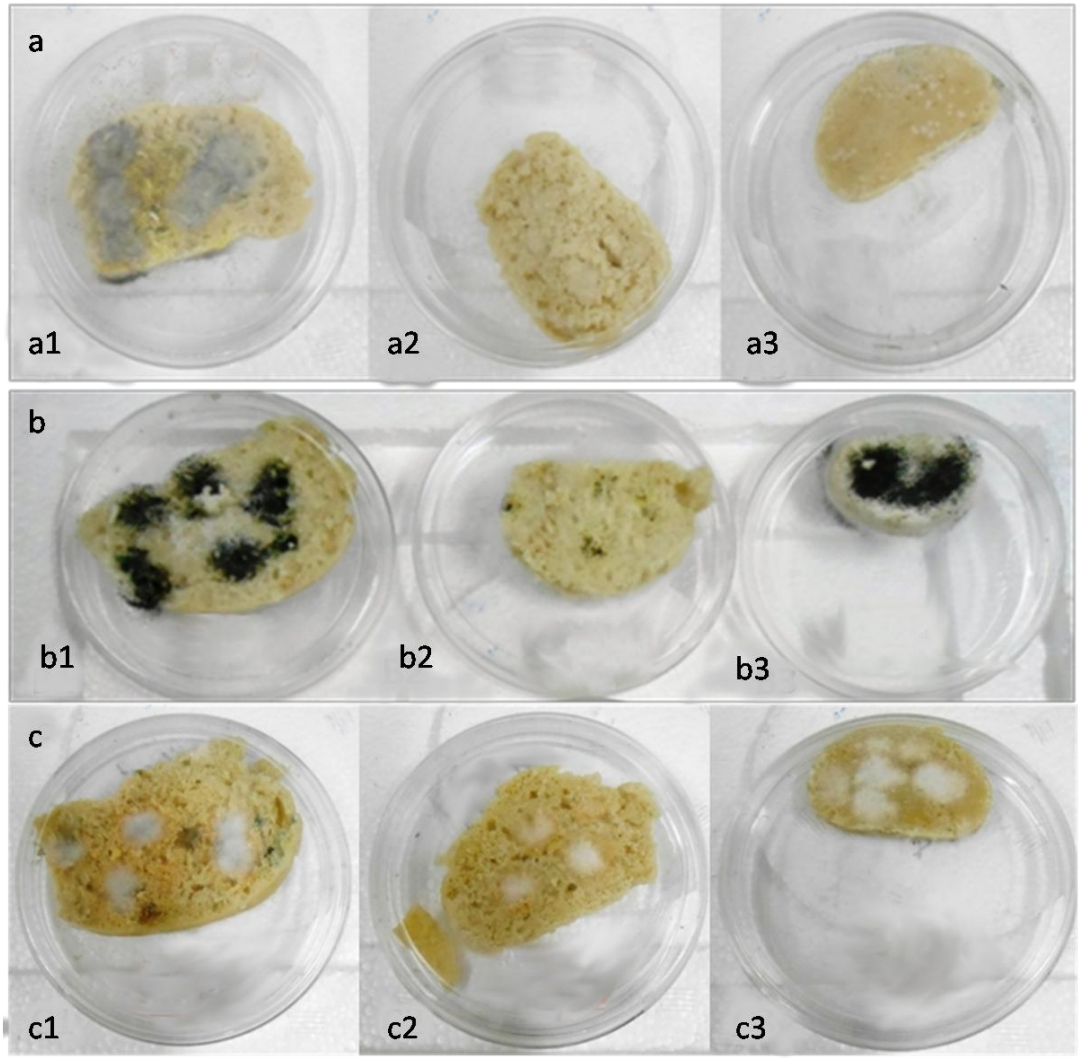


Fig.9.1 Antifungal activity of bread fermented with BSN307 and yeast on fifth day against *F. moniliforme* (a), *A. niger* (b) and *F. oxysporum* (c). a1, b1, and c1: bread treated with yeast alone, a2, b2, c2: bread treated with yeast and BSN307, a3, b3, c3: bread treated with BSN307 alone

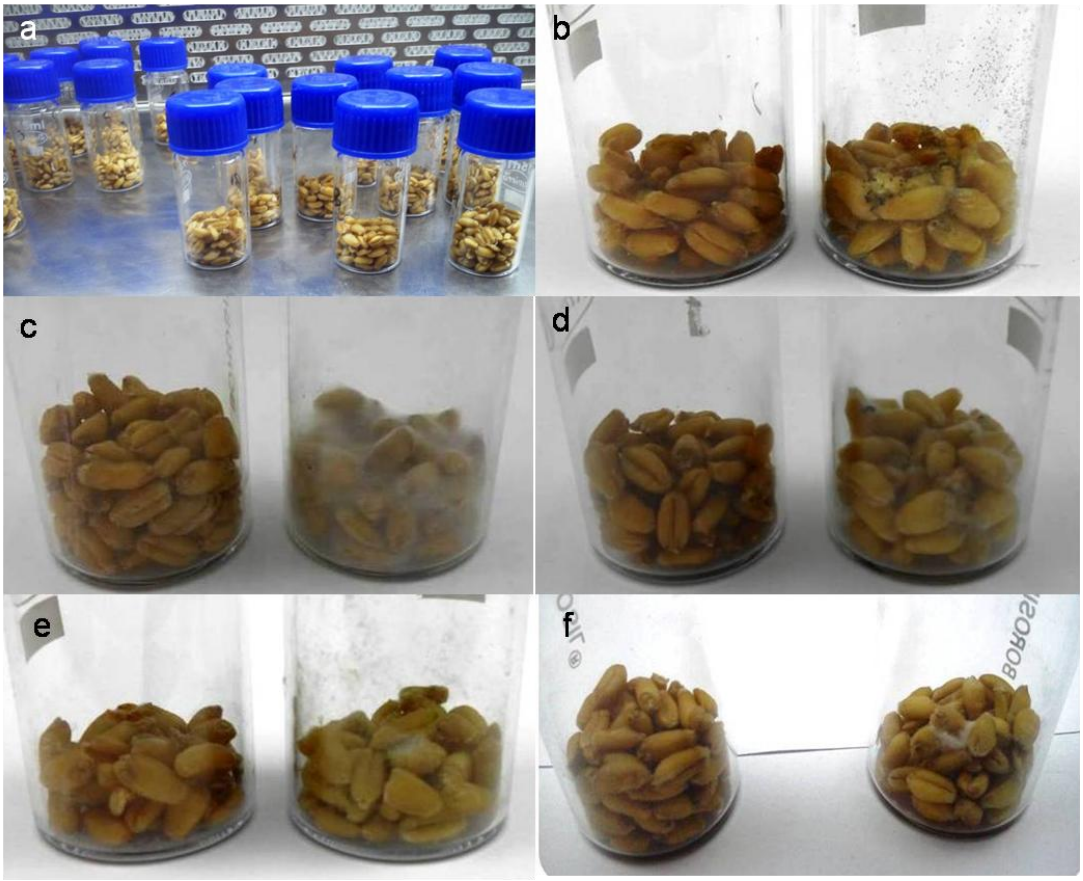


Fig.9.2 Biocontrol of fungi on wheat grains. Sterile vials with wheat grains (a). Antifungal activity of B1 fraction against *A. niger* (b), *F. chlamydosporum* (c), *F. oxysporum* (d), *F. moniliforme* (e), *F. graminearum* (f) along with untreated control on right side of each sub picture after 14 days of incubation

The B1fraction contains the phenazine compound, 2,4 DTBP and the bioactive peptide along with some other unidentified compounds. Biocontrol activity of 2,4 DTBP produced by *Flavobacterium johsoniae* strain GSE09 against *Phytophthora capsici* in pepper was reported before where the growth of pathogen was reduced to half with 100 µg/mL of this bioactive compound. This study demonstrates that the radicle formation in pepper seeds are not affected by 2,4 DTBP treatment but at the same time protected the infection by *P. capsici* (Sang and Kim, 2012) and the seeds could germinate after treatment. Recently

antimicrobial peptides from LAB were used to prevent the fungal attack on wheat grains along with maintaining the health of grains under storage (Gupta and Srivastava, 2014). Strains of *Pseudomonas aeruginosa* are the major producers of phenazine compounds and they are widely used to control the attack of fungi on crops. Phenazine compounds produced by *P. aeruginosa* are reported to control the Fusarium wilt of chick pea and Pythium damping of bean (Anjaiah et al., 1998) and are also used as colourants and antibacterial agents in food industry (Saha et al., 2008). Results from various research groups prove that 2,4 DTBP is not an unusual compound and naturally occurs in fruits, seeds and also produced by LAB (Varsha et al., 2015) and phenazine compounds are used as antimicrobial agents and colourants in food industry. The therapeutic effects caused by these compounds further supports their incorporation in adequate amounts into food products

9. 4. Conclusion

In sourdough baking where live *Lactococcus garvieae* subsp. *gaurensis* BSN307 cells were used along with yeast cells in an *in vivo* fermentation experiment revealed that they are capable producing antifungal compounds. The compounds produced were incorporated into the baked sourdoughs and could control fungal growth when challenged with different genera of fungi. This test shows the potential of this isolate to be selected as a good candidate in fermented food products to ensure safety and quality. Another experiment in which the compounds produced by BSN307 used to control fungal attack on wheat grains demonstrates the possibility of developing this antifungal compound fraction as a food additive so as to assure the safety as well as health promoting features of food. This would be of particular interest in grain or cereal based food products where fungal contamination is the main reason of food spoilage.

CHAPTER 10

Summary and Conclusion

Lactic acid bacteria (LAB) have a long tradition of being used in food fermentations and secured the position of food grade bacteria due to their generally regarded as safe (GRAS) status. The thesis represents the results of our investigations on the production of various antifungal compounds from new LAB isolates.

Eleven antifungal cultures out of 96 LAB isolates screened for antifungal activity against *Fusarium oxysporum*, were identified and belong to *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Pediococcus.* Preliminary screening of probiotic properties of these isolates was checked by growth in low pH and tolerance to bile salts. Probiotic potential of three isolates, *Pediococcus pentosaceus* (TG2), *Lactobacillus casei* (DY2) and *Lactococcus garvieae* subsp. *gaurensis* (BSN307) was further substantiated by *in vitro* transit tolerance to simulated conditions in the upper human gastrointestinal tract and bacterial adhesion capacity to HT-29 human intestinal cell lines.

Antifungal compounds from these three isolates were identified by ESI-MS and HPLC analysis as a range of carboxylic acids such as palmitic acid, salicylic acid along with some unidentified compounds. This concludes that organic acids produced by BSN307, TG2 and DY2 had a major role in the antifungal activity. Based on comparison of HPLC analysis of active fractions of three strains, *Lactococcus garvieae* subsp. *gaurensis* BSN307 was selected for further purification studies because of the presence of more number of compounds in the active fraction. The effect of pH and different enzymes on the antifungal activity of crude BSN307 bioactive fraction revealed that 20% of the activity was retained at pH 7 indicating to the existence of compounds other than organic acids. Effect of various carbon and nitrogen sources on growth and antifungal activity of BSN307 culture supernatant showed that dextrose, yeast

extract, ammonium citrate and beef extract are the major components contributing to both growth and antifungal compound production. Since all these ingredients a part of MRS medium, the same was selected as the production medium.

16S rDNA sequencing revealed BSN307 is closely related to *Lactococcus garvieae* but molecular and biochemical characterizations of the strain revealed that this isolate differed from its type strain significantly. Further experiments including phenotypic characteristics, rep-PCR, fatty acid methyl ester (FAME) analysis and DNA-DNA hybridization (DDH) techniques were performed to establish the taxonomic position of this novel isolate and found to carry considerable difference from the type strain *L. gravieae* CCUG 32208^T despite having a close 16S rRNA gene sequence similarity. At the same time comes under the reference strain based on DDH study. Based on the evidences provided in this study, the strain BSN307^T isolated from Indian gaur dung represents the type strain of a new subspecies within the species *L. garvieae*, for which the name *L. garvieae* subsp. *gaurensis* subsp. nov. (DSM 100577) is proposed.

Two antifungal compounds from BSN307 were purified by solvent extraction and reversed phase HPLC. Structure elucidation was done by NMR, MS and FTIR and one compound was identified as a phenazine compound (210 Da) and the other compound identified as 2, 4-di *tert*-butyl phenol (2,4 DTBP). Antagonistic activities of these compounds against various fungal pathogens were checked. Cytotoxicity against three cell lines and free radical scavenging activities were tested. Purified 2,4 DTBP (40 µg) had more than 75% free radical scavenging ability and displayed very remarkable cytotoxic activity against HeLa cell lines with an IC_{50} value of 10 µg/mL. IC_{50} against MCF-7 was achieved with 16 µg/mL. Against the normal cell line H9c2, a higher amount of 19 µg/mL was required to bring about IC_{50}. In the case of phenazine compound, 410 µg/mL compound was required to bring about more than 75% DPPH scavenging and 20 µg/mL and 24 µg/mL were required to bring abount IC_{50} in HeLa and MCF-7 cell lines respectively. At the same time no growth inhibition was observed against normal H9c2 cell lines up to an amount of 55 µg/mL. Both these compounds

showed more than 50% leucine and proline aminopeptidase inhibition with an amount of 500 µg in the reaction.

In this study, a novel bioactive peptide of 8595 Da size named niistin was purified from the cell free supernatant of BSN307. Peptide mass fingerprinting showed no significant similarity to any reported peptide from *Lactococcus* sp. or bacteria in general. Unlike other bacteriocins from LAB, niistin prevented the growth of Gram positive (*Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes*), Gram negative bacteria (*Escherichia coli*, *Serratia marcescens*) and the fungus *Fusarium oxysporum*. 200 µg/mL of niistin inhibited more than 70% growth of all the test organisms. Apart from broad spectrum antimicrobial activity it showed antioxidant activity by scavenging 50% of DPPH radical with an amount of 500 µg/mL. Another important biological activity of niistin is the highly selective cytotoxicity against the cancer cell lines HeLa and MCF-7 where the IC_{50} value was obtained with 17 µg/mL niistin and the normal H9c2 cells remained unaffected. The fluorescent microscopic analyses including DAPI staining showed characteristics of apoptosis such as cell shrinkage, blebbing and nuclear fragmentation in HeLa cells. Further experiments to check the pathway involved in apoptosis showed caspase-3 activation by caspase-3 fluorescence assay.

Antifungal and antioxidant property of 2,4 DTBP (206 Da) and the phenazine compound (210 Da) purified from BSN307 demonstrate the potential of these compounds to develop as food additive so as to improve the food safety as well as its health promoting characteristics. Cytotoxicity against cancer cell lines and aminopeptidase inhibitor potential revealed the diverse biological potential possessed by these bioactives and their potential in development of new therapeutic drugs. The bioactive peptide niistin purified from the cell free supernatant of BSN307 has a broad range of biological activities. Niistin showed wide spectrum antimicrobial activity against both bacteria and fungi and this could be exploited further to develop anti-infectives against bacterial and fungal infections. The selective cytotoxicity in cancer cells without affecting the normal

cells as revealed from *in vitro* assays proves it can be an excellent and safe candidate to develop anti-cancer therapeutic drugs. Moreover, the antioxidant activity confirmed by free radical scavenging gives an additional advantage when the peptide is consumed as drug.

An attempt to check out the shelf life extension of wheat bread without fungal spoilage was performed by fermenting the dough with BSN307 and the antifungal potential of baked sourdough was checked against *Aspergillus niger*, *Fusarium oxysporum* and *Fusarium moniliforme*. Comparison with the spoilage rate in the control sourdough revealed that the dough treated with BSN307 could delay the growth of *A. niger*, *F. oxysporum* and prevented the attack of *F. moniliforme.* The compounds produced were incorporated into the baked sourdoughs and could control fungal growth when challenged with different genera of fungi. This study shows the capability of this isolated to be selected as a good candidate in fermented food products to ensure safety and quality. Similarly, Wheat grains treated with the antifungal compounds prevented the growth of *A. niger*, *Fusarium graminearum*, *Fusarium chlamydosporum*, *F. oxysporum* and *F. moniliforme*. This experiment where the compounds produced by BSN307 was used to control fungal attack on wheat grains demonstrates the possibility of developing this antifungal fraction as a food additive so as to improve the safety as well as health promoting characteristics of food. This would be of particular interest in grain or cereal based food products where fungal contamination is the main reason of food spoilage.

For the first time, this study reports the purification of 2,4 DTBP and a phenazine compound from the culture medium of any LAB. Also for the first time compounds with anti-cancer and aminopeptidase inhibitory activity from LAB are reported here. The production of a bioactive peptide from LAB with apoptosis inducing potential is also the first kind of study. Presence of 2,4 DTBP, phenazine and the bioactive peptide in the LAB cell free supernatant (CFS) explains one of the reasons behind many of the health benefits including anticancer properties provided by LAB which represent the major human microbiota and the common

starter culture in food fermentation. Since LAB are normal part of our daily diet, their intake can bring along therapeutic effects considering they are source of natural antioxidants and may be particularly useful when long term treatment is required.

In vivo studies to confirm the probiotic potential of BSN307 are to be performed. Identification of the gene encoding the peptide and precise structural characterization of phenazine compound are yet to be done. Incorporation of the bioactive compounds into food systems and also as therapeutic agents are to be further confirmed by studies in animal models.

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Annexure I

Media composition

1. AP3 medium

Constituents	Concentration (g/L)
Sucrose	10
KH_2PO4	2
$MgSO_4$. $7H_2O$	1
NaCl	15
Na_2CO_3	0.5
Soybean powder	5
Tween 80	1.5
Distilled water	1000 mL
pH	7

2. Brain heart infusion (BHI) agar

Constituents	Concentration (g/L)
Calf brain, infusion from	200
Beef heart, infusion from	200
Proteose peptone	10
Disodium phosphate	2.5
Sodium Chloride	5
Dextrose	2
Agar	20
Distilled water	1000 mL
pH	7.4

3. Luria-Bertani (LB) medium

Constituents	Concentration (g/L)
Tryptone	10
Yeast extract	5
Sodium Chloride	10
Distilled water	1000 mL
pH	7

4. MRS (de Man Rogosa and Sharpe) medium

Constituents	Concentration (g/L)
Glucose	20
Yeast Extract	15
Beef Extract	5
Peptone	5
Ammonium Sulphate	5.5
Di-Potassium Hydrogen Phosphate	2
Ammonium Citrate	2
Sodium Acetate	5
Magnesium Sulphate	0.1
Manganese Sulphate	0.05
Tween 80	1
Distilled water	1000 mL
pH	6.5

5. Nutrient medium

Constituents	Concentration (g/L)
Peptic digest of animal tissue	5
Beef extract	1.5
Yeast extract	1.5
Sodium Chloride	5
Distilled water	1000 mL
pH	7.4

6. Potato dextrose agar (PDA) medium

Constituents	Concentration (g/L)
Potatoes, infusion from	200
Dextrose	20
Agar	20
Distilled water	1000 mL
pH	5.1

7. Tryptic soy agar

Constituents	Concentration (g/L)
Pancreatic digest of casein	17
Papaic digest of soyabean meal	3
Dextrose	2.5
Dibasic potassium phosphate	2.5
Sodium Chloride	5
Agar	20
Distilled water	1000 mL
pH	7.3

8. YEME (Yeast extract Malt extract) medium

Constituents	Concentration (g/L)
Yeast extract	3
Malt extract	5
Bacto peptone	3
Glucose	10
Distilled water	1000 mL
pH	6.8

Annexure II

List of major instruments

Instruments	Model
Autoclave	HVE-50, Hirayama, NewYork
Bioimager	BD Pathway^{TM} Bioimager Attovision 1.5.3-BD Biosciences, USA
Centrifuge	Eppendorf, Germany; Kuboto, Japan, Beckman Coulter, USA
Deep freezer (-86^{0}C)	Operon, Korea
DNA Sequencer	ABI 3100, Applied Biosystems, USA
Electron Microscope	JEOL JSM 5600LV, 115, Japan
Electrophoresis unit	Biorad, India
Fourier Transform-Infrared Spectrometer	PerkinElmer model Spectrum 100, USA
Gel documentation system	Chemi doc, Biorad, USA
Hot air Oven	Kemi Instruments, India
HPLC	Schimadzu, Japan
Incubating water bath	Julabo, Germany
Incubator	Infors HT, Ecotron, Switzerland, MCO-2OAIC-SANYO Electric Co Ltd,JAPAN
Laminar air Flow Chamber	Clean air System, India
Lyophilizer	hDB-5503, Operon, Korea
Mass spectrometer	Thermo Orbitrap LC/MS, USA
MALDI Spectrometer	Bruker Ultraflex TOF/TOF, USA
Microplate reader	Tecan NanoQuant, Switzerland, Synergy^{TM} 4 multimode Microplate Reader,BioTek instruments, USA
NMR Spectrometer	Bruker Avance II 500 spectrometer, USA
PCR machine	ep gradient-Eppendorf, India

pH meter	Systronics, India
Phase Contrast Microscope	Leica DMLS, Leica Microsystems, Germany, NIKON ECLIPSE TS 100, Japan
Protein purification System	BioLogic LP system, Bio-Rad, India
Rotavapor	Buchi, Switzerland
Scanning electron microscope	Carl Zeiss EVO 18, Germany
Sonicator	VCX-750, Sonics, USA
Thermostat	ThermoStat plus, Eppendorf, India
TLC scanner	CAMAG, Switzerland
UV-VIS Spectrophotometer	UV-160A, Schimadzu, Japan
Vacuum concentrator	5301-Eppendorf, Germany
Weighing balance	Mettler Toledo, Mumbai, India

Annexure III

List of publications

1. **Varsha K K**, Devendra L, Shilpa G, Priya S, Pandey A and Nampoothiri K M, 2,4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated *Lactococcus* sp, *International Journal of Food Microbiology*, **211**, (2015), 44-50.

2. **Varsha K K**, Priya S, Devendra L and Nampoothiri K, Control of Spoilage Fungi by Protective Lactic Acid Bacteria Displaying Probiotic Properties, *Applied Biochemistry and Biotechnology*, (2014), 1-12.

3. Divya J B, **Varsha K K** and Nampoothiri K M, Newly isolated lactic acid bacteria with probiotic features for potential application in food industry, *Applied Biochemistry and Biotechnology*, **167**, (2012), 1314-1324.

4. Divya J B, **Varsha K K**, Nampoothiri K M, Ismail B and Pandey A, Probiotic fermented foods for health benefits, *Engineering in Life Sciences*, **12**, (2012), 377-390.

5. Dilna S V, Surya H, Aswathy R G, **Varsha K K**, Sakthikumar D N, Pandey A, Nampoothiri K M, Characterization of an exopolysaccharide with potential health-benefit properties from a probiotic *Lactobacillus plantarum* RJF_4 . *LWT Food science and technology* (In press)

Manuscript Communicated

1. **Varsha K K**. Nampoothiri K M, *Lactococcus garvieae* subsp. *gaurensis* subsp. nov., a lactic acid bacterium isolated from wild gaur (Bos gaurus) dung. *Annals of Microbiology*, Manuscript number: ANMI-D-15-00590 (2015).

Manuscripts to be submitted

1. Antioxidant and anticancer potential of a novel antimicrobial peptide (AMP) purified from the cell free supernatant of *Lactococcus* BSN307 (2015)

2. Recent developments in biopreservatives based on lactic acid bacteria (2015)

3. A review on purification and therapeutic potential of antifungal compounds produced by lactic acid bacteria (2015)

Papers/Posters presented at International conferences

1. **Varsha K K**, Purification and characterization of 2,4 di-tert-butyl phenol from lactic acid bacteria. 2nd International conference on Bioenergy, Environment and Suatainable Technologies. Organized at Arunai Engineering College, Thiruvannamalai. January 28-31, 2015. (**Paper**)

2. **Varsha K K** and Nampoothiri K M, Identification and characterization of antifungal compounds from lactic acid bacteria, , Proceedings of the international conference on Advances in Biotechnologyand Bioinformatics ,Xconvention of the biotech research society, India. Organized at Dr. D. Y. Patil Vidyapeeth, Pune, Novemebr 25-27, 2013. (**Poster**)

3. **Varsha K K** and Nampoothiri K M, Identification and characterization of antifungal compounds from lactic acid bacteria , Proceedings of the international conference on industrial biotechnology ,XI convention of the biotech research society, india and the indo Italian workshop on food biotechnology:industrial processing , safety and health , organized at Punjabi University, Patiala, Novemebr 21-23 , 2012. (**Poster**)