Characterization of Bacteriocins from *Bacillus licheniformis* strain BTHT8 and *Bacillus subtilis* strain BTFK101 isolated from marine sediment

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CERTIFICATE

This is to certify that the research work presented in the thesis entitled "**Characterization of Bacteriocins from *Bacillus licheniformis* strain BTHT8 and *Bacillus subtilis* strain BTFK101 isolated from marine sediment"** is based on the original research work carried out by Ms. Smitha S under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any degree.

DR. SARITA G. BHAT
Supervising Guide

DECLARATION

I hereby declare that the thesis entitled "**Characterization of Bacteriocins from *Bacillus licheniformis* strain BTHT8 and *Bacillus subtilis* strain BTFK101 isolated from marine sediment"** is the authentic record of research work carried out by me at the Department of Biotechnology, Cochin University of Science and Technology for my doctoral degree, under the supervision and guidance of Dr. Sarita G. Bhat, Associate Professor, Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has previously formed the basis for the award of any degree or diploma, associateship or other similar titles or recognition.

Cochin-22 **Smitha S**

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ABBREVIATIONS

%	-	Percentage
°C	-	Degree Celsius
A_{570}	-	Absorbance at 570 nm
APS	-	Ammonium persulfate
ATP	-	Adenosine tri phosphate
AU	-	Activity units
BHI	-	Brain heart infusion broth
BLAST	-	Basic Local Alignment Search Tool
BLIS	-	Bacteriocin like inhibitory substance
bp	-	Base pair
BSA	-	Bovine serum albumin
CFS	-	Cell free supernatant
CFU	-	Colony Forming Units
cm	-	Centimetre
CTAB	-	Cetyl trimethyl ammonium bromide
Da	-	Dalton
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotide triphosphate
DTT	-	Dithiothreitol
DW	-	Distilled water
EDTA	-	Ethylene diamine tetra acetic acid
EtBr	-	Ethidium bromide
FDA	-	Food and Drug Administration
Fig	-	Figure

g	-	Grams
GRAS	-	Generally Recognized As Safe
h	-	Hours
HCl	-	Hydrochloric acid
HPLC	-	High performance liquid chromatography
IEF	-	Isoelectric focusing
ie.	-	that is
IPG	-	Immobilised pH Gradient
kb	-	Kilobase
kDa	-	Kilo Dalton
L	-	Litre
LAB	-	Lactic acid bacteria
Lac B	-	Lactose broth
LB	-	Luria Bertani
Log	-	Logarithm
M	-	Molar
m	-	Metre
MALDI-TOF	-	Matrix Assisted Laser Desorption Ionization- Time of Flight
mg	-	Milligram
MHB	-	Mueller Hinton broth
MIC	-	Minimum Inhibitory Concentration
Min	-	Minutes
mL	-	Millilitre
mm	-	Millimetre
mM	-	Millimolar
MRSA	-	Methicillin resistant *Staphylococcus aureus*

MS	-	Mass spectrometry
N	-	Normality
NA	-	Nutrient agar
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
NB	-	Nutrient broth
NCBI	-	National Center for Biotechnology Information
NCIM	-	National Collection of Industrial Microorganisms
ng	-	Nanogram
NGM	-	Nematode Growth Medium
Nm	-	Nanometer
No.	-	Number
OD	-	Optical density
OD_{600}	-	Absorbance at 600nm
PAGE	-	Polyacrylamide gel electrophoresis
PCR	-	Polymerase chain reaction
pH	-	Power of Hydrogen
pI	-	Isoelectric point
RNA	-	Ribonucleic acid
RP	-	Reverse phase
rpm	-	Revolutions per minute
rRNA	-	Ribosomal RNA
s	-	Seconds
SCD	-	Soyabean casein digest medium
SDS	-	Sodium dodecyl sulphate
sp.	-	Species

SRB	-	Sulphate reducing bacterium
TAE	-	Tris-acetate-EDTA
TCBS	-	Thiosulphate Citrate Bile salt Sucrose
TD	-	Time to death
TE	-	Tris-EDTA
TEMED-		N-N-N'-N'-Tetramethyl ethylene diamine
TSB	-	Tryptone soya broth
UF	-	Ultra filtration
UV	-	Ultraviolet
UV-VIS-		Ultraviolet-Visible
V	-	Volts
v/v	-	Volume/volume
viz.	-	Namely
w/v	-	Weight/volume
ZB	-	Zobell marine broth
μg	-	Microgram
μL	-	Microlitre
μM	-	Micromole
μM	-	Micromolar
μm	-	Micrometer
A-	Ala-	Alanine
R-	Arg-	Arginine
N-	Asn-	Asparagine
D-	Asp-	Aspartic acid
C-	Cys-	Cysteine
E-	Glu-	Glutamic acid

Q-	Gln-	Glutamine
G-	Gly-	Glycine
H-	His-	Histidine
I-	Ile-	Isoleucine
L-	Leu-	Leucine
K-	Lys-	Lysine
M-	Met-	Methionine
F-	Phe-	Phenyl alanine
P-	Pro-	Proline
S-	Ser-	Serine
T-	Thr-	Threonine
W-	Trp-	Tryptophan
Y-	Tyr-	Tyrosine
V-	Val-	Valine
Na	-	Sodium
Ca	-	Calcium
Mg	-	Magnesium
Fe	-	Iron
Mn	-	Manganese
N	-	Nickel
Ba	-	Barium
Cd	-	Cadmium
Zn	-	Zinc
Cu	-	Copper
Al	-	Aluminium
Co	-	Cobalt

1. INTRODUCTION

The soil microorganisms have been greatly exploited for their antibiotics to combat and destroy disease causing microbes. The water bodies are unexploited sources of biologically active substances. Given the fact that the oceans cover about 70 percent of the earth’s surface, the oceans hold great promise of novel pharmacologically active compounds.

The interaction of microbes, their competition for nutrients and survival, quorum sensing are all phenomenons having a biochemical basis. The antagonistic interrelationships among microorganisms have attracted attention since the dawn of bacteriology and are well studied. De Giaxa (1889) was probably the first to report the existence of bacteria antagonistic to anthrax bacilli and *Vibrio comma* in the sea. It was noted that natural sea water killed 80 percent of the organisms in sewage within half an hour (Carpenter *et al*., 1938). Zobell (1941) found that only 4 to 15 percent as many bacteria from soil, sewage and other fresh water or terrestrial sources formed colonies on nutrient agar prepared with sea water when compared to a similar medium prepared with distilled water. Lacking the properties of bacteriophage, the bactericidal property of sea water was primarily attributed to its content of antibiotic substances produced by microorganisms. In 1947, Rosenfeld and Zobell carried out detailed studies on the antibiotic producing marine microorganisms, wherein they found that most of these bacteria belonged to the genera *Bacillus* and *Micrococcus*. It was evident from their work that the marine environments may represent a reservoir of microbial antagonists of possible significance.

Since their discovery in the first half of the 19th century, antibiotics have been extensively used as therapeutic agents and growth promoters. Their efficacy and cost-

effectiveness contribute to their popularity. Nevertheless, the continuous use of antibiotics has resulted in the emergence of multidrug-resistant microbial strains that no longer respond to antibiotic therapy. A number of strategies have been explored to control microbial pathogens without the use of antibiotics. Among these bacteriocins, probiotic microorganisms and bacteriophages have been more extensively studied and proposed as potential alternatives to classic antibiotics in animal husbandry (Mantovani *et al*., 2011).

Bacteriocins are antimicrobial, proteinaceous compounds with a bactericidal mode of action against bacteria closely related to the producer strain. They are ribosomally synthesized antimicrobial peptides produced by microorganisms belonging to different eubacterial taxonomic branches. They are heterogeneous compounds that display variable molecular weights, biochemical properties, inhibitory spectra and mechanisms of action (O'Sullivan *et al*., 2002).

Bacteriocins were first discovered by Gratia in 1925. These compounds are produced by both Gram-negative and Gram-positive bacteria. Bacteriocins produced by Gram-positive and Gram-negative bacteria have diverse ecological and evolutionary aspects. In Gram-positive bacteria, the biosynthesis of bacteriocins is self-regulated and bacteriocin production is not a lethal event. In addition, the spectrum of antimicrobial activity is broader than the peptides from Gram-negative species and bacteriocin release is controlled by specific regulatory mechanisms. In Gram-positive bacteria, the gene clusters for bacteriocin production are generally organized in the chromosome and include genes encoding the pre-peptide and proteins responsible for post-translational modifications, regulation, immunity and transport across the cytoplasmic membrane. In contrast, Gram-negative bacteria are often killed by bacteriocin production, the release of the peptide being controlled by common

regulatory mechanisms (eg. SOS system) and specific genes encoding proteins responsible for cell lysis are common (Mantovani *et al*., 2011).

Some bacteriocins are peptides consisting of only 19 to 37 amino acids, whereas others are large peptides with molecular weights of up to 90 kDa. Some small bacteriocins contain unusual amino acids originating from modifications of conventional amino acids after translation. The activity spectrum of bacteriocins can be narrow and confined to inhibition of closely related species, or it can be relatively broad. Bacteriocins are often considered more natural because they are thought to have been present in many of the foods eaten since ancient times (Cleveland *et al*., 2001). Many bacteriocins are active at small concentrations, exhibiting both bactericidal and bacteriostatic activity toward sensitive cells. The major mechanisms of bacteriocin activity can vary from pore formation in cytoplasmic membrane to the inhibition of cell wall biosynthesis and enzyme activities (RNAse or DNAse) in target cells.

Bacteriocins are classified into different groups (Klaenhammer, 1993; Willey *et al*., 2007). Class I bacteriocins (lantibiotics) are small peptides that undergo extensive post-translational modification to produce the active peptide. Class II bacteriocins are heat-stable, low molecular weight, membrane-active peptides. Members of class III are large heat labile proteins, and a fourth class (complex bacteriocins) has also been suggested, requiring non-protein moieties for activity.

Currently, bacteriocins produced from lactic acid bacteria are studied extensively due to their generally recognized as safe (GRAS) status by the Food and Drug Administration (FDA) (O'Sullivan *et al*., 2002; Nes *et al*., 2002; Osmanagaoglu

& Beyatli, 2002). Nisin from *Lactococcus lactis* is the most studied and is a potential candidate for use in prophylaxis.

Bacteriocins can be introduced into food in three different ways: bacteriocins can be produced *in situ* in fermented food by bacterial cultures that substitutes for all or part of the starter culture; purified or semi-purified bacteriocins can be added directly to food; or added as an ingredient (Cotter *et al*., 2005; Calo-Mata *et al*., 2008). However, nisin-resistance is a phenotype that has already been demonstrated in some Gram-positive bacteria, including *Staphylococcus aureus*, *Streptococcus bovis* and *Listeria monocytogens* (Mantovani *et al*., 2011).

The bacteriocins from *E. coli* called colicins, are the longest studied bacteriocins. In fact, one of the oldest known so-called colicins was called colicin V and is now known as microcin V. It is much smaller, being produced and secreted in a different manner than the classic colicins (Riley and Wertz, 2002). Bacteriocins produced by *Enterococci* are termed as enterocins.

Bacillus sp. produce a large number of bacteriocins: subtilosin by *B. subtilis* (Zheng & Slavik, 1999), bacillocin 490 by *B. licheniformis* (Martirani *et al*., 2002), cerein by *B. cereus* (Oscariz *et al*., 1999), haloduracin by *B. halodurans* (Lawton *et al*., 2007), thuricin by *B. thuringiensis* (Gray *et al*., 2006 a & b), megacin by *B. megaterium* (Khalil *et al.,* 2009 a & b), bacteriocin like inhibitory substance (BLIS) by *B. amyloliquefaciens* (Lisboa *et al*., 2006) are some of them.

Bacillus is an interesting genus to investigate for inhibitory substances, being capable of producing a large number of antimicrobial peptides. Members of the genus *Bacillus* are Gram-positive, aerobic or facultative anaerobic and endospore forming bacteria that are characterized by their rod-shaped cell morphology, catalase

production and their ubiquitous distribution. They are found in diverse environments such as soil, clay, rock, dust, aquatic environments, vegetation, food and the gastrointestinal tracts of various insects and animals (Nicholson, 2002).

The ability of bacilli to survive and grow in such different ecosystems is based on the production of robust endospores, their diversity in physiological properties and their growth requirements. They exhibit quite diverse physiological properties such as the ability to degrade many different substrates derived from plant and animal sources (Lutz *et al*., 2006). This diversity in physiological properties is reflected by the considerable diversity of *Bacillus* strains, consequently allowing these bacteria to colonize a wide variety of ecological habitats.

The resistance of spores to heat, drying, disinfectants and other means of sterilization is of great relevance in food because of their economic concern in the food processing industry. Members of this genus hold tremendous importance because of their antimicrobial activity, since they produce a variety of peptide antibiotics representing several different basic chemical structures (von Dohren, 1995).

Most of the species from genus *Bacillus* are considered as industrially important bacteriocin producers, with a history of safe use and are also considered GRAS microorganisms (Martirani *et al*., 2002; Cladera-Olivera *et al.*, 2006). The capability to produce endospores allows *Bacillus* sp. to withstand extreme environmental conditions such as those encountered in food processing. The presence of *Bacillus* species in food does not always imply spoilage or food poisoning, and some species or strains are even used in human and animal food production such as, for example, *B. subtilis* strains that are used in Natto, an East Asian fermented food

production (Hosoi & Kiuchi, 2003). Furthermore, specific *B. subtilis* strains are also used as a starter culture for fermenting soybeans into the traditional West African condiment dawadawa (Terlabie *et al.*, 2006) or for fermenting African mesquite seeds in the production of the Nigerian food condiment okpehe (Oguntoyinbo *et al*., 2007). A non-toxinogenic *B. cereus* ssp. *toyoi* with probiotic properties is also used as animal feed additive (Lodemann *et al*., 2008). Some *Bacillus* strains are also used as probiotics in livestock to inhibit pathogenic bacteria, to improve the health status and performance of farm animals and poultry (eg. BioPlus 2B, containing a mixture of *B. licheniformis* and *B. subtilis* strains)

The aim of the present study was to characterize bacteriocins from marine (sediment and water) samples, study their production and potential applications which include therapeutic use, in food processing industry as biofilm control agents and also in controlling microflora in sea food industry, thus aiding in biopreservation.

OBJECTIVES OF THE STUDY

Bacteriocins are gaining more and more attention not only as an alternative therapeutic agent for the prevention and treatment of infections, but also as preservatives in food industries to avoid deterioration and spoilage of food. These antimicrobial peptides are generally recognized as naturally occurring food preservatives, with the ability to influence the quality and safety of foods (Settanni and Corsetti, 2008).

Thus the primary objectives of the study included

1. **Screening for bacteriocin producing bacteria from marine sediment and water samples**

2. **Characterization of the bacteriocin producers**

3. **Optimization of bioprocess variables for bacteriocin production by 'one-factor at-a-time' method**

4. **Purification and characterization of the bacteriocins**

5. **Application studies of the bacteriocins**

2. REVIEW OF LITERATURE

There is a growing awareness of the need for development of new antimicrobial agents for treatment of human, animal and plant diseases. Marine bacteria could represent a new source of antimicrobial compounds, which are currently needed to combat the emerging antibiotic-resistant pathogens. Many marine heterotrophic bacteria are known to produce antibacterial substances, which inhibit or kill other bacteria.

2.1 SEARCH OF MARINE ENVIRONMENT

Although the soil organisms have been greatly exploited for their antibiotics, the skein of complicating factors which influence the destruction of terrestrial organisms in marine environments has yet to be fully unravelled. There are innumerable reports on the bactericidal effects of sea water on the terrigenous microflora. De Giaxa (1889) observed that pathogens rapidly perish in raw sea water, although they may survive almost indefinitely in heat-treated sea water. Kofinek (1927) also observed that the multiplication of most non marine bacteria is inhibited by sea water. Sea water is also bactericidal for many non marine bacteria, Gram-positive organisms being more sensitive than Gram-negative forms (Beard & Meadowcroft, 1935; Zobell, 1936; Carpenter *et al.*, 1938; Zobell, 1941). Although Gram-negative organisms display resistance to the lethal action of sea water, their viability in this medium varies widely among different species. The bacteriostatic and bactericidal effects of sea water are greater than can be accounted for upon a basis of its salinity or osmotic pressure. Not only is natural sea water more bactericidal than synthetic sea water (Zobell & Feltham, 1933; Zobell, 1946), but it is also more bactericidal than heat-treated sea water (Zobell, 1936). Water from the Black sea was found by Krassilnikov (1938) to be germicidal for terrestrial bacteria until it was boiled. He confirmed the

observations of Beard and Meadowcroft (1935) and Zobell (1936) that the bactericidal potency of sea water was decreased but not destroyed by passing it through Berkefeld, Chamberland, Coors, or similar filters. Lacking the properties of bacteriophage, the bactericidal property of sea water is attributed primarily to its content of antibiotic substances produced by microorganisms. The first bacteriocin isolated from marine microorganisms was detected in *Vibrio harveyi* (formerly *Beneckea harveyi*). It was also determined that the killing range was limited to strains of *B. harveyi*. This bacteriocin was named harveyicin (McCall & Sizemore, 1979). Since then a large number of works have been conducted on marine bacteriocins.

According to Rosenfold & Zobell (1947), of the fifty eight species of marine microorganisms tested, nine have demonstrated antibiotic activity against non marine forms. The most actively antagonistic marine genera were *Bacillus* and *Micrococcus*. *Bacillus* sp. from sea water and marine bottom deposit are moderately halotolerant. They are able to propagate and metabolize under marine conditions. A total of twenty aerobic endospore-forming bacilli, isolated from marine invertebrates and sea water of different areas of the Pacific ocean, were taxonomically characterized (Ivanova *et al*., 1999). Most of the bacilli (11 strains) of marine origin belonged to the species *Bacillus subtilis*, two strains were *B. licheniformis*, others were *B. amyloliquefaciens, B. pumilus*, *B. firmus*, *B. lentus*, *B. sphaericus*, *B. fusiformis*, *B. megaterium* and *Paenibacillus* sp. A recent antimicrobial screening of 258 bacterial strains isolated from water and sediment in the Yucatan peninsula revealed that 46 strains belonging to the genera *Aeromonas*, *Bacillus, Burkholderia*, *Photobacterium*, *Pseudomonas*, *Serratia* and *Stenotrophomonas* possessed antimicrobial activity. Approximately fifty percent of this antimicrobial activity was due to bacteriocins or BLIS (De la Rosa-Garcia *et al*., 2007). A systematic study of *Bacillus* strains from diverse marine environment was carried out by Ki *et al*. (2009).

2.2 GENUS *BACILLUS*

Genus *Bacillus* is gaining momentum because of their ability to resist and survive under harsh industrial conditions. *Bacillus*, established by Cohn in 1872 is a heterogeneous group of Gram-positive, facultative anaerobic, endospore-forming bacteria. They are characterized by their rod-shaped cell morphology, catalase production and their ubiquitous distribution, even though soil is generally accepted as its natural reservoir. They are widely distributed in freshwater habitat and sea (Ruger, 1989); soil and clay, rock, dust, vegetation, food and the gastrointestinal tracts of various insects and animals (Nicholson, 2002). The ability to produce endospores allows *Bacillus* to withstand extreme environmental conditions.

They exhibit quite diverse physiological properties such as the ability to degrade many different substrates derived from plant and animal sources, including cellulose, starch, proteins, agar, hydrocarbons and also biofuels (Lutz *et al*., 2006). Furthermore, some *Bacillus* species are heterotrophic nitrifiers, denitrifiers, nitrogen fixers, iron precipitators, selenium oxidizers, oxidizers and reducers of manganese, facultative chemolithotrophs, acidophiles, alkalophiles, psychrophiles, thermophiles and others (Priest, 1993; Slepecky & Hemphill, 2006). This diversity in physiological properties, which is reflected by the considerable diversity of *Bacillus* strains, allowed these bacteria to colonize a wide variety of ecological habitats.

In fact, about two-thirds of the enzyme market (proteases, amylases, rennet substitutes, endonucleases, glucose-dehydrogenase and pullulanase) for industrial applications are produced by fermentation from *Bacillus* species (Queener & Lively, 1989). Members of the *Bacillus* group are considered to be good producers of antimicrobial substances, including peptide and lipopeptide antibiotics and bacteriocins (Stein, 2005).

2.2.1 PHYLOGENY AND CLASSIFICATION OF *BACILLUS*

Phylogenetically, bacteria belonging to the genus *Bacillus* belong to class I of the phylum Firmicutes ie. the bacilli. *Bacillus* has undergone considerable taxonomic changes. In the 2nd edition of the Taxonomic Outline of Bergey's Manual of Systematic Bacteriology (Ludwig *et al*., 2009) phylogenetic classification schemes, accomplished mainly by the analysis of 16S rDNA sequence similarities, they are included in the family of *Bacillaceae* and the genus is made up by 94 species. *Bacillus* species are historically clustered into six large groups based on numerous physiological, biochemical and morphological characters (Priest, 1993). Group I including *B. polymyxa* as a reference organism, comprises facultative anaerobic species that ferment a variety of sugars and have reasonably fastidious growth requirements in the form of vitamins and amino acids. These species secrete numerous extracellular carbohydrases such as amylases, glucanases including cellulases, pectinases and pullulanases. Group II includes *B. subtilis* and its relatives, *B. amyloliquefaciens*, *B. licheniformis* and *B. pumilus*. These species differentiate into oval endospores that do not distend the mother cell. Most of these bacteria are regarded as strict aerobes but many strains have a limited ability to ferment sugars and grow well anaerobically in the presence of glucose and nitrate as a terminal electron acceptor. *B. anthracis*, *B. cereus* and *B. thuringiensis* are facultative anaerobes. Group III species are perhaps taxonomically the least defined and are rather physiologically heterogeneous. This group is based on *Brevibacillus brevis* which is a strict aerobe that does not produce appreciable acid from sugars and differentiates into an oval endospore that distends the sporangium. Other species in this group might include *B. badius* and *B. freudenreichii*. Bacilli which differentiate into spherical endospores are allocated to group IV. This is a phylogenetically homogeneous group of species including *B. sphaericus*, the psychrophiles *B. insolitus*, *B. psychrophilus* and some other species. These bacteria are all strict aerobes distinguished from all other bacilli by the replacement of meso-diaminopimelic

acid in the peptidoglycan of their cell walls with lysine or ornithine. In particular, *B. sphaericus* does not use sugars for growth, metabolizing acetate, arginine, glutamate and histidine as carbon and energy sources. Group V includes the thermophilic bacilli, represented by *B. stearothermophilus.* This includes a physiologically and morphologically heterogeneous collection of species with various metabolic pathways ranging from strict aerobes to microaerophilic types. The acidophilic thermophiles (group VI) have recently been allocated to the new genus *Alicyclobacillus* in which thermophily appears to have independently evolved in many lineages.

2.2.2 *BACILLUS SUBTILIS*

Within the genus *Bacillus*, the *B. subtilis* group encompasses five physiologically similar species (Gordon *et al.*, 1973): *B. amyloliquefaciens*, the recently described *B. atrophaeus* (previously *B. subtilis var. aterrimus*; Nakamura, 1989), *B. licheniformis*, *B. pumilus* and *B. subtilis. B. subtilis* is a remarkably diverse bacterial species that is capable of growth within many environments, terrestrial and aquatic, making it seem that this species is ubiquitous and broadly adapted to grow in diverse settings within the biosphere. Recent microarray-based comparative genomic analyses have revealed that members of this species also exhibit considerable genomic diversity. Like all members of the genus *Bacillus*, *B. subtilis* can form highly resistant dormant endospores in response to nutrient deprivation and other environmental stresses. These spores are easily made airborne and dispersed by wind. Thus, spores might migrate long distances. When a strain is isolated from a particular environment there is no guarantee that it was actually growing at that location. Thus, the question of where *B. subtilis* grows has not been so simple to answer (Nicholson, 2002). *B. subtilis* is often referred to as a 'soil dweller'. Over 30 years ago, the use of fluorescent antibodies to distinguish vegetative and spore forms of *B. subtilis* in diverse soil samples revealed that the organism was most often in its vegetative form when associated with decaying

organic material (Siala *et al*., 1974). Further support for the idea that *B. subtilis* can lead a saprophytic lifestyle comes from recent experiments in which spores were inoculated into artificial soil microcosms saturated with filter-sterilized soluble organic matter extracted from soil. Under these conditions the spores not only germinated but the vegetative cells proliferated for several days until they again sporulated, probably in response to nutrient depletion. Soon after germination the cells formed bundled chains that moved on the surface in a flagella- independent fashion (Vilain *et al*., 2006).

B. subtilis can also grow in close association with plant root surfaces. In the laboratory, when *B. subtilis* was inoculated on the roots of *Arabidopsis thaliana*, formation of biofilms was observed (Fall *et al*., 2004). In addition, *B. subtilis* can be isolated in greater numbers than most other spore forming bacteria from the rhizosphere of a variety of plants (Pandey & Palini, 1997; Fall *et al*., 2004; Cazorla *et al*., 2007). There is evidence that through these associations, *B. subtilis* can promote plant growth. Possible explanations for this growth promotion are: (i) *B. subtilis* out competes other microbes that would otherwise adversely affect the plant (ii) *B. subtilis* activates the host defense system so that the plant is poised to resist potential pathogens or (iii) *B. subtilis* makes certain nutrients more readily available to the plant (e.g. phosphorus and nitrogen). Considering that *B. subtilis* is found on and around plants and that many animals consume plants, it is not surprising that this bacterium is often found in faeces.

B. subtilis has also been isolated repeatedly from marine environments (Miranda *et al*., 2008; Ivanova *et al*., 1999). Although growth in marine water might occur, the abundance of *B. subtilis* in these environments might also be explained by its observed association with the gastrointestinal tract of marine organisms (Newaj-Fyzul *et al*., 2007).

2.2.3 *BACILLUS LICHENIFORMIS*

Some classical biochemical tests can be used to distinguish *B. licheniformis* from other species of the *B. subtilis* group. These strains react positively for anaerobic growth, arginine dehydrolase production, starch hydrolysis and utilization of proprionate as a carbon source; no other species in the group displays this series of reactions (Priest *et al*., 1987). Thus, the distinction between *B. lichenifomis* and *B. subtilis* is now easily made.

B. licheniformis is a non-pathogenic soil organism that is mainly associated with plant and plant materials in nature but can be isolated from nearly everywhere due to its highly resistant endospores that are disseminated with the dust. *B. licheniformis* is commonly found on the feathers of ground dwelling birds (Burtt & Ichida, 1999). *B. licheniformis* dominated the feather degrading bacteria isolated and screened from the plumage of some wild-caught birds (Whitaker *et al*., 2005). A feather degrading *Bacillus licheniformis* PWD61 was isolated from the aerobic portion of a poultry waste digester (Wiliams *et al*., 1990). *B. licheniformis* is also common in foods including natural agricultural products such as cereals, which it presumably colonizes from wind-blow dust and soil particles. Large numbers of spores of this bacterium may be found in processed or dried foods and herbs such as cocoa and spices (reviewed by Priest, 1989 a & b). *Bacillus* species grew throughout fermentation and became the dominant microflora (107–108 CFU/g) after 48–72 h, when other species had died off. They remained at this high population until the fermentations were considered complete (Ardhana & Fleet, 2003). *B. licheniformis* was also found to be the dominant flora in raw milk (Janstova & Lukastova, 2001). Bacilli are capable of degrading several substrates and grow on a large diversity of nutrient sources due to its capacity to produce and secrete plenty of hydrolytic enzymes.

2.2.4 SPORULATION LIFE CYCLE OF *BACILLUS*

Under conditions of nutrient starvation, the growing vegetative cell will undergo a series of morphological changes that create a forespore /prespore within the mother cell (MC) of the sporangium. After an approximate of eight hours, the spore (S) is released by lysis of the MC (Fig: 2.1).

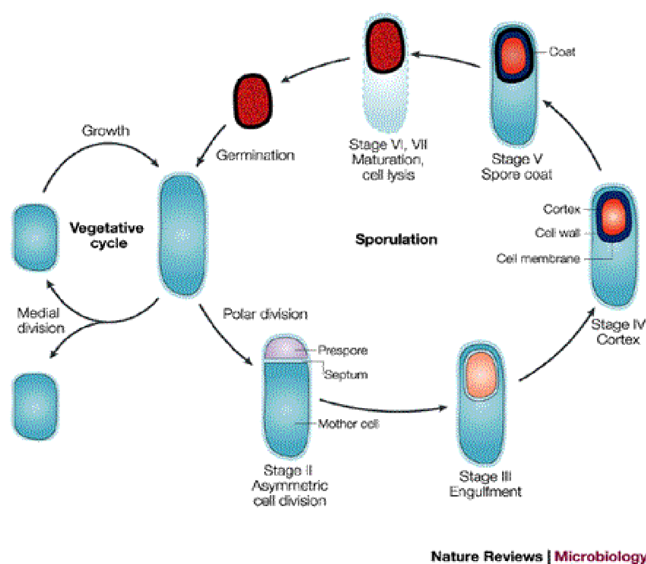

Fig: 2.1 Sporulation life cycle of *Bacillus* (Fig. adapted from Errington, 2003)

2.2.5 THE USE OF *BACILLUS* SPECIES FOR INDUSTRIAL PRODUCTION

Bacillus sp. have been the major workhorse industrial microorganisms, since the production of natto by solid-state fermentation of soybeans using *Bacillus subtilis* (natto) was first practiced in Japan, which dates back more than a thousand years ago (Hara & Ueda, 1982). These roles have continually expanded and evolved over the past century. The development of strains and production strategies has recently been influenced or facilitated by the application of

molecular biology techniques to strain development. *Bacillus* species are attractive industrial organisms for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into the extracellular medium, and the GRAS (generally regarded as safe) status with the Food and Drug Administration for species such as *B. subtilis* and *Bacillus licheniformis*. The uses of *Bacillus* species in industries include production of enzymes, insecticides, antibiotics, bacteriocins, D- ribose, vitamins and many more.

2.3 BACTERIOCINS

Microbes have an extraordinary array of defense systems. These include classical antibiotics, metabolic by-products such as lactic acid, numerous types of protein exotoxins and antimicrobial peptides like bacteriocins. The first description of bacteriocin-mediated growth inhibition was reported 85 years ago, when antagonism between strains of *Escherichia coli* was revealed (Gratia, 1925). The inhibitory substances were called 'colicins', to reflect the producer organism, whereas gene-encoded antibacterial peptides produced by bacteria are now referred to as 'bacteriocins'. Since their initial discovery in 1925, numerous bacteriocins have been isolated from various genera of bacteria.

Bacteriocins are ribosomally synthesized peptides with bactericidal activity towards species that are often closely related to the producer strain (Lisboa *et al.*, 2006). They are found lethal to other bacteria rather than the producing strains (Joerger, 2003). These substances are produced by various Gram-negative as well as Gram-positive bacteria. The most promising antimicrobial peptides are produced by lactic acid bacteria (LAB). Most LAB bacteriocins are non-toxic to eukaryotic cells and generally recognized as safe substances (GRAS), being active in the nanomolar range (Cotter *et al.*, 2005; Peschel & Sahl, 2006). LAB antimicrobial peptides typically exhibit antibacterial

activity against food-borne pathogens, as well as spoilage bacteria, thereby attracting attention as tools for food biopreservation (Collins *et al.,* 1998; O'Sullivan *et al*., 2002; Reid *et al*., 2003).

2.3.1 CLASSIFICATION OF BACTERIOCINS FROM LACTIC ACID BACTERIA (LAB)

The main classification scheme for antimicrobial peptides of ribosomal origin currently available is that for LAB bacteriocins. The classification of LAB bacteriocins was previously well established by Klaenhammer (1993) and then adaptations or reclassifications were performed by Nes *et al*. (2007).

Class I bacteriocins termed lantibiotics are small, membrane-active and heat-stable peptides that contain unusual thioether amino acids, like lanthionine and β-methyllanthione. Nisin from *Lactococcus lactis* is probably the most well-known and studied bacteriocins of this group (Cleveland *et al*., 2001).

Class II bacteriocins contain small (4-6 kDa), heat-stable, non-modified peptides (Nes and Holo, 2000; Cleveland *et al.,* 2001) which are subdivided into three sub classes. Class IIa is formed of listeria-active or anti-listerial peptides. These are 'pediocin-like' which are characterized by a well conserved YGNGVXaaC consensus motif at their N-terminal ends. These contain the recently described lactococcin MMFII (Ferchichi *et al*., 2001) and sakacin G (Simon *et al*., 2002). The Class IIb consists of the two–peptide bacteriocins wherein the association of two different peptides is essential for full activity, such as lactococcin G (Nissen-Meyer *et al.,* 1992) or lacticin F (Muriana & Klaenhammer 1991). A third subgroup Class IIc is suggested to contain sec-dependent secreted bacteriocins (Nes *et al.,* 1996) as it was shown more recently that enterocin P, which contains the IIa consensus motif, is also secreted in a sec-dependent manner (Cintas *et al*., 1997). On the other hand, bacteriocins like

enterocins B and I (Casaus *et al*., 1997; Floriano *et al*., 1998), which are sec-independent secreted one-peptide bacteriocin lacking the YGNGVXaaC motif, could not be clearly categorized within a particular subgroup. This classification scheme will therefore certainly be evolving based on new evidences, particularly the identification of new natural peptides.

Class III bacteriocins are secreted by the bacterial preprotein translocase (sec-pathway) and are large (>30kDa), heat-labile proteins (Nes *et al*., 1996). This group is not well documented. Only few large bacteriocins produced by LAB are described at the molecular level, such as helveticin J produced by *Lactobacillus helveticus* 481 (Joerger and Klaenhammer, 1990) or enterolysin A from *Enterococcus faecalis* LMG 2333. Nevertheless, some of the incompletely described antibacterial compounds could be suspected to be class III bacteriocins, although not yet classified, e.g. enterocins 1071A and 1071B from *E. faecalis* (Balla *et al.,* 2000), enterocin B (Casaus *et al.,* 1997), enterocins L50A and L50B (Cintas *et al.,* 1998) and enterocin Q from *Enterococcus faecium* (Cintas *et al.,* 2000).

Class IV includes circular bacteriocins. Due to the unique property of being circularized (head to tail), these bacteriocins have been proposed to be encompassed in a new class of bacteriocins (Kemperman *et al*., 2003). These circular peptide-bacteriocins are covalently linked head to tail and bacteriocin AS-48 from *E. faecalis* is so far the most thoroughly studied one (Diep *et al*., 1995). Bacteriocins identical to AS-48 have been reported, these include enterocin 4 (Joosten *et al*., 1996) and bacteriocin 21 (Tomita *et al.,* 1997). The genetic information for AS-48 bacteriocin is located in 7.8 kb region on a 68 kb pheromone-responsive conjugative plasmid. In two different *Lactobacillus* species, the two almost identical cyclic bacteriocins are found; they have been

termed gassericin A and reutericin 6. They are produced by *Lb. gasseri* and *Lb. reutrii* respectively (Kabuki *et al*., 1997; Kawai *et al.,* 1998).

Lactic acid bacteria have been a subject of intensive research as it is generally regarded as safe by the Food and Drug Administration (FDA). Of the bacteriocins identified to date, only nisin produced by *Lactococcus lactis* and related compounds such as pediocin are currently authorized for use as food preservatives (Brul & Coote, 1999). Nisin is a 34 residue antibacterial protein with the presence of lanthionines and uncommon amino acids. Nisin acts on vegetative bacteria by a four-step process of binding, insertion, aggregation and pore formation. Nisin binds to the target membrane by electrostatic interactions with the anionic phospholipid and inserts into the membrane by its hydrophobic patches (Bruelink *et al*., 1997). Nisin, however, acts on the sulfhydryl membrane groups for inactivation of germinated spores. Nisin is known to be reactive to Gram-positive bacteria and spores while being resistant to Gram-negative bacteria and yeast or filamentous fungi (Morris *et al.,* 1984).

2.3.2 BACTERIOCINS FROM GENUS *BACILLUS*

2.3.2.1 *Bacillus licheniformis*

Bacillocin 490 is a bacteriocin produced by the thermophilic strain *B. licheniformis* 490/5 isolated from a dairy product (Martirani *et al*., 2002). Bacteriocin like inhibitory substance (BLIS) with a broad spectrum of activity against pathogenic and spoilage bacteria like *L. monocytogenes*, *B. cereus* and clinical isolates of *Streptococcus* sp. was produced by *B. licheniformis* P40 that originated from the Amazon basin (Cladera-Olivera *et al*., 2004). Bacteriocins are also reported from *B. licheniformis* SN2 (El-Sersy *et al*., 2009) and *B. licheniformis* strain BTHT8 (Smitha & Bhat, 2013). Other uncharacterized antimicrobial substances are produced by *B. licheniformis* T6-5 isolated from an oil reservoir in Brazil (Korenblum *et al*., 2005) and by *B. lichenifomis* AnBa9

isolated from sediments of slaughterhouse sewage wastes (Anthony *et al*., 2009, 2010).

2.3.2.2 *Bacillus subtilis*

B. subtilis strains are known to produce a wide variety of antibacterial and antifungal compounds (Stein, 2005). Subtilin produced by *B. subtilis*, (Gross *et al*., 1973), shows antimicrobial activity in the nanomolar range against a broad spectrum of Gram-positive bacteria. Ericin S and ericin A are two related lantibiotics produced by *B. subtilis* A1/3 with strong similarities to subtilin. Ericins (mainly ericin S) are active against a variety of bacteria, including the causative agent of tomato bacterial canker, *Clavibacter michiganensis* (Stein *et al.*, 2002 a). Sublancin 168 produced by *B. subtilis* 168 exhibits bactericidal activity against other Gram-positive bacteria, including important pathogens such as *B. cereus*, *Streptococcus pyogenes* and *Staphylococcus aureus* (Paik *et al.,* 1998). Mersacidin produced by *B. subtilis* strain HILY-85, 54728 is a tetracyclic peptide active against methicillin- and vancomycin-resistant *S. aureus* (Bierbaum *et al*., 1995). Bacteriocins from *B. subtilis* strains termed subtilosins were reported (Zheng & Slavik, 1999; Zheng *et al*., 2000; Shelburne *et al*., 2007). *B. subtilis* LFB112 from Chinese herbs (Xie *et al*., 2009) produces a BLIS active against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases, including *E. coli*, *Salmonella* Pullorum, *Pseudomonas aeruginosa*, *Pasteurella multocida, Clostridium perfringens*, *Micrococcus luteus*, *Streptococcus bovis* and *S. aureus*. *B. subtilis* 14B isolated from the rhizosphere of healthy plants (bitter almond) produces a BLIS (Bac 14B) active against *Agrobacterium tumefaciens* (Hammami *et al*., 2009). Other strains that produce bacteriocins include *B. subtilis* BsGh-18 (El-Hamsary & Khattab, 2008), *B. subtilis* of reed bed (Collings & Phillips, 2001), *B. subtilis* KIBGE IB-17 (Ansari *et al*., 2012) and *B. subtilis* BS15 (Alam *et al*., 2011).

2.3.2.3 *Bacillus amyloliquefaciens*

The strain *B. amyloliquefaciens* LBM 5006 isolated from the Brazilian Atlantic forest also produces bacteriocin like inhibitory substance (BLIS) which inhibits the growth of *Listeria monocytogenes*, *B. cereus*, *Serratia marcescens* and *Pasteurella haemolytica* (Lisboa *et al*., 2006). *B. amyloliquefaciens* CECT 5940 produces a BLIS active against *E. coli* and *Clostridium perfringens* (Diaz, 2007). Other strains include the dairy product-derived *B. amyloliquefaciens* (Sutyak *et al*., 2008), *B. amyloliquefaciens* MBL27 (Vijayalakshmi *et al*., 2011 b) to name a few.

2.3.2.4 *Bacillus cereus*

B. cereus produces several BLIS or cereins such as cerein 8A produced by *B. cereus* 8A (Bizani & Brandelli, 2002; Bizani *et al*., 2005 a & b), cerein GN105 produced by *B. cereus* GN105 (Naclerio *et al*., 1993) and cereins produced by *B. cereus* strains 30/11, 15/5, 8/10 and 8/2 (Torkar & Matijasic, 2003). Cerein 8A inhibits several pathogenic and food-spoilage microorganisms such as *L. monocytogenes* and *B. cereus*, apparently by disturbing their membrane function. This BLIS is also active against intact spores of *B. cereus*. Cerein GN105 is active exclusively against other *B. cereus* strains. Bacteriocins from *B. cereus* are also reported by Risoen *et al*. (2004) and Sebei *et al*. (2007).

2.3.2.5 *Bacillus clausii*

The probiotic *B. clausii* O/C strain releases a pronase sensitive antimicrobial substance during the stationary growth phase coincident with sporulation. The antimicrobial spectrum of the inhibitor produced includes *S. aureus*, *Enterococcus faecium* and *Clostridium difficile* (Urdaci *et al*., 2004).

2.3.2.6 *Bacillus firmus*

B. firmus H2O-1 isolated from a Brazilian oil reservoir produces a BLIS that shows antimicrobial activity against *Desulfovibrio alaskensis* and a group of sulfate-reducing bacteria (SRB) obtained from the reservoir core (Korenblum *et al*., 2005).

2.3.2.7 *Bacillus lentus*

Sharma *et al.* (2006) reported a BLIS produced by a foodgrade *B. lentus* NG121 strain isolated from Khameera, a traditional fermented food from Himachal Pradesh which was active against *L. monocytogens* and *S. aureus*. Bacteriocin from *B. lentus* is also reported by Sharma *et al*. (2009 a).

2.3.2.8 *Bacillus megaterium*

The megacins produced by *B. megaterium* strains were divided into three groups A, B and C based on mode of action, activity spectrum and response to UV or mitomycin C induction (Holland & Roberts, 1964). Megacin19 produced by *B. megaterium* 19 isolated from a mixture of fermented vegetable wastes and megacin 22 produced by *B. megaterium* 22 isolated from soil, showed wide antimicrobial spectra against food-spoilage bacteria such as *Salmonella Typhimurium* and *S. aureus* (Khalil *et al*., 2009 a & b).

2.3.2.9 *Bacillus mycoides*

B. mycoides strain isolated from whey produced a BLIS active against food-borne pathogens such as *L. monocytogenes* and *L. mesenteroides* (Sharma & Gautam, 2008).

2.3.2.10 *Bacillus polyfermenticus*

A commercial probiotic *B. polyfermenticus* strain SCD used for the treatment of long-term intestinal disorders was reported to produce

polyfermenticin SCD, a BLIS with an estimated molecular weight of about 14.3 kDa by SDS-PAGE and with narrow spectrum of activity against Gram-positive bacteria like *Bacillus* sp., *S. aureus*, *Cl. perfringens* and *Micrococcus flavus* (Lee *et al*., 2001).

2.3.2.11 *Bacillus pumilus*

Pumilicin is a plasmid-encoded antimicrobial peptide produced by *B. pumilus* PL10 (Lovett *et al*., 1976). Pumilicin 4 produced by *B. pumilus* WAPB4 showed remarkable antibacterial activity against MRSA, vancomycin-resistant *E. faecalis* (VRE) and several Gram-positive test bacteria (Aunpad & Na-Bangchang, 2007).

2.3.2.12 *Bacillus thuringiensis*

Thuricin 7, a novel bacteriocin produced by *B. thuringiensis* BMG1.7 (Cherif *et al*., 2001), bacteriocin by *B. thuringiensis* strain B439 (Ahern *et al.,* 2003), thuricin 17 by *B. thuringiensis* NEB17 (Gray *et al*., 2006 b), BLIS produced by Mexican strains of *B. thuringiensis* (Barboza-Corona *et al*., 2007) are some of the bacteriocins reported from *B. thuringiensis*.

2.3.2.13 *Brevibacillus brevis* (formerly *Bacillus brevis*)

Bacillocin Bb produced by the *B. brevis* Bb from soil (Saleem *et al.,* 2009) is a BLIS active against *S. aureus*, *M. luteus*, *Corynebacterium diphtheriae*, *Corynebacteium xerosis* and *Corynebacterium hoffmanni*. Brevicin AF01 of *B. brevis* AF01 isolated from a wheat field is a lipoprotein active against several MRSA strains (Faheem *et al*., 2007).

2.3.2.14 *Lysinibacillus sphaericus* (formerly *Bacillus sphaericus*)

The production of BLIS by this species was reported in mosquito pathogenic and nonpathogenic strains (Cokmus & Yousten, 1993; Cetinkaya *et al*., 2003) and *Lysinibacillus sphaericus* SOPB1 (Aunpad *et al*., 2007).

2.3.2.15 *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*)

Four peptides named thermocins have been described in *G. stearothermophilus* strains (strains 17, 30, 31 and 32A) isolated from oil wells in Lithuania (Pokusaeva *et al*., 2009) which exhibited bactericidal activity against *Geobacillus* sp. as well as against several pathogenic bacteria such as *B. cereus* and *S. haemolyticus.* Thermocin 93 produced by *G. stearothermophilus* RS93 inhibited the growth of all thermophilic bacilli examined, but had no effect on mesophilic bacilli (Sharp *et al*., 1979).

2.3.2.16 *Geobacillus thermoleovorans* (formerly *Bacillus thermoleovorans*)

Thermoleovorin-S2 (estimated molecular weight of 42 kDa) and thermoleovorin-N9 produced by *G. thermoleovorans* S-II and *G. thermoleovorans* NR-9 respectively (Novotny & Perry, 1992) were found active against other *G. thermoleovorans* strains, *Salmonella* Typhimurium, *Branhamella catarrhalis* and *E. faecalis*.

2.3.2.17 *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*)

Paenibacillin is a lantibiotic produced by *P. polymyxa* OSY-DF, active against a broad range of foodborne pathogenic and spoilage bacteria including *Bacillus* sp., *Cl. sporogenes*, *Lactobacillus* sp., *L. lactis*, *Leuconostoc mesenteroides*, *Listeria* sp., *Pediococcus cerevisiae*, *S. aureus* and *Streptococcus agalactiae* (He *et al*., 2007). Polyxin is an antimicrobial peptide produced by *P. polymyxa* P13 isolated from Argentinean regional fermented sausages which is

active against a wide range of Gram-positive and Gram-negative bacterial species such as *Bacillus* sp., *Lactobacillus* sp., *E. coli*, *Proteus vulgaris* and *Klebsiella pneumoniae* (Piuri *et al*., 1998).

2.3.2.18 Other *Bacillus* sp.

The *Bacillus* sp. strain P34 isolated from the Amazon basin produces a BLIS P34 active against Gram positive bacteria including *L. monocytogenes* and Gram negative bacteria such as *E. coli* and *Salmonella Enteritidis* (Motta *et al.,* 2008). Another BLIS produced by *Bacillus* sp. MTCC 43 isolated from the rhizosphere of root crop radish (*Raphanus sativus*) was active against *S. aureus* and *Aeromonas hydrophila* (Sharma *et al*., 2009 b).

2.3.2.19 Bacteriocin like inhibitory substance with antibacterial and antifungal activity

Some of the BLIS produced by *Bacillus* strains also show inhibitory activity against yeasts and/or molds. *B. amyloliquefaciens* strain RC-2 produces a BLIS active against *Colletotrichum dematium*, mulberry anthracnose fungus and several other phytopathogenic fungi and bacteria (Yoshida *et al*., 2001). *B. brevis* strain isolated from kimchi, a Korean traditional fermented vegetable product produced a BLIS with a broad spectrum of antimicrobial activity against various Gram-positive bacteria, Gram-negative bacteria and some yeasts such as *Pichia anomala* and *Saccharomyces cerevisiae* (Mah & Hwang, 1998; Hyung *et al*., 2001). *B. licheniformis* strain ZJU12 isolated from soil produced a BLIS with broad spectrum antagonistic activity against various species of Gram-positive bacteria as well as pathogenic fungi (Lili *et al*., 2006). A low-molecular-weight peptide (1.5 kDa by SDS-PAGE) produced by *B. licheniformis* MKU3 (Kayalvizhi & Gunasekaran, 2008) exhibited bactericidal activity against Gram positive and Gram-negative bacteria, as well as different filamentous fungi and yeast (*Candida albicans*). *B. subtilis* strain MJP1 isolated from meju (Korean

fermented soybean) produces a BLIS with antimicrobial activity against various species of Gram-positive bacteria, yeasts and molds including food-spoilage microorganisms (Yang & Chang, 2007). *B. thuringiensis* ssp. *entomocidus* HD9 produces entomocin 9 (12.4 kDa) which is active against Gram-positive bacteria including *L. monocytogenes*, Gram-negative bacteria such as *P. aeruginosa* and against several fungi (Cherif *et al*., 2003). *Bacillus* sp. P45 isolated from the intestinal contents of the teleost fish Jaraqui (*Piaractus mesopotamicus*) of the Amazon basin produces an antimicrobial peptide (P45) that is active against Gram-positive and Gram-negative bacteria and also against yeasts (Motta *et al*., 2004; Sirtori *et al*., 2006).

2.3.3 CLASSIFICATION OF *BACILLUS* BACTERIOCINS

Bacteriocins and BLIS produced by *Bacillus* sp. may be considered as the second most important after bacteriocins produced by the LAB. Various strains of genus *Bacillus* produce a diverse array of antimicrobial peptides, with several different basic chemical structures (Gebhardt *et al*., 2002; Stein, 2005). To date, no classification scheme has been devised for *Bacillus* bacteriocins despite all the classification efforts made with the bacteriocins of LAB. This is most probably a result of the lack of information on many of these bacteriocin amino acid sequences or the considerable diversity of the peptides/proteins produced by the bacilli. A simple classification scheme for the bacteriocins/ BLIS produced by *Bacillus* species was proposed by Abriouel *et al*. (2011) which includes three classes.

Class I: includes antimicrobial peptides that undergo different kinds of post-translational modifications. This class can be subdivided into four subclasses. Subclasses I.1–I.3 include peptides with modifications typical of lantibiotics (e.g. formation of lanthionine and β-methyl lanthionine residues), while subclass I.4 includes other unique modifications.

Subclass I.1 includes type A lantibiotics with an enlongated structure such as subtilin, ericin S and ericin A.

Subclass I.2 includes the type B globular lantibiotic mersacidin and other lantibiotics such as sublancin 168 and paenibacillin.

Subclass I.3 includes the two-component lantibiotics such as haloduracin and lichenicidin.

Subclass I.4 includes the unique cyclic peptide subtilosin A that contains a head-to-tail peptide bond as well as particular sulfide bridges formed between cysteine groups and dehydrated amino acid residues.

Class II: includes small (0.77–10 kDa), ribosomally synthesized, non-modified and linear peptides, which are heat and pH stable. This class can be subdivided into four subclasses.

Subclass II.1 includes pediocin-like peptides with a conserved YGNGVXC motif near the N-terminus and the coagulin produced by *B. coagulans* I4, as well as the bacteriocins produced by *B. circulans* and *P. polymyxa* strains (SRCAM 37, SRCAM 602, SRCAM 1580) belong to this subclass.

Subclass II.2 includes thuricin-like peptides with a conserved DWTXWSXL motif near the N-terminus such as bacthuricin F4, thurincin H and thurincins S and 17 produced by *B. thuringiensis* strains; and cerein MRX1 produced by *B. cereus* strains.

Subclass II.3 includes other linear peptides, such as lichenin produced by *B. licheniformis*, or cereins 7A and 7B.

Class III: includes large proteins (430 kDa) with phospholipase activity such as megacins A-216 and A-19213 produced by *B. megaterium* strains.

Many other antimicrobial polypeptides of intermediate size (10–30 kDa) and other large antimicrobial proteins produced by bacilli are not included in this classification scheme due to the lack of data on their protein or gene sequences.

2.3.4 STRUCTURE OF *BACILLUS* BACTERIOCINS

Subtilin is a cationic, pentacyclic antimicrobial peptide (Fig. 2.2.a) belonging to type-A lantibiotics produced by *B. subtilis* and has been studied extensively in terms of its protein structure and genetic determinants (Gross *et al.,* 1973; Sahl & Bierbaum, 1998; Guder *et al*., 2000). The gene encoding the subtilin peptide (Banerjee & Hansen, 1988) encodes a 56-residue peptide precursor that is processed to yield the 32-residue mature peptide subtilin. The precursor contains serine, threonine and cysteine residues at positions that allow them to undergo a series of dehydration and cross-linking steps, to yield the mature subtilin, which contains the unusual amino acids such as lanthionine, β-methyllanthionine, D-alanine, dehydroalanine and dehydrobutyrine.

Although the precursors of ericin S and A show only 75% identity to subtilin, the mature lantibiotic peptides revealed highly similar properties. Ericin S differs from subtilin only by four amino acid residues and it elicits a subtilin-like, lanthionine bridging pattern (Fig. 2.2.a), which makes the antimicrobial activity and physico-chemical properties similar to subtilin. Ericin A has a different ring organization and 16 amino acid substitutions compared with ericin S (Fig. 2.2.a). Ericin A differs by two C-terminal rings from the lanthionine pattern of subtilin, and perhaps for this reason, it has a much lower antimicrobial activity (Stein *et al*., 2002 a).

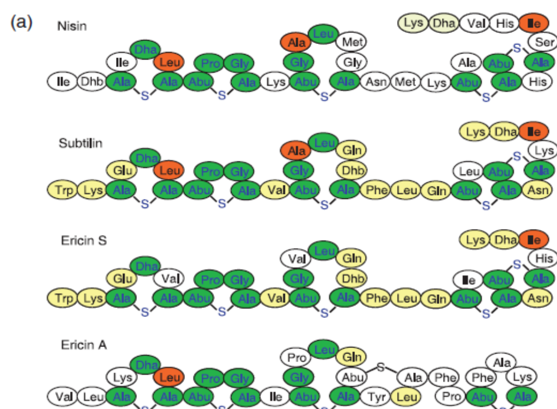

Fig: 2.2.a Comparison of subtilin and ericin structures with that of the lactococcal lantibiotic nisin A. Conserved residues at identical positions to all four bacteriocins are highlighted in green, while those conserved only in subtilin and ericins are depicted in yellow; other conserved residues are in light red (Fig. adapted from Abriouel *et al*., 2011)

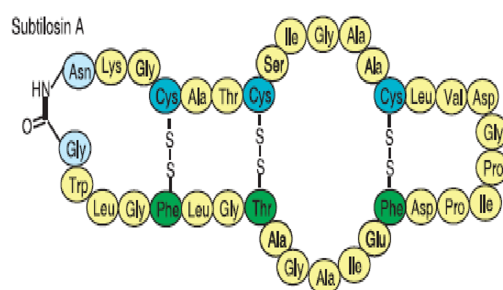

Fig: 2.2.b Structure of subtilosin A (Fig. adapted from Abriouel *et al*., 2011)

Subtilosin A is a highly post-translationally modified, macrocyclic, anion peptide. The 35-amino-acid mature peptide is cyclized by an unusual amide bond between N and C termini of asparagine and glycine respectively (Fig. 2.2.b). It also contains three intramolecular bridges formed between the sulfurs of Cys13, Cys7 and Cys4 and the α-carbons of Phe22, Thr28 and Phe31 respectively. Post

translational linkage of a thiol to the α-carbon of an amino acid residue in subtilosin A is unprecedented in ribosomally synthesized peptides or proteins. Such a unique nature of the cross-links in subtilosin A suggests that this bacteriocin (together with its lack of lanthionine and methyl lanthionine residues) should be classified separately from lantibiotics (Marx *et al*., 2001; Kawulka *et al*., 2003 & 2004).

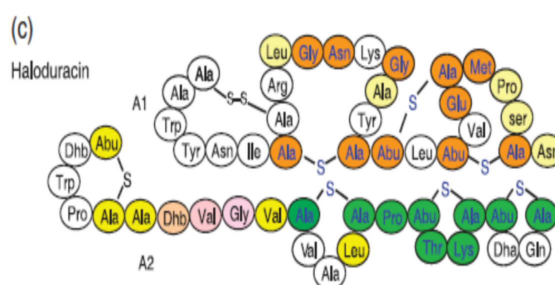

Fig: 2.2.c Structure of haloduracin (Fig. adapted from Abriouel *et al*., 2011)

Haloduracin (Fig 2.2.c) is produced by the alkaliphile *Bacillus halodurans* C-125. It consists of two post-translationally processed peptides Hala/A1 and Halb/A2.

2.3.5 GENETICS OF BACTERIOCINS FROM *BACILLUS* SP.

2.3.5.1 Class I: Post-translationally modified peptides

2.3.5.1.1 Subclass I. 1 Single-peptide, elongated lantibiotics

Subtilin is the paradigm lantibiotic produced by *B. subtilis* and has been studied extensively in terms of its protein structure and genetic determinants. Subtilin is a cationic, pentacyclic antimicrobial peptide belonging to type-A lantibiotics (Gross *et al*., 1973; Sahl & Bierbaum, 1998; Guder *et al*., 2000). The gene encoding the subtilin peptide (Banerjee & Hansen, 1988) encodes a 56-residue peptide precursor, processed to yield the 32-residue mature peptide

subtilin, with molecular mass of 3319.56 Da as revealed by MALDI-TOF MS (Stein, 2008). The precursor contains serine, threonine and cysteine residues at positions that allow them to undergo a series of dehydration and cross-linking steps, to yield the mature subtilin, which contains the unusual amino acids lanthionine, β-methyl lanthionine, D-alanine, dehydroalanine and dehydro butyrine. The precursor peptide contains a leader region that has an unusual hydropathic characteristic for an exported protein. The subtilin gene locus (Fig. 2.3.a) includes the structural gene *spaS*, which encodes the subtilin precursor and also genes encoding proteins involved in the post-translational modification of the precursor presubtilin viz. *spaB* and *spaC*, which encode a dehydratase and a cyclase respectively. The cluster also includes genes for the secretion of the modified precursor (*spaT*, which encodes the transporter SpaT) and genes for immunity against the cognate bacteriocin (*spaIFEG*). SpaFEG forms an ABC transporter that exports the lantibiotic from the cytoplasmic membrane, while SpaI is a lipoprotein that is thought to interfere with the binding of bacteriocin molecules to lipid II (Koponen *et al*., 2004; Stein *et al*., 2005). The modification and secretion components of subtilin are assembled in multimeric protein complexes (SpaB, SpaC and SpaT) that interact with the subtilin precursor (Siegers *et al*., 1996; Kiesau *et al*., 1997). The final processing steps that convert presubtilin to mature subtilin involves unspecific, non dedicated serine proteases secreted by *B. subtilis* (Corvey *et al*., 2003).

The production of subtilin appears to be regulated at the transcriptional level in a cell-density-dependent manner ie. by quorum sensing. This autoregulatory module includes the subtilin lantibiotic itself as an auto inducing agent. Signal transduction is by the corresponding two-component regulatory system made up by SpaKR and three independent transcriptional promoters (the lantibiotic-responsive) that precede *spaB*, *spaS* and *spaI*. After the extracellular subtilin reaches a certain threshold concentration, it activates the membrane-

located SpaK, which autophosphorylates. This in turn leads to phosphorylation of the cytosolic SpaR, which in its activated form can bind to a DNA motif promoting the expression of genes for subtilin biosynthesis (*spaS* and *spaBTC*) and immunity (*spaIFEG*) (Klein *et al*., 1993; Stein *et al*., 2002 b; Kleerebezem *et al*., 2004). In addition, SpaRK expression is controlled by the sporulation transcription factor SigH, which is repressed during the exponential phase of growth by the transition-state regulator AbrB (Fawcett *et al*., 2000). In this way, subtilin production is linked both to cell density and to sporulation. Subtilin is produced as an adaptive response to changes in the environment such as decreasing nutrient levels, allowing the cell to benefit optimally from the available resources. Therefore, high amounts of subtilin are produced under starvation conditions, compared with the lower amounts produced when nutrients are sufficient. It is believed that the role of subtilin is to increase the nutrient supply by liminating competing species and/or other *B. subtilis* cells. This would obviously result in the release of additional nutrients into the environment once they are killed.

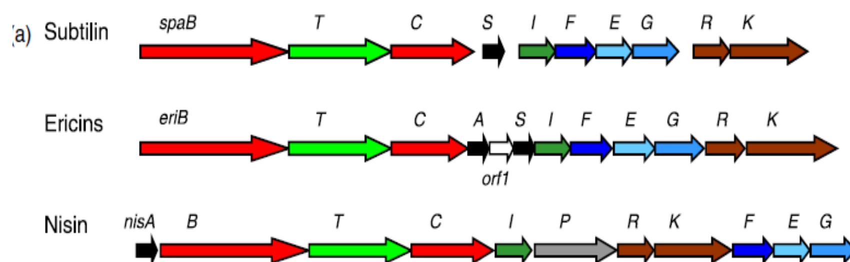

Fig: 2.3.a Gene loci of subtilin, ericin and nisin (Subclass I.1. Single-peptide, elongated lantibiotics) (Fig. adapted from Abriouel *et al*., 2011)

The ericin gene cluster (Fig. 2.3.a) contains two structural genes (*eriA*, *eriS*) and the ORFs are closely related to the corresponding genes of the subtilin

cluster. Both ericins are produced in equal quantities and share the single synthetase (EriBC), reflecting the flexibility of lantibiotic pathways (Stein *et al*., 2002 a).

2.3.5.1.2 Subclass I.2. Other single-peptide lantibiotics

Mersacidin produced by *B. subtilis* strain HILY-85 is a tetracyclic peptide of 1824 Da and belongs to the globular (type B) lantibiotics (Bierbaum *et al*., 1995). It is encoded in a region that corresponds to 3481 on the chromosome of *B. subtilis* (Chatterjee *et al*., 1992).

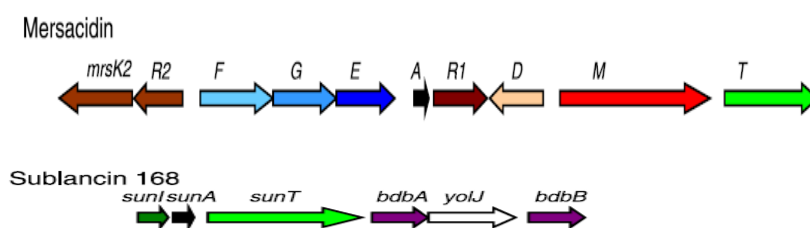

Fig: 2.3.b Genetic determinants of mersacidin and sublancin 168 (other single peptide lantibiotics) (Fig. adapted from Abriouel *et al*., 2011)

The mersacidin gene cluster is located on the bacterial chromosome and contains 10 genes spanning 12.3 kbp (Fig. 2.3.b). It includes the structural gene *mrsA*, two genes coding for enzymes involved in the post-translational modification of the prepeptide (*mrsM* and *mrsD*), one gene (*mrsT*) coding for a transporter with an associated protease domain and three genes (*mrsF*, *mrsG*, *mrsE*) encoding ABC transporter (group B) that could be involved in producer self-protection. MrsD is an oxidative decarboxylase responsible for the unique modification of the C-terminal cysteine of the peptide into S-[(Z)-2-aminovinyl] -3-methyl-D-cysteine (MeAviCys). In addition, among the three regulatory genes present; *mrsR2* and *mrsK2* encode a two component regulatory system that seems to be necessary for the transcription of the *mrsFGE* operon involved in bacteriocin

immunity and *mrsR1* encodes a protein with a similarity to response regulators and regulates mersacidin biosynthesis (Altena *et al*., 2000; Guder *et al*., 2002).

Sublancin 168 (3877.78 Da) is a chromosomally encoded lantibiotic produced by *B. subtilis* 168 (Paik *et al*., 1998). It contains a single lanthionine linkage and two disulfide bridges. The operon responsible for sublancin production and immunity contains five genes (Fig. 2.3.b): the structural gene *sunA*, which was identified by sequencing the SPβ prophage region of the *B. subtilis* 168 chromosome (Lazarevic *et al*., 1999) and four successive genes (*sunT*, *bdbA*, *yolJ* and *bdbB*) located downstream of *sunA* (Serizawa *et al*., 2005). ORF *sunT* encodes a bifunctional ABC transporter with an ATP binding cassette domain and a proteolytic domain (McAuliffe *et al*., 2001); *bdbA* and *bdbB* encode thiol-disulfide oxidoreductases (Bolhuis *et al*., 1999; Dorenbos *et al*., 2002; Kouwen *et al*., 2007); while the function of *yolJ* is unknown. More recently, the sublancin immunity gene (*sunI*) has been identified as an ORF upstream of the structural gene *sunA*. This gene encodes an immunity protein SunI, which is a membrane protein with a single N-terminal membrane-spanning domain (Dubois *et al*., 2009).

2.3.5.1.3 Subclass I.3. Two-peptide lantibiotics

Two-peptide lantibiotics owe their antimicrobial activity to the synergistic activities of two (β-methyl) lanthionine containing peptides (A1, A2). Haloduracin is produced by the alkaliphile isolate *B. halodurans* C-125. It consists of two post-translationally processed peptides Hala/A1 and Halb/A2 (Fig. 2.3.c). MALDI-TOF analysis showed that the mature Hala/A1 and Halb/A2 (processed by the enzymes HalM1 and HalM2, which perform the post-translational modification) have molecular masses of 3046 and 2332 Da respectively (McClerren *et al*., 2006). Bioinformatic analysis identified a total of 11 genes (Fig. 2.3.c) spanning a 15 kbp region potentially involved in haloduracin production (*halA1*, *A2* genes);

modification (*halM1, M2*); immunity and transport (Lawton *et al*., 2007). The ORF *halT* is likely to be responsible for export and leader cleavage of the antimicrobial peptides. The haloduracin gene cluster also contains two sets of *lanEFG* homologs (*halF1* to *halE2*) involved in self-protection/immunity.

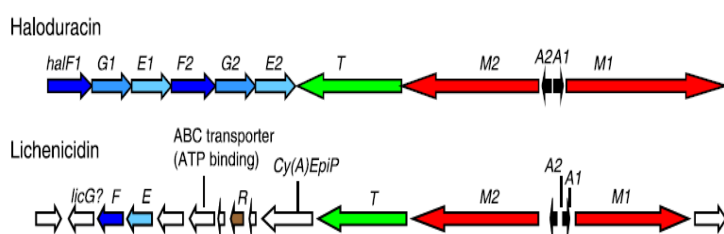

Fig: 2.3.c Gene loci of haloduracin and lichenicidin (Two-peptide lantibiotics) (Fig. adapted from Abriouel *et al*., 2011)

Lichenicidin is a two-peptide lantibiotic (Fig. 2.3.c) produced by *B. licheniformis* ATCC 14580 and *B. licheniformis* DSM 13 (Begley *et al*., 2009; Dischinger *et al*., 2009) with a high degree of homology to the two-peptide haloduracin from *B. halodurans* C-125 (Takami *et al*., 2000). The A subunit of lichenicidin also shows homology to mersacidin (Chatterjee *et al*., 1992; Bierbaum *et al*., 1995). The lichenicidin gene cluster resembles that of haloduracin (Fig. 2.3.c) as it contains two structural genes (*licA1,A2*) together with two genes (*licM1,M2*) encoding modification proteins of the LanM type, and a putative gene (*licT*) involved in the export and cleavage of the leader peptides for the mature lichenicidin A and B subunits. It also contains at least one set of genes of the *lanEFG* type involved in lantibiotic immunity.

2.3.5.1.4 Subclass I.4. Other post-translationally modified peptides

Subtilosin A is a macrocyclic, anionic, ribosomally synthesized and highly post-translationally modified peptide produced by *B. subtilis* strains, *B.*

amyloliquefaciens and *B. atrophaeus* (Babasaki *et al*., 1985; Zheng *et al*., 1999; Stein *et al*., 2004; Sutyak *et al*., 2008).

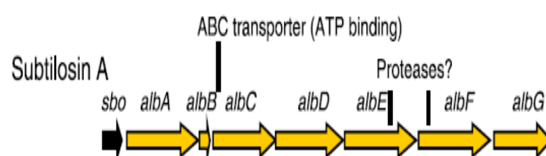

Fig: 2.3.d The operon encoding for subtilosin A (Other post-translationally modified peptides) (Fig. adapted from Abriouel *et al*., 2011)

The operon encoding for subtilosin A (Fig. 2.3.d) is composed of eight genes, with *sboA* encoding for the subtilosin prepeptide structural gene and *alb ABCDEFG* encoding the processing and immunity genes (Zheng *et al*., 1999 & 2000). The *sbo-alb* operon is induced under anaerobic conditions and is controlled by the transition state regulatory protein AbrB and the two-component regulatory proteins ResD and ResE that regulate gene expression in response to limiting oxygen supply (Nakano *et al*., 1996; Sun *et al*., 1996; Nakano & Zuber, 1998).

2.3.5.2 Class II: Non- modified peptides

2.3.5.2.1 Subclass II.1. Pediocin-like peptides

Coagulin is a pediocin-like peptide with antilisterial activity produced by *B. coagulans* I4 (Hyronimus *et al.*, 1998; Le Marrec *et al*., 2000). The genetic determinants of coagulin (*coa* operon) are located on the *B. coagulans* 14 kb plasmid pI4 and include the entire operon of four genes (Fig. 2.3.e). Interestingly, a putative plasmid mobilization module is located immediately downstream of the *coa* operon. It has been suggested that this module could be involved in plasmid transmission between bacteria. The structural gene for coagulin encodes a 44 amino acid peptide. The encoded peptide differs from pediocin PA-1/AcH only by a single C-terminal threonine residue at position 41, which is an asparagine in the

pediocin peptide (Asn41Thr). Bacteriocin immunity is assigned to *coaB*, while *coaC* and *coaD* code for a secretion system mediated by an ABC transporter and its accessory protein (Le Marrec *et al*., 2000).

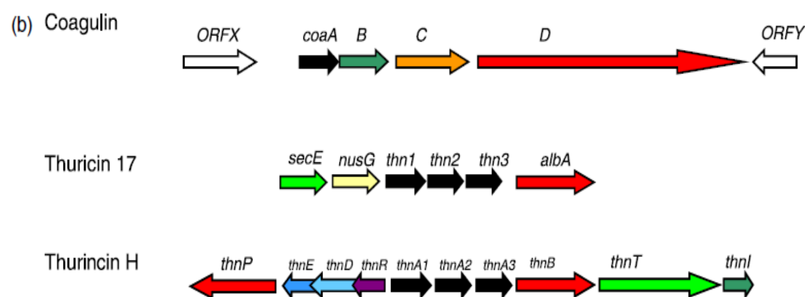

Fig: 2.3.e Genetic determinants of Class II (non- modified) peptides

(Fig. adapted from Abriouel *et al*., 2011)

2.3.5.2.2 Subclass II.2. Thuricin-like peptides

Thuricin is encoded by three copies of the same structural gene in tandem (Fig. 2.3.e), each of them coding for a 39 amino acid precursor (Lee *et al*., 2009 a). The N-terminal amino acid sequence determined by Edman degradation (DWTCWSCLVVAACSVELL) suggests that the first eight residues of the prepeptide are cleaved to produce the mature protein. The three-gene copy is flanked upstream by *secE* (encoding a protein translocase) and *nusG* (a transcriptional antitermination factor) and downstream by a sequence homologous to the *albA* gene of the subtilosin operon (which is probably involved in bacteriocin processing).

2.3.5.3 Class III: Large proteins

The A group megacins include the inducible megacins A-216 produced by *B. megaterium* 216 and A-19213 produced by *B. megaterium* ATCC 19213 (Ivanovics & Alfoldi, 1954; Von Tersch & Carlton, 1983 a). Both structural and

immunity genes of megacins are plasmid encoded on pBM309 (48 kb) and pBM113 (44 kb) in the *B. megaterium* 216 and ATCC 19213 strains respectively (Rostas *et al.*, 1980; Von Tersch & Carlton, 1983 b).

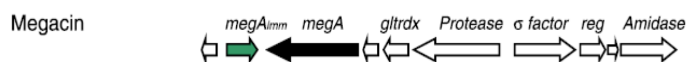

Fig: 2.3.f The genetic determinants of Class III bacteriocins (megacin A-216) (Fig. adapted from Abriouel *et al.*, 2011)

The genetic determinants of megacin A-216 (Fig. 2.3.f) are encoded by a 5.494 kbp plasmid region and include the structural gene *megA* encoding a 293-amino acid protein with sequence similarity to proteins with phospholipase A2 activity. Next to the *megA* gene is an ORF encoding a 91 amino acid protein responsible for the immunity of the producer strain against megacin A-216. At least two other genes, including ORF 73 and a gene encoding a 188 aminoacid protein sharing high sequence similarity to RNA polymerase s factors are required for the induction of megacin A-216 expression.

2.3.6 POST-TRANSLATIONAL MODIFICATIONS ENSUING ACTIVE BACTERIOCIN

Though bacteriocins are ribosomally synthesized, the resulting transcript must be modified before becoming active. Genes coding for the enzymes that facilitate the modifications are usually in close proximity to the structural gene. Lantibiotics experience the most extensive modification. LanB, a membrane spanning protein is transcribed by lantibiotic producers and enzymatically modifies the bacteriocin before transport out of the cell (Engelke *et al.*, 1992). LanC also participates in the formation of thioether bonds in lantibiotics (Kupke & Gotz, 1996).

The extensive post-translational modification of lantibiotics includes the formation of several unusual amino acids. Ingram (1969 & 1970) proposed that serine and threonine are modified to dehydroalanine and dehydrobutyrine respectively. These amino acids serve as precursors to lanthionine and methyl lanthionine, the formation of which occurs upon the addition of cysteine thiol groups. Over a dozen unusual amino acids are found in the lantibiotics and are summarized in a review by Kupke and Gotz (1996).

The prepeptides of non lantibiotics are also modified by cleavage of the leader sequence (Diep *et al*., 1996; Ehrmann *et al.,* 2000). These modifications are necessary for secretion and transport across the cell membrane.

2.3.7 BACTERIOCIN IMMUNITY

The immunity of the cell synthesizing the bacteriocin is a phenomenon that distinguishes bacteriocins from antibiotics. Genes coding for immunity proteins are in close proximity to the bacteriocin structural and processing genes (Siegers & Entian, 1995). It is common for the structural bacteriocin gene and the immunity gene to be located on the same operon and often next to each other (Nes *et al*., 1996; Klein & Entian, 1994). The immunity of lantibiotics was initially thought to be due to an immunity gene such as *nisI* for nisin and *spaI* for subtilin, which code for NisI and SpaI immunity proteins, respectively. It appears however that immunity to these bacteriocins is the result of the influence of several proteins, since the deletions of other genes result in altered host immunity (Klein & Entian, 1994). For example, non-nisin producing nisin-resistant strains of *L. lactis* do not have the genetic elements coding for NisI protein, but do have sequences similar to nisF, nisE and nisG (Duan *et al*., 1996). These are thought to render the strains resistant to nisin. The phenomenon of immunity is simpler in the non lantibiotics. One gene encodes for the immunity protein. Usually, it is a basic protein between 50 and 150 amino acid residues long that is loosely associated

with the membrane. The lactococcin A immunity protein (LcnI) is by far the most studied one, yet the basic mechanism behind the immunity is still not understood (Nissen-Meyer *et al*., 1993; Venema *et al*., 1994 & 1995; Saris *et al*., 1996).

2.3.8 TRANSPORT ACROSS THE CELL MEMBRANE

Most bacteriocins in class I and II are translocated to the outside of the cell by a dedicated ABC transporter system. The only exceptions are a few class II bacteriocins that are externalized by the sec-dependent system. The bacteriocins that are dependent on the ABC transporters can be divided into two major groups: bacteriocins with a double glycine-leader and bacteriocins with a different leader but not a sec-leader. The double-glycine leader bacteriocins are found mainly among the class II bacteriocins but also include some lantibiotics (Havarstein *et al*., 1994; Nes *et al*., 1996). These bacteriocins are secreted by a unique form of ABC transporters, which possess an N-terminal leader of approximately 150 amino acid residues exerting a specific proteolytic activity that cleaves the double glycine leader. Concomitant with the secretion, this specific ABC transporter cleaves the leader thereby activating the bacteriocin. An accessory protein is needed in this secretion process (Franke *et al*., 1996). The ABC transporters that secrete lantiobiotics with different type of leaders do not possess N-terminal proteolytic activity and the removal of the leader is carried out by a dedicated protease such as NisP in the nisin system.

2.3.9 MODE OF ACTION

Bacteriocins, particularly lantibiotics inhibit target cells by forming pores in the membrane, depleting the transmembrane potential and/ or the pH gradient, resulting in the leakage of cellular materials. Early studies suggest that in order for nisin to form pores, target cells require transmembrane potential which is negative inside and pH gradient which is alkaline inside (Okereke & Montville, 1992). Bacteriocins are positively charged molecules with hydrophobic patches.

Electrostatic interactions with negatively charged phosphate groups on target cell membranes are thought to contribute to the initial binding with the target membrane (Chen *et al*., 1997 a & b). The association of hydrophobic patches of bacteriocins with the hydrophobic membrane has also been modeled using computer simulation to predict the most favourable interaction, with the likelihood that the hydrophobic portion inserts into the membrane, forming pores (Lins *et al*., 1999).

Subtilin exhibits bactericidal activity based on pore formation in the cytoplasmic membrane, using cell wall precursors such as lipid II and undecaprenyl pyrophosphate as a docking module and central constituent of the pore (Breukink *et al*., 1999). It causes the dissipation of the transmembrane proton motive force as a result of pore formation and shows antimicrobial activity in the nanomolar range against a broad spectrum of Gram-positive bacteria.

Mersacidin targets the cell wall precursor lipid II and thereby inhibit cell wall synthesis (Brotz *et al*., 1998). However, it does not modify the bacterial cell membrane permeability like most other lantibiotics do. The pattern of lanthionine bridges of mersacidin is conserved in other lantibiotics such as the a/A1 subunits of haloduracin, lichenicidin, the a/A1 subunit of the lactococcal bacteriocin lacticin 3147 and others, suggesting that it may be required for binding to lipid II. Haloduracin mode of action by pore formation and binding membrane lipid II is similar to that exhibited by the two-peptide lantibiotic lacticin 3147 produced by *L. lactis* DPC3147 (Ryan *et al*., 1996).

There is evidence that class II bacteriocins actually inhibit septum formation in susceptible bacteria (Martinez *et al*., 2000). Because bacteriocins do not act equally against target species, researchers have examined the affinity of bacteriocins to specific species and strains.

2.3.10 RESISTANCE MECHANISMS

Once a new preservative is found to be safe and effective, it is critical to ensure the longevity of its use by preventing the proliferation of resistant cells. Already, cells exhibit resistance to several antibiotics and the transfer of resistance between organisms has been documented. Although bacteriocins are not antibiotics, there is concern that exposure to bacteriocins will render cells more resistant to antibiotics. Since antibiotics and nisin have different modes of action, it has shown that exposure to nisin has no effect on the frequency of resistance of *L. monocytogenes* Scott A to ampicillin and chloramphenicol (Tchikindas *et al*., 2000). In another study, several multi-drug resistant bacteria were subjected to up to 400 IU/mL nisin, and these organisms remained sensitive to nisin (Severina *et al.,* 1998). Cross-resistance between nisin and 33 other antimicrobials has also been studied, and penicillin resistant *S. aureus* was 50 times more sensitive to nisin (Szybalski, 1953). In addition to bacteriocins, other cationic peptides are active against antibiotic resistant organisms such as methicillin resistant *S. aureus* and vancomycin-resistant *S. haemolyticus* (Friedrich *et al*., 2000). Though bacteria exhibiting nisin resistance do not show cross-resistance with antibiotics, it is still important to understand the mechanism of resistance so that it can be avoided. Antibiotic resistance is usually associated with a genetic determinant, facilitating the transfer of resistance between cells, strains and species. Unlike most antibiotic resistance, bacteriocin resistance results from a physiological change in the target cell. For *L. monocytogenes*, the more rigid membrane usually having a lower C15:C17 ratio, results in increased tolerance to nisin (Crandall & Montville, 1998). Ming and Daeschel (1993) also found that nisin resistant *L. monocytogenes* have reduced amounts of phosphatidylglycerol, diphosphatidylglycerol and bisphosphatidylglyceryl phosphate. Though most researches show that a change in cell membrane composition accounts for resistance, some mutants produce nisinase, an enzyme which degrades nisin (Jarvis, 1967).

2.3.11 BACTERIOCINS vs. ANTIBIOTICS

Bacteriocins are often confused in the literature with antibiotics. This would limit their use in food applications from a legal standpoint. Bacteriocins, which are clearly distinguishable from clinical antibiotics, should be safely and effectively used to control the growth of target pathogens in foods. The major differences between bacteriocins and antibiotics are given in table 2.1

Table 2.1 Differences between bacteriocins and antibiotics

(Cleveland *et al*., 2001)

Characteristics	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Host cell immunity	Yes	No
Mechanism of target cell (resistance or tolerance)	Usually adaptation affecting cell membrane composition	Usually a genetic determinant affecting different sites depending on mode of action
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly pore formation, but in a few cases possibly cell wall biosynthesis	Cell membrane or intracellular targets
Toxicity/ side effects	None known	Yes

2.3.12 OPTIMIZATION OF CULTURE CONDITIONS AND MEDIA COMPOSITION

Optimization of culture conditions for the production of bacteriocins by *B. amyloliquefaciens* was studied by Vijayalakshmi *et al.* (2011 a) and Lisboa *et al.* (2006). Effect of shake and still culture conditions, different concentrations of inoculum, carbon sources and incubation temperature for maximum production was studied. Similarly, Ogunbanwo *et al.* (2003) studied bacteriocin production by *Lactobacillus brevis* OG1 and Paynter *et al.* (1997) studied plantaricin F production by *L. plantarum* under different incubation temperatures. Kim *et al.* (2006) optimized culture conditions and medium composition such as initial pH, incubation temperature, carbon sources, different media and concentration of di-potassium posphate and magnesium sulphate for the production of micrococcin GO5 by *Micrococcus* sp. GO5. Optimization of bacteriocin production by *B. megaterium* 19 was studied by Khalil *et al.* (2009b). El- Sersy *et al.* (2009) and Herranz *et al.* (2001) have studied the sodium chloride concentration for the maximum bacteriocin production by *Lactobacillus* sp. MSU3IR and *E. faceium* P13 respectively. Bacteriocin production studies using various inorganic and organic nitrogen sources were carried out by Iyapparaj *et al.* (2013). Effect of surfactants like tween 80 or tween 20 in bacteriocin production was studied by adding them in the growth medium (Garver & Muriana, 1994; Huot *et al*., 1996; Aymerich *et al.,* 2000).

2.3.13 ACTIVITY MEASUREMENTS

A major difficulty in bacteriocin research and applications is obtaining accurate quantification using bioassays which are based on the quantification of the inhibition produced in a sensitive microorganism (Turcotte *et al*., 2004). Although numerous other methods have also been described such as ELISA (Suarez *et al*., 1996; Bouksaim *et al*., 1999) ATP-bioluminometry (Waites & Ogden, 1987), radiometry (Culter *et al*., 1999), conductance measurements

(Giraffa *et al.*, 1990) or even sophisticated bioassays based on self-induction of the *nis* promoter and bioluminescence (Walstrom & Saris, 1999), they have not gained wide acceptance because of requirements for dedicated equipment, supplies and skills. Moreover, because the results produced by such methods cannot necessarily be correlated with antimicrobial activity. Therefore, growth inhibition techniques are still the most commonly used technique for quantitative estimation of bacteriocins in everyday trials.

Multiple procedures based on growth inhibition are described in the literature, relying on tests performed either in solid, eg. the plate agar diffusion assay or in liquid medium, eg. turbidometry. The agar diffusion assay, in which inhibition zones are produced in plates is a procedure similar to that of antibiograms and is undoubtedly the most commonly used despite the inconveniences and limitations of its application. The limitations of this method include 1. The performance of the method which is laborious and time-consuming 2. Depends largely on human ability 3. Judgment and precision cannot be obtained when inhibition zones are unclear or not perfectly circular 4. Diffusion-related difficulties of the active substance.

The need to eliminate diffusion-related problems associated with the agar techniques, introduced liquid medium methods, which make use of indicator organisms and quantify the bacteriocin concentration from the percentage of growth inhibition in the indicator organism. The method was introduced by Reeves (1965) in a study with colicins. Since then, applications of turbidometric assays can be found in a number of reports which show large variability regarding bacteriocin extraction, general experimental conditions and definition of the bacteriocin unit (Papagianni *et al.*, 2006).

2.3.14 PROTOCOLS FOR BACTERIOCINS PURIFICATION

2.3.14.1 Culture media

The composition of the growth medium greatly affects the production of individual bacteriocins (Tagg *et al*., 1976). Several media have been evaluated by numerous authors to improve bacteriocin synthesis because these peptides are not always produced in standard or enriched culture media. The isolation of a peptide in rich-medium supernatant is an additional problem, making the purification of the bacteriocin a relatively complicated process. Furthermore, many bacteriocin molecules tend to associate with other substances present in the culture medium, where they can display a high degree of hydrophobicity and form protein aggregates (Pingitore *et al*., 2007). In addition, incubation temperature, initial pH of the media, carbon sources, organic and inorganic nitrogen sources (Vijayalakshmi *et al*., 2011 b), incubation period (Hasan *et al*., 2012) as well as growth media and aeration (Khalil *et al.,* 2009 a) seem to play a crucial role in bacteriocin production. In general, the cultivation conditions directly affect bacteriocin production as such and indirectly through biomass production. This is to be explained by the fact that bacteriocin production is a growth-dependent physiological trait and hence follows primary metabolite kinetics.

2.3.14.2 Screening methods

There are several screening methods available to detect antimicrobial activity from supernatants of hypothetical producer strains: spot-on-lawn assay (Hoover & Harlander, 1993), disc diffusion (Bhunia *et al*., 1988), microtiter plate assay (Geis *et al*., 1983), agar well diffusion assay (Barefoot & Klaenhammer, 1983), cross streak method (Lemos *et al*., 1985) are a few. The ability to screen many strains in an easy, fast and reliable method is very important.

2.3.14.3 Concentration of the bacteriocin-containing supernatant

In order to begin characterization of a new antimicrobial peptide, it is important to decide and implement a purification strategy. Since most bacteriocins are not produced in large amounts by the producer strain, the initial concentration steps are very crucial. For this purpose, several protocols can be implemented: ammonium sulphate concentration, absorption-desorption, organic solvent extraction are a few to name.

2.3.14.4 Purification of bacteriocins

The methodology described above produces a partially purified extract of bacteriocins. These extracts must undergo other purification steps and the most frequenly used techniques are C18 Solid Phase exctraction (SPE), Ion Exchange Chromatography (cation or anion exchange resins), Gel filtration chromatography, Reverse-phase (RP) HPLC to name a few.

Three major purification methods for bacteriocins by LAB to homogeneity can be distinguished. Purification can by conventional methods based on rather laborious steps of ammonium sulfate precipitation, ion exchange, hydrophobic interaction, gel filtration, and reversed-phase high-pressure liquid chromatography (Mortvedt *et al*., 1991; Tichaczek *et al*., 1992; Parente & Ricciardi, 1999). Secondly, a simple three-step protocol (Callewaert *et al*., 1999), including (i) ammonium sulfate precipitation, (ii) chloroform/methanol extraction/precipitation, and (iii) reversed-phase high-pressure liquid chromatography, the sole chromatographic step involved can be used. Alternately, bacteriocins can be purified through a unique unit operation, i.e. expanded bed adsorption, using a hydrophobic interaction gel, after maximizing the bioavailable bacteriocin titer through pH adjustment of the crude fermentation medium (Callewaert & De Vyust, 1999).

2.3.14.5 Characterization of bacteriocins using SDS-PAGE

In addition to RP-HPLC, it is possible to analyze the partial or purified bacteriocin extracts by SDS-PAGE wherein, active extracts were electrophoresed using the Laemmli protocol (1970), or that modified by Schagger and von Jagow (1987) that utilizes the "discontinuous" tricine buffer, which is appropriate for the separation of low molecular weight peptides. After electrophoresis, the peptides are stained with Coomassie blue, silver-stain or with the luminescent stain SYPRO-Ruby (Molecular Probes-Invitrogen). This last dye stains proteins at concentration of less than 2 ng for band and is compatible with downstream analysis as mass spectrometry and sequencing. Therefore, the peptide band can be cut out and electroeluted for further processing. Recently, another fluorescent dye, Lumitein was introduced to the market (www.biotium.com). The manufacturer indicates that Lumitein is as sensitive as silver and could be a good alternative for bacteriocin staining; as unlike silver staining, this fluorescent dye is compatible with downstream processing (Pingitore *et al*., 2007).

2.3.14.6 Bioassay / bacteriocin activity on gel

When the peptide bands do not stain adequately in the previous protocols or just to confirm the identity of a bacteriocin, the application of bioassay is particularly useful to detect the antimicrobial compound in the gel. In this protocol, the SDS-PAGE gel is placed over a lawn of a sensitive strain/ gel is placed on a base agar plate followed by overlaying with soft agar containing the sensitive strain. A clear zone of inhibition is observed at the site of bacteriocin after appropriate incubation. Here, it is important to wash the gel previously with sterile water to reduce SDS content inorder to avoid inhibition of the test strain by the detergent. SDS is also removed from the gel by pre-treatment with an isopropanol (20%) and acetic acid (10%) mixture and subsequent washes, first with tween 80 (0.5%) and then in water as described by Bhunia and Johnson (1992).

If the sample is electrophoresed in duplicate, it is possible to process one strip by the bioassay and then compare the migration of the inhibitory compound in the second strip, cutting the band and electroeluting (Lei *et al*., 2007) for downstream processing.

2.3.14.7 Advanced strategies

The last two decades have shown an explosion in the publication describing new bacteriocins from LAB or related Gram positive microorganisms. It is therefore possible that the antimicrobial peptide intended for study has already been described previously. Consequently to confirm novelty of the bacteriocin to be studied, a good approach is PCR screening of the producer strain with "bacteriocin-specific" primers available in literature or designed based on the sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/).

2.3.14.8 Role of Bioinformatics tools in confirming novelty

The amino acid sequence of the peptide can be analyzed by BLAST tool in NCBI. In bioinformatics, Basic Local Alignment Search Tool (BLAST) is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences and identify library sequences that resemble the query sequence above a certain threshold. Different types of BLASTs are available according to the query sequences. BLAST is actually a family of programs (all included in the blastall executable). These include the most commonly used blastn and blastp programs.

Nucleotide-nucleotide BLAST (blastn): This program, given a DNA query, returns the most similar DNA sequences from the DNA database that the user specifies.

Protein-protein BLAST (blastp): This program, given a protein query, returns the most similar protein sequences from the protein database that the user specifies.

2.3.15 CHARACTERIZATION OF BACTERIOCINS

2.3.15.1 Molecular mass of bacteriocins

Molecular mass of the bacteriocins is determined by SDS-PAGE and MALDI-TOF mass spectrometry. The molecular mass of different bacteriocins from *Bacillus* sp. is given in Table 2.2.

2.3.15.2 Isoelectric point (pI) of bacteriocins

The isoelectric point (pI) is the pH at which a particular molecule or surface carries no net electrical charge (exists as Zwitter ion). Zwitter ion is a neutral molecule with a positive and a negative electrical charge, though multiple positive and negative charges can be present.

Amphoteric molecules contain both positive and negative charges depending on the functional groups present in the molecule. The net charge on the molecule is affected by pH of their surrounding environment and can become more positively or negatively charged due to the loss or gain of protons (H^+). The pI is the pH value at which the molecule carries no electrical charge or thenegative and positive charges are equal. Biological amphoteric molecules such as proteins contain both acidic and basic functional groups. Amino acids which make up proteins may be positive, negative, neutral or polar in nature and together give a protein its overall charge. At a pH below their pI, proteins carry a net positive charge; above their pI they carry a net negative charge.

Table 2.2 Molecular mass of bacteriocins from *Bacillus* species

***Bacillus* species**	**Bacteriocin**	**Mol.Wt (kDa)**	**Reference**
B. cereus	Cerein GN105	9.0	Naclerio *et al*., 1993
	Cerein 7A	3.9	Oscariz *et al.,* 1999
	Cerein 7B	4.9	Oscariz *et al*., 2006
B. thuringiensis	Thuricin 439	2.9	Ahern *et al*., 2003
	Thuricin 17	3.2	Gray *et al*., 2006 a
B. subtilis	Subtilin	3.3	Banerjee & Hansen, 1988
	Subtilin B	3.4	Chan *et al*., 1993
	Subtilosin A	3.4	Zheng *et al.,* 1999
	Mersacidin	1.8	Bierbaum *et al*., 1995
	Ericin S	3.4	Stein *et al*., 2002 a
	Ericin A	2.9	Stein *et al.,* 2002 a
	Bac 14B	21	Hammami *et al.,* 2009
	LFB112	6.3	Xie *et al*., 2009
B. licheniformis	Lichenin	1.4	Pattnaik *et al*., 2001
	Bacillocin 490	2.0	Martirani *et al.,* 2002
	Peptide A12C	0.7	Galvez *et al.,* 1993
B. amyloliquifaciens	BLIS 5006	5.0	Lisboa *et al*., 2006
B. lentus	BLIS	11	Sharma *et al*., 2009a
B. megaterium	Megacin 19	3.5-6.5	Khalil *et al.,* 2009 b
	Megacin 22	3.5- 6.5	Khalil *et al.,* 2009 a
B. polyfermenticus	Polyfermenticin SCD	14.3	Lee *et al.,* 2001
Brevibacillus brevis	BLIS	4.5-6	Hyung *et al*., 2001
Geobacillus stearothermophilus	Thermocin 17	6.9	Pokusaeva *et al.,* 2009
Geobacillus thermoleovorans	Thermoleovoran-N9	36	Novotny & Perry, 1992
Paenibacillus polymyxa	Paenibacillin	2.9	He *et al.,* 2007

The pI value can affect the solubility of a molecule at a given pH. Molecules have minimum solubility in water or salt solutions at the pH which corresponds to their pI and often precipitate out of solution. Proteins can thus be separated according to their isoelectric point (overall charge) on a polyacrylamide gel using a technique called isoelectric focusing (IEF), which uses a pH gradient to separate proteins.

Many reported bacteriocins from Gram positive bacteria have a pI value greater than 7 (Ray, 1992; Hancock *et al*., 1995). Subtilosin A has a pI value of approximately 4, based on its amino acid sequence (Babasaki *et al*., 1985). Bacteriocin produced by a *B. subtilis* strain also has a pI of 4.7 (Zheng and Slavik, 1999). The isoelectric point of the bulgaricin was about 6.2 (Hasan *et al.,* 2012).

2.3.15.3 N- Terminal sequencing

Automated N-terminal sequence analysis involves a series of chemical reactions that derivatize and remove one amino acid at a time from the N-terminus of purified peptides or intact proteins. At least several picomoles of a purified protein or 10 to 20 picomoles of a purified peptide with an unmodified N-terminus is required to obtain useful sequence information. However, N-terminal sequencing remains the method of choice for verifying the N-terminal boundary of recombinant proteins, determining the N-terminus of protease-resistant domains, identifying proteins isolated from species where most of the genome has not yet been sequenced and mapping modified or crosslinked sites in proteins that prove to be refractory to analysis by mass spectrometry. The major direct method of N-terminal protein sequencing is by Edman degradation reaction. Edman degradation is cyclic degradation of peptides based on the reaction of phenylisothiocyanate with the free amino group of the N -terminal residue such that amino acids are removed one at a time and identified as their phenylthiohydantoin derivatives (Edman *et al*., 1950; Fig. 2.4).

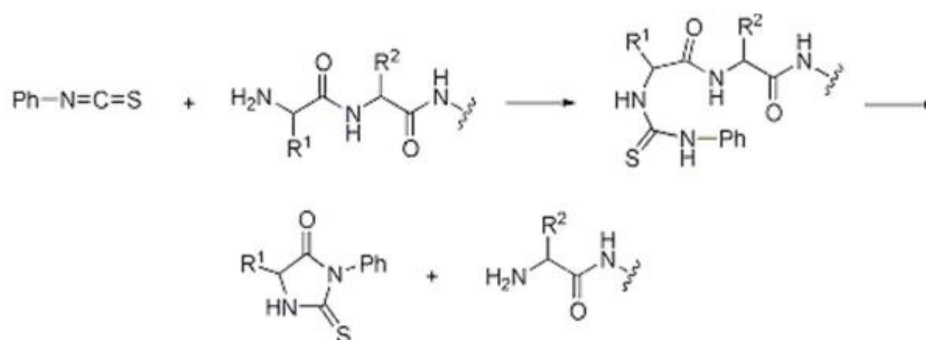

Fig: 2.4 Schematic representation of N-terminal sequencing by Edman degradation (Edman *et al*., 1950)

N- terminal sequencing by Edman degradation is the common method followed in characterisation of many bacteriocins. Then the aminoacid sequence is analyzed by BLAST in GenBank. Many authors followed the procedure mentioned to identify the bacteriocins or prove their novelty. This was the procedure followed in *B. thuringiensis* (Ahern *et al*., 2003), *Enterococcus durans* (Yanagida *et al*., 2005), *B. cerus* (Risoen *et al*., 2004; Sebei *et al*., 2007), *Lactobacillus salivarius* (Pilasombut *et al.,* 2006) etc.

2.3.15.4 Effect of pH, temperature and proteolytic enzymes on bacteriocin stability

Many bacteriocins produced by *Bacillus* sp. can tolerate broad pH ranges and are stable at high temperatures. *B. subtilis* strain MJP1 isolated from meju (Korean fermented soybean) produces a heat stable BLIS, with pH stabliliy between 6.0–10.0 and capability to withstand proteolytic treatment (Yang & Chang, 2007). Subtilosin A is high stable under extreme temperatures and pH stresses, with full activity retained even after an hour at 100°C or in the pH range of 2–10 (Sutyak *et al*., 2008).

The strain *B. amyloliquefaciens* LBM 5006 isolated from the Brazilian Atlantic forest produces a BLIS that is stable over the pH range of 3.0-8.0, heat stable (80°C, 30 min) and sensitive to proteolytic enzymes. Bacillocin Bb produced by the *B. brevis* Bb is a BLIS that is stable within the pH range of 1–9, resistant to heat (100°C for 30 min) as well as detergents and organic solvents (Saleem *et al*., 2009). Cerein 8A from *B. cereus* 8A isolated from soil of native woodlands of Southern Brazil (Bizani & Brandelli, 2002) is protease-sensitive, stable in the pH range of 2–11 and relatively thermostable, only losing activity at temperatures >75°C for 30 min (Bizani *et al*., 2005 a). *B. firmus* H2O-1 isolated from a Brazilian oil reservoir produces an antibacterial peptide that is resistant to proteases, heat stable (100°C for 1 h) and is stable at alkaline pH (Korenblum *et al*., 2005). Sharma *et al*. (2006) reported a BLIS produced by a food grade *B. lentus* NG121 strain isolated from Khameera, a traditional fermented food from Himachal Pradesh (India) which is characterized by its stability at high temperature (100°C for 10 min), over a wide range of pH (5–10) and its sensitivity towards proteolytic enzymes such as trypsin. Megacins 19 and 22 remain stable up to 100°C for 15 min but lose their activity at alkaline pH (Khalil *et al*., 2009 a & b). While megacin 19 is lipase and amylase sensitive, megacin 22 is sensitive to proteolytic enzymes and organic solvents. *B. mycoides* strain isolated from whey was shown to produce a BLIS stable at 100°C and over a wide pH range of 4–11 (Sharma & Gautam, 2008). *B. polyfermenticus* strain SCD was reported to produce polyfermenticin SCD, a BLIS which was heat-labile, proteinase K-sensitive and very stable throughout the pH range of 2.0–9.0 (Lee *et al*., 2001). Pumilicin 4 produced by *B. pumilus* WAPB4 (Aunpad & Na-Bangchang, 2007) is heat stable up to 121°C for 15 min and active in the pH range of 3–9.

Lysinibacillus sphaericus SOPB1 produced a BLIS, which is heat stable up to 121°C for 15 min and active in the pH range of 6–9 (Aunpad *et al*., 2007). The patented thuricin CD is sensitive to proteolytic enzymes, heat stable and

retains its inhibitory activity over a wide pH range (Hill *et al*., 2009). Polyxin produced by *P. polymyxa* P13 isolated from Argentinean regional fermented sausages is stable at high temperatures (at 90°C for 10 min) but sensitive to alkaline pH (Piuri *et al*., 1998). Thermocin 10 from *G. stearothermophilus* NU-10 is a glycoprotein that is stable at a high temperature and resistant to proteolytic enzymes (Fikes *et al*., 1983).

2.3.15.5 Effect of surfactants (detergents) on bacteriocins

Surfactants are surface-active agents containing a hydrophobic portion, which is more soluble in oil-like solutions and a hydrophilic portion, which is soluble in water. Surfactants will migrate to the interface of a solvent reducing its surface tension. Surfactants can be classified into non- ionic, Zwitterionic, anionic and cationic surfactants depending on their electrical charge. Triton X-100, Polysorbate 20 (common commercial brand names include Alkest TW 20 and Tween 20) and Polysorbate 80 (brand names include Alkest, Canarcel and Tween) are nonionic surfactants with wide applications in laboratories. Sodium dodecyl sulfate (SDS or NaDS), sodium laurilsulfate or sodium lauryl sulfate (SLS) is an anionic surfactant. It can be used to aid in lysing cells during DNA extraction and for unraveling proteins in SDS-PAGE. CTAB (Cetrimonium bromide or Hexadecyltrimethylammonium bromide) is a bactericidal, cationic detergent. CTAB is used in the extraction of genomic DNA from bacteria and for the isolation of high molecular weight DNA from plants.

Studies on the effect of surfactants like triton X-100, tween 20, tween 80 and SDS on different bacteriocins have been reported earlier. SDS inhibited the bacteriocin produced by *B. subtilis* LFB112 (Xie *et al*., 2009) but non ionic surfactants had no effect; treatment with tween 80 increased antimicrobial activity whereas SDS and Triton X-100 had no effect on the bacteriocin activity of bacteriocin G2 produced by the probiotic bacteria *Lactobacillus plantarum* G2

(Seatovic *et al*., 2011); surfactants did not affect the bacteriocin produced by *Lactoccous lactis* B14 (Ivanova *et al*., 2000); the antagonistic activity was greatly reduced when treated with SDS whereas triton X-100 and tween 20 completely inhibited the activity of plantaricin OL15, a bacteriocin produced by *Lactobacillus plantarum* OL15 (Mourad *et al.*, 2005); it was observed that tween 80 adverseley affected the activity of bacteriocin from *Leuconostoc* NT-1 (Maurya and Thakur, 2012); tween 80 and SDS increased the activity of the bacteriocin whereas triton X-100 lowered the activity of plantarcin SR18 produced by *Lactobacillus plantarum* SR18 (El-Shouny, 2013); SDS, tween 80 and triton X-100 stimulated th bacteriocin activity of *Lactobacillus lactis* (Rajaram *et al.,* 2010).

2.3.15.6 Effect of reducing agents, oxidizing agents and metal ions

Reducing agents act on any disulfide bonds to split it apart. Agents that break disulfide bonds by reduction include β-mercaptoethanol, dithiothreitol (DTT) etc. By breaking the S-S bonds, both the tertiary structure and the quaternary structure of some proteins can be disrupted. Because of their ability to disrupt the structure of proteins, they are used in the analysis of proteins, for instance, to ensure that a protein solution contains monomeric protein molecules instead of disulfide linked dimers or higher order oligomers. DTT reduced the activity of the bacteriocin produced by *B. subtilis* LFB112 (Xie *et al*., 2009). It was noticed that β-mercaptoethanol (0.2%) increased the activity of bacteriocin produced by *Lactobacillus plantarum* SR18 (*El-Shouny*, 2013) to 1.2-1.4 fold.

Disulfide bonds are formed by oxidation of the sulfhydryl groups on cysteine. Different protein chains or loops within a single chain are held together by the strong covalent disulfide bonds. Oxidation can occur when proteins are exposed to oxidizing agents such as hydrogen peroxide (H_2O_2), periodate, dimethyl sulfoxide, chloramine-T, *N*-chlorosuccinamide etc. DMSO is used as a

mild oxidant. Oxidation of methionine residues is common and it has been shown to cause a decline in the biological activity of the protein.

Heavy metal salts act to denature proteins in the same manner as acids and bases. Since salts are ionic they disrupt salt bridges in proteins. The reaction of a heavy metal salt with a protein usually leads to an insoluble metal protein salt. Few reports are available on the effect of oxidising agents and metal ions on bacteriocins.

2.3.16 APPLICATIONS OF *BACILLUS* BACTERIOCINS

The bacteriocins produced by *Bacillus* sp. find wide range of applications in medical field, dairy industry, food industry, sea food industry etc.

2.3.16.1 Bacteriocins for Human Health

The increasing bacterial resistance to conventional antibiotics of clinical application resulted in a growing interest to consider bacteriocins as alternative antimicrobials for the treatment of human (and possibly animal) infections (Lawton *et al.,* 2007). Cross-resistance between bacteriocins and conventional antibiotics of clinical use has seldom been reported, because these two groups of antimicrobials act on different cellular targets. Several bacteriocins or BLIS produced by *Bacillus* species show antimicrobial activity against pathogenic bacteria of concern such as MRSA or VRE. Some examples are the BLIS produced by *B. sphaericus*, pumilicin 4 or the lantibiotics lichenicidin, haloduracin and mersacidin. Haloduracin particularly seems to be of interest for medical applications because it is more stable at physiological pH values than nisin (Oman & van der Donk, 2009). Mersacidin shows strong antimicrobial activity against *S. aureus* both *in vitro* and in animal studies (Molitor *et al.,* 1996; Kruszewska *et al.*, 2004; Sass *et al*., 2008). The lantibiotic subtilosin A shows antimicrobial activity against pathogens such as *L. monocytogenes*, *G. vaginalis* and *S. agalactiae*

(Sutyak *et al*., 2008). Subtilosin A thus has a potential for application against vaginal pathogens such as *G. vaginalis* resistant to conventional antibiotic therapies. Besides their antibacterial activity, some bacteriocins/BLIS of *Bacillus* also show antifungal activity, which could be exploited in clinical applications. Bacteriocins from *Bacillus* may also have potential applications as natural contraceptives. One good example is subtilosin A, which shows spermicidal activity against spermatozoa from humans and a variety of farm animals (Sutyak *et al*., 2008).

2.3.16.2 Livestock applications

The application of *B. circulans* and *P. polymyxa* bacteriocins in poultry was reported by Stern *et al.* (2005). The bacteriocin producing *P. polymyxa* NRRL B-30507, NRRL B-30508 and NRRL B-30509 strains as well as *B. circulans* NRRL B-30644 were used to control *Campylobacter jejuni* as a non antibiotic treatment for animals carrying zoonoses. The dietary administration of bacteriocin preparations reduced the colonization of poultry by *C. jejuni*, decreasing the risk of public exposure and campylobacteriosis (Svetoch *et al.,* 2005). *Bacillus* bacteriocins with strong inhibitory activity against staphylococci could find practical applications in the control of mastitis in dairy cows. In a recent study, several BLIS from *B. thuringiensis* (morricin 269, kurstacin 287, kenyacin 404, entomocin 420 and tolworthcin 524) were tested against a collection of *S. aureus* isolates from dairy sources, which showed resistance to a variety of commercial antibiotics (Barboza-Corona *et al*., 2007). Despite *S. aureus* strain differences in sensitivity, the best results were reported for morricin 269, followed by kurstacin 287. Another antimicrobial substance of interest for application in animal health is the BLIS produced by *B. subtilis* LFB112 from Chinese herbs, which is active against several Gram-positive and Gram-negative bacteria involved in domestic animal diseases (Xie *et al*., 2009).

2.3.16.3 Food applications

Consumer demands for minimally processed foods or 'fresh foods' with no chemical preservatives, have stimulated research interest in natural antimicrobial agents such as bacteriocins. However, most studies concerning food applications have focused on LAB bacteriocins, mainly nisin and a few others (Galvez *et al.,* 2008). Although nisin is the only bacteriocin currently licensed as a biopreservative, its applications are restricted due to its very low activity at a neutral or an alkaline pH. Therefore, the search for new bacteriocins with improved physico-chemical properties (stability in a wide range of pH and temperature) and also a broad antimicrobial spectrum is of great interest for their application in foods. Inspite of the diverse array of bacteriocins produced by *Bacillus* species, the importance and industrial value of *Bacillus* bacteriocins has been largely under estimated and only a few number of applications were reported in foods. This fact is potentiated by the lack of GRAS status of some *Bacillus* species, except for some representatives such as *B. subtilis* and *B. licheniformis* (Sharp *et al*., 1989). Recently, the European Food Safety Authority (EFSA) introduced the concept of qualified presumption of safety (QPS) for acceptability of bacteria in foods if the taxonomic group did not raise safety concerns or, if safety concerns existed, but could be defined and excluded (EFSA, 2007 a & 2008). *Bacillus cereus* strains have already been approved by EFSA for animal feed (EFSA, 2004, 2005 & 2007 b). The qualification concerning QPS for *Bacillus* species is modified to 'absence of food poisoning toxins, absence of surfactant activities, absence of enterotoxic activities' (EFSA, 2008). Nevertheless, bacteriocin-producing strains or their bacteriocin preparations could still be used in food preservation provided they meet the criteria established by EFSA.

Bacteriocins from *Bacillus* have a potential preservative application in different food substrates like in dairy products such as milk and cheeses (Sharma *et al*., 2009 a & b). Two representative examples are bacillocin 490 and cerein 8A.

Bacillocin 490 was shown to be active against closely related *Bacillus* sp. both under aerobic and under anaerobic conditions and its bactericidal activity was maintained during storage at 4°C, over a wide pH range, at a high temperature and also in the food substrate i.e. milk (Martirani *et al*., 2002). These features may allow the use of bacillocin 490 during food processing performed at a high temperature and as a complementary antimicrobial agent to nisin (which displays a very low activity at a neutral or alkaline pH) against some *Bacillus* sp. in non acidic foods.

Another bacteriocin tested in dairy products (milk and soft cheese) to control the development of *L. monocytogenes* is cerein 8A produced by *B. cereus* 8A (Bizani & Brandelli, 2002; Bizani *et al.,* 2005 a & b). The addition of 160 AU/mL cerein 8A to ultrahigh-temperature milk resulted in a decrease of 3 log cycles in viable cells within the 14 day period at 4°C (Bizani *et al*., 2008). However, cerein-treated samples of cheese showed a 2 log cycle decrease in viable counts during 30 days at 4°C, when cerein 8A was used to control cheese surface contamination by *L. monocytogenes*. In Minas-type soft cheese, cerein caused only a delay in the start of the exponential growth phase (Bizani *et al*., 2008). Interestingly, the antimicrobial activity of cerein 8A against *Salmonella* Enteritidis was potentiated by EDTA and sodium lactate (Lappe *et al*., 2009). Therefore, the potency and spectrum of cerein 8A could be improved by combination with outer-membrane-destabilizing agents.

A further application reported was for the biopreservation of poultry meat using a BLIS produced by *B. amyloliquefaciens* strain GA1 (Halimi *et al*., 2010). One of the bottlenecks for the application of bacteriocins in foods is their industrial production at reasonably low costs. Cerein 8A can be obtained with high yields by cultivation of the producer strain in a brain–heart infusion broth (Bizani

& Brandelli, 2004) and in soybean protein medium, allowing a cost-effective production for possible applications (Dominguez *et al*., 2007).

Many other antimicrobial peptides show potential applications in food preservation. Most of them are stable to heat and pH conditions occurring during food processing, and can be degraded by proteases. Many of them also show broad spectra of antimicrobial activities, inhibiting Gram-positive and Gram-negative food borne pathogens, and in some cases, even fungi. In this respect, bacteriocins from *Bacillus* are superior to LAB bacteriocins. Good examples are the antimicrobial peptides P34 (Motta *et al.,* 2008), P45 (Sirtori *et al.,* 2006), paenibacillin (He *et al*., 2007), polyxin (Piuri *et al*., 1998) or the antibacterial/antifungal peptide produced by a *B. brevis* strain isolated from kimchi (Hyung *et al*., 2001). In addition, *Bacillus* strains play a central role in the manufacture of alkaline-fermented foods and beverages (Wang & Hesseltine, 1982; Odunfa & Oyeyiola, 1985; Yokotsuka, 1985; Wang & Fung, 1996). Application of bacteriocin-producing strains in these food substrates may offer new opportunities in food biopreservation. A subtilisin-producing *B. subtilis* strain was found to inhibit the growth of *B. cereus* in the production of the Nigerian alkaline fermented soup condiment okpehe (Oguntoyinbo *et al*., 2007).

2.3.16.4 Environmental applications

Bacilli are naturally associated with soil and plants. For this reason, strains producing bacteriocins or BLIS with antibacterial or antifungal activity could be amenable for application as biocontrol agents. Many of the bacteriocins or BLIS produced by bacilli inhibit plant pathogenic bacteria. Either the bacteriocin-producing strains or the partially purified bacteriocin preparations could be applied in the biological control of plant diseases. For example, ericin S is active against *Clavibacter michiganensis*, the causative agent of tomato bacterial canker. Purified ericin or its producer strain could be developed as a bioprotectant on

tomato plants. Another example is Bac 14B, a BLIS produced by *B. subtilis* 14B isolated from the rhizosphere of healthy plants. Because Bac 14B is active against *A. tumefaciens*, this BLIS could potentially be applied as a biocontrol agent for reducing infections in plants caused by *A. tumefaciens*. Rhizosphere bacteria can promote plant growth by several mechanisms. Plant growth-promoting rhizobacteria are of great interest for application in agriculture. Species of *Bacillus* are known to act as promoting agents for plant growth and/or promoting disease resistance in plants, as exemplified by the polypeptide produced by *B. thuringiensis* strain NEB17 (Smith *et al*., 2008). Strain NEB17 was isolated from soybean root nodules (Bai *et al*., 2002) and was shown to enhance nodulation when applied as a co inoculant with *Bradyrhizobium japonicum* 532C (Bai *et al.,* 2003). Subsequently, it was shown that this strain produces the antibacterial peptide thuricin 17 (Gray *et al*., 2006b). The plant growth-promoting activity of thuricin 17 has been demonstrated recently (Lee *et al*., 2009 a & b). Application of thuricin 17 induced root hair deformation curling response of soybeans, and was therefore involved in increasing nodulation. It was also shown that thuricin application to plant leaves or roots directly stimulated the growth of both soyabean (a C3 dicot) and corn (a C4 monocot). BLIS displaying antifungal activities or their producer strains could be applied in the biocontrol of plant decay and post harvest control of fruits and vegetables. One example is *B. amyloliquefaciens* strain RC-2, which produces a BLIS active against *C. dematium*, mulberry anthracnose fungus and several other phytopathogenic fungi and bacteria such as *Rosellinia necatrix*, *Pyricularia oryzae*, *A. tumefaciens* and *Xanthomonas campestris*.

Bacteriocin-producing bacilli may also be amenable for other environmental applications. Some interesting strains have been isolated from oil reservoirs, such as the cereicidin producing *B. cereus* Q1 strain isolated from a Brazilian oil reservoir with antimicrobial activity against SRB (Korenblum *et al*.,

2005). The BLIS produced by the *B. firmus* strain H2O-1 is a small peptide that is stable to heat and alkaline pH, and probably withstands the environmental conditions during oil drilling. Owing to its high antimicrobial activity, this BLIS offers potential use as a biocide in the petroleum industry for controlling the problems associated with these bacteria. In further studies, it was shown that the BLIS produced by strain H2O-1 reduced the viability and attachment of an SRB consortium biofilm (Korenblum *et al.*, 2008). The authors suggested that this strain or its antimicrobial peptide may have a potential for pipeline cleaning technologies to inhibit biofilm formation and consequently reduce biocorrosion.

2.3.16.5 Biofilm formation and prevention using bacteriocins

It is a natural tendency of microorganisms to attach to wet surfaces, to multiply and to embed themselves in a slimy matrix composed of extracellular polymeric substances (EPS) that they produce, forming a biofilm. More than 60 years after the first report on biofilms (Zobell, 1943), they are still a concern in a broad range of areas and specifically in the food, environmental and biomedical fields (Flint *et al.*, 1997; Maukonen *et al.*, 2003; Sihorkar & Vyas, 2001; Veran, 2002). Biofilms are problematic in particular food industry sectors such as brewing, dairy processing, fresh produce, poultry processing and red meat processing (Chen *et al.*, 2007; Frank *et al.*, 2003; Jessen & Lammert, 2003; Somers & Wong, 2004). Biofilms are more resistant to antimicrobials compared to planktonic cells and this makes their elimination from food processing facilities a big challenge (Simoes & Vieira, 2009; Simoes *et al.*, 2006). There are a number of mechanisms by which numbers of microbial species are able to come into closer contact with a surface, attach firmly to it, promote cell–cell interactions and grow as a complex structure (Breyers & Ratner, 2004; Gobbetti *et al.*, 2007).

Bacillus sp. particularly *B. cereus*, are implicated in food spoilage (Andersson *et al.*, 1995; Janneke *et al.*, 2007). In a commercial dairy plant *B.*

cereus accounted for more than 12% of the biofilms constitutive microflora (Sharma & Anand, 2002). As *B. cereus* is ubiquitously present in nature, it is easily spread through food production systems and contamination with this species is almost inevitable. Moreover, *B. cereus* spores are highly resistant to a large number of stresses and also very hydrophobic, which help them to adhere easily to food processing equipment (Lindsay *et al.*, 2006). *Cl. perfringens* is also one of the most ubiquitous bacteria in natural environments and can be found in all soils worldwide. Biofilm formation by *Cl. perfringens* has been reported by John *et al.* (2008).

Bacteriocins are gaining importance and have a unique potential in the food industry for the effective biocontrol and removal of biofilms (Sayem *et al.*, 2011). These newer biocontrol strategies are considered important for the maintenance of biofilm-free systems, for quality and safety of foods. Bacteriocins have several characteristics that make them ideal food preservatives. Many bacteriocins are capable of resisting inactivation at the relatively high temperatures used in food processing and can remain functional over a broad pH range. Bacteriocins are usually inactivated by one or more of the proteolytic enzymes present in the digestive tract of humans and would be digested just like any other protein in the diet. Bacteriocins are nontoxic, odorless, colorless, and tasteless. Finally bacteriocins may be perceived by consumers to be more natural than chemical preservatives. The efficacy of using bacteriocins as food preservatives need to be determined for each food system. Solubility, stability, sensory impact, heat and pH tolerance, and types and number of organisms inhibited will need to be evaluated for each bacteriocin in each food product category under a variety of storage conditions.

2.3.16.6 Biopreservation of fish

The application of nisin A in the preservation of fish products has been studied by Taylor *et al*. (1990) who showed that nisin treatment of cod, herring and smoked mackerel fillets inoculated with *Cl. botulinum* spores brought about a delay in toxin production of 5 days at 10°C, but only by half a day at 26°C. The effects of nisin Z, carnocin U149 and bavaricin A on bacterial growth and shelf life of brined shrimp was recently evaluated and compared with those of a benzoate-sorbate solution and a control with no added preservatives (Einarsson & Lauzon, 1995). The benzoate-sorbate solution preserves the brined shrimp for the whole storage period (59 days). The shelf life of the shrimp in the absence of preservatives was found to be 10 days. Carnocin U149 had no influence on shelf life, while crude bavaricin (a cell-free supernatant of *Lactobacillus bavaricus* MI401 extended the shelf life to 16 days. Significantly, when crude or purified nisin Z was applied to the same material the shelf life was extended to 31 days. Such results offer clear perspectives for the biopreservation of certain fish products with bacteriocins.

2.3.16.7 *Caenorhabditis elegans* as a model organism

Amidst ever growing concerns for the welfare of animals in scientific research, there is a heightened need to find organisms in which interactions can be studied ethically and at large scale. Therefore, the discovery of simple and genetically tractable model organisms such as *Arabidopsis thaliana* (Dodds and Rathjen, 2010), *Drosophila melanogaster* (Ferrandon *et al*., 2007), *Caenorhabditis elegans* (Millet & Ewbank, 2004) and Zebra fish (*Danio rerio*) (Trede *et al.,* 2004), susceptible to a number of human pathogens has been a remarkable advance in this field.

C. elegans is a free-living nematode that is found in soil and in compost heaps. The population is dominated by self-fertilising hermaphrodites (XX) with a

rare occurrence of males (X0) who have a distinct morphology. The animals were first adopted as a laboratory model by Sydney Brenner over 40 years ago (Brenner, 1974) for studies of development and behaviour; work which resulted in the award of Nobel Prize in Physiology or Medicine to Brenner and his colleagues in 2002 (Frangsmyr**,** 2003). In recent years, this tiny transparent nematode worm has been applied to the study of microbial pathogenesis and host innate immunity and for drug discovery and development. *C. elegans* is a self fertilizing hermaphrodite with a rapid generation time. Each adult worm grows to a length of approximately 1 mm and under laboratory conditions can produce 300 genetically identical progeny in a 3 day life cycle. This allows the rapid expansion of strains and the establishment of large homogeneous populations. In the laboratory, *C. elegans* is propagated on agar plates or in liquid media with the auxotrophic *Escherichia coli* mutant strain OP50, and can live up to 3 weeks at room temperature. The entire genome sequence for *C. elegans* is available and many functional genomic approaches have been developed. Nevertheless, it has well-differentiated muscles, nerves, gut cells, distinctive behaviour and so on. It therefore resembles larger and more complicated animals in fundamental ways. Permissive legislation ie. when *C. elegans* are used, there are no ethical concerns which are associated with the usage of higher animals. In light of the properties outlined above, *C. elegans* is a very convenient whole organism model in which to identify or assay antimicrobial compounds (Ewbank & Zugasti, 2011).

Currently the response to infection by four natural pathogens of this host has been described in detail: the Gram-negative bacteria *Microbacterium nematophilum,* the fungal parasite *Drechmeria coniospora*, the microsporidian parasite *Nematocida parisii* and most recently a nodavirus-like Orsay virus. There is an ever growing list of Gram positive, Gram negative and fungal pathogens that are known to infect *C. elegans*, many of which are of clinical relevance (Darby, 2005; Sifri *et al*., 2005; Powell & Ausubel, 2008). A prominent example is the

human opportunistic pathogen *P. aeruginosa*, which was the first microorganism shown to infect and kill *C. elegans.* Other microbes include *Salmonella enterica*,

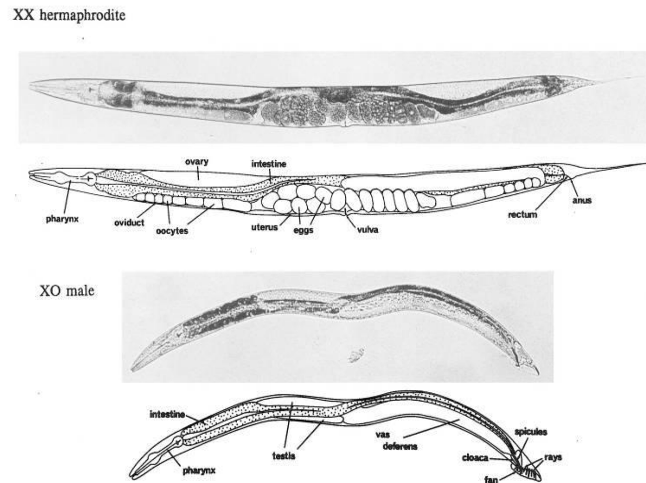

Fig: 2.5 Anatomy of adult *C. elegans* (Adapted from the power point presentation slides of Jonathan Hodgkin, Department of Biochemistry, University of Oxford)

Enterococcus faecalis, *Serratia marcescens*, *E. coli*, *S. aureus*, *Candida albicans*, *Cryptococcus neoformans etc. Pseudomonas aeruginosa* caused an infection-like process in the intestine of the animal killing over the course of several days, termed "slow" killing (Mahajan- Miklos *et al.*, 1999). *S*. Typhimurium has been extensively investigated in the *C. elegans* model, in which it produces a persistent and eventually fatal colonisation of the gut lumen (Aballay *et al.*, 2000), although there is a report of potential invasion into gut epithelial cells (Jia *et al.*, 2009). *E. faecalis* kills adults after establishing a persistent infection in the animals' intestine (Garsin *et al.*, 2001). *S. marcescens* establishes a persistent intestinal infection, arising from an avoidance of the pharyngeal grinder, leading to intestinal distension and death within 6 days (Kurz *et al.,* 2003). *S. aureus* is a common Gram-positive bacterium that causes a range of minor infections, which

occasionally become serious in many animals (Lindsay, 2010). In *C. elegans*, intact bacteria accumulate in the gut of the animal and it is this colonisation that eventually overwhelms the host, disrupting the gut epithelium and then destroying internal organs, ultimately leading to death (Garsin *et al.*, 2001; Irazoqui *et al.*, 2010).

***Caenorhabditis elegans* as a tool for therapeutic drug discovery and development**

Traditional *in vitro* assays measuring growth inhibition or killing of pathogens as well as whole-cell drug screens are not likely to meet the expected future needs of drug development. In light of the properties outlined above, *C. elegans* is a very convenient whole organism model to identify or assay antimicrobial compounds. A potent antagonist of quorum sensing in the bacteria *Chromobacterium violaceum* was shown to protect *C. elegans* from bacterial killing (Swem *et al.*, 2009). This validates the idea that compounds that block quorum sensing could be identified using *C. elegans* and potentially developed as antimicrobial drugs. Using a different approach, the antibiotic action of membrane-active cationic antimicrobial (CAMPs) from frog skin was recently evaluated in *C. elegans* (Uccelletti *et al.*, 2010). A liquid-based assay in 96-well microtiter plates was developed to test the potential of thousands of synthetic compounds and natural extracts to cure *C. elegans* following *Enterococcus faecalis* infection (Moy *et al.,* 2006). Since this pioneering work was published, the assay has been improved, miniaturized and fully automated. An important improvement involved the application of a fluorescent dye that allowed the researchers to discriminate between live and dead worms (Moy *et al.*, 2009). Similar high-throughput *in vivo* assays have been used to screen for antimicrobials that are effective against other human pathogens, including the fungus *Candida albicans* (Breger *et al.*, 2007; Okoli *et al.*, 2009). These assays also provided the

opportunity to assess the relative MIC and the effective concentration *in vivo* as well as the toxicity of these compounds in a single assay.

Nevertheless, *C. elegans* has many practical advantages. It is amenable to rapid, low-cost, large-scale *in vivo* screens and does not raise any of the ethical concerns associated with the use of monkeys, or even mice, for drug testing.

2.4 THE USE OF *BACILLUS* AS PROBIOTICS

Probiotics are live microbes, which when administered in adequate amounts confer a health benefit to the host (Araya *et al*., 2002). Several mechanisms of probiotic action have been described, with the most common relating to their abilities to strengthen the intestinal barrier, to modulate the host immune system and to produce antimicrobial substances. Indeed, the production of antimicrobials is often regarded as an important trait in the context of bacterial fitness and also in terms of probiotic efficacy. Several probiotic bacteria produce a variety of antimicrobial compounds (e.g. short-chain fatty acids, hydrogen peroxide, nitric oxide, bacteriocins) that may enhance their ability to compete against other gastro intestinal (GI) microbes and which could potentially inhibit pathogenic bacteria. Traditionally, bacteriocin production has been an important criterion in the selection of a probiotic strain, a few studies have definitively demonstrated the impact of bacteriocin production on the ability of a strain to compete within the GI tract and/or positively influence the health of the host (Dobson *et al*., 2012).

2.4.1 Contributions of Bacteriocins to Probiotic Functionality

It is possible that bacteriocins could contribute to probiotic functionality in a number of ways (Fig. 2.6). Bacteriocins may function as colonizing peptides, facilitating the introduction and/or dominance of a producer into an already occupied niche (Riley & Wertz, 2002). Alternatively, bacteriocins may act as

antimicrobial or killing peptides, directly inhibiting competing strains or pathogens (Majeed *et al*., 2011). Lastly, bacteriocins may function as signaling peptides, either signalling other bacteria through quorum sensing and bacterial cross talk within microbial communities or signaling cells of the host immune system (Czaran *et al*., 2002).

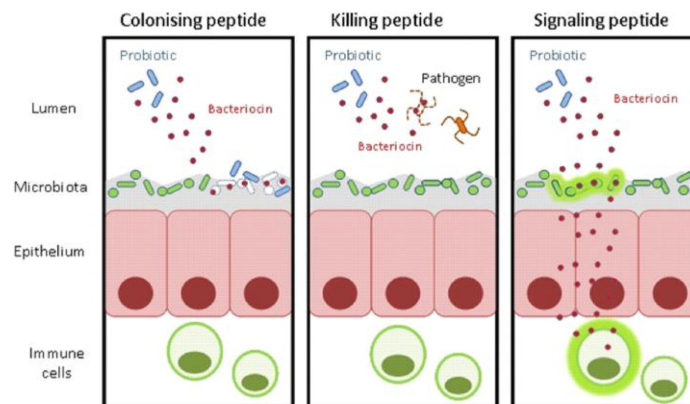

Fig: 2.6 Mechanisms through which bacteriocin production could contribute to probiotic functionality. Bacteriocins may act as colonizing peptides, facilitating the competition of a probiotic with the resident microbiota; they may function as killing peptides, directly eliminating pathogens; or they may serve as signaling peptides, signaling other bacteria or the immune system (Fig. adapted from Dobson *et al*., 2012)

Bacteriocins as colonizing peptides: The high cell density typically associated with the GI tract may result in close cell-cell contact between members of the same or different species, promoting both cooperative and antagonistic microbial interactions (Kreth *et al*., 2005). The production of antimicrobials may provide a mechanism by which producers can gain a competitive advantage over neighboring sensitive strains within this environment. In support of this hypothesis, Gillor *et al.* (2009) demonstrated that *E. coli*, producing the

bacteriocin colicin was able to persist in the large intestine of streptomycin-treated mice for an extended period of time relative to their non-colicin-producing counterparts.

Bacteriocins as killing peptides: The ability of bacteriocin producing microorganisms to inhibit pathogens *in vitro* has been well documented (Le Blay *et al*., 2007). The bacteriocin producing human isolates *P. acidilactici* MM33 and *L. lactis* MM19 were shown to reduce vancomycin-resistant enterococci (VRE) populations *in vivo* (Millet *et al*., 2008).

Bacteriocins as signaling peptides: Bacterial communication via extracellular diffusible signaling molecules (quorum sensing) allows populations of bacteria to synchronize group behavior and can facilitate coordinated multicellular functionality (Gillor & Ghazaryan, 2007). In Gram-negative bacteria, (*N*-acyl) homoserine lactone typically serves as a signal molecule whereas in Gram-positive bacteria, peptides including some bacteriocins, frequently serve as signalling agents. Thus, it has been suggested that at least some bacteriocins have a dual role, acting as inhibitors at high concentrations and as signaling compounds at lower concentrations (Fajardo & Martinez, 2008). Therefore, bacteriocins produced by probiotic strains may also act as quorum-sensing molecules or autoinducing peptides in the intestinal environment.

2.4.2 *Bacillus subtilis* and *Bacillus licheniformis* as probiotics

Bacillus species have been used as probiotics for at least 50 years with the Italian product known as Enterogermina® registered in 1958 in Italy as medicinal supplement. But the scientific interest in *Bacillus* species as probiotics has occurred only in the last 15 years and three principal reviews have covered the field (Hong *et al*., 2008; Mazza, 1994; Sanders *et al*., 2003). Species that have been most extensively examined are *B. subtilis*, *B. clausii*, *B. cereus*, *B. coagulans* and *B. licheniformis*. Spores being heat-stable have a number of advantages over

other non-spore formers such as *Lactobacillus* sp., that the product can be stored at room temperature in a desiccated form without any deleterious effect on viability. A second advantage is that the spore is capable of surviving the low pH of the gastric barrier (Barbosa *et al*., 2005; Spinosa *et al*., 2000) which is not the case for all species of *Lactobacillus* (Tuohy *et al*., 2007) and thus in principle, a specified dose of spores can be stored indefinitely without refrigeration and the entire dose of ingested bacteria will reach the small intestine intact.

B. subtilis has been extensively studied at a genetic and physiological level. Numerous probiotic products are labelled as carrying *B. subtilis* and in part, this probably results in assuming that most aerobic spore formers are *B. subtilis*. However, *B. subtilis* var. Natto is worthy of comment. This bacterium is used in the fermentation of soybeans that is used to prepare the Japanese staple known as Natto. Natto carries as many as 10^8 viable spores per gram of product and for decades health benefits have been associated with consumption of Natto including stimulation of the immune system (Hosoi & Kiuchi, 2004). A serine protease known as Nattokinase is secreted from vegetative cells of *B. subtilis* var Natto and has been shown to reduce blood clotting by fibrinolysis (Sumi *et al*., 1987; Sumi *et al*., 1995). In any event, Nattokinase has GRAS status as an enzyme produced from a bacterium in the US and is purified and sold as a health supplement worldwide. In poultry studies controlled trials have shown that oral administration of *B. subtilis* spores reduce infection by *Salmonella enterica* serotype Enteritidis, *Clostridium* perfringens and *Escherichia coli* O78:K80 (La Ragione *et al*., 2001; La Ragione & Woodward, 2003).

B. subtilis and *B. licheniformis* are used together in two products, BioPlus® 2B and Biosporin. BioPlus® 2B is used in animal feed while Biosporin is licensed as a medicine in the Ukraine and Russia. Biosporin is sold in glass vials that must be reconstituted in water before consumption. The two *Bacillus* strains,

B. subtilis 2335 and *B. licheniformis* 2336 are well characterized and a number of clinical studies have been used to demonstrate probiotic effects although none was performed with the rigour of a full clinical trial (Bilev, 2002; Osipova *et al*., 2003 & 2005; Sorokulova, 1997; Sorokulova *et al*., 1997).

BioPlus® 2B is an animal feed product that has been extensively studied with numerous efficacy studies focused on the suppression of gastrointestinal pathogens resulting in the registration of this product as a feed supplement in Europe (Scan, 2000 b). In piglets, ingestion of *B. subtilis* and *B. licheniformis* spores increases growth performance, feed conversion and meat quality (Kyriakis *et al*., 1999; Alexopoulos *et al*., 2004; Link & Kovac, 2006) and therefore, is an alternative to antibiotic growth promoters (Kritas & Morrison 2005). One reason for using bacterial spores as feed additives is their stability. The metabolically dormant spores are resistant not only to heat, radiation, desiccation, pH extremes, but also to toxic chemicals (Setlow 1994; Nicholson *et al*., 2000). Spores remain viable even after pelletizing of the feed at high temperature and pressure, ensuring the delivery of a viable product to the animals. *Bacillus* spores are tolerant to the acidic conditions in the stomach upon ingestion, but the stomach acidity may trigger germination (Ciffo *et al*., 1987). It remains unclear whether there is any added benefit in the combined use of the two species.

2.4.3 Safety

The safety of *Bacillus* species has been extensively reviewed elsewhere (De Boer & Diderichsen, 1991; Ishibashi & Yamazaki, 2001; Logan, 1988; Sanders *et al*., 2003; SCAN, 2000 a) and most incidences of illness associated with *Bacillus* appear to result for opportunistic infections or misdiagnosis. Extensive animal studies including acute and sub-chronic toxicity testing as well as *in vitro* studies have now been performed on a number of species including *B. subtilis* var. Natto (Hong *et al*., 2008), *B. indicus* (Hong *et al*., 2008), *B. coagulans*

(Endres *et al*., 2009), *B. subtilis* 2335 (Sorokulova *et al*., 2008) and *B. licheniformis* 2336 (Sorokulova *et al*., 2008). All of these bacilli appear to show no indications of adverse effects. This is important because it shows that these bacteria are not foreign, but rather may exert a unique symbiotic relationship with their host.

With the recent characterization of the genome of *B. subtilis* 168 and of some related strains, *Bacillus* sp. are poised to become the preferred hosts for the production of many new and improved products as we move through the genomic and proteomic era.

2.5 NATIONAL STATUS OF BACTERIOCIN RESEARCH

Only a few works on bacteriocins are ongoing in Kerala. Vizhinjam Research Centre of Central Marine Fisheries Research Institute (CMFRI) is involved in research work on bacteriocins. School of Biosciences at Mahatma Gandhi University, Kerala is another institute where research in bacteriocins is ongoing. There are many reports on bacteriocin studies from various institutes all over India. Develpoment of bacteriocin from *B. subtilis*, as a novel drug against diabetic foot ulcer bacterial pathogens is one of the research works reported from Malankara Catholic College and Hindusthan College of Arts and Science, Tamil Nadu. Characterization of bacteriocin from *B. pumilus* is a work ongoing at Vellore Institute of Technology University, Vellore, Tamil Nadu. Antimicrobial protein production by *Bacillus amyloliquefaciens* is the bacteriocin work at Central Leather Research Institute, Chennai, Tamil Nadu. Studies on bacteriocin like inhibitory substance (BLIS) from microalgal symbiotic *Vibrio* sp. are ongoing at Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Tamil Nadu. Isolation and production of bacteriocin from marine *Lactobacillus* is carried out at Annamalai University, Tamil Nadu. Bacteriocin production by *Enterococcus* sp. is being studied at Defence Food Research Laboratory, Mysore, Karnataka. Bacteriocin from lactic acid bacteria is carried out

at Central Food Technological Research Institute, Mysore, Karnataka. Work on bacteriocin from *Lactobacillus acidophilus* is being pursued at Bangalore University, Karnataka. Bacteriocin works are also reported from National dairy research institute, Bangaluru, Karnataka. Work on bacteriocin producing bacteria isolated from mango pulp is another work at *Sri* Venkateswara University, Tirupati, Andhra Pradesh. Bacteriocin produced by *Lactobacillus plantarum* is studied at Global Institute of Biotechnology, Hyderabad, Andhra Pradesh. Use of combination of bacteriocins from *Lactobacillus plantarum* and *Bacillus coagulans* is a work at Department of Biotechnology, Punjabi University, Patiala. Bacteriocin works are also reported from University of horticulture and forestry, Solan, Himachal Pradesh.

3. MATERIALS AND METHODS

3.1 SCREENING FOR BACTERIOCIN PRODUCING BACTERIA FROM MARINE SEDIMENT AND WATER SAMPLES

3.1.1 SAMPLE COLLECTION

Water and sediment samples off the coast of Kochi and salt brine from salt pans of Tuticorin, were collected. The sampling sites off the coast of Kochi included Fort Kochi, Thevara, Kumbalangi, Edakochi, and Palluruthy. Sediment samples were collected using a grab and water samples were collected using sterile glass bottle. The samples were transferred into sterile plastic covers and brought to the lab in ice cold condition.

3.1.2 ISOLATION OF BACTERIA

Sediment sample (10 g) collected from a sampling site was suspended in 100 mL physiological saline and incubated at 28°C in a rotary shaker (Scigenics, India) at 125 rpm for one hour. Both water and sediment samples after incubation were serially diluted in physiological saline (Appendix 1). 1 mL of the sample from each tube was plated on to Zobell marine agar (HiMedia, Mumbai, India; composition given in Appendix 1) by pour plate technique. The plates were incubated at 28° C for 24 h. Single colonies obtained on the plates were picked and stored on Zobell marine agar plates. The bacterial isolates were streaked (quadrant streaking) to get a monospecific culture based on uniform colony morphology and colour. Isolates were maintained as working stock preparations on Zobell marine agar slants for the purpose of screening. 20% glycerol stock was prepared and stored at -80°C for further analysis.

3.1.3 PRIMARY SCREENING FOR BACTERIOCIN PRODUCING BACTERIA

Direct plate assay method was used in the screening process. Mueller Hinton (MH) agar (HiMedia, Mumbai, India; Media composition given in Appendix 1) was used for the preparation of plates in the screening process.

3.1.3.1 Disc diffusion assay

Disc diffusion assay was used to screen the isolates for bacteriocin production (Bauer *et al.*, 1966). The test organism was swabbed on Mueller-Hinton (MH) agar plate (Appendix 1). Sterile discs were placed on the plate and 20 μL of culture supernatant was transferred on the disc. The plate was incubated at 37°C for 24 - 48 h and observed for the antimicrobial activity as clear regions indicating growth inhibition of the test organism around the disc.

3.1.3.1.1 Preparation of culture supernatant

The bacteria were inoculated in 10 mL Zobell marine broth (HiMedia, Mumbai, India) and incubated at 28°C for 24 h. The culture was centrifuged at 8000 rpm (Sigma, 3K30, Germany) for 10 min at 4°C. The supernatant was taken and filtered through 0. 22 μm membrane syringe filter (Millipore, USA) to remove any bacterial cells. This crude bacteriocin was used to evaluate antimicrobial activity using MH agar plate.

3.1.3.1.2 Test organisms used in antimicrobial activity testing

Gram positive and Gram negative bacteria were used as test organisms. The cultures were procured from National Centre for Industrial Microorganisms (NCIM, Pune, India). The vibrios were taken from the culture collection of the microbial genetics laboratory, Department of Biotechnology, CUSAT.

Table 3.1 Test organisms used to screen antibacterial activity of the bacteriocins

Test organisms	**NCIM no. / Isolate no.**
Pseudomonas aeruginosa	2863
Salmonella Typhimurium	2501
Escherichia coli	2343
Salmonella Abony	2257
Klebsiella pneumoniae	2957
Proteus vulgaris	2027
Clostridium perfringens	2677
Staphylococcus aureus	2127
Bacillus cereus	2155
Bacillus circulans	2107
Bacillus coagulans	2030
Bacillus macerans	2131
Bacillus pumilus	2189
Vibrio parahaemolyticus	CHAVA4(4)
V.cholerae	EKM16
V. alginolyticus	CHV2(2)
V. vulnificus	AF7
V. proteolyticus	AF4
V. harveyi	P6
V. furnisii	MUS13

3.1.3.2 Quantitative estimation of antibacterial activity by critical dilution assay

The quantitative estimation of the antibacterial activity of the culture supernatant was performed by the critical dilution assay (Enan *et al*., 1996). Twofold serial dilutions were made from CFS of the bacterial culture. From each dilution, 5µL was spotted on the surface of MH agar plates, swabbed with suspension of log-phase cells of test organism. Plates were incubated at 37°C for 18h.

One activity unit (AU) is defined as 5 µL of the highest dilution of bacteriocin yielding a definite zone of growth inhibition on the lawn of the test organism (in this study *S. aureus*). The highest dilution was multiplied by 200 to obtain the activity units per mL (AU/mL).

Highest dilution = 2^X (X is the number of times the sample was twofold diluted to give zone of growth inhibition)

Activity Units/mL = Highest dilution x 200

3.1.3.3 Protein estimation

Protein content was estimated according to the method of Bradford (1976) using bovine serum albumin (BSA) as the standard and the concentration was expressed in mg/mL.

Bradford reagent

Hundred milligrams of Coomassie Brilliant Blue G-250 (Sigma Aldrich, USA) was dissolved in 50 mL 95% ethanol, added 100 mL of 85% (w/v) phosphoric acid and diluted to 1L. When the dye was completely dissolved, this was filtered through Whatman no:1 filter paper.

Protein standards were prepared with 5 to 25 µg of bovine serum albumin (BSA) in 100 µL distilled water. 1 mL Bradford reagent was added, incubated for

5 min at 28°C and the absorbance was measured at 595 nm in a UV-Visible spectrophotometer (Shimadzu, Japan). Standard graph was plotted and the slope was calculated. 10 μL of test sample was taken and made up to 100 μL with distilled water. 1mL of Bradford reagent was added and the absorbance was measured as above. The protein concentration of the test was determined from the slope.

3.1.3.4 Specific Activity

Specific activity of the sample was calculated by dividing the inhibitory activity units (AU/mL) with the protein content (mg/mL) and was expressed as AU /mg protein.

$$\text{Specific activity} = \frac{\text{Activity Units (AU/mL)}}{\text{Protein (mg/mL)}}$$

As the values of specific activity obtained are very large, this was expressed as $\log_{10}$ (log specific activity) in this study.

3.1.4 SECONDARY SCREENING FOR BACTERIOCIN PRODUCERS

Following primary screening, the isolates with antibacterial activity were subjected to acetone precipitation and ammonium sulphate fractionation. Isolates that produce antibacterial compounds, that could be precipitated by acetone precipitation and ammonium sulphate were selected for further study.

3.1.4.1 Acetone precipitation

Protein samples commonly contain substances that interfere with downstream applications. Several strategies exist for eliminating these substances from samples. One strategy for removing undesirable substances is to add a compound that causes protein to precipitate. After centrifugation to pellet the precipitated protein, the supernatant containing the interfering substance is

removed and the protein pellet is re-dissolved in buffer. The procedure is as detailed below.

The required volume of acetone was cooled to -20°C. Placed the culture supernatant (prepared as described in section 3.1.3.1.1) in acetone- compatible tube and added four times the sample volume of cold (-20°C) acetone to the tube. Vortexed thoroughly and incubated for 60 min at -20°C, followed by centrifugation for 10 min at 16000 rpm. Decanted and properly disposed the supernatant, the pellet air dried, resuspended in 0.01 M phosphate buffer (pH 7.5; Appendix 2) and its antibacterial activity was checked.

3.1.4.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation of the culture supernatant (prepared as described in section 3.1.3.1.1) was carried out by the method of Englard and Siefter (1990). Fractionation using ammonium sulphate has the advantage of intermediate removal of unwanted proteins, with the simultaneous concentration of the protein of interest. Ammonium sulphate (SRL, India) required to precipitate the bacteriocin was optimized by adding varying concentrations (30%, 60% and 90% saturations) to the crude extract as detailed below.

To precipitate the bacteriocin, ammonium sulphate was slowly added, initially at 30% saturation to the crude extract, keeping in ice with gentle stirring. After complete dissolution of the ammonium sulphate, the solution was kept for precipitation at 4°C for 16 h (overnight). The precipitated protein was collected by centrifugation at 16,000 rpm for 15 min at 4°C. To the supernatant, required ammonium sulphate for next level of saturation was added and the procedure mentioned above was repeated. The precipitation was continued up to 90% of ammonium sulphate saturation.

3.1.4.2.1 Dialysis

The precipitate obtained as a result of ammonium sulphate precipitation was further dialyzed against 0.01 M phosphate buffer (pH 7.5), in order to remove the ammonium sulphate from the precipitate. The precipitated protein was resuspended in minimum quantity of 0.01 M phosphate buffer (pH 7.5). The solution was transferred into the benzoylated dialysis tube with cut off value of 2 kDa (Sigma-Aldrich, USA) and dialyzed against 0.01 M solution of phosphate buffer pH 7.5 for 24 h at 4°C with frequent changes of buffer. The partially purified bacteriocin obtained as a result was assayed for antibacterial activity, protein content and specific activity as described earlier

3.1.5 Stocking of cultures

Isolates that produced bacteriocins were purified and maintained as permanent stock cultures employing two methods viz. paraffin overlay method and glycerol stocking.

3.1.5.1 Paraffin oil overlay method

Nutrient agar autoclaved in glass stocking vials was inoculated with a single colony of the isolate and incubated for 18 hours at 37°C. Sterile liquid paraffin oil was dispensed on top and the vials were covered with sterile rubber stopper. The vials were kept in dark until further use.

3.1.5.2 Glycerol stocking

The isolates were grown in nutrient broth. After 18 h growth, the broth was mixed with sterile glycerol to make 30% glycerol stock and kept at -80°C.

3.1.5.3 Temporary stocking (working stock)

Cultures were grown overnight in nutrient broth and transferred onto nutrient agar slants. This was incubated at 37°C for 18 h. These cultures were kept at 4°C in refrigerator for short term use and were subcultured at 2 weeks interval.

3.2 CHARACTERIZATION OF THE BACTERIOCIN PRODUCERS

Characterization of the strains included identification of the strains by 16S ribotyping, plasmid isolation, antibiotic susceptibility test of the strains, hemolytic activity and growth curve analysis.

3.2.1 IDENTIFICATION OF THE ISOLATES SELECTED AFTER ACETONE PRECIPITATION

The strains that showed the ability to produce bacteriocins were identified at the molecular level by 16S rRNA gene sequence analysis. For this the chromosomal DNA was isolated, the 16S rRNA gene was amplified using specific primers, amplified gene sequenced and analysed.

3.2.1.1 DNA isolation

Chromosomal DNA was isolated using the method described by Ausubel *et al.* (1987). Log phase culture (2 mL) was taken in sterile microfuge tube and centrifuged at 6000 rpm for 10 min at 4ºC. The supernatant was discarded and the cell pellet was blot dried. The pellet was resuspended in 875 µL of Tris EDTA (TE) buffer (pH 8; Appendix 2). 5 µL proteinase K (10 mg/mL stock; Sigma-Aldrich, USA) and 100 µL of 10% SDS was added, mixed gently and incubated at 37ºC for 1 h. Equal volume of phenol-chloroform mixture (1:1) was added to this, centrifuged at 12000 rpm for 10 min at 4ºC. The supernatant was transferred to another sterile tube using sterile cut tip. The extraction with phenol-chloroform was repeated three times. To this supernatant, 0.1 volume of 5 M sodium acetate (pH 5.2) and double volume of ice-cold isopropanol were added. This was kept at -20ºC for about 1 h and then centrifuged at 12000 rpm for 10 min at 4ºC. The DNA pellet obtained was washed with 70% ethanol, air dried and dissolved in 50 µL TE buffer (pH 8). The concentration of the genomic DNA was estimated spectrophotometrically and appropriate dilutions (~80 -100 ng) were used as template for PCR reactions.

3.2.1.1.1 Agarose gel electrophoresis (Sambrook *et al*., 2000)

1% agarose in 1X TAE was taken and swirled to mix. This was microwaved for about 1 min to dissolve the agarose. When it was cooled to about 60°C, 2.5 µL of ethidium bromide (10 mg/mL) was added and swirled to mix. The gel was poured slowly into the tank. The comb was inserted in correct position. The gel was left to set for at least 30 min. The comb was removed carefully and the gel was placed in the gel tank with the wells closest to the cathode. 1X TAE buffer was poured into the gel tank to submerge the gel to 2–5mm depth. 5 µL sample was mixed with 1 µL loading dye and then loaded into the well of the gel taking care not to puncture the gel. The samples were run until the migrating dye (bromophenol blue) reached the anode. A voltage of 5 V/cm was given to run the gel. DNA ladder (Fermentas, India) was used as the marker. The gel was viewed and image captured with the help of Gel Doc system (Syngene, UK).

3.2.1.2 Polymerase chain reaction (PCR)

The 16S rRNA gene (~1.5 kb size) was amplified from the genomic DNA by PCR. The details of primers, composition of PCR mix and conditions for PCR are given in Table 3.2. Products after PCR amplification were subjected to sequencing, followed by homology analysis.

Table 3.2a Primers used to amplify 16S rRNA gene

Sequence	Amplicon	Reference
16SF5' AGTTTGATCCTGGCTCA 3'	1500 bp	(Shivaji *et al*., 2000)
16SR 5' ACGGCTACCTTGTTACGACTT 3'	”	(Reddy *et al*., 2002 a; Reddy *et al*., 2002 b)

Table 3.2b Composition of the PCR mix

Components	Volume
10X Taq buffer	2 µL
2 mM dNTP mix	2 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
Taq DNA polymerase	0.2 µL
Magnesium Chloride (25 mM)	1.2 µL
Template DNA (50 ng/µL)	1 µL
Sterile Milli Q water to a final volume of	20 µL

Table 3.2c The conditions used for PCR amplification of the 16S rRNA gene

Steps	Temperature	Time
Initial denaturation	94°C	for 90 s
Denaturation	94°C	for 30 s
Annealing	56°C	for 30 s
Primer extension	72°C	for 2 min
Repeated 34 times		
Final extension	72°C	for 10 min.

PCR was performed in a thermal cycler (Bio-Rad, USA). The PCR products were visualized using agarose gel electrophoresis as discussed under section 3.2.1.1.1

3.2.1.3 DNA sequencing and analysis

Nucleotide sequences of the amplicons were determined by the ABI Prism 310 genetic analyzer using the big dye Terminator kit (Applied Biosystems) at BioServe India Ltd., Hyderabad. The sequenced PCR products were analyzed online using Basic Local Alignment Search Tool (BLAST)

(http://www.ncbi.nlm.nih.gov/blast) and the identity of the sequences were determined (Altschul *et al.,* 1980). The sequences were deposited in the Genbank database and accession numbers were obtained for the submission.

3.2.1.4 Multiple sequence alignment and phylogenetic tree construction

All the nucleotide sequences were converted into FASTA format and multiple sequence alignment for the assembled nucleotide sequences was done using the Clustal W program (Thompson *et al.,* 1994) in BioEdit software (Hall, 1999). Aligned sequences were imported into MEGA 5 software (Molecular Evolutionary Genetics Analysis version 5.0; Tamura *et al.,* 2011) software for further analysis. The ends of the alignment were trimmed to obtain equal lengths for all sequences and the aligned sequences were converted into MEGA format for carrying out phylogenetic analysis. The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou & Nei, 1987) using nucleotide based TN84 evolutionary model for estimating genetic distances based on synonymous and non synonymous nucleotide substitutions. Statistical support for branching was estimated using 1000 bootstrap steps.

All further studies were conducted on the two selected strains BTFK101 and BTHT8.

3.2.2 PLASMID ISOLATION

Plasmids can be isolated by a variety of methods, many of which rely on the differential denaturation and reannealing of plasmid DNA compared to chromosomal DNA. One such method is the alkaline lysis method.

3.2.2.1 Alkaline lysis method

This is the technique developed by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). This method essentially relies on bacterial lysis by sodium hydroxide and sodium dodecyl sulfate (SDS), followed by neutralization

with a high concentration of low pH potassium acetate. This gives selective precipitation of the bacterial chromosomal DNA and other high molecular-weight cellular components. The plasmid DNA remains in suspension and is precipitated with ethanol.

Protocol

Test tube containing 3-5 mL of Luria Bertani broth (LB) was inoculated with a single isolated colony of the test strain picked from an LB agar plate. The broth culture was incubated at 37°C, overnight in a shaker at 125 rpm. 1.5 mL of this culture was taken in a microfuge tube and centrifuged for 10 min at 8000 rpm at 4°C and the supernatant was discarded. The bacterial pellet was resuspended in 100 µL of solution I (Appendix 2) and kept for 5 min in ice. 200 µL of solution II (Appendix 2) was added and gently mixed by inverting the tube. Tube was kept in ice for 10 min. 150 µL of ice cold solution III (Appendix 2) was added and mixed gently by inverting the tube. The tube was kept in ice for 15 min, followed by centrifugation at 14,000 rpm for 10 min. The supernatant was transferred to a fresh tube. The plasmid was precipitated by adding two volumes of ethanol to the supernatant, mixed well by inverting the tube several times, followed by keeping in ice for 30 min. The precipitated plasmid was collected by centrifuging at 14,000 rpm for 10 min and the supernatant was discarded. To the DNA pellet 1mL of ice cold 70% ethanol was added. Centrifuged again for 30 s, the supernatant discarded and the pellet air dried for 10-30 min. The pellet was resuspended in 50 µL of sterile deionised H_2O or TE buffer and stored at -20°C.

3.2.2.2 Plasmid isolation using Quicklyse ®Mini prep kit (Qiagen)

Plasmid isolation carried out as per manufacturer's instructions

The plasmids visualized using agarose gel electrophoresis as described under section 3.2.1.1.1

3.2.3 HEMOLYTIC ACTIVITY OF THE STRAINS

Hemolysis on blood agar plates is an indication of the potential for pathogenesis in bacteria. Hemolytic screening was performed on blood agar medium containing 5% human blood in agar base (Appendix 1), to test the pathogenicity of the stains producing bacteriocins. 16 h growth cultures in nutrient medium were spot inoculated onto the blood agar plates and incubated at 37°C. The blood agar plates were examined at regular intervals of 16 h, 24 h and 48 h. The formation of clearing zone/ green coloration of the medium around the colonies indicated the hemolytic nature of the isolates and they were considered as pathogenic (Isenberg, 1992).

3.2.4 ANTIBIOTIC SUSCEPTIBILITY TEST

Antibiotic susceptibility test was done according to disc diffusion method (Bauer *et al.,* 1966). With the help of a sterile cotton swab, a uniform bacterial smear was made on Mueller-Hinton agar plate. Antibiotic discs were placed on the plate, each plate holding not more than five discs. The discs were spaced to provide room for the development of the zone of inhibition. The plates were incubated at 37°C for 24 h before examination. The result was interpreted as resistant, intermediate or sensitive based on the size of the inhibition zones around each disc as provided by the manufacturer (Himedia, India). The antibiotics used were ampicillin (5 µg/disc), tetracycline (30 µg/disc), trimethoprim (5 µg/disc), azithromycin (15 µg/disc), ciprofloxacin (5 µg/disc), chloramphenicol (30 µg/disc), cefixime (5 µg/disc), cefuroxime (30 µg/disc), ceftriaxone (15 µg/disc), nalidixic acid (30 µg/disc) and gentamicin (10 µg/disc).

3.2.5 GROWTH CURVE AND BACTERIOCIN PRODUCTION

Hundred millilitres of Zobell marine broth was inoculated with 1 mL inoculum of BTFK101 and BTHT8. Inoculum was prepared as described in section 3.2.5.1.The culture broth were sampled at regular intervals of 45 min.

Optical density of the culture at 600 nm wavelength (OD_{600}) was checked in a UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). The cell suspension was centrifuged at 5000 rpm for 10 min at 4ºC to remove the cells and the supernatant was filter sterilized using 0.22 µm membrane syringe filter (Millipore, USA). The antibacterial activity of the culture supernatant was checked using critical dilution assay as mentioned in section 3.1.3.2. Growth curve was constructed to determine the phase of bacterial growth in which bacteriocin production started and reached its peak.

3.2.5.1 Inoculum preparation

The two selected strains were inoculated in 25 mL Zobell marine broth for inoculum preparation, followed by incubation at 30°C for 18 h in a shaker at 125 rpm. The culture was centrifuged, the cell pellet obtained was washed twice with physiological saline (pH 6) and resuspended in the same until the optical density at 600nm (OD_{600}) was ~1. When OD_{600} was ~ 1, there were 29x10^{7}CFU/mL for BTFK101 and 25x10^{7}CFU/mL for BTHT8.

3.3 BACTERIOCIN PRODUCTION BY *BACILLUS LICHENIFORMIS* STRAIN BTHT8 AND *BACILLUS SUBTILIS* STRAIN BTFK101: OPTIMISATION OF BIOPROCESS VARIABLES BY 'ONE-FACTOR AT-A-TIME' METHOD

Various physico-chemical and bioprocess parameters affecting bacteriocin production under submerged fermentation were optimized towards maximum bacteriocin production by one-factor at-a-time method. The parameters optimized included sodium chloride concentration, carbon sources, inorganic nitrogen sources, organic nitrogen sources, media, initial pH of medium, inoculum concentration, incubation temperature, surfactant, incubation time and agitation.

Inoculum preparation and extraction of bacteriocin were performed as detailed under sections 3.2.5.1 and 3.1.3.1.1 respectively. In each case, samples were analyzed for bacteriocin production and protein concentration as detailed under sections 3.1.3.2 and 3.1.3.3 respectively, unless otherwise mentioned. All the experiments were carried out in triplicates.

All the results are an average of triplicate experiments and standard deviation was determined using Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA). The graphs were plotted with the help of Sigma plot for Windows Version 11.0 (Systat Software Inc., Germany).

3.3.1 Optimization of different media for bacteriocin production

Seven different media were taken into account to check maximum bacteriocin production in the same. Zobell marine broth (ZB), nutrient broth (NB), Mueller Hinton broth (MH), lactose broth (Lac B), Luria Bertani broth (LB), brain heart infusion media (BHI), soyabean casein digest media (SCD) and minimal media (Mini M) were studied. Media preparation and their components are as given in appendix 1.The culture was incubated at 30°C, with agitation of 125 rpm. The analysis of the samples was done after 24 h incubation.

3.3.2 Optimization of sodium chloride (NaCl) concentration for bacteriocin production

Optimum sodium chloride concentration for maximum bacteriocin production was evaluated incorporating a range of sodium chloride concentrations (0.5%, 1%, 1.5%, 2%, 2.5%, 3% & 3.5%) in the minimal medium (Appendix 1). The culture was incubated at 30°C, at an agitation of 125 rpm. The analysis of the samples was done after 24 h incubation.

3.3.3 Optimization of carbon source for bacteriocin production

The best carbon source for maximum bacteriocin production was optimized by the addition of sucrose, glucose, lactose, mannitol, starch or pectin as carbon source of the minimal medium (0.8 % w/v). The culture was incubated at 30°C and at an agitation of 125 rpm. The samples were analysed after 24 h incubation.

3.3.4 Optimization of inorganic nitrogen sources for bacteriocin production

The inorganic nitrogen source for maximum bacteriocin production was optimized by the addition of ammonium chloride, ammonium sulphate, ammonium phosphate, ammonium nitrate or urea at 0.25 % (w/v) as nitrogen source in the minimal medium. The culture was incubated at 30°C and at an agitation of 125 rpm. The analysis of the samples was carried out after 24 h incubation.

3.3.5 Optimization of organic nitrogen sources for bacteriocin production

The best organic nitrogen source for maximum bacteriocin production was studied by incorporating tryptone, beef extract, peptone, yeast extract or malt extract at 0.25 % (w/v) as nitrogen source in the minimal medium. The culture was incubated at 30°C and at an agitation of 125 rpm. The analysis of the samples was done after 24 h incubation.

3.3.6 Optimization of inoculum concentration for bacteriocin production

Optimal inoculum concentration that supports maximum bacteriocin production was evaluated by adding different concentration of inoculum (2%, 4%, 6%, 8% & 10%), prepared as mentioned in section 3.2.5.1. The culture was incubated at 30°C and at an agitation of 125 rpm. The analysis of the samples was done after 24 h incubation.

3.3.7 Optimization of tween 80 (surfactant) concentration / its role in bacteriocin production

In order to check the effect of adding surfactants in the culture media used for bacteriocin production, tween 80 (HiMedia, Mumbai, India) at a concentration of 0.1%, 0.2%, 0.3%, 0.4% and 0.5% was added in the medium. Media without addition of tween 80 was kept as control. The culture was incubated at 30°C and an agitation of 125 rpm was provided. The analysis of the samples was done after 24 h incubation

3.3.8 Optimization of initial pH of the medium for bacteriocin production

Initial pH of the medium has an impact on the production of bacteriocins. For optimization of bacteriocin production at different pH, the initial pH of the media were adjusted to different levels (4, 5, 6, 7, 8, 9 & 10) using either 1 N HCl or 1 N NaOH. The culture was incubated at 30°C and at an agitation of 125 rpm. The analysis of the samples was done after 24 h incubation.

3.3.9 Optimization of incubation temperature for bacteriocin production

For the optimization of incubation temperature for maximum bacteriocin production, the culture was incubated at different temperature (20°C, 30°C, 40°C, 50°C or 60°C). The culture was provided with an agitation of 125rpm and the analysis of the samples was done after 24 h incubation.

3.3.10 Optimization of incubation period for bacteriocin production

Optimum incubation time required for maximum bacteriocin production was determined by incubating the culture for a total of 60 h and analyzing the samples at a regular interval of 6 h for bacteriocin production. The culture was incubated at 30°C in a shaker incubator at 125 rpm.

3.3.11 Optimization of agitation for bacteriocin production

The agitation required for maximum bacteriocin production was optimized by incubating the culture in static (0 rpm) and shake flask condition at 30°C in the environmental shaker at 50, 75, 100, 125 and 150 rpm. The analysis of the samples was done after 12 h incubation in the case of strain BTFK101 and 18 h incubation in the case of strain BTHT8.

3.4 PURIFICATION OF THE BACTERIOCINS

Bacteriocins isolated from *Bacillus licheniformis* strain BTHT8 and *Bacillis subtilis* strain BTFK101 were designated as BL8 and BS101 respectively. These bacteriocins were purified by standard protein purification methods, which included ammonium sulphate precipitation, followed by dialysis and gel filtration chromatography. All purification steps were carried out at 4°C unless otherwise mentioned.

3.4.1 AMMONIUM SULPHATE PRECIPITATION

Ammonium sulphate precipitation was carried out (Englard and Seifter, 1990) as described earlier under section 3.1.4.2.

3.4.2 GEL FILTRATION CHROMATOGRAPHY

The partially purified bacteriocins obtained as a result of ammonium sulphate fractionation were subjected to gel filtration chromatography using sephadex G-25 column to obtain homogenous bacteriocins.

3.4.2.1 Preparation of column

Ten grams of sephadex G-25 (Sigma-Aldrich, USA) was suspended in distilled water and allowed to hydrate for 1h at 90°C in a water bath, and the fine suspended particles were removed by decantation. Hydrated gel suspension was degassed under vacuum to remove the air bubbles. Gel suspension was carefully

poured into the column (50 X 1.5 cm) without air bubbles and allowed to settle under gravity while maintaining a slow flow rate through the column. Column was equilibrated by passing the eluent (0.15 M NaCl in deionised water) through the column bed, two times the bed volume.

3.4.2.2 Sample preparation and application

Four millilitres of ammonium sulphate fraction (30-60%) with protein content of 1.7 mg/mL for BS101 and 2.38 mg/mL for BL8 was concentrated using amicon UF-3 kDa membrane (Millipore, USA). This concentrated protein (0.5 mL) was applied to the column. Care was taken to ensure that the sample was completely free of undissolved substances. After the complete entry of sample into the column, the proteins were eluted using 0.15 M NaCl solution; with a flow rate of 18 mL/h; 5 mL fractions were collected. Twelve fractions were collected and each fraction from the column was concentrated using amicon UF-3 kDa membrane. This was assayed for antibacterial activity as described in section 3.1.3.2. The fraction with antibacterial activity was analyzed for its protein content and specific activity determined as described under sections 3.1.3.3 and 3.1.3.4 respectively.

3.4.3 ELECTRO ELUTION OF BACTERIOCIN

The partially purified protein was subjected to SDS-PAGE using 15% gel (Laemmli, 1970). After the electrophoretic run in a vertical slab electrophoresis system at 80 V, the gel was removed and cut into two. One portion containing the sample and molecular weight marker was silver stained. The other half with sample was washed thrice with 0.1% Tween 80 (30 min each), followed by washing with deionised water in order to remove the SDS. The gel portion corresponding to the active band was excised into small pieces. The gel pieces were transferred in a 2 kDa benzoylated dialysis tubing (Sigma Aldrich, USA). 2 mL 0.01 M phosphate buffer added and electro eluted in a electrophoretic tank

containing 0.01 M phosphate buffer. For this a voltage of 30 V was applied overnight at 4° C, followed by a reversal of the electrodes with voltage of 30 V applied for 30 min (Lei *et al.*, 2007). The solution containing the bacteriocin was concentrated in an amicon UF-3 kDa membrane to remove the salts. Then the sample was lyophilized (Yamato, Japan) and sent for molecular mass determination by MALDI-TOF MS (Vimta Labs Ltd, Hyderabad, India) and N-terminal sequence analysis at Indian Institute of Technology (IIT), Bombay.

3.4.4 Calculation of fold of purification

Fold of purification in each step was calculated by dividing the specific activity (section 3.1.3.5) of the respective fraction with that of the crude extract

$$\text{Fold of purification} = \frac{\text{Specific activity of the purified fraction}}{\text{Specific activity of the crude extract}}$$

3.5 CHARACTERIZATION OF THE BACTERIOCINS

Purified bacteriocins were further characterized for their biophysical and physicochemical properties like molecular weight, isoelectric point, stability at different temperature and pH, action of different proteases, effect of detergents, metal ions, reducing agents and oxidizing agent as described in the following sections. N-terminal sequences were also determined.

3.5.1 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The commonly used electrophoretic techniques for separating proteins were glycine SDS-PAGE (Laemmli, 1970) and tricine SDS-PAGE (Schagger and von Jagow, 1987) based on glycine-tris and tricine-tris buffer systems used respectively. The active fractions of the bacteriocins after ammonium sulphate fractionation and gel filtration chromatography were subjected to electrophoretic

analysis by SDS-PAGE in a vertical slab electrophoresis (Mini-PROTEAN Tetra cell, BioRad). Electrophoresis was carried out in a 15% polyacrylamide gel.

3.5.1.1 Glycine SDS-PAGE

The purified bacteriocins were subjected to SDS-PAGE for evaluating the nature of polypeptide. Reagents for glycine SDS-PAGE gels are given in table 3.3 and the preparation of reagents given in appendix 3.

Table 3.3 Reagents for the preparation for Glycine SDS-PAGE gel

Reagents	**Stacking gel**	**Resolving gel**
Stock acrylamide: bis-acrylamide (mL)	0.536	5
Stacking gel buffer stock (mL)	1	-
Resolving gel buffer stock (mL)	-	2.5
Water (mL)	2.4	2.32
10% SDS (µL)	40	100
Ammonium persulphate (APS) (µL)	20	75
TEMED (µL)	5	15

Gel preparation

The gel plates were cleaned and assembled.

Resolving gel - The resolving gel was prepared combining all reagents (table 3.3) except APS and TEMED in a beaker. Mixed gently, APS and TEMED added and again mixed well. Immediately poured the mixture into the cast and poured a layer of water over the gel and allowed to polymerize at least for 45 min.

Stacking gel - The components of stacking gel (table 3.3) except APS and TEMED were added into a beaker, mixed gently and finally APS and TEMED were added. Poured the contents into the cast above the resolving gel and

immediately inserted the comb between the glass plates. Allowed to polymerize for atleast 45 min.

Gel was placed in the electrophoresis apparatus, and reservoir was filled with running buffer for SDS-PAGE (Appendix 3). Protein samples were loaded to the gel. The gel was run at 80 V till the sample entered the resolving gel portion. When the dye front entered the resolving gel, the current was increased to 120 V. The run was stopped when the dye front reached 1 cm above the lower end of the glass plate. Removed the gel from cast and stained for at least 1 h in the staining solution. The gel was destained till the protein bands became clear.

Sample preparation

1X sample buffer (Appendix 3) was mixed with the protein samples and 20 μL sample was applied in the well of the gel.

3.5.1.2 Tricine–SDS-PAGE

Tricine SDS-PAGE is commonly used to separate proteins in the molecular mass range 1–100 kDa. It is the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa. Preparation of reagents is given in appendix 3.

First 16% separating gel prepared using the reagents given in table 3.4. After the polymerization of this gel, 10% gel prepared casted on its top. The polymerized separating gel (10% and 16%) is overlaid directly with a 4% sample (stacking) gel.

3.5.1.3 Coomassie staining

The gel was removed and put in the staining solution for 1h, followed by washing in destaining solution (Appendix 3) until the gel became clear, with blue colored protein bands.

Table 3.4 Reagents for the preparation of Tricine SDS-PAGE gel

Reagents	Stacking gel	Resolving gel	
	(4%)	10%	16%
AB-3 solution (mL)	1	6	10
Gel buffer (3×) (mL)	3	10	10
Water final volume (mL)	12	30	30
APS (10%) (μL)	90	150	100
TEMED (μL)	9	15	10

3.5.1.4 Silver staining

Silver staining of the gel after electrophoresis was performed by the method of Blum (1987) with slight modifications. The preparation of the solutions is provided in appendix 3.

Procedure

The SDS-PAGE gel was incubated in the fixer for 30 min. Then the gel was washed in wash solution for 15 min, followed by five washes (5 min interval) with deionised water. Incubated the gel in sensitizer for exactly 60 s and washed twice at 60 s intervals with deionised water. The gel was immersed in chilled silver nitrate solution for 25 min and washed two times for 60 s with deionised water. The gel was transferred to developer solution and kept until protein bands were developed. Reaction was arrested by adding stop solution.

3.1.5.5 Protein Markers for SDS-PAGE

Medium range molecular weight protein marker of GeNei (Bangaluru) and Broad range molecular weight protein marker mix of New England BioLabs (UK) was used for detecting the approximate size. Details of markers are given in appendix 3.

3.5.2 GLYCINE SDS-PAGE AND DETECTION OF ANTIBACTERIAL ACTIVITY TO DETERMINE APPROXIMATE MASS OF THE BACTERIOCINS

The partially purified protein was subjected to SDS-PAGE using 15% gel as described in section 3.5.1.1. The protein was run in two lanes along with protein molecular weight marker (GeNei, Bangaluru, India). After the electrophoretic run, the gel was removed and cut into two. One half containing the sample and molecular weight marker was coomassie / silver stained. The other half with sample was washed thrice with 0.1% tween 80 (30 min each), followed by washing with deionised water in order to remove SDS. The gel was then placed on MH agar base plate, overlaid with soft MH agar (0.8 % agar) seeded with 100 µL (OD_{600} ~ 1) of test organisms (Yamamoto *et al*., 2003) and checked for zone of clearance due to the antibacterial activity after overnight incubation at 37° C.

3.5.3 INTACT MASS BY MALDI-TOF MS

The purified bacteriocins were subjected to intact mass analysis by MALDI-TOF mass spectrometry. The intact molecular mass of the purified bacteriocins was determined by ABI 4800 MALDI TOF/TOF (Applied Biosystems). This was done at Vimta Labs Ltd, Hyderabad, India.

3.5.4 ISOELECTRIC FOCUSING

Isoelectric point (pI) of the purified bacteriocin proteins was determined by isoelectric focusing, performed using isoelectric focusing unit (Bio-Rad, USA). Immobilized pH gradient (IPG) strip of pH 3-10 (Bio-Rad, USA) was used for the purpose. The detailed procedure is as given below.

3.5.4.1 Rehydration of sample with IPG strip

The lyophilized sample (0.1 mg/ mL) was prepared in 2 mL rehydration buffer and loaded 125 µL of this prepared sample on to the equilibration tray. IPG

strip of pH 3-10 was gently placed gel side down in the equilibration tray. Removed air bubbles if any. The strips were overlaid with 2-3 mL of mineral oil to prevent evaporation during rehydration process. Covered the equilibration tray and left the tray overnight to rehydrate IPG strips.

3.5.4.2 Isoelectric focusing

Paper wicks were placed at both ends of the clean, dry IEF focusing tray covering the wire electrodes. IPG strips were taken out from the rehydration tray and drained the mineral oil by holding the strip vertically for 8 s. Paper wicks were made wet with nano pure water and placed the IPG strips in the focusing tray. Placed in the PROTEAN IEF cell, overlaid with mineral oil and closed the cover. Programmed the IEF cell as given below and run the electrophoresis.

Table 3.5 Steps involved in isoelectric focusing

	Voltage	Time	Volt-Hours	Ramp
Step 1	250	20 min	---	Linear
Step 2	4000	2 h	---	Linear
Step 3	4000	---	10,000 V-h	Rapid

The cell temperature was maintained at 20°C with maximum current of 50 µA/strip and no dehydration in all steps.

3.5.4.3 Staining of IPG strips after IEF

The gel was subjected to Coomassie staining followed by destaining to visualize the isoelectric points of the two bacteriocin proteins.

3.5.5 N-TERMINAL AMINOACID SEQUENCE ANALYSIS

The N- terminal amino acid sequence of the electro-eluted bacteriocin was resolved by automated Edman degradation (Applied Biosystems 494 Procise Protein Sequencing System) at IIT Bombay, Mumbai, India. The sequence obtained was compared to that of the sequences of bacteriocins from *Bacillus* sp. retrieved from the protein data base of NCBI (www.ncbi.nlm.nih.gov) by multiple sequence alignment using CLUSTAL W (Thompson *et al.*, 1994) in BioEdit software (Hall, 1999).

3.5.6 EFFECT OF PHYSICO-CHEMICAL PARAMETERS ON BACTERIOCIN STABILITY

3.5.6.1 Action of proteases on the bacteriocins

Sensitivity of bacteriocins to different proteases was assessed by incubating the purified bacteriocins with different concentrations of proteinase K, pepsin and trypsin (from Bovine pancreas, SRL, India) ranging from 20, 40, 60, 80 and 100 μg for 1 h at 37°C. The antibacterial activity was tested as described under section 3.1.3.2.

3.5.6.2 Effect of temperature on the stability of the bacteriocins

Thermal stability of the bacteriocins was studied by incubating the purified bacteriocins at different temperatures ranging from 4°C-100°C for 1 h. After this the antibacterial activity of each sample was assessed by conducting the critical dilution assay as described under section 3.1.3.2.

3.5.6.3 Effect of pH on the stability of bacteriocins

To study the effect of different pH on the stability of the bacteriocins, the purified bacteriocins were incubated with equal amount of buffers with pH range 2–13 and kept for 18 h at 4°C. The buffer systems used included hydrochloric acid- potassium chloride buffer (pH 2), citrate buffer (pH 3–6), phosphate buffer

(pH 7), Tris (hydroxy methyl amino methane) buffer (pH 8 and 9), carbonate-bicarbonate buffer (pH 10 and 11), sodium hydroxide- potassium chloride buffer (pH 12 and 13) (Vincent and John, 2009). Preparation of buffers is charted in appendix 2. The samples after incubation were tested for the antibacterial activity as described under section 3.1.3.2.

3.5.6.4 Effect of various detergents on stability of bacteriocins

Effect of various non-ionic and ionic detergents such as triton X-100, tween 80, tween 20, SDS and CTAB (0.1% each w/v) on bacteriocin activity was determined by incubating the purified bacteriocins in each detergent for 1h. This was followed by dialysis against 0.01 M phosphate buffer having pH 7.5. The sample was then concentrated in amicon UF-3 kDa membrane. Samples were tested for the antibacterial activity as described under section 3.1.3.2.

3.5.6.5 Effect of various metal ions on the activity of bacteriocins

Effect of various metal ions on activity of the bacteriocins was evaluated by incubating the purified bacteriocins along with 1 mM concentrations of various metals ions for 1 h followed by testing the antibacterial activity as described under section 3.1.3.2. The metal ions were supplied in the form of salts, which included sodium chloride, calcium chloride, magnesium sulphate, ferric chloride, manganese chloride, nickel chloride, barium chloride, cadmium sulphate, zinc sulphate, copper sulphate, cobalt chloride and aluminum sulphate which supplied the metal ions, Na^{+}, Ca^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, , Ba^{2+}, Cd^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+} and Al^{3+} respectively.

3.5.6.6 Effect of reducing agents on the stability of bacteriocins

The effect of reducing agents on the activity of bacteriocins was studied by incubating the bacteriocin BS101with β-mercaptoethanol at a concentration of 20, 40, 60, 80 & 100 mM and dithiothretol at a concentration of 2, 4, 6, 8 & 10

mM. Bacteriocin BL8 was treated with β-mercaptoethanol at a concentration of 200, 400, 600, 800 & 1000 mM and dithiothretol at a concentration of 20, 40, 60, 80 & 100 mM for 1 h followed by testing the antibacterial activity as described under section 3.1.3.2.

3.5.6.7 Effect of oxidizing agents on bacteriocins

Impact of oxidizing agents on the activity of bacteriocins was studied by incubating the purified bacteriocins with dimethyl sulfoxide (1-5%, v/v) for 1h followed by testing the antibacterial activity as described under section 3.1.3.2.

3.5.7 MINIMUM INHIBITORY CONCENTRATION (MIC)

The microtitre-plate based resazurin assay was modified to determine MIC values of the bacteriocin against *S. aureus* (Sarker *et al.*, 2007). For this 50 µL deionised water was added to each well. Then 50 µL purified bacteriocin was added in the first row and double diluted vertically below. 100 µL double strength nutrient broth was added in each well. 10 µL test organisms (OD at 600 nm ~1) was also added to each well. This was followed by the addition of 1 µL resazurin (1% stock prepared). The microtitre plate was incubated at 37°C for 18- 24 h and observed for colour change. Blue coloured resazurin turns pink in wells which contain living cells. From the highest dilution where colour changed from blue to pink, the MIC was calculated.

3.5.8 BROTH ASSAY TO TEST BACTERICIDAL/BACTERIOSTATIC NATURE OF THE BACTERIOCINS

In order to check the bacteriostatic/bactericidal nature of the bacteriocins, the method described by Wang & Kuramitsu (2005) was used, where 50 µL culture (OD_{600}~1) of the test organisms was added to 5 mL nutrient broth. In tubes taken as test, 100 µL partially purified bacteriocins also added. Suitable controls

were included in which, the bacteriocins were not added. Incubated at 37°C for 6 h, and OD_{600} of the cultures was measured using UV-Vis spectrophotometer.

3.6 APPLICATION STUDIES

3.6.1 Bacteriocins for the control of biofilms

The test organisms were checked for their capacity to form biofilms using microtitre plate assay of Rode *et al*. (2007). Two test organisms that were topmost biofilm producers were used in further studies incorporating the bacteriocins.

After the first experiment, *Cl. perfringens* and *B. cereus* were found to be good biofilm producers. So they were taken to test the role of the bacteriocins in biofilm control. The experiment was carried out in triplicates to determine the ability of the bacteriocins to inhibit biofilm formation. The wells of the sterile 96 well polystyrene microtitre plates were filled with 230 µL of tryptone soya broth (TSB). 20 µL test bacterial culture (OD_{600}~1) was added into each well separately (positive control). In wells taken as negative control, only TSB was added. In experimental wells, 10 µL of purified bacteriocins were also added. The microtitre plates were incubated aerobically for 24 h at 37°C. The contents of the plates were poured off and the wells were washed 3 times with 300 µL of phosphate buffer (0.1 M, pH 7.5). Remaining bacteria were fixed with 250 µL of methanol per well. After 15 min, the plates were emptied, air dried and stained with 250 µL of 1% crystal violet for 5 min. The excess stain was rinsed off by placing the microtitre plates under running tap water. After the plates were air dried, the dye bound to the adherent cells was extracted with 250 µL of 33% (v/v) glacial acetic acid per well and the absorbance of each well was measured at 570 nm using a UV-VIS spectrophotometer. The different treatments used in the experiment are given in table 3.6.

Table 3.6 Experimental design to study biofilm control by the bacteriocins

Sl.no.	Treatments
1	TSB only (negative control)
2	TSB + *B.cereus* (positive control)
3	TSB + *Cl. Perfringens* (positive control)
4	TSB + *B. cereus* + BS101/ BL8
5	TSB + *Cl. perfringens* + BS101/ BL8

Based on the absorbance (A_{570}) produced by the bacterial films (Christensen *et al*., 1985; Stepanovic *et al*., 2000);

$A = A_c$--------→ No biofilm producers

$A_c < A$--------→ Weak biofilm producers

$2A_c < A$--------→ Moderate biofilm producers

$4A_c < A$--------→ Strong biofilm producers

Where A_c = mean absorbance of the negative control.

3.6.2 Bacteriocins for the control of microflora of sea foods

Prawn and anchovies (10g) were ground using a mortar and pestle in normal saline (pH 6). These grinded sea foods were added to100 mL nutrient broth (NB). In the case of experimental flasks, 100 µL purified bacteriocins were also added along with sea food sample. In control flasks, bacteriocins were not added. This was incubated overnight at 37°C. Sample was serially diluted and plated. Plates were incubated at 37°C and colony forming units (CFU/mL) was calculated. Experiment was conducted in triplicates. The experimental design is given in table 3.7.

Table 3.7 Experimental design to study the control of microflora of sea foods by the bacteriocins

Sl.no.	Treatments
1	NB + Prawn sample (control)
2	NB + Prawn sample + BS101
3	NB + Prawn sample + BL8
4	NB + Anchovies sample (control)
5	NB + Anchovies sample + BS101
6	NB + Anchovies sample + BL8

3.6.3 Bioassay using model organism *Caenorhabditis elegans*: Pathogenesis and prophylaxis using the bacteriocins

Caenorhabditis elegans is a free-living, transparent, non-parasitic soil nematode (roundworm), about 1 mm in length. It has been used as a model system due to ease of manipulation and availability of detailed knowledge of its biology to study bacterial pathogenesis.

3.6.3.1 *C. elegans* strain and maintenance

The nematode *C. elegans* Bristol N2 wild type (kind courtesy to Dr. Sandhya P Koushika, National Centre for Biological Sciences, Bangaluru) was used for the study. It was propagated and maintained in modified Nematode Growth Medium (NGM) agar (Appendix 4) at 20ºC. Medium size plates (60 mm diameter) were used for general strain maintenance and small plates of 35 mm diameter were used for the bioassay. *E. coli* strain OP50 was used as the food source (Brenner, 1974). *E. coli* OP50 is a uracil auxotroph whose growth is limited on NGM plates. A limited bacterial lawn is desirable because it allows for easier observation of the worms. A starter culture of *E. coli* OP50 was prepared by aseptically inoculating Luria Bertani (LB) broth (Appendix 1) and incubated

overnight at 37ºC. This overnight culture was used in seeding NGM plates. The worms were observed under a simple dissecting stereo- microscope equipped with a transmitted light source.

3.6.3.2 Pathogenesis and prophylaxis using the bacteriocins

The experimental design to study the bacterial infection in *C. elegans* Bristol N2 wild type and the role of bacteriocins in controlling the infection consisted of seven groups (Table 3.8).

C. elegans killing assays were conducted as per Aballay *et al.* (2000). *B. circulans*, *S. aureus* and *E.coli* OP50 were grown over night in LB broth. The culture was spread on to the center of NGM agar media in 3.5 cm diameter plates. The plates were incubated at 37°C for 2–12 hours and allowed to equilibrate to room temperature. 15 to 20 worms (L-4 stage) were placed on each of these plates and were incubated at 25°C for 24 hours. After incubation, the worms from plates of group 1, 2 and 3 were transferred to NGM plates seeded with *E. coli* OP50. Nematodes from plates of group 5 and 6 were transferred to plate containing *E. coli* OP50 lawn in which bacteriocin BS101 was incorporated (conc. 25µg). Worms from group 4 and 7 plates were transferred onto plates with *E. coli* OP 50 lawn incorporated with bacteriocin BL8 (conc. 25 µg). All the plates were again incubated at 25°C for 24 hours. After incubation, the worms were transferred to fresh *E. coli* OP50 plate every 24 hours for the next 4 days of the assay or until no more progeny were evident. This was done in order to avoid losing track of the original worms due to crowding by reproduction. Worm mortality was scored for 10 days, and a worm was considered dead when it failed to respond to touch. Worms that died as a result of getting stuck to the wall of the plate were excluded from the analysis. The experiments were conducted in triplicates.

Table 3.8 Experimental design to study bacterial infection and prophylaxis using the bacteriocins in *C. elegans*

Group	Step 1- Infection : *C.elegans* transferred to NGM plates with	Step 2- Transfer of *C. elegans* of Step 1 to NGM plates with
1	*E. coli* OP50 (negative control)	*E.coli* OP50
2	*S. aureus* (positive control)	*E. coli* OP50
3	*B. circulans* (positive control)	*E. coli* OP50
4	*S. aureus*	*E. coli* OP50 + BL8
5	*S. aureus*	*E.coli* OP50 + BS101
6	*B. circulans*	*E. coli* OP50 + BS101
7	*B.circulans*	*E.coli* OP50+ BL8

Statistical analysis

The time taken for 50% of the nematodes to die (time to death 50, TD_{50}) was calculated using the PRISM (version 5.04) computer program using the equation: Y = Bottom + (Top – Bottom)/ (1 + 10^ ((LogEC50 – X)*Hill Slope), where X is the logarithm of days and Y is the average of dead worms. The data represents the mean ± the standard error.

4. RESULTS

4.1 SCREENING FOR BACTERIOCIN PRODUCING BACTERIA FROM MARINE SEDIMENT AND WATER SAMPLES

4.1.1 ISOLATION OF BACTERIA

One hundred and twelve isolates were obtained from the sediment and water samples collected from different locations. These isolates were screened first using disc diffusion assay (culture supernatant used) to short list bacteria capable of inhibiting the growth of test organisms, as this indicated their ability to produce antagonistic molecules.

4.1.2 PRIMARY SCREENING FOR BACTERIOCIN PRODUCING BACTERIA

The primary screening helped to segregate seventeen of the hundred and twelve isolates that produced some antibacterial compounds (Table 4.1). While sixteen isolates inhibited the growth of *Bacillus cereus*, representative of Gram positive test organism, one isolate inhibited the growth of *Escherichia coli*, representing Gram negative test organism.

4.1.3 SECONDARY SCREENING FOR BACTERIOCIN PRODUCING BACTERIA

Secondary screening was performed by testing the antibacterial activity of the acetone precipitated protein fraction and that of the ammonium sulphate precipitate. Acetone precipitation of culture supernatant of the seventeen isolates yielded protein concentrates, whose antibacterial activity was checked by disc diffusion assay against *B. cereus* and *E. coli*. Out of the seventeen isolates screened, five isolates (viz. BTFK101, BTHT8, BTKM4, BTEK16 and BTSB22) showed antimicrobial activity against *B. cereus.* No activity was observed against the Gram negative test bacteria.

Table 4.1 Primary screening by disc diffusion method for bacteriocin production

Isolate number	***B. cereus***	***E. coli***
BTFK101	+	-
BTFK103	+	-
BTFK105	+	-
BTFK108	+	-
BTEK2	+	-
BTEK4	+	-
BTEK7	+	-
BTEK8	+	-
BTEK16	+	-
BTEK22	+	-
BTSB3	-	+
BTSB14	+	-
BTSB22	+	-
BTKM2	+	-
BTKM4	+	-
BTHT5	+	-
BTHT8	+	-

+: growth inhibition of the test organism

- **:** no growth inhibition of the test organism

Ammonium sulphate precipitation (0-30%, 30-60% and 60-90%) of the culture supernatant of the above five isolates was carried out. The protein precipitated in the 30-60% fraction inhibited the growth of six Gram positive test organisms namely *B. cereus*, *B. circulans*, *B. pumilus*, *B. coagulans*, *Cl. perfringens* and *S. aureus* (Table 4.2; Fig. 4.2). However Gram negative organisms were not inhibited.

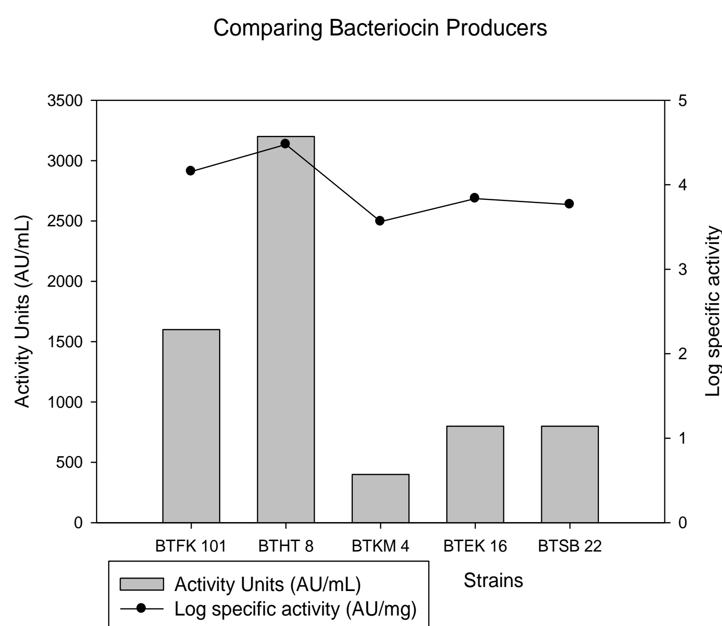

Fig: 4.1 Critical dilution assay for quantitative estimation of antibacterial activity of five isolates

The quantitative estimation of antibacterial activity of the five isolates (ammonium sulphate fraction) was determined by the critical dilution assay (Fig: 4.1). Two isolates BTFK101 and BTHT8 exhibited high activity of 1600AU/mL and 3200AU/mL respectively. These two isolates were selected for further study.

Table 4.2 Secondary screening for bacteriocin production by disc diffusion method (Antibacterial activity of ammonium sulphate precipitated protein fraction)

Strains / Test organisms	BTFK101	BTHT8	BTKM4	BTEK16	BTSB22
Pseudomonas aeruginosa	_	_	_	_	_
Salmonella Typhimurium	_	_	_	_	_
Escherichia coli	_	_	_	_	_
Salmonella Abony	_	_	_	_	_
Klebsiella pneumoniae	_	_	_	_	_
Proteus vulgaris	_	_	_	_	_
Clostridium perfringens	+	+	+	+	+
Staphylococcus aureus	+	+	+	+	+
B. cereus	+	+	+	+	+
B. circulans	+	+	+	+	+
B. coagulans	+	+	+	+	+
B. macerans	_	_	_	_	_
B. pumilus	+	+	+	+	+
Vibrio cholerae	_	_	_	_	_
V. parahaemolyticus	_	_	_	_	_
V. alginolyticus	_	_	_	_	_
V. vulnificus	_	_	_	_	_
V. proteolyticus	_	_	_	_	_
V. harveyi	_	_	_	_	_
V. furnisii	_	_	_	_	_

+ : growth inhibited; _ : growth not inhibited

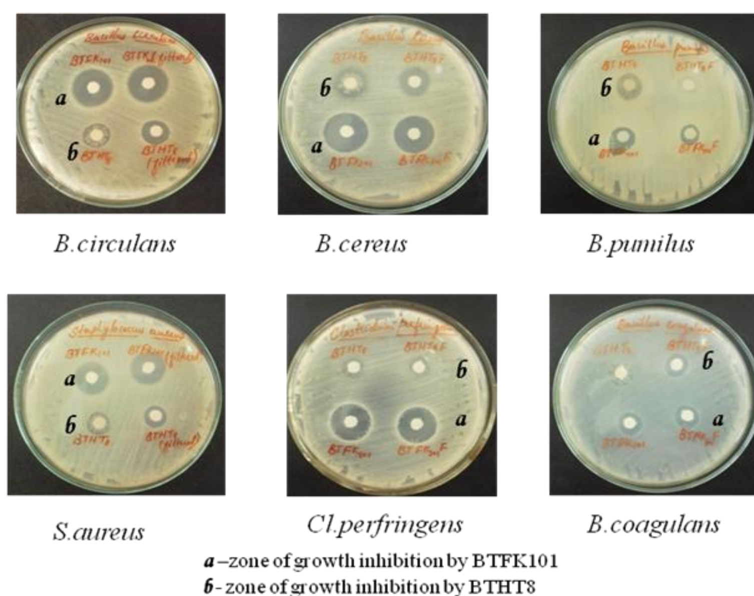

Fig: 4.2 Antimicrobial activity of BTFK101 and BTHT8 (ammonium sulphate fraction) against six test organisms by disc diffusion assay

4.2 CHARACTERIZATION OF THE BACTERIOCIN PRODUCERS

Characterization of the bacteriocin producing bacteria included molecular identification using 16S ribotyping, plasmid isolation, antibiotic susceptibility study, hemolytic activity and growth curve studies of the strains.

4.2.1 IDENTIFICATION OF THE ISOLATES SELECTED AFTER SECONDARY SCREENING

The five isolates which showed antibacterial activity after acetone precipitation were identified upto the molecular level by16S ribotyping.

Chromosomal DNA was isolated, purified and quantified. The agarose gel in which electrophoresis of genomic DNA isolated from the five bacteriocin producing isolates was performed is represented in fig. 4.3.

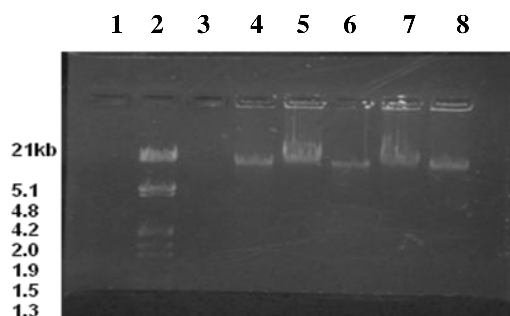

Fig: 4.3 Agarose gel electrophoresis of genomic DNA isolated from bacteriocin producing isolates; Lane 2- Lambda DNA / EcoR1/Hind III/ Double digest, Lane 4- DNA of BTFK101, Lane 5- DNA of BTHT8, Lane 6- DNA of BTKM4, Lane 7- DNA of BTEK16, Lane 8- DNA of BTSB22

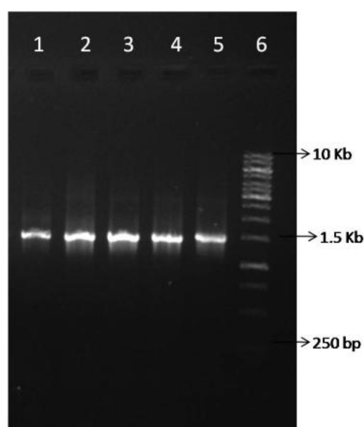

Fig: 4.4 Agarose gel showing amplified 16S rRNA gene from bacteriocin producing isolates; Lane 1 - 16 S rRNA gene amplicons of BTFK101; Lane 2- 16 S rRNA gene amplicons of BTHT8, Lane 3- 16 S rRNA gene amplicons of BTKM4, Lane 4- 16 S rRNA gene amplicons of BTEK16, Lane 5- 16 S rRNA gene amplicons of BTSB22, Lane 6- 1kb ladder**.**

This was followed by amplification of the 16S rRNA gene (~1.5kbsize) using PCR. The agarose gel containing amplified 16S rRNA gene of the bacteriocin producing isolates is represented in fig. 4.4. The amplicons were sequenced and

analysed by BLAST in NCBI database to reveal the identity of the isolates. The sequence analysis showed that the 16S rRNA gene sequences retrieved from the five isolates, showed maximum sequence similarity with two species of *Bacillus* namely *B. subtilis* and *B. licheniformis* after BLAST.

The sequences were submitted to GenBank and accession numbers were obtained.

1. BTFK101: *Bacillus subtilis* (Accession no. HM030818)
2. BTHT8 : *Bacillus licheniformis* (Accession no. HM030819)
3. BTKM4 : *Bacillus licheniformis* (Accession no. HM030820)
4. BTEK16 : *Bacillus licheniformis* (Accession no. HM030821)
5. BTSB22 : *Bacillus subtilis* (Accession no. HM030822)

4.2.2 MULTIPLE SEQUENCE ALIGNMENT AND PHYLOGENETIC TREE CONSTRUCTION

Based on the 16S rRNA gene sequences, the relatedness of the five isolates were studied by constructing a phylogenetic tree as depicted in fig. 4.5. From the phylogenetic tree, it can be observed that the two *B. subtilis* strains grouped together and the three *B. licheniformis* strains also grouped together. All the five isolates grouped together and form a single clade as they are related.

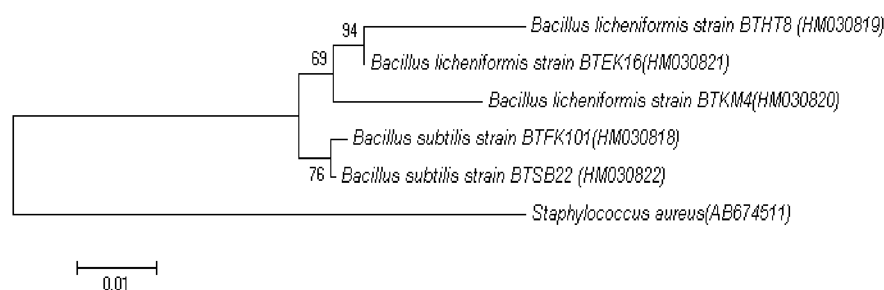

Fig: 4.5 Phylogenetic relationship of five bacteriocin producers based on 16S rDNA sequences. The number at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on neighbour- joining

analysis of 1000 resampled data set. *Staphylococcus aureus* was used as the outgroup.

All further studies were conducted on the two selected strains, *B. subtilis* strain BTFK101 and *B. licheniformis* strain BTHT8.

4.2.3 PLASMID DNA ISOLATION

Plasmid DNA was not obtained from both BTFK101 and BTHT8, despite the use of several protocols. This indicates that the strains do not harbour plasmids. This confirmed that the bacteriocins produced by these strains are not encoded by plasmid borne genes. They are encoded by the chromosomal genes.

4.2.4 HEMOLYTIC ACTIVITY

Strains BTHT8 and BTFK101 did not show hemolytic activity on blood agar plates. This point to their non- pathogenic nature. Most pathogenic strains possess hemolytic activity.

4.2.5 ANTIBIOTIC SUSCEPTIBILITY TEST

Eleven antibiotics were tested to study the antibiotic susceptibility of the two selected strains. Of the antibiotics tested (Table 4.3), BTFK101 was resistant only to cefixime. It was sensitive to all the other ten antibiotics tested viz. ampicillin, azithromycin, chloramphenicol, cefuroxime, ceftriaxone, ciprofloxacin, gentamicin, nalidixic acid, tetracycline and trimethoprim. BTHT8 was found sensitive to ciprofloxacin, gentamicin and trimethoprim; resistant to ampicillin, azithromycin, cefixime and cefuroxime; intermediate to chloramphenicol, ceftriaxone, nalidixic acid and tetracycline.

Table 4.3 Antibiogram of *B. subtilis* strain BTFK101and *B. licheniformis* strain BTHT8

Antibiotic	Concentration per disc (µg/disc)	Range R	Range I	Range S	BTFK101	BTHT8
Ampicillin	5	≤11	12-14	≥15	S	R
Azithromycin	15	≤13	14-17	≥18	S	R
Chloramphenicol	30	≤12	13-17	≥18	S	I
Cefixime	5	≤15	15-17	≥18	R	R
Cefuroxime	30	≤14	15-17	≥18	S	R
Ceftriaxone	15	≤13	14-20	≥21	S	I
Ciprofloxacin	5	≤15	16-20	≥21	S	S
Gentamicin	10	≤12	13-14	≥15	S	S
Nalidixic acid	30	≤13	14-18	≥19	S	I
Tetracycline	30	≤14	15-18	≥19	S	I
Trimethoprim	5	≤10	11-15	≥16	S	S

R-Resistant; I- Intermediate; S- Sensitive

4.2.6 GROWTH CURVE AND BACTERIOCIN PRODUCTION

The growth curve of *B. subtilis* strain BTFK101 and bacteriocin production is represented in fig. 4.6. The figure clearly shows that the organism entered the exponential phase after 1-2 h and reached stationary phase at about 12 h. It is also clear from the figure that bacteriocin production by BTFK101 started at early exponential phase (after 7 h) and the production peaked as the organism entered the stationary phase (at about 12 h) showing 1600AU/mL activity.

The growth curve and bacteriocin production of *B. licheniformis* strain BTHT8 is represented in fig. 4.7. It was observed that in this case also, bacteriocin production started at the early exponential phase and the production reached its peak at the stationary phase (at about 13-14 h), when production in terms of activity was 6400AU/mL.

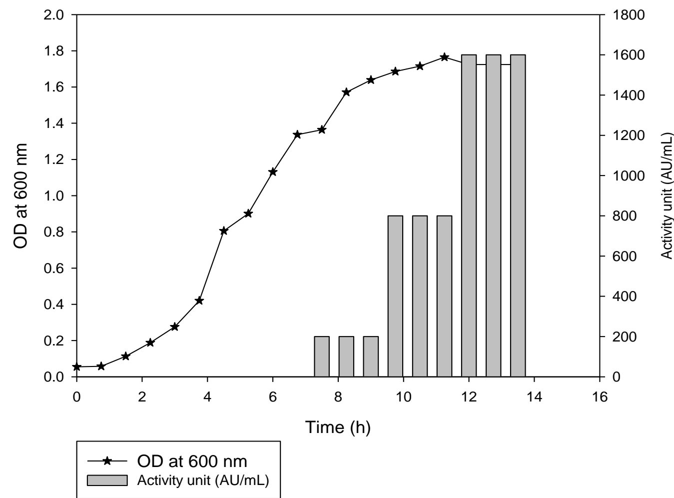

Fig: 4.6 Growth curve of *B. subtilis* strain BTFK101 **and bacteriocin production**

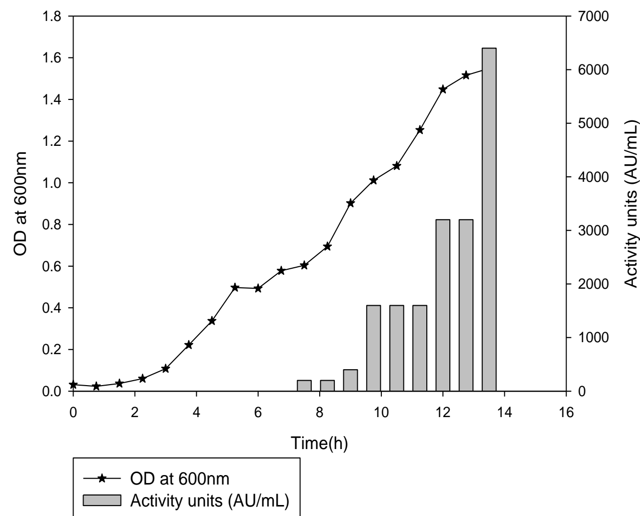

Fig: 4.7 Growth curve of *B. licheniformis* strain BTHT8 and **bacteriocin production**

4.3 BACTERIOCIN PRODUCTION BY *BACILLUS LICHENIFORMIS* STRAIN BTHT8 AND *BACILLUS SUBTILIS* STRAIN BTFK101: OPTIMIZATION OF BIOPROCESS VARIABLES BY 'ONE-FACTOR AT-A-TIME METHOD'

In order to achieve maximum production under laboratory conditions, the bioprocess variables were optimized. Eleven factors were taken into account viz. different media, sodium chloride concentration, carbon source, inorganic nitrogen source, organic nitrogen source, initial pH of the media, inoculum concentration, incubation temperature, surfactant concentration, incubation period and agitation.

4.3.1 Optimization of different media for bacteriocin production

The effect of eight different media on bacteriocin production by BTFK101 and BTHT8 were studied and the results depicted in fig. 4.8 & 4.9 respectively. Zobell marine broth was observed to be the best media for bacteriocin production by both the strains. No bacteriocin production was attained when lactose broth was used for BTFK101. Lactose broth and Mueller – Hinton broth did not promote bacteriocin production in the case of BTHT8. The media composition of minimal media can be altered as it is a defined media and also in both the strains bacteriocin production was observed when minimal media was used. Hence minimal medium was used in the study of optimization of bioprocess variables for bacteriocin production by BTFK101 and BTHT8.

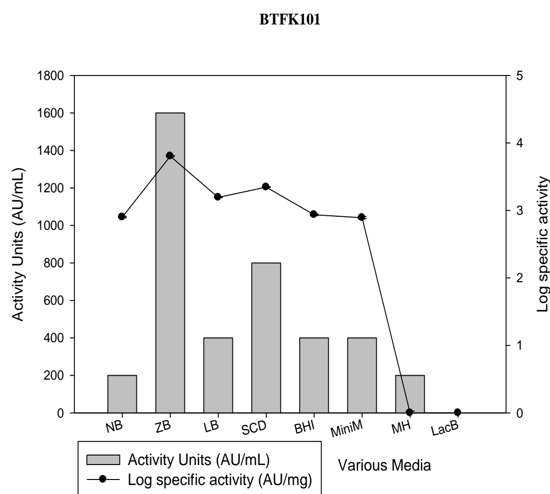

Fig: 4.8 Optimization of different media for bacteriocin production by *B. subtilis* strain BTFK101; NB- Nutrient broth, ZB- Zobell Marine broth, LB- Luria Bertani broth, SCD- Soyabean casein digest medium, BH- Brain heart infusion broth, Mini M- Minimal media, MH- Mueller Hinton broth, LacB- Lactose broth

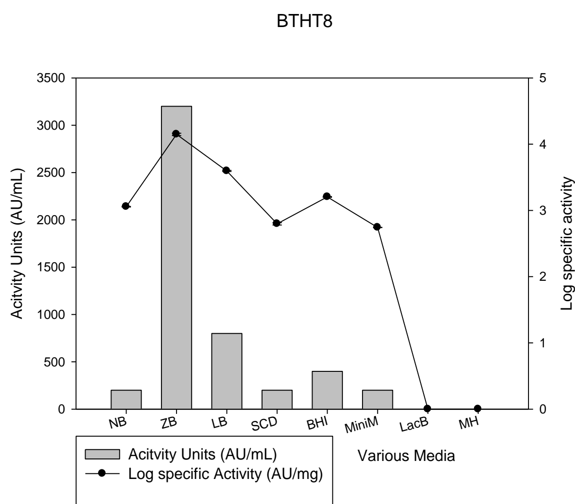

Fig: 4.9 Optimization of different media for bacteriocin production by *B. licheniformis* strain BTHT8; NB- Nutrient broth, ZB- Zobell Marine broth, LB- Luria Bertani broth, SCD- Soyabean casein digest medium, BH- Brain heart infusion broth, Mini M- Minimal media, MH- Mueller Hinton broth, LacB- Lactose broth

4.3.2 Optimization of sodium chloride concentration (NaCl) for bacteriocin production

In the case of BTFK101 (Fig. 4.10), maximum antibacterial activity (400AU/mL) was obtained when sodium chloride was supplied at a range of 0.5 - 2%. Specific activity was a little higher (log_{10} 2.87 ± 0.005 AU/mg protein) when 1.5% NaCl was provided. So this concentration of sodium chloride was considered optimum for bacteriocin production by BTFK101. As the concentration of sodium chloride increased, the production of bacteriocin decreased.

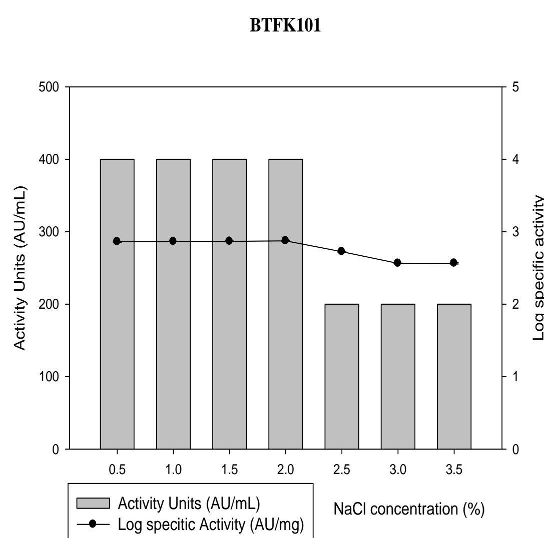

Fig: 4.10 Optimization of sodium chloride concentration for bacteriocin production by *B. subtilis* strain BTFK101

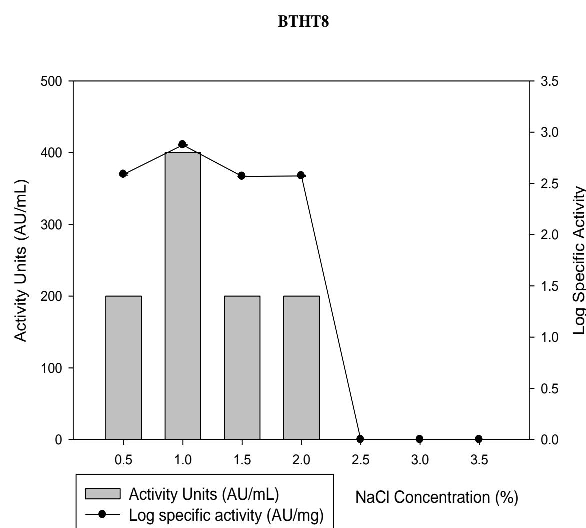

Fig: 4.11 Optimization of sodium chloride concentration for bacteriocin production by *B. licheniformis* strain BTHT8

In the case of strain BTHT8 (Fig. 4.11), bacteriocin production reached its peak (400AU/mL) with 1% NaCl concentration in the medium. Further increase in NaCl concentration reduced bacteriocin production, which stopped completely when NaCl was supplied at 2.5% or above.

4.3.3 Optimization of carbon sources for bacteriocin production

The results of optimization of different carbon sources for bacteriocin production by strains BTFK101 and BTHT8 are depicted in fig. 4.12 and fig. 4.13 respectively. For BTFK101 (Fig. 4.12) maximum bacteriocin production of 800AU/mL was obtained when 1% pectin was supplied as the carbon source. Glucose, mannitol, sucrose and starch also supported bacteriocin production when used as sole carbon source but the production was only 400AU/mL. No production was observed when lactose was used as the sole carbon source.

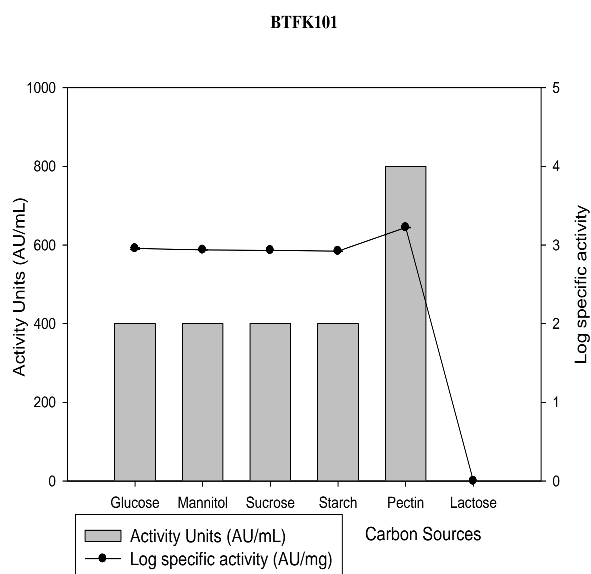

Fig: 4.12 Optimization of carbon sources for bacteriocin production by *B. subtilis* strain BTFK101

In the case of strain BTHT8 (Fig. 4.13), maximum specific activity ($\log_{10}$ 2.94 ± 0.001 AU/mg protein) was obtained with glucose as sole source of carbon although sucrose, starch and pectin also gave same rate of bacteriocin production (400AU/mL). Therefore glucose can be considered as the best carbon source. There was no bacteriocin production when lactose and mannitol were incorporated as carbon source.

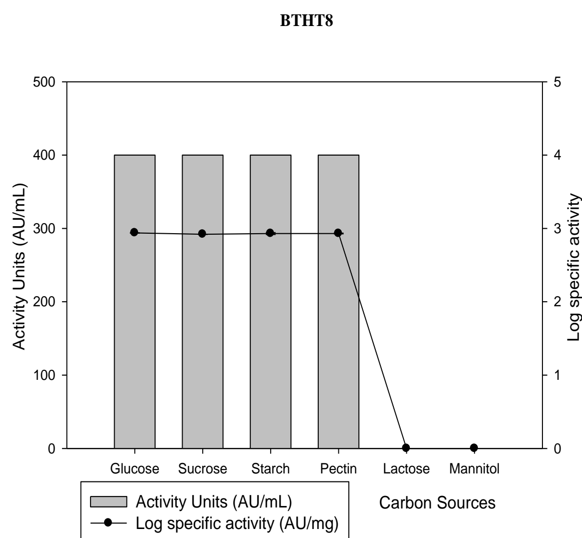

Fig: 4.13 Optimization of carbon sources for bacteriocin production by *B. licheniformis* strain BTHT8

4.3.4 Optimization of inorganic nitrogen sources for bacteriocin production

Five different inorganic nitrogen sources were incorporated in the media, for optimizing the inorganic nitrogen source for bacteriocin production by the strains. In the case of BTFK101 (Fig. 4.14), maximum production of 800AU/mL was obtained when ammonium sulphate was used as inorganic nitrogen source. When ammonium nitrate, ammonium chloride, ammonium phosphate and urea were used, bacteriocin production of 400AU/mL was obtained. Specific activity was highest when ammonium chloride ($\log_{10}$ 4.2 ± 0.03 AU/mg protein) was incorporated. When activity is taken into account, ammonium sulphate is the best inorganic nitrogen source for bacteriocin production.

BTFK101

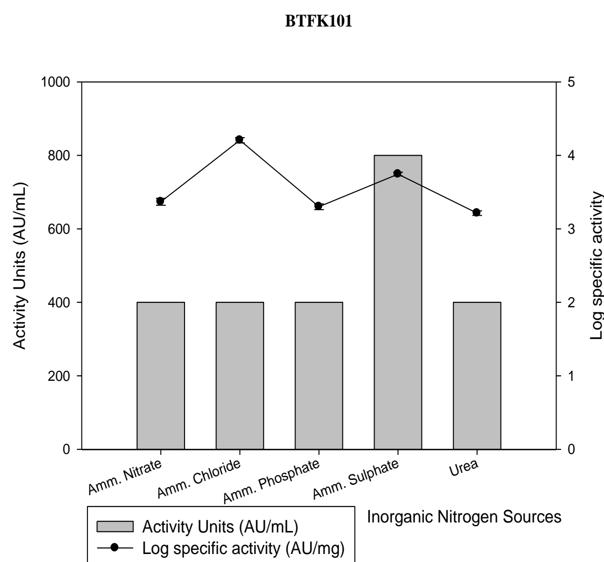

Fig: 4.14 Optimization of inorganic nitrogen sources for bacteriocin production by *B. subtilis* strain BTFK101

In the case of strain BTHT8 (Fig. 4.15), ammonium sulphate was observed to be the best inorganic nitrogen source for bacteriocin production as it helped to obtain highest activity of 1600AU/mL. The specific activity was also highest for ammonium sulphate with $\log_{10}$ 3.61 ± 0.001 AU/mg protein. Production was lowest when urea was supplied as the nitrogen source.

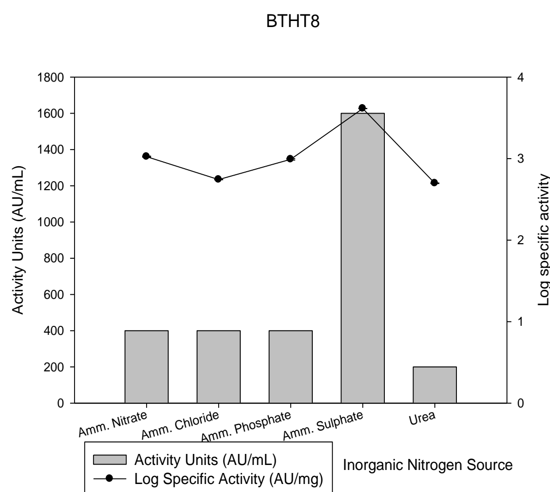

Fig: 4.15 Optimization of inorganic nitrogen sources for bacteriocin production by *B. licheniformis strain* BTHT8

4.3.5 Optimization of organic nitrogen sources for bacteriocin production

Malt, beef extract, yeast extract, tryptone and peptone were used to optimize the organic nitrogen source for bacteriocin production. For strain BTFK101 (Fig. 4.16), maximum bacteriocin production of 1600AU/mL was obtained when beef extract and yeast extract were incorporated as the organic nitrogen source. But specific activity was highest when beef extract ($\log_{10}$ 3.66 ± 0.006 AU/mg protein) was used and hence can be considered as the best organic nitrogen source. Lowest production was seen when malt was used (400AU/mL).

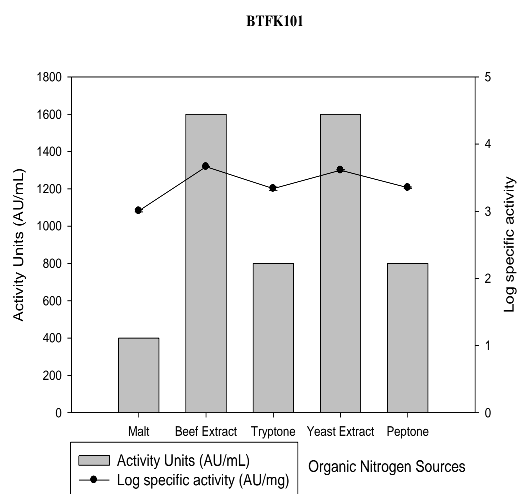

Fig: 4.16 Optimization of organic nitrogen sources for bacteriocin production by *B. subtilis* strain BTFK101

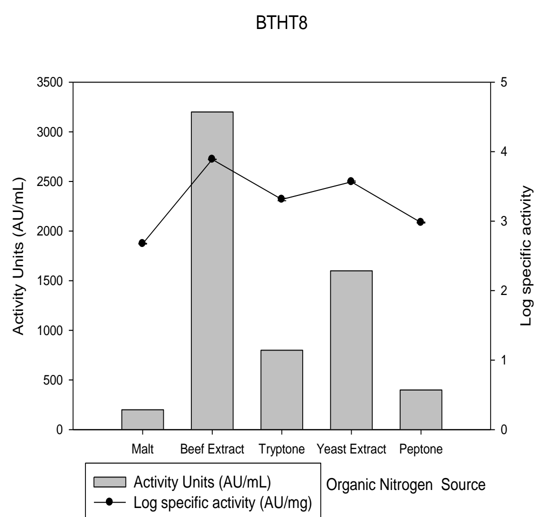

Fig: 4.17 Optimization of organic nitrogen sources for bacteriocin production by *B. licheniformis* strain BTHT8

For strain BTHT8 (Fig.4.17), production peaked when beef extract was used as the organic nitrogen source. Activity was estimated to be 3200AU/mL. Specific activity was also highest when beef extract was used (log_{10} 3.88 ± 0.003 AU/mg protein). Lowest production was observed with malt (200AU/mL). Thus beef extract could be the best organic nitrogen source for bacteriocin production

4.3.6 Optimization of inoculum concentration for bacteriocin production

The concentration of inoculum required for maximum bacteriocin production was studied and the results are depicted in fig. 4.18 & 4.19.

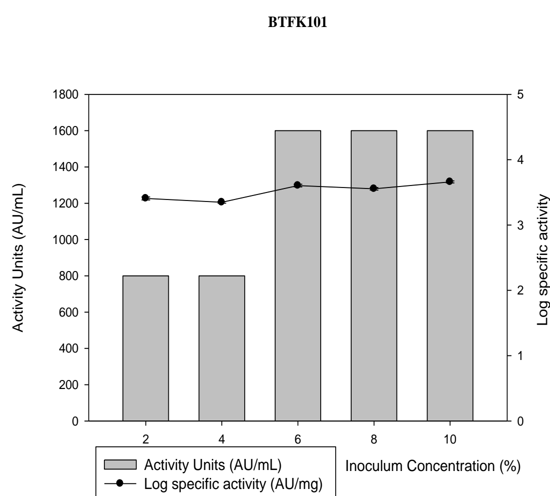

Fig: 4.18 Optimization of inoculum concentration for bacteriocin production by *B. subtilis* strain BTFK101

In the case of BTFK101 (Fig. 4.18), maximum production was obtained when concentration of inoculum in the range of 6- 10% was used. However, specific activity was highest (log_{10} 3.66 ± 0.01 AU/mg protein) when the media was inoculated with 10% inoculum. Therefore for maximum bacteriocin production by BTFK101, optimum inoculum concentration was determined as

10%. When concentration of inoculum used was below 5%, the production reduced.

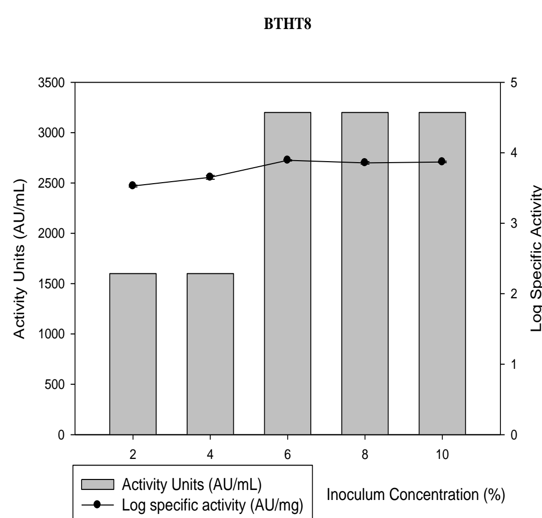

Fig: 4.19 Optimization of inoculum concentration for bacteriocin production by *B. licheniformis* strain BTHT8

For BTHT8 also, concentration of inoculum required for maximum production was in the range of 6-10% (Fig. 4. 19). However the specific activity was higher ($\log_{10}$ 3.89 ± 0.009 AU/mg protein) when inoculum at a concentration of 6% was used. Therefore 6% inoculum can be considered as the optimum for maximum output by BTHT8. In the case of BTHT8 also, production reduced when concentration of inoculum used was very low.

4.3.7 Optimization of tween 80 (surfactant) concentration / its role in bacteriocin production

The concentration of tween 80/ its role in bacteriocin production was studied. In the case of strain BTFK101 (Fig. 4.20), maximum bacteriocin production was observed in the absence of tween 80. It was observed that as

concentration of tween 80 added in the media increased, production reduced. Although the production remained at its maximal level at low concentrations of tween 80, the specific activity was found to be reduced. This indicated that incorporation of the surfactant had a negative impact on the production.

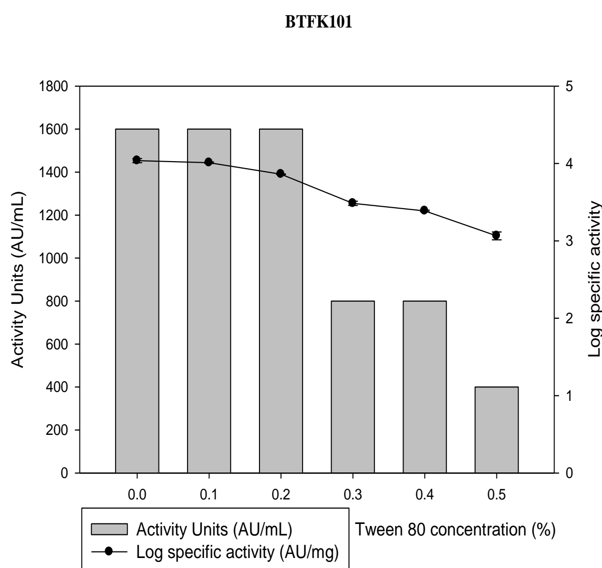

Fig: 4. 20 Optimization of tween 80 concentation for bacteriocin production by *B. subtilis* strain BTFK101

But for BTHT8 (Fig. 4.21), the surfactant had a positive impact on the bacteriocin production. Addition of 0.1% tween 80 in the media doubled the production when compared to the one in which no surfactant was added. But when the surfactant concentration was raised to 0.5%, the production decreased.

BTHT8

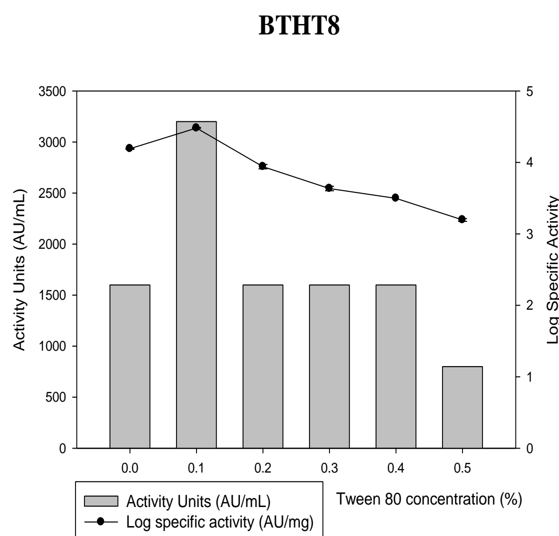

Fig: 4.21 Optimization of tween 80 concentation for bacteriocin production by *Bacillus licheniformis strain* BTHT8

4.3.8 Optimization of initial pH of the media for bacteriocin production

Initial pH of the media had a significant role in bacteriocin production by both the strains.

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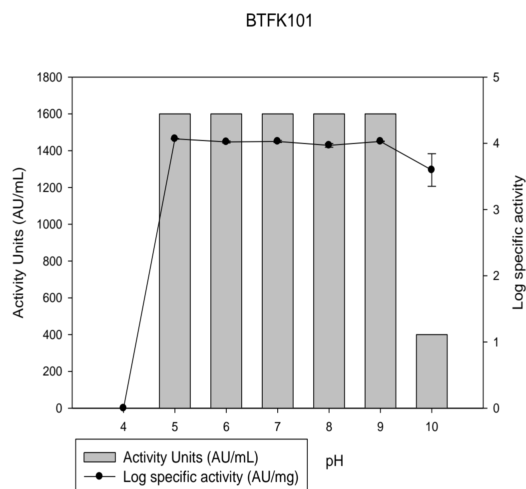


Fig: 4.22 Optimization of initial pH of the media for bacteriocin production by *B. subtilis* strain BTFK101

pH 5-9 was found to be the optimum pH range for bacteriocin production by strain BTFK101(Fig. 4.22). However since specific activity was highest ($\log_{10}$ 4.06 ± 0.005 AU/mg protein) with pH 5, it was taken as the optimum pH for the bacteriocin production by BTFK101. At pH 4 there was no production, while production decreased at pH 10.

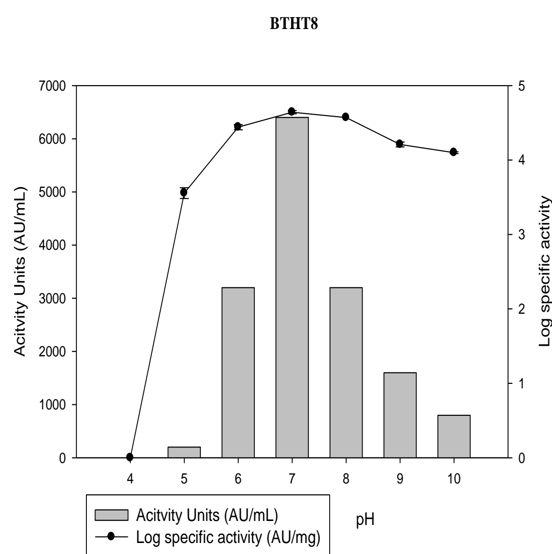


Fig: 4.23 Optimization of initial pH of the media for bacteriocin production by *B. licheniformis* strain BTHT8

In the case of strain BTHT8 (Fig. 4.23), maximum production was obtained when initial pH of the media was adjusted to 7. Production decreased when the pH was raised or lowered from 7.

4.3.9 Optimization of incubation temperature for bacteriocin production

The optimum incubation temperature for bacteriocin production was studied by taking into account the temperature range 20 - 60°C. The optimum incubation temperature for bacteriocin production by strain BTFK101 was determined to be 30°C (Fig. 4.24). Production decreased as incubation temperature was increased. Specific activity was also highest at 30°C. In the case of BTHT8 also (Fig. 4.25), 30°C was found to be the optimum temperature for maximum bacteriocin production.

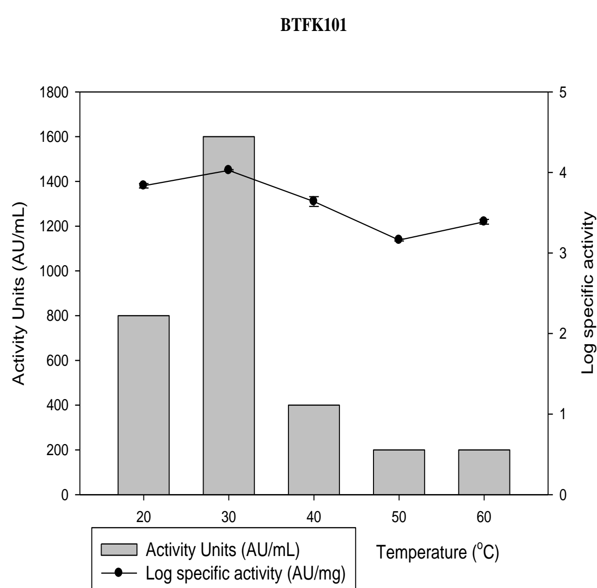


Fig: 4.24 Optimization of incubation temperature for bacteriocin production by *B. subtilis* strain BTFK101

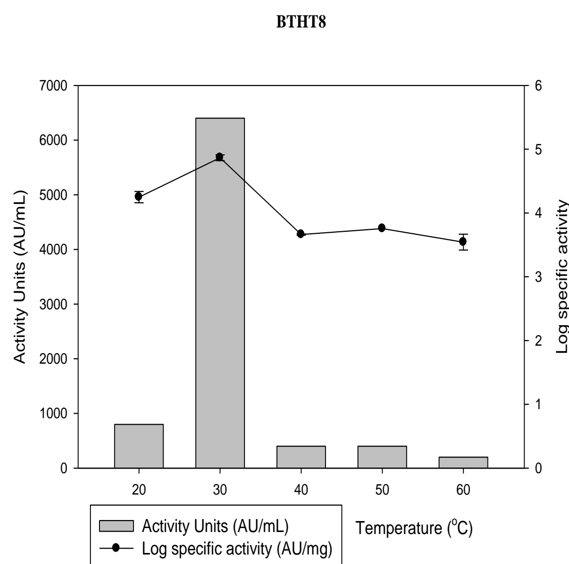


Fig: 4.25 Optimization of incubation temperature for bacteriocin production by *B. licheniformis strain* BTHT8

4.3.10 Optimization of incubation period for bacteriocin production

The incubation period required for maximum bacteriocin production was studied by incubating the cultures upto 60 h and sampling at 6 h interval. Although the bacteriocin production by strain BTFK101 started when the culture was incubated for 6 h (Fig. 4.26), maximum production (3200AU/mL) was observed at about 12 h. There was no further increase in production when the incubation period was prolonged. It was noted that the production decreased with increase in incubation period. No bacteriocin production was noted after 30 h.

In the case of BTHT8 (Fig. 4.27) also, production started at 6 h of incubation but it took 18 h to reach its maximum. The production decreased and remained in a stationary mode after 30 h.

BTFK101

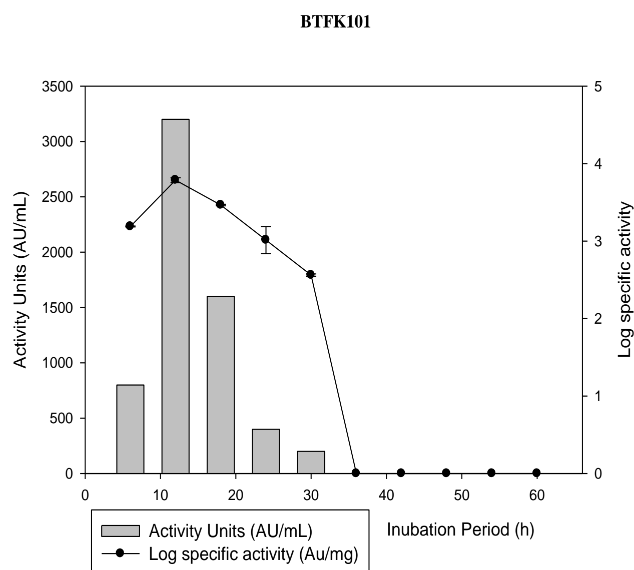


Fig: 4.26 Optimization of incubation period for bacteriocin production by *B. subtilis* strain BTFK101

BTHT8

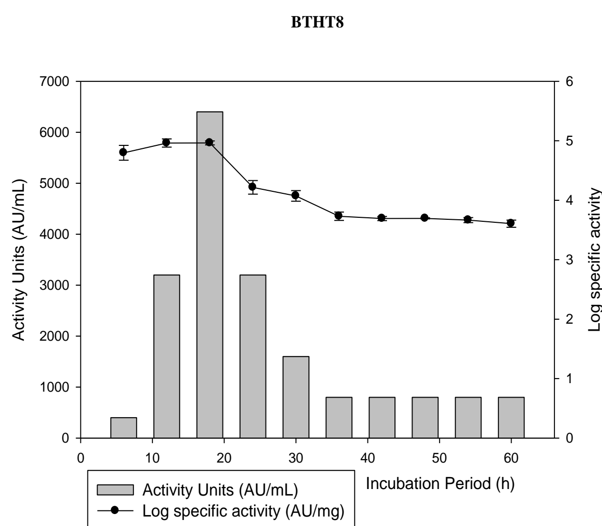


Fig: 4.27 Optimization of incubation period for bacteriocin production by

B. licheniformis strain BTHT8

4.3.11 Optimization of agitation for bacteriocin production

Agitation plays a major role in bacteriocin production as it allows aeration during production. In strain BTFK101 (Fig. 4.28), maximum production of 3200AU/mL was obtained when the culture was incubated with an agitation of 125 rpm. The bacteriocin production was very low (400AU/mL) when the culture was kept stationary.

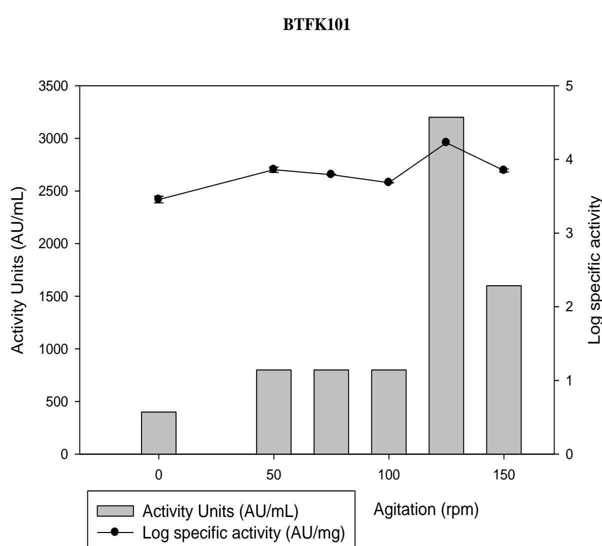


Fig: 4.28 Optimization of agitation speed for bacteriocin production by *B. subtilis* strain BTFK101

In the case of strain BTHT8 (Fig. 4.29) maximum bacteriocin production of 6400AU/mL was attained when the culture was incubated with an agitation of 100 rpm. Production was very low (only 200AU/mL) when the culture was incubated without shaking.

BTHT8

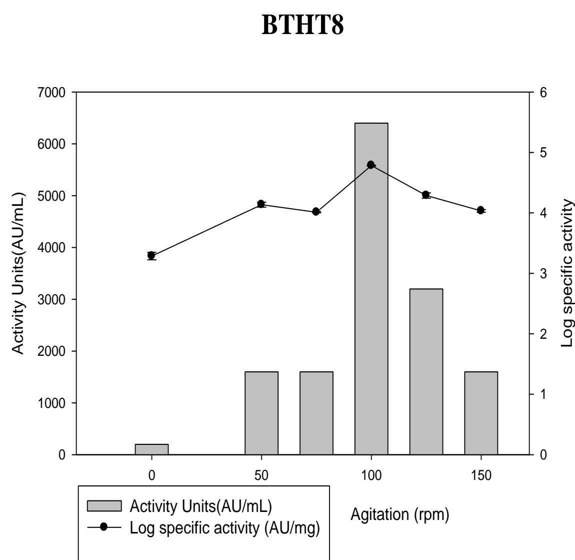


Fig: 4.29 Optimization of agitation speed for bacteriocin production by *B. licheniformis strain* BTHT8

The bioprocess variables for bacteriocin production were optimised by one factor-at-a-time method. Eleven factors were taken into account. Optimization of various media revealed Zobell marine broth as the best medium. So this media was used for production and purification. For optimization of various bioprocess variables minimal media was used. Production was found to be best when sodium chloride was supplied at a concentration of 1.5% and 1% in the media for BTFK101 and BTHT8 respectively. Pectin was the best carbon source for BTFK101 whereas it was glucose for BTHT8. Ammonium sulphate was the best inorganic nitrogen source for both the strains whereas beef extract proved to be the best organic nitrogen source. 10% inoculum (OD_{600}=1) was required for BTFK101 whereas only 6% inoculum was required for BTHT8 respectively to get maximum production. Tween 80 (surfactant) reduced the bacteriocin production by BTFK101 whereas addition of 0.1% tween 80 in the media promoted production of bacteriocin by BTHT8. Studies on initial pH of the media used for production

helped to understand that for BTFK101, the initial pH of the media must be 5 and for BTHT8 it must be 7 in order to get maximum production. Best incubation temperature was determined as 30°C for both the strains. Production reached its peak at about 12 h for BTFK101 but it took about 18 h for BTHT8 to get maximum production. The production was very low when the culture was kept stationary but when agitation at a speed of 125 rpm and 100 rpm was provided maximum bacteriocin yield was obtained from BTFK101 and BTHT8 respectively.

4.4 PURIFICATION OF THE BACTERIOCINS

The bacteriocin from *B. subtilis* strain BTFK101 was designated as BS101 and that from *B. licheniformis strain* BTHT8 was designated as BL8. The crude culture supernatant was concentrated and fractionated using ammonium sulphate precipitation. Only the 30- 60% fraction of ammonium sulphate precipitation showed antimicrobial activity. Therefore only this fraction was collected further. There was much increase in activity after this concentration step. This process was followed by gel filtration chromatography using sephadex G-25 column. The specific activity increased gradually after each purification process. The details of purification involved in BS101 and BL8 are depicted in table 4.4 and table 4.5 respectively.

Table 4.4 Fold of purification of bacteriocin BS101 at each step of purification

Purification step	Volume (mL)	Protein conc. mg/mL	Activity AU/mL	Specific activity AU/mg	Fold of purification
Crude	1000	0. 251	1600	6374.50	1*
Ammonium sulphate	11	1. 7	51200	30117.65	4.723
Gel filtration	1.5	0. 566	25600	45176.47	7.087

*Values taken arbitrarily

Table 4.5 Fold of purification for bacteriocin BL8 at each step of purification

Purification step	Volume (mL)	Protein conc. mg/mL	Activity AU/mL	Specific activity AU/mg	Fold of purification
Crude	1000	0. 227	3200	14096.90	1*
Ammonium sulphate	11	2. 38	205600	86386.55	6.128
Gel filtration	1.5	0. 25	102800	411200.00	29.169

*Values taken arbitarily

4.5 CHARACTERIZATION OF THE BACTERIOCINS

4.5.1 SDS- PAGE

Glycine SDS- PAGE and tricine SDS-PAGE were performed to check purity of the sample after each stage of purification. When ammonium sulphate fraction (30-60%) of the BS101 and BL8 were loaded onto tricine SDS-PAGE, a large number of bands were observed (Fig. 4.30). The protein band inhibiting the growth of test organisms was determined using the method of overlaying the gel with test organisms (zymogram). This is shown in fig. 4.31. From this, it was confirmed that the bacteriocins produced by the two strains are low molecular weight peptides, as they gave clearing zone near the dye front which is formed by inhibition of test organisms. After purification of the bacteriocins by gel filtration, single bands were obtained near the dye front which is depicted in the fig. 4.32. This infers complete purification of the bacteriocins which was in line with 2.4 kDa band (lowermost band) of NEB protein marker.

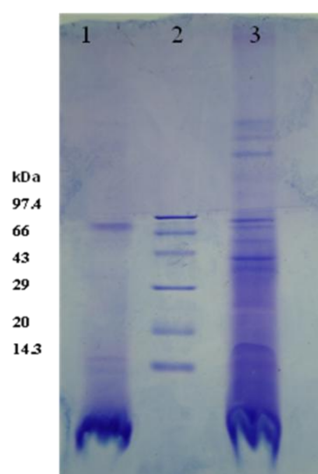


Fig: 4.30 Tricine SDS-PAGE of bacteriocins BS101 and BL8 partially purified by ammonium sulphate fractionation; Lane 1- BS101; Lane 2- GeNei Protein marker (Medium range); Lane 3- BL8

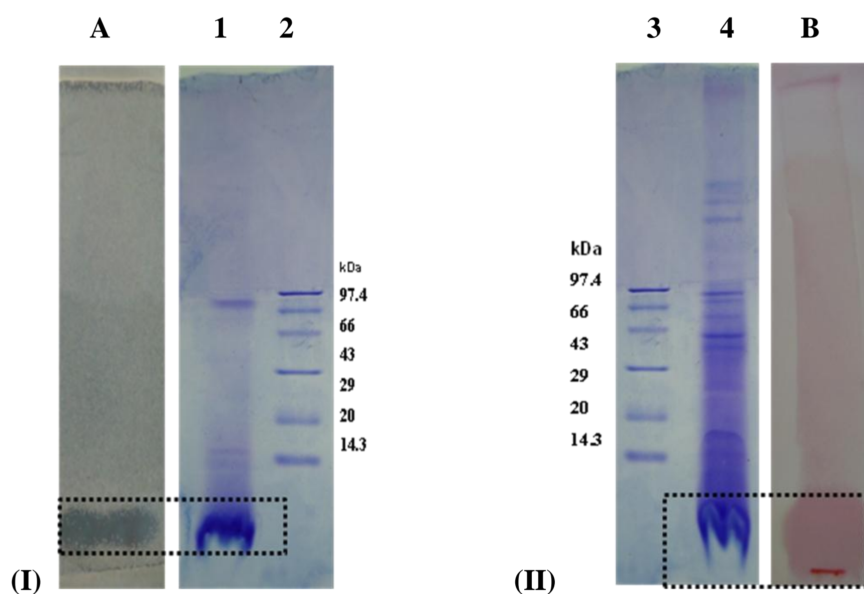


Fig: 4.31 Tricine SDS PAGE of (30-60%) ammonium sulphate fraction of the bacteriocins BS101 (I) and BL8 (II) and the overlay assay on gel to determine the protein band with antibacterial activity. Lane-1 BS101; lane-2 &3 GeNei protein marker; lane-4 BL8; lane A (BS101) & lane B (BL8) indicating clearing zone formed by growth inhibition of test organism (*S. aureus*) due to the corresponding bacteriocin bands

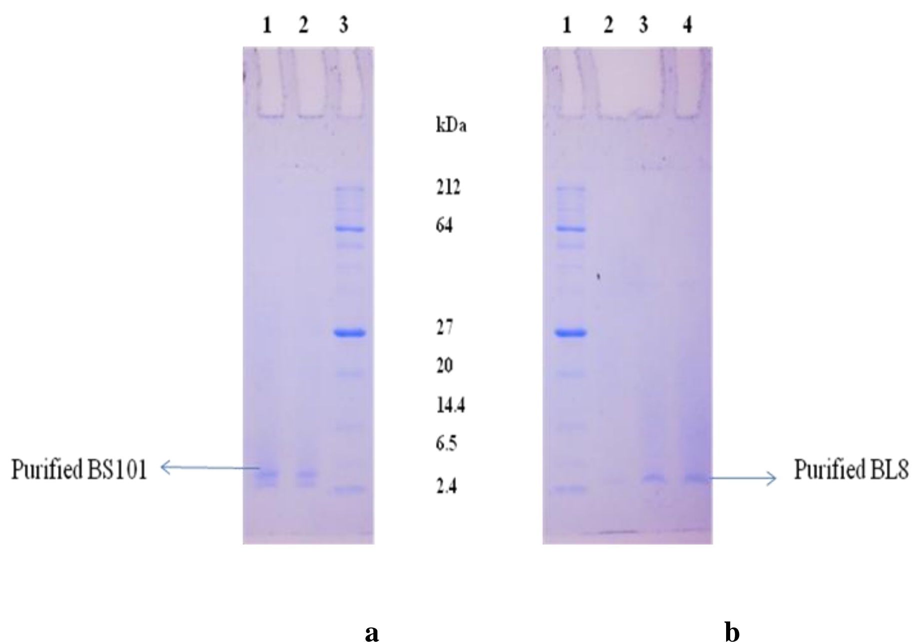


Fig: 4.32 Glycine SDS-PAGE of the bacteriocins after gel filtration
(a) BS101 Lane1 & 2 –Purified BS101; Lane 3- NEB protein marker
(b) BL8 Lane1- NEB protein marker; Lane 3&4 - Purified BL8

4.5.2 Intact mass determination by MALDI-TOF Mass spectrometry

The intact mass of the bacteriocins was determined by MALDI-TOF MS (Appendix 4). The mass of BS101 was determined as 3.3 kDa from the mass spectrum (Fig. 4.33). The mass of BL8 was determined as 1.4 kDa from the mass spectrum as depicted in fig. 4.34.

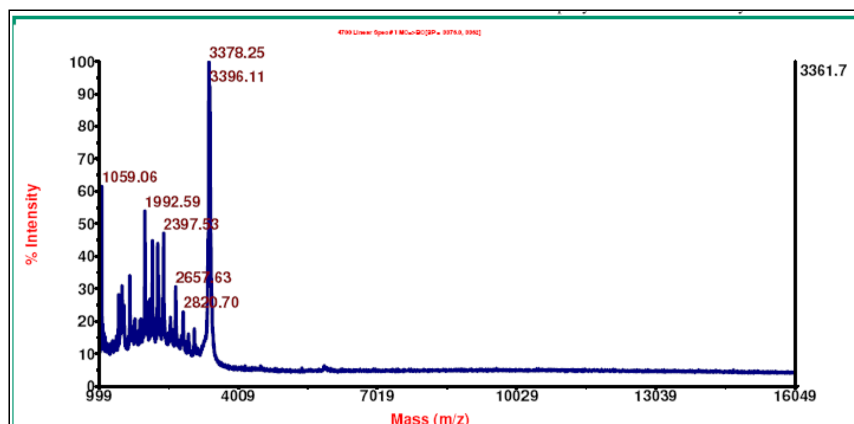


Fig: 4.33 Mass spectrum of bacteriocin BS101 obtained by MALDI-TOF MS

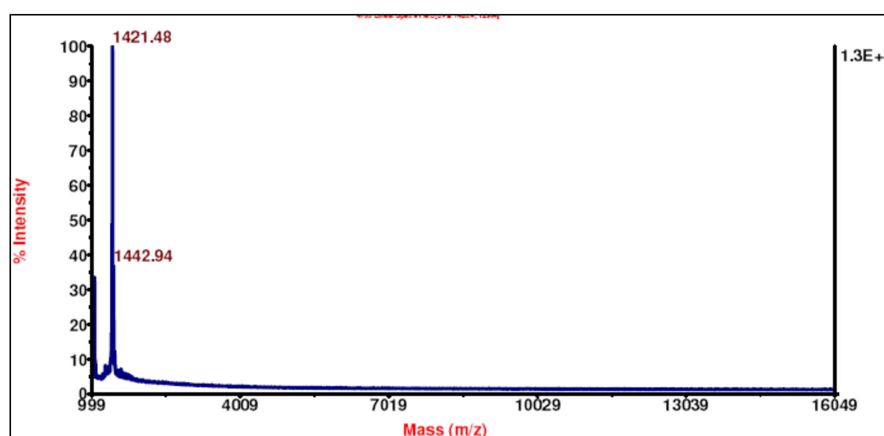


Fig: 4.34 Mass spectrum of bacteriocin BL8 obtained by MALDI-TOF MS

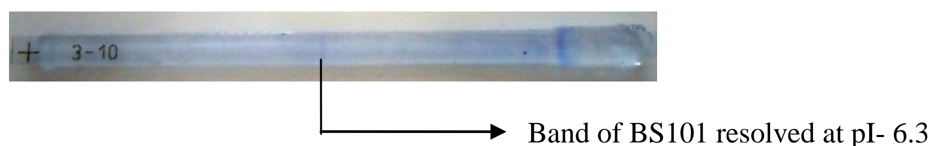
(Smitha & Sarita, 2013)

4.5.3 Isoelectric Focusing (IEF)

Isoelectric focusing was carried out in pre-casted IPG strips. After IEF, the strips were Coomassie stained. The bacteriocins were visualized as blue coloured bands on the strip. The 7cm IPG strip was equally divided into seven

points of pH. From the position of the bands, the isoelectric point (pI) was calculated. The pI of BS101was determined as 6.3 and that of BL8 was 5.7 (Fig. 4.35).

(a)



Band of BS101 resolved at pI- 6.3

(b)

Band of BL8 resolved at pI- 5.7

Fig: 4.35 IPG strips Coomassie stained after IEF; (a) IPG strip loaded with BS101 (b) IPG strip loaded with BL8

4.5.4 N-terminal aminoacid sequence analysis

The N-terminal amino acid sequence was determined by automated Edman degradation. N- terminal amino acid analysis of BL8 revealed a 13 amino acid sequence stretch (Appendix 4). In amino acid position 4, 5 and 8 Edman degradation gave blank signals which are observed when amino acid cysteine is present. So these three positions are assumed to contain cysteine and the sequence is as follows: NH_2-Ser-Trp-Ser-Cys-Cys-Gly-Asn-Cys-Ser-Ile-Ser-Gly-Ser-COOH. The sequence obtained was compared to the sequences of bacteriocins from *Bacillus* sp. retrieved from the protein database of NCBI by multiple sequence alignment using ClustalW. N- terminal amino acid sequences of BL8 showed no significant similarity with the sequences of bacteriocins of other

Bacillus sp. indicating novelty of the bacteriocin. The sequences also did not show similarity when compared with bacteriocins from other organisms by BLAST in protein database of NCBI. The multiple sequence alignment of the bacteriocins is depicted in Fig. 4.36.

Sequence	Alignment
YP006230889 Bacillus sp. JS	RMRTWKRIPKTTMLISLVSPFLLITPVLFYAALAFP
gbAEB23117 B.amyloliquefaciens	RMPTWKGIPKTTMLISTISPFLFITPVLFYAGLAFP
CAJ32354 B.cereus	GGGWWNSWGKCVA--GTIG------------GAGTG
AEP01267 B.coagulans	ASTLGISTAAAKKAIDIID--------------TAS
EAO54508 B.thuringiensis serovar israelensis	ALFVWMLFTTQKKNEQREE-----------------
ADP31478 B.atrophaeus	RLRQWKRIPKMTMLISLLSPFLLITPVLFYAGLAFP
gbAEK90981 B.amyloliquefaciens	GAGVWWMLSFTIS-----------------------
ZP 04069294 B.subtilis subsp. subtilis	AVGWGVAAASAIAAIVTAG-----------------
YP 004205572 B.thuringiensis	-----KGCATC-----SIG-----------------
B.licheniformis strain BTHT8	---SWSCCGNC-----SIS------------GS---

Fig: 4.36 Multiple sequence alignment of N-terminal aminoacid sequence of BL8 with the amino acid sequence of known bacteriocins of *Bacillus* sp. from protein data base of NCBI (Smitha & Sarita, 2013)

Despite several attempts to sequence the bacteriocin BS101, due to technical problems the N- terminal sequencing of the bacteriocin was not successful. However attempts are still under way to do so.

4.5.5 Effect of physico-chemical parameters on the stability of bacteriocins

4.5.5.1 Action of proteases on the bacteriocins

Action of three proteases viz. trypsin, pepsin and proteinase K on the stability of the bacteriocin was studied. The effect of proteases on bacteriocin BS101 is depicted in fig. 4.37. When BS101 was treated with trypsin at concentration ranging from 20-100 µg, the activity of the bacteriocin decreased and it was completely lost when the concentration of trypsin was elevated to 80 µg. When the bacteriocin was treated with pepsin in the same concentration range, the activity decreased, but complete loss of activity was not was observed upto 100 µg concentration of pepsin. The action of proteinase K showed a different pattern. Only about 40 µg of proteinase K was required to degrade BS101. The

activity was completely lost when the bacteriocin was treated with 40 µg proteinase K.

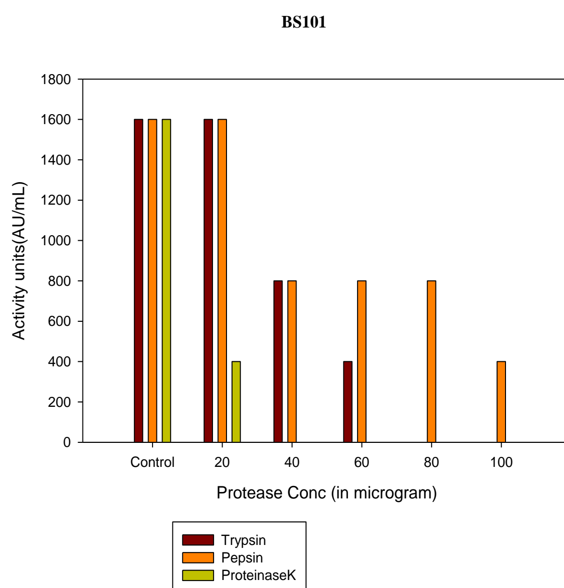


Fig: 4.37 Action of proteases like trypsin, pepsin and proteinase K on BS101

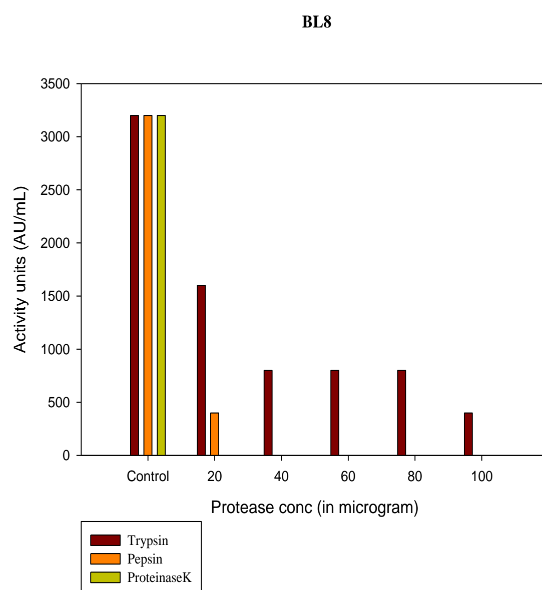


Fig: 4.38 Action of proteases like trypsin, pepsin and proteinase K on BL8

The effect of the proteases on BL8 is represented in fig. 4.38. Even very small concentrations of pepsin and proteinase K degraded BL8. The action of BL8 was completely lost when it was treated with proteinase K at concentration of 20 µg. Only 40 µg of pepsin was required to degrade BL8. The action of trypsin on BL8 was very different when compared to that of BS101. Trypsin did not completely inhibit the action of BL8 even at a concentration of 100 µg.

4.5.5.2 Effect of temperature on the stability of bacteriocins

The effect of temperature ranging from 20-100°C on bacteriocin stability was studied. Fig. 4.39 depicts the effect of temperature on the stability of BS101. It was noticed that the bacteriocin started degrading and the activity reduced when BS101 was kept at 40°C for 1 h. The antibacterial activity was completely lost when BS101 was incubated at a temperature of 50°C and above for 1 h.

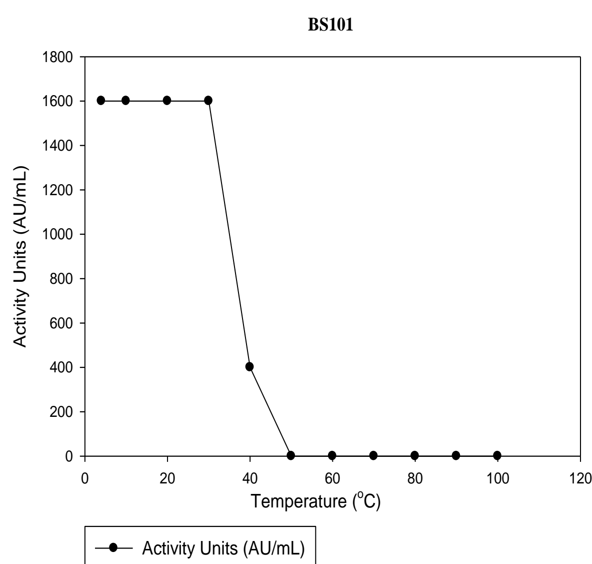


Fig: 4.39 Effect of temperature on the stability of BS101

The effect of temperature on the stability of BL8 is depicted in fig. 4.40. When compared to BS101, it was noticed that BL8 is more thermostable. The activity of BL8 was reduced only when it was incubated at 50°C for one hour. It was stable at 40°C. The activity was completely lost at 60°C and above.

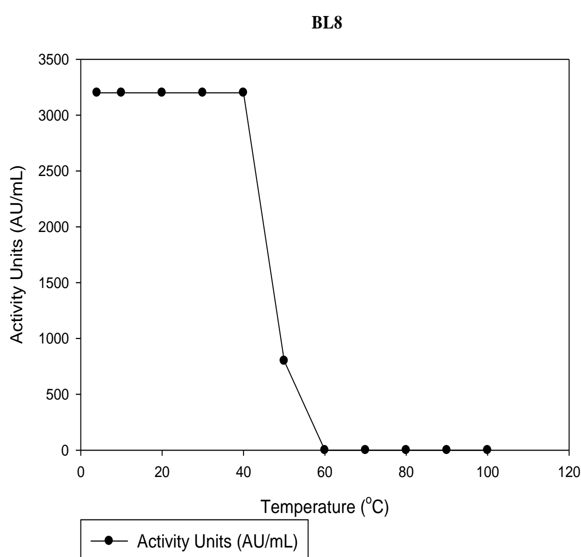


Fig: 4.40 Effect of temperature on the stability of BL8

4.5.5.3 Effect of pH on the stability of bacteriocins

In order to study the effect of pH on BS101 and BL8, the bacteriocins were treated with buffers having different pH in the range of 2-13. The effect of pH on the stability of bacteriocins is depicted in fig. 4.41 and 4.42. The bacteriocins were found to be very stable even at very low pH. But they were very unstable in alkaline pH range. For both BS101 and BL8, pH above 8 inhibited the antimicrobial action of the bacteriocins.

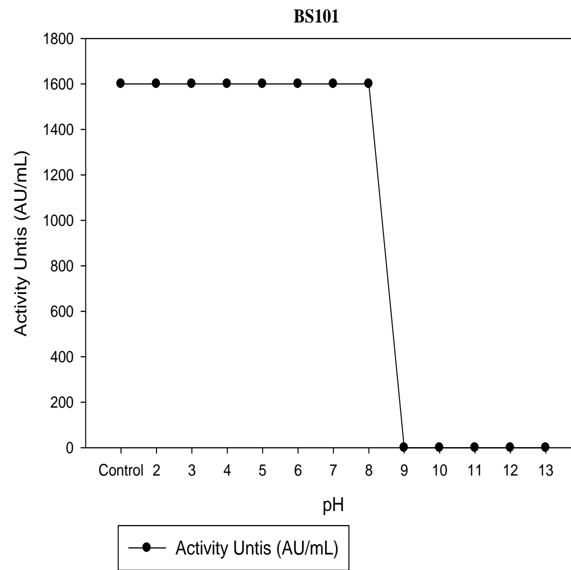


Fig: 4.41 Effect of pH on the stability of BS101

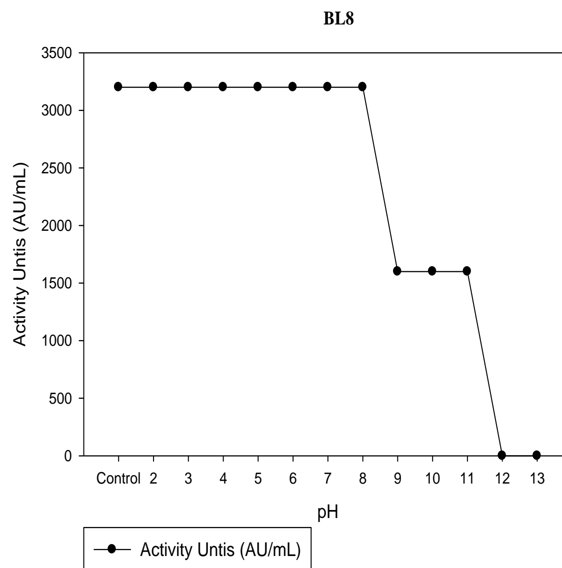


Fig: 4.42 Effect of pH on the stability of BL8

In the case of BS101, there was complete loss of activity when the bacteriocin was treated with buffers having pH 9 and above. For BL8, complete loss of activity occured only at pH 12. But we can see drastic reduction in activity from pH 9 onwards. The activity of both the bacteriocins was not affected in the pH range of 2-8.

4.5.5.4 Effect of detergents on the stability of bacteriocins

Stability of the two bacteriocins when treated with non-ionic detergents like tween 20, tween 80 and triton X 100 was tested and it was found that the bacteriocins were stable when exposed to these detergents or they did not reduce the activity of the bacteriocins (Fig. 4.43 and 4.44 respectively). But the ionic detergent SDS reduced the activity of both BS101 and BL8. CTAB had very prominent effect. It completely inactivated the two bacteriocins.

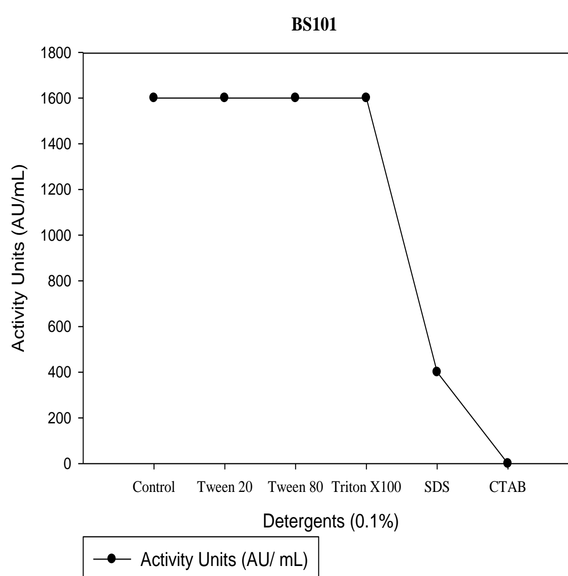


Fig: 4.43 Effect of detergents on the stability of BS101

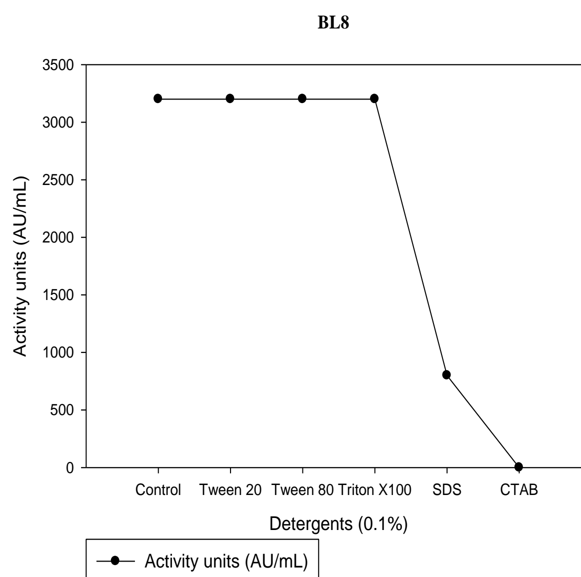


Fig: 4.44 Effect of detergents on the stability of BL8

4.5.5.5 Effect of metal ions on the activity of bacteriocins

The effect of twelve different metal ions on bacteriocin activity was studied. In the case of BS101 (Fig. 4.45) Mg, Al, Fe, Zn, Na, Cu, Ni, Ca, Mn and Co ions did not affect the activity of the bacteriocins. The activity remained the same when BS101was treated with 1mM concentration of these metal salts. The ions of Ba and Cd enhanced the activity of BS101.

For BL8 (Fig. 4.46) Mg, Al and Ba ions promoted the activity whereas Mn and Co ions reduced the activity. The ions of Cd, Fe, Zn, Na, Cu, Ni, and Ca did not affect the activity of the bacteriocin.

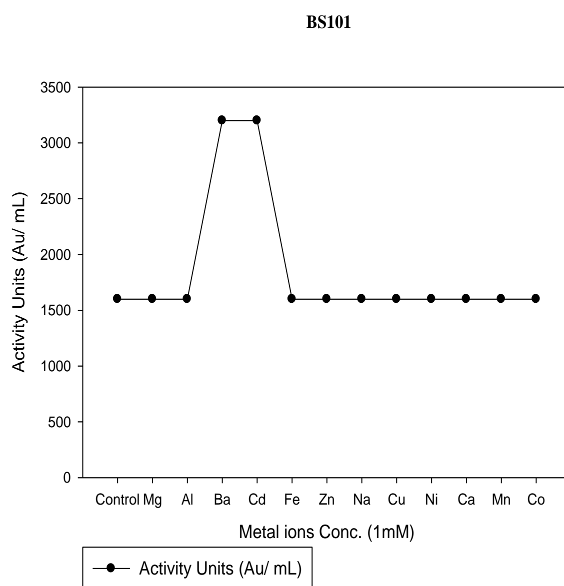


Fig: 4.45 Effect of metal ions on the stability of BS101

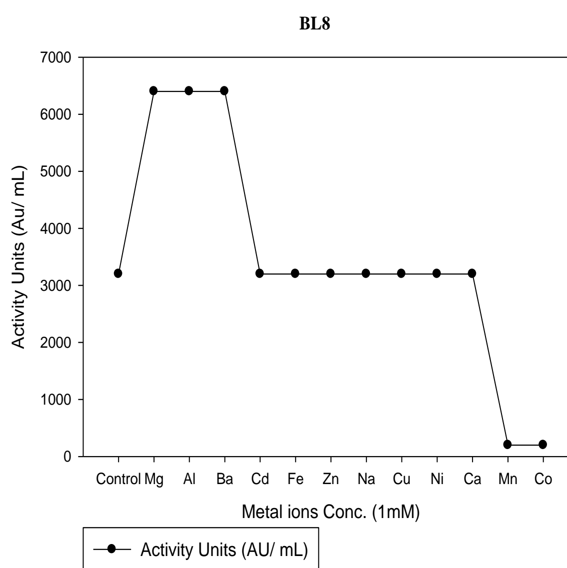


Fig: 4.46 Effect of metal ions on the stability of BL8

4.5.5.6 Effect of reducing agents on the stability of bacteriocins

The effect of two reducing agents, DTT and β- mercaptoethanol on the stability of the bacteriocins was tested. For BS101, the DTT at a concentration range of 1-10 mM was used and β- mercaptoethanol was used in the range of 10-100 mM concentration. BS101 remained stable when exposed upto 8 mM DTT. Above this concentration the activity reduced rapidly (Fig 4.47a). β - mercaptoethanol did not affect the activity of BS101 upto 60 mM concentration, but the activity reduced when exposed to concentration of 70 mM and above (Fig 4.47 b).

In the case of BL8, the concentration of DTT tested was in the range of 10-100 mM. β- mercaptoethanol was also taken at higher concentrations of 100-1000 mM (Fig. 4.48 a & b). The activity of BL8 was reduced to half when exposed to DTT at a concentration of 20 mM and above. The activity was not completely lost even when exposed to 100 mM concentration. Similarly, the effect of β- mercaptoethanol on the stability of BL8 was also studied and found that the activity reduced when the bacteriocin was treated with β- mercaptoethanol at a concentration of 200 mM and above. Complete activity loss was not observed even at 1000 mM concentration.

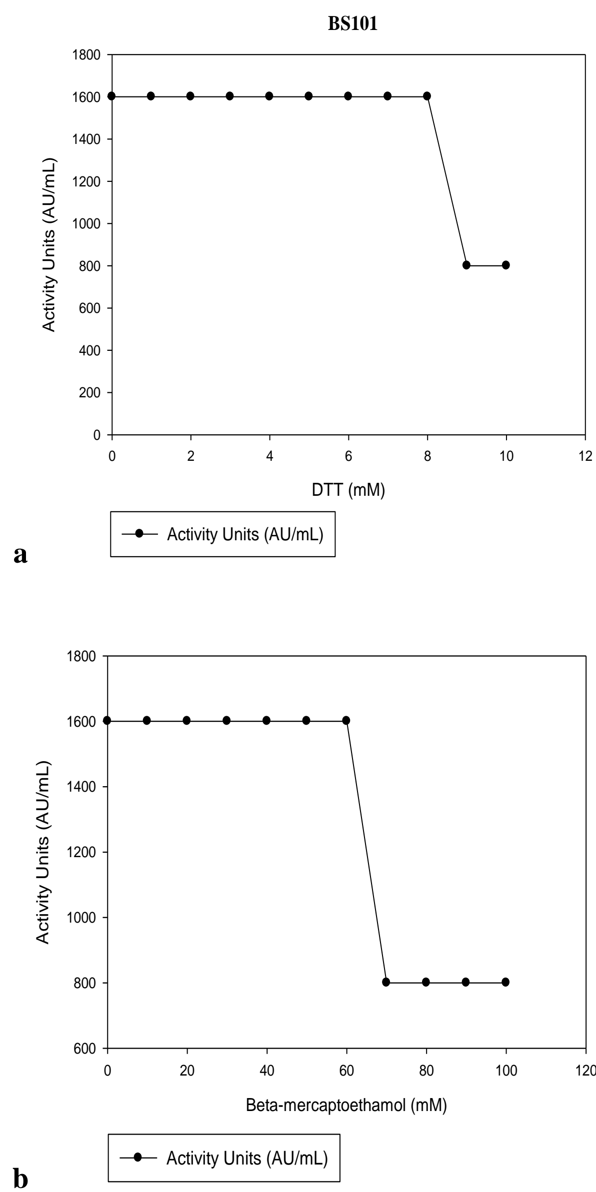
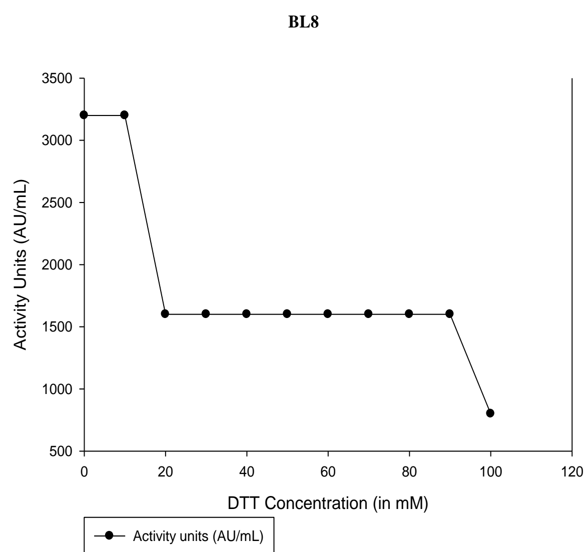
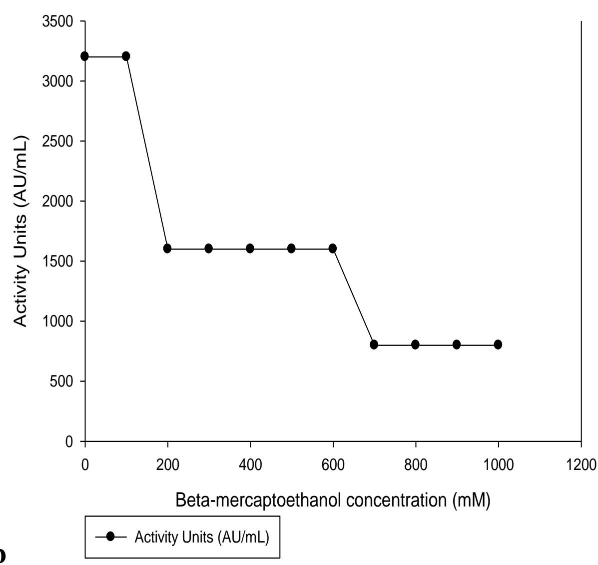


Fig: 4.47 Effect of reducing agents on the stability of BS101 (a) Effect of DTT (b) Effect of β -mercaptoethanol

BL8



a



b

Fig: 4.48 Effect of reducing agents on the stability of BL8 (a) Effect of DTT (b) Effect of β- mercaptoethanol

4.5.5.7 Effect of oxidising agent (DMSO) on the stability of bacteriocins

When the bacteriocins were treated with 1-5% DMSO (v/v), there was no noticeable effect on the activity. So the result is not represented by graphs.

Table 4.6 Summary of the effect of enzymes and physico-chemical parameters on the stability of bacteriocins

Parameters	BS101	BL8
Proteinase K	Complete inhibition (at 40µg conc)	Complete inhibition (at 20 µg conc.)
Pepsin	No complete inhibition	Complete inhibition (at 40µg conc.)
Trypsin	Complete inhibition (at 80µg conc.)	No complete inhibition
Temperature	Activity lost at 50°C	Activity lost at 60°C
pH	Unstable at basic pH	Unstable at basic pH
Detergents	Degraded by SDS and CTAB	Degraded by SDS and CTAB
Metal ions	Ba and Cd promoted activity. The metal ions did not reduce activity	Mg, Al, Ba promoted activity. Mn and Co reduced activity
DTT	Activity reduced when treated with 8 mM conc.	Activity reduced only when treated with 20 mm conc.
β-mercaptoethanol	Activity started reducing when treated with 70 mM conc.	Activity reduced only when treated with 200 mM conc.
DMSO	No effect	No effect

4.5.6 Minimum Inhibitory Concentration (MIC)

Minimum concentration of bacteriocins required to inhibit the growth of test organisms (*S. aureus*) was determined using microtitre plate assay incorporating resazurin. The microtitre well with highest dilution of the bacteriocins that inhibited the growth of test organisms remained blue. The well having bacteriocin concentration that does not inhibit the growth of test organisms turned pink. The protein concentration in the well was calculated by dividing the total protein content of purified bacteriocin in the first well with that of the dilution factor of the well (Fig. 4.49) and expressed in µg/mL. This value is considered as the MIC of the bacteriocin. Here, the total protein content of BS101 taken was 28.5 µg (50 µL purified BS101 having protein concentration of 570 µg/mL) and the dilution factor was found to be 4 and the MIC for BS101 was determined as 42.75 µg/mL. The total protein content of BL8 was 12.5 µg (50 µL purified BL8 having protein concentration of 250 µg/mL) and the dilution factor was found to be 4, the MIC was calculated as 18.75 µg/mL for BL8.

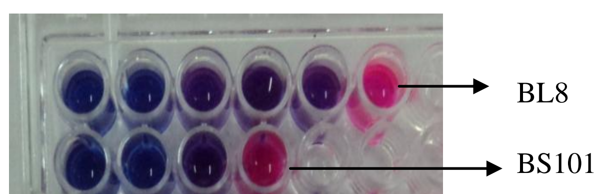


Fig: 4.49 Microtitre plate showing colour change of resazurin from blue to pink in the presence of living cells

4.5.7 Growth inhibition assay to test bacteriostatic/ bactericidal activity of the bacteriocins

From the experiment (Fig. 4.50) it was observed that the optical density (OD_{600}) of the four test organisms viz. *B. coagulans*, *B. circulans*, *B. cereus* and *S. aureus* did not increase drastically when the bacteriocins (at a concentration of

BS101: 34 µg/mL; BL8: 47.5 µg/mL) were added into the growth media at the time of their inoculation. This indicates that the growth of these test organisms was inhibited. But in the control, there was prominent growth and hike in OD_{600} after six hours of inoculation. If we take *B. pumilus* and *Cl. perfringens*, it was observed that the growth was controlled to a certain extent but was not completely inhibited. From the experiment it was concluded that both the bacteriocins are bacteriocidal to *B. coagulans*, *B. circulans*, *B. cereus* and *S. aureus* whereas they are bacteriostatic to *B. pumilus* and *Cl. perfringens.*

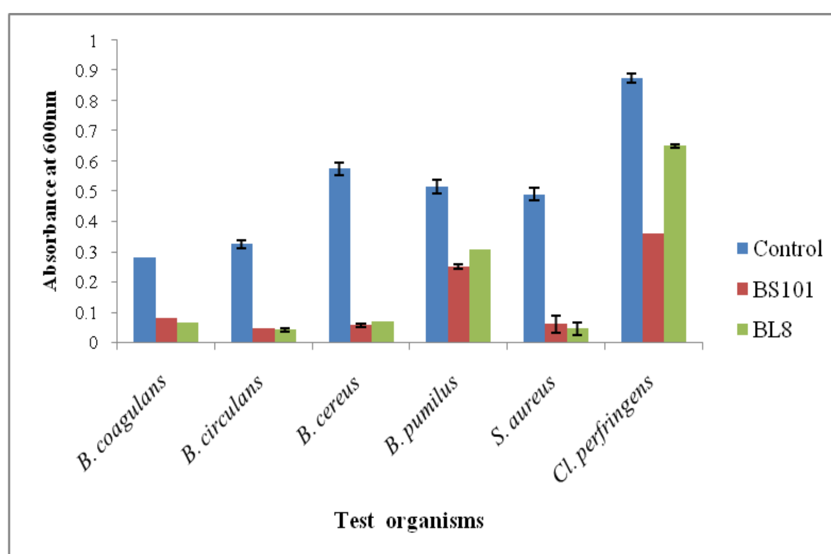


Fig: 4.50 Variation in OD_{600} when the test organisms were inoculated in media supplemented with BS101 and BL8

4.6 APPLICATION STUDIES

4.6.1 Bacteriocins for the control of biofilm formation

In vitro biofilm formation by the six test organisms under study was experimentally tested initially and the result is depicted in fig. 4.51. 2AC was calculated to be 0.024 ± 0.002 and 4AC was calculated as 0.048 ± 0.002. The

OD_{570} of *Cl. perfringens* was 0.0386 ± 0.002 and that of *B. cereus* was 0.045 ± 0.001 and hence categorised as moderate biofilm producers as OD_{570} was in between 2AC and 4AC. The other four test organisms were not very good biofilm formers. Next experiment was conducted to determine the ability of the bacteriocins, BS101 and BL8 to control biofilm formation by these two top most biofilm producers in this study. When the bacteriocins were added in the test, they inhibited the biofilm formation by *Cl. perfringens* and *B. cereus* (Fig. 4.52). When BS101 was applied the OD_{570} of *Cl. perfringens* and *B. cereus* was reduced to 0.009 and 0.008 ± 0.001 respectively. When BL8 was incorporated, the OD_{570} of *Cl. perfringens* and *B. cereus* was reduced to 0.013 ± 0.001 and 0.007 ± 0.001 respectively. Thus it was experimentally proved that the bacteriocins perform very well in the control of biofilm formation.

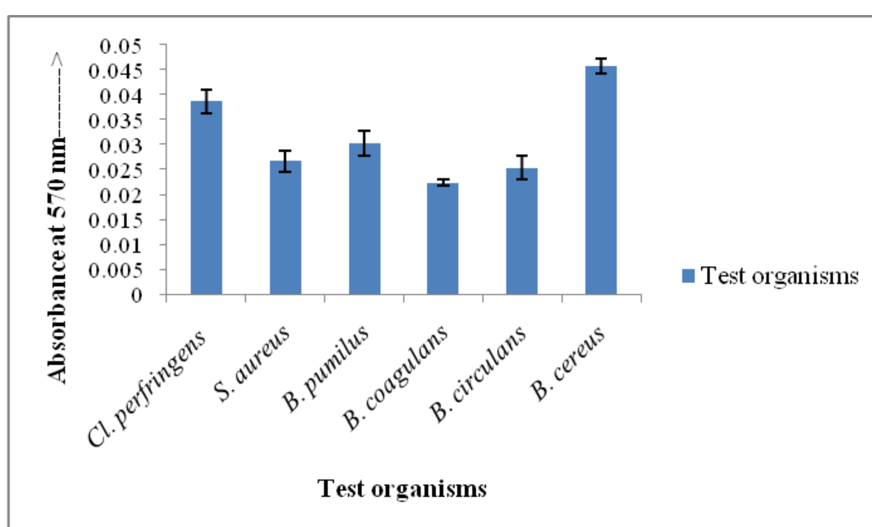


Fig: 4.51 *In vitro* biofilm formation by the six test organisms

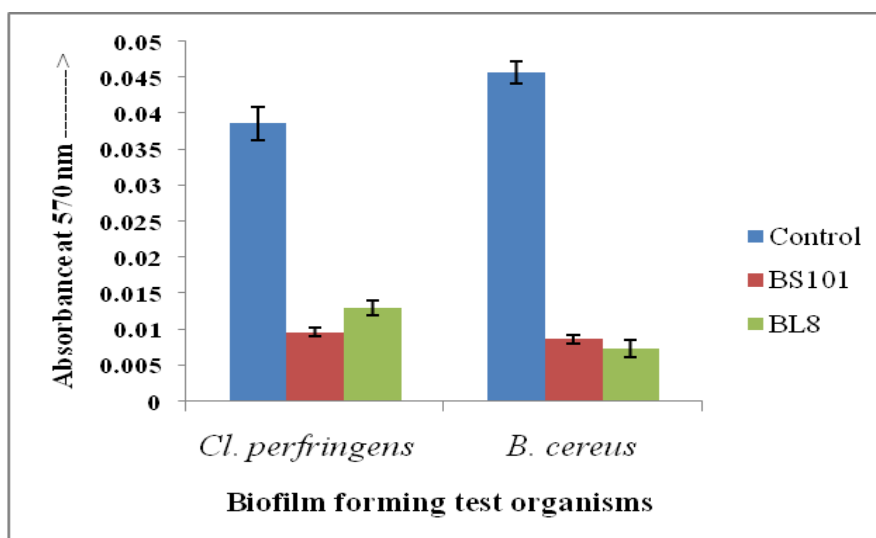


Fig: 4.52 Control of biofilm formation by the bacteriocins

4.6.2 Bacteriocins for the control of microflora of sea foods

BS101 and BL8 were also tested for their ability to control microflora in sea foods. Bacteriocins were found to be very efficient in controlling the microflora of sea foods like prawns and anchovies. The bacterial count in untreated anchovies was found to be 172 ± 12.02 x10^5 CFU/mL. The microbial count was reduced to 90 ± 4.24 x10^5 CFU/mL and 94 ± 3.53 x10^5 CFU/mL when the sample was treated with BS101 and BL8 respectively (Fig. 4.53). The bacterial count in untreated prawn was 842 ± 19.79 x10^5 CFU/mL. The microbial count was reduced to 176 ± 23.33 x10^5 CFU/mL and 111 ± 7.77 x10^5 CFU/mL when the sample was treated with BS101 and BL8 respectively. This drastic decrease in the microbial count of sea food samples treated with the bacteriocins indicates their ability to control the microflora of sea foods, supporting their application in biopreservation.

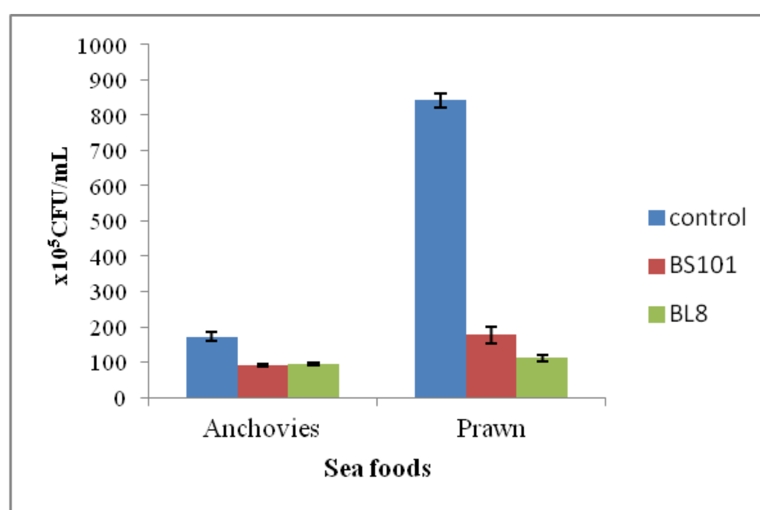


Fig: 4.53 Control of microflora of prawns and anchovies by the bacteriocins

4.6.3 Bioassay using model organism *Caenorhabditis elegans*: Pathogenesis and prophylaxis using the bacteriocins

The nematode *Caenorhabditis elegans* was used as a model system to study the pathogenesis of *S. aureus and B. circulans* and to confirm the ability of the bacteriocins BS101 and BL8 to confer protection against the bacterial infection. It was observed that the bacteriocin BL8 had a positive influence in increasing the life span of the infected worms. The figures (4.54 and 4.55) represent the percentage mortality of *C. elegans* over a span of 10 days when exposed to the pathogens *S. aureus and B. circulans* and the influence of the bacteriocins in maintaining the life span of worms to near normal levels when compared to the untreated (control) worms fed on *E. coli* OP50.

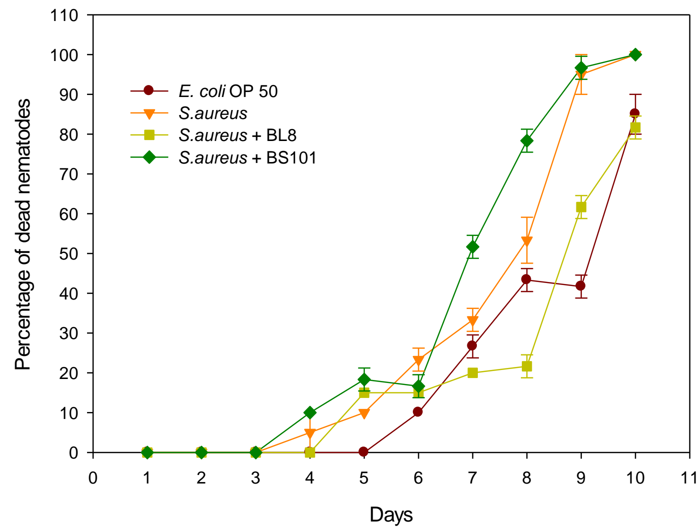


Fig: 4.54 Variation in TD_{50} of *C. elegans* infected with *S. aureus* in the presence and absence of bacteriocins

The time required for the death of 50% of the nematodes (TD_{50}) was calculated from the results obtained using the PRISM (version 5.04) computer program. TD_{50} was calculated in three independent experiments and values were represented in mean ± standard error. The time required for 50% of nematodes to die when fed on an *E. coli* OP50 lawn was 8.950 ± 0.236 days. TD_{50} of the nematodes when infected with the known pathogen *S. aureus* was 7.932 ± 0.173 days, while it was 7.932 ± 0. 113 days when the nematodes were fed with *B. circulans*, indicating the pathogenicity of these test organisms.

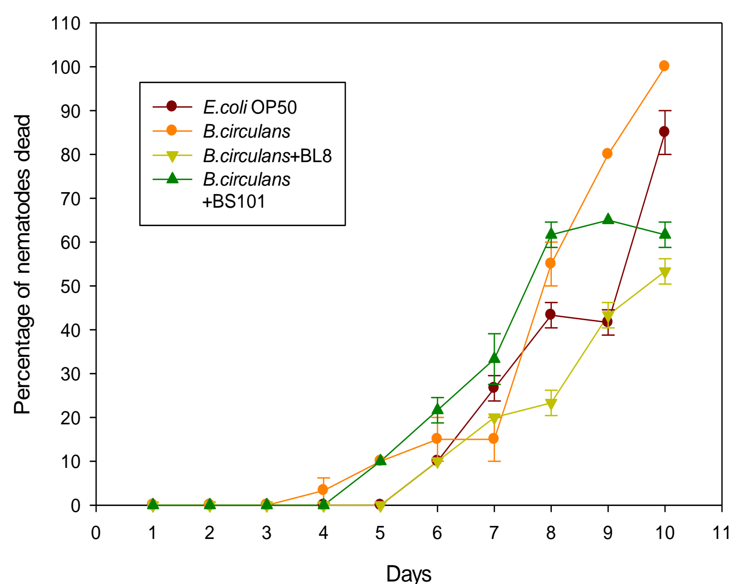


Fig: 4.55 Variation in TD_{50} of *C. elegans* infected with *B. circulans* in the presence and absence of bacteriocins

The outcome of the bacteriocin prophylaxis experiments was promising in the case of BL8. When the bacteriocin was applied, its ability to control the infection helped in reducing the mortality rate of infected worms. TD_{50} of worms infected with *B. circulans* followed by the treatment with BL8 was increased to 9.488 ± 0.231 days and TD_{50} of worms infected with *S. aureus* in presence of BL8 was calculated as 8.751 ± 0.169 days which denotes control of bacterial infection in the worms and thus increase in their life span.

The outcome of the bioassay was not so promising in the case of BS101. The TD_{50} of worms infected with *B. circulans* and *S. aureus* in the presence of BS101 was lower, illustrating its inability to reduce infection in live organisms. TD_{50} of worms infected with *B. circulans* in presence of BS101 was calculated as

7.69 ± 0.30 days and TD_{50} of worms infected with *S. aureus* in the presence of BL8 was calculated as 6.989 ± 0.13 days.

Thus two low molecular weight bacteriocins from *B. subtilis* strain BTFK101 and *B. licheniformis* strain BTHT8, isolated from the marine sediment were characterized in the present study. They were observed to exhibit an ability to participate in prophylaxis, biofilm control and also in sea food preservation.

5. DISCUSSION

Development and spread of antibiotic resistance in bacterial pathogens has been the driving force for scientists to seek alternatives. Several strains of human pathogens are known to contain upto 10 different genes coding for resistance (Henry, 2000). As bacteria continuously overcome the tools with which humans fight them, there is an urgent need to address the search for new products that affect the target. Great attention has been focused on bacteriophages, probiotics and antimicrobial peptides including bacteriocins (Asaduzzaman *et al*., 2009; Lin *et al*., 2009). Bacteriocins are ribosomally synthesized bacterial proteins or peptides with narrow or broad antimicrobial activity spectrum against other bacteria. They are produced by both Gram negative and Gram positive bacteria, and 99% of bacteria possess at least one bacteriocin in their repertoire for their microbial defense system (Klaenhammer, 1988).

Bacteriocins have a large degree of structural and biochemical diversity. Although a large numbers of bacteriocins have been identified and characterized, new bacteriocins are still being discovered and documented.

5.1 ISOLATION OF BACTERIOCIN PRODUCING BACTERIA FROM MARINE ENVIRONMENT

The present study was focused on the characterization of bacteriocins produced by bacteria isolated from the marine environment. With 70% of the earth's surface being covered by the ocean and representing 80% of life on earth, this enormous pool of microbial biodiversity is under utilized and therefore retains the potential for discovery of new natural products (Kennedy *et al*., 2008). Isolation of bacteria can originate not only from sediments, but also from open oceans or marine surfaces including marine living organisms (Jensen and Fenical, 1994). Antibiotic production by marine bacteria has been documented for a long

time (Rosenfeld & Zobell, 1947). Since these earlier reports, many low molecular weight antibiotic substances have been isolated from marine bacteria (Faulkner, 2001).

Of the one hundred and twelve isolates screened in the present study for their antibacterial property, only seventeen inhibited the growth of Gram positive/ Gram negative bacteria. After secondary screening using acetone precipitation and ammonium sulphate fractionation, the isolates were short listed to five that produced antibacterial compounds that were proteinaceous in nature. Marine microorganisms such as bacteria and fungi have been previously reported to produce antibacterial compounds. Competition among microbes for space and nutrient is a powerful selection pressure that endows marine microorganisms with the ability to produce natural products possessing industrial and medical values. De la Rosa-Garcia *et al*. (2007) screened 258 bacterial strains isolated from water and sediment in the Yucatan peninsula for antimicrobial activity and found 46 strains belonging to the genera *Aeromonas*, *Bacillus,* *Burkholderia*, *Photobacterium*, *Pseudomonas*, *Serratia* and *Stenotrophomonas* possessed antimicrobial activity. Approximately fifty percent of this antimicrobial activity was due to bacteriocins or bacteriocin like substances (BLIS). *B. subtilis* had also been isolated repeatedly from marine environments (Miranda *et al*., 2008; Ivanova *et al*., 1999).

Rosenfeld & Zobell (In 1947) carried out a detailed study on the antibiotic producing marine microorganisms. They found that most antibiotic-producing bacteria belonged to the genera *Bacillus* and *Micrococcus.* Although they did not attempt an isolation of specific antibiotics produced by marine bacteria, it was evident from their work that various species of microorganisms indigenous to the sea elaborate antimicrobial substances and they even suggested that the sea may represent a reservoir of microbial antagonists of possible importance.

5.2 CHARACTERIZATION OF BACTERIOCIN PRODUCING BACTERIA

The five bacteriocin producing isolates were identified using 16S ribotyping. The isolates were identified as bacilli belonging to two species, *B. subtilis* and *B. licheniformis*. 16S rRNA gene sequence analysis is a reliable method for identifying unknown bacterial isolates. The data generated using the universal 16S rRNA gene segment is of great accuracy and reproducibility. This method is less time consuming compared to the conventional phenotypic identification schemes. A 16S rRNA gene sequence similarity of ≥97% is a reasonable level for grouping bacteria into species (Hagstrom *et al*., 2000). Conventionally, bacilli have been identified in the laboratory through biochemical tests and fatty acid methyl ester profiling (Bobbie and White, 1980; Vaerewijck *et al*., 2001). These are technically complex and labour intensive procedures and the scarcity of reproducible and distinguishable phenotypic characteristics for several bacterial species often makes precise identifications difficult (Khamis *et al*., 2003). To date, the development of gene amplification and sequencing, especially that of the 16S rRNA gene sequences, has simplified the identification and the detection of specific bacteria, especially those lacking distinguishable phenotypic characteristics (Woese, 1987; Yamada *et al*., 1997; Kolbert and Persing, 1999; Shaver *et al*., 2002; Wang *et al*., 2003; Wu *et al*., 2006). The 16S rRNA gene is now used as a framework for the modern classification of bacteria, including those in the genus *Bacillus*.

Phylogram drawn with selected strains helped to study their relatedness or variability. Neighbour joining method was successfully used for this purpose. It was clear from the phylogram that the bacteriocin producing strains in the present study grouped together as clade. Within the clade they formed two groups, with the three *B. licheniformis* and the two *B. subtilis* strains grouping together, but

separately from each other. The three *B. licheniformis* strains showed relatedness as did the two *B. subtilis* strains.

Among diverse microbial species, isolates of marine *Bacillus* belong to phylogenetically and phenogenetically heterogeneous groups of bacteria. They are ubiquitous in the marine environment and can tolerate adverse conditions such as high temperature, pressure, salinity, and pH (Rampelotto, 2010). Generally, *Bacillus* strains need more nutrition and space to be the fastest growing bacteria for which they compete with other organisms. Due to the diluting effect of the ocean drives, marine organisms produce potent bioactive compounds to fight off their competitors or to escape from micropredation (Paul *et al*., 2007). Metabolically marine strains are different from their terrestrial counterparts, and thereby they may produce unique bioactive compounds which are not found in their terrestrial counterparts (Jensen & Fenical, 1994). The ability to produce diverse classes of antibiotics by *Bacillus* sp. has been evident by several genomic studies. For example, the genome sequence of the widely distributed *Bacillus* strains revealed that about 8% of genome is devoted to synthesizing antibiotics (Kunst *et al*., 1997; Chen *et al*., 2007).

The two strains *B. subtilis* strain BTFK101 and *B. licheniformis* strain BTHT8 were selected for the further study. Ivanova *et al*. (1999) characterized twenty aerobic endospore-forming bacilli, isolated from marine invertebrates and sea water of different areas of the Pacific ocean. Most of the bacilli (11 strains) of marine origin belonged to the species *B. subtilis*, two strains were *B. licheniformis*, others were *B. amyloliquefaciens, B. pumilus*, *B. firmus*, *B. lentus*, *B. sphaericus*, *B. fusiformis*, *B. megaterium* and *Paenibacillus* sp. Although growth in marine water might occur, the abundance of *B. subtilis* in these environments might also be explained by its observed association with the gastrointestinal tract of marine organisms (Newaj-Fyzul *et al*., 2007).

Both strains, BTFK101 and BTHT8 inhibited the growth of only Gram positive organisms, majority of which belonged to genus *Bacillus*. They also inhibited *Cl. perfringens* and *S. aureus*. The inhibitory spectrum of bacteriocins can be narrow and confined to closely related species or it can be relatively broad, inhibiting a range of target organisms including food-spoilage and pathogenic bacteria such as *Listeria monocytogenes*, *B. cereus*, *Clostridium tyrobutyricum*, methicillin-resistant *S. aureus* and vancomycin-resistant enterococci (Cotter *et al*., 2005; Galvez *et al*., 2008). The activity range of bacillocin 490 was rather narrow and limited to some Gram-positive bacteria like *B. stearothermophilus, B. smithii, B. subtilis*, *B. anthracis*, *B. cereus*, *B. licheniformis*, *Listeria innocua* and *S. aureus* (Martirani *et al*., 2002). A bacteriocin like inhibitory substance (BLIS) with a broad spectrum of activity against pathogenic and spoilage bacteria (*L. monocytogenes*, *B. cereus* and clinical isolates of *Streptococcus* sp.) was produced by *B. licheniformis* P40 that was isolated from the Amazon basin (Cladera-Olivera *et al*., 2004). *B. licheniformis* strain ZJU12 isolated from soil produces a 3 kDa BLIS that has a broad spectrum of antagonistic activity against various species of Gram-positive bacteria, as well as pathogenic fungi (Lili *et al*., 2006). A low-molecular-weight peptide (1.5 kDa by SDS-PAGE) produced by *B. licheniformis* MKU3 (Kayalvizhi & Gunasekaran, 2008) exhibited bactericidal activity against Gram positive and Gram negative bacteria as well as different filamentous fungi and yeast (*Candida albicans*).

Subtilin produced by *B. subtilis* showed antimicrobial activity in the nanomolar range against a broad spectrum of Gram-positive bacteria (Gross *et al*., 1973). Ericins (mainly ericin S) are active against a variety of bacteria including the causative agent of tomato bacterial canker, *Clavibacter michiganensis* (Stein *et al*., 2002 a). Sublancin 168 produced by *B. subtilis* 168 exhibits bactericidal activity against other Gram-positive bacteria including important pathogens such

as *B. cereus*, *Streptococcus pyogenes* and *S. aureus* (Paik *et al.,* 1998). Mersacidin produced by *B. subtilis* strain HILY-85, 54728 is a tetracyclic peptide active against methicillin and vancomycin-resistant *S. aureus* (Bierbaum *et al*., 1995). *B. subtilis* LFB112 from Chinese herbs produced a BLIS active against both Gram-positive and Gram-negative bacteria involved in domestic animal diseases including *E. coli*, *Salmonella Pullorum*, *P. aeruginosa*, *Pasteurella multocida, Cl. perfringens*, *M. luteus*, *Streptococcus bovis* and *S. aureus* (Xie *et al*., 2009). *B. subtilis* 14B isolated from the rhizosphere of healthy plants (bitter almond) produces a BLIS (Bac 14B) active against *Agrobacterium tumefaciens* (Hammami *et al*., 2009). *B. subtilis* strain MJP1 isolated from 'meju' (Korean fermented soybean) produces a BLIS with antimicrobial activity against various species of Gram-positive bacteria, yeasts and molds including food-spoilage microorganisms (Yang & Chang, 2007).

Antibacterial activity of the bacteriocins, BL8 and BS101 from the strains BTHT8 and BTFK101 respectively was tested against *S. aureus* in further studies. *S. aureus* has extensive genomic variability and easily acquires tools for resisting against antimicrobials, in particular against β-lactam antibiotics. Therefore it is one of the most successful and adaptable human pathogens (Daini and Akana, 2009). Methicillin resistant *S. aureus* (MRSA) has remained a major cause of nosocomial disease world-wide (Larsen *et al*., 2008).

As plasmid DNA was not obtained from the two strains BTFK101 and BTHT8, it was concluded that the genes encoding the bacteriocins are located on the chromosome. A survey of plasmid diversity among 50 natural isolates of *B. subtilis* estimated that only ~10% of strains harbour these extrachromosomal elements (Zawadzki *et al*., 1996). Lichenin is a chromosomally encoded bacteriocin (Pattnaik *et al*., 2005) produced under anaerobic conditions by *B. licheniformis* 26L-10/3RA isolated from water buffalo rumen (Pattnaik *et al*.,

2001). Plasmid encoded bacteriocins are also reported in other species of *Bacillus*. Megacin BII is encoded on plasmid pSE 203 in strains of *B. megaterium* (Stahl, 1989). *Lysinibacillus sphaericus* SOPB1 was shown to produce a BLIS encoded by the pSOPB1-19 plasmid. *B. thuringiensis* HD-2 produces a plasmid-encoded thuricin HD2 (Favret & Yousten, 1989).

There are several species of *Bacillus* that are known pathogens. These include *B. anthracis* which is pathogenic to humans and other animals, and *B. cereus* which is a common cause of food poisoning (Claus and Berkeley, 1986; Norris *et al*., 1981). *B. subtilis* and *B. licheniformis* appear to have a low degree of virulence to humans. Although *B. subtilis* thas been associated with outbreaks of food poisoning (Logan, 1988), the exact nature of its involvement has not been established. *B. licheniformis* can also cause food-borne gastro-enteritis. Dairy products are at increased risk of being contaminated with toxin-producing isolates of *B. licheniformis.* Cooked meats, raw milk, vegetables, and processed baby foods are also at risk. (Salkinoja- Salonen, 1999). de Boer *et al*. (1994) has documented in the review that authorities in the United States, Europe and Japan have approved production with and products from recombinant *B. licheniformis* strains and concluded that *B. licheniformis* is a safe host for the production of harmless, industrial products.

Hemolysis on blood agar plates is an indication of the potential of bacteria for pathogenesis. The two strains BTFK101 and BTHT8 tested on blood agar plate were non-hemolytic indicating their non-pathogenic nature. The non-pathogenic nature of the bacterium is one of the major criterions for selection of microbes for large scale production of any product (Byrom, 1992).

In the antibiotic susceptibility tests, both the strains were sensitive to the commonly used antibiotics like ciprofloxacin, gentamicin and trimethoprim. The strains which are sensitive to commonly used antibiotics can be regarded as safe

(De Vuyst *et al.*, 2003) especially when they are intended to be incorporated in food products.

B. subtlis strain BTFK101 and *B. licheniformis* strain BTHT8 start their bacteriocin production at early exponential phase and the production reached its peak at the end of exponential phase/ starting of stationary phase. Consequently, the bacteriocins were produced in the active growth phase. These characteristics of bacteriocin production clearly differentiate it from that of authentic secondary metabolites (Kleinkauf *et al.,* 1986; Martin & Liras, 1989). The bacteriocin production in *Bacillus* strain 8A, reported by Bizani and Brandelli (2002) started at the exponential phase and reached its peak at the stationary phase. Cherif *et al.* (2001) reported that thuricin 7, produced by *B. thuringiensis* BMG1.7 expressed in the exponential growth phase. Production of bacteriocins in the exponential growth phase of bacteria is also reported in *B. subtilis* (Alam *et al*., 2011; Xie *et al*., 2009); in *B. licheniformis* (Cladera-Olivera *et al*., 2004; He *et al*., 2006) and in *B. brevis* (Saleem *et al*., 2009). Bacteriocins of lactic acid bacteria, particularly lantibiotics are usually produced in the exponential phase (Horner *et al*., 1990). Evaluation of bacteriocin production along with the growth curve was also studied by Torkar and Matijasic (2003) on strains of *B. cereus* 15/5, *B. cereus* 8/10, *B. cereus* 30/11 and *B. cereus* 8/2. In all the four strains, bacteriocin production and secretion was observed in the stationary phase (after 10 to 16 h) of bacterial growth. Naclerio *et al.* (1993) also reported the production and activity of bacteriocin cerein by *B. cereus* strain in the early stationary phase.

5.3 OPTIMIZATION OF BIOPROCESS VARIABLES BY 'ONE-FACTOR AT-A-TIME' METHOD

Eleven factors were taken into account viz. different media, sodium chloride (NaCl) concentration, carbon source, inorganic nitrogen source, organic nitrogen source, initial pH of the media, inoculum concentration, incubation

temperature, surfactant concentration, incubation period and agitation. Bacteriocin is usually produced in liquid substrates. The optimum conditions for bacteriocin production are affected by growth phase of the bacterium, pH of the medium, incubation temperature, type of carbon and nitrogen sources, and concentration of NaCl (Kim *et al*., 2000). Likewise, the bacteriocin production was enhanced by optimization of culture conditions in *L. casei* (Vignolo *et al*., 1995) and *Leuconostoc mesenteroides* (Krier *et al.,* 1998).

Zobell marine broth was found to be the best media for bacteriocin production by both BTFK101 and BTHT8. Bacteriocin production was not attained when lactose broth was used for BTFK101. For BTHT8 also, bacteriocin production was not observed when lactose broth and Mueller-Hinton broth were used. The production of bacteriocin by *B. amyloliquefaciens* was studied by Lisboa *et al*. (2006) and maximum antibacterial activity was evaluated by cultivation in brain heart infusion (BHI). The effect of various media for the production of bacteriocin like inhibitory substance from microalgal symbiotic *Vibrio* sp. MMB2 was examined using media such as MRS, Luria broth, peptone enrichment media, peptic soya broth, TCBS, nutrient broth and Zobell marine broth (Selvendran and Michael, 2013). The maximum bacteriocin activity was recorded at MRS broth against *Vibrio harveyi.* Zobell marine broth stood next. The minimum bacteriocin activity was recorded in nutrient broth against *V. harveyi* and no activity was found when peptone enrichment broth and lactose broth were used.

While optimizing sodium chloride concentration, maximum bacteriocin production was obtained from BTFK101, when sodium chloride (NaCl) was supplied in concentrations ranging from 0.5 -2%. But specific activity was highest when 1.5% NaCl was provided. So 1.5% NaCl is optimum for BTFK101 for bacteriocin production. As the concentration of sodium chloride increased, the

production of bacteriocins lowered. In the case of BTHT8, bacteriocin production reached its peak when NaCl was supplied at a concentration of 1%. The bacteriocin production completely stopped when NaCl was supplied at 2.5% or above. El- Sersy *et al*. (2009) has optimised the production of antimicrobial agents from *B. licheniformis* SN2 and found that the optimum NaCl concentration was 0.3%. The maximum bacteriocin production by *Lactobacillus* sp. MSU3IR was achieved with 3% NaCl supplementation. NaCl could alter the osmolarity of the cell membrane of bacterium which favoured more extrusion of bacteriocin from cell to media (El- Sersy *et al*., 2009). In correlation, Herranz *et al.* (2001) also reported that bacteriocin production by *E. faceium* P13 was high at 3% NaCl and more than 7% of NaCl supplementation reciprocally affected the bacteriocin production.

With strain BTFK101 maximum bacteriocin production was obtained when pectin was supplied as the carbon source. No production was seen when lactose was used as the carbon source. In the case of BTHT8, maximum specific activity was found for glucose although sucrose, starch and pectin gave same rate of production. So glucose can be considered as the best carbon source. There was no production of bacteriocin when lactose and mannitol were used. The role of different carbon sources on bacteriocin production by *B. amyloliquifaciens* was studied by Vijayalakshmi *et al*. (2011 a). Maximal activity was observed when glucose was used as carbon source. Fructose, maltose, glycerol, sucrose and xylose gave same rate of bacteriocin production followed by lactose and galactose. Mannitol supported moderate activity whereas inositol was a poor carbon source for bacterial growth. Similar results were reported by Ogunbanwo *et al.* (2003) where bacteriocin by *L. brevis* OG1 were synthesized in larger amounts in medium supplemented with 1.0% (w/v) glucose. *Lactobacillus pentosus* ST151 BR showed maximum activity when glucose was used at a concentration of 1.0% (w/v) in the production medium (Todorov and Dicks, 2004). The response surface

methodology was effective in optimizing bacteriocin-like substance production by *B. cereus* XH25 (Zhong *et al.*, 2013). Optimal bacteriocin activity was recognized in *B. megaterium* 19 after 24 h of incubation in MRS supplemented with 4% sucrose, followed by glucose, maltose, fructose and finally lactose. Galactose did not stimulate the bacteriocin production (Khalil *et al.,* 2009 a & b).

The optimization studies of nitrogen sources showed maximum production when ammonium sulphate was used as inorganic nitrogen source for both the strains. Production was lowest when urea was opted as the inorganic nitrogen source. Beef extract proved to be the best organic nitrogen source for both the strains. Lowest production was seen when malt was used. However the effect of nitrogen source on bacteriocin production by *Lactobacillus* sp. MSU3IR revealed that, ammonium acetate favoured maximum bacteriocin production and minimum bacteriocin production was noticed in sodium nitrate (El- Sersy *et al*., 2009). Accordingly, the work of Iyapparaj *et al.* (2013) evidenced that the increment in bacteriocin production was attributed with organic nitrogen source. Cell growth and bacteriocin production was shown to be influenced by organic nitrogen source (Kim *et al.*, 1997).

Both the strains gave maximum production when inoculum at a concentration of 6-10% was used. For BTFK101 specific activity was highest when the media was inoculated with 10% culture and for BTHT8 it was 6% inoculum. In both the strains, when inoculum below 5% was used, the production reduced. Vijayalakshmi *et al*. (2011 a & B) studied on production of antimicrobial protein from *B. amyloliquefaciens* MBL27 and found that growth and bacteriocin production was minimal when 10^5cells/mL was added. Bacterial growth and production was maximal when 10^6cells/mL was inoculated to the medium. When the count was increased beyond this level, production and growth was decreased which may be due to the exhaustion of nutrients.

Tween 80 reduced the production in BTFK101. When tween 80 at a concentration up to 0.2% was supplemented in the media, the production remained the same as in the control but the specific activity decreased. Thus the surfactant has a negative impact on the production. But for BTHT8 the surfactant had a positive impact on the bacteriocin production. Addition of 0.1% tween 80 in the media doubled the production. But when the surfactant concentration was increased to 0.5%, the production decreased. Bacteriocin production by candidate bacterium emphasized that, the higher bacteriocin yield was attained in the medium supplied with tween 80 compared to other tested surfactants. Similar results were recorded for lactocin 705 (Vignolo *et al.,* 1995). Possibly, tween 80 could change the surface tension of the producer cell thereby increasing the rate of bacteriocin release from the cell surface. Previous reports have shown an increased yield of bacteriocin production when tween 80 or tween 20 was added to the growth medium (Garver and Muriana, 1994; Huot *et al*., 1996; Ayemerich *et al.*, 2000). Tween 80 increased the bacteriocin level by deadsorption of preformed bacteriocin and not by increasing its production or secretion, since the effect of the detergent was instantaneous. Non-ionic detergents such as tween 80 may mimic the effect of various food constituents inducing the production of bacteriocins and they are known to stimulate protein secretion by affecting membrane fluidity (Reese & Maguire, 1969). Tween-treated cultures also increased the supernatant activity relative to total activity, probably by desorption and disaggregation of the bacteriocin (Ayemerich *et al.*, 2000; Mortevedt *et al*., 1991). Keren *et al*. (2004) determined the minimal tween 80 concentration required for maximal deadsorption of lacticin RM to be 0.025% and that it does not stimulate growth or bacteriocin production. They found that tween 80 reduced the adsorption of lacticin RM to producer cells and this effect is instantaneous, leaving no significant residual bacteriocin adsorbed to the cells.

Initial pH of the media had significant role in bacteriocin production by both the strains. pH 5-9 was found to be the optimum pH range for bacteriocin production by BTFK101. Specific activity was highest when media had pH 5. So media pH 5 is the best for the bacteriocin production by BTFK101. At pH 4 and 10, the production decreased. In the case of BTHT8, maximum production was seen when initial pH of the media was adjusted to 7. Production reduced when pH was lowered or raised from 7. El- Sersy *et al*. (2009) have optimised the media pH for the production of antimicrobial agents by *B. licheniformis* SN2 as pH 5. The initial pH of the medium has a profound effect on the production of bacteriocin. Kim *et al.* (2006) also reported that micrococcin GO5 production was maximum when the initial pH was between 7.0 and 9.0. At pH 4.0 the activity was slightly lower. Bacteriocin production by *B. subtilis* LFE-1, *B.firmus* H2O-1 and *B.licheniformis* T6-5 (Korenblum *et al*., 2005) was at an efficient level upto pH 10. But at pH 11, bacterial growth and bacteriocin production were. The pH of the medium affects the cell growth, hence influencing the bacteriocin production. Among the tested pH of the media in *Lactobacillus* sp., the maximum bacteriocin production in terms of antagonistic activity was recorded at pH 5 (Iyapparaj *et al.*, 2013). However, further increase in pH found to mitigate the bacteriocin production. The minimum bacteriocin production was recorded at pH 9. De Vuyst and Vandamme (1992) found that nisin production at pH 6.8 showed an upward curvature and a sharp decrease in activity at the end of growth. Maximum production corresponded to maximum biomass concentration. Similar peaks in bacteriocin activity were obtained for helveticin J (Joerger and Klaenhammer 1986) and lactacin B (Barefoot & Klaenhammer 1984) at pH 6.0; mesenterocin 5 at pH 5.0 (Daba *et al*., 1993) or enterocin 1146 at pH 5.5-6.5 (Parente & Ricciardi, 1994). Similar patterns were observed among other lantibiotic-producing organisms like *Staphylococcus epidermidis* (Horner *et al.* 1989 & 1990).

The optimum incubation temperature for bacteriocin production by BTFK101 and BTHT8 was found to be 30°C. Production decreased as temperature of incubation was increased. Specific activity was also highest at 30°C. Galvez *et al.* (1993) recorded maximal bacteriocin production by *B. licheniformis* when incubated at 28°C. El- Sersy *et al.* (2009) optimised the temperature for bacteriocin production by *B. licheniformis* SN2 as 30°C. The production of bacteriocin from *B. amyloliquefaciens*, studied by Lisboa *et al*. (2006) was maximum when incubated at 37°C. A similar level of activity was observed at 30°C but growth was negligible and no antimicrobial activity was observed at 55°C. Maximum production of bacteriocin was observed at 30°C in *B. amyloliquifaciens* (Vijayalakshmi *et al.,* 2011 a & b). In many *Lactobacillus* sp. 30°C was found to be the best incubation temperature for maximum bacteriocin production as reported by Paynter *et al.* (1997), Ogunbanwo *et al.* (2003), Moonchai *et al.* (2005) etc. Decrease in bacteriocin production with increase in incubation temperature was observed by Iyapparaj *et al*. (2013).

In BTFK101, the bacteriocin production started when the culture was inoculated for 6 h but maximum production was attained at about 12 h. Production decreased as incubation period was increased. No bacteriocin production was noted after 30 h of incubation. In the case of BTHT8 also production started at 6 h of incubation but it took 18 h to reach its maximum. The production decreased and remained in a stationary mode after 30 h. Boe (1996) found that the growth of lactic acid bacteria increased logarithmically with the increase in incubation period. The increase in biomass of the bacteria was followed by a rise in bacteriocin production. When bacterial growth reached the stationary phase, bacteriocin production decreased. Galvez *et al.* (1993) recorded maximal bacteriocin production by *B. licheniformis* after 72 h of incubation. Production of antimicrobial protein by *B. amyloliquefaciens* MBL27 was studied by Vijayalakshmi *et al*. (2011 a & b) and observed that bacteriocin production was

growth associated and also biomass production was proportional to glucose utilization and it was maximal during the late exponential phase (24h), retaining its activity throughout the stationary phase.

Agitation plays a major role in bacteriocin production by both the strains. The bacteriocin production was very low when the cuture was kept stationary. Effect of shake and still culture conditions for bacteriocin production in *B. amyloliquefaciens* MBL27 (Vijayalakshmi *et al*., 2011 b) was studied by incubating the cultures under both shake and still culture conditions. Bacterial growth and antimicrobial activity was noticed in both the conditions but the rate of bacteriocin production under shake culture conditions was comparatively higher indicating the supportive role of oxygen in bacteriocin production.

5.4 PURIFICATION OF THE BACTERIOCINS

The purification process involved concentration and fractionation using ammonium sulphate precipitation of the crude culture supernatant. This was followed by gel filtration using sephadex G-25 column. Only the 30- 60% fraction of ammonium sulphate precipitation showed activity. So, only this fraction was collected further. The activity was increased drastically after this concentration step. The specific activity also increased step by step during purification process. There was a five fold increase in specific activity of BS101 after concentration using ammonium sulphate. For BL8, the specific activity increased six fold after ammonium sulphate fractionation. BS101 was seven fold purified and BL8 was 29 fold purified after gel filtration chromatography.

In order to study the biological activities and chemical structures of bacteriocins, it is necessary to develop appropriate purification procedures. Bacteriocins produced by Gram-positive bacteria are the most often secreted into

the growth medium. Therefore, the purification preferably starts by a concentration step using the cell-free culture supernatant. Then, several additional steps, the most often chromatographic steps are necessary to achieve a significant purity (Sebei *et al*., 2007).

Bacteriocin like inhibitory substance from *B. subtilis* BS15 was successfully precipitated by 80% ammonium sulphate precipitation. The total protein was estimated before and after the precipitation and specific-activity determined which was found markedly increased after ammonium sulphate precipitation (Alam *et al*., 2011). 80% Ammonium sulphate saturation was used to precipitate bacteriocins by *B. thuringiensis* subsp. *entomocidus*, *B. cereus* and *B. subtilis* LFB112 (Cherif *et al*., 2008; Risoen *et al*., 2004; Xie *et al*., 2009) respectively. The antimicrobial substance produced by *B. amyloliquefaciens* MBL27 was precipitated readily by adding 40% saturation with about 98% recovery of the antimicrobial protein (Vijayalakshmi *et al*., 2011 a & b). Ammonium sulphate fractionation was used as the first step in purification protocol in *B. thuringiensis* (Ahern *et al*., 2003) and in *B. lentus* (Sharma *et al*., 2006).

Purification of bacteriocins includes combination of several strategies. Subtilosin A was purified using 65% ammonium sulphate, followed by reversed-phase HPLC (Shelburne *et al*., 2007). Concentration using chloroform followed by HPLC using C18 was the procedure followed by Joseph *et al*. (2013) for purifying bacteriocin from *B. subtilis.* Bac thuricin F4 was purified ammonium sulphate followed by C18 reverse phase HPLC (Kamoun *et al*., 2005). Butanol extraction followed by C18 reverse phase HPLC was used by Gray *et al*. (2006 b) for the purification of thuricin 17. Ammonium sulfate fractionation - carboxymethyl (CM) sephadex column chromatography - sephadex G-100 gel filtration were performed on the culture filtrate *Bacillus amyloliquefaciens* J4 for purifying

bacteriocin J4 (Lim *et al.*, 2011). In purification of an antimicrobial peptide produced by a novel *Bacillus* sp. (Motta *et al*., 2007), concentration resulted in 57 fold purification and gel filtration made 80 fold increase of specific activity followed by ion exchange chromatography.

Bacillocin 490 from *B. licheniformis* was purified with a purification factor taking advantage of its ability to bind to the sensitive cells of *B. smithii* (Martirani *et al.*, 2002). In the purification of cerein MRX1 hydrophobic interaction chromatography (HIC) was used efficiently to concentrate the antimicrobial activity from the culture supernatant. Subsequently, more than two-fold increase in specific activity was obtained in this step. However, cation exchange chromatography (CEX) resulted in a significant loss of activity. RP-HPLC was carried out as final purification step and the fold of purity was increased. The ability of cerein MRX1 to adsorb to cellular surfaces was probably because of its high hydrophobicity.

5.5 CHARACTERIZATION OF THE BACTERIOCINS

The SDS-PAGE analysis of the purified bacteriocins helped to indicate purification of the bacteriocins to homogeneity. The approximate molecular mass of the bacteriocins were estimated based on SDS-PAGE analysis and activity on the gel. Appearance of clearing region on the gel near the dye front, brought about by inhibition of the test organism revealed that the bacteriocins have a very low molecular mass. Moreover, the intact molecular mass of BS101 and BL8 was determined by MALDI-TOF MS as 3.37 kDa and 1.4 kDa respectively. Many bacteriocins are reported from *Bacillus* sp. with low molecular mass. Subtilin produced by *B. subtilis* has a molecular mass of 3319.56 Da as revealed by MALDI-TOF MS (Stein, 2008). Ericin S (3442 Da) and ericin A (2986 Da) are two related lantibiotics produced by *B. subtilis* A1/3 with strong similarities to subtilin (Stein *et al*., 2002 a). Sublancin 168, the lantibiotic produced by *B. subtilis*

168 is having a molecular mass of 3877.78 Da (Paik *et al*., 1998). *B. subtilis* LFB112 from Chinese herbs produces a BLIS having 6.3 kDa, as estimated by SDS-PAGE (Xie *et al*., 2009). Bac 14B, a BLIS produced by *B. subtilis* 14B has an estimated molecular weight of 21 kDa by SDS-PAGE (Hammami *et al.*, 2009). *B. subtilis* strain MJP1 isolated from meju (Korean fermented soybean) produces a BLIS whose molecular weight is determined as ~ 2.4 kDa by PAGE (Yang & Chang, 2007). Subtilosin A has a molecular mass of 3399.7 Da (Marx *et al*., 2001). The molecular weight of lichenin from *B. licheniformis* 26L-10/3RA, estimated by SDS-PAGE and gel filtration chromatography was approximately 1400–1500 Da. Bacillocin 490 produced by a thermophilic strain of *B. licheniformis* 490/5 isolated from a dairy product is a 2-kDa peptide (Martirani *et al*., 2002). A low-molecular-weight peptide (1.5 kDa by SDS-PAGE) is found to be produced by *B. licheniformis* MKU3 (Kayalvizhi & Gunasekaran, 2008).

Using isoelectric focusing (IEF), the isoelectric points of the bacteriocins were determined. The pI of BS101 was found to be 6.3 and that of BL8 was found to be 5.7. Many reported bacteriocins from Gram positive bacteria have a pI value greater than 7 (Ray, 1992; Hancock *et al*., 1995). Subtilosin A has a pI value of approximately 4, based on its amino acid sequence (Babasaki *et al*., 1985). Bacteriocin produced by a *B. subtilis* strain also has a pI of 4.7 (Zheng & Slavik, 1999). The isoelectric point of the bulgaricin appeared to be around 6.2 (Hasan *et al.,* 2012).

N-terminal amino acid analysis of BL8 revealed the 13 amino acid sequence stretch. In amino acid position 4, 5 and 8 Edman degradation gave blank signals that are observed when cysteine residues are present (Yanagida *et al.,* 2005). So these three positions are assumed to contain cysteine and the sequence is as follows: NH2-Ser-Trp-Ser-Cys-Cys-Gly-Asn-Cys-Ser-Ile-Ser-Gly-Ser-COOH. Comparison of the sequence with the amino acid sequence of bacteriocins

in the NCBI protein database by multiple sequence alignment showed no significant similarity to any of the sequences, indicating the novelty of the bacteriocin. N-terminal sequencing of the thuricin 439A and B peptides by Edman degradation showed identical amino acid sequences. A sequence of 20 amino acids was obtained for each fraction: G-W-VA- X-V-G-A-X-G-T-V-V-L-A-S-G-G-V-V (the X indicates an amino acid for which the identity could not conclusively be established; however, these two amino acids are likely to correspond to cysteine residues). The obtained sequence was compared to sequences present in the non redundant NCBI database. No significant similarity to any sequence was observed; indicating that thuricin 439 is a novel BLIS compound (Ahern *et al.,* 2003). N-terminal amino acid sequencing was carried out for cerein 7A: NH_2- GWGDVL- and cerein 7B: NH_2- GWWNSWGH- by (Oscariz *et al*. (2006); cerein MRX1: NH_2- DWTCWSCLVCAACSVELL by Sebei *et al.* (2007). The determined 18-amino acid sequence of cerein MRX1 is different from the sequences of cereins 7A and 7B but very similar to the 18-amino acid sequence of a recently discovered thuricin 17 produced by *B. thuringiensis* NEB17 (Gray *et al*., 2006 b). The difference between the two sequences resides in the residue in position 10 which is a cysteine in cerein MRX1 while it is a valine in thuricin 17.

The action of proteases viz. proteinase K, pepsin and trypsin on the bacteriocins was studied. There was complete loss/ reduction in activity of the bacteriocins when treated with proteases confirming their proteinaceous nature. Similar results are reported in bacteriocins of many *Bacillus* sp. *B. subtilis* LFB112 from chinese herbs produces a BLIS, sensitive to proteinase K and pronase E, but resistant to papain, trypsin and pepsin (Xie *et al.,* 2009). Bac 14B, a BLIS produced by *B. subtilis* 14B was found sensitive to proteases. BLIS from *B. amyloliquefaciens* LBM 5006 (Lisboa *et al*., 2006); Brevicin AF01 from *B. brevis* AF01 (Faheem *et al*., 2007); Cerein 8A from *B. cereus* 8A (Bizani & Brandelli,

2002); BLIS produced by *B. lentus* (Sharma *et al*., 2009 a); Megacins19 (Khalil *et al*., 2009a); polyfermenticin SCD (Lee *et al*., 2001); thuricin CD (Hill *et al*., 2009); thuricin 7 (Cherif *et al*., 2001); thermoleovorins (Novotny & Perry, 1992); entomocin 9 (Cherif *et al.,* 2003) and P45 by *Bacillus* sp. P45 (Sirtori *et al*., 2006) all are reported as protease sensitive bacteriocins. But *Bacillus firmus* H2O-1(Korenblum *et al*., 2005); *B. subtilis* strain MJP1 (Yang & Chang, 2007) and *G. stearothermophilus* NU-10 (Fikes *et al*., 1983) produce bacteriocins resistant to proteolytic enzymes.

The bacteriocins BS101 and BL8 are able to tolerate wide pH range and temperature stresses. BS101 starts deactivating at 40°C and when it reaches 50°C it loses its activity completely, whereas BL8 was stable up to 60°C. Both BS101 and BL8 could tolerate a pH range of 2-9. Many bacteriocins are able to tolerate temperature and pH stresses. *B. subtilis* LFB112 from Chinese herbs produces a BLIS that is heat and pH (3–10) stable (Xie *et al.,* 2009). *B. subtilis* 14B produces a BLIS (Bac 14B), stable to heat (up to 100°C for 2 h) (Hammami *et al*., 2009). *B. subtilis* strain MJP1 produces a BLIS stable in the pH range of 6.0–10.0 and heat stable (Yang & Chang, 2007). Lichenin produced by *B. licheniformis* 26L-10/3RA is heat stable, retaining biological activity even after boiling for 10 min and is active over a wide pH range of 4.0–9.0 (Pattnaik *et al*., 2001). Bacillocin 490 is produced by a thermophilic strain of *B. licheniformis* 490/5 isolated from a dairy product, with a high stability at 4 and 100°C and stable over a wide pH range (Martirani *et al.,* 2002). *B. licheniformis* P40 that originated from the Amazon basin (Cladera-Olivera *et al.,* 2004) produces a BLIS, stable over a very wide pH range (3–11) and at a high temperature of 100°C, but lost its activity at 121°C for 15 min.

In both BS101 and BL8, non-ionic detergents viz. tween 20, tween 80 and triton X-100 did not affect the activity of the bacteriocins. The ionic detergent

SDS however reduced the activity of both BS101 and BL8. CTAB has very prominent effect. It completely deactivated both the bacteriocins. SDS inhibited the bacteriocin produced by *B. subtilis* LFB112 (Xie *et al*., 2009) but non ionic surfactants had no effect. Treatment with tween 80 increased antimicrobial activity whereas SDS and triton X-100 had no effect on the bacteriocin activity of bacteriocin G2 produced by the probiotic bacteria *Lactobacillus plantarum* G2 (Seatovic *et al*., 2011). Surfactants did not affect the bacteriocin or bacteriocin like substance produced by *Lactoccous lactis* B14 (Ivanova *et al*., 2000). The antagonistic activity was greatly reduced when treated with SDS whereas triton X-100 and tween 20 completely inhibited the activity of plantaricin OL15 a bacteriocin produced by *Lactobacillus plantarum* OL15 (Mourad *et al.*, 2005). It was observed that tween 80 adverseley affected the activity of bacteriocin from *Leuconostoc* NT-1 (Maurya & Thakur, 2012). It was noticed that tween 80 and SDS increased the activity of the bacteriocin whereas Triton X-100 lowered the activity of plantarcin SR18 produced by *Lactobacillus plantarum* SR18 (*El-Shouny et al*., 2013). SDS, tween 80 and triton X-100 stimulated the bacteriocin activity of *Lactobacillus lactis* (Rajaram *et al.,* 2010).

BS101 remained stable when it was exposed up to 8 mM DTT. Above this concentration, the activity reduced drastically. β - mercaptoethanol did not affect the activity of BS101 up to a concentration of 60 mM but the activity reduced when it was exposed to β-mercaptoethanol at a concentration of 70 mM and above. The activity of BL8 was reduced to half when it was exposed to 20 mM and above. The activity of BL8 was reduced when treated with β- mercaptoethanol at a concentration of 200 mM. A few reports are available on the study of the effect of reducing agents on bacteriocins. DTT reduced the activity of the bacteriocin produced by *B. subtilis* LFB112 (Xie *et al*., 2009). It was noticed that β-mercaptoethanol (0.2%) increased the activity of bacteriocin produced by *Lactobacillus plantarum* SR18 (*El-Shouny*, 2013) to 1.2-1.4 fold. Oxidation can

occur when proteins are exposed to oxidizing agents such as hydrogen peroxide. DMSO is used as a mild oxidant. Oxidation of methionine residues is common and it has been shown to cause a decline in the biological activity of the protein. No reduction in activity of the bacteriocins was observed when treated with DMSO, which may be due to the absence of methionine residues in the bacteriocins. In the case of BL8 this is supported by N-terminal sequencing.

In the present study the ions of Na, Ca, Mg, Fe, Mn, Ni, , Ba, Cd, Zn, Cu, Co and Al were evaluated for their impact on the activity of bacteriocins. For BS101ions of metals like Mg, Al, Fe, Zn, Na, Cu, Ni, Ca, Mn and Co ions did not affect the stability and activity. The ions of two metals Ba and Cd increased the activity of BS101. They acted as activity promoters for BS101. For BL8 ions of Mg, Al and Ba increased the activity. Mn and Co ions decreased the activity. The metal ions of Cd, Fe, Zn, Na, Cu, Ni, and Ca had no role on the activity of the bacteriocin. Studies have indicated that metal ions impart thermodynamic stabilization to the native state of the protein by binding to the active site of the enzyme (Wyman and Gill, 1990). Ca ions have been reported to bind to the inner surface and autolytic sites of protein molecules thereby strengthening the interaction inside the molecule (Ghorbel *et al.,* 2003).

The action of bacteriocins was found to be bactericidal for *S. aureus*, *B. circulans*, *B. coagulans* and *B. cereus*. But both the bacteriocins were bacteriostatic for *B. pumilus* and *Cl. perfringens*. Most bacteriocins exert bactericidal mode of action against the sensitive microorganisms, although some of the bacteriocins have been shown to act in a bacteriostatic manner. Most of bacteriocins from *Bacillus* sp. showed a bactericidal effect (Naclerio *et al*., 1993; Hyronimus *et al*., 1998; Cherif *et al*., 2003; Gray *et al.,* 2006 b; Sharma *et al*., 2006; Aunpad and Na- Bangchang, 2007). Bacteriocin from *Lactococcus lactis* (Ivanova *et al*., 2000) was found to be bacteriostatic.

5.6 APPLICATION STUDIES

It was experimentally proved that both the bacteriocins perform very well in the control of biofilm formed by *B. cereus* and *Cl. perfringens*. *Bacillus* sp. particularly *B. cereus*, are implicated in food spoilage (Andersson *et al*., 1995; Janneke *et al*., 2007). Sharma & Anand (2002) studied the biofilm constitutive microflora in a commercial dairy plant and found that *B. cereus* accounted for more than 12% of the microflora. As *B. cereus* is ubiquitously present in nature, it is easily spread through food production systems and contamination with this species is almost inevitable. Moreover, *B. cereus* spores are both highly resistant to a large number of stresses and very hydrophobic, which causes them to adhere easily to food processing equipment (Lindsay *et al.*, 2006). *Cl. perfringens* is also one of the most ubiquitous bacteria in natural environments. Biofilm formation by *Cl. perfringens* has been reported by John *et al*. (2008). Bacteriocins are gaining importance and have a unique potential in the food industry for the effective biocontrol and removal of biofilms. These newer biocontrol strategies are considered important for the maintenance of biofilm-free systems and thus improve the quality and safety of foods. Bacteriocins have several characteristics that make them ideal food preservatives.

Both the bacteriocins proved to be very efficient in controlling the microflora of sea foods like prawns and anchovies. Taylor *et al.* (1990) studied the application of nisin A in the preservation of fish products. The cod, herring, and smoked mackerel fillets inoculated with *Cl. botulinum* spores brought about a delay in toxin production when they were treated with nisin. The bacterial growth of brined shrimp was put to a control and shelf life extended when treated with with nisin Z, carnocin U149 and bavaricin (Einarsson & Lauzon, 1995). Such results offer clear perspectives for the biopreservation of certain fish products with bacteriocins.

In order to study the effectiveness of bacteriocins in combating the bacterial pathogen *in vivo*, *C. elegans* was chosen as the model organism. *C. elegans* is a very convenient whole organism model to identify or assay antimicrobial compounds (Ewbank & Zugasti, 2011). It is a small, free-living soil nematode that feeds primarily on bacteria. Its usefulness as a model organism is due to its genetic tractability, rapid generation time, ease of propagation, transparent body, a well-defined cell lineage map and a fully sequenced genome that contains a large number of vertebrate orthologues (Portal-Celhay & Blaser, 2012). Several human pathogens including *Pseudomonas aeruginosa, Salmonella* Typhimurium, *Serratia marcescens*, *Staphylococcus aureus*, *Vibrio cholerae,* and *Burkholderia pseudomallei* kill *C. elegans* when supplied as a food source (Tan *et al.,* 1999; Aballay *et al*., 2000; Kurz & Ewbank, 2000; Labrousse *et al.,* 2000).

Both *S. aureus* and *B. circulans* were able to infect *C. elegans* and reduce the TD_{50}. *S. aureus* is a common Gram-positive bacterium that causes a range of minor infections, which occasionally become serious in many animals (Lindsay, 2010). In *C. elegans*, intact bacteria accumulate in the gut of the animal and it is this colonisation that eventually overwhelms the host, disrupting the gut epithelium and then destroying internal organs ultimately leading to death (Garsin *et al.*, 2001; Irazoqui *et al*., 2010).

The ability of the bacteriocin BL8 to confer protection against the bacterial infection by *S. aureus* and *B. circulans* were proved by this study. It was observed that the bacteriocin BL8 succeeded in controlling the infection of *C. elegans* and extent the life span of the worms when compared to the untreated (control) worms fed on *E. coli* OP50. *In vivo* assays of many antimicrobial agents have been studied using *C. elegans*. Compounds that block quorum sensing were identified using *C. elegans* and potentially developed as antimicrobial drugs (Swem *et al*., 2009). Uccelletti *et al*. (2010) studied the antibiotic action of

membrane-active cationic antimicrobial peptides (CAMPs) from frog skin in *C. elegans*. Moy *et al.* (2006) developed a liquid-based assay using microtiter plates to test the potential of thousands of synthetic compounds and natural extracts to cure *C. elegans* following *Enterococcus faecalis* infection. Similar high-throughput *in vivo* assays have been used to screen for antimicrobials that are effective against other human pathogens, including the fungus *Candida albicans* (Breger *et al*., 2007; Okoli *et al*., 2009). These assays also provided the opportunity to assess the relative MIC, the effective concentration *in vivo* as well as the toxicity of these compounds in a single assay.

This work was envisaged to understand the potential of bacteriocins as biocontrol agents. During the course of this study, two potent bacteriocins, BS101 and BL8 capable of inhibiting the growth of many Gram positive pathogenic bacteria including *S. aureus* and *Cl. perfringens* were characterised. These small molecular weight peptides exhibited excellent properties like high stability and sturdiness under various physical and chemical exposures.

6. SUMMARY AND CONCLUSION

Pathogenic microorganisms such as *Bacillus cereus*, *Listeria monocytogenes* and *Staphylococcus* sp. have caused serious diseases, and consequently contributed to considerable economic loss in the food and agricultural industries. Antibiotics have been practically used to treat these pathogens since penicillin G was discovered more than half a century ago. Many different types of antibiotics have been discovered or synthesized to control pathogenic microorganisms. Repetitive use and misuse of antibiotics by the agricultural and pharmaceutical industries have caused the emergence of multidrug-resistant microorganisms, even to the strongest antibiotics currently available; therefore, the rapid development of more effective antimicrobial compounds is required to keep pace with demand.

Bacteria were isolated from marine water and sediment samples collected from various locations off the coast of Cochin and salt pans of Tuticorin using pour plate technique. One hundred and twelve isolates were obtained. Seventeen isolates exhibiting antimicrobial activity were segregated after primary screening. The secondary screening which was aimed at selection of bacteria that produce proteinaceous inhibitory compounds, helped to select five strains viz. BTFK101, BTHT8, BTKM4, BTEK16 and BTSB22.

The five isolates inhibited the growth of six Gram positive test organisms viz. *B. cereus*, *B. circulans*, *B. coagulans*, *B. pumilus*, *Staphylococcus aureus* and *Clostridium perfringens.* After quantitative estimation of the bacteriocin production, the two strains BTFK101 and BTHT8 were selected for further study.

Molecular identification of the five isolates using 16S ribotyping revealed the identity of the isolates as *Bacillus subtilis* and *Bacillus licheniformis*. The nucleotide sequences were submitted in GenBank and accession numbers obtained. The best bacteriocin producers in this study, BTFK101 and BTHT8 were identified as *B. subtilis* and *B. licheniformis* respectively.

Plasmids were not obtained from the two strains which suggest that the bacteriocin production is chromosomally encoded. The two strains were not hemolytic on blood agar plates indicating their non-pathogenic nature. Antibiotic susceptibility tests revealed that both the strains were sensitive to ciprofloxacin, gentamicin and trimethoprim. From the studies on correlation of growth and bacteriocin production of the two strains, it was clear that the bacteriocin production started at an early exponential phase and reached its peak at the stationary phase.

The bioprocess variables for bacteriocin production by strain BTFK101 and BTHT8 were optimised. Eleven factors were taken into account. The optimised conditions are given in Table 6.1.

Purification protocol of bacteriocin production included concentration and partial purification of the culture supernatant using ammonium sulphate. 30-60% fraction of ammonium sulphate precipitation inhibited the test organisms under study, indicating the presence of the bacteriocins in this fraction. This was followed by gel filtration chromatography which gave pure bacteriocins. The bacteriocins produced by BTFK101 and BTHT8 were designated as BS101 and BL8 respectively. BS101 was seven fold purified whereas BL8 was twenty nine fold purified after the purification process.

Table 6.1 Optimized bioprocess variables for bacteriocin production by BTFK101 and BTHT8

Bioprocess Variables	BTFK101	BTHT8
Media	Zobell marine broth	Zobell marine broth
NaCl Concentration	1.5%	1%
Carbon source	Pectin	Glucose
Inorganic nitrogen source	Ammonium sulphate	Ammonium sulphate
Organic Nitrogen source	Beef extract	Beef extract
Inoculum concentration	10%	6%
Surfactant (Tween 80)	Reduced production	0.1% promoted production
Initial pH of media	5	7
Incubation temperature	$30^{o}C$	$30^{o}C$
Incubation period	12 h	18 h
Agitation	125 rpm	100 rpm

The protein band inhibiting the growth of test organisms was determined using the method of overlaying the SDS-PAGE gel with test organisms (zymogram). From this experiment, it was confirmed that the bacteriocins produced by the two strains are low molecular weight peptides as clearing zone was obtained near the dye front by the inhibition of the test organism. After purification of the bacteriocins by gel filtration, single bands were obtained near the dye front which helped to confirm that the bacteriocins were completely purified.

The intact mass of BS101 was determined as 3.3 kDa and that of BL8 was determined as 1.4 kDa from the mass spectrum of MALDI-TOF mass spectrometry. The isoelectric points were determined as 5.7 for BS101 and 6.3 for BL8. The N-terminal amino acid sequencing of BL8 by automated Edman degradation analysis revealed a 13 amino acids sequence stretch: NH2-Ser-Trp-Ser-Cys-Cys-Gly-Asn-Cys-Ser-Ile-Ser-Gly-Ser-COOH. Comparison of this sequence to the sequences of bacteriocins from *Bacillus* sp. retrieved from the protein database of NCBI by multiple sequence alignment using Clustal W showed no significant similarity indicating its novelty.

The action of proteases on the bacteriocins was studied. The activity of the bacteriocins was reduced or lost when treated with proteases like proteinase K, pepsin and trypsin. BS101 was lost completely degraded when treated with proteinase K and trypsin whereas proteinase K and pepsin promoted complete degradation of BL8. 40 µg of proteinase K was required for complete degradation of BS101 whereas only 20 µg was required for BL8. This confirmed the proteinaceous nature of the bacteriocins.

The effect of temperature on the stability of BS101 revealed that BS101 was stable only up to 40°C. Above this temperature it started degrading and the activity was completely lost at 50°C and above. BL8 was more thermostable when compared to BS101. The activity of BL8 was reduced only when it was incubated at 50°C for one hour and complete degradation occurred at 60°C and above.

The bacteriocins were found stable at acidic pH whereas they were very unstable at alkaline pH. The activity of BS101 was completely lost when treated with buffers having pH 9 and above. For BL8 there was drastic reduction in activity when the pH reached 9 and above but complete degradation occurred only when it was exposed to pH 12.

The bacteriocins were stable in non-ionic detergents like tween 20, tween 80 and triton X-100. The ionic detergent like SDS reduced the activity of both the bacteriocins and CTAB completely deactivated them.

When exposed to ions of metals like Mg, Al, Fe, Zn, Na, Cu, Ni, Ca, Mn and Co, the activity of BS101 was not affected. But Ba and Cd ions increased the activity of BS101. For BL8 Mg, Al and Ba ions increased the activity. Mn and Co ions decreased the activity. The metal ions of Cd, Fe, Zn, Na, Cu, Ni, and Ca did not affect the activity of the bacteriocin.

The effect of two reducing agents DTT and β- mercaptoethanol were tested. The activity of BS101 reduced when it was exposed to β-mercaptoethanol at a concentration of 70 mM and above and to DTT at a concentration of 8 mM and above. In the case of BL8, the activity was reduced when exposed to β-mercaptoethanol at 200 mM level and above and to DTT at 20 mM and above. When the bacteriocins were exposed to oxidising agent (1-5% DMSO), the activity was not affected.

The MIC was determined as 42.75μg/mL for BS101 and it was 18.75 μg/mL for BL8 against *S. aureus*. Both the bacteriocins were bactericidal to *B. coagulans*, *B. circulans*, *B. cereus* and *S. aureus*, whereas they are bacteriostatic to *B. pumilus* and *Cl. perfringens.*

Application studies of the bacteriocins included control of biofilm formation, control of microflora of sea foods and studies on pathogenesis and prophylaxis in *C. elegans* (Bioassay using model organism). Both BS101 and BL8 inhibited the biofilm formation by *Cl. perfringens* and *B. cereus* which were experimentally proved as moderate biofilm producers. When the bacteriocins were

applied the OD_{570} of *Cl. perfringens* and *B. cereus* was reduced drastically. Thus it was experimentally proved that both the bacteriocins performed very well in the control of biofilm formation. When the fish samples were treated with BS101 and BL8, the microflora present in the sea foods were reduced efficiently which indicates their ability to aid biopreservation. The outcome of the bacteriocin prophylaxis experiments in *C. elegans* was promising in the case of BL8. When the bacteriocin was applied, the *S. aureus* and *B. circulans* infection was controlled and the mortality rate of infected worms was reduced. TD_{50} of worms infected with *B. circulans* followed by the treatment with BL8 was raised to near control level of 9.488 ± 0.231 days and TD50 of worms infected with *S. aureus* in presence of BL8 was also extended to 8.751± 0.169 days.

CONCLUSION

The two bacteriocins BS101 and BL8 from *B. subtilis* strain BTFK101 and *B. licheniformis* strain BTHT8 respectively were purified and characterized in the present study. These bacteriocins with low molecular weight are highly efficient, active at very low (in µg) concentrations, inhibiting an array of Gram positive pathogenic organisms including *S. aureus* and *Cl. perfringens* in the study, all of which are implicated either in gastroenteritis and / or food poisoning. *C. elegans* was explored as a model organism and used to test the therapeutic effect of BS101 and BL8 in the bioassay.

Food-grade bacteria producing antimicrobial peptides may present a greater opportunity for application in the food and agricultural industries. Currently, only nisin is approved by the US Food and Drug Administration for application as a natural preservative in food. Compared with bacteriocins produced by lactic acid bacteria, antimicrobial peptides from the genus *Bacillus* have been relatively less recognized despite their broad antimicrobial spectra. This

makes bacteriocins from *Bacillus* sp. promising for application in the food, agricultural and pharmaceutical industries. Consequently, it may be advantageous to study bacteriocins from *Bacillus* sp. for the development of more efficient, biologically safe antimicrobial compounds to replace those that cause serious side effects and the emergence of antibiotic-resistant pathogens.

The outcome of the experiments involving the application of these bacteriocins, indicate the various fields where these small molecules can be applied. Bacilli are generally recognized as safe (GRAS). The bacteriocins isolated from the bacilli in the present study, with their ability to withstand high temperatures and very low pH, enable their applications in food processing industry. Their role in the control of biofilm forming pathogens in food industry also supports this application. Their role in controlling the microflora of sea foods is also being highlighted and this could be exploited in sea food industry for biopreservation.

Thus to conclude, these bacteria with the ability to produce bacteriocins may be used in probiotic applications after suitable study.

7. REFERENCES

Aballay A, Yorgey P, Ausubel FM (2000): *Salmonella* Typhimurium proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. Current Biology **10**, 1539-1542.

Abriouel H, Franz CMAP, Omar NA, Galvez A (2011): Review article - Diversity and applications of *Bacillus* bacteriocins. FEMS Microbiology Reviews **35**, 201–232.

Ahern M, Verschueren S, van Sinderen D (2003): Isolation and characterisation of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. FEMS Microbiology Letters **163**, 229–236.

Alam SI, Kamran M, Sohail M, Ahmad A, Khan SA (2011): Partial characterization of bacteriocin like inhibitory substance from *Bacillus subtilis* BS15, a local soil isolate. Pakistan Journal of Botany **43**, 2195–2199.

Alexopoulos C, Georgoulakis IE, Tzivara A, Kyriakis CS, Govaris A, Kyriakis SC (2004): Field evaluation of the effect of a probiotic containing *Bacillus licheniformis* and *Bacillus subtilis* spores on the health status, performance, and carcass quality of grower and finisher pigs. Journal of Veterinary medicine, Physiology, Pathology and Clinical medicine **51**, 306-312.

Altena K, Guder A, Cramer C, Bierbaum G (2000): Biosynthesis of the lantibiotic mersacidin: organization of a type B lantibiotic gene cluster. Applied and Environmental Microbiology **66**, 2565–2571.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990): Basic local alignment search tool. Journal of Molecular Biology **215**, 403–410.

Andersson A, Ronner U, Granum PE (1995): What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*? International Journal of Food Microbiology **28**, 145–155.

Ansari A, Aman A, Siddiqui NN, Iqbal S, Ali ul Qader S (2012): Bacteriocin (BAC-IB17): Screening, isolation and production from *Bacillus subtilis* KIBGE IB-17. Pakistan Journal of Pharmaceutical Sciences **25**, 195-201.

Anthony T, Chellappa GS, Rajesh T, Gunasekaran P (2010): Functional analysis of a putative holin-like peptide-coding gene in the genome of *Bacillus licheniformis* AnBa9. Archives of Microbiology **192**, 51–56.

Anthony T, Rajesh T, Kayalvizhi N, Gunasekaran P (2009): Influence of medium components and fermentation conditions on the production of bacteriocin(s) by *Bacillus licheniformis* AnBa9. Bioresource Technology **100**, 872–877.

Araya M, Morelli L, Reid G, Sanders ME, Stanton C (2002): Joint FAO/WHO working group report on guidelines for the evaluation of probiotics in food. Ontario, London.

Ardhana MM, Fleet GH (2003): The microbial ecology of cocoa bean fermentations in Indonesia. International Journal of Food Microbiology **86**, 87– 99.

Asaduzzaman SM, Sonomoto K (2009): Lantibiotics: Diverse activities and unique modes of action. Journal of Bioscience and Bioengineering **107**, 475-487.

Aunpad R, Na-Bangchang K (2007): Pumilicin 4, a novel bacteriocin with anti-MRSA and anti-VRE activity produced by newly isolated bacteria *Bacillus pumilus* strain WAPB4. Current Microbiology **55**, 308–313.

Aunpad R, Na-Bangchang K, Pipatsatitpong D (2007): Bacteriocins with anti-MRSA activity produced by water and soil isolated bacteria. Annals of Microbiology **57**, 9–14.

Ausubel FM, Brent R, Kingston RE, Moore DD, Smith JA, Seidman JG, Struhl K (1987): Current Protocols in Molecular Biology. Greene Publishing Associates & Wiley Interscience, New York.

Aymerich T, Artigas MG, Garriga M, Monfort JM, Hugas M (2000): Effect of sausage ingredients and additives on the production of enterocin A and B by *Enterococcus faecium* CTC492. Optimization of *in vitro* production and anti-listerial effect in dry fermented sausages. Journal of Applied Microbiology **88,** 686–694.

Babasaki K, Takao T, Shimonishi Y, Kurahashi K (1985): Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: Isolation, structural analysis and biogenesis. Journal of Biochemistry **98**, 585–603.

Bai Y, D'Aoust F, Smith DL, Driscoll BT (2002): Isolation of plant growth-promoting *Bacillus* strains from soybean root nodules. Canadian Journal of Microbiology **48**, 230–238.

Bai Y, Zhou X, Smith DL (2003): Enhanced soybean plant growth resulting from co-inoculation of *Bacillus* strains with *Bradyrhizobium japonicum.* Crop Science **43**, 1774–1781.

Balla E, Dicks LMT, Du Toit M, van der Merwe MJ, Holzapfel WH (2000): Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by *Enterococcus faecalis* BFE 1071. Applied and Environmental Microbiology **66**, 1298–1304.

Banerjee S, Hansen JN (1988): Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. Journal of Biological Chemistry **263**: 9508–9514.

Barbosa TM, Serra CR, La Ragione RM, Woodward MJ, Henriques AO (2005): Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Applied and Environmental Microbiology **71**, 968–978.

Barboza-Corona JE, Vazquez-Acosta H, Bideshi DK, Salcedo-Hernandez R (2007): Bacteriocin-like inhibitor substances produced by Mexican strains of *Bacillus thuringiensis*. Archives of Microbiology **187**, 117–126.

Barefoot SF, Klaenhammer TR (1983): Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus.* Applied and Environmental Microbiology **45**, 1808-1815.

Barefoot SF, Klaenhammer TR (1984): Purification and characterization of the *Lactobacillus acidophilus* bacteriocin lactacin B. Antimicrobial Agents and Chemotherapy **26**, 328-334.

Bauer AW, Kirby WMM, Sherris JC, Turck M (1966): Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology **36**: 493-496.

Beard PJ, Meadowcroft NF (1935): Survival and rate of death of intestinal bacteria in sea water. American Journal of Public Health **25**, 1023-1026.

Begley M, Cotter PD, Hill C, Ross RP (2009): Identification of a novel two-peptide lantibiotic, lichenicidin, following rational genome mining for LanM proteins. Applied and Environmental Microbiology **75**, 5451–5460.

Bhunia AK, Johnson MC, Ray B (1988): Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. The Journal of Applied Bacteriology **65**, 261- 268.

Bhunia AK, Johnson MG (1992): A modified method to directly detect in SDS-PAGE the bacteriocin of *Pediococcus acidilactici*. Letters in Applied Microbiology **15**, 5–7.

Bierbaum G, Brotz H, Koller KP, Sahl HG (1995): Cloning, sequencing and production of the lantibiotic mersacidin. FEMS Microbiology Letters **127**, 121–126.

Bilev AE (2002): Comparative evaluation of probiotic activity in respect to *in vitro* pneumotropic bacteria and pharmaco dynamics of biosporin-strain producers in patients with chronic obstructive pulmonary diseases. Voenno-Meditsinskii Zhurnal **323**, 54-57.

Birnboim H, Doly J (1979): A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research **7**, 1513–1523.

Bizani D, Brandelli A (2002): Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. strain 8A. Journal of Applied Microbiology **93**, 512–519.

Bizani D, Brandelli A (2004): Influence of media and temperature on bacteriocin production by *Bacillus cereus* 8A during batch cultivation. Applied Microbiology and Biotechnology **65**, 158–162.

Bizani D, Dominguez APM, Brandelli A (2005a): Purification and partial chemical characterization of the antimicrobial peptide cerein 8A. Letters in Applied Microbiology **41**, 269–273.

Bizani D, Motta AS, Morrissy JAC, Terra RM, Souto AA, Brandelli A (2005b): Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by *Bacillus cereus*. International Microbiology **8**, 125–131.

Bizani D, Morrissy JAC, Domingue APM, Brandelli A (2008): Inhibition of *Listeria monocytogenes* in dairy products using the bacteriocin-like peptide cerein 8A. International Journal of Food Microbiology **121**, 229–233.

Blum H, Beier H, Gross H J (1987): Silver staining of proteins in polyacrylamide gels. Electrophoresis **8**, 93-99.

Bobbie RJ, White DC (1980): Characterization of benthic microbial community structure by high solution gas chromatography of fatty acid methyl esters. Applied and Environmental Microbiology **39**, 1212–1222.

Boe (1996): Evaluation of optimum production for bacteriocin from *Lactobacillus* sp. JB 42 isolation from Kimichi. Journal of Microbiology and Biotechnology **6**, 63-67.

Bolhuis A, Venema G, Quax WJ, Bron S, van Dijl JM (1999): Functional analysis of paralogous thiol-disulfide oxidoreductases in *Bacillus subtilis*. Journal of Biological Chemistry **274**, 24531–24538.

Bouksaim M, Lacroix C, Bazin R, Simard RE (1999): Production and utilization of polyclonal antibodies against nisin in an ELISA and for immunolocation of nisin in producing and sensitive bacterial strains. Journal of Applied Microbiology **87**, 500-510.

Bradford MM (1976): A dye binding assay for protein. Analytical Biochemistry **72**, 248–254.

Breger J, Fuchs BB, Aperis G, Moy TI, Ausubel FM, Mylonakis E (2007): Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. PLOS Pathogens **3**, e18.

Brenner S (1974): Genetics of *Caenorhabditis elegans*. Genetics **77**, 71-94.

Breukink E, Wiedemann I, van Kraaij C, Kuipers OP, Sahl H, de Kruijff B (1999): Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science **286**, 2361–2364.

Breyers JD, Ratner JP (2004): Bioinspired implant materials befuddle bacteria. ASM News **70**, 232–237.

Brotz H, Bierbaum G, Leopold K, Reynolds PE, Sahl HG (1998): The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. Antimicrobial Agents and Chemotherapy **42**, 154–160.

Bruelink E, van Kraaij C, Demel RA, Siezen RJ, Kuipers OP, de Kruijff B (1997): The C-terminal region of nisin is responsible for the initial interaction of nisin with the target membrane. Biochemistry **36**, 6968−6976.

Brul S, Coote P (1999): Preservative agents in food. Mode of action and microbial resistance mechanisms. International Journal of Food Microbiology **50**, 1−17.

Burtt EH, Ichida JM (1999): Occurrence of feather degrading bacilli in the plumage of birds. Auk **116**, 364-372.

Byrom D (1992): Production of polyhydroxybutyrate: polyhydroxyvalerate copolymers. FEMS Microbiology Reviews **103**, 247-250.

Callewaert R, De Vuyst L (1999): Expanded bed adsorption as a unique unit operation for the isolation of bacteriocins from fermentation media. Bioseparation **8**, 159–168.

Callewaert R, Holo H, Devreese B, Van Beeumen J, Nes I, De Vuyst L (1999): Characterization and production of amylovorin L471, a bacteriocin purified from *Lactobacillus amylovorus* DCE 471 by a novel three-step method. Microbiology **145**, 2559–2568.

Calo-Mata P, Arlindo S, Boehme K, de Miguel T, Pascoal A, Barros-Velazquez J (2008): Current applications and future trends of lactic acid bacteria and their bacteriocins for the biopreservation of aquatic food products. Food Bioprocess Technology **1**, 43-63.

Carpenter LV, Settr LR, Weinberg M (1938): Chloramine treatment of sea water. American Journal of Public Health **28**, 929-934.

Casaus P, Nilsen T, Cintas LM, Nes IF, Hernandez PE, Holo H (1997): Enterocin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin. Applied Microbiology **143**, 2287-2294.

Cazorla FM, Romero D, Perez-Garc A, Lugtenberg BJJ, Vicente AD, Bloemberg G (2007): Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. Journal of Applied Microbiology **103**, 1950- 1959.

Cetinkaya S, Osmanagaoglu O, Cokmus C (2003): Bacteriocin diversity in *Bacillus sphaericus*. Folia Microbiologica **48**, 157–161.

Chan WC, Bycroft BW, Leyland ML, Lian L-Y, Roberts GCK (1993): A novel posttranslational modification of the peptide antibiotic subtilin: isolation and characterization of a natural variant from *Bacillus subtilis* ATCC 6633. Journal of Biochemistry **291**, 23–27.

Chatterjee S, Chatterjee S, Lad SJ, Phansalkar MS, Rupp RH, Ganguli BN, Fehlhaber HW, Kogler H (1992): Mersacidin, a new antibiotic from *Bacillus:* fermentation, isolation, purification and chemical characterization. Journal of Antibiotics **45**, 832–838.

Chen J, Rossman ML, Pawar DM (2007): Attachment of enterohemorragic *Escherichia coli* to the surface of beef and a culture medium. Journal of Food Science and Technology **40**, 249–254.

ChenY, Ludescher RD, Montville TJ (1997 a): Electrostatic interactions, but not the YGNGV consensus motif, govern the binding of pediocin PA-1 and its

fragments to phospholipid vesicles. Applied and Environmental Microbiology **63**, 4770–4777.

ChenY, Shapira R, Eisenstein M, Montville TJ (1997 b): Functional characterization of pediocin PA-1 binding to liposomes in the absence of a protein receptor and its relationship to a predicted tertiary structure. Applied and Environmental Microbiology **63**, 524–531.

Cherif A, Chehimi S, Limem F, Hansen BM, Hendriksen NB, Daffonchio D, Boudabous A (2003): Detection and characterization of the novel bacteriocin entomocin 9 and safety evaluation of its producer, *Bacillus thuringiensis* subsp. *entomocidus* HD9. Journal of Applied Microbiology **95**, 990-1000.

Cherif A, Ouzari H, Daffonchio D, Cherif H, Ben Slama K, Hassen A, Jaoua S, Boudabous A (2001): Thuricin 7: A novel bacteriocin produced by *Bacillus thuringiensis* BMG1.7, a new strain isolated from soil. Letters in Applied Microbiology **32**, 243–247.

Cherif A, Rezgui W, Raddadi N, Daffonchio D, Boudabous A (2008): Characterization and partial purification of entomocin 110, a newly identified bacteriocin from *Bacillus thuringiensis* subsp. *entomocidus* HD110. Microbiological Research **163**, 684–692.

Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM, Beachey EH (1985): Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for adherence of staphylococci to medical devices. Journal of Clinical Microbiology **22**, 996-1006.

Ciffo F, Dacarro C, Giovanetti M, Mazza PG (1987): Gastric resistance of *Bacillus subtilis* spores used in oral bacteriotherapy: *in vitro* studies. Farmacia Terapia **4**, 163-169.

Cintas LM, Casaus P, Havarstein LS, Hernandez PE, Nes IF (1997): Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Applied and Environmental Microbiology **63**, 4321–4330.

Cintas LM, Casaus P, Herranz C, Havarstein LS, Holo H, Hernandez PE (2000): Biochemical and genetic evidence that *Enterococcus faecium* L50 produces enterocins L50A and L50B, the sec-dependent enterocin P and a novel bacteriocin secreted without an N-terminal extension termed enterocin Q. Journal of Bacteriology **182**, 6806–6814.

Cintas LM, Casaus P, Holo H, Hernandez PE, Nes IF, Havarstein LS (1998): Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to staphylococcal hemolysins. Journal of Bacteriology **180**, 1988–1994.

Cladera-Olivera F, Caron GR, Brandelli A (2004): Bacteriocin like substance production by *Bacillus licheniformis* strain P40. Letters in Applied Microbiology **38**, 251–256.

Cladera-Olivera F, Lorenzini DM, Tsai S-M, Brandelli A (2006): Purification and partial characterization of an antimicrobial peptide produced by *Bacillus* sp. strain P45, a bacterium from the Amazon basin fish *Piaractus mesopotamicus*. Journal of General and Applied Microbiology **52**, 357–363.

Claus D, Berkeley RCW (1986): Genus *Bacillus* Cohn 1872. In Sneath PHA, Mair NS, Sharpe ME, Holt JG (Eds.): Bergey's Manual of Systematic Bacteriology.Vol **2**. Williams and Wilkins Co., Baltimore, MD, pp. 1105-1139.

Cleveland J, Montville TJ, Nes IF, Chikindas ML (2001): Bacteriocins: safe natural antimicrobials for food preservation. International Journal of Food Microbiology **71**, 1–20.

Cohn O (1872): Untersuchungen über Bakterien. Beitrage zur Biologie der Pflanzen Heft **2**, 127-224.

Cokmus C, Yousten AA (1993): Bacteriocin production by *Bacillus sphaericus*. Journal of Invertebrate Pathology **61**, 323–325.

Collings JM, Phillips CA (2001): Bacteriocin- Like inhibition of *Bacillus subtilis* by an indigenous reed bed bacterium. World Journal of Microbiology and Biotechnology **17**, 321-324.

Collins JK, Thornton G, Sullivan GO (1998): Selection of probiotic strains for human applications. International Dairy Journal **8**, 487-490.

Corvey C, Stein T, Dusterhus S, Karas M, Entian KD (2003): Activation of subtilin precursors by *Bacillus subtilis* extracellular serine proteases subtilisin (AprE), WprA, and Vpr. Biochemical and Biophysical Research Communications **304**, 48–54.

Cotter PD, Hill C, Ross RP (2005): Bacterial lantibiotics: strategies to improve therapeutic potential. Current Protein and Peptide Science **6**, 61-75.

Crandall AD, Montville TJ (1998): Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Applied and Environmental Microbiology **64**, 231-237.

Culter RR, Wilson P, Clarke FV (1999): Evaluation of a radiometric method for studying bacterial activity in the presence of antimicrobial agents. Journal of Applied Bacteriology **66**, 515-522.

Czaran TL, Hoekstra RF, Pagie L (2002): Chemical warfare between microbes promotes biodiversity. Proceedings of National Academy of Sciences USA **99**, 786–790.

Daba H, Lacroix C, Huang J, Simard RE (1993): Influence of growth conditions on production and activity of mesenterocin 5 by a strain of *Leuconostoc mesenteroides.* Applied Microbiology and Biotechnology **39**, 166-173.

Daini OA, Akana SA (2009): Plasmid-mediated antibiotic resistance in *Staphylococcus aureus* from patients and non-patients. Scientific Research and Essay **4**, 346-350.

Darby C (2005): Interactions with microbial pathogens. In Fitch DHA (Ed.): WormBook, The *C. elegans* Research Community, doi/10.1895/ wormbook.1.21.1, http://www.wormbook.org

de Boer AS, Priest F, Diederchsen B (1994): On the industrial use of *Bacillus licheniformis* a review. Applied Microbiology and Biotechnology **40**, 595-598.

de Boer AS, Diderichsen B (1991): On the safety of *Bacillus subtilis* and *B. amyloliquefaciens*: a review. Applied Microbiology and Biotechnology **36**, 1-4.

De Giaxa (1889): About the behavior of some pathogenic micro-organisms in the sea water. Zentralblatt fur Hygiene Infectious Diseases **6**, 162-225.

De la Rosa-Garcia SA, Munoz-Garcia AA, Barahona-Perez LF, Gamboa-Angulo MM (2007): Antimicrobial properties of moderately halotolerant bacteria from cenotes of the Yucatan peninsula. Letters in Applied Microbiology **45**, 289–294.

De Vuyst L, Vandamme EJ (1992): Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentations. Journal of General Microbiology **138**, 571–5786.

De Vuyst L, Moreno FMR, Revets H (2003): Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. International Journal of Food Microbiology **84**, 299–318.

Diaz D (2007): Effect of *Bacillus amyloliquefaciens* CECT-5940 spores on broiler performance and digestibility. Available at http://en.engormix.com/MA-poultry-industry/articles/ effect-bacillus-amyloliquefaciens-cect5940_795.htm

Diep DB, Havarstein LS, Nes IF (1995): A bacteriocin-like peptide induces bacteriocin synthesis in *Lb. plantarum* C11. Molecular Microbiology **18**, 631-639.

Diep DB, Havarstein LS, Nes IF (1996): Characterization of the locus responsible for the bacteriocin production in *Lactobacillus plantarum* C11. Journal of Bacteriology **178**, 4472–4483.

Dischinger J, Josten M, Szekat C, Sahl HG, Bierbaum G (2009): Production of the novel two-peptide lantibiotic lichenicidin by *Bacillus licheniformis* DSM 13. Plos One 4: e6788. DOI: 10.1371/journal.pone.0006788.

Dobson A, Cotter PD, Ross RP, Hill C (2012): Minireview Bacteriocin Production: a Probiotic Trait? Applied and Environmental Microbiology **78**, 1-6.

Dodds PN, Rathjen JP (2010): Plant immunity: towards an integrated view of plant-pathogen interactions. Nature Reviews Genetics **11**, 539-548.

Dominguez APM, Bizani D, Cladera-Olivera F, Brandelli A (2007): Cerein 8A production in soybean protein using response surface methodology. Biochemical Engineering Journal **35**, 238–243.

Dorenbos R, Stein T, Kabel J, Bruand C, Bolhuis A, Bron S, Quax WJ, van Dijl JM (2002): Thiol-disulfide oxidoreductases are essential for the production of the lantibiotic sublancin 168. Journal of Biological Chemistry **277**, 16682–16688.

Duan K, Harvey ML, Liu CQ, Dunn NW (1996): Identification and characterization of a mobilizing plasmid, pND300, in *Lactococcus lactis* M189 and its encoded nisin resistance determinant. Journal of Applied Bacteriology **81**, 493–500.

Dubois JYF, Kouwen TRHM, Schurich AKC, Reis CR, Ensing HT, Trip EN, Zweers JC, van Dijl JM (2009): Immunity to the bacteriocin sublancin 168 is

determined by the SunI (YolF) protein of *Bacillus subtilis*. Antimicrobial Agents and Chemotherapy **53**, 651–661.

Edman P, Erik H, Lars Gunnar S, Per-Olof K (1950): Method for determination of the amino acid sequence in peptides. Acta Chemica Scandinavica **4**, 283–293.

EFSA (2004): Opinion of the scientific panel on additives and products or substances used in animal feed (FEEDAP) on the efficacy of product Toyocerin for pigs in fattening. EFSA Journal **62**, 1-5.

EFSA (2005): Scientific opinion of the panel on additives and products or substances used in animl feed (FEEDAP) on the modification of terms of authorisation of the micro-organism preparation of *Bacillus cereus* var. *toyoi* (NCIMB 40112/CNCM I-1012) (Toyocerins) authorised as a feed additive in accordance with Directive 70/524/EEC. EFSA Journal **288**, 1-7.

EFSA (2007 a): Introduction of a qualified presumption of safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA Journal **587**, 1-16.

EFSA (2007 b): Scientific opinion of the panel on additives and products or substances used in animl feed (FEEDAP) on the safety and efficacy of Toyocerins (*Bacillus cereus* var. *toyoi*) as feed additive for turkeys. EFSA Journal **549**, 1–11.

EFSA (2008): The maintenance of the list of QPS microorganisms intentionally added to foods or feeds. Scientific opinion of the panel on biological hazards. EFSA Journal **923**, 1–48.

Ehrmann MA, Remiger A, Eijsink VG, Vogel RF (2000): A gene cluster encoding plantaricin 1.25 beta and other bacteriocin- like peptides in *Lactobacillus plantarum* TMW1.25. Biochimica et Biophysica Acta **1490**, 355–361.

Einarsson H, Lauzon HL (1995): Biopreservation of brined shrimp (*Pandalus borealis*) by bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology **61**, 669- 676.

El-hamshary OIM, Khattab AA (2008): Evaluation of Antimicrobial Activity of *Bacillus subtilis* and *Bacillus cereus* and Their Fusants Against *Fusarium solani*. Research Journal of Cell and Molecular Biology **2**, 24-29.

El- Sersy NA, Hassan AH, Ebrahim AH, Abou- Elela GM (2009): Response surface methodology as a tool for optimising the production of antimicrobial agents from *Bacillus licheniformis* SN2. Current research in bacteriology **3**, 1-14.

El-Shouny W, Amal AK, Suzan R (2013): Characterization of the partially purified Plantarcin SR18 produced by *Lactobacillus plantarum* SR18. Journal of Microbiology, Biotechnology and Food science **2**, 2301 – 2305.

Enan G, El-Essawy AA, Uyttendaele M, Debeverea J (1996): Antibacterial activity of *Lactobacillus plantarum* UGl isolated from dry sausage: Characterization, production and bactericidal action of plantaricin UG1. International Journal of Food Microbiology **30**, 189-215.

Endres JR, Clewell A, Jade KA, Farber T, Hauswirth J, Schauss AG (2009): Safety assessment of a proprietary preparation of a novel probiotic, *Bacillus coagulans*, as a food ingredient. Food and Chemical Toxicology **47**, 1231-1238.

Engelke G, Gutowski-Eckel Z, Hammelmann M, Entian KD (1992): Biosynthesis of the lantibiotic nisin: genomic organization and membrane localization of the NisB protein. Applied and Environmental Microbiology **58**, 3730-3743.

Englard S, Seifter S (1990): Precipitation Techniques. Methods in enzymology **182**, 285-300.

Errington J (2003): Regulation of endospore formation in *Bacillus subtilis*. Nature Reviews Microbiology **1**, 117-126.

Ewbank JJ, Zugasti O (2011): *C. elegans*: model host and tool for antimicrobial drug discovery. Disease Models & Mechanisms **4**, 300-304.

Faheem F, Saeed S, Rasool SA (2007): Studies on Brevicin AF01: a bacteriocin like inhibitory substance active against methicillin resistant *Staphylococcus aureus*. Pakistan Journal of Botany **39**, 1293–1302.

Fajardo A, Martinez JL (2008): Antibiotics as signals that trigger specific bacterial responses. Current Opinions in Microbiology **11**, 161–167.

Fall R, Kinsinger RF, Wheeler KA (2004): A simple method to isolate biofilm-forming *Bacillus subtilis* and related species from plant roots. Systematic and Applied Microbiology **27**, 372–379.

Faulkner DJ (2001): Marine natural products. Natural Product Reports **18**, 1-49.

Favret ME, Yousten AA (1989): Thuricin: the bacteriocin produced by *Bacillus thuringiensis*. Journal of Invertebrate Pathology **53**, 206–216.

Fawcett P, Eichenberger P, Losick R, Youngman P (2000): The transcriptional profile of early to middle sporulation in *Bacillus subtilis*. Proceedings of the National Academy of Sciences of USA **97**, 8063–8068.

Ferchichi M, Frere J, Mabrouk K, Manai M (2001): Lactococcin MMFII, a novel class IIa bacteriocin produced by *Lactococcus lactis* MMFII, isolated from a Tunisian dairy product. FEMS Microbiology Letters **205**, 49-55.

Ferrandon D, Imler JL, Hetru C, Hoffmann JA (2007): The Drosphila systemic immune response: sensing and signalling during bacterial and fungal infections. Nature Reviews Immunology **7**, 862-874.

Fikes JD, Crabtree BL, Barridge BD (1983): Studies on the mode of action of a bacteriocin produced by *Bacillus stearothermophilus*. Canadian Journal of Microbiology **29**, 1576–1582.

Flint SH, Bremer PJ, Brooks JD (1997): Biofilms in dairy manufacturing plant - description, current concerns and methods of control. Biofouling **11**, 81–97.

Floriano B, Ruiz-Barba JL, Jimenez-Diaz R (1998): Purification and genetic characterization of enterocin I from *Enterococcus faecium* 6T1, a novel antilisterial plasmid-encoded bacteriocin which does not belong to the pediocin family of bacteriocins. Applied and Environmental Microbiology **64**, 4883- 4890.

Frangsmyr T (2003): Les Prix Nobel. In Frangsmyr T (Ed.): The Nobel Prizes 2002. The Nobel Foundation, Stockholm, Sweden.

Frank JF, Ehlers J, Wicker L (2003): Removal of *Listeria monocytogenes* and poultry soil-containing biofilms using chemical cleaning and sanitizing agents under static conditions. Food Protection Trends **23**, 654–663.

Franke CM, Leenhouts KJ, Haandrikman AJ, Kok J, Venema G, Venema K (1996): Topology of LcnD, a protein implicated in the transport of bacteriocins from *Lactococcus lactis*. Journal of Bacteriology **178**, 1766–1769.

Friedrich CL, Moyles D, Beveridge TJ, Hancock RE (2000): Antibacterial action of structurally diverse cationic peptides on Gram-positive bacteria. Antimicrobial Agents and Chemotherapy **44**, 2086–2092.

Galvez A, Lopez RL, Abriouel H, Valdivia E, Ben Omar N (2008): Application of bacteriocins in the control of food borne pathogenic and spoilage bacteria. Critical Reviews in Biotechnology **28**, 125-152.

Galvez A, Valdivia E, Gonzalez-Segura A, Lebbadi M, Martinez Bueno M, Maqueda M (1993): Purification, characterization and lytic activity against *Naegleria fowleri* of two amoebicins produced by *Bacillus licheniformis* A12. Applied and Environmental Microbiology **59**, 1480–1486.

Garsin DA, Sifri CD, Mylonakis E, Qin X, Singh KV, Murray BE, Calderwood SB, Ausubel FM (2001): A simple model host for identifying Gram positive virulence factors. Proceedings of the National Academy of Sciences of USA **98**, 10892-10897.

Garver KI, Muriana PM (1994): Purification and partial amino acid sequence of Curvaticin FS47, a heat-stable bacteriocin produced by *Lactobacillus curvatus* FS47. Applied and Environmental Microbiology **60**, 2191–2195.

Gebhardt K, Schimana J, Muller J (2002): Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. FEMS Microbiology Letters **217**, 199–205.

Geis A, Singh J, Teuber M (1983): Potential of lactic streptococci to produce bacteriocin. Applied and Environmental Microbiology **45**, 205-211.

Ghorbel B, Kamoun A S, Nasri M. (2003): Stability studies of protease from *Bacillus cereus* BG1. Enzyme and Microbial Technology **32**, 513-518.

Gillor O, Ghazaryan L (2007): Recent advances in bacteriocin application as antimicrobials. Recent Patents on Anti-Infective Drug Discovery. **2**, 115–122.

Gillor O, Giladi I, Riley MA (2009): Persistence of colicinogenic *Escherichia coli* in the mouse gastrointestinal tract. BMC Microbiology **9**, 165.

Giraffa G, Neviani E, Veneroni A (1990): Use of conductance to detect bacteriocin activity. Journal of Food Protection **53**, 772-776.

Gobbetti M, De Angelis M, Di Cagno R, Minervini F, Limitone A (2007): Cell-cell communication in food related bacteria. International Journal of Food Microbiology **120**, 34 – 45.

Gordon RE, Haynes WC, Pang CHN (1973): Genus *Bacillus*. In Agricultural Handbook Number 427, US Department of Agriculture, Washington DC.

Gratia A (1925): On unremarquable example antagonism between deuxsouches of *E. coli*. Rendered accounts of meetings of the Society of Biology and its Subsidiaries **93**, 1040-1041.

Gray EJ, Di Falco M, Souleimanov A, Smith DL (2006 a): Proteomic analysis of the bacteriocin thuricin 17 produced by *Bacillus thuringiensis* NEB17. FEMS Microbiology Letters **255**, 27–32.

Gray EJ, Lee KD, Souleimanov AM, Di Falco MR, Zhou X, Ly A, Charles TC, Driscoll BT, Smith DL (2006 b): A novel bacteriocin, thuricin 17, produced by plant growth promoting rhizobacteria strain *Bacillus thuringiensis* NEB17: isolation and classification. Journal of Applied Microbiology **100**, 545–554.

Gross E, Kiltz HH, Nebelin E (1973): Subtilin VI: Structure of subtilin. Hoppe-Seyler's Zeitschrift fur Physiologische Chemie **354**, 810–822.

Guder A, Schmitter T, Wiedemann I, Sahl HG, Bierbaum G (2002): Role of the single regulator MrsR1 and the two component system MrsR2/K2 in the regulation of mersacidin production and immunity. Applied and Environmental Microbiology **68**, 106–113.

Guder A, Wiedemann I, Sahl HG (2000): Post translationally modified bacteriocins: the lantibiotics. Biopolymers **55**, 62–73.

Hagstrom A, Pinhassi J, Zweifel UL (2000): Biogeographical diversity among marine bacterioplankton. Aquatic Microbial Ecology **21**, 231–244.

Halimi B, Dortu C, Arguelles-Arias A, Thonart P, Joris B, Fickers P (2010): Antilisterial Activity on poultry meat of amylolysin, a bacteriocin from *Bacillus amyloliquefaciens* GA1. Probiotics and Antimicrobial Proteins **2**, 120–125.

Hall TA (1999): Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series **41**, 95-98.

Hammami I, Rhouma A, Jaouadi B, Rebai A, Nesme X (2009): Optimization and biochemical characterization of a bacteriocin from a newly isolated *Bacillus subtilis* strain 14B for biocontrol of *Agrobacterium* spp. strains. Letters in Applied Microbiology **48**, 253–260.

Hancock REW, Falla T, Brown M (1995): Cationic bacteriocidal peptides. Advances in Microbial Physiology **37**, 135–175.

Hara T, Ueda S (1982): Regulation of polyglutamate in *Bacillus subtilis* (natto): transformation of high PGA productivity. Agricultural and Biological Chemistry **46**, 2275–2281.

Hasan HG, Arif HH, Musleh RM (2012): Partial purification of bulgaricin antibacterial from *Lactobacilli* isolates in Iraqi Kurdish dairy product. International Proceedings of Chemical, Biological and Environmental Engineering. Vol **48**. IACSIT Press, Singapore.

Havarstein LS, Holo H, Nes IF (1994): The leader peptide of colicin V shares consensus sequences with leader peptides that are common among peptide bacteriocins produced by Gram positive bacteria. Microbiology **140**, 2383–2389.

He L, Chen W, Liu Y (2006): Production and characterization of bacteriocin – like peptides by *Bacillus licheniformis* ZJU12. Microbiological Research **161**, 321-326.

He Z, Kisla D, Zhang L, Yuan C, Green-Church KB, Yousef AE (2007): Isolation and identification of a *Paenibacillus polymyxa* strain that coproduces a novel lantibiotic and polymyxin. Applied and Environmental Microbiology **73**, 168–178.

Henry CM (2000): Antibiotic resistance. Chemical & Engineering News **6**, 41–58.

Herranz C, Martinez JM, Rodriguez JM, Hernandez PE, Cintas LM (2001): Optimization of enterocin P production by batch fermentation of *Enterococcus faecium* P13 at constant pH. Applied Microbiology and Biotechnology **56**, 378–383.

Hill C, Rea M, Ross P (2009): Thuricin CD, an antimicrobial for specifically targeting *Clostridium difficile*. Patent: WO2009068656-A1 13 04-JUN-2009; TEAGASC, The Agriculture and Food Development Authority (IE), University College, Cork-National University of Ireland, Cork (IE).

Holland IB, Roberts CF (1964): Some properties of a new bacteriocin formed by *Bacillus megaterium*. Journal of General Microbiology **35**, 271–285.

Hong HA, Huang JM, Khaneja R, Hiep LV, Urdaci MC, Cutting SM (2008): The safety of *Bacillus subtilis* and *Bacillus indicus* as food probiotics. Journal of Applied Microbiology **105**, 510-520.

Hoover DG, Harlander SK (1993): Screening methods for detecting bacteriocin activity. In Hoover DG, Steenson LR (Eds.): Bacteriocins of Lactic Acid Bacteria. Academic Press, NewYork, London, pp. 23- 39.

Horner T, Ungerman V, Zfihner H, Fiedler HP, Utz R, Kellner R, Jung G (1990): Comparative studies on the fermentative production of lantibiotics by staphylococci. Applied Microbiology and Biotechnology **32**, 511-517.

Horner T, Zfihner H, Kellner R, Jung G (1989): Fermentation and isolation of epidermin, a lanthionine containing polypeptide antibiotic from *Staphylococcus epidermidis.* Applied Microbiology and Biotechnology **30**, 219-225.

Hosoi T, Kiuchi K (2003): Natto - a food made by fermenting cooked soybeans with *Bacillus subtilis* (natto). In Farnworth ER (Ed.): Handbook of Fermented Functional Foods. CRC Press, Boca Raton, FL, pp. 227–245.

Hosoi T, Kiuchi K (2004): Production and probiotic effects of Natto. In Ricca E, Henriques AO, Cutting SM (Eds.): Bacterial spore formers: Probiotics and emerging applications. Horizon Bioscience Publishers, Wymondham, UK, pp. 143-154.

Huot E, Barrena-Gonzalez C, Petitdemange H (1996): Tween 80 effect on bacteriocin synthesis by *Lactococcus lactis subsp. cremoris* J46. Letters in Applied Microbiology **22**, 307–310.

Hyronimus B, Le Marrec C, Urdaci MC (1998): Coagulin, a bacteriocin-like inhibitory substance produced by *Bacillus coagulans* I4. Journal of Applied Microbiology **85**, 42–50.

Hyung MJ, Kwang-Soo K, Jong-Hyun P, Myung-Woo B, Young-Bae K, Han-Joon H (2001): Bacteriocin with a broad antimicrobial spectrum, produced by

Bacillus sp. isolated from Kimchi. Journal of Microbiology and Biotechnology **11**, 577–584.

Ingram L (1970): A ribosomal mechanism for synthesis of peptides related to nisin. *Biochimica et Biophysica Acta* **224**, 263–265.

Ingram LC (1969): Synthesis of the antibiotic nisin: formation of lanthionine and beta-methyl-lanthionine. *Biochimica et Biophysica Acta* **184**, 216–219.

Irazoqui JE, Urbach JM, Ausubel FM (2010): Evolution of host innate defence: insights from *Caenorhabditis elegans* and primitive invertebrates. Nature Reviews Immunology **10**, 47-58.

Isenberg HD (1992): Interpretation of aerobic bacterial growth on primary culture media. In Isenberg HD (Ed): Clinical microbiology procedures Handbook. Vol.1. American Society for Microbiology, Washington DC, USA, pp. 1.61-1.67.

Ish-Horowicz D, Burke JF (1981): Rapid and efficient cosmid cloning. Nucleic Acids Research **9**, 2989–2998.

Ishibashi N, Yamazaki S (2001): Probiotics and safety. American Journal of Clinical Nutrition **73**, 465 S - 470 S.

Ivanova EP, Vysotskii MV, Svetashev VI, Nedashkovskaya OI, Gorshkova NM, Mikhailov VV, Yumoto N, Shigeri Y, Taguchi T, Yoshikawa S (1999): Characterization of *Bacillus* strains of marine origin. International Microbiology **2**, 267–271.

Ivanova V, Kabadjova P, Pantev A, Danova S, Dousset X (2000): Detection, purification and partial characterization of a novel bacteriocin substance produced by *Lactoccous lactis* subsp. *lactis* B14 isolated from boza | Bulgarian traditional cereal beverage. Biocatalysis-2000: Fundamentals & Applications, 47-53.

Ivanovics G, Alfoldi L (1954): A new anti-bacterial principle: Megacin. Nature **174**, 465.

Iyapparaj P, Maruthiah T, Ramasubburayan R, Prakash S, Chandrasekaran K, Immanuel G, Palavesam A (2013): Optimization of bacteriocin production by *Lactobacillus* sp. MSU3IR against shrimp bacterial pathogens. Aquatic Biosystems **9**, 12.

Janneke G, Wijman E, Patrick P, de Leeuw LA, Mooezelaar R, Zwietering MH, Abee T (2007): Air–liquid interface biofilms of *Bacillus cereus*: formation, sporulation, and dispersion. Applied and Environmental Microbiology **73**, 1481–1488.

Janstova B, Lukasova J (2001): Heat resistance of *Bacillus* spp. spores isolated from cow's milk and farm environment. Acta Veterinaria Brno **70**, 179–184.

Jarvis B (1967): Resistance to nisin and production of nisin-inactivating enzymes by several *Bacillus* species. Journal of General Microbioogy **47**, 33–48.

Jensen PR, Fenical W (1994): Strategies for the discovery of secondary metabolites from marine bacteria: Ecological perspectives. Annual Review of Microbiology **48**, 559–584.

Jessen B, Lammert L (2003): Biofilm and disinfection in meat processing plants. International Biodeterioration & Biodegradation **51**, 265–269.

Jia K, Thomas C, Akbar M, Sun Q, Adams-Huet B, Gilpin C, Levine B (2009): Autophagy genes protect against *Salmonella* Typhimurium infection and mediate signaling-regulated pathogen resistance. Proceedings of the National Academy of Sciences of USA **106**, 14564-14569.

Joerger MC, Klaenhammer TR (1986): Characterization and purification of helveticin J and evidence for a chromosomally encoded bacteriocin produced by *Lactobacillus helveticus* 481. Journal of Bacteriology **167**, 439- 446.

Joerger MC, Klaenhammer TR (1990): Cloning, expression and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. Journal of bacteriology **171**, 6339–6347.

Joerger R (2003): Alternatives to Antibiotics: Bacteriocins, Antimicrobial peptides and bacteriophages. Poultry Science **82**, 640-647.

John JV, Therit B, Melville SB (2008): Type IV Pili and the CcpA Protein. Infection and Immunity **76**, 4944 – 4951.

Joosten HM, Nunez M, Devreese B, Van Beeumen J, Marugg JD (1996): Purification and characterization of enterocin 4, a bacteriocin produced by *Enterococcus faecalis* INIA 4. Applied and Environmental Microbiology **62**, 4220-4223.

Joseph B, Dhas B, Hena V, Raj J (2013): Bacteriocin from *Bacillus subtilis* as a novel drug against diabetic foot ulcer bacterial pathogens. Asian Pacific Journal of Tropical Biomedicine **3**, 942-946.

Kabuki T, Saito T, Kawai Y, Uemura J, Itoh T (1997): Production, purification, and characterization of reutericin 6, a bacteriocin with lytic activity produced by *Lactobacillus reuteri* LA6. International Journal of Food Microbiology **34**, 145-156.

Kamoun F, Mejdoub H, Aouissaoui H, Reinbolt J, Hammami A, Jaoua S (2005): Purification, amino acid sequence and characterization of Bacthuricin F4, a new bacteriocin produced by *Bacillus thuringiensis.* Journal of Applied Microbiology **98**, 881–888.

Kawai Y, Saito T, Kitazawa H, Itoh T (1998): Gassericin A; an uncommon cyclic bacteriocin produced by *Lactobacillus gasseri* LA39 linked at N- and C-terminal ends. Biosciences Biotechnology and Biochemistry **62**, 2438-2440.

Kawulka K, Sprules T, McKay RT, Mercier P, Diaper CM, Zuber P, Vederas JC (2003): Structure of subtilosin A, an antimicrobial peptide from *Bacillus subtilis* with unusual posttranslational modifications linking cysteine sulfurs to alpha-carbons of phenylalanine and threonine. Journal of the American Chemical Society **125**, 4726–4767.

Kawulka KE, Sprules T, Diaper CM, Whittal RM, McKay RT, Mercier P, Zuber P, Vederas JC (2004): Structure of subtilosin A, a cyclic antimicrobial peptide from *Bacillus subtilis* with unusual sulfur to alpha-carbon cross-links: formation and reduction of alpha-thio-alpha-amino acid derivatives. Biochemistry **43**, 3385–3395.

Kayalvizhi N, Gunasekaran P (2008): Production and characterization of a low-molecular-weight bacteriocin from *Bacillus licheniformis* MKU3. Letters in Applied Microbiology **47**, 600–607.

Kemperman R, Jonker M, Nauta A, Kuipers OP, Kok J (2003): Functional analysis of the gene cluster involved in production of the bacteriocin circularin A by *Clostridium beijerinckii* ATCC 25752. Applied and Environmental Microbiology **69**, 5839-5848.

Kennedy J, Marchest JR, Dobson ADW (2008): Marine metagenomics: Strategies for the discovery of novel enzymes with biotechnological applications from marine environments. Microbial Cell Factoris **7**, 27–28.

Keren T, Yarmus M, Halevy G, Shapira R (2004): Immunodetection of the bacteriocin lacticin RM: analysis of the influence of temperature and Tween 80 on its expression and activity. Applied and Environmental Microbiology **70**, 2098–2104.

Khalil R, Djadouni F, Elbahloul Y, Omar S (2009 a): The influence of cultural and physical conditions on the antimicrobial activity of bacteriocin produced by a newly isolated *Bacillus megaterium* 22 strain. African Journal of Food Science **3**, 011–022.

Khalil R, Elbahloul Y, Djadouni F, Omar S (2009 b): Isolation and partial characterization of a bacteriocin produced by a newly isolated *Bacillus megaterium* 19 strain. Pakistan Journal of Nutrition **8**, 242–250.

Khamis A, Colson P, Raoult D, Scola BL (2003): Usefulness of *rpoB* gene sequencing for identification of *Afipia* and *Bosea* species, including a strategy for choosing discriminative partial sequences. Applied and Environmental Microbiology **69**, 6740– 6749.

Ki J, Zhang W, Qian P (2009): Discovery of marine *Bacillus* species by 16S rRNA and *rpoB* comparisons and their usefulness for species identification. Journal of Microbiological Methods **77**, 48–57

Kiesau P, Eikmanns U, Gutowski-Eckel Z, Weber S, Hammelmann M, Entian KD (1997): Evidence for a multimeric subtilin synthetase complex. Journal of Bacteriology **179**, 1475–1481.

Kim CH, Ji GE, Ahn C (2000): Purification and molecular characterization of a bacteriocin from *Pediococcus* sp. KCA 1202-10 isolated from fermented flat fish. Food Science and Biotechnology **9**, 270-276.

Kim MH, Kong YJ, Baek H, Hyun HH (2006): Optimisation of culture conditions and medium composition for production of micrococcin GO5 by *Micrococcus* sp. GO5. Journal of Biotechnology **121**, 54-61.

Kim WS, Hall RJ, Dunn NW (1997): The effect of nisin concentration and nutrient depletion on nisin production of *Lactococcus lactis*. Applied Microbiology and Biotechnology **48**, 449–453.

Klaenhammer TR (1988): Bacteriocins of lactic acid bacteria. Biochimie **70**, 337-349.

Klaenhammer TR (1993): Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiology Reviews **12**, 39–86.

Kleerebezem M, Bongers R, Rutten G, de Vos WM, Kuipers OP (2004): Autoregulation of subtilin biosynthesis in *Bacillus subtilis*: the role of the *spa*-box in subtilin-responsive promoters. Peptides **25**, 1415–1424.

Klein C, Entian KD (1994): Genes involved in self-protection against the lantibiotic subtilin produced by *Bacillus subtilis* ATCC 6633. Applied and Environmental Microbiology **60**, 2793–2801.

Klein C, Kaletta C, Entian KD (1993): Biosynthesis of the lantibiotic subtilin is regulated by a histidine kinase/response regulator system. Applied and Environmental Microbiology **59**, 296–303.

Kleinkauf H, Von Dohren H, Dornauer H, Nesemann G (Eds.) (1986): Regulation of Secondary Metabolite Formation. VCH, Weinheim, Germany.

Kolbert CP, Persing DH (1999): Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. Current Opinion in Microbiology **2**, 299–305.

Koponen O, Takala TM, Saarela U, Qiao M, Saris PE (2004): Distribution of the NisI immunity protein and enhancement of nisin activity by the lipid-free NisI. FEMS Microbiology Letters **231**, 85–90.

Korenblum E, der Weid I, Santos AL, Rosado AS, Sebastian GV, Coutinho CM, Magalhaes FC, Paiva MM, Seldin L (2005): Production of antimicrobial substances by *Bacillus subtilis* LFE-1, *B. firmus* HO-1 and *B. licheniformis* T6-5 isolated from an oil reservoir in Brazil. Journal of Applied Microbiology **98**, 667–675.

Korenblum E, Sebastian GV, Paiva MM, Coutinho CM, Magalhaes FC, Peyton BM, Seldin L (2008): Action of antimicrobial substances produced by different oil reservoir *Bacillus* strains against biofilm formation. Applied Microbiology and Biotechnology **79**, 97–103.

Kofinek J (1927): Ein Beitrag zur Mikrobiologie des Meeres. Zentr. Bakt. Parasitenk., II **71**, 73-79.

Kouwen TR, van der Goot A, Dorenbos R, Winter T, Antelmann H, Plaisier MC, Quax WJ, van Dijl JM, Dubois JY (2007): Thiol-disulphide oxidoreductase modules in the low-GC Gram-positive bacteria. Molecular Microbiology **64**, 984–999.

Krassilnikov NA (1938): The bactericidal action of sea water. Mikrobiologiia **7**, 329-334.

Kreth J, Merritt J, Shi W, Qi F (2005): Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. Journal of Bacteriology **187**, 7193-7203.

Krier F, Revol-Junelles AM, Germain P (1998): Influence of temperature and pH on production of two bacteriocins by *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 during batch fermentation. Applied Microbiology and Biotechnology **50**, 359–363.

Kritas SK, Morrison RB (2005): Evaluation of probiotics as a substitute for antibiotics in a large pig nursery. Veterinary Record **156**, 447–448.

Kruszewska D, Sahl HG, Bierbaum G, Pag U, Hynes SO, Ljungh A (2004): Mersacidin eradicates methicillin-resistant *Staphylococcus aureus* (MRSA) in a mouse rhinitis model. Journal of Antimicrobial agents and Chemotherapy **54**, 648–653.

Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, Bertero MG, Bessières P, Bolotin A, Borchert S (1997): The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature **390**, 249–256.

Kupke T, Gotz F (1996): Post-translational modifications of lantibiotics. Antonie van Leeuwenhoek **69**, 139–150.

Kurz C, Ewbank JJ (2000): *Caenorhabditis elegans* for the study of host-pathogen interactions. Trends Microbiology **8**, 142-144.

Kurz CL, Chauvet S, Andres E, Aurouze M, Vallet I, Michel GPF, Uh M, Celli J, Filloux A, de Bentzmann S, Steinmetz I, Hoffmann JA, Finlay BB, Gorvel JP, Ferrandon D, Ewbank JJ (2003): Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. European Molecular Biology organization Journal **22**, 1451-1460.

Kyriakis SC, Tsiloyiannis VK, Vlemmas J, Sarris K, Tsinas AC, Alexopoulos C, Jansegers L (1999): The effect of probiotic LSP 122 on the control of post-weaning diarrhoea syndrome of piglets. Research in Veterinary Science **67**, 223–228.

La Ragione RM, Casula G, Cutting SM, Woodward M (2001): *Bacillus subtilis* spores competitively exclude *Escherichia coli* 070:K80 in poultry. Veterinary Microbiology **79**, 133-142.

La Ragione RM, Woodward MJ (2003): Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. Veterinary Microbiology **94**, 245-256.

Labrousse A, Chauvet S, Couillault CL, Ewbank JJ (2000): *Caenorhabditis elegans* is a model host for *Salmonella* Typhimurium. Current Biology **10**, 1543-1545.

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

Lappe R, Motta AS, Sant'Anna V, Brandelli A (2009): Inhibition of *Salmonella* Enteritidis by cerein 8A, EDTA and sodium lactate. International Journal of Food Microbiology **135**, 312–316.

Larsen AR, Bocher S, Stegger M, Goering R, Pallesen LV, Skov R (2008): Epidemiology of European community-associated methicillin-resistant *Staphylococcus aureus* clonal complex 80 type IV strains isolated in Denmark from 1993 to 2004. Journal of Clinical Microbiology **46**, 62-68.

Lawton EM, Ross RP, Hill C, Cotter PD (2007): Two-peptide lantibiotics: a medical perspective, Mini Review. Mini-Reviews in Medicinal Chemistry **7**, 1236–1247.

Lazarevic V, Dusterhoft A, Soldo B, Hilbert H, Mauel C, Karamata D (1999): Nucleotide sequence of the *Bacillus subtilis* temperate bacteriophage SPbetac2. Microbiology **145**, 1055–1067.

Le Blay G, Lacroix C, Zihler A, Fliss I (2007): *In vitro* inhibition activity of nisin A, nisin Z, pediocin PA-1 and antibiotics against common intestinal bacteria. Letters in Applied Microbiology **45**, 252–257.

Le Marrec C, Hyronimus B, Bressollier P, Verneuil B, Urdaci MC (2000): Biochemical and genetic characterization of coagulin, a new antilisterial

bacteriocin in the pediocin family of bacteriocins, produced by *Bacillus coagulans* I (4). Applied and Environmental Microbiology **66**, 5213–5220.

Lee KD, Gray EJ, Mabood F, Jung WJ, Charles T, Clark SR, Ly A, Souleimanov A, Zhou X, Smith DL (2009 a): The class IId bacteriocin thuricin-17 increases plant growth. Planta **229**, 747–55.

Lee H, Churey JJ, Worobo RW (2009 b): Biosynthesis and transcriptional analysis of thurincin H, a tandem repeated bacteriocin genetic locus, produced by *Bacillus thuringiensis* SF361. FEMS Microbiology Letters **299**, 205–213.

Lee KH, Jun KD, Kim WS, Paik HD (2001): Partial characterization of polyfermenticin SCD, a newly identified bacteriocin of *Bacillus polyfermenticus*. Letters in Applied Microbiology **32**, 146–151.

Lei Z, Anand A, Mysore KS, Sumner LW (2007): Electroelution of intact proteins from SDS-PAGE gels and their subsequent MALDI-TOF MS analysis. Methods in Molecular Biology **355**, 353–363.

Lemos ML, Toranzo AE, Barja JL (1985): Antibiotic activity of epiphytic bacteria isolated from intertidal sea weeds. Microbial Ecology **11**,149- 163.

Lili He, Chen W, Liu Y (2006): Production and partial characterization of bacteriocin-like peptides by *Bacillus licheniformis* ZJU12. Microbiological Research **161**, 321–326.

Lim J, Jeong H, Kim S (2011): Characterization of the Bacteriocin J4 produced by *Bacillus amyloliquefaciens* J4 isolated from Korean traditional fermented soybean Paste. Journal of the Korean Society for Applied Biological Chemistry **54**, 468-474.

Lin J, Wang Y, Hoang KV (2009): Systematic identification of genetic loci required for polymyxin resistance in C*ampylobacter jejuni* using an efficient *in vivo* Transposon Mutagenesis System. Food borne Pathogens and Disease **6**, 173-185.

Lindsay D, Brozel VS, von Holy A (2006): Biofilm-spore response in *Bacillus cereus* and *Bacillus subtilis* during nutrient limitation. Journal of Food Protection **69**, 1168–1172.

Lindsay JA (2010): Genomic variation and evolution of *Staphylococcus aureus*. International Journal of Medical Microbiology **300**, 98-103.

Link R, Kovac G (2006): The effect of probiotic BioPlus 2B on feed efficiency and metabolic parameters in swine. Biologia **61**, 783–787.

Lins L, Ducarme P, Breukink E, Brasseur R (1999): Computational study of nisin interaction with model membrane. *Biochimica et Biophysica Acta* **1420**, 111–120.

Lisboa MP, Bonatto D, Bizani D, Henriques JAP, Brandelli A (2006): Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic forest. International Microbiology **9**, 111-118.

Lodemann U, Lorenz BM, Weyrauch KD, Martens H (2008): Effects of *Bacillus cereus* var. *toyoi* as probiotic feed supplement on intestinal transport and barrier function in piglets. Archives of Animal Nutrition **62**, 87–106.

Logan NA (1988): *Bacillus* species of medical and veterinary importance. Journal of Medical Microbiology **25,** 157-165.

Lovett PS, Duvall EJ, Keggins KM (1976): *Bacillus pumilus* plasmid pPL10: properties and insertion into *Bacillus subtilis* 168 by transformation. Journal of Bacteriology **127**, 817–828.

Ludwig W, Schleifer KH, Whitman WB (2009): Revised road map to the phylum Firmicutes. In De Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer K-H, Whitman WB (Eds.): Bergey's Manual of Systematic Bacteriology. Vol 3, The Firmicutes. Springer-Verlag, New York, pp. 1- 17.

Lutz G, Chavarria M, Arias ML, Mata-Segreda JF (2006): Microbial degradation of palm (*Elaeis guineensis*) biodiesel. International Journal of Tropical Biology **54**, 59–63.

Mah J-H, Hwang H-J (1998): Microflora during cabbage-kimchi fermentation. Journal of Science and Technology **16**, 173–187.

Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM (1999): Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. Cell **96**, 47-56.

Majeed H, Gillor O, Kerr B, Riley MA (2011): Competitive interactions in *Escherichia coli* populations: the role of bacteriocins. ISME Journal **5**, 71–81.

Mantovani HC, Cruz AMO, Paiva AD (2011): Bacteriocin activity and resistance in livestock pathogens. In Mendez-Vilas A (Ed.): Science against microbial

pathogens: Communicating current research and technological advances. Formatex Publisher, Spain, pp. 853-863.

Martin JF, Liras P (1989): Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. Annual Review of Microbiology **43,** 173-206.

Martinez B, Rodriguez A, Suarez JE (2000): Lactococcin 972, a bacteriocin that inhibits septum formation in lactococci. Microbiology **146**, 949–955.

Martirani L, Varcamonti M, Naclerio G, De Felice M (2002): Purification and partial characterization of bacillocin 490, a novel bacteriocin produced by a thermophilic strain of *Bacillus licheniformis*. Microbial Cell Factories **1**, 1–5.

Marx R, Stein T, Entian K-D, Glaser SJ (2001): Structure of the *Bacillus subtilis* peptide antibiotic subtilosin A determined by 1H-NMR and matrix assisted laser desorption/ionization time-of-flight mass spectrometry. Journal of Protein Chemistry **20**, 501–506.

Maukonen J, Matto J, Wirtanen G, Raaska L, Mattila-Sandholm T, Saarela M (2003): Methodologies for the characterization of microbes in industrial environments: a review. Journal of Industrial Microbiology and Biotechnology **30**, 327–356.

Maurya AP, Thakur RL (2012): Inhibition spectrum, purification and characterization of bacteriocin from *Leuconostoc* NT-1. Current Science **103**, 1405-1407.

Mazza P (1994): The use of *Bacillus subtilis* as an antidiarrhoeal microorganism. Bollettino Chimico Farmaceutico **133**, 3-18.

Mc Auliffe O, Ross RP, Hill C (2001): Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiology Reviews **25**, 285–308.

Mc Call JO, Sizemore RK (1979): Description of a bacteriocinogenic plasmid in *Beneckea harveyi.* Applied and Environmental Microbiology **38**, 974–979.

Mc Clerren AL, Cooper LE, Quan C, Thomas PM, Kelleher NL, van der Donk WA (2006): Discovery and *in vitro* biosynthesis of haloduracin, a two-component lantibiotic. Proceedings of National Academy of Sciences of USA **103**, 17243–17248.

Millet ACM, Ewbank JJ (2004): Immunity in *Caenorhabditis elegans*. Current Opinion in Immunology **16**, 4-9.

Millette M, Cornut G, Dupont C, Shareck F, Archambault D, Lacroix M (2008): Capacity of human nisin- and pediocin-producing lactic acid bacteria to reduce intestinal colonization by vancomycin resistant enterococci. Applied and Environmental Microbiology **74**, 1997–2003.

Ming X, Daeschel M (1993): Nisin resistance of foodborne bacteria and the specific resistance responses of *Listeria monocytogenes* Scott A. Journal of Food Protection **56**, 944–948.

Miranda CA, Martins OB, Clementino MM (2008): Species-level identification of *Bacillus* strains isolates from marine sediments by conventional biochemical, 16S rRNA gene sequencing and inter-tRNA gene sequence lengths analysis. Antonie van Leeuwenhoek **93**, 297–304.

Molitor E, Kluczny C, Brotz H, Bierbaum G, Jack R, Sahl HG (1996): Effects of the lantibiotic mersacidin on the morphology of staphylococci. Zentralblatt Fur Bakteriologie **284**, 318–328.

Moonchai S, Madlhoo W, Jariyachavalit K, Shimizu H, Shioya S, Chauvatcharin S (2005): Application of a mathematical model and dierential evolution algorithm approach to optimization of bacteriocin production by *Lactococcus lactis* C7. Bioprocess and Biosystems Engineering **28**, 15–26.

Morris SL, Walsh RC, Hansen JN (1984): Identification and characterization of some bacterial membrane sulfhydryl groups which are targets of bacteriostatic and antibiotic action. Journal of Biological Chemistry **259**, 13590−13594.

Mortvedt CI, Nissen-Meyer J, Sletten K, Nes IF (1991): Purification and amino acid-sequence of lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. Applied and Environmental Microbiology **57**, 1829–1834.

Motta AS, Cladera-Olivera F, Brandelli A (2004): Screening for antimicrobial activity among bacteria isolated from the Amazon basin. Brazilian Journal of Microbiology **35**, 307–310.

Motta AS, Flores FS, Souto AA, Brandelli A (2008): Antibacterial activity of a bacteriocin-like substance produced by *Bacillus* sp. P34 that targets the bacterial cell envelope. Antonie van Leeuwenhoek **93**, 275–284.

Motta AS, Lorenzini DM, Brandelli A (2007): Purification and partial characterization of an antimicrobial peptide produced by a novel *Bacillus* sp. isolated from Amazon basin. Current Microbiology **54**, 282–286.

Mourad K, Zadi-Karam H, Nour-Eddine K (2005): Detection and activity of plantaricin OL15 a bacteriocin produced by *Lactobacillus plantarum* OL15 isolated from Algerian fermented olives. Grasasy Aceites **56**, 192-197.

Moy TI, Ball AR, Anklesaria Z, Casadei G, Lewis K, Ausubel FM (2006): Identification of novel antimicrobials using a live-animal infection model. Proceedings of the National Academy of Sciences of USA **103**, 10414-10419.

Moy TI, Conery AL, Larkins-Ford J, Wu G, Mazitschek R, Casadei G, Lewis K, Carpenter AE, Ausubel FM (2009): High-throughput screen for novel antimicrobials using a whole animal infection model. ACS Chemical Biology **4**, 527-533.

Muriana PM, Klaenhammer TR (1991): Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. Applied and Environmental Microbiology **57**, 114–121.

Naclerio G, Ricca E, Sacco M, Felice M De (1993): Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. Applied and Environmental Microbiology **59**, 4313- 4316.

Nakamura LK (1989): Taxonomic relationship of black-pigmented *Bacillus subtilis* strains and proposal for *Bacillus atrophaeus* sp. *nov*. International Journal of Systematic Bacteriology **39**, 295-300.

Nakano MM, Zuber P (1998): Anaerobic growth of a strict aerobe. Annual Review of Microbiology **52**, 165–190.

Nakano MM, Zuber P, Glaser P, Danchin A, Hulett FM (1996): Two-component regulatory proteins ResD-ResE are required for transcriptional activation of

fnr upon oxygen limitation in *Bacillus subtilis*. Journal of Bacteriology **178**, 3796–3802.

Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H (1996): Biosynthesis of bacteriocins in lactic acid bacteria. Antonie van Leeuwenhoek **70**, 113–128.

Nes IF, Diep DB, Holo H (2007): Bacteriocin diversity in *Streptococcus* and *Enterococcus*. Journal of Bacteriology **189**, 1189–1198.

Nes IF, Holo H (2000): Class II antimicrobial peptides from lactic acid bacteria. Biopolymers **55**, 50-61.

Nes IF, Holo H, Fimland G, Hildeng-Hauge H, Nissen-Meyer J (2002): Unmodified peptide-bacteriocins (Class II) produced by lactic acid bacteria. In Dutton CJ, Haxell MA, Mc Arthur HAI,Wax RG (Eds.): Peptide antibiotics. Marcel Decker, New York, pp. 81–115.

Newaj-Fyzul A, Adesiyun AA, Mutani A, Ramsubhag A, Brunt J, Austin B (2007): *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). Journal of Applied Microbiology **103**, 1699-1706.

Nicholson WJ, Munakata N, Horneck G, Melosh HJ, Setlow P (2000): Resistance of *Bacillus* endospores to extreme terrestial and extraterrestrial environments. Microbiology and Molecular Biology Reviews **64**, 548-572.

Nicholson WL (2002): Roles of *Bacillus* endospores in the environment. Cellular and Molecular Life Sciences **59**, 410–416.

Nissen-Meyer J, Havarstein LS, Holo H, Sletten K, Nes IF (1993): Association of the lactococcin A immunity factor with the cell membrane: purification and characterization of the immunity factor. Journal of General Microbiology **139**, 1503–1509.

Nissen-Meyer J, Holo H, Håvarstein SL, Sletten K, Nes IF (1992): A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. Journal of Bacteriology **174**, 5686–5692.

Norris JR, Berkeley RCW, Logan NA, Donnell AGO (1981): The genera *Bacillus* and *Sporolactobacillus*. In Starr MP, Stolp H, Triiper HG, Balows A, Schlegel HG (Eds.): The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria. Vol. 2. Springer-Verlag, Berlin, pp. 1711-1742.

Novotny JF, Perry JJ (1992): Characterization of bacteriocins from two strains of *Bacillus thermoleovorans*, a thermophilic hydrocarbon-utilizing species. Applied and Environmental Microbiology **58**, 2393–2396.

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

Odunfa SA, Oyeyiola GF (1985): Microbiological study of the fermentation of ugba, a Nigerian indigenous fermented food flavour. Plant Foods for human nutrition **6**, 155–163.

Ogunbanwo ST, Sanni AI, Onilude AA (2003): Influence of cultural conditions on the production of bacteriocin by *Lactobacillus brevis* OG1. African Journal of Biotechnology **2**, 179-184.

Oguntoyinbo A, Sanni AI, Franz CMAP, Holzapfel WH (2007): *In vitro* fermentation studies for selection and evaluation of *Bacillus* strains as starter cultures for the production of okpehe, a traditional African fermented condiment. International Journal of Food Microbiology **113**, 208–218.

Okereke A, Montville TJ (1992): Nisin dissipates the proton motive force of the obligate anaerobe *Clostridium sporogenes* PA 3679. Applied and Environmental Microbiology **58**, 2463–2467.

Okoli I, Coleman JJ, Tampakakis E, An WF, Holson E, Wagner F, Conery AL, Larkins-Ford J, Wu G, Stern A (2009): Identification of antifungal compounds active against *Candida albicans* using an improved high-throughput *Caenorhabditis elegans* assay. PLOS One **4**, e7025.

Oman TJ, van der Donk WA (2009): Insights into the mode of action of the two-peptide lantibiotic haloduracin. ACS Chemical Biology **4**, 865–874.

Oscariz JC, Cintas L, Holo H, Lasa I, Nes IF, Pisabarro AG (2006): Purification and sequencing of cerein 7B, a novel bacteriocin produced by *Bacillus cereus* Bc7. FEMS Microbiology Letters **254**, 108–115.

Oscariz JC, Lasa I, Pisabarro AG (1999): Detection and characterization of cerein 7, a new bacteriocin produced by *Bacillus cereus* with a broad spectrum of activity. FEMS Microbiology Letters **178**, 337–341.

Osipova IG, Makhailova NA, Sorokulova IB, Vasileva EA, Gaiderov AA (2003): Spore probiotics. Zhurnal Mikrobiologii, Epidemiologii, Immunobiologii **3**, 113-119.

Osipova IG, Sorokulova IB, Vasileva EA, Budanova EV (2005): Pre-clinical trials of new spore probiotics. Vestnik Rossiiskoi akademii meditsinskikh nauk **12**, 36-40.

Osmanagaoglu O, Beyatli Y (2002): The use of bacteriocins produced by lactic acid bacteria in food biopreservation. Turk Mikrobiyoloji Cemiyeti Dergisi. **32**, 295-306.

Paik SH, Chakicherla A, Hansen JN (1998): Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. Journal of Biological Chemitry **273**, 23134–23142.

Pandey A, Palni LM (1997): *Bacillus* species: the dominant bacteria of the rhizosphere of established tea bushes. Microbiological Research **152**, 359–365.

Papagianni M, Avramidis N, Filioussis G, Dasiou D, Ambrosiadis I (2006): Determination of bacteriocin activity with bioassays carried out on solid and liquid substrates: assessing the factor indicator microorganism. Microbial Cell Factories **5**, 30. doi:10.1186/1475-2859-5-30.

Parente E, Ricciardi A, Addario G (1994): Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140NWC during batch fermentation. Applied Microbiology and Biotechnology **41**, 388–394.

Parente E, Ricciardi A (1999): Production, recovery and purification of bacteriocins from lactic acid bacteria. Applied Microbiology and Biotechnology **52**, 628–638.

Pattnaik P, Kaushik JK, Grover S, Batish VK (2001): Purification and characterization of a bacteriocin-like compound (lichenin) produced anaerobically by *Bacillus licheniformis* isolated from water buffalo. Journal of Applied Microbiology **91**, 636–645.

Pattnaik P, Sunita G, Virender KB (2005): Effect of environmental factors on production of lichenin, a chromosomally encoded bacteriocin like compound produced by *Bacillus licheniformis* 26L-10/3RA. Microbiological Research **160**, 213—218

Paul, VJ, Arthur KE, Williams RR, Ross C, Sharp K (2007): Chemical defenses: From compounds to communities. Biological Bulletin **213**, 226–251.

Paynter MJB, Brown KA, Hayasaka SS (1997): Factors affecting the production of an antimicrobial agent, plantaricin F, by *Lactobacillus plantarum* BF001. Letters in Applied Microbiology **24**, 159-165.

Peschel A, Sahl HG (2006): The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nature Reviews Microbiology **4**, 529–536.

Pilasombut K, SakpuaramT, Wajjwalku W, Nitisinprasert S, Swetwiwathana A, Zendo T, Fujita K, Nakayama J, Sonomoto K (2006): Purification and amino acid sequence of a bacteriocin produced by *Lactobacillus salivarius* K7 isolated from chicken intestine. Songklanakarin Journal of Science and Technology **28**, 121-131.

Pingitore EV, Salvucci E, Sesma F, Nader-Macias ME (2007): Different strategies for purification of antimicrobial peptides from Lactic Acid Bacteria (LAB). In Mendez-Vilas A (Ed.): Communicating Current Research and Educational

Topics and Trends in Applied Microbiology. Formatex publishers, Spain, pp. 557-568.

Piuri M, Sanchez-Rivas C, Ruzal SM (1998): A novel antimicrobial activity of a *Paenibacillus polymyxa* strain isolated from regional fermented sausages. Letters in Applied Microbiology **27**, 9–13.

Pokusaeva K, Kuisiene N, Jasinskyte D, Rutiene K, Saleikiene J, Chitavichius D (2009): Novel bacteriocins produced by *Geobacillus stearothermophilus*. Central European Journal of Biology **4**, 196–203.

Portal-Celhay C, Blaser MJ (2012): Competition and resilience between founder and introduced bacteria in the *Caenorhabditis elegans* gut. Infection and Immunity **80**, 1288-1299.

Powell JR, Ausubel FM (2008): Models of *Caenorhabditis elegans* infection by bacterial and fungal pathogens. In Ewbank J, Vivier E (Eds.): Methods in Molecular Biology. Humana Press, New York, pp. 403-427.

Priest FG (1989 a): Products and applications. In Harwood CR (Ed.): Biotechnology handbooks. Vol 2, *Bacillus*, Plenum press, New York, pp. 293-320.

Priest FG (1989 b): Isolation and identification of aerobic, endospore-forming bacteria. In Harwood CR (Ed.): Biotechnology handbooks: *Bacillus.* Vol 2. Plenum press, New York, pp. 27-56.

Priest FG (1993): *Bacillus subtilis* and other Gram-positive bacteria. In Sonenshein AL, Hoch JA, Losick R (Eds.): Systematics and ecology of *Bacillus*. American Society for Microbiology, Washington DC, pp. 3–16.

Priest FG, Goodfellow M, Shute LA, Berkeley RCW (1987): *Bacillus amyloliquefaciens* sp. *nov*., nom. *rev*. International Journal of Systematic and Evolutionary Bacteriology **37**, 69-71.

Queener SW, Lively DH (1989): Screening and selection for strain improvement. In Domain AL, Solomon NA (Eds.): Manual of Industrial Microbiology and Biotechnology. American Society for Microbiology, Washington DC, pp. 155–169.

Rajaram G, Manivasagan P, Thilagavathi B, Saravanakumar A (2010): Purification and characterization of a bacteriocin produced by *Lactobacillus lactis* isolated from marine environment. Advance Journal of Food Science and Technology **2**, 138-144.

Rampelotto PH (2010): Resistance of microorganisms to extreme environmental conditions and its contribution to astrobiology. Sustainability **2**, 1602–1623.

Ray B (1992): Bacteriocins of starter culture bacteria as food biopreservatives: an overview. In Ray B, Daeschel M (Eds.): Food Biopreservatives of Microbial Origin. CRC Press, Boca Raton, FL, pp. 177–205.

Reddy GSN, Prakash JSS, Matsumoto GI, Stackebrandt E, Shivaji S (2002 a): *Arthrobacter roseus* sp. *nov*. A psychrophilic bacterium isolated from an Antarctic cyanobacterial mat sample. International Journal of Systematic and Evolutionary Microbiology **52**, 1017–1021.

Reddy GSN, Prakash JSS, Vairamani M, Prabhakar S, Matsumoto GI, Shivaji S (2002 b): *Planococcus antarcticus* and *Planococcus psychrophilus* spp. *nov*. isolated from cyanobacterial mat samples collected from ponds in Antarctica. Extremophiles **6**, 253–261.

Reese ET, Maguire A (1969): Surfactants as stimulants of enzyme production by microorganisms. Applied Microbiology **17**, 242–245.

Reeves P (1965): The bacteriocins. Bacteriological Reviews **29**, 24-45.

Reid G, Sanders ME, Gaskins HR, Gibson GR, Mercenier A, Rastall R, Roberfroid M, Rowland I, Cherbut C, Klaenhammer TR (2003): New scientific paradigms for probiotics and prebiotics. Journal of Clinical Gastroenterology **37**, 105-118.

Riley M A, Wertz JE (2002): Bacteriocins: evolution, ecology, and application. Annual Review of Microbiology **56**, 117-137.

Risoen PA, Ronning P, Hegna IK, Kolsto AB (2004): Characterization of a broad range antimicrobial substance from *Bacillus cereus*. Journal of Applied Microbiology **96**, 648-655.

Rode TM, Langsrud S, Holck A, Moretro T (2007): Different patterns of biofilm formation in *Staphylococcus aureus* under food-related stress conditions. International Journal of Food Microbiology **116**, 372-383.

Rosenfeld WD, Zobell CE (1947): Antibiotic production by marine microorganisms. Journal of Bacteriology **54**, 393-398.

Rostas K, Dobritsa SV, Dobritsa AP, Koncz CS, Alfoldi L (1980): Megacinogenic plasmid from *Bacillus megaterium* 216. Molecular Genetics and Genomics **180**, 323-329.

Ruger HJ (1989): Benthic studies of the northwest African upwelling region of psychrophilic bacterial communities from areas with different upwelling intensities. Marine Ecology Progress Series **57**, 45-52.

Ryan MP, Rea MC, Hill C, Ross RP (1996): An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. Applied and Environmental Microbiology **62**, 612–619.

Sahl HG, Bierbaum G (1998): Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from Gram positive bacteria. Annual Review of Microbiology **52**, 41–79.

Saitou N, Nei M (1987): The Neighbor-Joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4**, 406-425.

Saleem F, Ahmad S, Yaqoob Z, Rasool SA (2009): Comparative study of two bacteriocins produced by representative indigenous soil bacteria. Pakistan Journal of Pharmaceutical Sciences **22**, 252–258.

Salkinoja-Salonen S, Vuorio R, Andersson MA, Kampfer P, Andersson MC, Honkanen-Buzalski T, Scoging AC (1999): Toxigenic strains of *Bacillus licheniformis* related to food poisoning. Applied and Environmental Microbiology **65**, 4637–4645.

Sambrook J, Fritsch E, Maniatis (2000): Molecular cloning, A laboratory manual. C. S. H. Laboratory, USA.

Sanders ME, Morelli L, Tompkins TA (2003): Spore formers as human probiotics: *Bacillus*, *Sporolactobacillus*, and *Brevibacillus*. Comprehensive Reviews in Food Science and Food Safety **2**, 101-110.

Saris PE, Immonen T, Reis M, Sahl HG (1996): Immunity to lantibiotics. Antonie van Leeuwenhoek **69**, 151–159.

Sarker SD, Lutfun N, Yashodharan K (2007): Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. Methods **42**, 321–324.

Sass P, Jansen A, Szekat C, Sass V, Sahl HG, Bierbaum G (2008): The lantibiotic mersacidin is a strong inducer of the cell wall stress response of *Staphylococcus aureus*. BMC Microbiology **8**, 186.

Sayem SMA, Manzo E, Ciavatta L, Tramice A, Cordone A, Zanfardino A, de Felice M, Varcamonti M (2011): Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of *Bacillus licheniformis*. Microbial Cell Factoris **10**, 74.

SCAN (2000 a): Opinion of the Scientific Committee on Animal Nutrition on the safety of the use of *Bacillus* species in animal nutrition. European Commission, Health and Consumer Protection Directorate-General.

SCAN (2000 b): Report of the Scientific Committee on Animal Nutrition on product BioPlus 2B® for use as feed additive. European Commission, Health and Consumer Protection Directorate-General.

Schagger H, von Jagow G (1987): Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical Biochemistry **166**, 368- 379.

Seatovic SL, Novakovic JSJ, Zavisic GN, Radulovic ZC, Gavrovic-Jankulovicc M, Jankov RM (2011): The partial characterization of the antibacterial peptide bacteriocin G2 produced by the probiotic bacteria *Lactobacillus plantarum* G2. Journal of the Serbian Chemical Society **76**, 699–707.

Sebei S, Zendo T, Boudabous A, Nakayama J, Sonomoto K (2007): Characterization, N-terminal sequencing and classification of cerein MRX1, a novel bacteriocin purified from a newly isolated bacterium: *Bacillus cereus* MRX1. Journal of Applied Microbiology **103**, 1621–1631.

Selvendran M, Michael BM (2013): Studies on Novel bacteriocin like inhibitory substance (BLIS) from microalgal symbiotic *Vibrio* spp MMB2 and its activity against aquatic bacterial pathogens. Journal of Applied Pharmaceutical Science **3**, 169-175.

Serizawa M, Kodama K, Yamamoto H, Kobayashi K, Ogasawara N, Sekiguchi J (2005): Functional analysis of the Yvr GHb two-component system of *Bacillus subtilis*: identification of the regulated genes by DNA microarray and northern blot analyses. Bioscience Biotechnology and Biochemistry **69**, 2155–2169.

Setlow P (1994): Mechanisms which contribute to the long term survival of spores of *Bacillus* species. Journal of Applied Bacteriology- Symposium Supplement **76**, 49 S–60 S.

Settanni L, Corsetti A (2008): Application of bacteriocins in vegetable food biopreservation, Review. International Journal of Food Microbiology **121**, 123-138.

Severina E, Severin A, Tomasz A (1998): Antibacterial efficacy of nisin against multidrug-resistant Gram-positive pathogens. Journal of Antimicrobial Agents and Chemotherapy **41**, 341- 347.

Sharma M, Anand SK (2002): Biofilms evaluation as an essential component of HACCP for food/dairy industry – a case. Food Control **13**, 469- 477.

Sharma N, Attri A, Gautam N (2009 a): Purification and characterization of bacteriocin like substance produced from *Bacillus lentus* with perspective of a new biopreservative for food preservation. Pakistan Journal of Scientific and Industrial Research **52**, 191–199.

Sharma N, Kapoor G, Gautam N, Neopaney B (2009 b): Characterization of partially purified bacteriocin of *Bacillus* sp. MTCC 43 isolated from rhizosphere of radish (*Raphanus sativus*) and its application as a potential food biopreservative. Journal of Scientific and Industrial Research **68**, 881–886.

Sharma N, Gautam N (2008): Antibacterial activity and characterization of bacteriocin of *Bacillus mycoides* isolated from whey. Indian Journal of Biotechnology **8**, 117–121.

Sharma N, Kapoor G, Neopaney B (2006): Characterization of a new bacteriocin produced from a novel isolated strain of *Bacillus lentus* NG121. Antonie van Leeuwenhoek **89**, 337–43.

Sharp RJ, Bingham AHA, Comer MJ, Atkinson A (1979): Partial characterization of a bacteriocin (thermocin) from *Bacillus stearothermophilus* RS93. Journal of General Microbiology **111**, 449–451.

Sharp RJ, Scawen MD, Atkinson T (1989): Fermentation and downstream processing of *Bacillus.* In Harwood CR (Ed.): Biotechnology Handbook: *Bacillus*. Plenum Press, New York, pp. 255–292.

Shaver YJ, Nagpal ML, Rudner R, Nakamura LK, Fox KF, Fox A (2002): Restriction fragment length polymorphism of rRNA operons for discrimination and intergenic spacer sequences for cataloging of *Bacillus subtilis* sub-groups. Journal of Microbiological Methods **50**, 215–223.

Shelburne CE, An FY, Dholpe V, Ramamoorthy A, Lopatin DE, Lantz MS (2007): The spectrum of antimicrobial activity of the 18 bacteriocin subtilosin A. Journal of Antimicrobial Chemotherapy **59**, 297–300.

Shivaji S, Bhanu NV, Aggarwal RK (2000): Identification of *Yersinia pestis* as the causative organism of plague in India as determined by 16S rDNA sequencing and RAPD-based genomic fingerprinting. FEMS Microbiology Letters **189**, 247–252.

Siala A, Hill IR, Gray TRG (1974): Populations of spore-forming bacteria in an acid forest soil, with special reference to *Bacillus subtilis*. Journal of General Microbiology **81**, 183–190.

Siegers K, Entian KD (1995): Genes involved in immunity to the lantibiotic nisin produced by *Lactococcus lactis* 6F3. Applied and Environmental Microbiology **61**, 1082–1089.

Siegers K, Heinzmann S, Entian KD (1996): Biosynthesis of lantibiotic nisin. Post-translational modification of its prepeptide occurs at a multimeric membrane-associated lanthionine synthetase complex. Journal of Biological Chemistry **271**, 12294–12301.

Sifri CD, Begun J, Ausubel FM (2005): The worm has turned-microbial virulence modeled in *Caenorhabditis elegans*. Trends in Microbiology **13**, 119-127.

Sihorkar V, Vyas SP (2001): Biofilm consortia on biomedical and biological surfaces: delivery and targeting strategies. Pharmaceutical Research **18**, 1254–1427.

Simoes M, Simoes LC, Machado I, Pereira MO, Vieira MJ (2006): Control of flow-generated biofilms using surfactants-evidence of resistance and recovery. Food and Bioproducts Processing **84**, 338–345.

Simoes M, Vieira MJ (2009): Persister cells in *Pseudomonas fluorescens* biofilms treated with a biocide. In Proceedings of the international conference processes biofilms: Fundamentals to applications, Davis, CA, USA, pp 58–62.

Simon L, Fremaux C, Berjeaud JM (2002): Sakacin G, a new type of antilisterial bacteriocin. Applied and Environmental Microbiology **68**, 6416- 6420.

Sirtori LR, Cladera-Olivera F, Lorenzini DM, Tsai SM, Brandelli A (2006): Purification and partial characterization of an antimicrobial peptide produced by *Bacillus* sp. strain P45, a bacterium from the Amazon basin fish *Piaractus mesopotamicus*. Journal of General and Applied Microbiology **52**, 357–363.

Slepecky R, Hemphill E (2006): The genus *Bacillus*-Nonmedical. The Prokaryotes **4**, 530–562.

Smith D, Lee KD, Gray E, Souleimanov A, Zhou X (2008): Use of bacteriocins for promoting plant growth and disease resistance. US Patent Application number: 20080248953.

Smitha S, Bhat SG (2013): Thermostable Bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment. Journal of Applied Microbiology **114,** 688-694.

Somers EB, Wong AC (2004): Efficacy of two cleaning and sanitizing combinations on *Listeria monocytogenes* biofilms formed at low temperature on a variety of materials in the presence of ready-to-eat-meat residue. Journal of Food Protection **67**, 2218–2229.

Sorokulova IB (1997): A comparative study of the biological properties of Biosporin and other commercial *Bacillus*-based preparations. Mikrobiologicheskii Zhurnal **59**, 43-49.

Sorokulova IB, Beliavskaia VA, Masycheva VA, Smirnov VV (1997): Recombinant probiotics: Problems and prospects of their use for medicine and veterinary practice. Vestnik Rossiiskoi akademii meditsinskikh nauk **3**, 46-49.

Sorokulova IB, Pinchuk IV, Denayrolles M, Osipova IG, Huang JM, Cutting SM, Urdaci MC (2008): The safety of two *Bacillus* probiotic strains for human use. Digestive Diseases and Sciences **53**, 954-963.

Spinosa MR, Braccini T, Ricca E, De Felice M, Morelli L, Pozzi G, Oggioni MR (2000): On the fate of ingested *Bacillus* spores. Research in Microbiology **151**, 361-368.

Stahl S (1989): A new bacteriocinogenic activity: Megacin BII encoded by plasmid pSE 203 in strains of *Bacillus megaterium*. Archives of Microbiology **151**, 159–165.

Stein T (2005): *Bacillus* subtilis antibiotics: Structures, syntheses and specific functions. Molecular Microbiology **56**, 845–857.

Stein T (2008): Whole-cell matrix-assisted laser desorption/ ionization mass spectrometry for rapid identification of bacteriocin/lantibiotic-producing bacteria. Rapid Communications in Mass Spectrometry **22**, 1146–1152.

Stein T, Borchert S, Conrad B, Feesche J, Hofemeister B, Hofemeister J, Entian K-D (2002 a): Two different lantibiotic like peptides originate from the ericin gene cluster of *Bacillus subtilis* A1/3. Journal of Bacteriology **184**, 1703–1711.

Stein T, Borchert S, Kiesau P, Heinzmann S, Kloss S, Klein C, Helfrich M, Entian KD (2002 b): Dual control of subtilin biosynthesis and immunity in *Bacillus subtilis*. Molecular Microbiology **44**, 403-416.

Stein T, Dusterhus S, Stroh A, Entian KD (2004): Subtilosin production by two *Bacillus subtilis* subspecies and variance of the *sbo-alb* cluster. Applied and Environmental Microbiology **70**, 2349- 2353.

Stein T, Heinzmann S, Dusterhus S, Borchert S, Entian K-D (2005): Expression and functional analysis of the subtilin immunity genes *spaIFEG* in the subtilin-sensitive host *Bacillus subtilis* MO1099. Journal of Bacteriology **187,** 822–828.

Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M (2000): A modified microtiter-plate test for quantification of staphylococcal biofilm formation. Journal of Microbiological Methods **40**, 175–179.

Stern NJ, Svetoch EA, Eruslanov BV, Kovalev YN, Volodina LI, Perelygin VV, Mitsevich EV, Mitsevich IP, Levchuk VP (2005): *Paenibacillus polymyxa* purified bacteriocin to control *Campylobacter jejuni* in chickens. Journal of Food Protection **68**, 1450–1453.

Suarez AM, Rodriguez JM, Hernandez PE, Azcona-Olivera JI (1996): Generation of polyclonal antibodies against nisin: immunization strategies and immunoassay development. Applied and Environmental Microbiology **62**, 2117-2121.

Sumi H, Hamada H, Tsushima H, Mihara H, Muraki H (1987): A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese Natto; a typical and popular soybean food in the Japanese diet. Experientia **43**, 1110-1111.

Sumi H, Yatagai C, Wada H, Yoshida E, Maruyama M (1995): Effect of *Bacillus* natto-fermented product (BIOZYME) on blood alcohol, aldehyde concentrations after whisky drinking in human volunteers, and acute toxicity of acetaldehyde in mice. Arukoru Kenkyuto Yakubutsu Ison **30,** 69-79.

Sun G, Sharkova E, Chesnut R, Birkey S, Duggan MF, Sorokin A, Pujic P, Ehrlich SD, Hulett FM (1996): Regulators of aerobic and anaerobic respiration in *Bacillus subtilis*. Journal of Bacteriology **178**, 1374–1385.

Sutyak KE, Wirawan RE, Aroutcheva AA, Chikindas ML (2008): Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-

derived *Bacillus amyloliquefaciens*. Journal of Applied Microbiology **104**, 1067–1074.

Svetoch EA, Stern NJ, Eruslanov BV, Kovalev YN, Volodina LI, Perelygin VV, Mitsevich EV, Mitsevich IP, Pokhilenko VD, Borzenkov VN, Levchuk VP, Svetoch OE, Kudriavtseva TY (2005): Isolation of *Bacillus circulans* and *Paenibacillus polymyxa* strains inhibitory to *Campylobacter jejuni* and characterization of associated bacteriocins. Journal of Food Protection **68**, 11–17.

Swem LR, Swem DL, O'Loughlin CT, Gatmaitan R, Zhao B, Ulrich SM, Bassler BL (2009): A quorum-sensing antagonist targets both membrane-bound and cytoplasmic receptors and controls bacterial pathogenicity. Molecular and Cellular Biology **35**, 143-153.

Szybalski W (1953): Genetic studies on microbial cross resistance to toxic agents. Antibiotics and Chemotherapy **3**, 1095–1103.

Tagg JR, Dajani AS, Wannamaker LW (1976): Bacteriocins of Gram-positive bacteria Bacteriological Reviews **40**, 722 - 756.

Takami H, Nakasone K, Takaki Y, Maeno G, Sasaki R, Masui N, Fuji F, Hirama C, Nakamura Y, Ogasawara N, Kuhara S, Horikoshi K (2000): Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Research **28**, 4317–4331.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011): MEGA5 : Molecular Evolutionary Genetics Analysis using Maximum Likelihood,

Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution **28**, 2731-2739.

Tan MW, Rahme LG, Sternberg JA, Tompkins RG, Ausubel FM (1999): *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. Proceedings of the National Academy of Sciences USA **96**, 2408-2413.

Taylor L, Cann DD, Welch BJ (1990): Antibotulin properties of nisin in fresh fish packaged in an atmosphere of carbondioxide. Journal of Food Protection **53**, 953- 959.

Tchikindas M, Cleveland J, Li J, Montville T (2000): Unrelatedness of nisin resistance and antibiotic resistance in *Listeria monocytogenes*. Program and Abstract Book. International Association for Food Protection, **55**.

Terlabie NN, Esther SD, Wisdom KA (2006): The comparative ability of four isolates of *Bacillus subtilis* to ferment soybeans into dawadawa. International Journal of Food Microbiology **106**, 145-52.

Thompson JD, Higgins DG, Gibson TJ (1994): CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research **22**, 4673-4680.

Tichaczek PS, Meyer JN, Nes IF, Vogel RF, Hammes WP (1992): Characterization of the bacteriocins curvacin A from *Lactobacillus curvatus* LTH1174 and

sakacin P from *L. sake* LTH673. Systematic and Applied Microbiology **15**, 460- 468.

Todorov SD, Dicks LMT (2004): Effect of medium components on bacteriocin production by *Lactobacillus pentosus* ST151BR, a strain isolated from beer produced by the fermentation of maize, barley and soy flour. World Journal of Microbiology and Biotechnology **20**, 643-650.

Tomita H, Fujimoto S, Tanimoto K, Ike Y (1997): Cloning and genetic and sequence analyses of the bacteriocin 21 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pPD1. Journal of Bacteriology **179**, 7843-7855.

Torkar KG, Matijasic BB (2003): Partial characterisation of bacteriocins produced by *Bacillus cereus* isolates from milk and milk products. Food Technology and Biotechnology **41**, 121–129.

Trede NS, Langenau DM, Traver D, Look AT, Zon LI (2004): The use of zebrafish to study immunity. Immunity **20**, 367-379.

Tuohy KM, Pinart-Gilberga M, Jones M, Hoyles L, McCartney AL, Gibson GR (2007): Survivability of a probiotic *Lactobacillus casei* in the gastrointestinal tract of healthy human volunteers and its impact on the faecal microflora. Journal of Applied Microbiology **102**, 1026-1032.

Turcotte C, Lacroix C, Kheadr E, Grignon L, Fliss I (2004): A rapid turbidometric microplate bioassay for accurate quantification of lactic acid bacteria bactriocins. International Journal of Food Microbiology **90**, 283-293.

Uccelletti D, Zanni E, Marcellini L, Palleschi C, Barra D, Mangoni ML (2010): Anti-*Pseudomonas* activity of frog skin antimicrobial peptides in a *Caenorhabditis elegans* infection model: a plausible mode of action *in vitro* and *in vivo*. Antimicrobial Agents in Chemotherapy **54**, 3853-3860.

Urdaci MC, Bressolier P, Pinchuk I (2004): *Bacillus clausii* probiotic strains: antimicrobial and immunomodulatory activities. Journal of Clinical Gastroenterology **38**, S 86–S 90.

Vaerewijck MJ, De Vos P, Lebbe L, Scheldeman P, Hoste B, Heyndrickx M (2001): Occurrence of *Bacillus sporothermodurans* and other aerobic spore-forming species in feed concentrate for dairy cattle. Journal of Applied Microbiology **91**, 1074–1084.

Van Belkum MJ, Stiles ME (2000): Non lantibiotic antibacterial peptides from lactic acid bacteria. Natural Product Reports **17**, 323-335.

Venema K, Haverkort RE, Abee T, Haandrikman AJ, Leenhouts KJ, de Leij L, Venema G, Kok J (1994): Mode of action of LciA, the lactococcin A immunity protein. Molecular Microbiology **14**, 521–532.

Venema K, Venema G, Kok J (1995): Lactococcal bacteriocins: Mode of action and immunity. Trends in Microbiology **3**, 299–304.

Veran J (2002): Biofouling in food processing: biofilm or biotransfer potential? Food and Bioproducts Processing **80**, 292–298.

Vignolo GM, Kairuz MN, Ruiz-Holgado AAP, Oliver G (1995): Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705. Journal of Applied Bacteriology **78**, 5–10.

Vijayalakshmi K, Premalatha A, Suseela RG (2011 a): Antimicrobial protein production by *Bacillus amyloliquefaciens* MBL27: Optimization of culture conditions using Taguchi's experimental design. Indian Journal of Science and Technology **4**, 931- 937.

Vijayalakshmi K., Premalatha A, Suseela R (2011 b): Production and antimicrobial potential of a broad spectrum antimicrobial protein from a new strain of *Bacillus amyloliquefaciens* MBL27. International Journal of Pharmacy and Pharmaceutical Sciences **3**, 243-249.

Vilain S, Luo Y, Hildreth MB, Brozel VS (2006): Analysis of the life cycle of the soil saprophyte *Bacillus cereus* in liquid soil extract and in soil. Applied and Environmental Microbiology **72**, 4970 - 4977.

Vincent SS, John SB (2009): Buffers: principles and practice. In Richard RB, Murray PD (Eds.): Methods in Enzymology. Elsevier Science & Technology Books, MA, USA, pp. 43–56.

Von Dohren H (1995): Peptides. In Vining LC and Stuttard C (Ed.): Genetics and Biochemistry of antibiotic production, Butterworth-Heinemann Newton Publishers, MA, USA, pp. 129-171.

Von Tersch M, Carlton B (1983 a): Bacteriocin from *Bacillus megaterium* ATCC 19213: Comparative studies with megacin A-216. Journal of Bacteriology **155**, 866-871.

Von Tersch MA, Carlton BC (1983 b): Megacinogenic plasmids of *Bacillus megaterium*. Journal of Bacteriology **155**, 872–877.

Waites MJ, Ogden K (1987): The estimation of nisin using ATP-bioluminometry. Journal of the Institute of Brewing **93**, 30-32.

Walstrom G, Saris PE (1999): A nisin bioassay based on bioluminescence. Applied and Environmental Microbiology **65**, 3742-3745.

Wang BY, Kuramitsu HK (2005): Interaction between oral bacteria: Inhibition of *Streptococcus mutans* bacteriocin production by *Streptococcus gordonii*. Applied and Environmental Microbiology **71**, 354-362.

Wang HL, Hesseltine CW (1982): Oriental fermented foods. In Reed R (Ed.): Prescott and Dunn's Industrial Microbiology. AVI Publishing Company, Hartford, CT, pp. 492–538.

Wang J, Fung DYC (1996): Alkaline-fermented foods: A review with emphasis on pidan fermentation. Critical Reviews in Microbiology **22**, 101–138.

Wang X, Heazlewood SP, Krause DO, Florin THJ (2003): Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. Journal of Applied Microbiology **95**, 508–520.

Whitaker JW, Cristol DA, Forsyth MH (2005): Prevalence and genetic diversity of *Bacillus licheniformis* in avian plumage. Journal of Field Ornithology **76**, 264–270.

Willey JM, van der Donk WA (2007): Lantibiotics: Peptides of diverse structure and function. Annual Review of Microbiology **61**, 477–501.

Williams CM, Richter CS, MacKenzie JM, Shih JCH (1990): Isolation, identification and characterization of a feather-degrading bacterium. Applied and Environmental Microbiology **56**, 1509-1515.

Woese CR (1987): Bacterial evolution. Microbiology and Molecular Biology Reviews **51**, 221–271.

Wu XY, Walker MJ, Hornitzky M, Chin J (2006): Development of a group-specific PCR combined with ARDRA for the identification of *Bacillus* species of environmental significance. Journal of Microbiological Methods **64**, 107–119.

Wyman L, Gill S (1990): Binding and linkage, Functional Chemistry of biological macromolecules. University Science Books, Mill Valley, California.

Xie J, Zhang R, Shang C, Guo Y (2009): Isolation and characterization of a bacteriocin produced by an isolated *Bacillus subtilis* LFB112 that exhibits antimicrobial activity against domestic animal pathogens. African Journal of Biotechnology **8**, 5611–5619.

Yamada Y, Hoshino K, Ishikawa T (1997): The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus Gluconoacetobacter to the genetic level. Bioscience Biotechnology and Biochemistry **61**, 1244–1251.

Yamamoto Y, Togawa Y, Shimosaka M, Okazaki M (2003): Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* strain RJ-11. Applied and Environmental Microbiology **69**, 5746–5757.

Yanagida F, Chen Y, Onda T, Shinohara T (2005): Durancin L28-1A, a new bacteriocin from *Enterococcus durans* L28-1, isolated from soil. Letters in Applied Microbiology **40**, 430–435.

Yang EJ, Chang HC (2007): Characterization of bacteriocin-like substances produced by *Bacillus subtilis* MJP1. Korean Journal of Microbiology and Biotechnology **35**, 339–346.

Yokotsuka T (1985): Fermented protein foods in the orient, with emphasis on shoyu and miso in Japan. In Wood BJB (Ed.): Microbiology of Fermented Foods. Elsevier Applied Science, London, pp. 197–247.

Yoshida S, Hiradate S, Tsukamoto T, Hatakeda K, Shirata A (2001): Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology **91**, 181–187.

Zawadzki P, Riley AM, Cohan FM (1996): Homology among nearly all plasmids infecting three *Bacillus* species. Journal of Bacteriology **178**, 191–198.

Zheng G, Hehn R, Zuber P (2000): Mutational analysis of the *sbo-alb* locus of *Bacillus subtilis*: Identification of genes required for subtilosin production and immunity. Journal of Bacteriology **182**, 3266–3273.

Zheng G, Slavik MF (1999): Isolation, partial purification and characterization of bacteriocins produced by newly isolated *Bacillus subtilis* strain. Letters in Applied Microbiology **28**, 363-367.

Zheng G, Yan LZ, Vederas JC, Zuber P (1999): Genes of the *sbo-alb* locus of *Bacillus subtilis* are required for production of the antilisterial bacteriocin subtilosin. Journal of Bacteriology **181**, 7346–7355.

Zhong XT, Zhang Q, Lin K, Yuan CH, Li K, Jia BH, Che ZM, Xiang WL (2013): Optimization of bacteriocin-like substance production by *Bacillus cereus* XH25 using response surface methodology. Research Journal of Biotechnology **8**, 42-48.

Zobell CE, Feltham CB (1933): Are there specific marine bacteria? Proceedings of Fifth Pacific Science Congress **3**, 2097-2100.

Zobell CE (1936): Bactericidal action of sea water. Proceedings of the Society for Experimental Biology and Medicine **34**, 113-116.

Zobell CE (1941): Studies on marine bacteria. I. The cultural requirement of heterotrophic aerobes. Journal of Marine Research **4**, 42-75.

Zobell CE (1943): The effect of solid surfaces upon bacterial activity. Journal of Bacteriology **46**, 39-56.

Zobell CE (1946): Marine microbiology: A monograph on hydrobacteriology. Chronica Botanica Co., Waltham, MA, USA.

APPENDIX – 1

NUTRIENT MEDIUM

Ingredients		g/L
Peptone	-	5
Sodium chloride	-	5
Beef extract	-	1
Yeast extract	-	2

Suspended 13g of media (Himedia, Mumbai, India) in 1000mL distilled water. Mixed well, autoclaved at 15 lbs pressure for 15 minutes and cooled to 50-55°C. When used as solid agar medium, 2.0% agar (w/v) was added to the medium. Final pH 7.4 ± 0.2.

LACTOSE BROTH

Ingredients		g/L
Peptone	-	5
Beef extract	-	3
Lactose	-	5

Suspended 13g of media (Himedia, Mumbai, India) in 1000 mL distilled water. Sterilized by autoclaving at 15 lbs pressure for 15 minutes. Final pH- 6.9 ± 0.2.

LURIA BERTANI BROTH

Ingredients		g/L
Casein enzymic hydrolysate	-	10
Yeast extract	-	5
Sodium chloride	-	10

Suspended 25 grams of media (Himedia, Mumbai, India) in 1000 mL distilled water. Heated to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Final pH is 7.5±0.2.

SOYABEAN CASEIN DIGEST MEDIUM (TRYPTONE SOYA BROTH)

Ingredients		g/L
Pancreatic Digest of Casein	-	17
Papaic digest of soyabean meal	-	3
Sodium chloride	-	5
Dipotassium hydrogen phosphate	-	2.5
Dextrose (Glucose)	-	2.5

Suspended 30 grams of media (Himedia, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 10 minutes. Final pH is 7.3 ± 0.2.

MUELLER HINTON BROTH

Ingredients		g/L
Beef infusion	-	300
Casein acid hydrolysate	-	17.5
Starch	-	1.5

Suspended 21 grams of media (Himedia, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. When used as solid agar medium, 2.0% agar (w/v) was added to the medium for agar plate preparation. Final pH (at 25°C) -7.4 ± 0.2.

ZOBELL MARINE BROTH

Ingredients		g/L
Peptic digest of animal tissue	-	5
Yeast extract	-	1
Ferric citrate	-	0.1
Sodium chloride	-	19.45
Magnesium chloride	-	8.8
Sodium sulphate	-	3.24
Calcium chloride	-	1.8
Potassium chloride	-	0.55
Sodium bicarbonate	-	0.16
Potassium bromide	-	0.08
Strontium chloride	-	0.034
Boric acid	-	0.022

Sodium silicate	-	0.004
Sodium fluorate	-	0.0024
Ammonium nitrate	-	0.0016
Disodium phosphate	-	0.008

Suspended 40.25 grams of media (Himedia, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. When used as solid agar medium, 2.0% agar (w/v) was added to the medium for agar plate preparation. Final pH -7.6 ± 0.2.

BRAIN HEART INFUSION MEDIUM

Bovine brain and heart tissue powder

Suspended 20 grams of media (Himedia, Mumbai, India) in 1000 mL distilled water. Heated to boiling to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

MINIMAL MEDIA

Ingredients		**g/ 100 mL**
Sodium phosphate	-	1.28
Dipotassium phosphate	-	0.3
Sodium chloride	-	0.5
Ammonium chloride	-	0.5

Suspended the above ingredients in 100 mL distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

To this add filter sterilised

1M Magnesium sulphate	-	400 µL
1M Calcium chloride	-	20 µL
20% Glucose	-	4 mL

BLOOD AGAR MEDIUM

Ingredients		**g/L**
Enzymatic digest of Casein	-	15
Enzymatic digest of animal tissue	-	4
Yeast extract	-	2

Starch	-	1
Sodium Chloride	-	5
Agar	-	14

Suspended 42 g of the blood agar base in one liter of purified water. Heated the agar base with frequent agitation and boiled for one minute to completely dissolve the medium. Final pH: 7.0 ± 0.2 at 25°C. Autoclaved the medium at 121°C for 15 minutes and prepared 10% blood agar by aseptically adding the appropriate volume of sterile defibrinated blood to melted sterile agar medium, cooled to 45 – 50°C and poured into sterile petriplates.

PHYSIOLOGICAL SALINE

NaCl	-	0.85g
Distilled water	-	100 mL

Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

APPENDIX – 2

0.01M Phosphate buffer (pH 7.5)

Solution A: 0.2 M NaH_2PO_4

Solution B: 0.2 M Na_2HPO_4

Mixed 16 mL of solution A with 84 mL of solution B and the volume was made up to 200mL with distilled water. This is made upto 1L to get 0.01 M buffer.

Sodium dodecyl sulphate (SDS) – 10%

SDS -10 g

Distilled water -100 mL

Gently swirl.

3M Sodium acetate (pH 5.2)

Sodium acetate.$3H_2O$ - 408.3 g

Distilled water - 800 mL

pH adjusted to 5.2 with glacial acetic acid. Adjusted the volume to 1 litre with distilled water. Sterilized by autoclaving.

Ethidium Bromide (10mg/mL)

Ethidium bromide - 10 mg

Distilled water - 1mL

Dissolved well and stored by wraping in aluminium foil (to be kept in dark)

TE buffer

1M Tris-Cl (pH 8) - 10mL

500mM EDTA (pH 8.0) - 2mL

1M Tris-HCl (pH 8)

Tris base - 60.57 g

Deionised water - 500mL

Adjusted to desired pH using concentrated HCl

0.5M EDTA (pH 8)

EDTA - 18.6 g

Deionised water - 100mL

Adjust the pH to 8.0 with NaOH

50X TAE Buffer

Tris base - 121 g

Glacial acetic acid - 28.6mL

0.5M EDTA pH 8.0 - 50 mL

Deionised water added to make volume to 500mL.

1X TAE Buffer

50X TAE buffer - 10mL

Deionised water - 490mL

6x Gel-loading buffer

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

40% (w/v) sucrose in H_2O

Stored at 4°C.

SOLUTIONS FOR PLASMID ISOLATION

Solution I

25mM Tris - HCl - pH 8.0

50mM glucose

10mM EDTA

Autoclaved the solution and stored at 4°C.

Solution II

0.2N NaOH (freshly diluted from a 10N stock)

1%SDS

Solution II prepared as fresh and used at room temperature.

Solution III

5.0M Potassium Acetate - 60 mL

Glacial acetic acid - 11.5 mL

Water - 28.5 mL

Prepared and stored at 4°C. Transfered to an ice bucket just before use.

Hydrochloric acid- potassium chloride buffer (pH 2)

Solution A: 0.2 M KCl

Solution B: 0.2 M HCl

Mixed 50 ml of solution A with 10.6 ml of solution B and made up to 200ml with distilled water.

Citrate buffer (pH 3 - 6)

Solution A: 0.1 M Citric acid

Solution B: 0.1 M sodium citrate

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to make up volume to 200 ml and then filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
3	46.5	3.5
4	33.0	17
5	20.5	29.5
6	9.5	41.5

Phosphate buffer (pH 7)

Solution A: 0.2 M NaH_2PO_4

Solution B: 0.2 M Na_2HPO_4

Mixed 39 ml of solution A with 61 ml of solution B and the volume was made up to 200ml with distilled water, followed by filter sterilization.

Tris (hydroxymethylamino methane buffer system (pH 8 and 9)

Solution A: 0.2 M Tris buffer

Solution B: 0.2 M HCl

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 ml and filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
8	50	26.8
9	50	5

Carbonate – bicarbonate buffer (pH 10 and 11)

Solution A: 0.2 M Na_2CO_3

Solution B: 0.2M $NaHCO_3$

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 ml and filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
10	27.5	22.5
10.7	45.0	5

Sodium hydroxide - Potassium chloride buffer (pH 12 and 13)

Solution A: 0.2 M KCl

Solution B: 0.2M NaOH

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 ml and then filter sterilized.

Desired pH	Solution A (ml)	Solution B (ml)
12	50	12
13	50	132

APPENDIX 3

REAGENTS FOR GLYCINE SDS-PAGE

1. Stock acrylamide- bisacrylamide solution (30% T and 0.8% C)

Acrylamide (T)	-	30 g
Bis-acrylamide (C)	-	0.8 g
Distilled water (DW)	-	100 mL

Stored at 4°C in amber coloured bottle

2. Stacking gel buffer stock

Tris buffer (0.5 M)	-	6.05 g in 40 mL DW

Titrated to pH 6.8 with 1M HCl and made up to 100 mL with DW. Filtered through Whatman No: 1 (Whatman, England) filter paper and stored at 4°C.

3. Resolving gel buffer stock

Tris buffer (1.5 M)	-	18.15 g

Titrated to pH 8.8 with 1M HCl and made up to 100 mL with DW. Filtered through Whatman No: 1 filter paper and stored at 4°C.

4. Running buffer for SDS-PAGE (pH 8.3)

Tris buffer	-	3 g
Glycine	-	14.4 g
SDS	-	1 g

Dissolved and made up to 1L with DW. Prepared in 10X concentration and stored at 4°C.

5. Sample buffer for Non- Reductive SDS-PAGE

Tris-HCl (pH 6.8)	-	1.25 mL
Glycerol	-	2.5 mL
SDS (10%, W/v)	-	2 mL
Deionised water	-	3.55 mL
Bromophenol blue (0.5%, w/v)	-	0.2 mL

Samples were diluted with sample buffer prior loading into the gel

6. SDS (10%) - 1 g in 10 mL DW

7. **Ammonium persulfate** (10%, w/v) **-** 0.1 g of ammonium persulfate was dissolved in 1mL DW (prepared freshly).

REAGENTS FOR TRICINE SDS-PAGE

Table: The electrode and gel buffers for Tricine–SDS-PAGE

Reagents	Anode buffer (10×)	Cathode buffer (10×)	Gel buffer (3×)
Tris (M)	1.0	1.0	3.0
Tricine (M)	-	1.0	-
HCl (M)	0.225	-	1.0
SDS (%)	-	1.0	0.3
pH	8.9	~8.25	8.45

AB-3 stock solution

For the acrylamide-bisacrylamide AB-3 stock solution which is normally used, dissolved 48 g of acrylamide and 1.5 g of bisacrylamide in 100 mL of water.

COOMASSIE STAINING SOLUTIONS

Protein staining solution

Coomassie brilliant blue (0.1%)	-	100 mg
Methanol (40%)	-	40 mL
Glacial acetic acid (10%)	-	10 mL
DW	-	50 mL

Destaining solution

Methanol (40%)	-	40 mL
Glacial acetic acid (10%)	-	10 mL
DW	-	50 mL

REAGENTS OF SILVER STAINING

1. Fixer

Methanol (50%, v/v)	-	50 mL
Acetic acid (5%, v/v)	-	5 mL
Milli Q water	-	45 mL

2. Wash

Methanol (50%, v/v)	-	50 mL
Milli Q water	-	50 mL

3. Sensitizing solution

Sodium thiosulfate (0.02%, w/v)	-	20 mg
Milli Q water	-	100 mL

4. Silver nitrate solution

Silver nitrate (0.2%, w/v)	-	200 mg
Milli Q water	-	100 mL

5. Developer

Sodium carbonate (6%, w/v)	-	3 g
Formaldehyde	-	12.5 μL
Milli Q water	-	100 mL

6. Stop solution

Sodium-EDTA	-	1.4 g
Milli Q water	-	100 mL

PROTEIN MARKERS

a. Medium range protein molecular weight marker (GeNei) was mixed by tapping and 10µL taken. Mixed with 10µL sample buffer and 30µL distilled water. Incubated in boiling water bath for one minute and loaded on to SDS-PAGE.

Protein	MW (Da)
Phosphorylase b	97,400
Bovine serum albumin	66,000
Ovalbumin	43,000
Carbonic anhydrase	29,100
Soyabean trypsin inhibitor	20,100
Lysozyme	14,300

b. Broad range molecular weight protein marker mix from New England BioLabs (UK) is a ready to load marker. The protein marker was mixed and 7µl taken in a tube. Heated for 5 min at 100°C. After a quick microcentrifuge spin, loaded directly on to a gel. The composition of the marker mix is as given below.

Components		MW in Da
Myosin	-	212,000
MBP-β- galactosidase	-	158,194
β- galactosidase	-	116, 351
Phosphorylase b	-	97,184
Serum albumin	-	66,409
Glutamic dehydrogenase	-	55,561
MBP2	-	42,710
Thioredoxin reductase	-	34,622
Triosephosphate isomerase	-	26,972
Trypsin inhibitor	-	20,000
Lysozyme	-	14,313
Aprotinin	-	6,517
Insulin A	-	3,400
B chain	-	2,340

APPENDIX 4

Nematode Growth Medium

NaCl	-	3g
Agar	-	17g
Peptone	-	3.5g
Distilled water	-	975 ml

Autoclaved and cooled the flask in 55°C water bath for 15 minutes and added

1 M CaCl2	-	1ml
Cholesterol in ethanol (5 mg/ml)	-	1ml
1 M MgSO4	-	1ml
1 M KPO4 buffer	-	25 ml

Swirled to mix well before use.

1 M KPO_4 buffer (pH 6)

KH_2PO_4	-	108.3 g
K_2HPO_4	-	35.6 g

Distilled water - 1000ml

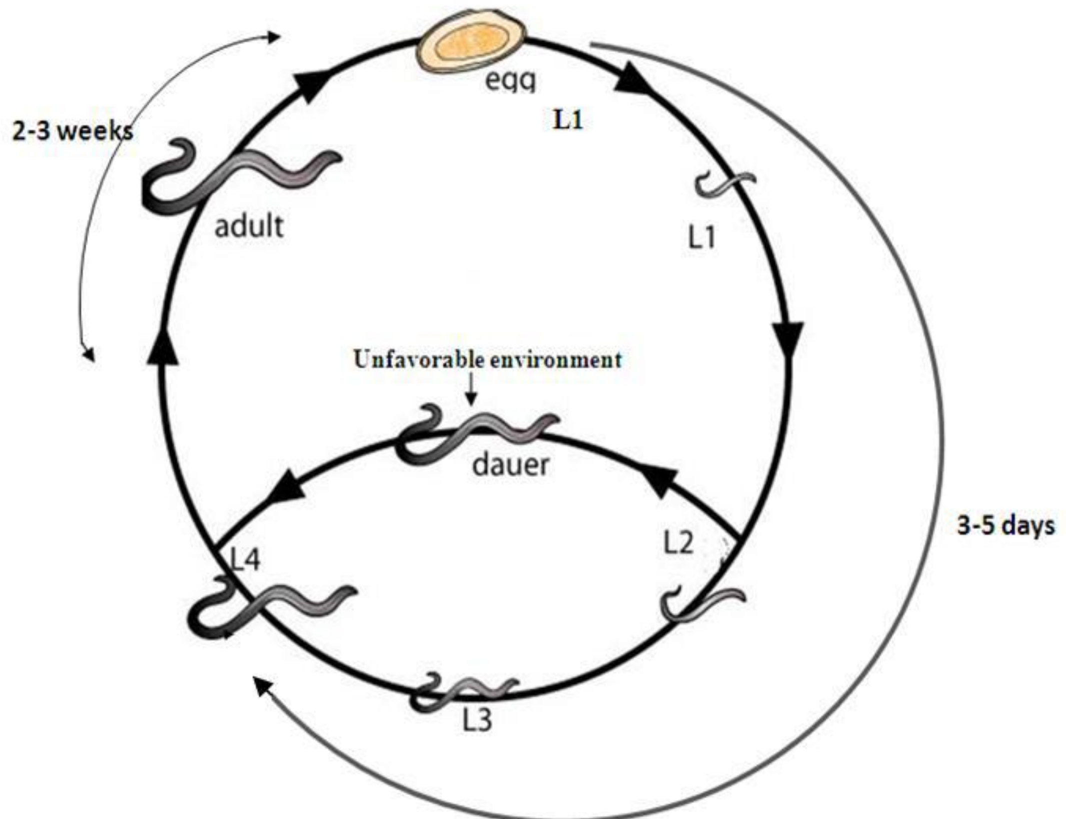
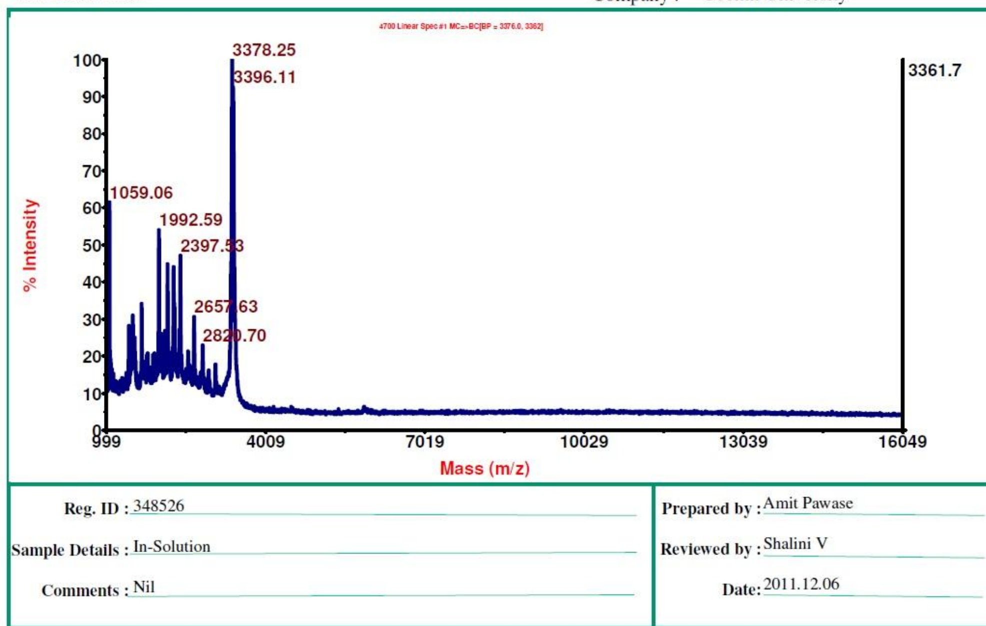


Fig. 8.1 Life cycle of *C. elegans* (figure adapted from http://www.scq.ubc.ca/genetic-studies-of-aging-and-longevity-in-model-organisms - with modification)

The life cycle of *C. elegans* is comprised of the embryonic stage, four larval stages (L1-L4) and adulthood. The end of each larval stage is marked with a molt. Under favorable environments, *C. elegans* will develop rapidly to reproductive maturity, but in unfavorable environments, animals will arrest at the dauer diapause, a larval stage geared for survival. Dauer larvae are thin and can move but their mouths are plugged and they cannot eat. When animals reach adulthood, they produce about 300 progeny each. They live a total of up to 3 weeks depending on the temperature.



Advanced Molecular Biology,
Vimta Labs Limited
www.vimta.com, 040-39848484, Ext. 4207

BTFK101 IMD

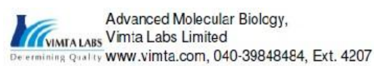
Customer : Smitha
Company : Cochin University

Reg. ID : 348526
Sample Details : In-Solution
Comments : Nil

Prepared by : Amit Pawase
Reviewed by : Shalini V
Date: 2011.12.06

2

Fig. 8.2 Report of intact mass determination of BS101 at Vimta Labs Ltd., Hyderabad, India



Customer : Smitha
BTH8IMD Company : Cochin University

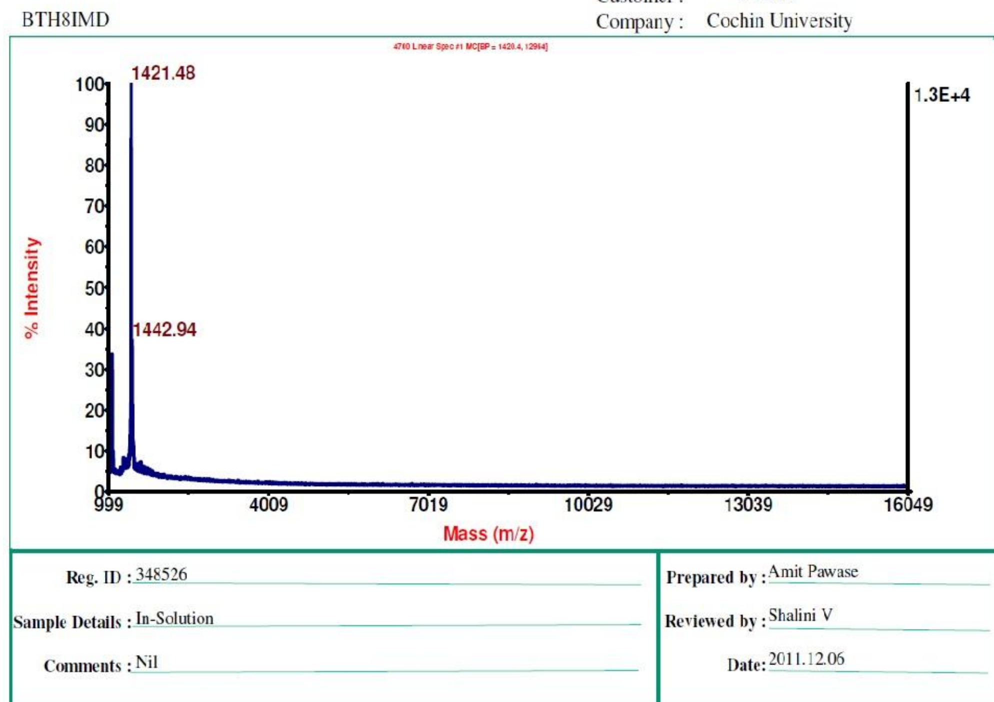


Fig. 8.3 Report of intact mass determination of BL8 at Vimta Labs Ltd., Hyderabad, India

National Facility for Protein Sequencing

Indian Institute of Technology Bombay

26th June 2012

N-terminal Protein Sequencing
(Report)

METHOD

Automated Edman Degradation was carried out using an Applied Biosystems 494 Procise Protein Sequencing System.

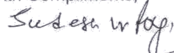
RESULTS

Sequence - S W S X X G/E N X S I S G S

NOTES ON INTERPRETING THE RESULTS

The following notes apply to all N-terminal sequencing results:
1. Sequencing is achieved by repeated cycles of chemical reaction, and an amino acid (residue) is detected or called by its increase in signal during a particular cycle.
2. In each amino acid position there can be more than one residue apparent. This reflects inherent background effects such as the internal cleavage of the full protein during the sequencing procedure which creates a 2nd, 3rd, etc N-terminus.
3. The first and second positions are subject to higher background signal and as such may be difficult to call.
4. Residues where no clear signal is detected are designated X.
5. Cysteine is undetectable by sequencing (without special derivitisation) and so X can imply Cysteine.

With Compliments,



Department of Science and Technology

Scanned copy of the report of N-terminal sequencing of BL8 from IIT Bombay

LIST OF PUBLICATIONS

FULL PAPER IN PEER-REVIEWED JOURNALS

1. **Smitha S**, Bhat SG (2013): Thermostable bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment, Journal of Applied Microbiology **114**, 688- 694.

FULL PAPERS IN THE PROCEEDINGS OF INTERNATIONAL/ NATIONAL SYMPOSIA, CONFERENCES AND SEMINARS

1. **Smitha S**., Jeena Augustine, Raghul Subin S. and Sarita G Bhat (2011) "Antimicrobial protein from *Bacillus licheniformis* isolated from Cochin backwaters" Proceedings of UGC sponsored two day national seminar on " Emerging trends in biopharmaceuticals" organized by St. Mary's College, Thrissur.

2. **Smitha S.**, Rekhamol K.R and Sarita G Bhat, (2011) Partial characterization of antibacterial proteins from *Bacillus licheniformi*s. Proceedings of 2 day National symposium on "Emerging trends in Biotechnology" conducted by Department of Biotechnology, CUSAT, 1st & 2nd September, 2011. **ISBN number : 978-93-80095-30**

3. Rekhamol K.R, **Smitha S,** Manzur Ali P P, Sapna K, Abraham Mathew, Sarita G Bhat and Elyas K.K (2011) "Screening of various biological sources for antibacterial peptides". Proceedings of 2 day National symposium on "Emerging trends in Biotechnology" conducted by Department of

Biotechnology, CUSAT, 1st & 2nd September, 2011. **ISBN number : 978-93-80095-30-1**

4. Raghul Subin S, **Smitha S.** and Sarita G Bhat (2008) Exploration of marine benthic Vibrios as a promising source of bioactive molecules. Paper presented at National Seminar on "Bioactive compounds from the marine organisms" from 14th to 15th March, 2008, organized by the Department of Marine Biology, Microbiology and Biochemistry, School of Ocean Science and Technology.

POSTERS / ABSTRACTS IN NATIONAL / INTERNATIONAL SYMPOSIA

1. **Smitha S.** and Sarita G Bhat, (2008) Exploitation of marine bacteria for novel bioactive compounds. Proceedings of BIOCAM 2008, Cochin, Kerala. International conference on biodiversity conservation and management, Kochi, from3-6th Feb, 2008.

2. Helvin Vincent, **S. Smitha** and Sarita G Bhat (2009) Isolation of a novel protease gene using metagenomic approach, Book of abstracts of MECOS 09, Cochin, Kerala. International symposium on Marine Ecosystems challenges and Opportunities, Cochin from 9-12 Feb, 2009.

GENBANK SUBMISSIONS

1. Smitha, S., Jeena, A. and Sarita, B.G. (2010) **HM030818**- *Bacillus subtilis* subsp. *subtilis* strain BTFK101 16S ribosomal RNA gene, partial sequence.

2. Smitha, S., Raghul, S.S. and Sarita, B.G. (2010) **HM030819**- *Bacillus licheniformis* strain BTHT8 16S ribosomal RNA gene, partial sequence.

3. Smitha, S., Helvin, V., Linda, L. and Sarita, B.G (2010) **HM030820**- *Bacillus licheniformis* strain BTKM4 16S ribosomal RNA gene, partial sequence.

4. Smitha, S., Siju, V.M., Alphonsa, V.J. and Sarita, B.G (2010) **HM030821**- *Bacillus licheniformis* strain BTEK16 16S ribosomal RNA gene, partial sequence.

5. Smitha, S., Manzur, A.P.P. and Sarita, B.G. (2010) **HM030822**- *Bacillus subtilis* subsp. *subtilis* strain BTSB22 16S ribosomal RNA gene, partial sequence.

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ORIGINAL ARTICLE

Thermostable Bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment

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Department of Biotechnology, Cochin University of Science and Technology, Cochin, India

Keywords
ammonium sulphate fractionation, *Bacillus licheniformis*, bacteriocin, electroelution, Gram positive, mass spectrometry, thermostable.

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Abstract

Aim: To isolate and characterize bacteriocin, BL8, from the bacteria identified as *Bacillus licheniformis* from marine environment.

Methods and Results: One-hundred and twelve bacterial isolates from sediment and water samples collected off the coast of Cochin, India, were screened for antibacterial activity. Strain BTHT8, identified as *Bacillus licheniformis*, inhibited the growth of Gram-positive test organisms. The active component labelled as bacteriocin BL8 was partially purified by ammonium sulphate fractionation and was subjected to glycine SDS-PAGE. The band exhibiting antimicrobial activity was electroeluted and analysed using MALDI-TOF mass spectrometry, and the molecular mass was determined as 1·4 kDa. N-terminal amino acid sequencing of BL8 gave a 13 amino acid sequence stretch. Bacteriocin BL8 was stable even after boiling at 100°C for 30 min and over a wide pH range of 1–12.

Conclusion: A novel, pH-tolerant and thermostable bacteriocin BL8, active against the tested Gram-positive bacteria, was isolated from *Bacillus licheniformis*.

Significance and Impact of the Study: This study reports a stable, low molecular weight bacteriocin from *Bacillus licheniformis*. This bacteriocin can be used to address two important applications: as a therapeutic agent and as a biopreservative in food processing industry.

Introduction

Bacteriocins are ribosomally synthesized peptides which kill bacteria that are often closely related to the producer strain (Lisboa *et al.* 2006). They are heterogeneous compounds that display variable molecular weights, biochemical properties, inhibitory spectra and mechanisms of action (O'Sullivan *et al.* 2002).

The genus *Bacillus* is a heterogeneous group of Gram-positive, facultative anaerobic, endospore-forming bacteria widespread in the environment, although soil is generally accepted as its natural reservoir (Galvez *et al.* 1993). *Bacillus* spp. produce a large number of bacteriocins, subtilosin by *B. amyloliquifaciens* (Sutyak *et al.* 2008), bacillocin 490 by *B. licheniformis* (Martirani *et al.* 2002), cerein by *B. cereus* (Oscariz *et al.* 1999), haloduracin by *B. halodurans* (Lawton *et al.* 2006), thuricin by *B. thuringiensis* (Gray *et al.* 2006) and subtilin by *B. subtilis* (Banerjee and Hansen 1988), all of which are mostly active against Gram-positive organisms.

A single *Bacillus* strain often has the ability to produce several different molecules partially resistant to enzyme treatments, with stability over a wide range of pH and temperature (Baruzzi *et al.* 2011). The capability to produce endospores allows *Bacillus* sp. to withstand extreme environmental conditions such as those encountered in food processing. Like the lactic acid bacteria (LAB), the genus *Bacillus* includes representatives that are 'generally recognized as safe' (GRAS), such as *B. subtilis* and *B. licheniformis* (Sharp *et al.* 1989), and can hence find application in the control of food pathogens and spoilage micro-organisms during food processing. The concept of qualified presumption of safety (QPS) for acceptability of bacteria in foods if the

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taxonomic group did not raise safety concerns, or if safety concerns did exist could be defined and excluded, was recently introduced by the European Food Safety Authority (EFSA 2007, 2008). The qualification concerning QPS for *Bacillus* species is modified to 'absence of food poisoning toxins, absence of surfactant activities, absence of enterotoxic activities' (EFSA 2008). Nevertheless, bacteriocin-producing strains or their bacteriocin preparations could still be used in food preservation provided they meet the criteria established by EFSA.

This study was undertaken to isolate and characterize the bacteriocin-like substance active against *Bacillus* sp. and other Gram-positive bacteria produced by strain of *B. licheniformis*.

Materials and methods

Bacterial strains

Water and sediment samples collected off the coast of Cochin, India, and serially diluted were plated on prepared tryptone soya agar plates (HiMedia, Mumbai, India) to obtain isolated bacterial colonies. Standard test organisms availed from National Collection of Industrial Microorganisms (NCIM, NCL, Pune) were included in this study (Table 1).

Bacteriocin production

Bacteria were grown in Zobell marine broth 2216 (HiMedia), incubated at 28°C for 24 h in environmental shaker (Scigenics, Chennai, India) at 150 rev min^{-1}. The culture broth was centrifuged at 9400 ***g*** for 15 min at 4°C (Sigma 3K30, Osterode, Germany), the supernatant collected and filtered through 0·22-μm membrane (Millipore, Billerica, MA, USA). This filtrate with an alkaline pH (pH 8) was used to evaluate antimicrobial activity without any heat treatment or change in the pH.

Table 1 The antibacterial activity of the bacteriocin BL8

Test organisms	NCIM No.	Zone size in mm
Pseudomonas aeruginosa	2863	–
Salmonella Typhimurium	2501	–
Escherichia coli	2343	–
Salmonella Abony	2257	–
Klebsiella pneumoniae	2957	–
Proteus vulgaris	2027	–
Clostridium perfringens	2677	13 ± 0·5
Staphylococcus aureus	2127	11 ± 1
Bacillus cereus	2155	15 ± 0·6
Bacillus circulans	2107	10 ± 1
Bacillus coagulans	2030	10 ± 0·5
Bacillus macerans	2131	–
Bacillus pumilus	2189	11 ± 1

Experiments conducted in triplicate and values given with standard deviation.

Detection of antimicrobial activity

Antibacterial activity was determined by agar well diffusion assay (Tagg and McGiven 1971). Mueller-Hinton agar (HiMedia) plates were swab-inoculated with the test organisms (Table 1) grown in nutrient broth (HiMedia) for 12 h and wells were cut. 20 μl of culture filtrate was added into the wells. Plates were incubated for 24 h at 37°C and the inhibition zones were measured. Each time, the antibacterial activity was tested against all six Gram-positive test organisms. The assay was conducted in triplicate.

Bacterial identification

Bacterial DNA was isolated according to the protocol described by Ausubel *et al.* (1987). 16S rRNA gene (1·5 kb size) was amplified from the genomic DNA using thermal cycler (Bio-Rad, CA, USA) with universal primers for 16S rDNA (Shivaji *et al.* 2000) with an initial denaturation at 94°C for 1·5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 2 min and final extension at 72°C for 10 min. Nucleotide sequence of the PCR amplicon was determined by the ABI Prism 310 genetic analyzer (Applied Biosystems, Carlsbad, CA, USA) using big dye terminator kit. The identity of the sequence was determined by comparing the sequences in the NCBI database using Basic Local Alignment Search Tool (BLAST) software (Altschul *et al.* 1990). The sequence was submitted to GenBank and accession number obtained.

Partial purification of bacteriocin BL8

As a first step towards purification of the active compound, the supernatant collected after centrifugation was fractionated using ammonium sulphate (0–30%, 30–60% and 60–90%). The precipitate obtained was dialysed using a 2-kDa benzoylated dialysis tubing (Sigma Aldrich, St Louis, MO, USA) against deionized water at 4°C for 36 h with six changes of water.

Protein quantification and Minimum Inhibitory Concentration (MIC)

The total protein content in the partially purified BL8 was estimated according to the protocol described by Bradford (1976). The resazurin assay utilizing microtitre plate (Sarker *et al.* 2007) has been modified to determine

the minimum inhibitory concentration (MIC) values of the bacteriocin against the test organisms. To 50 μl of deionized water, 2 μl of resazurin (1%) was added. 50 μl of BL8 was added in the first row and double-diluted vertically below. Then 100 μl of double-strength nutrient broth was added in each well followed by 50 μl of test organisms (OD_{600} = 1) and incubated at 37°C for 18–24 h.

Glycine SDS-PAGE and detection of bacteriocin activity

The partially purified protein was subjected to glycine SDS-PAGE using 15% resolving gel (Laemmli 1970). The protein was run in two lanes along with protein molecular weight marker (GeNei, Bangalore, India) in a vertical slab electrophoresis system (Genei) at 80 V, and the gel was cut vertically into two halves. One half containing the sample and molecular weight marker was silver-stained. The other half with sample was washed thrice with 0·1% Tween 80 (30 min each), followed by washing with deionized water to remove SDS. The gel was then placed on Mueller-Hinton agar (HiMedia) base plate, overlaid with soft Mueller-Hinton agar (0·8% agar) seeded with 100 μl (OD_{600} = 1) of test organisms (Yamamoto *et al.* 2003) and checked for zone of clearance due to the antibacterial activity after overnight incubation at 37°C.

Electroelution of bacteriocin

The protein band with antibacterial activity was excised and electroeluted in a 2-kDa benzoylated dialysis tubing (Sigma Aldrich) against 0·01 mol l^{-1} phosphate buffer. For this, a voltage of 30 V was applied overnight at 4°C, after which the electrodes were reversed and voltage of 30 V was applied for 30 min (Lei *et al.* 2007).

Mass spectroscopy

Intact mass of the electroeluted fraction of bacteriocin BL8 was determined using mass spectrometer having specification of ABI4800MALDI-TOF/TOF (Applied Biosystems).

N-terminal aminoacid sequence analysis

The N-terminal amino acid analysis of the electro-eluted protein was resolved by automated Edman degradation (Applied Biosystems 494 Procise Protein Sequencing System). The sequence obtained was compared to bacteriocins from *Bacillus* sp. retrieved from the protein database of NCBI by multiple sequence alignment using ClustalW (Larkin *et al.* 2007).

Effect of heat and pH on bacteriocin activity

Partially purified bacteriocin was used for the study. To evaluate heat stability, bacteriocin was exposed to temperatures ranging from 40 to 100°C for 30 min and 121°C per 105 kPa for 15 min. To study the effect of different pH, samples of bacteriocin were treated with equal amount of buffers with pH range 1–12 and kept for 18 h at 4°C. The buffer systems used included hydrochloric acid/potassium chloride buffer (pH 1–2), citric acid/sodium citrate buffer (pH 3–5), phosphate buffer (pH 6–7), Tris amino methane/hydrochloric acid buffer (pH 8–9), sodium bicarbonate/sodium hydroxide buffer (pH 10), sodium phosphate dibasic/sodium hydroxide buffer (pH 11–12) (Vincent and John 2009). The samples after the treatment were tested for the antimicrobial activity against the test organisms. All assays were conducted in triplicate.

Results

One-hundred and twelve strains were isolated from sediment and water samples. All strains were screened for antibacterial activity. Of these, strain BTHT8 exhibited broad range of antibacterial activity against the test organisms considered to be the major pathogens in food-borne illnesses and other infections, and also showed consistency and reproducibility. The bacterial strain BTHT8 was identified to be *Bacillus licheniformis* by 16S rDNA sequence analysis. The sequence obtained was submitted to GenBank (accession no. HMO30819).

Maximum antibacterial activity was exhibited by the 30–60% ammonium sulphate fraction. The total protein concentration was found to be 798 μg ml^{-1}. The MIC of bacteriocin BL8 ammonium sulphate fraction for *B. circulans* and *Staphylococcus aureus* was found to be 40 μg ml^{-1}, for *B. coagulans* it was 80 μg ml^{-1}, for *B. cereus* it was 160 μg ml^{-1} and for *Clostridium perfringens* and *B. pumilis* it was 250 μg ml^{-1}.

Glycine SDS-PAGE and silver staining of the partially purified bacteriocin showed numerous protein bands on gel. A clearing zone on gel overlaid with Mueller-Hinton soft agar seeded with test organisms indicated antibacterial activity (Fig. 1b) at a region with molecular mass below 3 kDa (Fig. 1a).

The band with antibacterial activity was electroeluted, and the protein analysis by SDS-PAGE showed a single band whose intact mass was determined to be 1·4 kDa by mass spectrometric analysis (Fig. 2).

N-terminal amino acid analysis revealed the 13 amino acid sequence stretch: NH_2-Ser-Trp-Ser-Cys-Cys-Gly-Asn-Cys-Ser-Ile-Ser-Gly-Ser-COOH. Comparison with the bacteriocins from protein database by multiple sequence

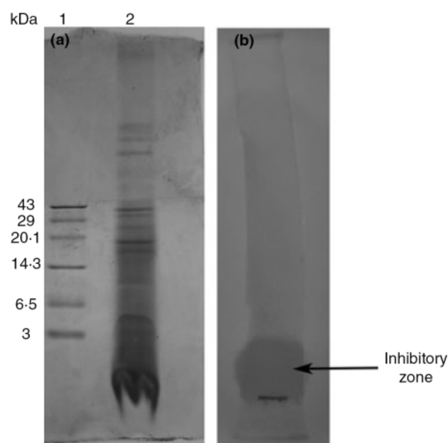

Figure 1 (a) Silver-stained SDS-PAGE, Lane 1: protein molecular weight marker (43–3 kDa); Lane 2: partially purified bacteriocin BL8 using 30–60% ammonium sulphate fractionation. (b) Gel overlaid with test organisms showing a clearing region (inhibitory zone) indicating antibacterial activity associated with 30–60% ammonium sulphate fraction of bacteriocin BL8.

alignment showed no significant similarity indicating its novelty (Fig. 3).

Thermal stability studies revealed that the antibacterial activity of bacteriocin BL8 was lost when exposed to high temperatures of 121°C per 105 kPa for 15 min but was retained when exposed to temperatures ranging from 40 to 100°C for 30 min. The bacteriocin activity was stable over a broad pH range of 1–12.

Discussion

Bacteriocins and BLIS produced by the genus *Bacillus* are considered as significant as the bacteriocins produced by the lactic acid bacteria (LAB). A diverse array of antimicrobial peptides with several different basic chemical structures is produced by genus *Bacillus* (Gebhardt *et al.* 2002; Stein *et al.* 2005).

The few bacteriocins reported from *B. licheniformis* have their antibacterial activity restricted to Gram-positive organisms. Bacillocin 490 produced by thermophilic *B. licheniformis* 490/5 from a dairy product had antibacterial activity against the closely related species *Geobacillus stearothermophilus*, *B. smithii*, *B. subtilis*, *B. anthracis*, *B. cereus* and *B. licheniformis* (Martirani *et al.* 2002). A bacteriocin-like inhibitory substance (BLIS) produced by *B. licheniformis* P40 from the Amazon Basin inhibited *Listeria monocytogenes*, *B. cereus* and clinical isolates of *Streptococcus* sp. (Cladera-Olivera *et al.* 2004), whereas other uncharacterized antimicrobial substances were produced by *B. licheniformis* T6-5 from an oil reservoir in Brazil (Korenblum *et al.* 2005) and by *B. lichenifomis* AnBa9 from sediments of slaughterhouse sewage (Anthony *et al.* 2009). The spectrum of antibacterial activity of bacteriocin BL8 from *B. licheniformis* strain BTHT8 clearly shows that it inhibited not only phylogenetically related species but also other Gram positives.

Bacteriocins active against Gram-negative organisms have been previously reported from other *Bacillus* spp; *B. amyloliquefaciens* LBM 5006 from the Brazilian Atlantic forest produces a broad antibacterial spectrum BLIS that included activity against *L. monocytogenes*, *B. cereus*, *Serratia marcescens* and *Pasteurella haemolytica* (Lisboa *et al.* 2006). Megacin 19 by *B. megaterium* 19 from fermented vegetable wastes and megacin 22 by *B. megaterium* 22 from soil show wide antimicrobial spectra against food spoilage bacteria like *Salmonella* Typhimurium and *Staph. aureus* (Khalil *et al.* 2009a,b). BLIS by *B. subtilis* LFB112 from Chinese herbs was active against domestic animal disease caused by Gram-positive and Gram-negative bacteria including *Escherichia coli*, *Salmonella* Pullorum, *Pseudomonas aeruginosa*, *Pasteurella multocida*,

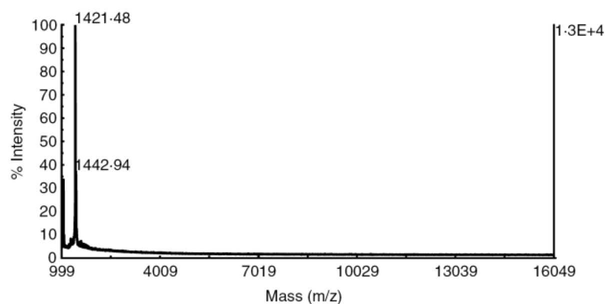

Figure 2 Mass spectrum of bacteriocin BL8 obtained by mass spectrometry (MALDI-TOF/TOF).

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YP006230889 Bacillus sp. JS                        RMRTWKRIPKTTMLISLVSPFLLITPVLFYAALAFP
gbAEB23117 B.amyloliquefaciens                     RMPTWKGIPKTTMLISTISPFLFITPVLFYAGLAFP
CAJ32354 B.cereus                                  GGGWWNSWGKCVA--GTIG------------GAGTG
AEP01267 B.coagulans                               ASTLGISTAAAKKAIDIID--------------TAS
EAO54508 B.thuringiensis serovar israelensis       ALFVWMLFTTQKKNEQREE-----------------
ADP31478 B.atrophaeus                              RLRQWKRIPKMTMLISLLSPFLLITPVLFYAGLAFP
gbAEK90981 B.amyloliquefaciens                     GAGVWWMLSFTIS-----------------------
ZP 04069294 B.subtilis subsp. subtilis             AVGWGVAAASAIAAIVTAG-----------------
YP 004205572 B.thuringiensis                       -----KGCATC-----SIG-----------------
B.licheniformis strain BTHT8                       ---SWSCCGNC-----SIS------------GS---
```

Figure 3 Alignment of amino acid sequence of BL8 from *Bacillus licheniformis* strain BTHT8 with the amino acid sequence of known bacteriocins from protein database of NCBI.

Cl. perfringens, *Micrococcus luteus*, *Streptococcus bovis* and *Staph. aureus* (Xie *et al.* 2009).

The present study is on bacteriocin from *B. licheniformis* of marine origin. Bacteriocins reported earlier from marine bacteria were extracellular inhibitory substances produced by the marine *Alteromonas* strain P-31 (Barja *et al.* 1989) and antimicrobial protein from marine bacterium *Pseudoalteromonas* sp. strain X153 (Longeon *et al.* 2004).

BL8 with 1·4 kDa size can be grouped with small molecular peptides. Although some bacteriocins are small peptides with 19–37 amino acids, others have molecular weights up to 90 kDa (Joerger 2003). Bacillocin 490 reported from *B. licheniformis* 490/5 is a 2-kDa peptide (Martirani *et al.* 2002). BLIS with 3–5 kDa size from *B. subtilis* (Alam *et al.* 2011), 6·3 kDa from *B. subtilis* (Xie *et al.* 2009) and 5 kDa from *B. amyloliquefaciens* LBM 5006 have been reported (Lisboa *et al.* 2006). Cerein GN105 from *B. cereus* GN105 has an apparent molecular weight of 9 kDa by SDS-PAGE (Naclerio *et al.* 1993), while that from *B. lentus* was 11 kDa (Sharma *et al.* 2009a,b). Pumilicin 4 from *B. pumilus* with molecular mass of 1·9 kDa (Aunpad and Na-Bangchang 2007) and tochicin with 10·5 kDa from *B. thuringiensis* ssp. *tochigiensis* HD868 (Paik *et al.* 1997) are other small molecular weight bacteriocins.

BL8 activity was stable at 100°C and over wide pH range. Bacillocin 490 from *B. licheniformis* 490/5 exhibited high stability at 4 and 100°C over a wide pH range (Martirani *et al.* 2002). BLIS from *B. licheniformis* P40 showed stability at pH 3–11 and at higher temperature of 100°C but lost its activity on exposure to 121°C for 15 min (Cladera-Olivera *et al.* 2004). Heating at 100°C for 60 min had no apparent effect on subtilosin (Sutyak *et al.* 2008). Bacillocin Bb, a BLIS by *B. brevis* Bb from soil (Saleem *et al.* 2009), is stable not only over pH range of 1–9, but also at 100°C for 30 min. Megacins 19 and 22 remained stable up to 100°C for 15 min (Khalil *et al.* 2009a,b) as did Pumilicin 4 but up to 121°C for 15 min and in the pH range of 3–9 (Aunpad and Na-Bangchang 2007). Some bacteriocins lose their activity at very high temperatures. Cerein 8A from *B. cereus* 8A had broad pH stability (pH 2–11) and was relatively thermostable, losing activity only at temperatures above 75°C for 30 min (Bizani *et al.* 2005). BLIS from *B. amyloliquefaciens* LBM 5006 has stability at 80°C for 30 min, but the residual activity decreased at 100°C and all activity lost at 121°C (Lisboa *et al.* 2006). On the other hand, tochicin from *B. thuringiensis* was relatively heat stable at 90°C, but activity undetected after boiling for 30 min (Paik *et al.* 1997).

Production of antimicrobial substances and sporulating capacity confers a double advantage on *Bacillus* strains for their survival in diverse habitats. The presence of these bacteria in food does not always imply spoilage or food poisoning, with several species or strains used in human and animal food production. *B. subtilis* strains are used in the production of Natto, an East Asian fermented food (Hosoi and Kiuchi 2003), as a starter culture for fermentation of soya beans into the traditional West African condiment dawadawa (Terlabie *et al.* 2006) or African mesquite seeds for the Nigerian food condiment okpehe (Oguntoyinbo *et al.* 2007). Bacteriocins from *Bacillus* have a potential preservative application in dairy products like milk and cheeses (Sharma *et al.* 2009a,b).

Bacteriocin BL8 from *B. licheniformis* strain BTHT8 with its thermostable and antibacterial activity over a broad pH range can be used to address two important aspects: as a therapeutic agent and as a biopreservative in food processing industry considering its antibacterial activity against pathogenic Gram-positive bacteria that cause foodborne illnesses.

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References

Alam, S.I., Kamran, M., Sohail, M., Ahmad, A. and Khan, S.A. (2011) Partial characterization of bacteriocin like inhibitory substance from *Bacillus subtilis* BS15, a local soil isolate. *Pak J Bot* **43**, 2195–2199.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Anthony, T., Rajesh, T., Kayalvizhi, N. and Gunasekaran, P. (2009) Influence of medium components and fermentation conditions on the production of bacteriocin(s) by *Bacillus licheniformis* AnBa9. *Biores Technol* **100**, 872–877.

Aunpad, R. and Na-Bangchang, K. (2007) Pumilicin 4, a novel bacteriocin with anti-MRSA and anti-VRE activity produced by newly isolated bacteria *Bacillus pumilus* strain WAPB4. *Curr Microbiol* **55**, 308–313.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G. and Struhl, K. (1987) *Current Protocols in Molecular Biology*. New York: Greene Publishing Associates/Wiley Interscience.

Banerjee, S. and Hansen, J.N. (1988) Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. *J Biol Chem* **263**, 9508–9514.

Barja, J.L., Manuel, L.L. and Toranzo, A.E. (1989) Purification and characterization of an antibacterial substance produced by a marine *Alteromonas* Species. *Antimicrob Agents Chemother* **33**, 1674–1679.

Baruzzi, F., Quintieri, L., Morea, M. and Caputo, L. (2011) Antimicrobial compounds produced by *Bacillus* spp. and applications in food. Science against microbial pathogens: communicating current research and technological advances **2**, 1102–1111.

Bizani, D., Dominguez, A.P.M. and Brandelli, A. (2005) Purification and partial chemical characterization of the antimicrobial peptide cerein 8A. *Lett Appl Microbiol* **41**, 269–273.

Bradford, M.M. (1976) A dye binding assay for protein. *Anal Biochem* **72**, 248–254.

Cladera-Olivera, F., Caron, G.R. and Brandelli, A. (2004) Bacteriocin like substance production by *Bacillus licheniformis* strain P40. *Lett Appl Microbiol* **38**, 251–256.

EFSA (2007) Introduction of a qualified presumption of safety (QPS) approach for assessment of selected microorganisms referred to EFSA. *EFSA J* **587**, 1–16.

EFSA (2008) The maintenance of the list of QPS microorganisms intentionally added to foods or feeds. Scientific opinion of the panel on biological hazards. *EFSA J* **923**, 1–48.

Galvez, A., Valdivia, E., Gonzalez-Segura, A., Lebbadi, M., Martinez Bueno, M. and Maqueda, M. (1993) Purification, characterization and lytic activity against *Naegleria fowleri* of two amoebicins produced by *Bacillus licheniformis* A12. *Appl Environ Microbiol* **59**, 1480–1486.

Gebhardt, K., Schimana, J. and Muller, J. (2002) Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. *FEMS Microbiol Lett* **217**, 199–205.

Gray, E.J., Lee, K.D., Souleimanov, A.M., Di Falco, M.R., Zhou, X., Ly, A., Charles, T.C., Driscoll, B.T. *et al.* (2006) A novel bacteriocin, thuricin 17, produced by plant growth promoting rhizobacteria strain *Bacillus thuringiensis* NEB17: isolation and identification. *J Appl Microbiol* **100**, 545–554.

Hosoi, T. and Kiuchi, K. (2003) Natto – a food made by fermenting cooked soybeans with *Bacillus subtilis* (natto). In *Handbook of Fermented Functional Food* ed. Farnworth, E.R. pp. 227–245. Boca Raton, FL: CRC Press.

Joerger, R.D. (2003) Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poult Sci* **82**, 640–647.

Khalil, R., Djadouni, F., Elbahloul, Y. and Omar, S. (2009a) The influence of cultural and physical conditions on the antimicrobial activity of bacteriocin produced by a newly isolated *Bacillus megaterium* 22 strain. *Afr J Food Sci* **3**, 011–022.

Khalil, R., Elbahloul, Y., Djadouni, F. and Omar, S. (2009b) Isolation and partial characterization of a bacteriocin produced by a newly isolated *Bacillus megaterium* 19 strain. *Pakistan J Nutr* **8**, 242–250.

Korenblum, E., der Weid, I., Santos, A.L., Rosado, A.S., Sebastian, G.V., Coutinho, C.M., Magalhaes, F.C., Paiva, M.M. *et al.* (2005) Production of antimicrobial substances by *Bacillus subtilis* LFE-1, *B. firmus* HO-1 and *B. licheniformis* T6–5 isolated from an oil reservoir in Brazil. *J Appl Microbiol* **98**, 667–675.

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

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I. M. *et al.* (2007) ClustalW and ClustalX version 2. *Bioinformatics* **23**, 2947–2948.

Lawton, E.M., Paul, D., Cotter, C.H. and Ross, P.R. (2006) Identification of a novel two-peptide lantibiotic, Haloduracin, produced by the alkaliphile *Bacillus halodurans* C-125. *FEMS Microbiol Lett* **267**, 64–71.

Lei, Z., Anand, A., Mysore, K.S. and Sumner, L.W. (2007) Electroelution of intact proteins from SDS-PAGE gels and their subsequent MALDI-TOF MS analysis. *Methods Mol Biol* **355**, 353–363.

Lisboa, M.P., Bonatto, D., Bizani, D., Henriques, J.A.P. and Brandelli, A. (2006) Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic Forest. *Intl Microbiol* **9**, 111–118.

Longeon, A., Peduzzi, J., Barthelemy, M., Corre, S., Nicolas, J. L. and Guyot, M. (2004) Purification and partial identification of novel antimicrobial protein from marine

bacterium *Pseudoalteromonas* Species Strain X153. *Mar Biotechnol* **6**, 633–641.

Martirani, L., Varcamonti, M., Naclerio, G. and Felice, M.D. (2002) Purification and partial characterization of bacillocin 490, a novel bacteriocin produced by a thermophilic strain of *Bacillus licheniformis*. *Microb Cell Fact* **1** doi: 10.1186/1475-2859-1-1.

Naclerio, G., Ricca, E., Sacco, M. and de Felice, M. (1993) Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. *Appl Environ Microb* **59**, 4313–4316.

Oguntoyinbo, A., Sanni, A.I., Franz, C.M.A.P. and Holzapfel, W.H. (2007) *In vitro* fermentation studies for selection and evaluation of *Bacillus* strains as starter cultures for the production of okpehe, a traditional African fermented condiment. *Int J Food Microbiol* **113**, 208–218.

Oscariz, J.C., Lasa, I. and Pissabarro, A.G. (1999) Detection and characterization of cerien 7, a new bacteriocin produced by *Bacillus cereus* with a broad spectrum of activity. *FEMS Microbiol Lett* **178**, 337–341.

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

Paik, H.D., Bae, S.S., Park, S.H. and Pan, J.G. (1997) Identification and partial characterization of tochicin, a bacteriocin produced by *Bacillus thuringiensis* subsp. *tochigiensis*. *J Ind Microbiol Biotechnol* **19**, 294–298.

Saleem, F., Ahmad, S., Yaqoob, Z. and Rasool, S.A. (2009) Comparative study of two bacteriocins produced by representative indigenous soil bacteria. *Pak J Pharm Sci* **22**, 252–258.

Sarker, S.D., Lutfun, N. and Yashodharan, K. (2007) Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. *Methods* **42**, 321–324.

Sharma, N., Attri, A. and Gautam, N. (2009a) Purification and characterization of bacteriocin like substance produced from *Bacillus lentus* with perspective of a new biopreservative for food preservation. *Pak J Sci Ind Res* **52**, 191–199.

Sharma, N., Kapoor, G., Gautam, N. and Neopaney, B. (2009b) Characterization of partially purified bacteriocin of *Bacillus* sp. MTCC43 isolated from rhizosphere of radish (*Raphanus sativus*) and its application as a potential food biopreservative. *J Sci Ind Res* **68**, 881–886.

Sharp, R.J., Scawen, M.D. and Atkinson, T. (1989) Fermentation and downstream processing of *Bacillus*. In *Biotechnology Handbook Bacillus* ed. Harwood, C.R. pp. 255–292. New York: Plenum Press.

Shivaji, S., Bhanu, N.V. and Aggarwal, R.K. (2000) Identification of *Yersinia pestis* as the causative organism of plague in India as determined by 16SrDNA sequencing and RAPD-based genomic fingerprinting. *FEMS Microbiol Lett* **189**, 247–252.

Stein, T., Heinzmann, S., Dusterhus, S., Borchert, S. and Entian, K.D. (2005) Expression and functional analysis of the subtilin immunity genes spaIFEG in the subtilin-sensitive host *Bacillus subtilis* MO1099. *J Bacteriol* **187**, 822–828.

Sutyak, K.E., Wirawan, R.E., Aroutcheva, A.A. and Chikindas, M.L. (2008) Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*. *J Appl Microbiol* **104**, 1067–1074.

Tagg, J.R. and McGiven, A.R. (1971) Assay system for bacteriocins. *Appl Microbiol* **21**, 943–944.

Terlabie, N.N., Sakyi-Dawson, E. and Amoa-Awua, W.K. (2006) The comparative ability of four isolates of *Bacillus subtilis* to ferment soybeans into dawadawa. *Int J Food Microbiol* **106**, 145–152.

Vincent, S.S. and John, S.B. (2009) Buffers: principles and practice. In *Methods in Enzymology* ed. Richard, R.B. and Murray, P.D. pp. 43–56. Massachusetts: Elsevier Science & Technology Books.

Xie, J., Zhang, R., Shang, C. and Guo, Y. (2009) Isolation and characterization of a bacteriocin produced by an isolated *Bacillus subtilis* LFB112 that exhibits antimicrobial activity against domestic animal pathogens. *Afr J Biotechnol* **8**, 5611–5619.

Yamamoto, Y., Togawa, Y., Shimosaka, M. and Okazaki, M. (2003) Purification and characterization of a novel bacteriocin produced by *Enterococcus faecalis* strain RJ-11. *Appl Environ Microbiol* **69**, 5746–5757.