Bacteriocins BaCf3 and BpSl14 with anticancer and antibiofilm potential from probiotic *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14 isolated from gut of marine fishes: Enhanced production, Purification and Characterization

Thesis submitted to the
Cochin University of Science and Technology
Under the Faculty of Science
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

By

BINDIYA. E. S

Reg no: 4957

Microbial Genetics Laboratory
Department of Biotechnology
Cochin University of Science and Technology
Cochin - 682 022, Kerala, India.

AUGUST 2017

DEPARTMENT OF BIOTECHNOLOGY

COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY

COCHIN-682 022, INDIA Phone : 91-484-2576267

e-mail: saritagbhat@gmail.com, Fax: 91-484-2577595

Dr. SARITA G. BHAT

Professor

Date: 30/8/2017

CERTIFICATE

This is to certify that the research work presented in the thesis entitled "**Bacteriocins BaCf3 and BpSl14 with anticancer and antibiofilm potential from probiotic *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14 isolated from gut of marine fishes: Enhanced production, Purification and Characterization**" is based on the original research work carried out by Ms. Bindiya. E. 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. Also certified that all the relevant corrections and modifications suggested by the audience during the Pre-synopsis seminar and recommended by the Doctoral Committee have been incorporated in the thesis.

Sarita G Bhat

DR. SARITA G BHAT

(Supervising Guide)

URKUND

Urkund Analysis Result

Analysed Document:	full thesis without image.pdf (D30275731)
Submitted:	2017-08-29 08:09:00
Submitted By:	beenacherukuth@gmail.com
Significance:	3 %

Certified that the contents in the thesis entitled "Bacteriocins BaCf3 and BpSl14 with anticancer and antibiofilm potential from probiotic *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14 isolated from gut of marine fishes: Enhanced production, Purification and Characterization" submitted by Bindiya. E. S was subjected to plagiarism detection using the software 'URKUND' and that the significant level of plagiarism was less than the limit permitted (20%) by Cochin University of Science and Technology.

DR. BEENA. C

(University Librarian)

DR. SARITA G BHAT

(Supervising Guide)

DECLARATION

I hereby declare that the thesis entitled "Bacteriocins BaCf3 and BpSl14 with anticancer and antibiofilm potential from probiotic *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14 isolated from gut of marine fishes: Enhanced production, Purification and Characterization" 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, 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, associate ship or other similar titles or recognition.

Cochin-22 Bindiya. E. S
30/8/2017

ACKNOWLEDGMENT

In the name of God, most gracious and most merciful

I thank God, under whose patronage I was born to my parents, brought up in a well built family and guided me towards submitting this thesis. It is He who made my acquaintance with those people who helped me in the successful completion of the project.

The work presented in this thesis would not have been possible without them who were always there to help me when I was in a need. I take this opportunity to acknowledge each end every one of them and extend my sincere gratitude.

In this journey of my life through my dreams there is a guiding light which I followed to complete my work successfully, with immense pleasure and modesty I express my sincere deep sense of gratitude to my supervising guide and mentor, Dr. Sarita G Bhat. I am deeply grateful to her for providing me necessary facilities, excellent supervision and allowing me to explore my own and concurrently redirected me when my steps were stumbled. I am wordless to express my earnest appreciation for her patience and broad-mindedness throughout this period. Without her kind and serene instructions, it would be really impossible for me to finalize this thesis. I am really obliged to her more than words could ever pass on. Much thanks to you Ma'am, for being my dearest educator it is to you I owe this work..... Thank you from the bottom of my heart for the immense support as a guiding light to complete my thesis up to the requirements and standards and also being in my life forever as a mentor. With your support I am able to keep my dreams alive and your faith and belief in me and your hard work motivated me to complete my work with determination and dedication.

Every successful action begins as a dream, I am thankful to Dr. M. Chandrasekharan for inducing the passion of research into my mind. I also thank him for assembling a self sufficient Microbial Technology Lab for the future students to work in. I also thank all the seniors of the lab for setting up a wonderful platform.

A bouquet of gratitude and my heartfelt prayers to the HOD during our post graduate times, Dr.C.S Poulose for his support as always has benefitted me from the time of registration. My prayers for you Sir for your heavenly abode.

Dr.Padma Nambisan, who was the HOD during my registration, my doctoral committee member, her support to fulfil the requirement of this course was unmatchable and I am thankful to her for the cooperation extended to me during the entire period. Dr. P.M. Shereef, Adjunct Faculty, has shown keen interest and his valuable suggestions were very much helpful for me and I am thankful to him for his kind support.

My special thanks to Dr. I.S. Bright Singh, National Centre for Aquatic Animal Health for providing the animal culture facility in his lab. I am grateful to Dr. Salini, for helping me with the cell culture work. A special note for Dr. Sreekala of RCC and Unni for the help rendered to me for anticancer work.

I take this opportunity to sincerely acknowledge CMLRE-MoES for the financial support in the form of project, and Cochin University of Science and Technology in the form of fellowship. My thanks to all the present and past office staffs of our department, for their help and co-operation. I also thank staff of the administrative office for their great support and care.

I thankfully remember Dr. Elyas. K.K, Dr. Baby Chakrapani, Dr. Ajith, Dr. Mohanan, Dr. K.J Joy, Dr. Vijayan, Dr. Anusha in this occasion.

My college teacher and a great friend in my life, Dr. Roselin Alex whose support to start my work has helped me to complete my first publication. She was an inspiration during my entire work. Dr. Manjula whose support as an experienced teacher and a co-researcher always helped me to reach my heights. Both these personalities helped me in recognizing myself.

Dr. Raghul Subin's contribution to the thesis is countless. I thank you for the support to start my work, collecting samples from the cruise, for teaching me how to write a publication, making a work plan for me and so on.... Your contribution and contagious energy can never be forgotten. Dr.Anju who has been a motivation to complete confocal experiments and her support as post doctoral fellow is beyond words and has contributed a lot to complete my work with satisfaction.

I am constantly obliged to my dear Tina K Johny, my lab mate and more like a younger sister who was with me all the time and made me bold in all difficult situations. She generally appeared to be empowering, patient and kind which helped me in a greater extent to finish my research work. I pray to God that we remain best friends forever. Thank you Tina for the extra time you spent for me irrespective of your busy work and supported me up amid my harsh times. I am grateful to Venetia for taking care of my organisms for future work. I also thank her for the help she rendered during my thesis correction and binding. I would also like to thank Honey for her kind support and the future studies she is planning to undertake with the bacteriocins. I thankfully remember the good times I had with Anu during the tenure of my PhD.

My colleagues of MGL require a special acknowledgment for their support and encouragement. Rinu, who supported me in the project; Sritha, an inspiration; Nandita, a helpful friend and the post doctoral fellow Dr. Priji, all of them helped me a lot during the correction and binding of the thesis. Dr. Harisree, my friend and companion for her helping hand in proof reading and support. Dr. Laxmi needs a special mention. My special thanks to Dr. Smitha, Dr. Helvin, Dr.Mridula, Dr.Siju, Dr. Vijaya, Dr. Noble, Mr. Cikesh, Dr. Manjusha for their love, support and care towards me throughout my research.

I am highly owed to Dr. Soumya, my student, for her unconditional support and care for me. I am wordless to express my gratitude for the help she had rendered during the proof reading, cover page design, corrections and binding of the thesis. I am grateful to Ms. Kiran Lakshmi, Ms. Arrinya, Ms. Anala, Ms. Nayana Ms. Aiswarya and Dr. Jikku for their love, encouragement and assistance throughout the

work. I could never forget the contributions by Dr. Sapna, Dr. Mansoor, Dr. Rekha. I thank Dr. Anoop for the sample analysis from RGCB and Dr. Soorej for MALDI.

I recognise the help rendered by Dr.Karthikeyan, Dr. Doles, Mr. Sajan, Dr. Lailaja and all the seniors of MTL. I also recognise the research scholars of neuroscience lab Dr. Anita, Dr Chinthu, Dr. Jayanarayan, Dr. Shilpa, Dr. Roshini, Dr. Naigil George and Mr. Ajayan for their cooperation and friendship. I kindly remember the new generation research scholars Bins, Prabha, Sreelakshmi, Gayathri for their support.

I also thank all the post doctoral fellows in the department Dr. Sreekanth, Dr. Soumya, Dr. Deepa, Dr. Sreeja, Dr. Smitha. I thank all the biotech alumina, pBAC for their encouragement.

I would like to keep a note on the assistance by Anitha Raj, Dr. Anila, and Jaya, who were my trainees during the period.

I would like to acknowledge all the teachers who taught me since my childhood. I would not have been here without their guidance, blessing and support.

I sincerely thank my husband, Mr. Navas for the kind support, inspiration and unconditional love towards me. I feel privileged to be his better half and I am indebted to him for his cooperation throughout my research career, right from transportation to writing the thesis. I thank him for being with me and for covering up all my tensions, frustrations and emotions. My daughter Alayna Rukhia (Rukku), our 'vava' was the worst affected during my PhD period. I thank her for the patience and sacrifice. She even helped me with the proof reading.

My parents are my best well wishers. I, though wordily gratitude is not enough, express my thanks to them for being the pillars of my achievements, taking care of my child like they took care of me, spending their valuable time for me. I also thank my brother Roshan and his wife Shahlaa for their support and affection.

I thank my in-laws, Umma, Vappa, Nasir Ali, Dr. Sapna, Bahadur and Adil for their support and cooperation. A special thanks to Umma for delightful dishes she used to serve for my friends.

I specially thank, Jeena, my friend and well wisher for her support.

I take this opportunity to thank all my relatives- uncles, aunts and cousins for their love, prayers and encouragement. I thank all my well wishers who I may not have mentioned unknowingly, for their generous help and prayers for the successful completion of my research work.

Bindiya. E. S

ABBREVIATIONS

%	-	Percentage
≈	-	Approximately
<	-	Less than
>	-	Greater than
°C	-	Degree Celsius
AMS	-	Antimicrobial Substance
AMP	-	Antimicrobial peptide
ANOVA	-	Analysis of Variance
APS	-	Ammonium persulfate
AU/mg	-	Activity Units/milligram
AU/mL	-	Activity Units/milliliter
BLAST	-	Basic Local Alignment Search Tool
Bp/bp	-	Base pair
BHIB	-	Brain heart infusion broth
BSA	-	Bovine serum albumin
cm	-	Centimeter
COI	-	Cytochrome Oxidase I
Da	-	Dalton
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotide triphosphate
DTT	-	Dithiothreitol
DW	-	Distilled water
EDTA	-	Ethylene diamine tetra acetic acid
e.g.	-	for example
et al.,	-	and others
EtBr	-	Ethidium bromide
FDA	-	Food and Drug Administration
Fig	-	Figure
FTIR	-	Fourier Transform Infra Red
g	-	Grams
GRAS	-	Generally Recognized As Safe
GI	-	Gastrointestinal
h	-	Hours
HCl	-	Hydrochloric acid
H_2O_2	-	Hydrogen peroxide
i.e.	-	that is

IEF	-	Iso electric focusing
kb	-	Kilobase
kDa	-	Kilo Dalton
L	-	Litre
LAB	-	Lactic acid bacteria
LB	-	Luria Bertani
Log	-	Logarithm
M	-	Molar
m	-	Meter
MALDI	-	Matrix Assisted Laser Desorption Ionization
MCC	-	Microbial Culture Collection
mg	-	Milligram
µg	-	Microgram
MH	-	Mueller Hinton
min	-	Minutes
mL	-	Millilitre
µL	-	Microlitre
mm	-	Millimeter
mM	-	Millimolar
µm	-	Micrometre
µM	-	Micromolar
MRSA	-	Methicillin resistant *Staphylococcus aureus*
MS	-	Mass Spectroscopy
N	-	Normality
NA	-	Nutrient Agar
NaCl	-	Sodium chloride
NaClO	-	Sodium hypochlorite
NaOH	-	Sodium hydroxide
NB	-	Nutrient Broth
NCBI	-	National Center for Biotechnology Information
NCIM	-	National Collection of Industrial Microorganisms
NCCS	-	National Centre for Cell Science
ng	-	Nanogram
nm	-	nanometer
No.	-	Number
OD	-	Optical density
PAGE	-	Polyacrylamide gel electrophoresis
PB	-	Plackett Burman

PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
pH	-	Power of Hydrogen
pI	-	Isoelectric point
rpm	-	Revolutions per minute
rDNA	-	Ribosomal DNA
s	-	Seconds
SDS	-	Sodium dodecyl sulphate
SmF	-	Submerged fermentation
sp. /spp	-	Species
Sp.	-	Specific
TAE	-	Tris-acetate-EDTA
TE	-	Tris-EDTA
TEMED	-	N-N-N'-N'-Tetramethyl ethylene diamine
TOF	-	Time Of Flight
Tm	-	melting temperature
UV-VIS	-	Ultraviolet-Visible
V	-	Volts
v/v	-	Volume/volume
viz.	-	Namely
w/v	-	Weight/volume
ZMB	-	Zobell Marine Broth
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

List of Figures

List of Tables

Contents

Contents

Contents

Contents

Contents

Contents

Contents

Contents

Chapter 1

INTRODUCTION

Antimicrobial resistance due to overuse and misuse of antibiotics causes untreatable infections to persist, thereby increasing the risk of contagion. This threat to our ability to treat common infectious diseases, can prolong illness, cause disability and death, subsequently increasing the risk of cancer chemotherapy, diabetes management, medical procedures such as organ transplantation, and major surgeries like caesarean sections or hip replacements; all conditions prone to secondary infections. Antimicrobial resistance increases cost of health care due to lengthier hospital stays and intensive treatments. According to WHO, 480,000 people world over develop multi-drug resistant TB each year, while drug resistance has complicated the fight against HIV, malaria and many other ailments.

In May 2017, WHO adopted a global action plan to increase investment in new medicines, diagnostic tools, vaccines and other interventions. Accordingly, member states were encouraged to participate in international collaborative research to support the development of new medicines, diagnostic tools and vaccines. This is through prioritized support of basic scientific research on infectious diseases through investigation of natural resources of biodiversity and biorepositories for the development of new drugs. Besides the emphasis is also on creating new as well as strengthening existing public-private partnerships to encourage research and development of new therapeutics and diagnostics, besides adopting new market models to encourage investment and ensure access to new antimicrobial products.

Broad spectrum antibiotics such as amoxicillin, levofloxacin, gatifloxacillin, streptomycin, tetracycline and chloramphenicol are prescribed for bacterial resistance, but the use of these increase the risk of childhood asthma

(Jedrychowski *et al*., 2011). These antibiotics even disturb the normal balance of the intestinal flora, when they kill the healthy bacteria in the body. Therefore scientific communities propose friendlier alternatives such as vaccines, antibiotic substitutes or the use of probiotics.

Bacteriocins portrayed as superior alternative to conventional antibiotics, are found in all major lineages of bacteria (Klaenhammer, 1999). They are different from classical antibiotics as they are ribosomally synthesized and have a narrow action spectrum. Those produced by Gram positive and Gram negative organisms are diverse with respect to their size, target, mode of action, release and immune mechanisms. Bacteriocins of Gram-positive bacteria are as abundant as and even more diverse than those of Gram-negative bacteria. The Gram-positive bacteriocins resemble the many antimicrobial peptides (AMPs) of eukaryotic origin which are generally cationic, amphiphilic, membrane-permeabilizing molecules with sizes ranging from 2 to 6 kDa (Heng *et al.,* 2007).

Bacteriocins are regarded to be natural being present in many food items eaten from ancient times (Cleveland *et al.,* 2001). The bacteriocin nisin has been used as a food preservative and has GRAS (Generally recognized as safe) status (21 CFR 184.1538).

In microbial communities, bacteriocins serve as anti-competitors enabling the invasion of the producer strain into an established microbial community. Their defensive role is to inhibit the invasion of other strains or species into an occupied niche or limit the advance of neighboring cells. Moreover, the Gram-positive bacteriocins mediate quorum sensing (Miller and Bassler, 2001), a cell-density dependent regulatory system in which cell-to-cell communication is mediated by auto-inducing signal molecules; also one of the well-studied systems involved in bacteriocin gene control (Diep *et al*., 1995; Eijsink *et al*., 1996).

Production of Gram-positive bacteriocins generally occurs during the shift from log to stationary phase as in the case of nisin (Breukink and de Kruijff, 1999). The regulation of expression of Gram-positive bacteriocins is culture density dependent and not cell cycle dependent and that nisin A acts as a protein pheromone in regulating its own expression (Dufour *et al.,* 2006; Hechard and Sahl, 2002).

Several bacteriocins reportedly have anticancer and antibiofilm activity (Sadekuzzaman *et al*., 2015; Kaur and Kaur, 2015). The complex microbial communities of biofilms are highly resistant to antibiotics thereby persists despite several conventional approaches to get rid of them. Development of bacteriocin coated surfaces, especially where the propensity for biofilm formation is high, is considered a superior method to combat biofilms (Pimentel-Filho *et al*., 2014).

The effect of bacteriocins on eukaryotic cells was first reported by Farkas-Himsley and Cheung (1976). Bacteriocins are generally membrane destabilizing agents. The membrane of cancer cells are different from normal cells in the loss of asymmetry with respect to phospholipid types, are negatively charged with higher fluidity, besides having significantly higher number of microvilli that increase their surface area. All these factors help bacteriocins to effectively bind cancer cells causing their destruction.

Another approach to substitute antibiotics smartly and sustainably is the selection of bacteriocinogenic and anti-pathogen strains from animal-associated microorganisms to exploit as probiotics. Bacteriocinogenic strains serve dual purposes, with the bacteriocin as an antibiotic substitute and the producer bacteria a potent probiotic. The producer strain is established into a niche by the bacteriocin which inhibits invasion of competing strains and pathogens, whereby

the composition of the microbiota is modulated which in turn influences the host immune system.

There are reports that bacteriocins function in a number of ways within the gastrointestinal tract (Bhardwaj *et* al., 2010). They function as colonizing peptides to support the introduction or dominance of a producer into an already occupied niche (Czárán *et al*., 2002). Bacteriocins also work as antimicrobial or killing peptides directly by preventing competing strains or pathogens. They may act as signaling peptides (quorum sensing), in interspecies communication, in bacterial cross talk within microbial diversity or impact signaling cells of host immune system (Meijerink *et al*., 2010). Thus in essence, bacteriocins directly prevent the attack of competing pathogens or adjust the composition of microbiota to influence the host immune system.

Marine environments represent an under exploited source for new biologically active molecules, especially antibiotics. In addition marine bacteria and fungi are prominent sources for antibiotic discovery due to their diversity, ability to grow rapidly and their sustained and enhanced production in bioreactors. Other sources like sponges, corals and other marine animals also supply very interesting scaffolds for antibiotic discovery. Marine environment is a repository of diversity and diverse niches, hence novel characteristics are expected in these products from marine subjects.

Fish gut and gills too are much exposed to these same environments hence the microbes in these organs may have the potential to produce novel bioactive substances. Although the bacterial intake from water, sediment, and/or food happen constantly in fish, they are protected by the acid in gastric juices, by the bile acids and lysozyme secreted in the intestines, besides their immune responses. Moreover, it is recognized that the ability to adhere to enteric mucus

and wall surfaces is indispensable for bacteria to establish in fish intestines. Consequently microorganisms in the fish intestine endure severe competition and struggle for existence. During this process, the many inhibitory substances produced harm other bacteria, and benefit the host. Most of the inhibitory compounds are proteinaceous and are classified under bacteriocins.

Generally, Gram positive bacteriocins are highly thermostable and pH tolerant due to their peculiar structure as well as the presence of unusual amino acids. Some bacteriocins undergo post translational modifications accounting for their high stability. Bacteriocins generally target cytoplasmic membrane and form pores resulting in efflux of ions and ATP. Various models proposed for pore formation, include the "wedge" model, the "barrel-stave" model and the "carpet" model (Driessen *et al.,* 1995; Héchard and Sahl, 2002; Moll *et al.,* 1999; Sahl, 1991; van den Hooven *et al.,* 1996). The "wedge" model was proposed for lantibiotics, while the "barrel-stave" and "carpet" models were for the class II bacteriocins (Moll *et al.,* 1999).

Considering the various facts, this study was envisaged to explore the marine fauna in the search for novel bacteriocins and probiotic producer strains. The strategy was to isolate and characterize bacteriocin producing bacteria from marine fish gut, purify the bacteriocins and characterize them for biological applications. To this end, the bacteriocins were also tested for their anticancer and antibiofilm activities. The bacteria with antagonistic activity were also characterized for their probiotic potential. The objectives to achieve these aims are as given below,

Objectives

1. Screening and characterization of bacteriocin producing bacteria from gut of marine fishes - *Centroscyllium fabricii* and *Sardinella longiceps*.
2. Optimization of process conditions for bacteriocins' production by one - factor - at - a time.
3. Purification and characterization of the bacteriocins and elucidation of their mechanism of action.
4. *De novo* sequencing and modeling of the bacteriocins.
5. Probiotic characterization of bacteriocin producing bacteria.
6. Antibiofilm and anticancer activity of purified bacteriocins.

Chapter 2

REVIEW OF LITERATURE

2.1 Introduction

Gratia discovered bacteriocin while looking for ways to kill bacteria in 1925, but Jacob and Wollman coined the term in 1953. This discovery led to development of microbial antibiotics and the detection of bacteriophages within a span of few years. Gratia called his first discovery a colicin since it killed *E. coli.* Others like halobacteria produce their own version, the halocins (Torreblanca *et al.,* 1994).

Many bacteria produce toxins with bacteriocin-like features, which are only partially characterized, called as bacteriocin-like inhibitory substances or BLIS. A precise definition of bacteriocins is therefore futile. Conventional criteria for defining bacteriocins are based on colicin characteristics in varying combinations, applied with different degrees of consistency such as,

(i) a narrow inhibitory spectrum of activity centered on the homologous species;
(ii) bactericidal mode of action;
(iii) presence of an essential, biologically active protein moiety;
(iv) attachment to specific cell receptors;
(v) plasmid-borne genetic determinants of bacteriocin production and of host cell bacteriocin immunity;
(vi) production by lethal biosynthesis (i.e., commitment of the bacterium to produce bacteriocin will ultimately lead to cell death) (Tagg *et al.,* 1976).

The above criteria generally applicable to the colicins however, show incongruity in bacteriocins produced by Gram-positive bacteria. Some atypical features associated with bacteriocins of Gram-positive bacteria include a wider spectrum of activity against different bacterial species and less-solid host cell

immunity to the homologous bacteriocin (Hamon and Peron, 1963). These toxins play a critical role in maintaining microbial population or community interactions.

2.2 Bacteriocin nomenclature

There are two difficulties with the term 'bacteriocin': a) there is no universally accepted definition for this group of substances and b) many of the described inhibitory substances are yet uncharacterized sufficiently to fulfil any classification. They were originally named based on the producer species, e.g. colicins by *Escherichia coli,* pyocins of *Pseudomonas aeruginosa* (formerly named *pyocyania*), cerecins of *Bacillus cereus*, cloacins of *Enterobacter cloacae* and pesticins of *Yersinia pestis.* Frederico created the first classification and hence nomenclature focussed on the colicins of *E. coli* (Frederico, 1957).

Nomenclature of bacteriocins is chaotic at times, as it is based on the generic and at other times the species designation of producer strains (Tagg *et al*., 1976). In Gram-positive bacteria, this lack of uniformity is evident in the alternate designations of bacteriocins of *Listeria monocytogenes* as listeriocins or monocins, those of *S. aureus* as either staphylococcins or aureocins and those of *Corynebacterium diphtheria* as corycins or diphthericins; whereas mesentericin Y 105, leucocin A UAL-187 and leuconocin Lcm1 are from *Leuconostoc mesenteroides*, *Leuconostoc gelidum* and *Leuconostoc carnosum* strains, respectively. Similarly, bacteriocins of genus *Clostridium* were termed clostocins or clostridiocins or, have received individual species designations such as boticin, butyricin or perfringocin, welchicin. Pediocin PA-1 and pediocin AcH are chemically identical bacteriocins from *Pediococcus acidilactici*. In many cases, authors have added a terminal "e" to the name of the bacteriocin; for example, staphylococcine, listeriocine and corycine (Jack *et al*., 1995; Tagg *et al*., 1976)

Additional labelling is essential when a species produces different bacteriocins (Tagg *et al.,* 1976). These are generally arbitrary, consecutive letters of the alphabet. For precise specification of a particular bacteriocin, it was suggested that the trivial designation of the producing strain be included within the bacteriocin name. This recommendation gained widespread acceptance by investigators of the colicins (e.g., colicin E1-K30 is a colicin of type El produced by *Escherichia coli* strain K30).

2.3 Bacteriocin classification

Bacteriocins include proteins diverse in terms of size, modes of action, microbial targets and immunity mechanisms. In general, classification is based on Gram designation of the producer species, i.e. Gram-negative vs Gram-positive. Furthermore, a few from archaeal species have also been characterized (Table 2.1).

2.3.1 Bacteriocins of Gram-negative bacteria

Bacteriocins of Gram-negative bacteria are of four main classes: colicins, colicin-like bacteriocins, microcins, and phage-tail like bacteriocins (Chavan and Riley, 2007). Colicins are thermo-sensitive, protease sensitive proteins varying in size from 25 to 90 kDa (Pugsley and Oudega, 1987); they are used as models to study bacteriocin structure, function and evolution (Cascales *et al.,* 2007; Riley and Gordon, 1999; Riley and Wertz, 2002a; 2002b).

Colicins are of two major types based on their mode of killing - nuclease and pore former colicins. Nuclease colicins (Colicins E2, E3, E4, E5, E6, E7, E8, E9) kill by acting as DNases, RNases, or tRNAses while pore former colicins (colicins A, B, E1, Ia, Ib, K) kill by forming pores in the cell membrane. Proteinaceous bacteriocins by other Gram-negative species with similar structural and functional characteristics are termed colicin-like. They may be nucleases (pyocins S1, S2) or pore-formers (pyocin S5) like colicins

(Michel-Briand and Baysse, 2002; Cascales *et al.,* 2007). S-pyocins of *Pseudomonas aeruginosa*, Klebicins of *Klebsiella* species, and alveicins of *Hafnia alvei* are the most studied in this category.

Pore-forming colicins have 449 to 629 amino acids, but the nuclease bacteriocins with 178 to 777 amino acids have an even broader size range. The central domain comprising 50% of colicin protein and involved in specific cell surface receptors recognition, is translocated into the target cell by the N-terminal domain (> 25 % of the protein), while the remaining short sequence is involved in immunity protein binding. The killing domain and the immunity region are present here. Although pyocins share similar domain structure, the order of the translocation and receptor recognition domains are reversed (Riley and Wertz, 2002b).

Phage-tail like bacteriocins are larger structures resembling bacteriophage tails, and are argued to be defective phage particles (Bradley, 1967). R and F pyocins of *P. aeruginosa* are some thoroughly studied phage-tail like bacteriocins (Michel-Briand and Baysse, 2002; Nakayama, 2000; Liu *et al.,* 2013).

The smaller (<10 kDa) peptide bacteriocins produced by Gram-negative bacteria called microcins, are of three classes - the post-translationally modified (microcins B17, C7, J25, and D93) (Gillor *et al.,* 2004), the unmodified (microcins E492, V, L, H47, and 24) (Pons *et al*., 2004) and the Class IIc bacteriocins (such as microcin E492). The last are non-ribosomal siderophore-type with post-translational modification at the serine-rich carboxy-terminal region (De Lorenzo and Pugsley, 1985).

2.3.2 Bacteriocins of Gram-positive bacteria

Bacteriocins of Gram-positive bacteria are more abundant and diverse than those in Gram-negative bacteria (Jack *et al.,* 1995), but differ in two fundamental ways.

1. Bacteriocin production is not necessarily a lethal event as in Gram-negative bacteria.

This vital difference is due to the transport mechanisms encoded by Gram-positive bacteria to release bacteriocin toxin. Some have evolved a bacteriocin-specific transport system, whereas others employ the *sec*-dependent export pathway (De Vos *et al*., 1991).

2. While Gram-positive bacteria have evolved bacteriocin-specific regulation, bacteriocins of Gram-negative bacteria solely rely on host regulatory networks.

2.3.3 Classification of bacteriocins of Gram-positive bacteria

Size, morphology, physical, and chemical properties form the basis for bacteriocin classification of Gram-positive bacteria into four (Lee and Kim, 2011).

Class I bacteriocins are post-translationally modified small peptides (<5 kDa) with non-traditional amino acids like dehydrobutyrine (Fig 2.1a), dehydroalanine (Fig 2.1b) and methyl-lanthionin (Fig 2.1c), called lantibiotics (Cleveland *et al*., 2001). This class is subdivided into Type A and B; with Type A being positively charged, linear peptides (nisin) (Flaherty *et al*., 2014), whereas Type B are rigid globular peptides (mersacidin), either negatively or neutrally charged, e.g. labyrinthopeptins such as labyrinthopeptin A2 (Meindl *et al*., 2010), and sactibiotics for instance subtilosin A (Kawulka *et al*., 2004).

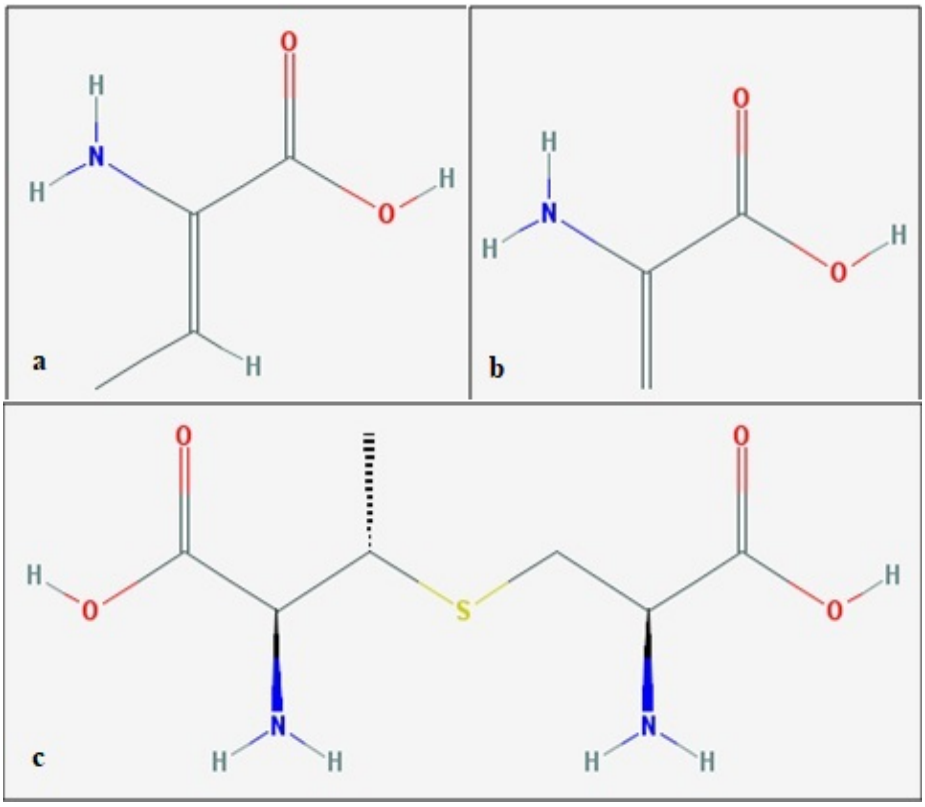


Fig 2.1 Pictorial representation of a) dehydrobutyrin b) dehydroalanine c) methyl-lanthionine (Images adapted from PubChem, https://pubchem.ncbi.nlm.nih.gov/)

Table 2.1 Classification of bacteriocins with examples (Bindiya and Bhat, 2016)

	Bacteriocins	**Type/Class**	**Size**	**Example**	**Reference**
Gram negative Bacteria	Colicins	Pore Formers	20-80	Colicins A, B	Cascales *et al.,* 2007
		Nucleases		Colicins E2, E3	Cascales *et al.,* 2007
	Colicin-like		20-80	S-pyocins Klebicins	Michel-Briand and Baysse, 2002
	Phage-tail like		>80	Maltocin P28	Liu *et al*., 2013

	Microcins	Post-translationally modified	<10	Microcin C7 Microcin B17	Gillor *et al*., 2004
		Unmodified		Colicin V	Gratia, 1925
		Class IIc - non-ribosomal siderophore-type post-translation modification		Microcin E492	De Lorenzo and Pugsley, 1985
Gram positive Bacteria	Class I	Type A- Linear peptides, positively charged	<5	Nisin	Cleveland *et al*., 2001
		Type B- Rigid globular peptides, negatively or neutrally charged		Subtilosin A	Meindl *et al*., 2010
	Class II	IIa - contain YGNGVxCxxxxCxV, Narrow spectrum of activity	<10	Pediocin, enterocin	Balciunas *et al*., 2013 Heng *et al*., 2007
		IIb – require concerted activity of 2 peptides		Lactacin F, Lactococcin G	Nissen-Meyer *et al*., 1992
		IIc – circular peptide bacteriocins		Carnocyclin A	Gong *et al*., 2009
		IId – linear, non-pediocin like, single peptide		Epidermicin NIO1	Sandiford and Upton, 2012
	Class III	IIIa – bacteriolysin	>10	Enterolysin A	Nilsen *et al*., 2003

		IIIb – non-lytic bacteriocin		Helveticin A and J	Joerger and Klaenhammer, 1986
	Class IV	Require lipid or carbohydrate moieties		Leuconocin S8, Lactocin 27	Carolissen-Mackay *et al*., 1997
Archea	Halocins	Microhalocins	<10	Halocin A4, C8, G1	Price and Shand, 2000
		Protein Halocins	>10	Halocin H1, H4	Cheung *et al*., 1997
	Sulfolobicin	Membrane associated proteins	~20	Sulfolobicin	Prangishvili *et al*., 2000

Class II bacteriocins are small (<10 kDa), heat-stable positively charged peptides having 30–60 amino acids, but not post-translationally modified, (Heng *et al*., 2007); and subdivided into four subgroups. The class IIa *Listeria*-active or pediocin-like peptides with a conserved N-terminal sequence (YGNGVxCxxxxCxV) or "pediocin box" with two cysteine residues forming disulphide bridge, are the most extensively studied group with a narrow spectrum of activity (Balciunas *et al*., 2013). Lactacin F and lactococcin G are Class IIb bacteriocins requiring the concerted action of two peptides for fully activity (Nissen-Meyer *et al*., 1992). Class IIc are circular peptide bacteriocins like carnocyclin A (Gong *et al*., 2009), while Class IId are linear, non-pediocin-like, single-peptide such as epidermicin NI01 (Sandiford and Upton, 2012).

Class III bacteriocins are generally large (>10 kDa), heat-sensitive peptides, subdivided into two subtypes. Type IIIa bacteriolysins are enzymes like Enterolisin that kill sensitive strains by cell wall lysis (Nilsen *et al*., 2003). Helveticin J (37 kDa) produced by *Lactobacillus helveticus* is a Type IIIb, non-lytic bacteriocin (Joerger and Klaenhammer, 1986).

Class IV bacteriocins are also known as complex bacteriocins with unique structural characteristics, requiring lipid or carbohydrate moieties for activity. The first and last amino acids of these bacteriocins, e.g. leuconocin S 8 and lactocin 27, are covalently linked giving cyclic structures (Carolissen-Mackay *et al*., 1997). Enterocin AS-48 by *Enterococcus faecalis* subsp. *liquefaciens* S-48 was the first characterized in this class (Maqueda *et al*., 2004).

2.3.4 Bacteriocins of archaea

Archaea too produce unique bacteriocin-like antimicrobial compounds called archaeocins (Shand and Leyva, 2007), but much less scrutinized. Two major types of archaeocins identified are the halocins of halobacteria and sulfolobicins of *Sulfolobus* genus. Halocins are classified into two based on size- the smaller microhalocins (3.6 kDa) and larger halocins of 35 kDa (O'Connor and Shand, 2002). S8, the first halocin discovered is a short, 36 amino acid hydrophobic peptide, processed from a larger 34 kDa pro-protein. Halocin production is a universal feature of halobacteria (Torreblanca *et al*., 1994) and their genes are located on megaplasmids (or minichromosomes). Halocins H4 and S8 are located on ~300 kbp and ~200 kbp plasmids, respectively (Price and Shand, 2000). Their activity is usually detected at the late exponential to early stationary growth phase.

Sulfolobicins however are not extensively studied, but its production was reported from *Sulfolobus islandicus* isolated from volcanic vents through-out Iceland (Prangishvili *et al.,* 2000). This study predicted sulfolobicin to be a membrane-associated protein. Sulfolobicins are associated with membranous vesicles ranging in size from 90 to 180 nm in diameter. Like many bacteriocins, they are thermostable and sensitive to protease treatment, but their mode of action is still unknown (Ellen *et al*., 2011).

2.4 *Bacillus* bacteriocins

Strains from Genus *Bacillus* produce diverse antimicrobial peptides with several different basic chemical structures (Gebhardt *et al*., 2002; Stein, 2005), which are poorly characterized and some are bacteriocin-like in nature. *Bacillus* species that produce "antibiotics" include *B. laterosporus*, *B. pumilus*, *B. circulans*, *B. polymyxa* and *B. cereus*. *B.cereus* also produces a bacteriolytic principle identical to phospholipase A. Studies of *B. cereus* have categorized the inhibitory substances as bacteriocins. Other *Bacillus* sp. that have bacteriocin-like inhibitors are *B. stearothermophilus*, *B. licheniformis, B. thuringiensis, B. subtilis* (Abriouel, 2011) and *B. amyloliquefaciens* (Lisboa *et al*., 2006). The bacteriocins of *B. licheniformis* and *B. subtilis* are of the defective phage type. A novel thermostable bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment has been reported (Smitha and Bhat, 2012).

Many *Bacillus* bacteriocins are lantibiotics, a category of post-translationally modified peptides widely disseminated among different bacterial clades. Lantibiotics are the best-characterized antimicrobial peptides at the levels of genetic determinants, peptide structure and biosynthesis mechanisms. Members of Genus *Bacillus* also produce many other non-modified bacteriocins, some resembling the pediocin-like bacteriocins of the lactic acid bacteria (LAB), while others show completely novel peptide sequences. *Bacillus* bacteriocins are gaining importance due to their broader spectra of inhibition, which include Gram-negative bacteria, fungi or yeasts, in addition to some Gram-positive species, that are known human and/ or animal pathogens.

The best-studied and characterized bacteriocins are megacins produced by *B. megaterium.* It was first reported by Ivanovics and Alfoldi in 1954 from *B. megaterium* strain 216. Megacin possess several characteristics of bacteriocin, including a narrow spectrum of activity, production by lethal

biosynthesis and a proteinaceous composition. The original megacin by strain 216, is now known as megacin A, since megacin types B and C were also identified later. Another megacin that has production characteristics similar to megacin C, but a diverse mode of action, is referred to as killer principle (Marjari and Ivanovic, 1962) or megacin Cx-337.

The 2009 release of the BACTIBASE dataset (version 2, July 2009) http://bactibase.pfba-lab-tun.org/main.php, contains 177 (44% more) bacteriocin sequences, of which 156 are the products of Gram-positive organisms and 18 of Gram-negative organisms (Hammami *et al*., 2010). Three bacteriocins from the Archaea domain are also included. The database now comprises 31 genera, as shown in Fig 2.2. Without surprise, the lactic acid bacteria (order Lactobacillales) make up the predominant group of producers with 113 bacteriocins.

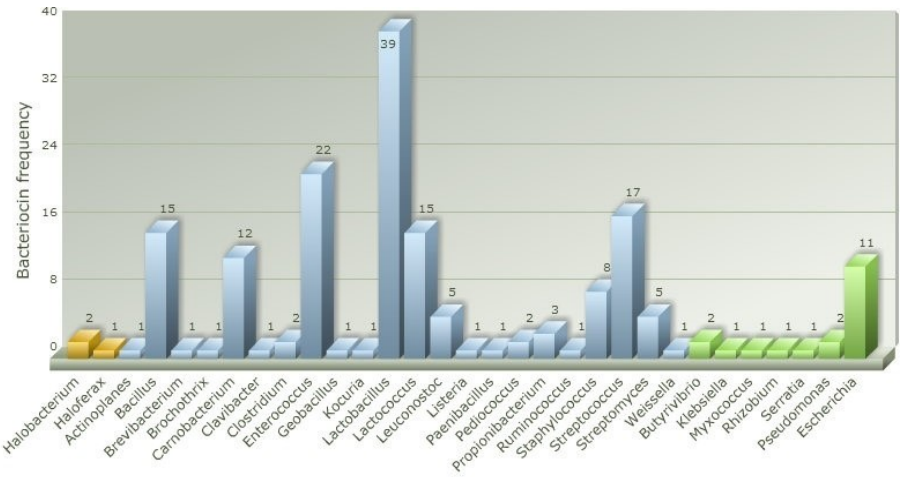


Fig 2.2 Distribution of bacteriocins among the producer genera in the BACTIBASE database (Archea (yellow), Gram positive (Blue) and Gram-negative (green))

2.5 Bacteriocin mode of action

The variety of their chemical structures allow bacteriocins to affect different essential functions of the living cell (transcription, translation, replication and cell wall biosynthesis), but most form membrane channels or

pores, destroying the energy potential of sensitive cells. Research on their mode of action focussed on two distinct aspects of bacteriocin action on susceptible bacteria: the kinetics of physical interaction between bacteriocin and susceptible cells, and the detection of specific biochemical lesions within the affected organisms. The mode of action of bacteriocin with sensitive cells is widely hypothesized to be a two stage process (Reeves, 1972). An initial reversible phase corresponds to physical adsorption of bacteriocin to cell-envelope receptors. Bacteriocin removal during this stage leaves the cell unscathed, without permanent physiological damage. The second stage causes irreversible pathological changes affected via specific biochemical lesions after a measurable time.

Although bacteriocin adsorption is highly specific for susceptible bacteria, some like staphylococcins 414 and 1580, lactocin LP27 and streptococcin B-74628 lack this and adsorb to bacteria resistant to its killing action. This nonlethal binding is a reflection of the high surface activity of some bacteriocins. Polypeptide antibiotics like polymyxin B adsorb nonspecifically to bacteria. Nonspecific adsorption to susceptible or resistant bacteria was also demonstrated by bacteriocin 28 of *C. perfringens* (Mahony and Butler, 1972), staphylococcin 462 (Hale and Hinsdili, 1975) and viridin B (Law and Dajani, 1978).

Antagonism of bacteriocins from Gram-positive bacteria is the effect due to their interaction with bacterial membrane, which is the target for most bacteriocins, despite diverse modes of action (Bizani *et al*., 2005). Nearly all class II bacteriocins disturb the proton motive force (PMF) by pore formation. The subclass IIc comprises miscellaneous peptides with varied modes of action like membrane permeablization, specific inhibition of septum formation and pheromone activity (Héchard and Sahl, 2002).

Besides inhibiting pore formation and cell wall biosynthesis, bacteriocins like nisin and cationic lantibiotic Pep5 induce autolysis of

susceptible staphylococcal cells by causing massive cell wall degradation. Some others release two cell-wall hydrolysing enzymes - an N-acetylmuramoyl-L-alanine amidase and an N-acetylglucosaminidase; these strong cationic proteins bind the cell wall via electrostatic interactions, displacing enzymes from the cell-wall by a cation exchange-like process, resulting in apparent enzyme activation and rapid cell lysis (Bierbaum and Sahl, 1985; 1987). Furthermore, nisin and subtilin inhibit bacterial spore germination.

2.5.1 Bacteriocin-induced cell damage

Susceptibility to the lethal action of bacteriocins is influenced by the physiological state of indicator organism. Actively multiplying cells were most sensitive to streptococcin A-FF22, staphyloccin 1580, bacteriocin 28 of *C. perfringens*, and bacteriocin E-1 and bacteriocin X-14 (hemolysin) of *S. faecalis* subsp. *Zymogenes,* indicating active cellular metabolism as prerequisite to affect killing. A time dependent morphological shift was demonstrated by the action of pediocin from *P. acidilactici* to the sensitive strain *L. monocytogenes.* Bacteriocin-treated *L. monocytogenes* V7 were destroyed after 6 h. The major morphological changes were in the cell wall, which relaxed and ruptured after just 0.5 h of treatment with bacteriocin (6,400 AU/mL). After 1 h and 3 h of treatment, ruptures in the cell wall and plasma membrane were more evident with more cell contents escaping. After 6 h of treatment, the cell wall was completely irregular and damaged (Heo *et al*., 2007).

2.6 Other biological activities of bacteriocins

2.6.1 Anticancer activity

The growing population with cancer has created the need for new anticancer medications, to make available medicines affordable and increased research in novel cancer therapeutics. The Indian Council of Medical Research

(ICMR) report (2016) put the new cancer cases around 14.5 lakh, with the figure likely to reach nearly 17.3 lakh new cases by 2020. Over 8.8 lakh people could succumb to cancer by 2020. Data also revealed that only 12.5 per cent of patients avail treatment in early stages of the disease (ICMR., 2016).

These statistics show that cancer is a wide spread disease and exploits humans physically, mentally and economically. It is the uncontrolled division of cells or tissues leading to malignancy, hence apoptosis is hindered or restricted in these cells. Anticancer drugs target these abnormal cells which have certain biochemical markers.

Felgner (2016) compiled the phenomenon of spontaneous regression of tumours associated with bacterial infections over the past centuries. The relationship between bacterial infection and cancer regression led the American physician, Coley to the discovery of a killed bacterial vaccine for cancer known as "Coley's toxin" in late 1891. This led to the development of new therapeutic anticancer modalities based on live bacteria and their purified products namely bacterial toxins, proteins, peptides, and enzymes. Recently, a number of bacterial proteins and peptides demonstrated anticancer activity toward diverse cancer cells.

Nisin, a well-accepted food preservative and bacteriocin, induced preferential apoptosis and decreased cell proliferation in head and neck squamous cell carcinoma (HNSCC) cells (Joo *et al*., 2012). Plantaricin A (PlnA), a pheromone generated by *Lactobacillus* (L.) *plantarum*, not only exhibits antibacterial activity but also kills some human cancer cell lines through both apoptosis and necrosis processes (Sand *et al*., 2010). PlnA had a higher ability to penetrate the cell membrane of a tumor than that of normal cells (Sand *et al*., 2007). The design and research on AMPs with antitumor properties have high medical value because they selectively kill the tumor cells. For example, pediocin PA-1, a recombinant pediocin produced from *Pichia pastoris*, inhibited human lung carcinoma cell line (A-549) and colon

cancer cells (DLD-1) growth (Beaulieu, 2005); pyocin S2 inhibited the proliferation of hepatocarcinoma cell lines (Abdi-Ali *et al*., 2004).

The antimicrobial and anticancer activities of antimicrobial peptide (AMP) KL15 obtained by *in silico* sequence modification of two bacteriocins m2163 and m2386 from *Lactobacillus casei* ATCC 334 were studied (Tsai *et al*., 2015). Apart from the significant bactericidal effect on pathogens like *Listeria*, *Escherichia*, *Bacillus*, *Staphylococcus*, *Enterococcus* species, the AMP could also kill human adenocarcinoma cells, SW480 and Caco-2 (Tsai *et al*., 2015).

2.6.2 Antibiofilm activity of bacteriocins

Biofilms are irreversible assemblage of surface associated microbial cells enclosed within extracellular polymeric matrix substance that contaminate food products by introduction of pathogenic microorganisms or spoilage bacteria. Biofilm formation is depicted in figure 2.3. According to Costerton *et al*. (1987) biofilm is a functional consortium of micro-organisms attached to a surface and embedded in the extracellular polymeric substances (EPS). They are difficult to eradicate completely as they are often resistant to normal sanitation procedures, thereby causing other detrimental process effects.

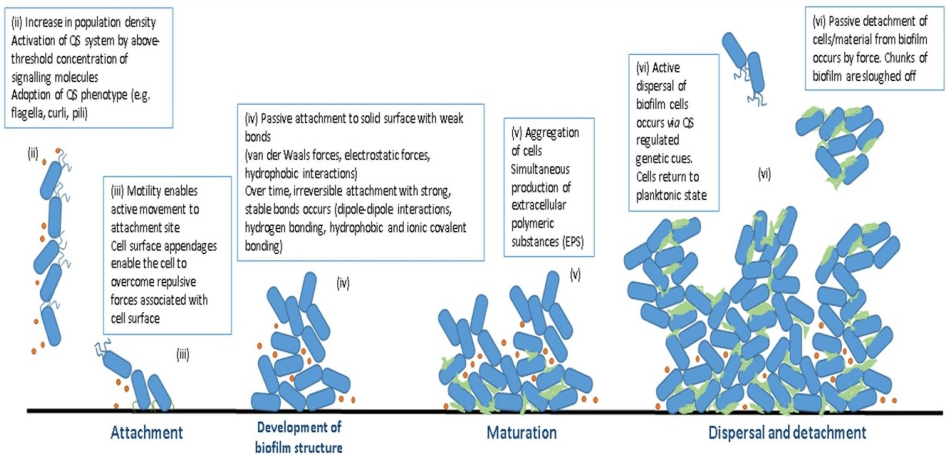


Fig 2. 3 Different stages of biofilm formation (Coughlan *et al*., 2016)

Biofilm formation is also associated with virulence of pathogenic bacteria, and cells within a biofilm are more resistant (up to 1,000-fold) to antibiotics and disinfectants than free-living bacteria (Donlan and Costerton, 2002). Biofilms are a major concern in hospital environments where nosocomial (hospital acquired) infections prevail, as well as in other domains where their growth constitutes a source of contamination for humans or animals (food industry, cooling towers, and water pipes, etc.) or leads to economical loss (due to biofouling of boats and immersed structures; material biocorrosion, etc.). In almost all cases, implanted medical devices were implicated in nosocomial infections. Regardless of the sophistication in the biomedical implant, all medical devices or tissue engineering constructs are susceptible to microbial colonization and infection (Bryers and Ratner, 2004; Castelli *et al*., 2006).

Even when a surface appears to be clean, the presence of biofilms is a potential hazard needing elimination, to prevent recurrent contamination. An antibiofilm drug can either facilitate the dispersion of preformed biofilms or inhibit the formation of new biofilms *in vivo*. The marine bacteriocin BL8 showed antibiofilm ability (Laxmi *et al*., 2016). The biopreservative effect of sonorensin coated film showing growth inhibition of spoilage bacteria in chicken meat and tomato samples, demonstrated the potential of sonorensin as an alternative to current antibiotics/ preservatives (Chopra *et al*., 2015).

Nisin A inhibited biofilm formation by *L. monocytogenes* on stainless steel coupons (García-Almendárez *et al*., 2008). A combination of lactic acid and nisin A, both produced by *L. lactis* UQ2 successfully restricted *L. monocytogenes* biofilms by competitive exclusion. In another study, a bioengineered variant of nisin not only impaired biofilm formation, but also reduced pre-existing biofilms of *Staphylococcus pseudintermedius* (Field *et al*., 2015). *Lactobacillus sakei*, a bacteriocin producing lactic acid bacteria is

commonly used in the preservation and fermentation of meat products (Champomier-Verges *et al*., 2001).

2.7 Marine organisms as a potent source of bacteriocins

Marine environment differs substantially from terrestrial and fresh water habitats by its exigent, competitive and aggressive nature. Bacterial density in seawater and sediment range from 10^5 - 10^7/mL and 10^8 - 10^{10}/g respectively (Austin, 1988). Little is known about marine microbial diversity, with species estimates as low as 10^4 - 10^5 to as high as 10^6 - 10^7 (Glöckner, 2012). Marine bacteriocins are primarily of interest to researchers for their application as probiotics and antibiotics in the seafood industry and marine aquaculture (Galvez, 2008; García, 2010; Pilet and Leroi, 2011). The first marine bacteriocin isolated was from *Vibrio harveyi* (formerly *Beneckea harveyi*) by McCall and Sizemore (1979) when they screened 795 strains of *Vibrio* sp. from Galveston Island, Texas. The identification of harveyicin led to numerous other bacteriocin-screening studies that focussed on biochemical characterization of bacteriocins and BLIS.

Approximately 10% of surface-attached marine bacteria from Sydney Harbor, Australia possessed antibacterial activity (Wilson *et al*., 2010). Proteinase K treatment attributed this inhibitory activity to proteinaceous substances such as bacteriocins or BLIS. Antimicrobial screening of 258 bacterial strains from water and sediment in the Yucatan peninsula revealed 46 strains of genera *Aeromonas*, *Burkholderia*, *Photobacterium*, *Bacillus*, *Pseudomonas*, *Serratia* and *Stenotrophomonas* with antimicrobial activity. Fifty percent of this antimicrobial activity was attributed to bacteriocins or BLIS (La Rosa-Garcia *et al*., 2007). A thermostable bacteriocin BL8 from *Bacillus licheniformis* from marine sediment (Smitha and Bhat, 2012), and halocin SH10 produced by an extreme haloarchaeon *Natrinema* sp. BTSH10 from saltpans of South India (Karthikeyan *et al*., 2013) were reported.

Some bacteria, particularly those in the digestive tract, produce inhibitory compounds to control colonization by potential pathogens in fish (Ringø *et al*., 2000; Makridis *et al*., 2005). For instance, a heat-labile and proteinaceous substance with a molecular mass of <5 kDa was recovered from *Vibrio* sp. obtained from the intestine of spotnape pony fish (Sugita *et al*., 1997a). Similarly, bacteria inhibiting growth of pathogenic *Vibrio* sp. were isolated from the digestive tract of halibut (*Hippoglossus hippoglossus*) larvae (Bergh, 1995). In another study, of the 1,055 intestinal bacteria derived from 7 coastal fish in Japan, 28 isolates (2.7% of the total) inhibited the human and eel pathogen *V. vulnificus* (Sugita *et al*., 1998). Marked inhibition of 15 isolates, consisting of 11 Vibrionaceae representatives, 3 coryneforms, and 1 *Bacillus* strain NM 12 was observed; the latter demonstrating the most pronounced antimicrobial activity. A heat labile siderophore of <5 kDa molecular weight inhibited 227 of 363 (62.5% of the total) intestinal bacterial isolates from 7 fish (Austin, 2006). Bacteriocin producer was also reported from the deep sea shark gut, where a *Bacillus amyloliquefaciens* BTSS3 was shown to produce thermostable, pH tolerant bacteriocin (Bindiya *et al*., 2015). Table 2.2 reviews some characterized marine bacteriocins and their sources.

Fifteen isolates from Irish seaweeds as well as sand and seawater, with confirmed, consistent antimicrobial activity were all spore-forming *Bacillus* sp. While PCR screening identified three of the marine bacteria as lichenicidin producers, the rest did not harbour structural genes for any of the known *Bacillus* bacteriocins for which PCR primers could be designed. These negative PCR outcomes strongly suggest that these isolates produce novel bacteriocins (Prieto *et al*., 2012).

Bacteriocins and their producer bacteria from marine environment can play a pivotal role in areas requiring reduced use of chemical antibiotics. Though the spectrum of action is small for bacteriocins, the probiotic bacteria can be an alternative. Thus, a better under understanding of bacteriocins, their

classification and mechanism of action is worthwhile. The use of nisin and pediocin as food preservative is well studied and applied in many countries. The requirement is still open and hence exploration of marine resources for bacteriocin is still in the lime light.

Table 2.2 Some characterized marine bacteriocins and their sources (Bindiya and Bhat, 2016)

Bacteriocin	**Producer strain**	**Molecular weight**	**Killing breadth**	**Source of isolation**	**Reference**
BLIS	*Lactobacillus pentosus* 39	-	*Aeromonas hydrophila Listeria monocytogenes*	Salmon llets	Pineiro and Stanton, 2007
Carnocin U149	*Carnobacterium* sp.	4.5-5kDa	*Lactobacillus, Lactococcus, Pediococcus, Carnobacterium*	Fish	Stoffels *et al*., 1992
Divergicin M35	*Carnobacterium divergens* M35	~4.5 kDa	*Carnobacterium, Listeria*	Frozen smoked mussel	Tahiri *et al*., 2004
Divercin V41	*Carnobacterium divergens* V41	4.5 kDa	*Carnobacterium, Listeria, Enterococcus*	Fish viscera	Metivier *et al*., 1998
Carnobacteriocin B2	*Carnobacterium pisciocola* A9b	~4.5 kDa	*Listeria*	Cold smoked salmon	Nilsson *et al*., 1997
Piscicocin CS526	*Carnobacterium pisciocola* CS526	~4.4 kDa	*Tetragenococcus, Leuconostoc, Listeria, Enterococcus, Pediococcus*	Frozen surimi	Yamazaki *et al*., 2005; Suzuki *et al*., 2005
Piscicocin V1a	*Carnobacterium pisciocola* V1	4.4 kDa	*Lactobacillus, Listeria, Enterococcus, Pediococcus, Carnobacterium*	Fish	Bhugaloo-Vial *et al*., 1996
BLIS	*Enterococcus faecium* CHG 2-1 and Ch 1-2	-	*Enterococcus*	Venus clams	Valenzuela *et al*., 2010
BLIS	*Enterococcus faecium* C-K, C-S, M 2-1, and PEF 2-2	-	*Listeria*	Anchovy, shark fillet, Swordfish fillet	Valenzuela *et al*., 2010
Enterocin B like BLIS	*Enterococcus faecium* ALP7	<6.5 kDa	*Listeria, Staphylococcus, Bacillus, Enterococcus, Lactobacillus, Lactococcus, Leukonostoc*	Non-fermented shellfish	Pinto *et al*., 2009

Table 2.2 Some characterized marine bacteriocins and their sources (Continued)

Bacteriocin	**Bacteria**	**Molecular Wt**	**Killing breadth**	**Source**	**Reference**
Bacteriocin BL8	*Bacillus licheniformis*	<3kDa	*Staphylococcus aureus*, *Bacillus* sp.	Sediment	Smitha and Bhat, 2012
BLIS	*Vibrio* sp.	<5kDa	*Bacillus* sp., *Vibrio* sp. *Pseudomonas* sp.	Spot nape pony fish	Sugita *et al*., 1997a
BLIS	*Vibrio* sp.			Halibut larvae (*Hippoglossus hippoglossus)*	Bergh, 1995
Bacteriocin	*Bacillus* sp. NM12	Siderophore, <5kDa	Fish pathogens	Coastal fish	Sugita *et al*., 1998
Bacteriocin Bacf3	*Bacillus amyloliquefaciens* BTSS3	~ 3kDa	*Bacillus* sp., *Staphylococcus aureus*	Deep sea shark (*Centroscyllium fabricii*)	Bindiya *et al*., 2015
BLIS	*Proteus* sp.CT1.1	-	*Vibrio*	Cobia	Nguyen *et al*., 2014
BLIS	*Proteus* sp. G1	-	*Vibrio*	Ornate spiny lobster	Nguyen *et al*., 2014
BLIS	*Bacillus cereus* D9	-	*Vibrio*	Subnose pompano	Nguyen *et al*., 2014
BLIS	*Lactobacillus lactis*	94 kDa	*Bacillus, Staphylococcus, Enterococcus, E. coli, Pseudomonas, Shigella*	Sediment sample	Rajaram *et al*., 2010

2.8 Production of bacteriocins

Production conditions strongly influence bacteriocin yield. For many strains, ill-defined factors diminished yield and hence optimum conditions need to be determined empirically. An experimental fractional factorial design for optimization of production medium maximizes bacteriocin activity.

2.8.1 Composition of the growth medium

Certain medium components may be critical for production of individual bacteriocins. Addition of 2 % yeast extract to Trypticase medium enhanced Bacteriocin production by strains of *S. mutans* (Rogers, 1972). Similarly, butyricin 7423 formation in a semi-defined medium was dependent upon casein hydrolysate (Clarke *et al*., 1975). Tryptone was the key nitrogen source for bacteriocin ST341LD production. For plantaricin 423, optimal production was in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract (Verellen *et al*., 1998). Pediocin AcH (Bhunia *et al*., 1988) and helveticin J (Joerger and Klaenhammer, 1986) production was stimulated by yeast extract and meat extract. Although maximum activity of cerein 8A was in brain/heart infusion broth, production was also observed in peptone, MRS, Mueller–Hinton and nutrient broth, but in thioglycollate or tryptic soy broth, there was no observable activity (Bizani and Brandelli, 2004). Meat extract or yeast extract as sole nitrogen source, or a combination of the two (1 : 1) in MRS broth, stimulated bacteriocin production of LAB. With tryptone as sole nitrogen source, only 50% increase in activity (3,200 AU/mL) was recorded, whereas a combination of tryptone, meat extract and yeast extract yielded 6,400 AU/mL (Todorov and Dicks, 2004)

Bacteriocin production by several corynebacteria (Meitert, 1969) and a staphylococcus (Lachowicz and Brodzicki, 1973) was dependent upon the amino acid content of culture medium. Manganese ions were necessary in

chemically defined medium for megacin production. The optimal concentration of $MgSO_4.7H_2O$ was 0.5 % for micrococcin GO5 production (Kim *et al.,* 2006). Addition of 0.5 % mannitol to brain heart infusion broth improved yield of staphylococcin 462, but reduced that of staphylococcin 414 (Hale and Hinsdill, 1975). Similarly, glucose increased streptococcin A-FF22 production but decreased that of streptococcin B-74628 (Tagg *et al*., 1975). Others have shown that addition of either glucose or mannitol enhanced production of a phage type inhibitor by staphylococci in tryptic soy digest broth (Tagg *et al*., 1976). Maximal bacteriocin production by *Lactobacillus plantarum* ST13BR (6,400 AU/ml) was in medium with 2 % (w/v) maltose (Todorov and Dicks, 2004).

The yield of bacteriocins by *Streptococci* isolated from the oral cavity of humans and rodents (Kelstrup and Gibbons, 1969) increased due to increased viscosity of liquid media due to added agar, glycerol or starch and dextran. In another study, staphylococcin 1580 yield was 20 times greater in a semisolid medium than in liquid medium (Jetten *et al*., 1972).

2.8.2 Conditions of incubation

Variations in culture conditions like temperature, pH, incubation time and aeration profoundly affect active bacteriocin yield. Generally, bacteriocin production is higher at growth temperature optima of producer strain. Growth at elevated temperatures can suppress bacteriocin production (Tagg *et al*., 1975), sometimes leading to an irreversible loss of the property (Jetten and Vogels, 1972).

Maximum yields may occur at different phases of the growth cycle. Schlegel and Slade (1973) showed that streptocin STH production was best during exponential growth phase, with a sharp decline before the stationary phase. Similarly, staphylococcin C55 production commenced during logarithmic phase, reached a maximum between 24 and 48 h of growth before

gradually declining. By contrast, streptococcin AFF22 production started late in the logarithmic phase and activity decreased slowly on prolonged incubation. Butyricin 7423 secretion was during late exponential phase; however, perfringocin 11105 appeared only at the beginning of stationary phase, and its production or release was coincidental with partial lysis of the producer cells (Tagg *et al*., 1976).

Some strains of *C. diphtheriae* release bacteriocin continuously whereas others produce it in bursts, with the latter effect signifying spontaneous inducibility of the bacteriocin (Meitert, 1969). Lachowicz (1965) studied the dynamics of staphylococcin A-1262a production on solid media, wherein activity first detected after 8 h, reached a maximum at 18 to 24 h, and subsequently fell to zero. Other studies have also reported substantial losses of bacteriocin activity on prolonged incubation. This effect may be due to specific bacteriocin inactivators or enzymatic digestion or could be attributed to re-adsorption of the bacteriocin to the producer cells. Aeration of cultures greatly increased yield of staphylococcal bacteriocins (Lachowicz, 1965). Addition of antifoaming agents minimized mechanical denaturation of bacteriocins (Jetten *et al.,* 1972).

There is evidence of pH influencing production of some bacteriocins. In colicin K production, control of medium pH was a critical factor (Goebel *et al*., 1956). In another study, streptococcin A-FF22 production on Todd-Hewitt agar increased when initial pH of the medium was 6.5. A lower terminal pH (below 4.0) along with a large cell mass facilitated better pediocin AcH production. Reaction(s) necessary for active pediocin AcH synthesis probably occur at low pH (Biswas *et al*., 1991). The effect of pH and temperature on bacteriocin production has been studied for several lactic acid bacteria like *Lactococcus lactis* (Vuyst, 1995), *Lactobacillus casei* (Vignolo *et al*., 1995), *Leuconostoc mesenteroides* (Krier *et al*., 1998) and *Bacillus licheniformis* P40 (Cladera-Olivera *et al*., 2004a).

2.9 Purification of bacteriocins

The bacteriocin purification methods are those of protein biochemistry, with a wide variety of procedure combinations utilized, with varying success. The extreme heterogeneity of substances, necessitate specific purification protocols to be empirically designed for each bacteriocin. Often crude bacteriocin preparations are initially concentrated by fractional precipitation with acids, salts, ethanol, or various solvent mixtures. Subsequent purification is based on size (gel filtration chromatography, ultrafiltration or centrifugation) or charge differences (ion-exchange chromatography, electrophoresis, isoelectric focussing).

A common problem is the loss (often massive) of activity, even as purification progresses. Hence, it is important to monitor specific bacteriocin activity (units of bacteriocin/milligram of protein) after each purification step, in order to modify those leading to excessive activity loss. Protein concentration is determined by Folin phenol reagent (Lowry *et al*., 1951); however, differential absorption at 280 and 260 nm is a convenient method of protein estimation while dealing with multiple samples, as in column fractionation.

2.10 Physicochemical properties of bacteriocins

Different physicochemical properties are examined to provide information regarding bacteriocin composition and structure.

2.10.1 Chemical composition

Since bacteriocins are a heterogeneous group of substances, essential protein component is the one unifying property. Tests of sensitivity to specific enzymes (proteinases, amylases, etc.) help identify important chemical components of bacteriocin. It was on this basis that streptocin STH, was

recognized to be a complex molecule containing essential protein, lipid, and phosphate groups.

Chemical analyses indicate that while some bacteriocins are simple proteins; others including certain from *Staphylococcus*, *Clostridia*, and *Lactobacillus*, seem to be complex molecules with lipid and carbohydrate components in addition to protein. The staphylococcin 414 composition was like that of the staphylococcal cell membrane. Investigations show than in several colicins, the biologically active protein components are complexed with lipopolysaccharide antigens on the producer cell surface (Klaenhammer, 1993).

Butyricin 7423 and perfringocin 11105 are amphiphilic proteins, whose hydrophobic regions are postulated to facilitate interaction with cell membranes of susceptible organisms. Similarly, a few colicins and entero-bacteriocins were inactivated by anionic detergents, but were relatively unaffected by cationic detergents and nonionic detergents (Hamon and Peron, 1963). It was speculated that these bacteriocins cause local disorganization of the cytoplasmic membranes of susceptible cells due to their weak detergent activity.

2.10.2 Antigenicity

Their protein compositions implicate most bacteriocins to be antigens. However, only little is available on antigenicity of bacteriocins by Gram-positive species. Megacin A-216 evoked an antibody capable of neutralizing its own killing effect. Tubylewicz (1966) could distinguish antigenic differences between four bacteriocins of *C. perfringens* in double-diffusion tests. By contrast, three apparently dissimilar monocins gave cross-neutralizing antibodies. Studies of different staphylococcins have both failed and succeeded in the demonstration of neutralizing antibodies (Tagg *et al*., 1976).

Rabbits immunized with staphylococcin C55 produced specific immunoglobulin M neutralizing antibodies. In addition, normal serum from humans, guinea pigs, and some rabbits showed nonspecific neutralizing factors for this bacteriocin. The low molecular weight of staphylococcin A-FF22 did not invoke antibody production, but the reason for failures in other cases is unclear. Antigenicity of bacteriocins by the Gram-positive bacteria warrants further exploration. Studies of antibodies to bacteriocin in human sera may be an interesting area for future investigation.

Antigenicity of BLIS is a controversial subject, as BLIS of high molecular weight have elicited neutralizing antibodies. However, low molecular weight BLIS from *Staphylococcus* were not antigenic (Iandolo and Crupper, 1997).

2.10.3 Physical properties

Bacteriocins range from simple, low-molecular-weight proteins like streptococcin A-FF22 with a molecular weight of 8,000 dalton, to complex defective phage particles with molecular weight > 106 kilodalton. Low-molecular- weight forms are generally susceptible to trypsin digestion, but are less sensitive to heat inactivation. It was suggested that the high-molecular-weight forms are likely phage related whereas the low molecular forms were not (Bradley, 1967). Electron microscopy of several high-molecular-weight bacteriocins attempted to correlate bacteriocin activity with some visible structure (Tagg *et al*., 1976).

Bacteriocins by Gram-positive species commonly exist in two or more distinct physical forms. Different molecular species of several of these bacteriocins exist in equilibrium; the relative proportions of small units and aggregates of these monomers influenced by either the pH and/or ionic strength of the preparation (Tagg *et al*., 1975).

2.10.4 Stability

The stability of bacteriocin preparations often decrease dramatically with greater purification. Addition of bovine serum albumin protects some purified bacteriocins from excessive inactivation by acting as molecular chaperons (Hastings *et al*., 1991; Finn *et al*., 2012). Bacteriocins such as staphylococcin 1580 as well as an "antibiotic" produced by *S. aureus* were extremely sensitive to mechanical denaturation. They differed in their sensitivity to inactivation by pH changes with many bacteriocins and bacteriocin-like substances considerably more tolerant to acid than alkaline pH extremes (Ellison and Kautter, 1970).

A *Lactobacillus lactis* bacteriocin retained its activity even at 121 °C for 10 min, was stable in pH from 4 to 7, but was inactive in alkaline pH. Bacteriocin from *Lactobacillus plantarum* was active up to 100 °C and within pH 4 to 7. Trypsin and pepsin treatment inactivated both bacteriocins (Lade, 2006). Bacteriocin by LAB *Carnobacterium piscicola* JG126 isolated from spoiled ham was stable at low pH but high pH values saw loss of activity. Proteolytic enzymes like trypsin and chymotrypsin inactivated it, but not catalase, lipase or lysozymes (Jack *et al*., 1996). *Carnobacterium piscicola* is a facultative psychrotrophic bacteriocin producing lactic acid bacterium from fresh fish (Stoffels *et al*., 1992), whose maximum bactericidal activity was at 34 °C, but declined until 15 °C, after which there was no activity.

Bacteriocins produced by LAB *W. cibaria* N23 were completely inactivated after treatment with the proteolytic enzymes trypsin, actinase, protease XIII, ficin, trypsin from porcine pancreas, achymotrypsin and pepsin, confirming their proteolytic nature (Pringsulaka *et al*., 2012). Exposure to different pH values showed that bacteriocin N23 remained fully active in pH from 2.0 to 8.0; however, activity was completely lost at pH 10. Even after heat treatment at 60 to 100 °C for 20 min bacteriocin was stable, but retained only 50 % activity at 100 °C for more than 30 min or 121 °C for 15 min

(Pringsulaka *et al*., 2012). Naclerio and co-workers in 1993, reported cerein, a protein with bactericidal activity in culture supernatant of *B. cereus*; with activity only against other similar strains from lab collections and food isolates, but not against other bacteria tested. A physiological and biochemical characterization of this 9,000 Da cerein showed that it was inducible at the beginning of stationary phase and stable even after various chemical and physical treatments.

Bacillus licheniformis MKU3, a bacteriocin-producing strain from slaughterhouse sediments exhibited bacteriocin-like activity against Gram-positive and negative bacteria, different fungi as well as yeast. SDS–PAGE analysis of its extracellular proteins revealed protein with molecular mass of 1.5 kDa, stable between pH 3.0 to 10.0, temperatures up to 100 °C for 60 min, but inactivated by proteinase K, trypsin or pronase E (Kayalvizhi and Gunasekaran, 2008). Martirani *et al*. (2002) isolated a thermophilic strain of *Bacillus licheniformis* from dairy products, which produced bacillocin 490 with some novel features. This too was inactivated by pronase E and proteinase K and was effective only against related *Bacillus* sp. both under aerobic and anaerobic conditions. Bactericidal activity was retained when stored at 4 °C, as well as in a wide pH range (4.5 to 9.0). Bacillocin 490 was partially purified by elution after adhesion to *Bacillus smithii*, a food isolate strain and had a molecular mass of 2 kDa.

The antimicrobial substance produced by *Bacillus licheniformis* strain P40 was sensitive to pronase E, but not papain or trypsin. It was also stable at all temperatures tested, except 121 °C and 103.5 kPa for 15 min; lost its activity after treatment with TCA and butanol, while all other chemicals used caused no effect on antimicrobial activity (Acetone, 1-Butanol, Chloroform, Methanol, Toluene, Trichloroacetic acid). The antimicrobial substance was stable in all pH tested (3 to 11), retaining all of its initial activity (Cladera-Olivera *et al*., 2004b). BL8 from *Bacillus licheniformis* BTHT8 lost its

antibacterial activity at high temperature of 121 °C per 105 kPa for 15 min; however activity was retained at temperatures from 40 to 100 °C for 30 min. BL8 activity was stable at pH from 1to12 (Smitha and Bhat, 2012).

Criteria for thermostability of bacteriocins are difficult to define, as this not only dependent on state of purification, but also other factors like pH, ionic strength, and presence of protective molecules.

2.11 Applications of bacteriocins

Global production of fish, crustaceans, molluscs and other aquatic animals reached 167 million tonnes in 2014. Aquaculture production continued to show strong growth, with an average annual growth rate of 6.1 percent from 36.8 million tonnes in 2002 to 74 million tonnes in 2014 according to Food and Agriculture Organization of United Nations (FAO) 2016. Consumer demand for fish is high, especially in affluent nations, which imported around 33 million tonnes of fish in 2012. Moreover, fish is also an ingredient in pet food, health supplements, fishmeal and many non-food products manufactured on a global scale. Hence, pressure on seafood resources is set to increase further, such that fisheries being poorly managed may quickly collapse with spoilage and disease both due to microorganisms emerging as the greatest challenges in seafood industry.

2.11.1 Food preservation

Lactococcus lactis subsp. *lactis* KT2W2L, a nisin Z producer was used for biopreservation of cooked, peeled and ionized tropical shrimps during storage at 8 °C (Hwanhlem *et al*., 2015). The efficacy of *Lactobacillus* sp. AMET1506 as a biopreservative for shrimp under different storage conditions was evaluated (Karthik *et al*., 2013). Nisin-coated plastic films suppressed growth of aerobic and anaerobic spoilage microorganisms in a concentration-dependent manner (Neetoo *et al*., 2008). *L. lactis* subsp. *lactis* strain CWBIB1410, another nisin producer used as a starter culture to improve the

traditional Senegalese fish fermented guedj, helped enhance its safety (Diop *et al*., 2009). *Lactobacillus pentosus* 39, a BLIS producing strain controlled the growth of *Aeromonas hydrophila* ATCC14715 and *Listeria monocytogenes* under simulated cold chain break conditions (Anacarso *et al*., 2014). One area of active research in seafood aquaculture is the utilization of bacteriocins as antimicrobials.

2.11.2 Probiotics in aquaculture

Probiotics are "live microorganisms" consumed in adequate amounts to confer health benefits on the host (Pineiro and Stanton, 2007) and include species of lactic acid bacteria (LAB), including lactobacilli, bifidobacteria, nonpathogenic *Escherichia coli*, bacilli as well as yeasts like *Saccharomyces boulardii* (Dobson *et al*., 2012). Antibiotic over use in aquaculture disease control led rise of bacterial resistance that can be spread by horizontal gene transfer via plasmids or bacteriophages. This highlights the need for alternatives for antibiotics in aquatic disease management. Use of probiotics in aquaculture for elimination of antimicrobial drug is increasing, with successful use of bacteria like *Vibrio* sp., *Pseudomonas* sp., *Bacillus* sp. and several *Lactobacilli* sp. as probiotics in mollusk, crustacean, and finfish aquaculture; with most obtained from aquatic animals, their culture environment or from the intestine of different aquatic species (Nikapitiya, 2013). Since probiotic research in aquaculture is in its infancy and only just gaining acceptance in the industry, much work is essential to understand and resolve controversies related to real environmental demonstrations on successful use of probiotics, their mode of action, and mechanism *in vivo*. Application of terrestrial bacteria in aquaculture has limited success. Hence, identification of potential probiotic bacteria from marine environments where they grow optimally is a better approach.

Aeromonas media A199 controlled *Vibrio tubiashii* infection in Pacific oyster, *Crassostrea gigas* larvae by producing bacteriocin-like inhibitory

substances, which antagonized several pathogenic bacteria in culture (Gibson, 1999; Gibson *et al*., 1998). *Alteromonas macleodii* 0444 as a probiotic controlled *V. coralliilyticus* and *Vibrio pectenicida* in flat oyster, *Ostrea edulis* larvae (Kesarcodi-Watson *et al*., 2012), as well as *Vibrio splendidus* infection in Greenshell mussel *Perna canaliculus* larvae, leading to increased survival (Kesarcodi-Watson *et al*., 2010).

Bacteriocinogenic bacteria were isolated from ornate spiny lobsters (*Panulirus ornatus*), black tiger shrimp (*Penaeus monodon*), cobia (*Rachycentron canadum*) and snubnose pompano (*Trachinotous blochii*). Two candidate probiotic formulations with bactericinogenic bacteria proved their beneficial effects on aquaculture-raised juveniles of ornate spiny lobsters (*Panulirus ornatus*) challenged with *V. owensii* DY05 (Nguyen *et al*.,2014). Thus in all aspects either the bacteriocin or the producer organism can be a food preservatives or immune enhancer in marine food industry.

2.11.3 Applications in human health

The increasing bacterial resistance to antibiotics used in clinical application, promulgated a growing interest in bacteriocins as alternative therapeutics for human (and possibly animal) infections (Lawton *et al.*, 2007). Since bacteriocins and conventional antibiotics act on different cellular targets, cross-resistance is rarely reported between the two. Several bacteriocins or BLIS produced by *Bacillus* sp. are effective against MRSA or VRE pathogenic bacteria; pumilicin 4 or the lantibiotics: lichenicidin, mersacidin and haloduracin produced by *B. sphaericus*. Haloduracin being more stable than nisin at physiological pH is of particular interest for medical applications (Oman and van der Donk, 2009). Mersacidin has antimicrobial activity against *S. aureus* both *in vitro* and *in vivo* (Molitor *et al.,* 1996; Kruszewska *et al., 2*004; Sass *et al., 2*008). The lantibiotic subtilosin A has antimicrobial activity against *G. vaginalis*, *L. monocytogenes* and *S. agalactiae* (Sutyak *et al.,* 2008b). Subtilosin A is thus a potential agent against vaginal pathogens like *G.*

vaginalis that is resistant to conventional antibiotics. Besides their antibacterial activity, some bacteriocins/BLIS of *Bacillus* also show antifungal activity, which can be exploited in clinical applications (Abriouel *et al.,* 2011).

Their inhibitory action against intestinal pathogens like *C. perfringens*, *C. difficile* and others make bacteriocin-producing *Bacillus* strains effective as human probiotics. For example, *B. polyfermenticus* SCD (polyfermenticin SCD producer) is a commercial probiotic used for treatment of long-term intestinal disorders due to *C. perfringens* (Lee *et al., 2*001). The probiotic strain *B. clausii* O/C produces inhibitory substances against *S. aureus*, *E. faecium* and *C. difficile* (Urdaci *et al., 2*004), while thuricin CD specifically targets *C. difficile* (Hill *et al.,* 2014). Bacteriocins from *Bacillus* may also be used as natural contraceptives; for example subtilosin A which showed spermicidal activity against spermatozoa from humans as well as several farm animals (Sutyak *et al., 2*008a).

2.11.4 Livestock applications

Several *Bacillus* strains are marketed as probiotics for livestock based on functional properties like improvement in body weight of farm animals or poultry; e.g. BioPlus2B®, a probiotic formulation of *B. licheniformis* and *B. subtilis* strains (Mutus *et al.,* 2006). Lichenin A producing *B. licheniformis* has antibacterial effect against *S. bovis* and *Eubacterium ruminantium*, as well as hydrolytic activities against several polysaccharides, indicating its use as digestive aid to improve rumen fermentation (Pattnaik *et al., 2*001). Spores of BLIS-producing *B. amyloliquefaciens* CECT 5940 in poultry feeds (Ecobiol®, Norel and Nature Nutrition) reduce pathogenic bacteria like *C. perfringens*, *E. coli* and *Yersinia* in the intestinal tract of poultry, diminishing poultry mortality (Diaz, 2007). *B. circulans* and *P. polymyxa* bacteriocins function similarly in poultry (Stern *et al*., 2005). The *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* zoonosis in animals. The

dietary administration of bacteriocin preparations in poultry reduced intestinal colonization by *C. jejuni*, thereby decreasing risk to public exposure as well as campylobacteriosis (Svetoch *et al*., 2005).

Disease control using bacteriocin is cheap, effective, and non-toxic to non-target organisms including animals and humans (Lewus *et al*., 1991). For example, nisin used topically is as effective as a 1% iodophor dip in controlling both Gram-positive and Gram-negative bacteria inoculated onto teat skin of live cows (Sears *et al*., 1992). Moreover, treatment with nisin does not irritate skin, even after multiple applications. In addition, bacteriocins are GRAS (i.e., generally recognized as safe by the Food and Drug Administration), hence milk from bacteriocin-treated cows is not considered adulterated, consequently not requiring a holding period before marketing (Cao *et al.,* 2007).

Bacillus bacteriocins with strong inhibitory activity against *Staphylococci* could control 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 MAR (multiple antibiotic resistance) *S. aureus* isolates from dairy sources (Barboza-Corona *et al*., 2009). Despite differences in sensitivity of *S. aureus* strain, morricin 269 followed by kurstacin 287 had best inhibitory action. The authors suggested the use of these antimicrobial peptides as an alternative to control bovine mastitis. They also proposed over-expression of these BLIS, as well as development of purified, concentrated bacteriocin preparations for prevention and treatment of mastitis. Another antimicrobial substance for application in domestic animal health is the BLIS by *B. subtilis* LFB112 isolated from Chinese herbs, which is active against disease causing Gram-positive and Gram-negative bacteria (Xie *et al*., 2009).

2.11.5 Environmental applications

Many bacteriocins or BLIS produced by *Bacilli* inhibit plant-pathogenic bacteria. Either the bacteriocin-producing strains or the partially purified bacteriocin preparations can be used to control plant diseases. Ericin S is active against *C. michiganensis, which* causes bacterial tomato canker. Purified ericin or its producer strain could be a suitable bioprotectant for tomato plants. Bac 14B, a BLIS produced by *B. subtilis* 14B isolated from the rhizosphere of healthy plants can be applied as a biocontrol agent as it can reduce *A. tumefaciens* infections in plants (Hammami *et al*., 2012).

Several mechanisms help rhizosphere bacteria that are of great interest in agriculture to promote plant growth. *Bacillus* species promote plant growth and disease resistance. A polypeptide from *B. thuringiensis* strain NEB17 (Smith *et al*., 2006), isolated from soybean root nodules enhanced nodulation when applied as a coinoculant with *Bradyrhizobium japonicum* 532C (Bai *et al*., 2003). This strain also produced thuricin 17, an antibacterial peptide (Gray *et al*., 2006), with plant growth-promoting activity (Lee *et al*., 2009). Thuricin 17 induced root hair deformation curling response of soybeans, and increased nodulation. Its application to plant leaves or roots directly stimulated the growth of both soybean (a C3 dicot) and corn (a C4 monocot). The N-terminal amino acid sequences is identical or highly homologous to that of other bacteriocins from *Bacillus* such as thuricin S, thurincin H, bacthuricin F4 or cerein MRX1 (He, 2009). Thus, it is reasonable to suspect that these similar bacteriocins may also promote plant growth. These bacteriocins with a dual function and broad inhibitory spectra, may be exploited not only to improve plant growth and crop yield, but also to inhibit plant-pathogenic bacteria as well as to decrease plant contamination with human pathogens like *L. monocytogenes* or *Salmonella*. BLIS with antifungal activities, or their producer strains can be used to control plant decay and postharvest control of fruits and vegetables. One example is *B. amyloliquefaciens* RC-2, which

produces a BLIS active against *C. dematium*, a mulberry anthracnose fungus as well as several other phytopathogenic fungi and bacteria, such as *R. necatrix*, *P. oryzae*, *A. tumefaciens* and *X. campestris* pv. *campestris* (Yoshida *et al*., 2001).

Bacteriocin-producing *Bacilli* are also amenable for other environmental applications. Some interesting strains isolated from oil reservoirs (Korenblum *et al*., 2005) like the *B. firmus* strain H2O-1, produces a small, heat and alkali stable BLIS peptide, capable of withstanding environmental conditions during oil drilling. Its antimicrobial activity against sulphate reducing bacteria (SRB), offers potential use as a biocide in the petroleum industry to control these bacteria. Further, BLIS by strain H2O-1 reduced viability and attachment of an SRB consortium biofilm (Korenblum *et al*., 2008). The authors suggest that this strain or its antimicrobial peptide may have a potential for pipeline cleaning technologies to inhibit biofilm formation and consequently reduce biocorrosion.

2.11.6 Biotechnological applications

There are significant benefits to employing modern cutting-edge bioengineering to advance the traditional peptide discovery, description and production, because of the gene-encoded nature of bacteriocins. One of the greatest advantages of bioengineering in the lantibiotic field is creation of strains producing larger amounts of lantibiotic peptides (Suda *et al*., 2010). Another strategy to improve lantibiotic-producing strains used conjugation of multiple large bacteriocin-encoding plasmids into a single strain (Collins *et al*., 2010), thereby improving its ability kill the undesired target more effectively than the wild type (O'Sullivan *et al*., 2003). It is also possible to achieve this goal by amplifying and cloning lantibiotic-encoding genes into shuttle vectors and its heterologous production in other strains. Such approaches improved lacticin 3147 production by *Enterococcus* host (Ryan *et al*., 2001). Bioengineering of existing peptides can also create lantibiotics with

improved activity and/or suitable for specific applications (Collins *et al*., 2010).

The amino acid composition of lantibiotics can be modified via protein engineering to gather important clues concerning structure-function relationships (Chen *et al*., 1998). Production of engineered mutacin II with normal gene dosage and regulatory responses was attained with a gene replacement strategy in a streptococcal expression system. A similar approach was used to engineer analogs of subtilin (Liu and Hansen, 1992), nisin (Dodd *et al*., 1996), gallidermin (Ottenwalder *et al*., 1995), and epidermin. In addition, peptides were developed with enhanced characteristics to improve the activity or inhibitory spectrum. For example, solubility and stability of nisin Z at different experimental conditions was improved by peptide engineering without dramatically reducing specific activity (Rollema *et al*., 1995). Other than lantibiotics, non-lantibiotic peptides can be altered by introducing new post-translational modifications using specific enzymes. The cyclase of nisin (NisC) protects non-lantibiotic peptides against peptidases and proteases by cyclization (Rink *et al*., 2007), a property which is particularly useful from a drug design standpoint; while the dehydratase of nisin (NisB) introduces dehydro residues leading to the formation of thioether bridges into various peptides easier (Kluskens *et al*., 2005).

Bioengineering of bacteriocins is not limited to lantibiotics. The well-conserved cationic N-terminal half of pediocin like bacteriocins mediates its initial binding to target cells through electrostatic interactions (Chen *et al*., 1997). The possibility that the N-terminal half of the pediocin-like bacteriocin, sakacin P may influence the target-cell specificity, has been investigated by the use of site-directed *in vitro* mutgenesis (Kazazic *et al.,* 2002). Though variants generated in these types of studies are useful from an academic standpoint, none of them displayed increased activity against several microorganisms.

2.11.7 Applications in the pharmaceutical industry

With the availability of powerful and effective arsenal of drugs, most pharmaceutical companies abandoned their antimicrobial drug development programs, as there seemed little need for new drug compounds (Knowles, 1997). Bacterial resistance to antimicrobials emerged right after their initial wide-scale use (Levin *et al*., 1998). Since then, the resistance levels continued to rise dramatically. Antibiotic resistance was thought to be confined to hospital settings, where the use of antibiotics was most intensive; approximately one third of all hospitalized patients receive antibiotics with at least half of those prescriptions being unnecessary, poorly chosen or incorrectly administered (Van Houten *et al.,* 1998).

Compounding the problem further, an almost exclusive reliance on broad-spectrum antibiotic agents contributed to a rapid emergence of multi resistant pathogens (Wester *et al*., 2002). In the agricultural industry, antibiotics for disease control, prophylaxis and growth promotion, contributed significantly to resistant pathogens in animals (Barton and Hart, 2001) and plants (McManus *et al*., 2002). Additionally, bacteria isolated from animals in environments unrelated to clinical or agricultural management settings have also acquired high levels of antibiotic resistance (Sherley *et al*., 2000). Paradoxically, it is the extensive use of antibiotics that has contributed to the limited array of effective drugs available today for treating multi-resistant bacteria (Lowy, 2003).

Numerous antibacterial agents are being considered as alternatives, such as bacteriophages (Alisky *et al*., 1998), probiotic bacteria (Macfarlane and Cummings, 2002), antimicrobial peptides (Joerger, 2003), and bacteriocins (Twomey *et al*., 2002). In order to exploit the desired activities of these varied antimicrobial leads optimally, researchers often employ chemical or genetic engineering methods (Lien and Lowman, 2003).

The pediocin-like bacteriocin, a class IIa like microcin V, was used as a possible alternative to control Gram-negative bacteria (Duquesne *et al.,* 2007). Three different proteins serve as a specific receptor for microcins, namely the membrane component F0 of the ATP synthase, SdaC, and the mannose permease, required by MccH47, MccV, and MccE492, respectively (Biéler *et al.,* 2010; Gérard *et al.,* 2005). Because of the Gram-negative cell wall structure, the class IIa microcins requires an outer membrane (OM) transporter system to reach the plasma membrane receptor. This requires an additional step of modification. Enterocin CRL35, a pediocin-like bacteriocin has potent antilisterial activity but is inactive against Gram-negative bacteria (Farías *et al.,* 1996). Asymmetrical PCR fused the required portions of genes encoding enterocin CRL35 and microcin V, namely munA and cvaC, to obtain a peptide with a broader antimicrobial spectrum (Acuña *et al.,* 2012). The hybrid bacteriocin purified from *E. coli* extracts, named Ent35eMccV, showed inhibitory activity against enterohemorrhagic *E. coli*, *L. monocytogenes*, and other pathogenic Gram-positive and Gram-negative bacteria (Acuña *et al*., 2012).

Chapter 3

Screening and characterization of bacteriocin producing bacteria from gut of marine fishes- Centroscyllium fabricii and Sardinella longiceps

3.1 Introduction

Many of the antibacterial properties elucidated by bacteria may be ascribed to antibiotics, bacteriocins, lysozymes, siderophores, proteases, and/or hydrogen peroxide as well as the alteration of pH values by organic acids produced either singly or in combination. Bacteriocins which are potent antimicrobial polypeptides and/or proteins elaborated by every bacterial species examined to date are important. These tens or hundreds of bacteriocins (Riley, 1992), play key roles in regulating microbial colonization of animals or fish.

In fish, colonization by microorganisms may start at the egg and/or larval stage, continuing with the development of the fish (Olafsen, 2001). Thus the number and diversity of microorganisms present in the larval stage, in feed and water influence the microflora of the developing fish. Several functions including digestion of algal cells, provision of amino acids, and possibly the colonization by bacterial pathogens (Westerdahl *et al*., 1991) are assigned to the resident symbiotic gut microflora. Bacteria in the digestive tract produce inhibitory compounds to regulate colonization by pathogens in fish (Ringø *et al*., 2000; Makridis *et al*., 2005).

Several potent bacteriocins and bacteria with antimicrobial properties have been reported from fish gut. For instance, a proteinaceous, heat-labile substance with molecular mass <5 kDa was recovered from *Vibrio* sp. isolated from the intestine of a spotnape pony fish (Sugita *et al*., 1997). Likewise, bacteria inhibiting pathogenic *Vibrio* sp. were isolated from the digestive tract of halibut (*Hippoglossus hippoglossus*) larvae (Bergh, 1995). In another study

28 of 1,055 intestinal bacterial isolates from seven coastal fish in Japan, (2.7% of the total) inhibited the human and eel pathogen *V. vulnificus* (Sugita *et al*., 1998). Marked inhibition was displayed by 15 isolates, consisting of 11 *Vibrionaceae* representatives, 3 *Coryneforms*, and 1 *Bacillus* strain NM 12; the latter demonstrated the most pronounced antimicrobial activity (Austin, 2006).

This chapter deals with the isolation, screening and identification of potent bacteriocin producers from gut of marine fish. The fishes as well as the bacteriocin producers were identified using molecular techniques.

3.2 Materials and methods

Deep sea shark and Sardines were used as samples for isolating bacteriocin producers. The composition of the media used is given in Appendix – I.

3.2.1 Sampling of Deep sea fish

The deep sea shark caught during the cruise no. 305 of FORV Sagar Sampada using HSDT (High Speed Demersal Trawl) net from station number 17 (8° 11.4” N and 75° 54.9” E) at 1000m depth on 10/10/2012 was used for isolating bacteriocin producers. The sample was given voucher number SSDSS1.

3.2.2 Sampling of Indian oil Sardine

Sardines were collected from local fishermen from Thoppumpadi harbor, (Ernakulam district, Kerala, India) pre-monsoon, immediately after the catch. Though the fish can be identified easily by morphological aspects, it was confirmed by molecular identification. The sample was given voucher code SFF.

3.2.3 Identification of fishes by barcoding

DNA from tissue samples (mostly white muscle) obtained from fish specimens (deep sea shark and Indian sardine) was extracted using standard phenol–chloroform procedures (Sambrook *et al*., 2006). Approximately 655 bp were amplified from the 5′ region of the *cox1* gene from mitochondrial DNA using the primers (Ward *et al*., 2005)

FishF2-5′TCGACTAATCATAAAGATATCGGCAC3′ and
FishR2-5′ACTTCAGGGTGACCGAAGAATCAGAA3′

PCR was performed in thermal cycler (BioRad, USA) with an initial denaturation at 94 °C for 2 min, followed by 40 cycles of [94 °C/30 s, 56.5 °C/30 s, 72 °C/1’30 s] and a final extension at 72 °C for 10 min. The amplicons obtained were processed as in section 3.2.8.

3.2.4 Bacterial isolation and primary screening for antagonistic activity

The gut contents of the fish was isolated aseptically, diluted in a tenfold series, after which 100 µL sample of each dilution was spread onto Nutrient agar (NA) plates (HiMedia, Mumbai , India). The plates were incubated at 28 °C for 1 week and all the isolated colonies were purified and maintained on nutrient agar slants at 4 °C.

The isolates were screened for antimicrobial activity by cross streak method (Gardner, 1950) using standard pathogens from NCIM (*Escherichia coli* 2343, *Salmonella typhimurium* 2501 and *Staphylococcus aureus* 2127). The experiment was repeated for three consecutive subcultures. The bacteria showing antagonistic activity even after three subcultures/transfers were selected and further screened for bacteriocin production.

3.2.5 Secondary screening: Quantitative estimation of antibacterial titres by critical dilution assay

In the secondary screening, all bacterial isolates with antagonistic activity (after three sub culturing/transfers) in the primary screening went

through the critical dilution assay (Mayr-Harting *et al*., 1972) for quantitative estimation of their antibacterial titres. For this, the cell free supernatant of the culture in Zobell marine broth (HiMedia), obtained by filtering through 0.22 µm filter was serially diluted using twofold dilutions. From each dilution, 5 µL was spotted on an indicator strain (Table 3.1), swab-inoculated on Mueller Hinton agar plate (Nakano *et al*., 1968). The plates were incubated at the optimum growth temperature of the indicator strain. The bacteriocin activity was expressed in activity units per mL (AU/mL).

One activity unit (AU) of bacteriocin was defined as 5 µL of the highest dilution yielding a zone of growth inhibition on the indicator lawn. The reciprocal of the highest dilution was multiplied by 200 (1 mL/5 µL) to obtain activity units per mL (AU/mL) (Enan *et al*., 1996). Protein concentration was estimated by Lowry's method (1951). Specific activity was obtained by dividing activity with protein concentration and expressed as AU/mg.

3.2.6 Confirming protein nature of bacteriocin

The cell free supernatants of the cultures in Zobell marine broth (HiMedia) were used to confirm the protein nature, by ammonium sulphate precipitation and action of proteolytic enzymes. To confirm the presence of active peptide, enzymes such as proteinase K, trypsin, and pepsin (Sigma-Aldrich) were mixed with the active ammonium sulphate fraction at a final concentration of 5 mg/mL of enzyme and 5±0.5 mg/mL of ammonium sulphate fraction; incubated for 1 h at 37 °C and checked for bacteriocin activity.

Table 3.1 List of standard cultures used for antibacterial screening

Test organisms	NCIM No.
Pseudomonas aeruginosa	2863
Salmonella Typhimurium	2501
Escherichia coli	2343
Proteus vulgaris	2027
Clostridium perfringens	2677
Staphylococcus aureus	2127
Bacillus cereus	2155
Bacillus circulans	2107
Bacillus macerans	2131
Bacillus pumilus	2189

3.2.7 Bacterial characterization and identification

The bacteriocin producer strains were characterized and identified using both phenotypic and genotypic or molecular characterization.

Phenotypic characterization: Gram staining was performed using a Gram stain kit (Himedia, Mumbai) according to the manufacturer's instructions. Cell motility was observed by the hanging-drop method (Skerman, 1967). Different biochemical tests were carried out according to standard methods (Dong and Cai, 2001). The biochemical tests included IMViC, sugar fermentation (TSI), oxidative/fermentative (MoF), and enzyme productions like catalase, urease, amylase, protease and lipase.

Antibiotic susceptibility tests: The bacteriocin producers were tested for antibiotic susceptibility by using Combi 69 and G VIII plus octo-discs (Himedia, Mumbai). The antibiotics, their concentrations per disc and zone size interpretation are given in table 3.2.

Table 3.2 List of antibiotics used for antibiotic sensitivity, their concentration and zone size interpretations

Antibiotic	Concentration	Zone size of growth inhibition (mm)		
		R	I	S
Ciprofloxacin (CIP)	5µg	≤15	16-20	≥21
Ofloxacin (OF)	5µg	≤12	13-16	≥16
Sparfloxacin (SPX)	5µg	≤15	16-18	≥19
Gatifloxacin (GAT)	5µg	≤14	15-17	≥18
Aztreonam (AT)	30µg	≤15	16-21	≥22
Azithromycin (AZM)	15µg	≤13	14-17	≥18
Vancomycin (VA)	30µg	≤14	15-16	≥17
Doxycycline Hydrochloride (DO)	30µg	≤12	13-15	≥16
Bacitracin (B)	10Unit	≤8	9-12	≥13
Chloramphenicol (C)	30µg	≤12	13-17	≥18
Co-Trimoxazole (COT)	25µg	≤10	11-15	≥16
Penicillin-G (P)	10Unit	≤28	-	≥29
Polymyxin-B (PB)	300Unit	≤11	-	≥12
Gentamicin (GEN)	10µg	≤12	13-14	≥15
Neomycin (N)	30µg	≤12	13-16	≥17
Tetracycline (TE)	30µg	≤14	15-18	≥19

Genotypic identification: Bacterial DNA was isolated according to the protocol described by Ausubel *et al*., (1987) (Appendix-II). 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). The sequences for the primer pair used were as follows:

Forward primer - 5' AGAGTTTGATCCTGGCTCAG 3'

Reverse primer - 5'ACGGCTACCTTGTTACGACTT 3'

The conditions used for PCR amplification are listed in

Step	Temperature	Time
1.Initial Denaturation	94 °C	1.5 min
2. Denaturation	94 °C	30 s
3. Annealing	56 °C	30 s
4. Extension	72 °C	2 min
5. Final extension	72 °C	10 min

* Steps 2, 3and 4 are repeated in 30 cycles

3.2.8 Agarose gel electrophoresis and sequencing

PCR products were visualized on 1.5 % agarose gels and sequenced by Sanger sequencing using ABI Prism 310 genetic analyzer (Applied Biosystems, Carlsbad, CA, USA) using big dye terminator kit. The identity of the sequences was determined by comparing the sequences in the NCBI database using Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990). The sequences were submitted to GenBank and accession numbers were secured.

Phylogenetic analysis was performed using MEGA version 6.0 (Tamura *et al*., 2013). Clustering using the neighbour-joining (Saitou and Nei, 1987) were determined using bootstrap values based on1,000 replicates.

3.2.9 Growth curve of the two bacteriocin producing microorganisms

Zobell marine broth was inoculated with strain *Bacillus amyloliquefaciens* BTSS3 and strain *Bacillus pumilus* SDG14 each (inoculum 1 % (v/v)), and incubated at 30 °C at 120 rpm for 24 h in a rotary shaker. Growth was assessed by measuring the optical density at 600 nm (Schimadzu,

Germany) at 2 h intervals. The generation time (G) and growth rate (μ) were calculated by the formula:

$$G = t/n$$

$$n = 3.3 \log (b/B)$$

$$\mu = 0.693\ G$$

Where,

t = time interval in minutes

B = number of bacteria at the beginning of a time interval

b = number of bacteria at the end of a time interval

n = number of generations

3.3 Results and discussion

The deep sea shark caught during the cruise no. 305 of FORV Sagar Sampada at a depth of 1000 m was barcoded as was the Sardine sampled from the fishing harbour.

3.3.1 Identification of fishes by barcoding

3.3.1.1 Identification of deep sea fish

A 750 bp amplicon was obtained after PCR of the mitochondrial cytochrome oxidase (COI) gene from the deep sea shark (Fig 3.1 A.). BLAST analysis of the sequenced PCR amplicons showed 98 % identity with the mitochondrial cytochrome oxidase (COI) gene of *Centroscyllium fabricii* (Accession no. KC015264.1). The phylogenetic tree using Cox I genes clearly shows *Centroscyllium fabricii* voucher no. SSDSS1 clading with other *Centroscyllium fabricii* voucher samples, confirming the identity of the deep sea shark. The sequences were submitted in GenBank and accession number KJ888145.1 was obtained (Fig 3.1 B).

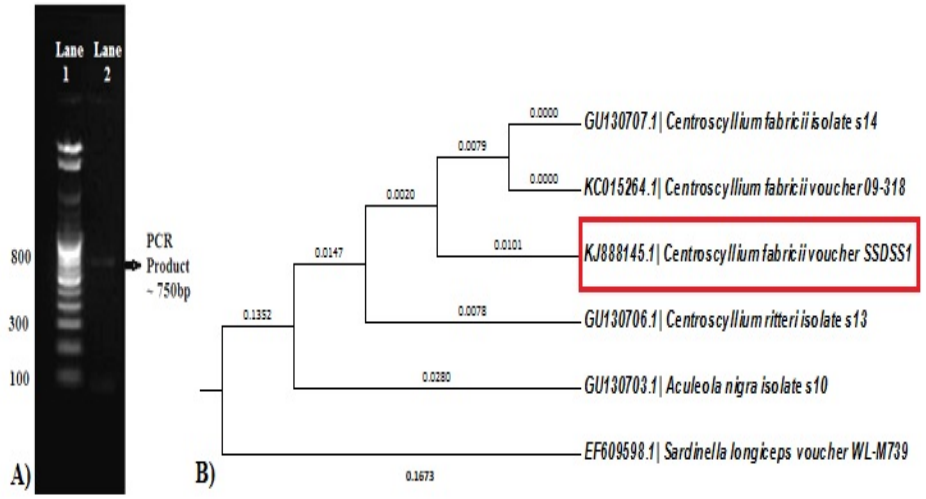


Fig 3.1. A) Agarose gel electrophoresis of COX1 PCR product. Lane 1- 1kb plus ladder, Lane 2- PCR product. B) Phylogenetic tree of Cox I gene of *Centroscyllium fabricii* voucher no. SSDSS1 and 5 other sample sequences. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distance was computed and is in the units of the number of base substitutions per site. Accession numbers are given along with the name. Evolutionary analyses were conducted in MEGA6. *Sardinella longiceps* voucher WL-M739 served as out group.

3.3.1.2 Identification of Indian oil sardine

A 750 bp amplicon was obtained after PCR of the mitochondrial cytochrome oxidase (COI) gene from sardine sample (Fig 3.2 A.) The BLAST analysis of the sequenced PCR amplicons confirmed that this partial gene sequence encoded mitochondrial cytochrome oxidase (COI) gene of *Sardinella longiceps.* This was further proved after multiple sequence alignment involving 9 other sequences and in the phylogenetic tree the mitochondrial cytochrome oxidase (COI) genes of various *Sardinella longiceps* voucher specimens and *Sardinella longiceps* Voucher No. SFF (this study) claded together, also confirming the identity of the test sample. The sequences were deposited in GenBank under accession number KF151867 (Fig 3.2 A and B).

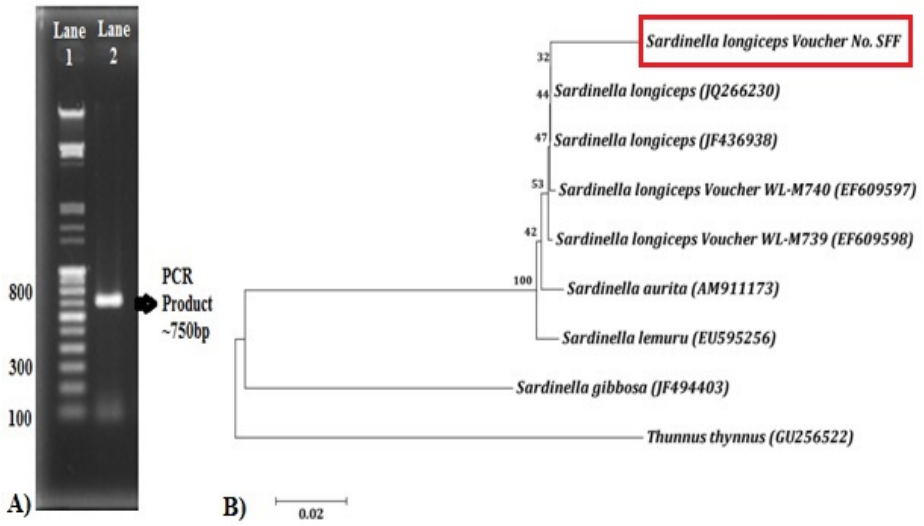


Fig 3.2 A) Agarose gel electrophoresis of COX1 PCR product. Lane 1- 100bp ladder, Lane 2- PCR product. B) Phylogenetic tree of Cox I gene of *Sardinella longiceps* Voucher No. SFF. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed and are in the units of the number of base substitutions per site. The analysis involved 9 nucleotide sequences and *Thunnus thynnus* (accession number GU256522) was the out-group. Accession numbers are given in parentheses. Evolutionary analyses were conducted in MEGA6.

The mitochondrial cytochrome c oxidase subunit I (COI) DNA sequences act as unrepeatable signatures and constitute a unique DNA barcode. This short DNA sequence provides sufficient identification labels in terms of nucleotide positions (Hebert *et al*., 2003). Bucklin *et al*., (1999) sequenced mitochondrial gene, cytochrome c oxidase subunit I (COI), in eight species from three genera of planktonic copepods and found the method could reliably discriminate even among sibling species. DNA barcodes had been obtained for more than 8000 species of fish and the COI sequences were deposited in the Barcode of Life Data Systems (BOLD) online repository (Ratnasingham and Hebert 2007).

3.3.2 Bacterial isolation and primary screening for antagonistic activity

Seventy two isolates were obtained by culture dependent methods from gut of *Centroscyllium fabricii* (SSDSS1) and twenty five from gut of *Sardinella longiceps* (SFF). Two isolates (BTSS2 and BTSS3) from *Centroscyllium fabricii* and three (SDG 14, GUT 13 and GUT22) from *Sardinella longiceps* showed antagonistic activity against various test organisms (Fig 3.3).

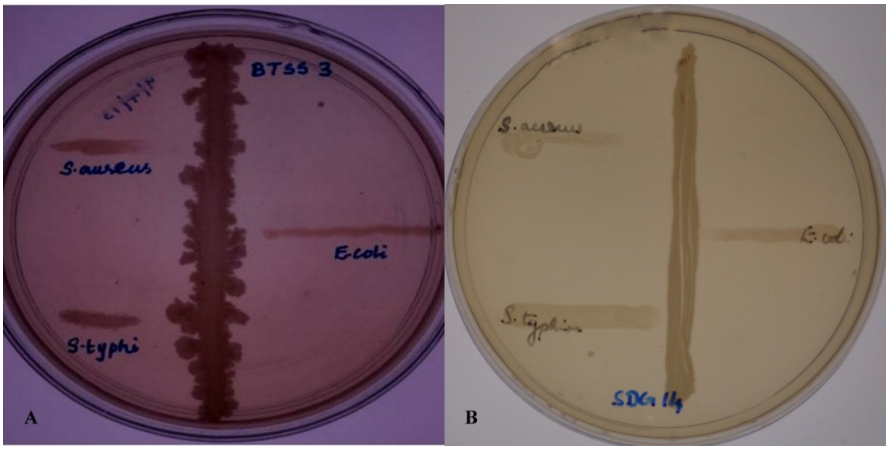


Fig 3.3 Antibacterial screening of A) BTSS3 and B) SDG14 by cross streak method against *S. aureus* NCIM2127, *S.* Typhimurium NCIM2501 and *E. coli* NCIM2343.

These organisms were sub-cultured three times with intermittent activity screening to prove sustained bacteriocin production. Two isolates, GUT13 and GUT22 lost their activity after the second transfer while BTSS2 could not retain its activity after the third subculture (Table 3.3). Only two isolates labelled BTSS3 and SDG14 showed consistent activity against most of the Gram positive organisms tested even after repeated sub-culturing. These were selected for further studies.

The loss of antibacterial activity during sub-culturing was previously reported in plasmid-associated bacteriocin production by *Lactobacillus* LMG 21688 (Kouakou *et al*., 2010).

Table 3.3 Activities of the antibacterial isolates from fish gut after sub-culturing

Isolate	Test Organism*	Activity		
		1st Subculture	2nd Subculture	3rd Subculture
GUT13	*Escherichia coli*	-	-	-
	Staphylococcus aureus	-	-	-
	Salmonella Typhimurium	+	-	-
	Bacillus circulans	+	+	-
GUT22	*Escherichia coli*	-	-	-
	Staphylococcus aureus	-	-	-
	Salmonella Typhimurium	+	-	-
	Bacillus circulans	-	-	-
SDG14	*Escherichia coli*	+	+	+
	Staphylococcus aureus	+	+	+
	Salmonella Typhimurium	+	+	+
	Bacillus circulans	+	+	+
BTSS3	*Escherichia coli*	-	-	-
	Staphylococcus aureus	+	+	+
	Salmonella Typhimurium	+	+	+
	Bacillus circulans	+	+	+
BTSS2	*Escherichia coli*	-	-	-
	Staphylococcus aureus	+	+	-
	Salmonella Typhimurium	-	-	-
	Bacillus circulans	-	-	-

* Activity was checked only against *Escherichia coli, Staphylococcus aureus, Salmonella* Typhimurium *and Bacillus circulans* initially, + indicates growth inhibition, - indicates No activity

3.3.3 Secondary screening: Quantitative estimation of antibacterial titres by critical dilution assay

The secondary screening included only two isolates BTSS3 and SDG14 and involved quantification of the antimicrobial activity. It was observed that the cell free supernatant had antimicrobial activity and could be quantified by critical dilution assay. The zone of inhibition can be visualized in Fig 3.4.

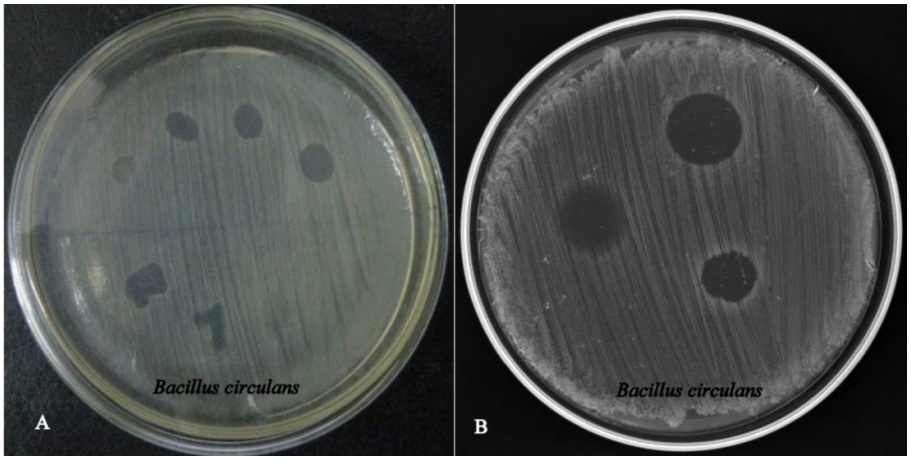


Fig 3.4 Antibacterial assay by spot on lawn method. The cell free supernatant (cfs) was spotted on Muller Hinton agar plate previously swab inoculated with the test organism, *B. circulans*. A) Zone of inhibition formed by different dilutions of BTSS3 supernatant, B) Zone of inhibition formed by different dilutions of SDG14 supernatant.

Marine environment represents a largely unexploited source for isolation of new microorganisms with the ability to produce bioactive compounds. The host organism generates these compounds as primary or secondary metabolites, as a defence mechanism and to maintain homeostasis in their environment. Marine bacteria represent approximately 10 % of the living carbon biomass in the biosphere (Parkes *et al*., 1994). However, only a small percentage among them has been studied for their bioactive potential.

3.3.4 Confirming protein nature of bacteriocin

Cell free supernatants of strain BTSS3 and SDG14 showed anti-bacterial activity after ammonium sulphate precipitation. For BTSS3, the activity was obtained in the 0-30 % fraction while that for SDG14 was in the 0-80 % fraction of ammonium sulphate precipitation. The use of ammonium sulphate precipitation to separate and purify, suggested that the active compound was a protein. Addition of ammonium sulphate alters the ionic strength of the solution, affecting the solubility of proteins, causing them to aggregate and precipitate. The concentration of ammonium sulphate depends on the ionic strength of the proteins precipitated.

The effect of action of proteolytic enzymes on bacteriocin activity is depicted in Table 3.4. Bacteriocins being proteinaceous are sensitive to one or more proteolytic enzymes. BTSS3 supernatant lost its antimicrobial activity completely on treatment with 5 mg/mL of Trypsin, and Proteinase K, while there was 50 % reduction in activity on treatment with Pepsin (pH-2). The action of these enzymes reduced the antimicrobial activity present in SDG14 supernatant to 50 %.

The action of proteolytic enzymes on the antibacterial compounds produced by BTSS3 and SDG14 strongly pointed to the presence of peptide substances, suggesting that the compound may be bacteriocins. The sensitivity to proteases is an important criterion in the characterization of bacteriocins. The well recognized bacteriocin nisin of *L. lactis* subsp. *lactis* ATCC11454 was either fully or partially inactivated by proteases like pronase E, pepsin, proteinase K and α-chymotrypsin (Moreno *et al*., 2000).

Table 3.4 Action of proteases on antibacterial compound from BTSS3 and SDG14

Enzyme	Activity (AU/mL)	
	BTSS3	SDG14
Trypsin	0	200
Pepsin	200	200
Proteinase K	0	200
Untreated Control	400	400

Strain BTSS3 showed antibacterial activity towards all Gram positive organisms and some Gram negative organisms, while the antibacterial spectrum of SDG14 was somewhat broad, showing activity towards both Gram positive and Gram negative organisms including *Escherichia coli* and *Pseudomonas aeruginosa* (Table 3.5).

Struggle for existence among microbes for space and nutrient is a powerful selection pressure that endows marine microorganisms, especially those in the gut, with the ability to produce natural products possessing industrial and medicinal values. According to Sugita *et al*. (1997), 11.2 % of the bacteria isolated from fish intestine could inhibit fish pathogens. Austin *et al*. (1995) too observed a similar phenomenon when *Vibrio alginolyticus* used as a probiotic strain, reduced diseases caused by *A. salmonicida*, *V. anguillarum* and *Vibrio ordalii*. Smith and Davey (1993) reported that *Pseudomonas fluorescence* isolated from fish, diminished diseases caused by *Aeromonas salmonicida*.

Table 3.5 Antibacterial activities of cell free supernatants of BTSS3 and SDG14

Test organisms	NCIM No.	BTSS3*		SDG14	
		Activity (AU/mL)	Sp. Activity (AU/mg)	Activity (AU/mL)	Sp. Activity (AU/mg)
Pseudomonas aeruginosa	2863	---	---	400	164.39±14.7
Salmonella Typhimurium	2501	400	103.19 ± 17.05	200	82.2±7.35
Escherichia coli	2343	---	----	400	164.39±14.7
Proteus vulgaris	2027	400	103.19 ± 17.05	400	164.39±14.7
Clostridium perfringens	2677	800	206.38 ± 34.1	----	----
Staphylococcus aureus	2127	800	206.38 ± 34.1	200	82.2±7.35
Bacillus cereus	2155	400	103.19 ± 17.05	800	328.79±29.4
Bacillus circulans	2107	800	206.38 ± 34.1	1600	657.58±58.81
Bacillus macerans	2131	400	103.19 ± 17.05	400	164.39±14.7
Bacillus pumilus	2189	200	51.5959 ± 8.53	----	----

* Published data: ***Bindiya ES***, *Tina KJ, Raghul SS, Bhat SG. Characterization of deep sea fish gut bacteria with antagonistic potential, from Centroscyllium fabricii (Deep sea shark). Probiotics Antimicrob Proteins 2015; 7:157–163.*

3.3.5 Bacterial characterization and identification

Phenotypic characteristics: Strains BTSS3 and SDG14 were Gram-stain positive, spore-forming, rod-shaped motile bacteria, which could grow at varying concentrations (0-5 %) of NaCl and 8 – 42 °C, (optimum range 30 - 42 °C). Strains BTSS3 and SDG14 could also tolerate range of pH from pH 4 - 9. The detailed biochemical characteristics are mounted in Table 3.6.

Table 3.6 Biochemical characteristics of Bacteriocin producers

Biochemical Characteristics of the organism	BTSS3*	SDG14
Gram Staining	+	+
Shape	Short rod	rod
Spore formation	+	+
Indole Production	-	-
Methyl Red	-	-
Voges Proskauer	-	-
Citrate Utilization	-	-
Triple Sugar Iron test	Alkaline	Alkaline
MoF test	Fermentative	Non fermentative
Catalase	+	+
Urea Utilization	+	-
Starch Utilization	+	-
Casein hydrolysis	+	-
Lipid hydrolysis	+	+

* Published data: ***Bindiya ES****, Tina KJ, Raghul SS, Bhat SG. Characterization of deep sea fish gut bacteria with antagonistic potential, from Centroscyllium fabricii (Deep sea shark). Probiotics Antimicrob Proteins 2015; 7:157–163.*

Strain BTSS3 was urease positive, produced amylase, protease and lipase, while SDG14 did not produce the other enzymes tested for except lipase.

Antibiotic sensitivity: The antibiotic susceptibility test showed that the organism BTSS3 was sensitive to all the antibiotics tested, except bacitracin, while strain SDG14 was resistant only to Aztreonam (AT). The result is shown in Table 3.7. The sensitivity of these microorganisms to antibiotics implied the absence of resistance genes and hence the safety in their applications as probiotic. These organisms may not harbour any of the tested antibiotic resistance gene to many of the commonly used broad spectrum antibiotics, thereby not participating in the dissemination of antibiotic resistance in a niche by horizontal gene transfer.

Table 3.7 Antibiogram of BTSS3 and SDG14

Antibiotic	Concentration	BTSS3*	SDG14
	Combi 69		
Ciprofloxacin (CIP)	5µg	S	S
Ofloxacin (OF)	5µg	S	S
Sparfloxacin (SPX)	5µg	S	S
Gatifloxacin (GAT)	5µg	S	S
Aztreonam (AT)	30µg	S	R
Azithromycin (AZM)	15µg	S	S
Vancomycin (VA)	30µg	S	S
Doxycycline Hydrochloride (DO)	30µg	S	S
	G VIII Plus		
Bacitracin (B)	10Unit	R	S
Chloramphenicol (C)	30µg	S	S
Co-Trimoxazole (COT)	25µg	S	S
Penicillin-G (P)	10Unit	S	S
Polymyxin-B (PB)	300Unit	S	S
Gentamicin (GEN)	10µg	S	S
Neomycin (N)	30µg	S	S
Tetracycline (TE)	30µg	S	S

**R stands for resistant and S stands for Sensitive

Genotypic Identification: The amplified product of 1.5 kb of the 16S rRNA gene of strain BTSS3 was obtained and represented in Fig 3.5A. Comparative 16S rRNA gene sequence analysis indicated that strain BTSS3 isolated from *Centroscyllium fabricii* belonged to the Genus *Bacillus*, with high (98%) similarity to 16S rDNA sequences of *Bacillus amyloliquefaciens*. The sequence was submitted to Genbank and the accession number KF018921.1 was obtained. The phenotypic characterization also confirmed the identity of strain BTSS3 as *Bacillus amyloliquefaciens.*

The phylogenetic tree based on partial 16S rDNA sequences of strain BTSS3 and related *Bacillus* sp. is shown in Fig 3.5B. The optimal tree with the sum of branch length = 5.61153123 is shown. The analysis involved 6 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 894 positions in the final dataset. It showed that the organism was *Bacillus amyloliquefaciens*.

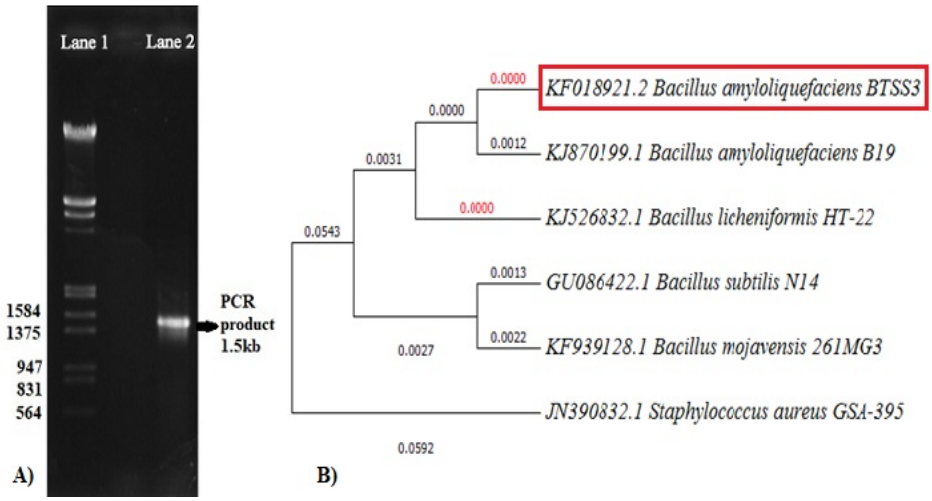


Fig 3.5 A) Agarose gel electrophoresis of PCR product of 16S rRNA gene. Lane 1- 1000bp ladder, Lane 2- PCR product of BTSS3 B) Phylogenetic analysis of strain BTSS3. The optimal tree with the sum of branch length = 5.61153123 is shown. The organism shows similarity to *Bacillus amyloliquefaciens* B19 (Bindiya *et al.,* 2015).

The amplified product of 1.5 kb of the 16S rRNA gene of SDG14was obtained and represented (Fig 3.6A). Comparative 16S rDNA gene sequence analysis indicated that strain SDG14 isolated from *Sardinella longiceps* also belonged to the genus *Bacillus*, with high (100 %) similarity to 16S rDNA sequences of *Bacillus pumilus*. The sequence was submitted to Genbank with KP878546. The phenotypic characterization also confirmed strain SDG14 as *Bacillus pumilus.* The phylogeny based on partial 16S rDNA sequences of strain SDG14 and related *Bacillus* sp. is shown in Fig 3.6B. The evolutionary history was inferred using the Neighbor-Joining method (Siatou and Nei, 1987). The optimal tree with the sum of branch length = 0.14811186 is shown. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2007) and are in the units of the number of base substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 456 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

Both the cultures were deposited in Microbial Culture Collection, National Centre for Cell Science, Pune (MCC, NCCS, Pune) to have a public access of the bacteria. *Bacillus amyloliquefaciens* BTSS3 was allocated the accession number MCC 2981 and *Bacillus pumilus* SDG14 was allocated the strain number MCC 3070. The bacteriocin produced by *Bacillus amyloliquefaciens* BTSS3 was designated as BaCf3, while that produced by *Bacillus pumilus* SDG14 as BpSl14 and will be referred to as such in the text.

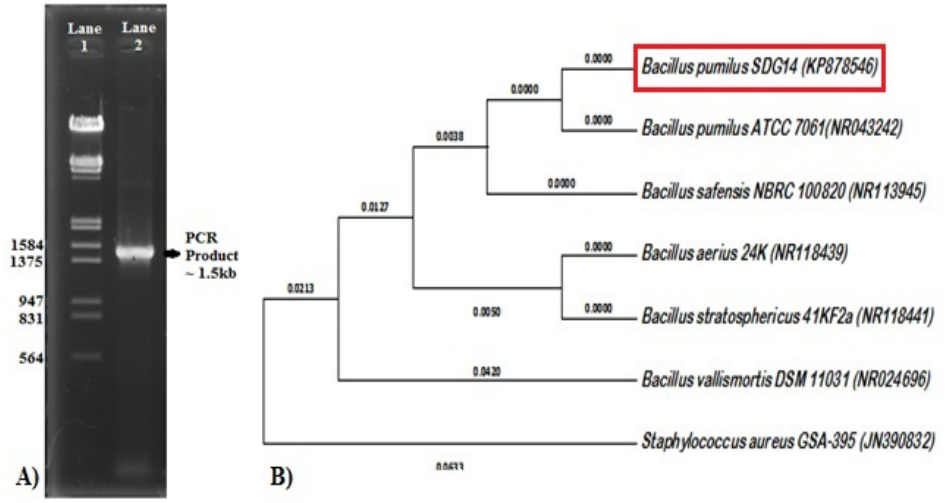


Fig 3.6 A) Agarose gel electrophoresis of PCR product of 16S rRNA gene. Lane 1- Lambda DNA HindIII/EcoR1 digest ladder, Lane 2- PCR product of SDG14. B) Phylogenetic analysis of SDG14. The optimal tree with the sum of branch length = 0.35712151 is shown. The organism showed similarity to *Bacillus pumilus* ATCC7061

Conventionally, bacilli have been identified in the laboratory through biochemical tests (Vaerewijck *et al*., 2001). This involves tedious laboratory work and sometimes misleading interpretations. 16S rDNA sequence analysis had been a widely accepted method for identifying unknown bacteria (Bosshard *et al*., 2003). The data generated by 16S rDNA sequencing is highly reliable and reproducible. *Bacillus* species are phenotypically and genotypically heterogeneous; they exhibit diverse physiological properties such as the ability to degrade many different substrates derived from plant and animal sources (Lutz *et al*., 2006). 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 many more (Priest, 1993; Slepecky and Hemphill, 2006). This diversity in physiological properties and diversity of *Bacillus* strains allow them to colonize a wide variety of ecological habitats.

Bacillus species which are omnipresent in both terrestrial and marine environments (Siefert *et al*., 2000; Oguntoyinbo 2007; Ki *et al*., 2009; Ivanova *et al*., 2010) are widely employed in industrial and agricultural production because of their ability to synthesize various enzymes (Reiss *et al*., 2011), antimicrobial compounds (Mukherjee *et al*., 2009) and many other molecules. Many genomic studies also reveal the ability of *Bacillus* sp. to produce antibiotics. Almost all the species of *Bacilli* are known to produce bacteriocins or other inhibitory substances. *Bacillus subtilis* strains are known to produce a wide variety of antibacterial and antifungal compounds (Stein, 2005). *Bacillus subtilis* ATCC 6633 is a producer of several lantibiotics (Bierbaum *et al*., 1995; Paik *et al*., 1998), rhizocticin (Kugler *et al*., 1990) lipopeptides, surfactin and mycosubtilin (Duitman *et al*., 1999, 2007). Strains of *B. thuringiensis* also produce an array of antibacterial proteins and peptides known as thuricins, tochicins or entomocins etc. (Paik *et al*., 1997). *Bacillus amyloliquefaciens* strain RC-2 produces a BLIS active against *Colletotrichum dematium*, a mulberry anthracnose fungus and several other phytopathogenic fungi (Yoshida *et al*., 2001).

3.3.6 Growth curve of the two bacteriocin producing microorganisms

The growth curve of *Bacillus amyloliquefaciens* BTSS3 is depicted in Fig 3.7. It was observed that exponential growth started after two hours of incubation and the generation time (G) was calculated to be 46.73 minute, with a specific growth rate (μ) of 32.38 per min.

From the growth curve of *Bacillus pumilus* SDG14 (Fig 3.7), it is inferred that the logarithmic phase started after two hours; the generation time (G) was 53.58 minute at a specific growth rate (μ) of 37.13 per min.

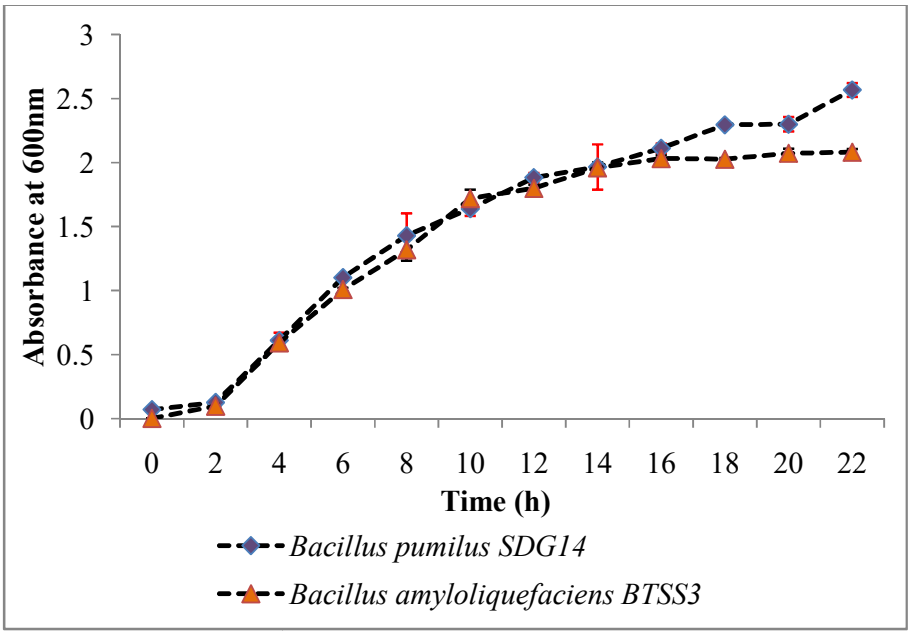


Fig 3.7 Growth curves of the bacteriocin producers. --▲-- shows growth curve of *Bacillus amyloliquefaciens* BTSS3 and --◆-- shows growth curve of *Bacillus pumilus* SDG14

The specific growth rate depends on the growth media supplied. The specific growth rate of *B. subtilis* grown in M9 medium containing glucose 0.1 % was calculated as 0.481 per h. The Generation time was calculated to be 1.44 h. The specific growth rate and generation time of *B. subtilis* in M9 minimal medium containing 0.1 % of cellulose was 0.046 (per h) and 15.07 h respectively (Mageshwaran *et al*., 2014). *B. subtilis* has a doubling time of 26 minutes under optimal conditions, where as 45 minutes is the normal generation time. In this study we can see that the generation time of *B. amyloliquefaciens* BTSS3 is 46.73 minutes and that of *B. pumilus* SDG14 is 53.58 minutes which are very close to that for *B. subtilis*.

3.4 Summary

Fish gut is a microenvironment facilitating the growth of a blend of microbes both pathogenic and probiotic. Deep sea fishes are the most unexplored in the search for bacteriocins and other biomolecules. The

environments in which they endure are also least exploited due to difficulties of access. This screening has pointed the presence of bacteria with antimicrobial activity in the deep sea fish gut. Two strains were selected for further study, wherein strain BTSS3 had antagonistic activity against all the Gram positive bacteria tested, while SDG14 showed activity even against Gram negative bacteria. The reduction/inhibition of the antibacterial activity of the ammonium sulphate fractions implied the presence of bacteriocins in the culture supernatants. The bacteriocin producers from *Centroscillium fabricii* (deep sea shark) was identified as *Bacillus amyloliquefaciens* BTSS3 and that from *Sardinella longiceps* (Indian oil sardine) as *Bacillus pumilus* SDG14.

From the observations made in this chapter, we can conclude that marine fish gut act as a repository of antagonistic organisms waiting to be discovered, with myriad future applications. These studies suggest a role for the antibiotic-producing bacteria in effective control of microflora in the fish intestine. Moreover, these antibacterial substances may be supplied into aquaculture waters or the organism itself included as a probiotic, expanding the scope of its application.

Chapter 4

Optimization of process conditions for bacteriocin production by one-factor-at-a-time (OFAT) method

4.1 Introduction

Medium formulation is an essential stage in the design of successful laboratory pilot-scale development and manufacturing process. Medium constituents must satisfy all the elemental requirements for cell biomass as well as metabolite production, besides ensuring an adequate supply of energy for biosynthesis and cell maintenance. For an aerobic fermentation process:

carbon and energy source + nitrogen source + O_2 + other requirements

↓

biomass + products + CO_2 + H_2O + heat

Medium optimization is an integral part of biopharmaceutical process development which involves the addition of different supplements to an existing basal medium. Detailed investigation is needed to establish the most suitable medium for an individual fermentation process. Every microorganism requires water, sources of energy, carbon, nitrogen, mineral elements, possibly vitamins as well as oxygen if aerobic, for growth. On a small scale it is relatively simple to devise a medium containing pure compounds, but the same may be unsuitable for use in large scale processes (Stanbury, 2013). But certain microorganisms are unable to synthesize specific nutrients like amino acids, vitamins or nucleotides. Once identified these nutrients can then be incorporated into the medium in adequate amounts as a pure compound or as a component of a complex mixture to aid growth or production processes.

Medium optimization processes must meet the following eight criteria as much as possible:

i. Produce maximum yield of product or biomass per gram of substrate used.
ii. Produce the maximum concentration of product or biomass.
iii. Permit the maximum rate of product formation.
iv. Give the minimum yield of undesired products.
v. Should have consistent quality
vi. Should be readily available throughout the year.
vii. Should cause minimal problems during media preparation and sterilization
viii. Should cause minimal problems in other aspects of the production process particularly in - aeration and agitation, extraction, purification and waste treatment.

The meaning of optimization needs careful consideration. When only the biomass increase is considered, it should be noted that efficiently grown biomass produced by an optimized high productivity growth phase is not necessarily best suited for synthesizing the desired product. Different combinations and sequences of process conditions need to be investigated to determine growth conditions which produce the biomass with the physiological state best suitable for product formation.

Media optimization include techniques such as borrowing, component swapping, biological mimicry, one-factor-at-a-time, factorial design, Plackett and Burman design, central composite design, response surface methodology, evolutionary operation, evolutionary operation factorial design, artificial neural network, fuzzy logic and genetic algorithms.

One-factor-at-a-time is a close-ended system for fermentation process optimization. This method can be applied for optimization of medium components as well as for process condition since it is based on the classical method of changing one independent variable while fixing all others at a definite level. This strategy has the advantage of simplicity, ease as well as the

advantage of visualizing the individual effects of medium components and process conditions using graphs without the aid of high end statistical analysis. The limitations of this method are interactions between the components are ignored; extremely time consuming, expensive for large number of variables. Irrespective of all these disadvantages one-factor-at-a-time method is the most popular method for improving fermentation medium and process conditions.

Each optimization technique has its own advantages and disadvantages. There is an on-going debate within the industry as to the various advantages and disadvantages of both defined and undefined media as well as media components. The choice of the type of system to employ is often motivated by reducing adverse effect with respect to consistency of performance, against the highest possible product titers for any given system. The field application of various biomolecules requires their production in large quantities which necessitates optimization.

In this chapter the effect of various process parameters like media, incubation time, pH, inoculum concentration, nitrogen sources, carbon sources, NaCl concentration, Tween 80 %, additional inorganic nitrogen source and Mg^{2+} ion concentration on production of two bacteriocins by their respective producer strains were studied. Activity was checked at each step, protein concentrations were estimated according to Lowry *et al*. (1951) to calculate specific activity (AU/mg). Thus the optimal media was selected based on the activity and specific activity of the bacteriocins. The optimized medium for production and other parameters were added in each subsequent optimization steps. Finally, the time course experiment was conducted to obtain yield.

4.2 Materials and Methods

4.2.1 Bacterial strains and Medium

The bacteriocin BaCf3 producing strain, *Bacillus amyloliquefaciens* BTSS3, and BpSl14 producing *Bacillus pumilus* SDG14, were used for optimizing production conditions. *Bacillus circulans* (NCIM No. 2107) was used as the indicator strain in all experiments to test the antimicrobial activity of the bacteriocin. The stock cultures were maintained at −70 °C in nutrient broth (Himedia, Mumbai, India) containing 30 % (v/v) glycerol. Modified minimal medium -M9G (Miller, 1992) was used unless otherwise specified, whose constituents include (per litre) 0.6 g Na_2HPO_4, 0.3 g KH_2PO_4, 2 g NaCl, 0.5 g Tryptone, 1 g glucose, 4 mM $MgSO_4$ and 200 µM $CaCl_2$. All the media components were purchased from Himedia (India) and/or Merck (Germany).

4.2.2 Inoculum preparation

A single colony of each producer strain from Zobell agar (Himedia) was inoculated into 20 mL of Zobell broth and incubated at 30 °C at 150 rpm in orbital shaker (Orbitek®, India), cells collected by centrifugation (7000 X g, 10 min) (Hermle, Germany) when culture OD_{600} = 1, CFU/mL = 3 x 10^8 (Schimadzu, Japan), pellet was washed twice with sterile physiological saline (pH 7.4) and resuspended in physiological saline.

4.2.3 Quantitative estimation of antibacterial titre by critical dilution assay

Bacteriocin activity was determined by spot-on-agar method (van Reenen *et al.*, 1998). The activity and specific activity was quantified as described in section 3.2.5. The reciprocal of the highest dilution was multiplied by 200 (1 mL/5 µL) to obtain activity units per mL (AU/mL). Colony forming units per millilitre (CFU/mL) was determined using standard

methods for every medium. These estimations were conducted for optimization of each process parameter.

4.2.4 Effect of culture media on bacteriocin production

Five different culture media, Tryptone Soya broth (TSB), Nutrient broth (NB), Luria Bertani (LB) broth, Zobell Marine broth and M9G (Mineral salt) medium (Himedia) supplemented with 1 % glucose and 0.5 % tryptone were compared for bacteriocin production by the two producer organisms, *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14. 100 mL of each medium taken in 250 mL Erlenmeyer flasks was inoculated with 1.0 % inoculum prepared as described earlier. The initial pH of all media was adjusted to 7. The flasks were incubated with agitation of 150 rpm at 30 °C for 24 h (Orbitek®, India). The medium effecting optimum production of bacteriocin was then used for further experiments.

4.2.5 Effect of incubation time on bacteriocin production

Minimal medium supplemented with glucose and tryptone was inoculated with 1 % inoculum in individual flasks, incubated at 30 °C and 150 rpm for 36 h in an environmental shaker. Samples were drawn regularly at 6 h intervals and optimum incubation time for bacteriocin production was determined.

4.2.6 Optimization of inoculum concentration for bacteriocin production

To optimize the inoculum concentration of *B. amyloliquefaciens* BTSS3 and *B. pumilus* SDG14 on bacteriocin production, the inocula were prepared and added to the optimized medium at varying concentrations ranging from 1 % to 10 % (v/v) of the culture medium. The broth was incubated at the above optimized conditions and checked for activity as described in section 4.2.3.

4.2.7 Effect of media pH on production of bacteriocins

The initial pH of the production medium was adjusted to pH 2 to 9 with 1 M HCl or 0.1 M NaOH. The flasks were then inoculated with 1 % (w/v) inoculum and incubated at 30 °C for optimized incubation period, with agitation of 150 rpm. The culture free supernatants were assayed for activity (as described in section 4.2.3) to determine optimum pH for bacteriocin production.

4.2.8 Optimization of nitrogen sources for bacteriocin production

The influence of different organic nitrogen sources on bacteriocin production was determined by adding ammonium chloride, beef extract, yeast extract, tryptone, and peptone (Himedia) each to the basal medium at 0.5 % (w/v) and incubating at 30 °C. The medium, pH and incubation period are as optimized previously. Glucose was the carbon source in the basal medium. Medium without nitrogen source was the control.

4.2.9 Optimization of carbon sources for bacteriocin production

The effect of different carbon sources on bacteriocin production by *B. amyloliquefaciens* BTSS3 and *B. pumilus* SDG14 was examined. 5 % solutions of glucose, fructose, maltose, lactose, starch, sorbitol, inositol were individually sterilized at 10 lbs for 10 minutes and added at 1 % (w/v) concentration to basal M9 broth without an added carbon source. All other conditions were as previously optimized. M9 medium without carbon source served as control.

4.2.10 Effect of temperature on bacteriocin production

The effect of different incubation temperatures (25, 30, 35, 40 and 45 °C) on bacterial growth and bacteriocin production was studied with agitation of 150 rpm. All other conditions were as previously optimized.

4.2.11 Effect of NaCl concentration

The mineral content of the water is very important and critical in fermentation. In many media, magnesium, phosphorous, potassium, sulphur, calcium and chlorine are essential components and must be added as distinct components. Others such as cobalt, copper, iron, manganese, molybdenum and zinc are also essential but are usually present as impurities in other major ingredients.

To study the effect of sodium chloride, different concentrations (10, 20, 30, 40, 50 mg/mL) were added to the media, inoculated and kept for production in optimized conditions. Medium without NaCl acted as control.

4.2.12 Effect of additional inorganic nitrogen sources

The effect of additional inorganic nitrogen sources like ammonium chloride (NH_4Cl), potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3) were also studied by adding in combination with tryptone in 1:1 proportion. Medium without inorganic nitrogen served as control.

4.2.13 Effect of Tween 80 and Mg^{2+} ion concentration

Different concentrations (0.02, 0.04, 0.06, 0.08 and 0.1 %) of Tween 80 were added to previously optimized medium to investigate the effect of Tween 80, while varying concentrations (200, 300, 400 and 500 mM) of magnesium sulphate were added to the medium to study its effect on bacteriocin production. Medium without Tween 80 and without $MgSO_4$ acted as controls respectively.

4.2.14 Time course study

To study the effect of optimized medium and production parameters on the production time of bacteriocins, the organisms were inoculated in respective optimized medium and incubated at the optimized conditions.

Samples were withdrawn every 2 h. The activity and biomass of each sample was noted. A graph was plotted with time in hour on X-axis and activity on Y-axis.

4.2.15 Statistical analysis

Bacteriocin production (AU/mL) and specific activity (AU/mg) were determined for all the samples under different experimental conditions, conducted in triplicate. All the specific activity calculations and standard deviations were done in Microsoft Excel 2010 and the graphs were plotted by GraphPad Prism 6.0.

4.3 Results and discussion

Bacteriocin BaCf3 production by *Bacillus amyloliquefaciens* BTSS3 and BpSl14 production by *Bacillus pumilus* SDG14, were optimized by one-factor-at-a-time. The optimization of bacteriocin production by modification of growth conditions can greatly benefit efficient commercial application.

4.3.1 Effect of culture media on bacteriocin production

When different culture media were examined for BaCf3 production by *B. amyloliquefaciens* BTSS3, Zobell marine broth and mineral salt medium M9G supplemented with glucose and tryptone gave maximum activity of 800 AU/mL, however but a higher specific activity of 125.658±0.939 AU/mg was obtained in M9G All media tested however showed bacteriocin production to a lesser extent. Thus M9G mineral salt medium was selected for further studies considering also the ease of purification and optimization (Fig 4.1a).

For the production of BpSl14, M9G medium gave maximum activity of 3200 AU/mL followed by Tryptone soya broth (TSB), Zobell marine broth and nutrient broth. In M9G media the specific activity was 1595.273±51.58 AU/mg (Fig 4.1b).

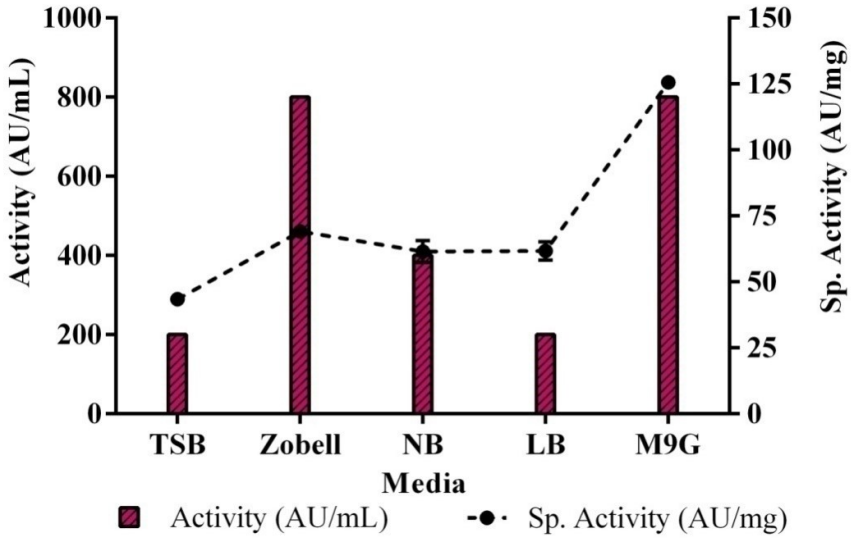


Fig 4.1a. Effect of different media on bacteriocin BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

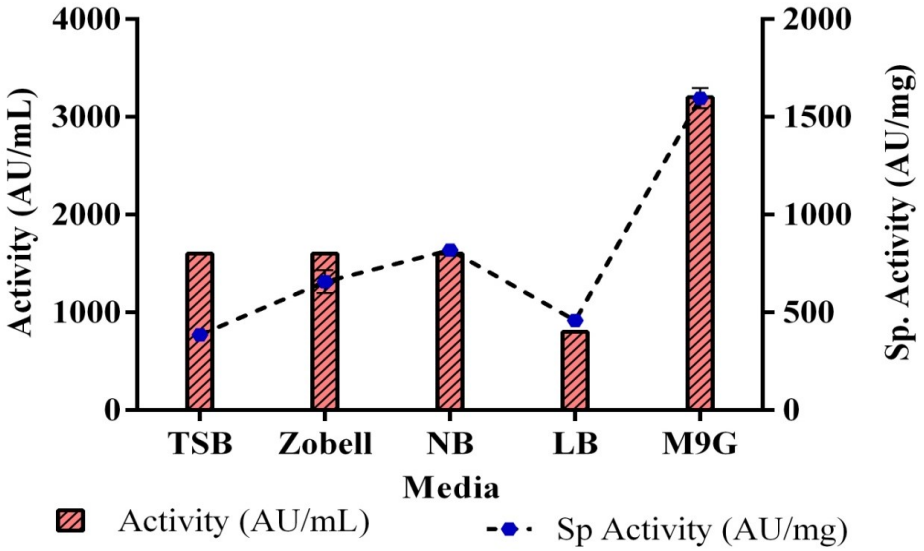


Fig 4.1b. Effect of different media on bacteriocin BpSl14 production by *Bacillus pumilus* SDG14

From literature we can understand that different types of media promote bacteriocin production and clear cut evidence in support of a particular media could not be chalked out. Thus the optimized media depends on the producer organism as well as the type of bacteriocin produced.

Bacteriocin production by *Bacillus subtilis* 14 was in Luria Bertani broth supplemented with glucose (Anthony *et al.,* 2009) where as cultivation in brain heart infusion broth promoted the production of cerein 8A by *Bacillus cereus* 8A (Bizani and Brandelli, 2004).

Since both organisms gave maximum activity and specific activity in M9G medium, this was selected as production medium for further studies. The added advantage of using M9G is that the medium composition can be amended for further optimization of production.

4.3.2 Effect of incubation time on bacteriocin production

BaCf3 production increased with time and maximum activity of 800 AU/mL was obtained for BaCf3 after 18 h of incubation (Fig 4.2a). A maximum specific activity of 119.581±10.401 AU/mg was observed at this time. After that the activity and specific activity decreased with further increase in incubation time. This decrease of bacteriocin activity was ascribed to proteolytic inactivation, protein aggregation, or adsorption of the bacteriocin molecules to the cell surface of the producer cells (Parente *et al.*, 1997).

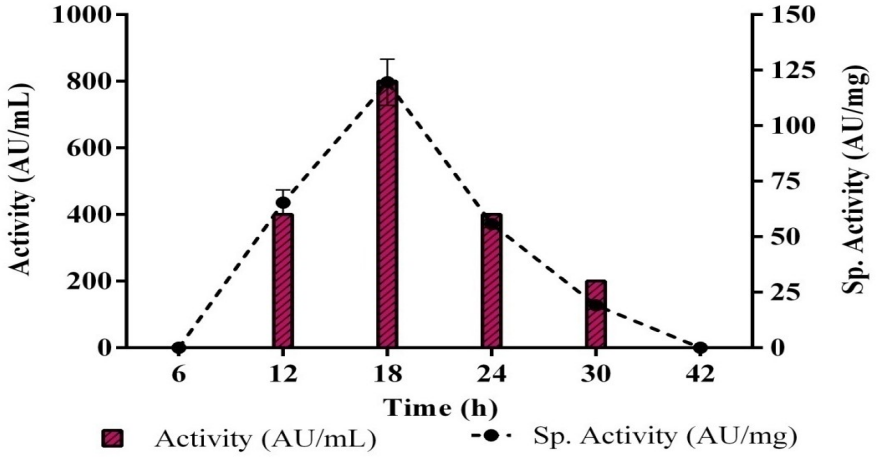


Fig 4.2a. Effect of incubation time on BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

For BpSl14 the activity increased with increase in time, reached a maximum of 6400 AU/mL after 24 h of incubation and then maintained a steady activity. When we consider the specific activity we can see that it was maximum after 24 h of incubation, 2562.16±93.13 AU/mg (Fig 4.2b).

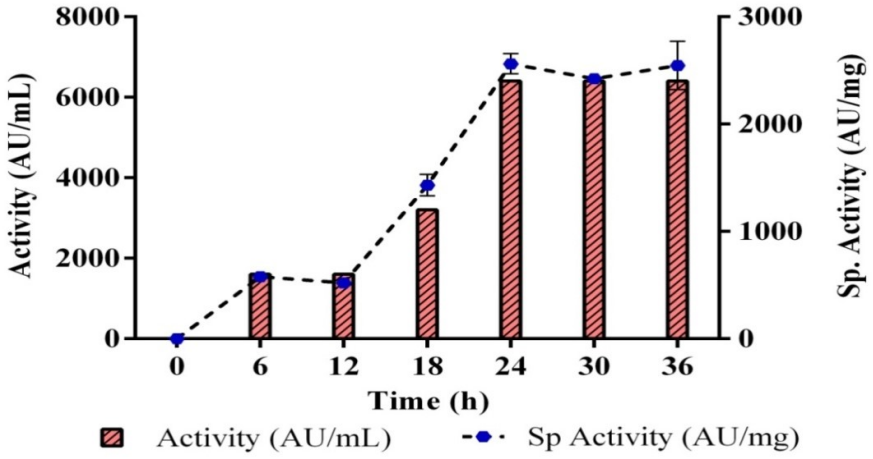


Fig 4.2b. Effect of incubation time on BpSl14 production by *Bacillus pumilus* SDG14

Production of pediocin AcH by *Pediococcus acidilactici* H started after 8h of incubation at 30 °C and 40 °C, and continued till 24 h of incubation (Biswas *et al*., 1991). Balasubramanyam and Varadaraj (1998) studied the effect of incubation period on bacteriocin production by an active culture of *Lact. delbruecki* ssp. *bulgaricus* CFR 2028. The presence of antibacterial activity was detected only after 24 h, which increased up to 48 h of incubation. Maximum production of lichenin by *B. licheniformis* 26L-10/3RA isolated from water buffalo rumen was after 72 h of incubation (Pattnaik *et al*., 2005). Bac-IB17 production by *B. subtilis* KIBGE IB-17 from soil samples maximized at 37 °C after 24 h in medium with initial pH 7.0 (Ansari *et al*., 2012). Bacteriocin activity of *Bacillus* sp. Sh10 was undetected during the exponential phase, but was detected at the end of this phase (18 h), reaching 1200 AU mL^{-1} during the mid-stationary phase (30 h), and declining at the end of this phase (42 h) (Shayesteh *et al*., 2014).

4.3.3 Optimization of inoculum concentration for bacteriocin production

The inoculum concentration also affected the production of bacteriocins. BaCf3 production was maximum of 800 AU/mL with 2, 6 and 7 % inoculum, but 2 % inoculum was considered as optimum with a specific activity of 144.473 AU/mg (Fig 4.3a).

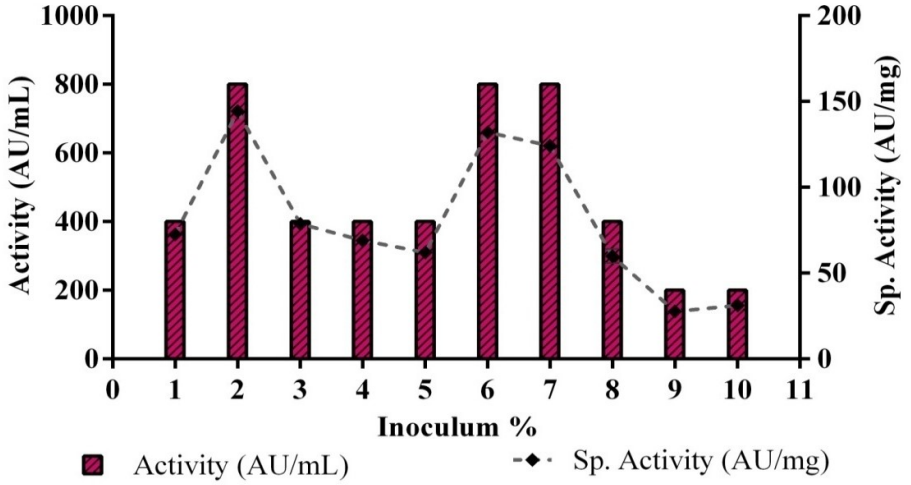


Fig 4.3a. Effect of inoculum concentration on BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

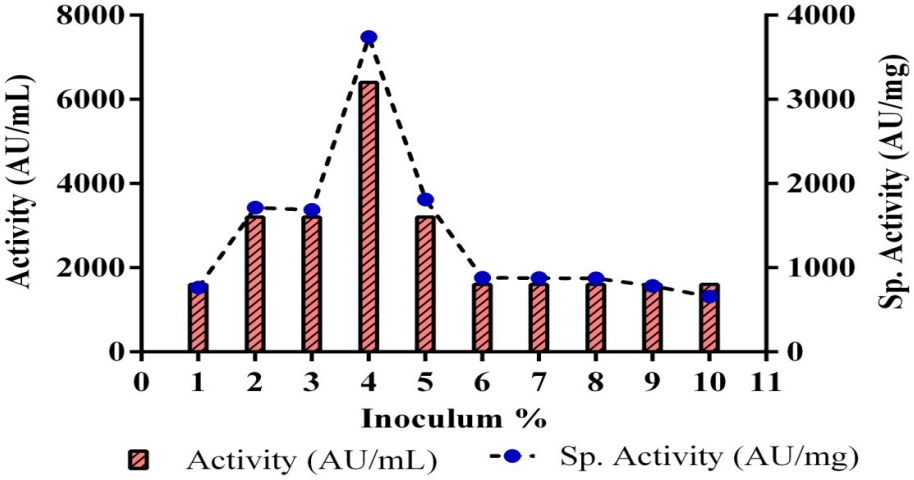


Fig 4.3b. Effect of inoculum concentration on BpSl14 production by *Bacillus pumilus* SDG14

BpSl14 production by *Bacillus pumilus* SDG14 was at maxima with 4% inoculum (Fig 4.5b). The activity obtained when inoculated with 4 % inoculum was 6400 AU/mL, with specific activity of 3742.27±18.935 AU/mg. 2 %, 3 % and 5 % of inoculum was also comparable with an activity of 3200 AU/mL.

4.3.4 Effect of media pH on bacteriocin production

The control of pH is extremely important if optimal productivity is to be achieved. Several media are buffered at about pH 7.0 and phosphates play a key role in buffering.

Although maximum activity of 800 AU/mL was observed for BaCf3 at pH 6, 7 and 8, maximum specific activity of 195.677±7.714 AU/mg was obtained at pH 6 with the specific activity decreasing along the increase in pH (Fig 4.4a). pH 8 was the optimal pH for BpSl14 production with an activity of 6400 AU/mL followed by pH 7 with an activity of 1600 AU/mL (Fig 4.4b). The specific activity was 3807.077±34.29 AU/mg at pH 8.

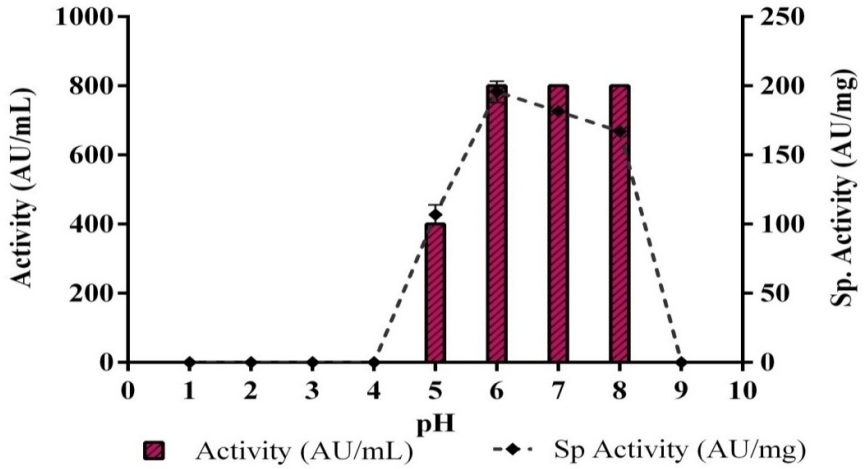


Fig 4.4a. Effect of media pH on BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

A pH dependent increase in bacteriocin production can be observed for both *B. amyloliquefaciens* BTSS3 and *B. pumilus* SDG14. The production

of both bacteriocins was good between pH 6 and 9. Similarly, cerein 8A production by *Bacillus cereus* 8A was achieved at pH between 6.5 and 9.0 (Dominguez *et al*., 2007). Motta and Brandelli (2008) too observed maximum antimicrobial activity production at initial pH values between 6.0 and 8.0. This also suggests an alkaline pH requirement for BaCf3 production.

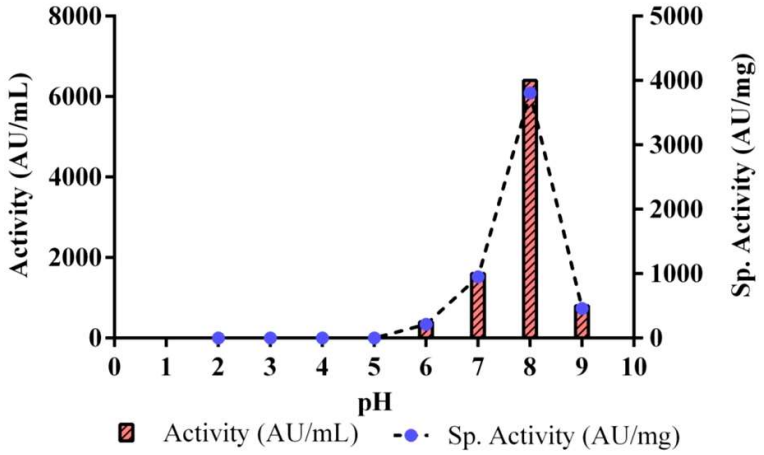


Fig 4.4b. Effect of media pH on BpSl14 production by *Bacillus pumilus* SDG14

4.3.5 Optimization of nitrogen sources for bacteriocin production

Antibiotic production by many microorganisms is influenced by the type and concentration of the nitrogen source in the culture medium. The use of complex nitrogen sources for antibiotic production is commonly recommended. They are helpful in creating the correct physiological state of the microorganism and prepare them for production. However the complex nitrogenous sources may not be utilized by certain microorganisms and create problems in downstream processing and effluent treatment. These factors must be borne in mind before selecting a nitrogen source for production.

Despite testing several organic nitrogen sources, tryptone proved to be the best source for bacteriocin production by *Bacillus amyloliquefacience* BTSS3, with activity and specific activity being 800 AU/mL and

279.67±2.115 AU/mg respectively. Tryptone was followed by peptone with an activity of 400 AU/mL and specific activity of 152.922±1.265 AU/mg. The addition of yeast extract and ammonium chloride did not promote bacteriocin production (Fig 4.5a). Tryptone was the best organic nitrogen source for bacteriocin production also for ST341LD production (Todorov and Dicks, 2006), as well as for plantaricin 423 (van Reenen *et al*., 1998).

Even though BpSl14 production was promoted by all the nitrogen sources tested, maximum production was observed with yeast extract with an activity of 6400 AU/mL (Fig 4.5b). All other nitrogen sources accounted a production of 3200 AU/mL. The specific activity while using yeast extract was also high, i.e., 2644.488±9.685 AU/mg. For commercially viable nisin production, pasteurized milk added with yeast extract is treated with a protease and used as a substrate in batch fermentation at controlled pH and temperature (Parente and Ricciardi, 1999). A De Vuyst and Vandamme (1992) comparison of nitrogen sources reported that nisin production was high (>2000 IU/mL) when using yeast extract and fish meal.

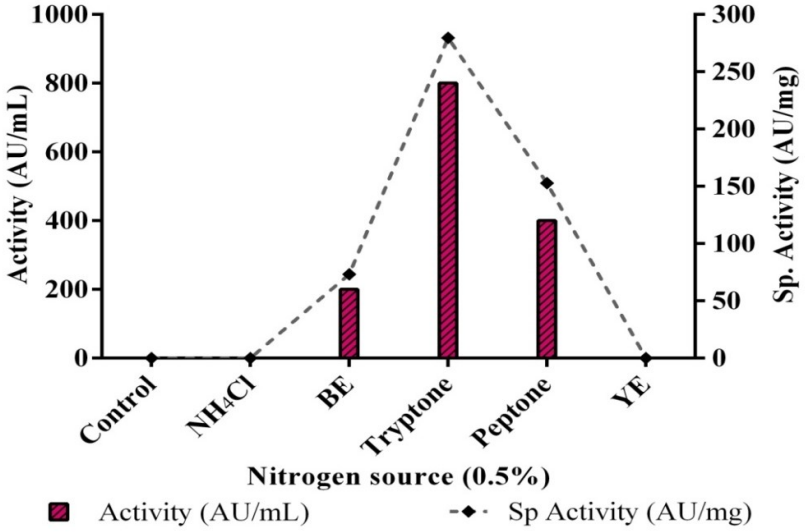


Fig 4.5a. Effect of nitrogen sources on BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

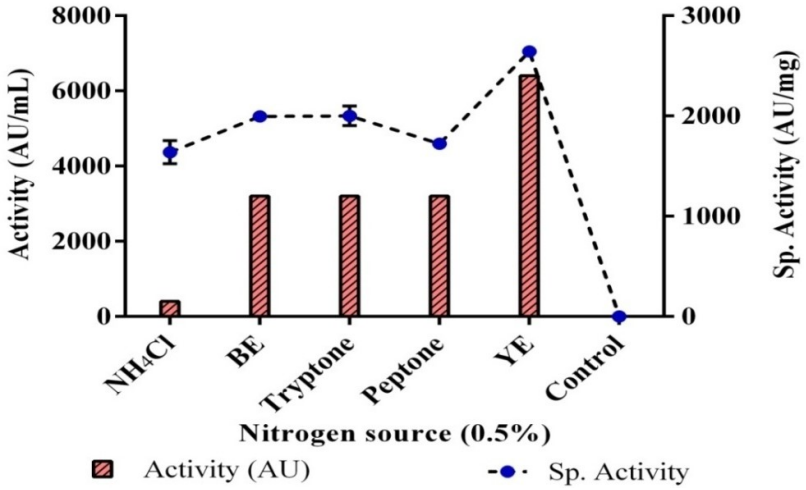


Fig 4.5b. Effect of nitrogen sources on BpSl14 production by *Bacillus pumilus* SDG14

4.3.6 Optimization of carbon sources for bacteriocin production

The main product of a fermentation process will often determine the choice of carbon source, hence an adequate supply of the carbon source is essential. The purity of the carbon source may also influence the choice of substrate. The method of media preparation, particularly sterilization may limit the suitability of carbohydrates for individual fermentation processes. The best choice is to sterilize sugars separately as they may react with ammonium ions and amino acids to form black nitrogen containing compounds which will partially inhibit the growth of many microorganisms (Wang and Hsiao, 1995).

The change in the carbon source increased the bacteriocin production by *Bacillus amyloliquefaciens* BTSS3. The addition of monosaccharides (glucose and fructose) and disaccharides (maltose and lactose) produced 800 AU/mL of bacteriocin with slight variations in specific activities, whereas the addition of polysaccharide like starch reduced the production. But there was a pronounced rise in BaCf3 production due to the addition of polyols like sorbitol and inositol as carbon sources (Fig 4.6a). The activities for sorbitol

and inositol were 800 AU/mL and 1600 AU/mL respectively with a high specific activity of 574.768±8.932 AU/mg and 639.193±30.817 AU/mg respectively.

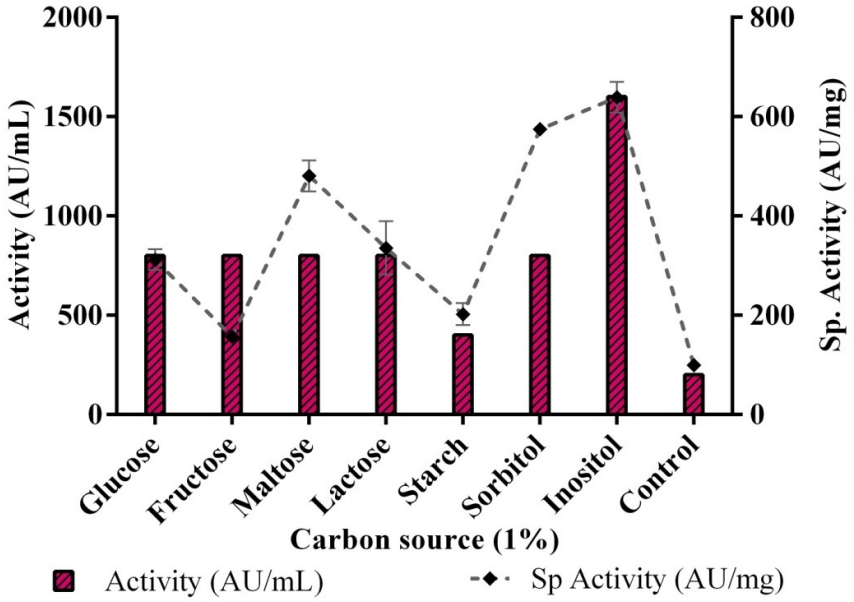


Fig 4.6a. Effect of carbon sources on BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

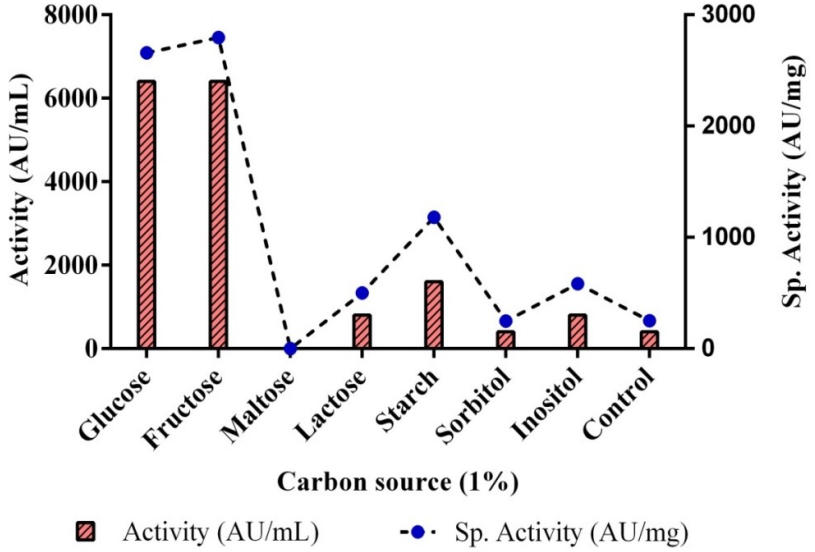


Fig 4.6b. Effect of carbon sources on BpSl14 production by *Bacillus pumilus* SDG14

Inositol, a pseudovitamin compound was the best carbon source for bacteriocin production by *Bacillus amyloliquefaciens* BTSS3. Inositol is present in cereals and vegetables as phytic acid, which is a combination of inositol and phosphorus, a major component of the phospholipid lecithin. Inositol phosphate utilizing bacteria are found in mammalian gut and are considered beneficial in mediating cross-kingdom dialog in cell signalling (Stentz *et al*., 2014). This is an indication in favour of the application of this bacterium as a probiotic.

In the case of BpSl14, the activity was high with monosaccharides like glucose and fructose (Activity = 6400 AU/mL), while the production reduced drastically with disaccharides, polysaccharides and polyols (Fig 4.6b). The specific activity was 2658.181±23.883 AU/mg for glucose and 2796.708±15.863 AU/mg for fructose as carbon sources in the fermentation medium. Owing to the high specific activity, fructose was selected as the best carbon source for production of BpSl14 by *Bacillus pumilus* SDG14.

For the production of bacteriocin by *Leuconostoc mesenteroides* E131, glucose and fructose were equally good as carbon sources as is the case of BpSl14 by *B. pumilus* SDG14 (Dorsinos *et al*., 2005). Fructose was the second best carbon source for bacteriocin production by *Pediococcus pentosaceus* KC692718 (Suganthi and Mohanasrinivasan, 2015). Barcena *et al*., (1998) investigated production of Class I bacteriocin, Plantaricin C by *Lactobacillus plantarum* LL441, in continuous culture with different carbon sources. Fructose and sucrose as carbon sources showed activity much higher than glucose. Supplementation of MRS medium with fructose doubled the bacteriocin production by enteric *Bifidobacterium* isolate B2 (Al-Wendawi and Al-Saady, 2012).

4.3.7 Effect of temperature on bacteriocin production

The incubation at 35 °C and 40 °C showed maximum activity of 1600 AU/mL for BaCf3 and specific activity of 887.87±89.08 AU/mg and

995.219±6.695 AU/mg respectively (Fig 4.7a). Hence 40 °C was selected as the optimum temperature for BaCf3 production by *Bacillus amyloliquefaciens* BTSS3.

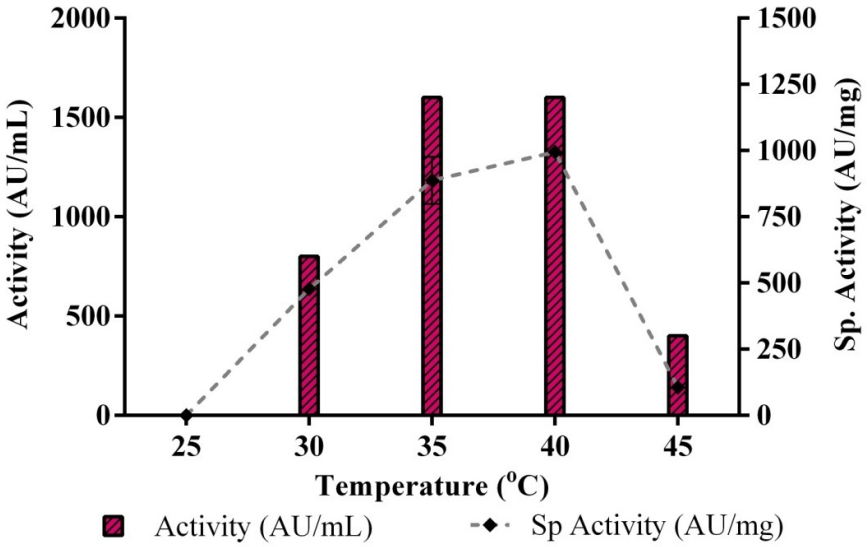


Fig 4.7a. Effect of temperature on BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

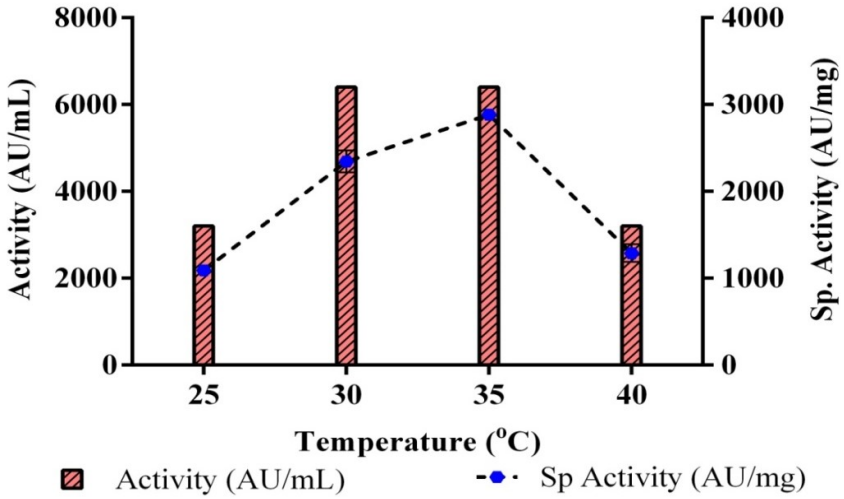


Fig 4.7b. Effect of temperature on BpSl14 production by *Bacillus pumilus* SDG14

Incubation at 30 °C and 35 °C showed maximum activity of 6400 AU/mL for BpSl14 and specific activity of 2345.958±126.317 AU/mg and

2883±2.810 AU/mg respectively (Fig 4.7b). Hence 35 °C was selected as the optimum temperature for BpSl14 production by *Bacillus pumilus* SDG14.

Growth at optimal temperature usually results in optimal bacteriocin production but sometimes temperature stress and growth at sub-optimal temperature may result in an increased yield of bacteriocins (Lejeune *et al*., 1998). Thus in both the cases we can see that the temperature stress may be a reason for increased production. Studies conducted on bacteriocins from some lactic acid bacteria, e.g. pediocin PD-1 (Nel *et al*., 2001), enterocin AS-48 (Abriouel *et al*., 2001), enterocin P (Herranz *et al*., 2001), sakP (Aasen *et al*., 2000), and bacteriocins, mesenterocin 52A and mesenterocin 52B, produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 (Krier *et al*., 1998) have shown that production is often regulated by growth temperature. Maximum production of lichenin by *Bacillus licheniformis* 26L-10/3RA was at 39 °C (Pattnaik *et al*., 2005). *Bacillus subtilis* KIBGE IB-17 can produce bacteriocin Bac-IB17 at 37 °C (Ansari *et al*., 2012). Bacteriocin production by *Bacillus* sp. SH10 from marine clams was maximum at 30 °C and the production continued up to 40 °C (Shayesteh *et al.,* 2014).

4.3.8 Effect of NaCl concentration

BaCf3 production increased with increasing NaCl concentration. The addition of 30, 40 and 50 mg/mL of NaCl showed a maximum bacteriocin production of 1600 AU/mL with specific activity 1198.8±40.78 AU/mg, 1128.33±5.164 AU/mg and 1033.747±20.22 AU/mg correspondingly (Fig 4.8a). In this study, 30 mg/mL NaCl was considered as optimum for production of BaCf3 since it was having maximum specific activity.

BpSl14 production decreased with increasing concentration of NaCl. The control without NaCl, addition of 10 mg/mL and 20 mg/mL NaCl showed same activity of 6400 AU/mL. The addition of 30, 40 and 50 mg/mL of NaCl reduced bacteriocin production (Fig 4.8b). Specific activity was higher

without the addition of NaCl (Specific activity = 3437.709±28.26 AU/mg) and hence we concluded that NaCl is not required in the optimized production medium for BpSl14 even though it is a marine organism. The bacteriocin production diminished although *Bacillus pumilus* SDG14 could grow at higher salt concentrations.

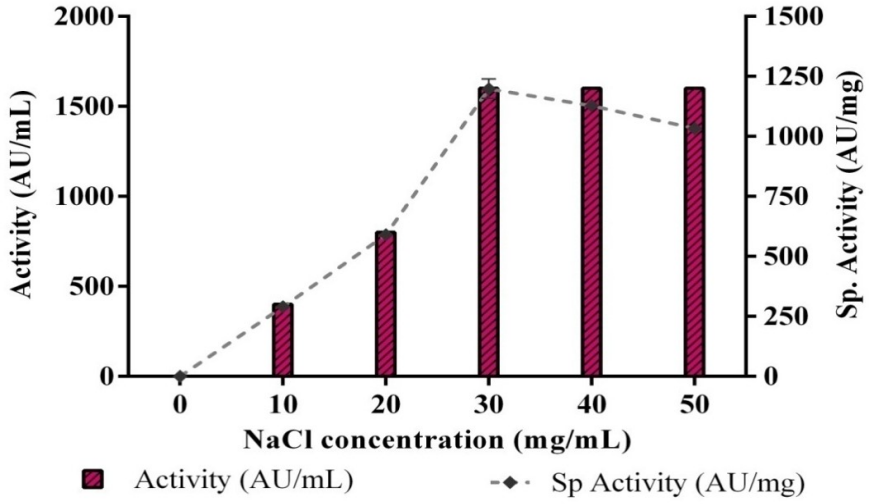


Fig 4.8a. Effect of NaCl on BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

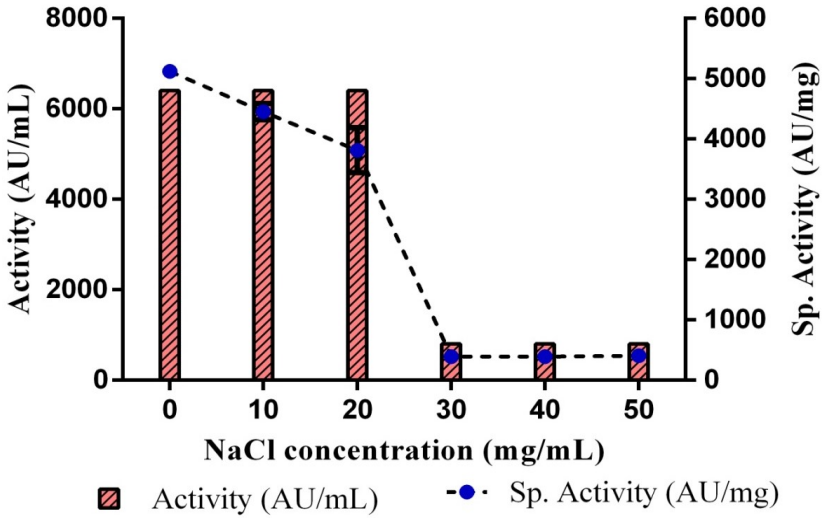


Fig 4.8b. Effect of NaCl on BpSl14 production by *Bacillus pumilus* SDG14

Positive effect of NaCl on production of bacteriocin BaCf3 is in congruent with an earlier study on *B. licheniformis* AnBa9 antibacterial peptide (Anthony *et al*., 2009). NaCl concentration has a positive effect in the release of extra cellular bacteriocin from bacteria (Verluyten *et al*., 2004; Delgado *et al*., 2005). Bacteriocin production by marine *Bacillus* sp. Sh10 required addition of 2 % NaCl, but production decreased with increasing concentration of NaCl (Shayesteh *et al.,* 2014). The reduction in activity of BpSl14 at higher concentration (>2 %) of NaCl can be due to the interference with induction factors as seen in the case of *Enterococcus faecium* CTC492 (Aymerich *et al*., 2000).

4.3.9 Effect of additional inorganic nitrogen sources

The addition of inorganic nitrogen source did not increase the activity of BaCf3 any further. Although activity of 1600 AU/mL remained the same as that obtained in the previous step, but there was a slight increase in specific activity to 1770.416±46.601 AU/mg with the addition of ammonium chloride (Fig 4.9a).

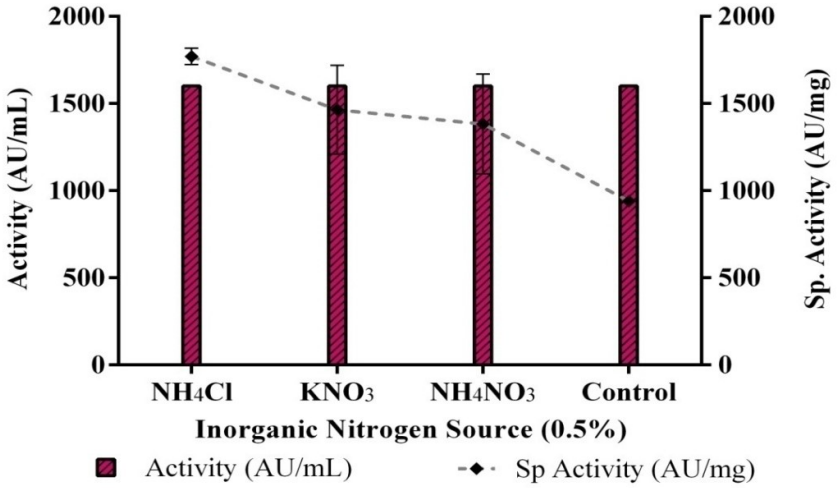


Fig 4.9a. Effect of additional nitrogen sources on BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

The addition of inorganic nitrogen source did not increase production of BpSl14 further. The activity 6400 AU/mL was the same as that activity obtained in the previous optimization step, but there was a slight increase in specific activity to 4729.212±128 AU/mg with the addition of ammonium nitrate (Fig 4.9b).

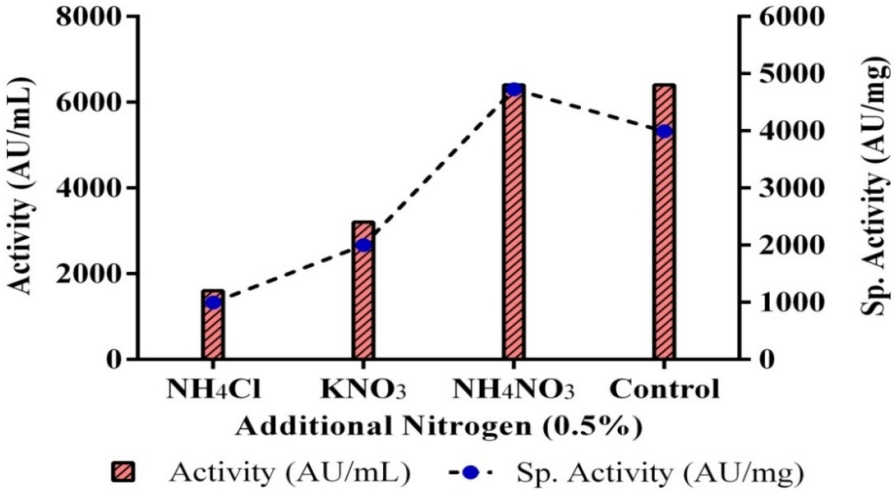


Fig 4.9b. Effect of additional nitrogen sources on BpSl14 production by *Bacillus pumilus* SDG14

Micrococcus MCCB 104, a marine isolate identified as a probiotic and antagonistic to *Vibrio harveyi* was positively affected by the concentration of ammonium chloride used in the medium (Preetha *et al*., 2007). Ammonium nitrate as a supplemental nitrogen source in the medium enhanced antimicrobial protein production by *Bacillus amyloliquefaciens* MBL27, (Vijayalakshmi *et al*., 2011). Among the different nitrogen sources tested by Verluyten *et al*. (2004), ammonium nitrate gave maximum bacteriocins activity, which may be attributed to the presence of two nitrogen atoms in NH_4NO_3.

4.3.10 Effect of Tween 80 and Mg^{2+} ion concentration

Addition of Tween 80 in the medium reduced the production of BaCf3 to 50 % (Fig 4.10a). Thus for production of BaCf3 medium without Tween 80

showed an activity of 3200 AU/mL and specific activity of 1765.42±18.962 AU/mg. Addition of Tween 80 in the medium was also a cause of >50 % reduction in bacteriocin ST151BR production (Todorov and Dicks, 2004).

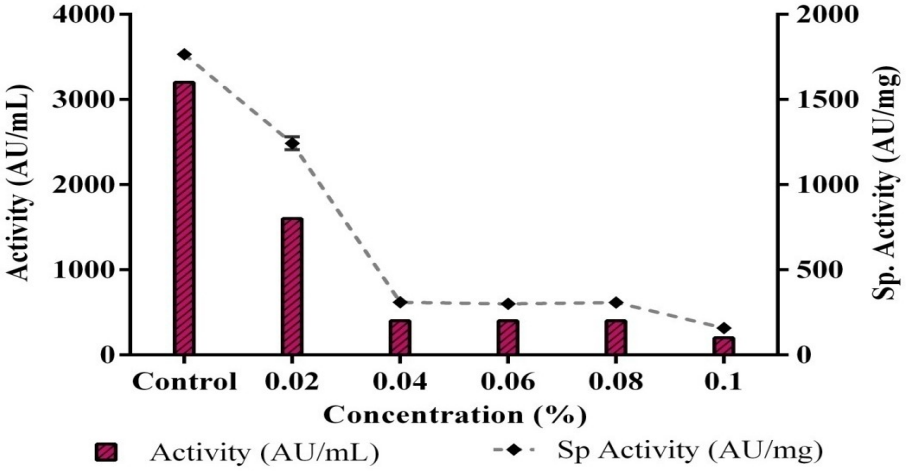


Fig 4.10a. Effect of Tween 80 on BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

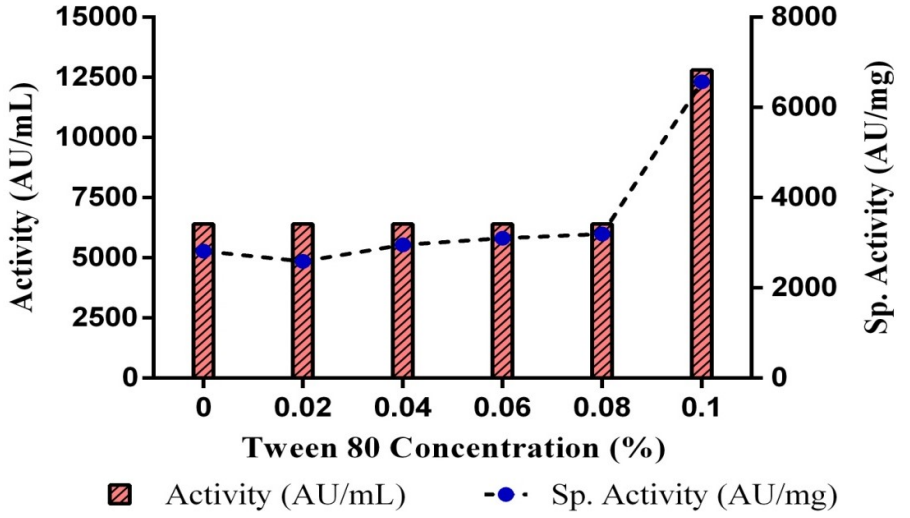


Fig 4.10b. Effect of Tween 80 on BpSl14 production by *Bacillus pumilus* SDG14

The addition of 0.1 % Tween 80 increased the production of BpSl14, with an increased activity of 12800 AU/mL and specific activity of 6569.02±116.67 AU/mg (Fig 4.10b). This is also supported by previous

studies for plantaricin 423 (Verellen *et al*., 1998), pediocin AcH (Biswas *et al*., 1991), lactacin B (Barefoot and Klaenhammer, 1984) and lactocin 705 (Vignolo *et al*., 1995), proving that the effect of Tween 80 depends on the nature of the bacteriocin being produced. The addition of Tween 80, a surfactant, is thought to be helpful in discharging the bacteriocin molecules bound to the bacterial cell surface.

BaCf3 production was almost constant with 200 to 500 mM Mg^{2+} concentration. The activity remained 3200 AU/mL for these concentrations where as the specific activity was slightly increased in 300mM $MgSO_4$. It was 2065.109±14.415 AU/mg. The optimum concentration of Mg^{2+} ions for BaCf3 production was 300 mM (Fig 4.11a).

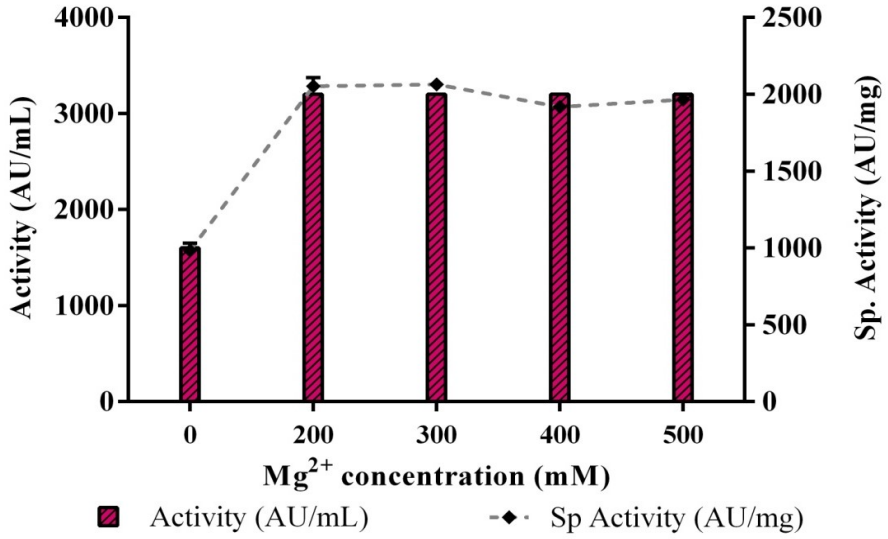


Fig 4.11a. Effect of Mg^{2+} concentration on BaCf3 production by *Bacillus amyloliquefaciens* BTSS3

BpSl14 production was maximal without Mg^{2+} ions (Fig 4.11b). The addition of Mg^{2+} ions in the fermentation medium reduced the activity to 6400 AU/mL. This observation is backed by previous studies that metal ions regulate bacteriocin production in several bacterial strains. The production of Streptococcal bacteriocin SA-FF22 was controlled by magnesium

concentration (Jack and Tagg, 1992). This may be the case with BpSl14 also. The production of BpSl14 was diminished by the addition of magnesium sulphate. Thus the medium without Mg^{2+} ions was selected for production.

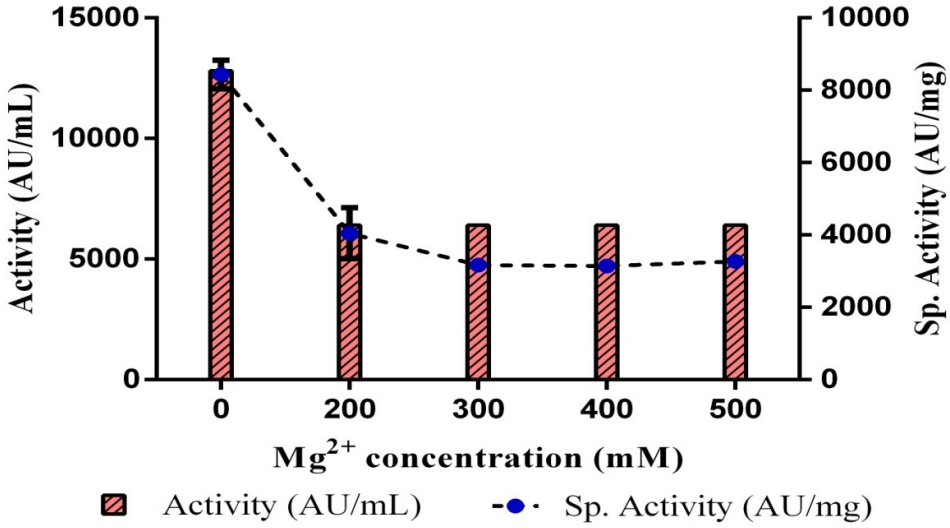


Fig 4.11b. Effect of Mg^{2+} concentration on BpSl14 production by *Bacillus pumilus* SDG14

Table 4.1 Optimized Media composition and conditions

Component	BaCf3	BpSl14
Na_2HPO_4 (%)	0.6	0.6
KH_2PO_4 (%)	0.3	0.3
NaCl (mg/mL)	30	-
$MgSO_4$ (mM)	300	-
$CaCl_2$ (µM)	200	200
Carbon Source (1%)	Inositol	Fructose
Nitrogen Source (0.5%)	Tryptone	Yeast Extract
Additional Nitrogen Source (0.5%)	NH_4Cl	NH_4NO_3
pH	6	8
Inoculum Concentration (%)	2	4
Temperature (°C)	40	35
Incubation Period (h)	18	24
Activity (AU/mL)	3200	12800
Specific Activity (AU/mg)	2065.109±14.415	8435.919±396.4716

4.3.11 Time course study

The result of optimization studies is summarized in Table 4.1. These optimized conditions were incubated at 150 rpm for the time course study. The bacteriocin production increased with time in both cases. The production time of BaCf3 remained unchanged from the optimized time (Fig 4.12a) where as

the production in terms of activity showed a fourfold increase (6400 AU/mL) with specific activity of 4514.307±96.417 AU/mg.

The optimum incubation period for production of BpSl14 decreased from 24 h to 22 h for BpSl14 (Fig 4.12b). The final activity was 12800 AU/mL while there was only a slight increase in specific activity 9339.276±88.445 AU/mg.

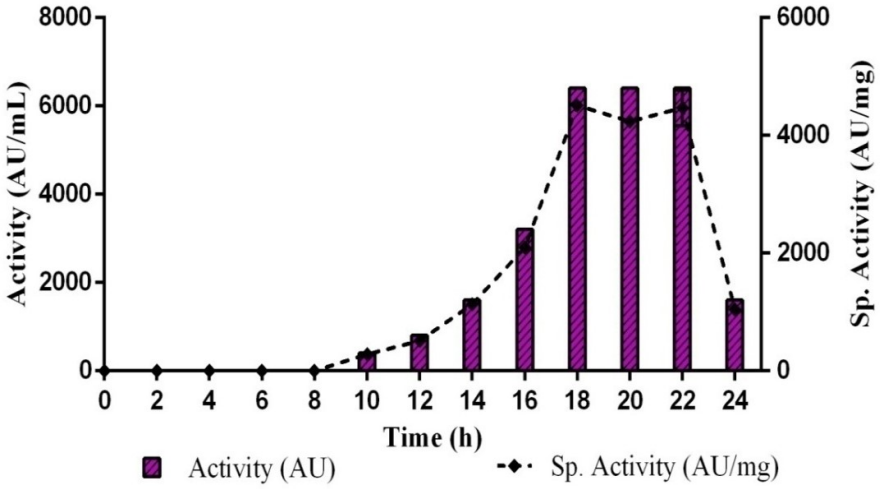


Fig 4. 12a. Time course study of BaCf3 production by *Bacillus amyloliquefaciens* BTSS3 using optimized media

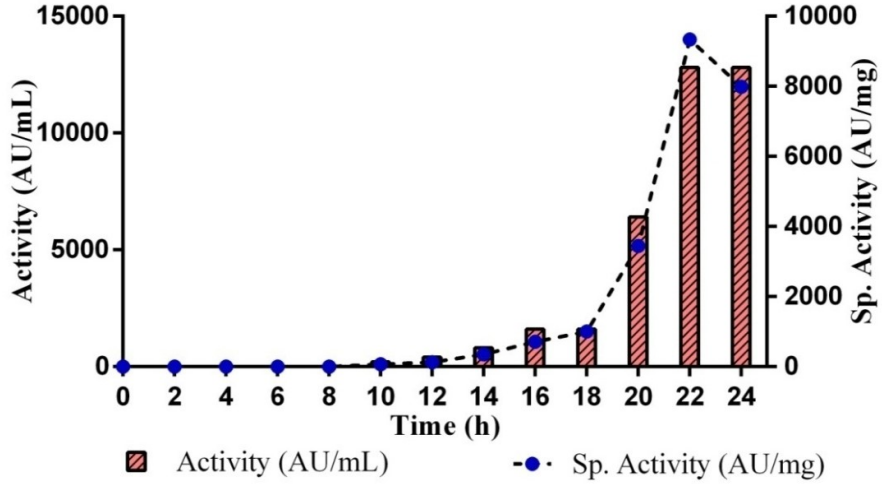


Fig 4. 12b. Time course study of BpSl14 production by *Bacillus pumilus* SDG14 using the optimized media

4.4 Summary

We can see that before optimization the initial activity of BaCf3 was only 800 AU/mL with specific activity of 125.658±0.939 AU/mg. The activity remained steady in the subsequent optimization steps, but with only slight observable increase in the specific activity. When the carbon source was modified, there was a steep increase in the activity (1600 AU/mL) and specific activity (639.193±30.816 AU/mg). This increasing trend was observed in the final optimization step with varying concentrations of $MgSO_4$ and the activity reached 3200 AU/mL with specific activity 2065.109±14.415 AU/mg. After time course study the activity was 6400 AU/mL with a specific activity of 4514.307±96.417 AU/mg. Thus we can conclude that there was a four-fold increase in activity and 36 fold increase in specific activity after the media optimization for the production of BaCf3.

The initial activity of BpSl14 was 3200 AU/mL with a specific activity of 1595.273±51.587 AU/mg. There was an increase in activity and specific activity when the incubation period was optimized but there was no further increase in activity for the seven optimization steps though the slight increase in specific activity cannot be ignored. The activity and specific activity increased steeply by the addition of 0.1 % Tween 80 to the fermentation medium. Thus after the final optimization, including time course study, for the production of BpSl14, the activity was 12800 AU/mL with a specific activity of 9339.276±88.445 AU/mg. There was a four-fold increase in activity and more than five-fold increase in specific activity. In conclusion, we can reaffirm that bioprocess optimization is an inevitable step for the production of bacteriocins.

Chapter 5

Purification and characterization of the bacteriocins

5.1 Introduction

Protein purification is one of the first steps towards understanding the function, characteristics, structure, and interactions of proteins. When a fermentation broth is analyzed, it is possible to detect a specific protein present in very low concentration among the 10,000 - 20,000 different proteins in an aqueous solution that contains intact microorganisms, cell fragments, soluble and insoluble media components and other metabolic products. The desired product may intracellular, heat labile or may easily be degraded by contaminating microorganisms. All these factors tend to increase the complexity of product recovery. To ensure good recovery or purification, speed of operation may be the overriding factor because of the labile nature of a product.

The choice of the recovery process is based on the following criteria (Stanbury *et al.,* 2013):

1. The intracellular or extracellular location of the product.
2. The concentration of the product in the fermentation broth.
3. The physical and chemical properties of the desired product (as an aid to selecting separation procedures).
4. The intended use of the product.
5. The minimal acceptable standard of purity.
6. The magnitude of bio-hazard of the product or broth.
7. The marketable price for the product.

The final degree of purity required depends upon the purpose for which the protein will be used. A sample is defined as ‘pure protein’ when it contains

only a single protein species, although it is more or less impossible to attain 100% purity. Many studies on proteins can be carried out on samples that contain as much as 5 % -10 % impurities of other proteins. A 90 % pure protein is sufficient for amino acid sequence determinations as the contaminants can be analyzed quantitatively.

The main objective of the first stage for the recovery of an extracellular product is the removal of large solid particles and microbial cells usually by centrifugation or filtration. Afterwards, the product containing cell free supernatant is purified by fractional precipitation, chromatographic techniques and crystallization to obtain a product which is highly concentrated and essentially free from impurities. During protein purification specific activity guided purification is always advised.

De novo is Latin word for, "over again", or "anew". A popular definition for "de novo peptide sequencing" is, peptide sequencing performed without prior knowledge of the amino acid sequence. The introduction of MALDI-MS/MS (Matrix Assisted Laser Desorption Ionization - Mass spectroscopy/Mass spectroscopy) has strengthened the role of MALDI for amino acid sequence determination. In the case of peptides, although a wide variety of fragmentations may occur, there is a predominance of peptide bond cleavages. This means that the peptide fragments by losing one amino acid residue at a time give rise to peaks in the spectrum which differs sequentially by amino acid residue mass. These sequential mass differences represent exactly the amino acid sequence known as primary structure of the peptide. The post translational modifications are also part of primary structure.

The biological properties of proteins are dependent on the tertiary structure of the proteins. The primary structure can form hydrogen bonds between

the amino acids which lead to the formation of secondary structure. The two main types of secondary structure are α helices and β sheets. The overall three dimensional structure of the entire protein/peptide molecule is the tertiary structure. The formation of disulphide linkages for stabilization is also grouped under this. The primary structure can be converted to tertiary structure or their spatial models. These models can be used for virtual screening of the molecule for bioactivities.

This chapter discusses the purification, characterization and the mechanism of action of the bacteriocins. The bacteria grown in optimized production media was used for purification. The purification steps involved collection of supernatant, precipitation, dialysis and chromatography. SDS PAGE, zymogram and Matrix Assisted Laser Desorption Ionization- Mass Spectroscopy (MALDI-MS) were employed for characterization. Other characterizations included the effect of pH, temperature, metal ions, oxidizing and reducing agents on the activity and stability of the bacteriocins. The mechanism of action was studied by microscopic techniques. The primary sequences of the bacteriocins BpSl14 and BaCf3 were obtained from their MS/MS data, which was used to predict their secondary and tertiary structures. The sequences were searched for similarity using BLAST in the databases and multiple sequence alignment was done. The sequences were also searched for the formation of disulphide linkages for stabilization.

5.2 Materials and methods

5.2.1 Purification of bacteriocins

Purification of bacteriocins involved the steps in protein purification such as ammonium sulphate precipitation, dialysis and gel filtration chromatography (Fig 5.1).

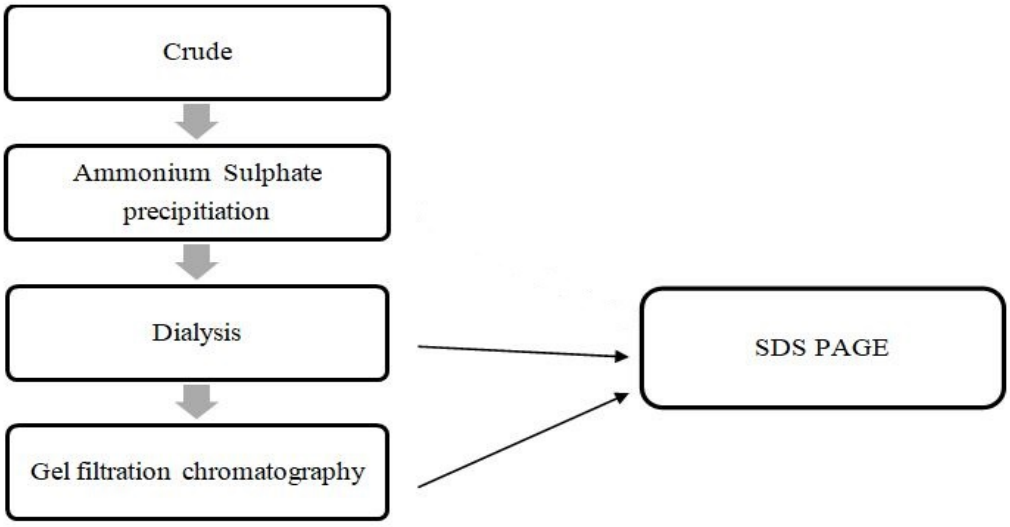


Fig 5.1 Flow chart representing the purification steps followed in the present study

5.2.1.1 Ammonium sulphate precipitation and dialysis

Ammonium sulphate precipitation is a simple and effective means of protein fractionation. It is based on the fact that at high salt concentrations, the surface charges of proteins are neutralized and the proteins get aggregated. Due to charge neutralization the proteins tend to bind together, form large complexes and hence easy to precipitate out by centrifugation. Since each protein aggregates at a characteristic salt concentration, this approach provides a simple way of enriching a specific protein in a mixture.

The organisms were grown in the appropriate optimized production medium as described in section 4.3.10 and 4.3.11. The cell free supernatant from the optimized production medium was collected by centrifugation (Sigma, India) at 10000 rpm for 15 minutes. Ammonium sulphate was added to the supernatant to obtain 0-30 %, 30-60 % and 60-90 % saturation, and mixed well using magnetic stirrer overnight at 4 ºC. After precipitation the pellet was collected by

high speed centrifugation, 20000 rpm (36000 X g) for 20 minutes at 4 ºC and dissolved in minimum volume of 0.01 M phosphate buffer of pH 7.

The pellet dissolved in phosphate buffer was dialyzed using 2 kDa benzoylated dialysis tube (Sigma-Aldrich) against 0.01 M phosphate buffer of pH 7 at 4 ºC, overnight with continuous stirring. The buffer was changed every hour to remove the salt from the sample. After dialysis, activity and total protein content of the sample was determined as described in section 3.2.5.

5.2.1.2 Gel filtration chromatography

The active fraction obtained after dialysis was further purified by gel filtration chromatography using Sephadex® G50 column. Econo Column (Biorad, India) was packed with Sephadex® G50 which was previously washed with water and subsequently equilibrated with thrice the column volume of 0.01 M phosphate buffer of pH 7. The dialyzed sample was filtered through 0.22 µm Millex® sterile filter (Merck Millipore, Germany) and added slowly through the sides of the column and eluted with the same buffer at a flow rate of 0.25 mL/min. 5 mL fractions were collected in labeled tubes and the elution was done until the column volume of sample was collected.

The antimicrobial activity of each fraction was determined against *Bacillus circulans* NCIM 2107 by spotting 5 µL of each fraction on a MH agar plate. The protein concentration of active fractions was determined by measuring the absorbance at 280 nm using BioSpectrometer (Eppendorf, India) and elution profile of the bacteriocin was plotted against time. Samples which showed activity were pooled, checked the activity and protein concentration as per section 3.2.5.

$$\text{Specific activity (AU/mg)} = \frac{\text{Total activity(AU/mL)}}{\text{Total protein(mg/mL)}}$$

$$\text{Fold of purification} = \frac{\text{Specific activity (AU/mL)}}{\text{Initial specific activity (AU/mL)}}$$

$$\text{Yield of protein (\%)} = \frac{\text{Total activity (AU/mL)}}{\text{Initial total activity (AU/mL)}} \times 100$$

5.2.2 SDS-PAGE and silver staining

The purity of the gel filtration fractions were checked using SDS-PAGE.

5.2.2.1 Sample preparation

Purified bacteriocin samples were mixed with sample buffer (5X) containing bromophenol blue as the tracking dye, in such a way that the final volume becomes 20 µL (protein concentration = 0.5 mg/mL). These samples were loaded carefully into the wells. The composition of sample loading buffer is given in Appendix III.

5.2.2.2 Protein markers for SDS PAGE

GeNei (Bengaluru) Medium range molecular weight protein marker and broad range molecular weight protein marker of New England Biologicals® (UK) was used for detecting the approximate size. Details of markers are given in appendix- III.

5.2.2.3 Gel preparation

The gel was prepared according to method described by Laemmli (1970). The composition of the gel is given in table 5.1. The gel was cast in mini-PROTEAN® (Biorad, India) casting unit. Standard molecular weight markers were also loaded according to the manufacturer's instructions. Electrophoresis was conducted at a constant voltage (80 V in stacking gel and 100 V in resolving

gel). Gel was separated from the running unit and silver staining was carried out for visualization of protein bands (Appendix III).

Table 5.1 Composition of gel preparation

Constituents	12% separating gel	5% stacking gel
Acrylamide –bisacrylamide	2 mL	0.425 mL
1.5 M Tris HCl buffer (pH 8.8)	2.5 mL	-
0.5 M Tris HCL buffer (pH 6.8)	-	0.625 mL
MilliQ water	0.405 mL	1.425 mL
SDS	50 µL	25 µL
TEMED	7.5 µL	2.5 µL
10 % Ammonium persulphate	37.5 µL	25 µL

Silver staining of the gel after electrophoresis was performed by the method of Blum (1987) with slight modifications. The steps involved were as follows

1. Fixed the gel with 50 % methanol and 5 % acetic acid for 30 minutes and incubated in 50 % methanol.
2. Washed the gel five times in milliQ water for five minutes each to remove methanol.
3. Incubated in sodium thiosulphate solution (20 %, freshly prepared) for 60 seconds
4. Rinse the gel twice with milliQ for 60 seconds.
5. Submerged in silver nitrate solution (200 %) chilled to 4 ºC for 25 minutes.
6. Developer, anhydrous solution of Na_2CO_3 (3 %) containing 25 µL formaldehyde was added and waited till the bands became visible.

7. Stop solution Na_2EDTA (14 g/100 mL) was added to stop the staining reaction.
8. The gel was visualized and image was captured in ChemiDoc™ XRS System (Biorad, India).

5.2.3 In-gel activity assay of bacteriocin activity

The ammonium sulphate precipitated fraction after dialysis was subjected to SDS PAGE as described in section 5.2.2. The protein was loaded in two wells alongside the protein marker. After the electrophoresis, the gel was cut into two halves – one containing the marker lane and protein lane and the other with test protein lane alone. The portion of gel with test protein lane alone was washed thrice for thirty minutes with 0.01 % Tween 80 to remove SDS, followed thrice again with milliQ water for 30 minutes to remove Tween80. After washing the gel was carefully placed in a sterile petri-dish and overlaid with 0.8 % MH agar containing 1 % test organism (*Bacillus circulans* NCIM 2107). The plate was incubated at 37 °C overnight and examined for zone of inhibition (Yamamoto *et al*., 2003).

The other half of the gel was used for silver staining as described in section 5.2.3.

5.2.4 MALDI-TOF mass spectrometry

The molecular mass of the purified antimicrobial peptides were determined by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. MALDI-TOF was performed by Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with smart beam solid state laser (337 nm) in reflectron positive ion mode using 19 KV acceleration voltage. The eluted peptides were co-crystallized with α-cyano-4-

hydroxycinnamic acid matrix on the target plate (384-well ground steel plate, Bruker Daltonics, Germany) and external peptide mass calibration was applied (Peptide mixture 1) as per the manufacturer's instructions.

5.2.5 Characterization of bacteriocins

Bacteriocins are an extremely heterogeneous group of substances. Though they are chemically diverse, the unifying property is the presence of an essential protein component. Tests of sensitivity to specific enzymes (proteinases, amylases, etc.) are often used to identify important chemical components of bacteriocin molecules. Low molecular weight bacteriocins are generally more susceptible to trypsin digestion but are less sensitive to heat inactivation. Bacteriocins differ greatly with respect to their sensitivity to changes in pH. Many of the bacteriocins and bacteriocin-like substances are considerably more tolerant to acid than alkaline pH.

5.2.5.1 Action of hydrolytic enzymes

Ammonium sulphate precipitated fraction of bacteriocins (Activity = 12800 AU/mL) were used to test the susceptibility of bacteriocin to enzymatic digestion, enzymes like proteinase K, trypsin, pepsin and α-amylase, were mixed with the bacteriocin at final concentrations of 1mg/mL and 5 mg/mL. The mixture was incubated for 1h at 37 °C and tested for bacteriocin activity as described in section 3.2.5.

5.2.5.2 Effect of temperature on bacteriocins

To evaluate heat stability, ammonium sulphate fraction of bacteriocin (Activity = 12800 AU/mL) was exposed to temperatures ranging from 40 °C to 100 °C for 1 h and 40 °C to 121 °C per 105 kPa for 15 min. The treated samples

were tested for residual antimicrobial activity against the test organism as described in section 3.2.5.

5.2.5.3 Effect of pH on bacteriocins

To study the effect of different pH, equal volume of purified bacteriocin (Activity = 51200 AU/mL) were added to buffers with pH range 2 - 13 and kept for 18 h at 4 °C. The buffer systems (Appendix-II) used included hydrochloric acid/potassium chloride buffer (pH 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 - 11), sodium phosphate dibasic/sodium hydroxide buffer (pH 12 -13) (Vincent and John, 2009). The treated samples were tested for residual antimicrobial activity against the test organism as described in section 3.2.5.

5.2.5.4 Effect of metal ions on bacteriocins

Equal volumes of 1mM solutions of Na^{2+}, Ca^{2+}, Mg^{2+}, Ba^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+} and purified bacteriocins (Activity =512000 AU/mL) were mixed and incubated at 4 °C for 1 h. The untreated sample served as control. The treated samples were tested for residual activity against *Bacillus circulans* NCIM 2107.

5.2.5.5 Effect of oxidizing and reducing agents on bacteriocins

To examine the effect of oxidizing and reducing agents on bacteriocin activity 50 and 100 mM of DTT, β-mercaptoethanol and DMSO were prepared. Equal volume of purified bacteriocin samples (Activity = 512000 AU/mL) were mixed with these oxidizing/reducing agents and incubated for 1 h and assayed for activity.

Percentage residual activity was calculated by

$$\% \text{ Residual activity} = \frac{\text{Activity(t)}}{\text{Activity(c)}} \times 100$$

5.2.6 Mechanism of action of bacteriocins

The main target of bacteriocins is usually the lipid bilayer, where peptides can promote the cell depolarization and/or internalize in the cytosol in order to interact with inner targets. Many biophysical techniques can be conducted using model membranes in order to understand the peptide – membrane interaction mechanism. An understanding of the mechanisms of action of the bacteriocins against susceptible bacterial strains would be important for their effective utilization. Many microscopic techniques can also be used to find out the changes on target cell membrane. In this study we have used scanning electron microscopy (SEM), transmission electron microscopy (TEM) and confocal laser microscopy to understand the mechanism of action of the bacteriocins.

5.2.6.1 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations of the bacteriocins, BaCf3 and BpSl14 were determined according to the protocol recommended by Sarker *et al*. (2007). The MIC was calculated for all the sensitive test organisms. To each well of a sterile 96-well U-shaped microtitre plate, 100 µL of double strength LB broth was added. Then, 100 µL of purified bacteriocin of known concentration (1 mg/mL) was added to the first well of every row, mixed well, 50 µL was taken from the first well and serially diluted. Bacterial suspensions of 1 OD_{600} prepared in LB broth (10 µL) was added to each well. The plate was incubated at 37 ºC for 18 hours. After the incubation 2 µL of rezasurin was added into each well. Wells with live organisms convert blue rezasurin into pink color. The lowest

concentration of bacteriocin that inhibited the growth of the organisms was considered as the MIC.

5.2.6.2 Bactericidal/Static mode of action

The test organisms were grown in LB broth. To 1 mL of bacterial cultures of optical density 0.1 at 600 nm, purified bacteriocins were added at their MIC concentration. Tubes were incubated at 37 ºC, 120 rpm for 6 h and absorbance was noted at 600 nm. Tubes without bacteriocins acted as control.

$$\% \text{ Inhibition } = \left[\frac{\text{OD control} - \text{OD of test}}{\text{OD of control}}\right] 100$$

5.2.6.3 Action of bacteriocins on bacterial membrane

5.2.6.3.1 Scanning electron microscopy

Overnight cover slip cultures of *Bacillus circulans* NCIM2107 was treated with MIC concentration of bacteriocin for 1 h and 2 h at 37 ºC. The cover slip without bacteriocin was used as control. The cover slips were fixed with 2.5 % gluteraldehyde in PBS, washed twice with PBS and dehydrated in a graded series of ethanol (50, 70, 80, 90, 100 %). These were dried in desiccators and used for scanning electron microscopy (Tescan VEGA3 SB).

5.2.6.3.2 Transmission electron microscopy

Bacillus circulans NCIM2107 culture of OD_{600} = 1 was mixed with bacteriocin, (BaCf3 and BpSl14 each) at its MIC concentrations and incubated at 37 ºC, 120 rpm for 1 h. The liquid culture was mixed with 2 % solution of phosphotungstic acid in phosphate buffer of pH 7.4. This solution was loaded on the grid, dried and analyzed by transmission electron microscopy (Jeol/JEM 2100). The culture without bacteriocin served as control.

5.2.6.3.3 Confocal laser scanning microscopy

Bacillus circulans NCIM2107 cells (1 x 10^4 CFU/mL) were allowed to bind on StarFrost® glass slides (Knittel, Germany) with activated surface for 30 minutes. The slide was then covered by bacteriocins at $1/4^{th}$ of their MIC for 30minutes. After the treatment, the slides were washed thrice with PBS, fixed with 2.5 % gluteraldehyde in PBS. The cells were stained with To-Pro-3 stain diluted 1:1000 in PBS for 20 minutes in dark at room temperature. They were observed and photographed using confocal imaging system (Leica TCS SP 5) (Anju *et al*., 2013). To-pro-3 is a nuclear stain; hence membrane compromised cells can be seen as red spots due to the far red fluorescence of the dye. Therefore, the pixel intensity of the red spots appearing in the images before (Control) and after (Treated) treatment with bacteriocin BaCf3 and BpSl14 was quantified using ImageJ software.

5.2.7 *De novo* sequencing and modelling of the bacteriocins BpSl14 and BaCf3

5.2.7.1 Sample preparation

The protein band excised from silver stained SDS-PAGE gel (Section 5.2.2) was used. The in-gel digestion of bacteriocins was done following the protocol of Shevchenko *et al*. (2006). Added 500 µL of acetonitrile and incubated tubes for 10 min until gel pieces became opaque and stuck together. The gel pieces were spun down to remove all liquid. 30 - 50 µL of DTT solution was added to completely cover gel pieces and incubated for 30 min at 56 °C. The tubes were chilled to room temperature (22 °C), again added 500 µL of acetonitrile and incubated for 10min. The liquid was removed from gel and added 30 - 50 µL of the iodoacetamide solution in such a way as to cover the gel pieces and incubated for 20 min at room temperature in the dark. Again, shrunk the gel

pieces with acetonitrile and removed all liquid. Enzymatic digestion was carried out by incubating the reaction mixture with trypsin (40 ng/L of 25 mM NH_4HCO_3, Promega, Madison, WI, USA) and incubated out overnight at 37 °C. After digestion, the gel pieces were treated with 10 µL of 50 % acetonitrile/1 % trifluoroacetic acid (TFA). This extract was transferred to the primary supernatant, and repeated the extraction. The digests were concentrated using vacuum centrifuge.

5.2.7.2 *De novo* sequencing by MS/MS

The peptide mass fingerprinting (PMF) data was acquired in the mass range of 700-3500 m/z. The MS/MS fragmentation was carried out for the selected peptide in the LIFT™ mode of the instrument. This was performed by Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with smart beam solid state laser (337 nm) in reflectron positive ion mode using 19 KV acceleration voltage. The in-gel digested peptides from previous section was co-crystallized with α-cyano-4-hydroxycinnamic acid matrix on the target plate (384-well ground steel plate, Bruker Daltonics, Germany) and external peptide mass calibration was applied (Peptide mixture 1) as per the manufacturer's instructions.

The MS/MS data of selected trypsin digested fragments were analysed by mMass 5.5.0 (Niedermeyer and Strohalm, 2012). The sequence of the peptide fragments were derived manually by looking at the ‘a’, ‘b’ and ‘y’ fragments and hence the corresponding amino acids from their molecular mass (Wilson and Walker, 2000). The sequences obtained from MS/MS data was validated for accuracy in Fragment Ion Calculator a free program for calculating masses of fragmentation ions from peptides provided by the institute for systems biology (http://db.systemsbiology.net:8080/proteomicsToolkit/).

5.2.7.3 Multiple sequence alignment

The derived sequences were used for multiple sequence alignment with many known bacteriocins using Mega 6.0 (Tamura *et al*., 2013). The sequences were searched for similar sequences in PDB using PDBsum an online web server. PDBsum is a pictorial database that provides an at-a-glance overview of the contents of each 3D structure deposited in the Protein Data Bank (PDB). It shows the molecule(s) that make up the structure (ie., protein chains, DNA, ligands and metal ions).

5.2.7.4 Amino acid composition of bacteriocins

The amino acid composition of the bacteriocins BpSl14 and BaCf3 was calculated using protein analysis tool in ExPASy (http://web.expasy.org/ protparam/), the ProtParam tool (Gasteiger *et al*., 2005). ProtParam is a tool which allows the computation of various physical and chemical parameters for a given protein stored in Swiss-Prot or TrEMBL or for a user entered protein sequence.

5.2.7.5 Secondary and tertiary structure prediction from the derived partial sequence

The structure prediction of the *de novo* sequence was done using I-TASSER (Iterative Threading ASSEmbly Refinement, https://zhanglab.ccmb. med.umich.edu/I-TASSER/), which is a hierarchal approach to protein structure and function prediction (Yang *et al*., 2015). This server provides the most accurate structural and functional predictions for small peptide sequences (Zhang, 2008).

I-TASSER modeling starts from the structure templates identified by LOMETS which is a meta-server threading approach containing multiple

threading programs, from the PDB library. I-TASSER uses only the templates of the highest significance in the threading alignments, which are measured by the Z-score (the difference between the raw and average scores in the unit of standard deviation). The top 10 templates are the 10 templates selected from the LOMETS threading programs.

5.2.7.6 Prediction of disulphide bridge

Disulphide bridges are involved in the stabilization of tertiary structures. DiANNA (DiAminoacid Neural Network Application) is a web server that provides disulfide connectivity prediction. DiANNA 1.1 determines the disulfide connectivity involving a novel architecture neural network (Ferre and Clote, 2006). The neural network input includes evolutionary as well as secondary structure information.

5.3 Results and discussion

5.3.1 Purification of bacteriocins

The initial volume of crude cell free extract of both bacteriocin BaCf3 and BpSl14 used for purification was 500 mL with a protein concentration of 1.36 mg/mL and 1.4 mg/mL respectively.

5.3.1.1 Ammonium sulphate precipitation and dialysis

The initial activity of BaCf3 extract was 6400 AU/mL with a specific activity of 4705.88 AU/mg. The bacteriocin BaCf3 was precipitated in the 0-30 % ammonium sulphate fraction, dissolved in 0.01 M phosphate buffer of pH 7.4 and kept for dialysis in the same buffer. After dialysis the volume, activity and specific activity were determined to be 14 mL, 12800 AU/mL and 640000 AU/mg

respectively. The yield of BaCf3 after this purification step was 5.6 % with a 136 fold increase in purity.

The crude culture supernatant of BpSl14 with activity 12800 AU/mL and specific activity 9175.63 AU/mg was partially purified using 0 – 80 % ammonium sulphate precipitation; this was dissolved in 0.01 M phosphate buffer of pH 7.4 and dialysed using the same buffer. The volume, activity and protein concentration of the extract were noted. After this step the activity and specific activity were 12800 AU/mL and 128000 AU/mg respectively. The yield of BpSl14 obtained after this step of purification was 6 %.

Concentration by precipitation is the first step of purification of all bacteriocins. Ammonium sulphate precipitation is a common step for bacteriocin purification. It is a method used for fractionation of proteins. Bacteriocin ISK-1 was purified initially by addition of ammonium sulphate to 70 % saturation (Kimura *et al*., 1998) with 9.3 fold of purification. A bacteriocin like substance produced by *Bacillus licheniformis* 26L-10/3RA was also purified initially by ammonium sulphate precipitation with a yield of 50 % (Pattnaik *et al*., 2001). Entomocin 9, produced by *Bacillus thuringiensis* HD9 was also precipitated by the addition of 80 % ammonium sulphate. This shows that ammonium sulphate precipitation is an easy and reliable method for concentration of bacteriocins (Cherif *et al*., 2003). The bacteriocin from *Lactobacillus murinus* AU06 with broad antibacterial spectrum was concentrated by ammonium sulphate precipitation (Elayaraja *et al*., 2014). Bacteriocin produced by *Weissella confusa* A3 of dairy origin was precipitated with 80 % ammonium sulphate (Goh and Philip, 2015). A novel bacteriocin Paracin 1.7, synthesized by *Lactobacillus papacasei* HD1-7 isolated from Chinease sauerkraut juice required 70 % ammonium sulphate precipitation (Ge *et al*., 2016) for initial purification. Salivaricin CRL 1328, from *Lactobacillus (Lb.) salivarius* CRL1328 a probiotic

isolate from healthy human vagina, was precipitated to a yield of 21.7 % with 70 % ammonium sulphate (Pingitore *et al*., 2007).

5.3.1.2 Gel filtration chromatography

The bacteriocins were further purified by gel filtration chromatography. Fractions of 5 mL each were collected, measured in BioSpectrometer® (Eppendorf) and elution profile was plotted. The elution profile showed that the bacteriocin BaCf3 was obtained in the 10 to 15 minutes time (Fig 5.2). Activity of BaCf3 after gel filtration chromatography was 51200 AU/mL and the specific activity was 1024000 AU/mg. The yield of BaCf3 after gel filtration chromatography was 1.6 % and the fold of purification after the purification was 217.6. The details are depicted in Table 5.2.

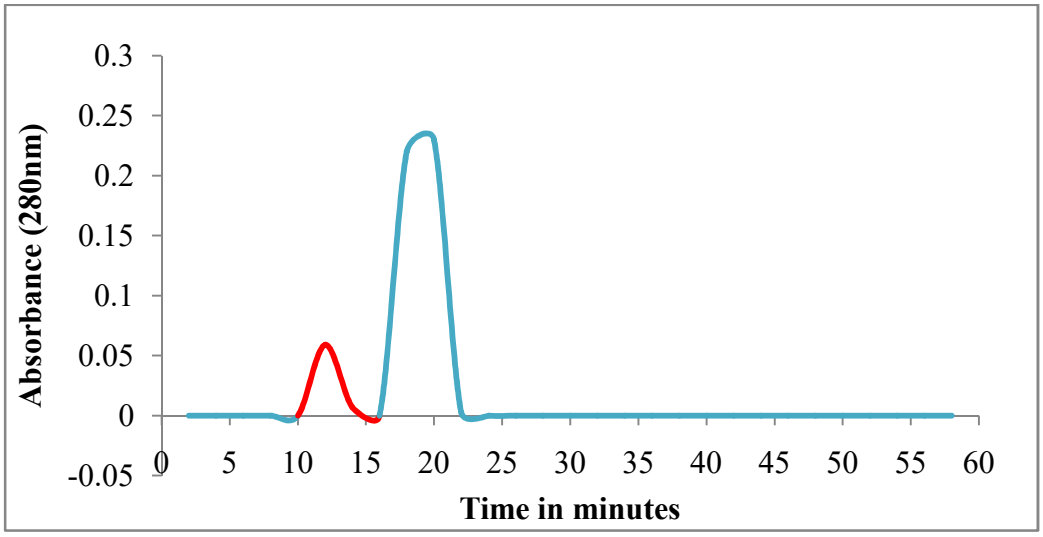


Fig 5.2 Elution profile of BaCf3. BaCf3 was eluted between 10 to 15 minutes

The elution profile was also plotted for BpSl14 extract. The active component was obtained in fractions of 10 to 25 minutes (Fig 5.3). Activity of BpSl14 after gel filtration chromatography was 51200 AU/mL and the specific activity was 223581 AU/mg. The yield of BpSl14 after gel filtration

chromatography was 4 % and the fold of purification was 24.37. The details are depicted in Table 5.3.

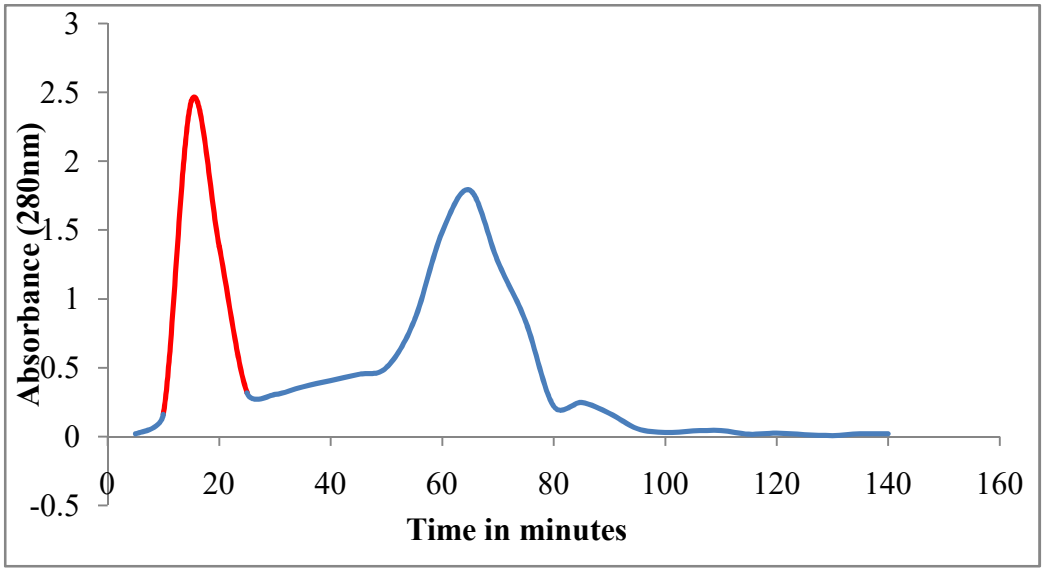


Fig. 5.3 Elution profile of BpSl14. BpSl14 was eluted between 10 to 25 minutes

The BLIS produced by *Bacillus licheniformis* P40 was purified with 1.5M NaCl by gel filtration chromatography on Sephadex G-100 (Cladera-Olivera *et al*., 2004b). Lactacin F was concentrated by the addition of 40 % solid ammonium sulfate and was sized using 50 mM sodium phosphate (pH 7.2) on Sephacryl S-300 gel filtration column with 369 fold of purity (Muriana and Klaenhammer, 1991). Bacteriocins of *Bacillus* and *Paenibacillus* isolates from stomach contents of birds were purified from the crude antimicrobial preparations by gel filtration on a Superose® 12HR 16/50 column (Svetoch *et al*., 2005). The antimicrobial peptide BLS P34 produced by *Bacillus* sp. isolated from Amazon Basin was purified by ammonium sulphate precipitation, gel filtration, and ion exchange chromatography, and single band suggesting the homogeneity was obtained after the final purification step (Motta *et al*., 2007).

Gel filtration chromatography was also used for the purification of the bacteriocin Linocin M18 obtained from *Brevibacterium linens* and a single band was observed with a molecular mass of approximately of 31 kDa in silver stained SDS-polyacrylamide gels after the purification step (Valdés-Stauber and Scherer, 1994). After gel filtration chromatography using Superdex G-75, a single 2 kDa band showing bactericidal activity was obtained by 10 - 25 % acrylamide SDS-PAGE electrophoresis with a very high yield (60 %) and increased specific activity (from 33.7 to 237.8 AU per mg of total proteins) with a purification factor of approximately 7 (Martirani *et al*., 2002).

Bacteriocins Piscicocin CS526, Macedocin and Enterocin CRL35 purified by gel filtration chromatography gave yields of 7 %, 6 % and 2 % respectively (Farias *et al*., 1996; Georgalaki *et al*., 2002; Yamazaki *et al*., 2005). The novel bacteriocin Paracin 1.7 after Sephadex G-10 column gave 40 % yield with 2.87 fold of purification (Ge *et al*., 2016).

Table 5.2 Purification table of bacteriocin BaCf3

Sample	Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Activity (AU/mL)	Total Activity (AU)	Sp. Activity (AU/mg)	Fold of purification	%yield
Crude	500	1.36	680	6400	3200000	4705.88	1	100
0-30%$(NH_4)_2SO_4$	14	0.02	0.28	12800	179200	640000	136	5.6
Gel Filtration Sephadex G50	1	0.05	0.05	51200	51200	1024000	217.6	1.6

Table 5. 3 Purification table of bacteriocin BpSl14

Sample	Volume (ml)	Protein (mg/mL)	Total Protein (mg)	Activity (AU/mL)	Total Activity (AU)	Sp. Activity (AU/mg)	Fold of purification	%yield
Crude	500	1.395	697.5	12800	6400000	9175.63	1	100
0-80% $(NH_4)_2SO_4$	30	0.1	3	12800	384000	128000	13.95	6
Gel filtration Sephadex G50	5	0.229	1.145	51200	256000	223581	24.37	4

5.3.1.3 SDS PAGE and silver staining

SDS PAGE of the dialyzed ammonium sulphate precipitated fraction and the purified fraction from gel filtration chromatography is depicted in Fig 5.4 and 5.5. In both bacteriocins, multiple bands were visible in the ammonium sulphate precipitated fraction but there was only single band in the gel filtration chromatography fraction. This demonstrates that purified bacteriocins were obtained after gel filtration chromatography. The molecular weight as estimated from SDS PAGE was 3kDa for BaCf3 and 6.5 kDa for BpSl14 (Fig 5.4 and 5.5).

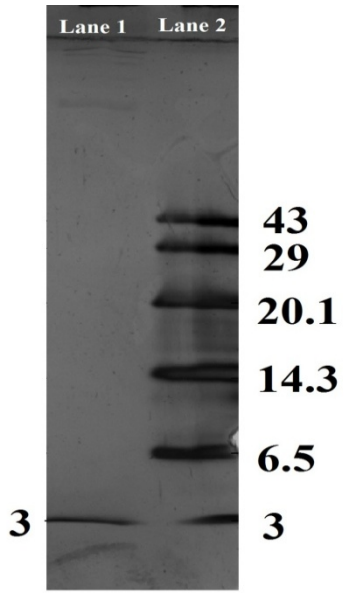


Fig 5.4 SDS PAGE of BaCf3 after silver staining. Lane 1- Purified fraction from gel filtration chromatography, Lane2- GeNei broad range protein marker. A single band was obtained in the 3 kDa region in lane 1.

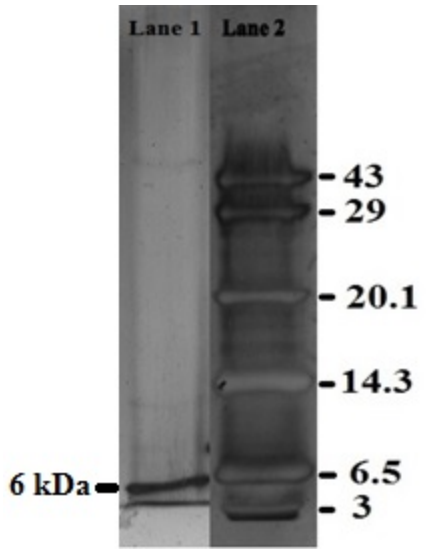


Fig 5.5 SDS PAGE of BpSl14 after silver staining. Lane 1- Purified fraction from gel filtration chromatography, Lane2- GeNei broad range protein marker. A single band was obtained in the 6 kDa region in lane 1.

The molecular weight of Paracin 1.7 based on Tricine-SDS-PAGE was identified as 10 kDa (Ge *et al*., 2016). Purified bacteriocin from *Weissella confusa* A3 was about 2.5 kDa on SDS PAGE compared to the marker (Goh and Philip, 2015).

5.3.1.4 In-gel activity assay for detection of bacteriocin activity

In-gel activity assay conducted for both the bacteriocins confirmed that the molecular weight of the bacteriocin BaCf3 was between 3 and 10 kDa (Fig 5.6 a and b). Zymogram and SDS PAGE shows that the molecular weight of the bacteriocin BpSl14 was approximately 6 kDa (5.7 a andb).

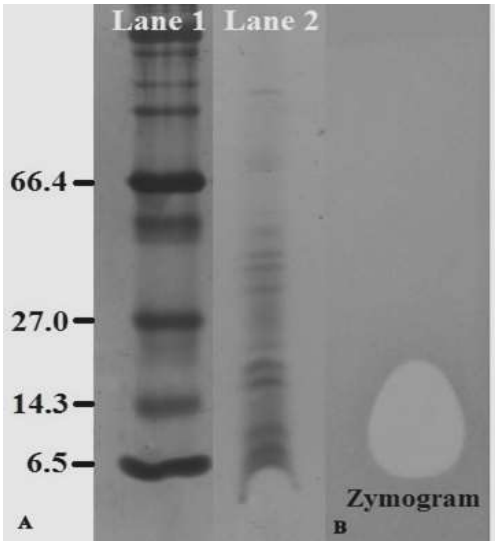


Fig 5.6 A) SDS PAGE of 0-30 % ammonium sulphate fraction of BaCf3. Lane 1- NEB broad range protein marker; Lane 2- 0-30 % ammonium sulphate fraction of BaCf3 B) Zymogram of BaCf3 showing a clearing zone between 3 and 10 kDa; the sample used was 0-30 % ammonium sulphate fraction of BaCf3.

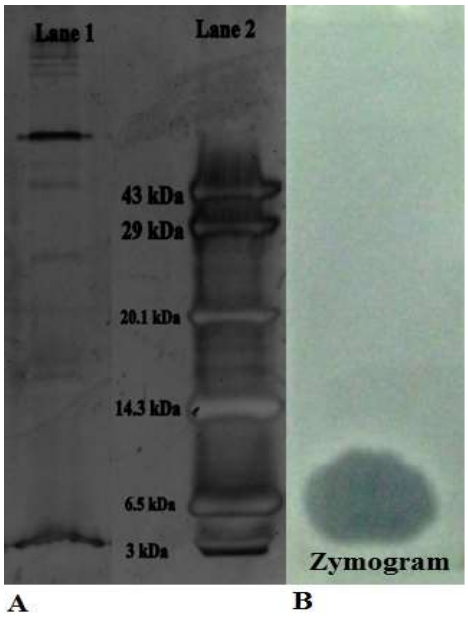


Fig 5.7 A) SDS PAGE and Zymogram of BpSl14. a) Lane-1 Ammonium sulphate precipitate of BpSl14, Lane-2 GeNei low molecular weight marker. B) Zymogram of BpSl14 showing a clearing zone at a region of molecular weight 3 kDa and 10 kDa

The antibacterial activity of cerein 7 could be demonstrated by overlaying a similar gel with an indicator strain which exposed a growth inhibitory zone at the gel position where the single protein was visualized in the silver-stained control gel (Oscáriz *et al*., 1999). Antimicrobial activity of bacteriocin-like substance produced by *B. amyloliquefaciens* was also detected on polyacrylamide gels (Lisboa *et al*., 2006) and the apparent molecular mass was identified to be 5 kDa. In- gel activity of BLS P34 produced by *Bacillus* sp. was observed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Motta *et al*., 2007).

5.3.1.5 MALDI-TOF mass spectrometry

From the MALDI-TOF mass spectroscopy the intact molecular weight of BaCf3 was 3028.422 Da (Fig 5.8) while that of BpSl14 as 6061.2 Da (Fig 5.9).

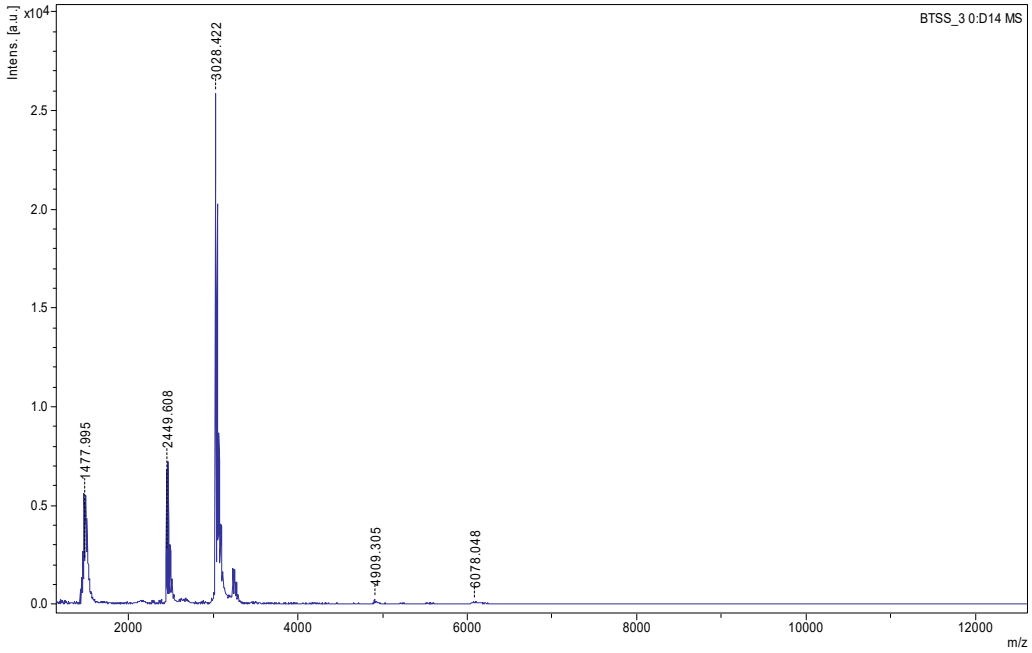


Fig.5.8 MALDI TOF MS of purified sample of BaCf3

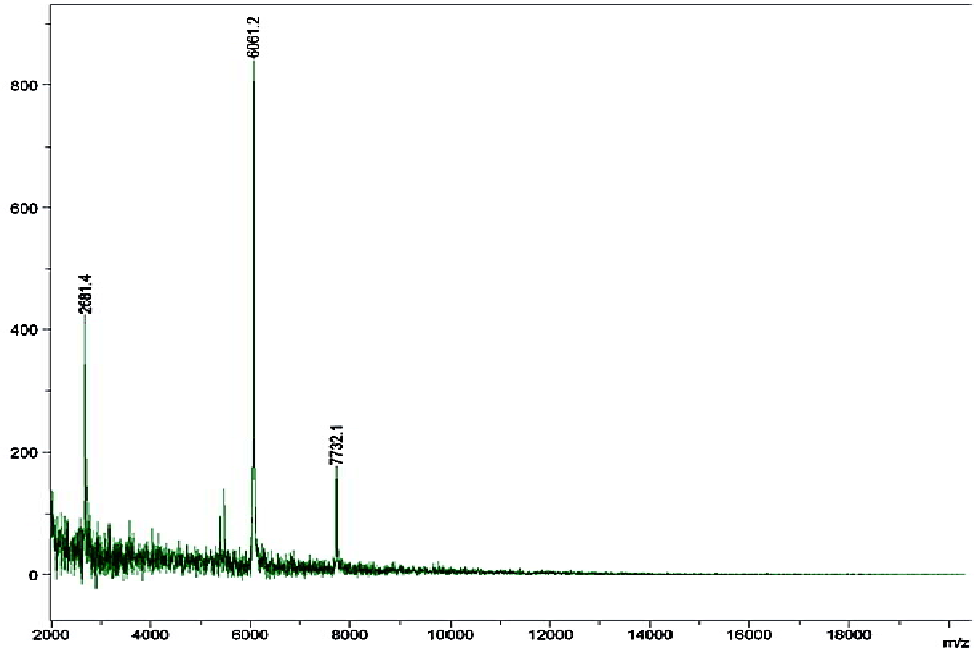


Fig. 5.9 MALDI TOF MS of purified sample of BpSl14

The molecular weights of many bacteriocins were identified by MALDI TOF MS. Subtilosin A1 has a molecular mass of 3412.5 Da as revealed by MALDI MS analysis (Sebei *et al*., 2007). Cerein MRX1, a heat-stable bacteriocin detected in a *B. cereus* isolate from water plant roots (Lewus *et al*., 1992), had a molecular mass of 3137.93 Da (according to MALDI-TOF MS analysis) was capable of withstanding exposure to a wide range of pH values. MALDI-TOF analysis of bacteriocin from *Weissella confusa* A3 of dairy origin gave a mass of 2.7 kDa (Goh and Philip, 2015). From literature it is noted that Class II bacteriocins of Gram positive bacteria are <10 kDa in size.

5.3.2 Characterization of bacteriocins

Bacteriocins are an extremely heterogeneous group of substances. Although chemically diverse, the one unifying property is the presence of an essential protein component. Chemical analyses indicate that some bacteriocins

may be simple proteins; while many others, including certain *Staphylococcal*, *Clostridial* and *Lactobacillus* bacteriocins may be quite complex molecules with lipid and/or carbohydrate components in addition to protein.

5.3.2.1 Effect of temperature on the activity of bacteriocins

Bacteriocin BaCf3 was observed to be highly thermostable, as 100 % activity was retained even after treatment at 121 °C for 15 min (Fig 5. 10). But the activity was reduced to 50 % after an extended temperature treatment at 70 °C to 100 °C for 1 h.

The stability of bacteriocin BpSl14 at various temperatures ranging from 40 ºC-100 ºC was studied. It was amazing that the bacteriocin retained complete activity even after incubating in these temperatures for 1 hour (Fig 5.10). The bacteriocin also retained 100 % of its activity after autoclaving at 15lbs, i.e. at 121 °C for 15 minutes.

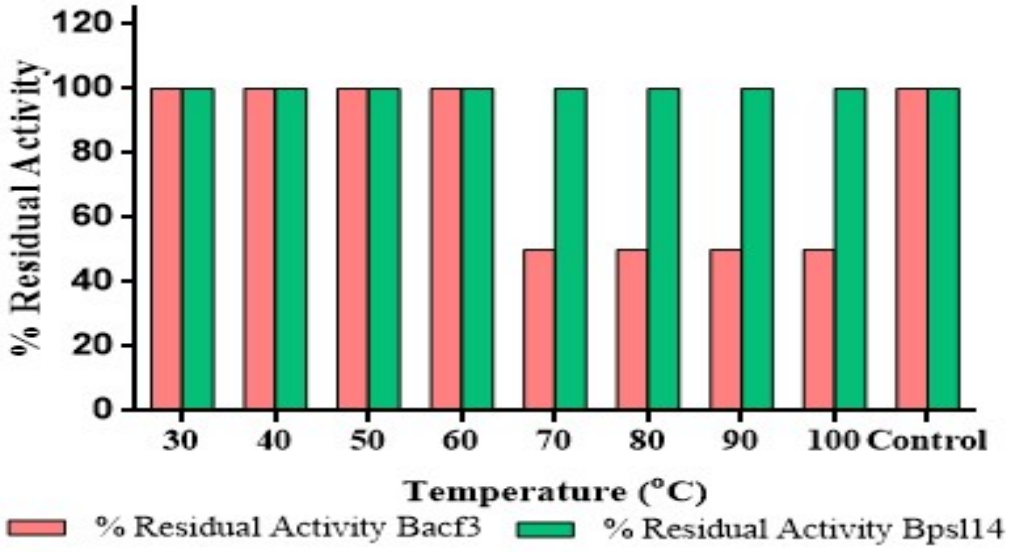


Fig. 5.10 Temperature stability of bacteriocins BaCf3 and BpSl14

Martirani *et al*. (2002) isolated a thermophilic strain of *Bacillus licheniformis* (from dairy products) which produced bacillocin 490, inactivated by both pronase E and proteinase K. Bactericidal activity of bacteriocin-like substance produced by *B. licheniformis* P40 was stable at all temperatures tested, except 121°C and 103.5 kPa for 15 min and was remarkably stable in a wide pH range (Cladera-Olivera *et al*., 2004b). Thermal stability studies of bacteriocin BL8 produced by *B. licheniformis* revealed loss of activity only when exposed to very high temperature 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 (Smitha and Bhat, 2013). Curvacin A was also stable at pH 4.8 to 7.0 and resistant to temperature of 100 °C for 3 min (Messens *et al*., 2003), while sakacin P was tolerant to 100 °C for 20 min. Criteria of thermostability of bacteriocins are difficult to define, particularly since this is dependent on the state of purification and also upon other factors such as pH, ionic strength, and presence of protective molecules (Albano *et al*., 2007). Although, bacteriocin of *L. murinus* AU06 was stable over temperature ranging from 30 - 80 °C, it retained only 60 % of its original activity at 60 °C for 30 min, and declined thereafter (Elayaraja *et al*., 2014). The antibacterial activity of Paracin 1.7 was not significantly affected by heat treatment at 40, 50, 60, 70, or 100 °C for 20 min; 82.48 % of its original inhibitory activity was retained even at 121 °C after 20 min. (Ge *et al*., 2016).

5.3.2.2 Effect of pH on bacteriocin activity

The bacteriocin BaCf3 retained its activity over a broad pH range (2.0 - 13.0) (Fig 5.11). Its activity was reduced to 50 % at very high pH of 11 to 13.

The BpSl14 sample showed no loss of activity in all different pH tested, indicating the broad range pH stability of this bacteriocin. Therefore it is clear that this bacteriocin can withstand both alkaline and acidic pH (Fig 5.11).

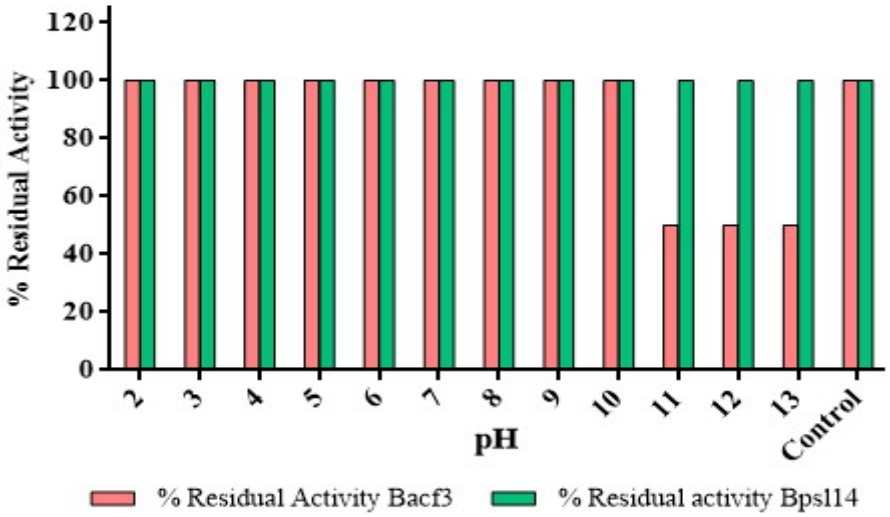


Fig. 5.11 Effect of pH on stability of bacteriocins. Both the bacteriocins are stable in a wide range of pH.

Bacteriocins differ greatly in their sensitivity to changes in pH. Most bacteriocins and bacteriocin-like substances are considerably more tolerant of acid than alkaline pH extremes (Ellison and Kautter, 1970). But both the bacteriocins, BaCf3 and BpSl14, were stable at all the pH tested, suggesting suitability for applications in acid and alkaline conditions.

Activity of BLIS produced by *Bacillus amyloliquefaciens* LBM5006 isolated from Brazilian Atlantic forest was retained over a pH range of 3.0−8.0, but lost at alkaline pH values (pH 9.0 and 10.0) (Lisboa *et al*., 2006). Bacteriocins BS101 and BL8 isolated from *Bacillus subtilis* BTFK101 and *Bacillus licheniformis* BTHT8 could tolerate a pH range of 2 - 9 (Smitha and Bhat, 2013). Many more pH tolerant bacteriocins or bacteriocin like substances were isolated such as BLIS from *B. subtilis* LFB112 (Xie *et al*., 2009), BLIS Bac 14B from *B. subtilis* 14B (Hammami *et al*., 2009), Lichenin from *B. licheniformis* 26L-10/3RA (Pattnaik *et al*., 2001) and so on. The BLIS isolated by Cladera-Olivera *et al*., (2004) from *Bacillus licheniformis* P40 could also retain 100% of its activity in a

wide range of pH (3 - 11). The temperature and pH stability of antimicrobial peptides were also reported for Pumilicin 4 by *B. pumilus* (Aunpad and Na-Bangchang, 2007).

5.3.2.3 Effect of metal ions on the activity of bacteriocins

Antibacterial activity of BaCf3 was nullified by the addition of Ca^{2+}, Mg^{2+} and Mn^{2+} salts. Ba^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+} and Al^{3+} also diminished the activity of BaCf3 (Fig 5.12).

Significant changes were not observed in the activity of BpSl14 after treatment with metal ions; except with Cu^{2+}, Mn^{2+} and Al^{3+} when activity was reduced to 50% for the treated samples (Fig 5.12).

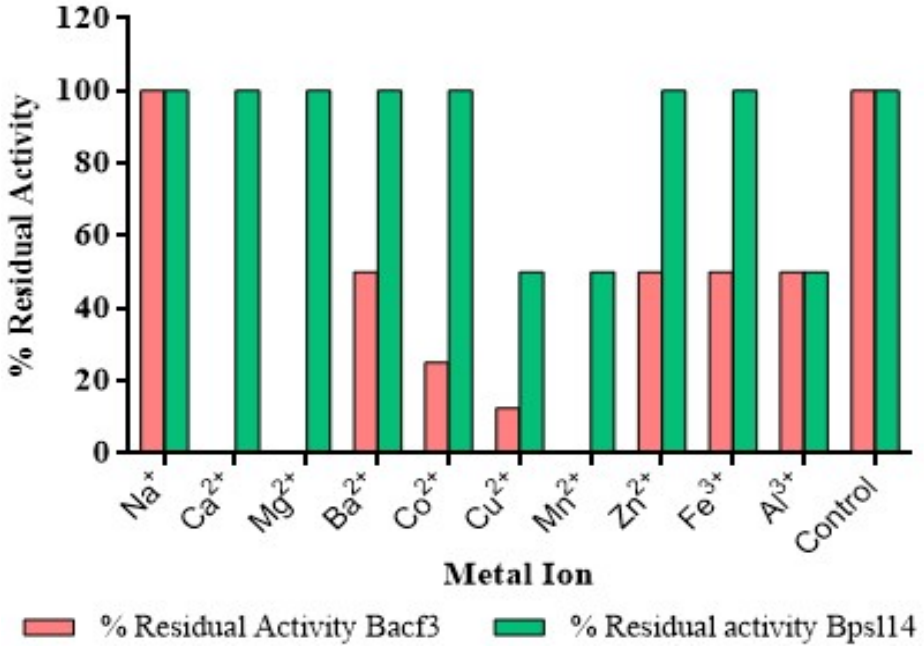


Fig.5.12 Effect of metal ions on the activity of bacteriocins

Thus we can conclude that the treatment of BaCf3 with alkaline earth metals (Ca^{2+}, Mg^{2+} and Ba^{2+}) and transition metals (Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+} and

Fe^{3+}) affected its activity. Al^{3+}reduced the activity of both the bacteriocins. $Cu^{2+}$and Mn^{2+} reduced the activity of BpSl14 to 50 %.

Metal atoms, especially the divalent ions, disturb salt bridges of proteins there by destabilize and precipitate them. Many metal ions can chelate amino acids of the bacteriocins and cause conformation changes. The effect of metal ions on bacteriocins are least studied, but their effect on enzymes and other biological peptides are well known.

It can be inferred that BpSl14 is more metal ion tolerant than BaCf3 which may be due to the presence of disulphide bridge in BaCf3 (Section 5.3.2.5 and 5.3.8). The broad spectrum bacteriocin isolated from *Bacillus subtilis* RLID12.1 also retained 100 % of its activity when treated with eight different metal ions (Ramachandran *et al*., 2014).

5.3.2.4 Effect of hydrolytic enzymes on bacteriocins

The activity of bacteriocin BaCf3 was affected by all the enzymes used. Activity was completely lost on treatment with trypsin and proteinase K, while fifty percent bacteriocin activity was retained with treatment of 1 mg/mL pepsin. On the other hand, 75 % activity was lost on treatment with α-amylase (1 mg/mL), where after, with increasing enzyme concentrations, activity disappeared completely (Fig 5.13A).

Bacteriocin BpSl14 was more stable than BaCf3 to enzyme treatment. Pepsin affected the bacteriocin even at a low concentration of 1 mg/mL. All other enzymes reduced the activity of BpSl14 to 50 % at a concentration of 5 mg/mL (Fig 5.13B).

Trypsin cleaves peptide chains at the carboxyl side of the amino acids lysine or arginine when not followed by proline. Pepsin is efficient in

cleaving peptide bonds between hydrophobic and aromatic amino acids such as phenylalanine, tryptophan, and tyrosine preferentially after the N-terminal. The predominant site of cleavage of proteinase K is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups. Action of α-amylase shows the presence of carbohydrates in the active site of the bacteriocins.

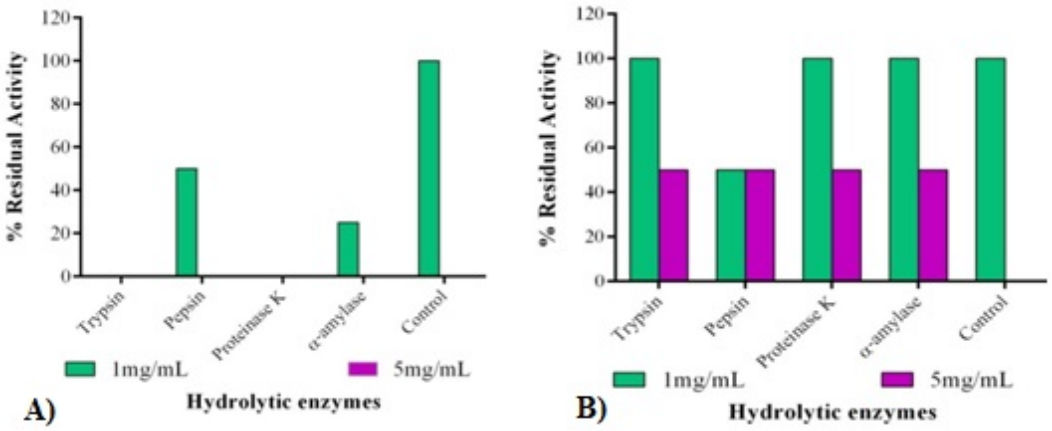


Fig. 5.13 Effect of hydrolytic enzymes on the activity of bacteriocins A) BaCf3 B) BpSl14.

Tests of sensitivity to specific enzymes (proteinases, amylases, etc.) are often used to identify important chemical components of bacteriocin molecules. The sensitivity of bacteriocin BaCf3 towards proteolytic enzymes proved its proteinaceous nature. The action of trypsin on BaCf 3 pointed to the presence of Argenine or Lysine residues in the peptide. Trypsin is a paradigm for the family of serine proteases that have evolved to cleave peptide bonds after Arg and Lys amino acid residues. But the sensitivity of BaCf3 to amylase indicates the presence of carbohydrates as a component of this bacteriocin. The loss of antibacterial activity of some bacteriocins following treatment with either carbohydrate- or lipid-hydrolyzing enzymes has been considered in some studies to be a sign of a carbohydrate or lipid moiety which is essential for antibacterial

activity. According to Klaenhammer (1993), such bacteriocins are classified as class IV, complex bacteriocins that contain essential lipid or carbohydrate moieties in addition to protein. An essential glycosidic bond was identified in plantaricin S, leuconocin S, lactocin 27 and pediocin SJ-1 bacteriocins. Its stability in pepsin and at low pH indicated its potential probiotic applications (Shukla and Goyal, 2014). While bacteriocin of *L. murinus* AU06 was completely inactivated by proteinase K, chymotrypsin, trypsin and pepsin confirming its proteinaceous nature (Elayaraja *et al*., 2014). Bacteriocin from dairy origin *Weissella confusa* A3 showed reduced activity after treatment with proteinase K, trypsin and peptidase (Goh *et al*., 2015). Paracin 1.7 produced by *L. paracasei* HD1-7 was degraded by trypsin and proteinase K, moderately sensitive to papain and insensitive to amylase (Ge *et al*., 2016). There was no loss of activity of *Bacillus subtilis* RLID 12.1 bacteriocin upon exposure to pronase E, trypsin, α-amylase, and lipase at a concentration of 10 mg/mL. However the 72 % activity was lost when treated with 10 mg/mL proteinase K (Ramachandran *et al*., 2014).

5.3.2.5 Effect of oxidizing and reducing agents

The effect of oxidizing and reducing agents on bacteriocin activity was analyzed by incubating the sample with 50 mM and100 mM concentration of DTT, β-mercaptoethanol and DMSO at 4 ºC for 1 hour. There was no observable reduction in the activity of BpSl14 after the treatment (Table 5.4). But the activity of BaCf3 was reduced due to the treatment.

Table 5.4 Effect of oxidising and reducing agents on the activity of bacteriocins

	Oxidising and reducing agents	% Residual activity in 50 mM	% Residual activity in 100 mM	% Residual activity Control
BpSl14	DMSO	100	100	100
	β-mercaptoethanol	100	100	100
	DTT	100	100	100
BaCf3	DMSO	12.5	12.5	100
	β-mercaptoethanol	12.5	12.5	100
	DTT	12.5	No activity	100

From the table 5.3 we can conclude that the activity of BaCf3 was affected by both oxidizing agents and reducing agents, and a higher concentration (100 mM) of DTT caused complete loss of activity which points to the presence of a crucial disulphide bond in the active region of the bacteriocin, whose reduction diminished the activity. This was supported by the *de novo* sequencing data (Section 5.3.4). The loss of activity on treatment with mild oxidizing agent DMSO also indicates of the presence of oxidation labile amino acids like asparagine, glutamine and methionine in its active site. The resistance of BpSl14 may be attributed to the fold stabilization of the molecule. The folding stabilization may be due to the formation of hydrogen bonds and Vander Waals forces within the molecule. The solvent accessible areas are more in BaCf3 than in BpSl14. This accounts for the higher stability of BpSl14 than BaCf3.

5.3.3 Mechanism of action of bacteriocins

Microscopy techniques are suitable to study the behaviour of bacteriocins in intact cells. Combination of fluorescence techniques (CLSM) with electronic microscopy can give a very detailed picture of the mechanism of action of these

peptides. Transmission (TEM) and scanning electron microscopy (SEM) are conventional procedures that allow visualizing morphological changes at the membrane and cell wall ultrastructure at native conditions, while confocal microscopy provides, at a first glance, an overall picture of the viability of cells.

5.3.3.1 Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of BaCf3 and BpSl14 against various test organisms were determined by microtitre plate assay incorporating resazurin. *E. faecalis* required minimum amount of bacteriocins to inhibit its growth, where as *Proteus vulgaris* required highest concentration of bacteriocins. *E. coli* was not inhibited by BaCf3 and hence could not find out MIC for it. A detailed report of MIC of both the bacteriocins is given in table 5.5.

Table 5.5 Minimum Inhibitory Concentration (MIC) of the bacteriocins

Bacteria	BpSl14 (µg/mL)	BaCf3 (µg/mL)
Bacillus circulans	43.63±0.18	1.84±0.005
Bacillus macerens	87.25±0.35	7.36±0.228
Staphylococcus aureus	87.25±0.35	1.84±0.005
Enterococcus faecalis	0.69±0.01	0.014±0.001
Escherichia coli	174.50±0.70	-
Proteus vulgaris	174.50±0.70	7.36±0.228

5.3.3.2 Bactericidal/Static mode of action

The effect of bacteriocins on the growth of different organisms was studied by incubating 0.1 OD_{600} culture of test organisms with bacteriocins at

their MIC concentration for six hours. The % inhibition was calculated for each organism and a graph was plotted.

From the graph (Fig 5.14), it was evident that ≥80 % inhibition in growth of *B. circulans*, *S. aureus* and *P. vulgaris* by both bacteriocins. We can infer from that the bacteriocins have a bactericidal mode of action on these organisms. Both BaCf3 and BpSl14 act on *E. coli, E. feacalis* and *B. macerans* in a bacteriostatic manner. Thus the mechanism of action of bacteriocin can be concluded as bacteriostatic for *E. coli, E. faecalis* and *B. macerans* and bactericidal for *B. circulans, S. aureus* and *P. vulgaris*.

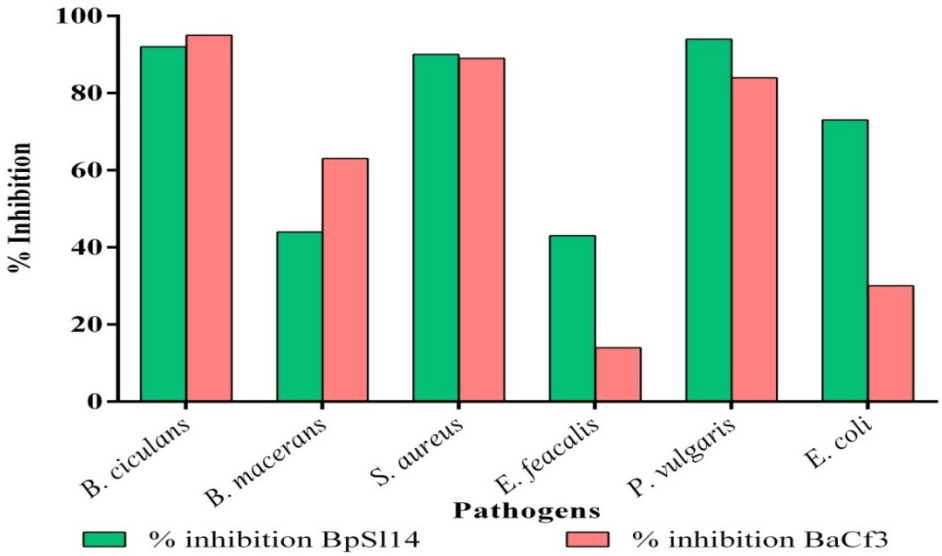


Fig 5.14 Inhibition assay of bacteriocins on test organisms. The % inhibition was plotted on Y axis and organisms on X axis.

Most of the bacteriocins are reported to have bactericidal mode of action. Mesentericin Y105 was shown to exhibit a bactericidal mode of action towards *L. monocytogenes* (Maftah *et al*., 1993). Plantaricin A, a 26 amino acid peptide synthesized by *L. plantarum* C11 exhibits both bactericidal and pheromone activities (Hauge *et al*., 1998). Subtilosin A (molecular mass of 3399.7 Da),

produced by *Bacillus* sp., showed by ATP efflux assay the bactericidal activity against *Listeria monocytogenes*, *Gardnerella vaginalis* and *S. agalactiae* and also against Gram-negative bacteria (Sutyak *et al*., 2008b).

5.3.3.3 Action of bacteriocins on bacterial membrane

5.3.3.3.1 Scanning electron microscopy

The gold plated cover slips were observed under 15000X magnification. The changes in cell morphology in control and treated cells were compared (Fig.15 and Fig. 16). From the SEM analysis, it can be visualized that the cell wall of organism were deformed in the treated samples indicating the action of bacteriocins on cell wall (Fig 5.15 B and C; Fig 5.16 B and C). After 1h of incubation with the bacteriocins, the cell wall deformation is clearly visible and after 2 hours the cell wall ruptures in both the cases.

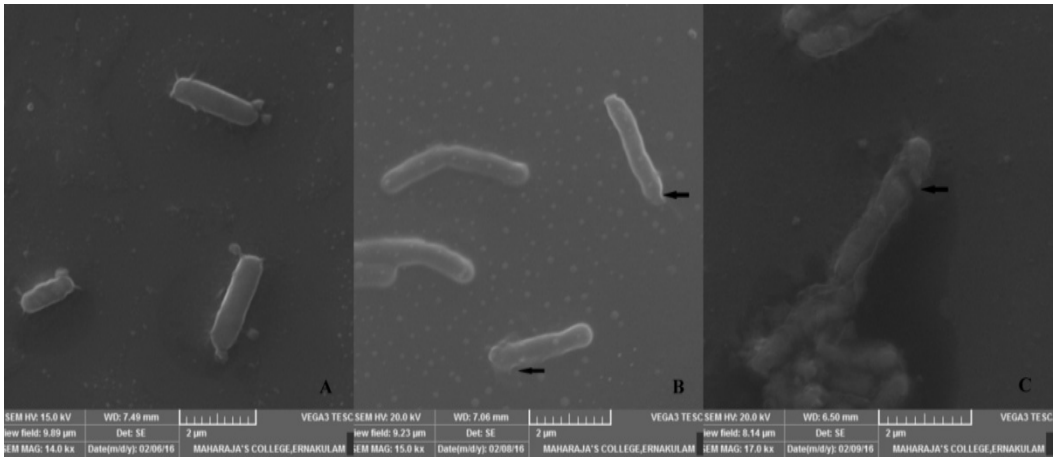


Fig. 5.15 Action of BaCf3 on test organism by SEM. A) Control cells of *B. circulans* B) and C) After treatment with bacteriocin for 1h and 2 h respectively. The image was captured at a magnification of 15000X.

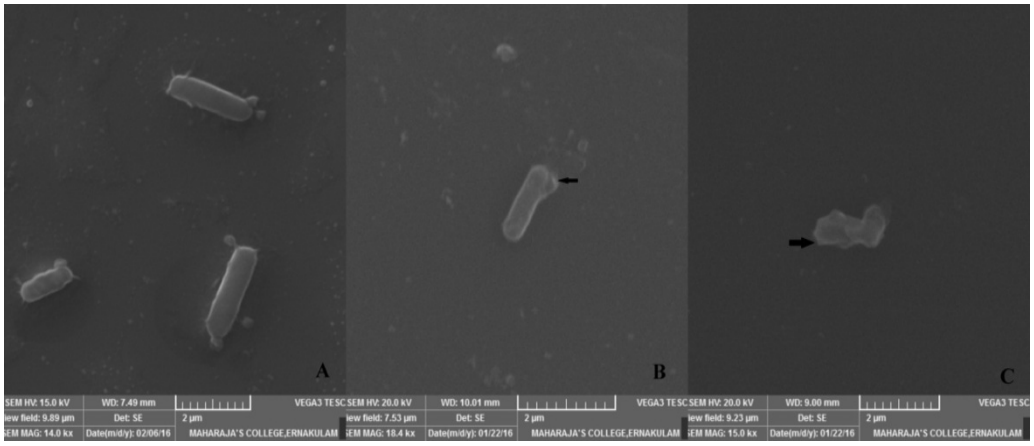


Fig. 5.16 Action of BpSl14 on test organism by SEM. A) Control cells of *B. circulans* B) and C) After treatment with bacteriocin for 1h and 2 h respectively. The image was captured at a magnification of 15000X.

From the scanning electron micrographs, it can be noted that the deformation of target cells occurred in a time-dependent manner. Cells appeared collapsed, flat, and empty after the bacteriocin treatment for 2 h (Fig 5.16C). All these changes may reflect the efflux of the cell content through the pores created in the cell membrane. Similar observations were attained after the action of a nisin and pediocin mixture on *Listeria monocytogenes* (Kalchayanand *et al*., 2004). The morphological changes visualized by scanning electron microscopy showed that BaCf3 and BpSl14 act as bactericidal bacteriocins on *Bacillus circulans* thus confirming the results of inhibition assay.

Subtilin exhibits bactericidal activity based on pore formation in the cytoplasmic membrane. Sublancin 168 (3877.78 Da) is a lantibiotic produced by *B. subtilis* 168 which exhibits bactericidal activity against other Gram-positive bacteria, including pathogens such as *B.cereus, Streptococcus pyogenes* and *Staphylococcus aureus* (Paik *et al.*, 1998; Stein, 2005). Sonorensin, a bacteriocin obtained from *Bacillus sonorensis* MT93 isolated from marine soil sample, induced an increase in the permeability of *S. aureus* cytoplasmic

membrane over time. SEM image of sonorensin treated *S. aureus* displayed roughening of cell surface with accumulation of cell debris (Chopra *et al*., 2015). To gain insight into the mode of bactericidal action of the laterosporulin on indicator strain, Singh *et al*. (2012) performed scanning electron microscopy (SEM) of *E. coli* treated with lethal dose of bacteriocin. They have also noticed roughening of cell surface and accumulation of cell debris.

5.3.3.3.2 Transmission electron microscopy

Morphological changes were better observed with TEM micrographs. The changes in the cell wall integrity and formation of ghost cells could be clearly visualized (Fig 5.17 and Fig 5.18). The accumulation of membranous structures in the cytoplasm of the treated cells is visible in images. Disruptions with release of intracellular material associated with cells losing their cytoplasm (empty and flaccid cells) were also observed. The distortion of the physical structure of the cell caused the expansion and destabilization of the membrane and increased membrane fluidity, which in turn increases the passive permeability and manifest as a leakage of various vital intracellular constituents, such as ions, ATP, nucleic acids, sugars, enzymes and amino acids. Cell ghost is an empty intact cell envelope structure devoid of cytoplasmic content including genetic material.

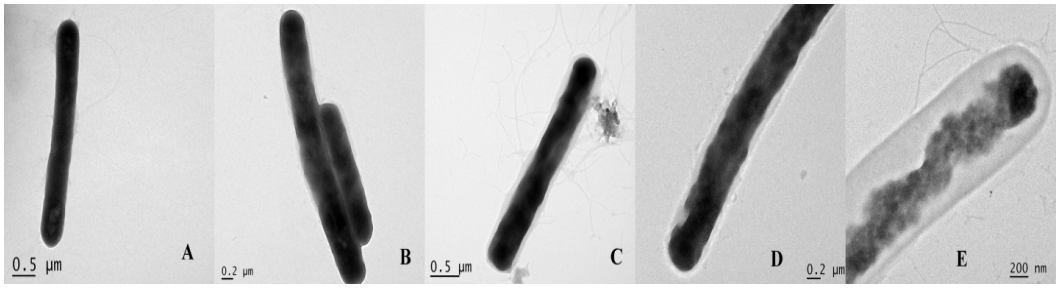


Fig. 5.17 Transmission electron microscopy showing different stages of bacteriocin BaCf3 action. The change in cell wall integrity is clearly visible. C) Shows the release of cell contents. E) Shows the ghost cell.

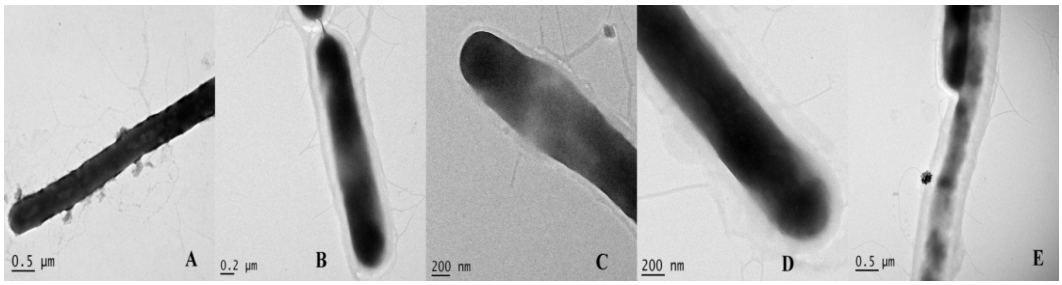


Fig. 5. 18 Transmission electron microscopy showing different stages of bacteriocin BpSl14 action. The changes in cell wall integrity is clearly visible

The cell membrane separated from the cell wall and part of the cytoplasm appears concentrated and dispersed (Fig. 5.17E and 5.18E). After treatment for 2 h, the cell surface collapsed with obvious debris around the cell. The microscopic studies show that the treatment of bacteriocins affects the membrane integrity of the target cells. Transmission electron microscopy (TEM) was used to study the mechanism of action of BLS P34 on *L. monocytogenes* and was indicative of cytoplasmic membrane alteration (Motta *et al*., 2008). Transmission electron microscopy also showed cell lysis of *B.s cereus* and *Listeria monocytogenes* after treatment with cerein 8A (Bizani *et al*., 2005). Similarly, nisin, pediocin, and epidermin, and many other amphiphilic antimicrobial peptides which belong to Type A lantibiotics, exert their activity by disrupting the functional barrier of microbial cytoplasmic membranes (Dalmau *et al*., 2002; McCafferty *et al*., 1999). The hydrophobic bacteriocin cerein 7, produced by *B. cereus* Bc7, has also been characterized as a membrane-active compound (Oscáriz *et al*., 1999).

5.3.3.3.4 Confocal laser microscopy (CLSM)

Confocal laser microscopy (CLSM) showed that the membrane damage occurs after 30 minutes of treatment. The red fluorescence was clearly visible in the treated cells indicating the membrane damage and To-pro-3 binding to the DNA (Fig 5.19). The pixel intensity also increased in the treated samples.

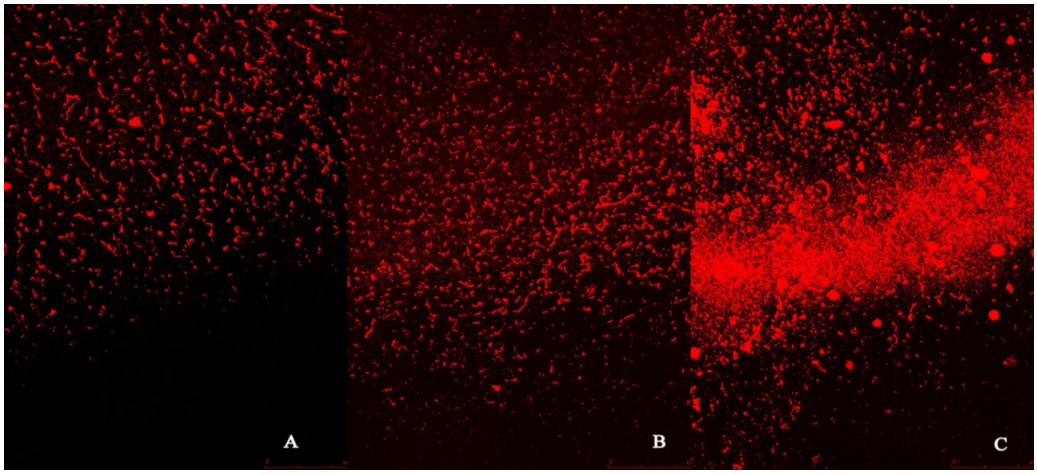


Fig 5.19 Confocal imaging to study the action of bacteriocins on membranes of *Bacillus circulans*. A) Untreated *B. circulans* B) BaCf3 treated *B. circulans* C) BpSl14 treated *B.s circulans*. Membrane disruption occurred after 30 minutes of incubation with bacteriocins. This can be observed by increase in pixel intensity of treated samples due to the binding of To-Pro-3 on DNA of membrane compromised cells (scale bar - 250 μm).

Viable cells having intact membrane do not allow penetration of ToPro3 and thus are not stained, where as dead cells or cells with permeable and disrupted membrane give a fluorescent signal due to penetration of the dye and intercalation into cellular DNA or RNA. Thus the pixel intensity obtained is a measure of permeability of the target cells.

Sample	Pixel Intensity
Control	4.312±0.776
BaCf3	12.98±1.131
BpSl14	16.525±3.077

The assessment of bacterial cell viability by confocal microscopy is a good indicator to characterize the action of bacteriocins (Torrent *et al*., 2010). One of the advantages of CLSM is that statistical analysis can be performed in

order to derive quantitative measurements about viability by measuring the pixel intensity.

5.3.4 *De novo* sequencing by MS/MS

From the MS/MS pattern of BaCf3, it was observed that trypsin digested the proteins in to seven fragments having mass of 1173, 1304, 1640, 2451, 2474, 2632, 2654 (Fig 5.20).

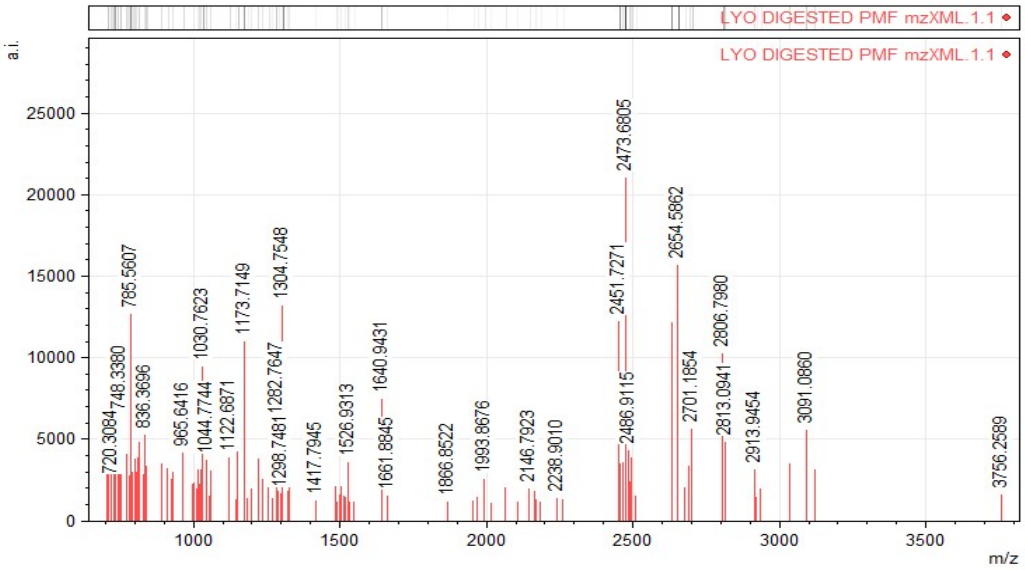


Fig. 5.20 PMF of BaCf3 from mMass

From the MS/MS pattern of 1173 and 1304 (Appendix IV), the partial peptide sequences were derived by manual calculation (Hunt *et al*., 1992). The other fragments, 2451, 2474, 2632, 2654, were overlapping and hence made the manual sequencing process difficult. The intensity of fragment 1640 was less hence omitted from sequence derivation.

The amino acid sequence of BaCf3 was derived as GNHDCCMGQ LPKCMLGPGQGAATXQGK. The primary structure of BaCf3 was created using Discovery Studio 2016, is shown in fig. 5.21. The sequence could not find any

match in BLAST showing its novelty. MALDI-TOF MS/MS was used for partial sequencing of acidocin D20079 (Deraz *et al*., 2005).

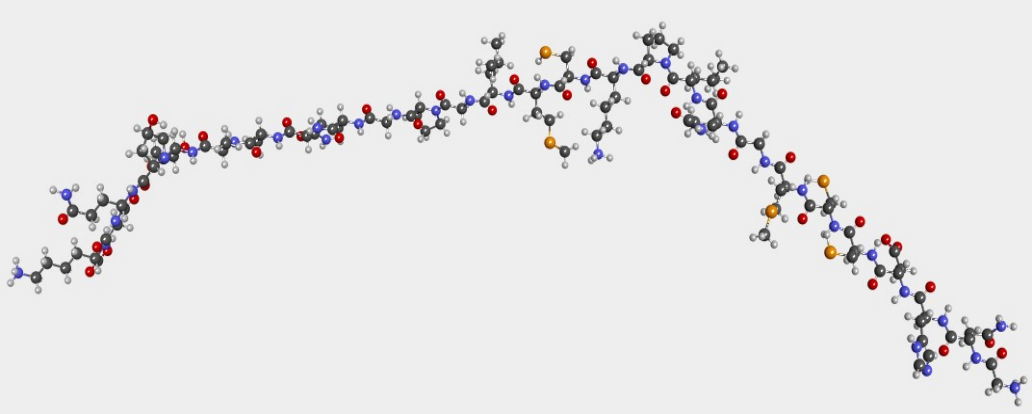


Fig 5.21 Primary structure of BaCf3. Blue indicates Nitrogen atoms, black balls indicate Carbon atoms, red balls indicate Oxygen atoms, orange balls indicate Sulphur atoms, and small grey balls indicate hydrogen atoms.

From the MS/MS pattern of BpSl14, it was found that trypsin digested the protein in to seven fragments such as 1769, 1941, 1997, 2083, 2569 (Fig 5.22).

The partial sequence of BpSl14 was derived from 1769 and 1941 fragments (Appendix IV). In the PMF of BpSl14, we can see that the peaks of large molecular weight are missing even though the molecular weight of the bacteriocin is 6061 Da. This may be due to the presence of Tween 80 in the production medium. This may get co-purified with the bacteriocin and interfere with mass spectrometry analysis leading to suppression of the peptide and protein signals (Deraz, *et al*., 2005). This may also be the case for tryptic digest and hampered sequence analysis of the purified sample, limiting its success to a partial sequence.

The sequence obtained for BpSl14 was AGGGFMEHEGSEPVGPGGP-KGDHMQDGKIPNDLCC which could be aligned to some known amino acid sequences of bacteriocins. But the peaks in the MS/MS spectrum of BpSl14 were

not pronounced enough to derive further sequences. Though Tween 80 is an essential component for BpSl14 production, it interferes with mass spectrometry analysis leading to suppression of peptide signals.

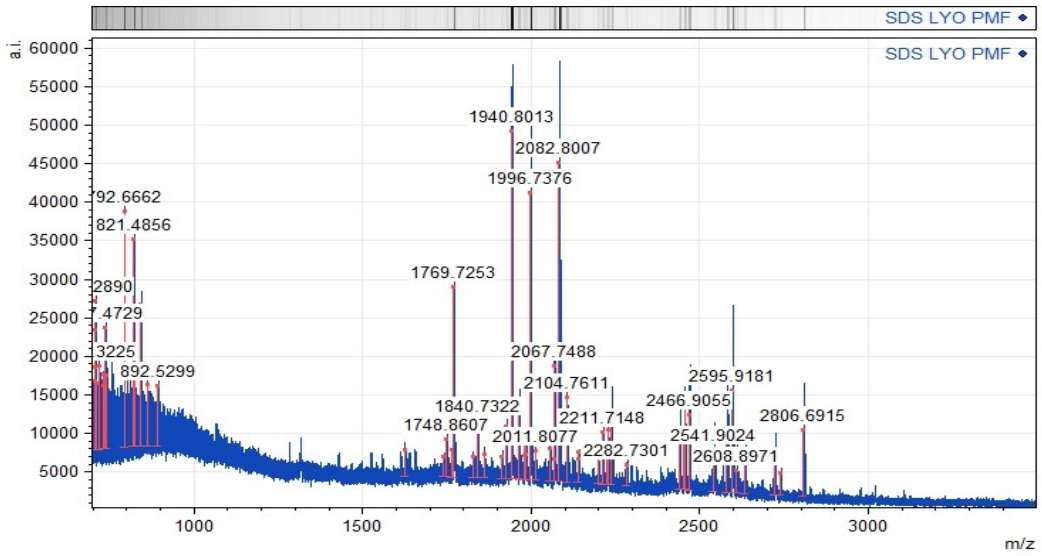


Fig 5.22 PMF of BpSl14 from mMass

The primary structure of BpSl14 constructed in Discovery Studio 2016, is shown in fig. 5.23. But the sequence could not find any match in BLAST showing its novelty.

The fragment ion calculator server gave the result for BaCf3 sequence as shown in Fig 5.24, indicating that the MS/MS peaks and the fragments obtained from the calculator coincides and the derived sequence is of least error.

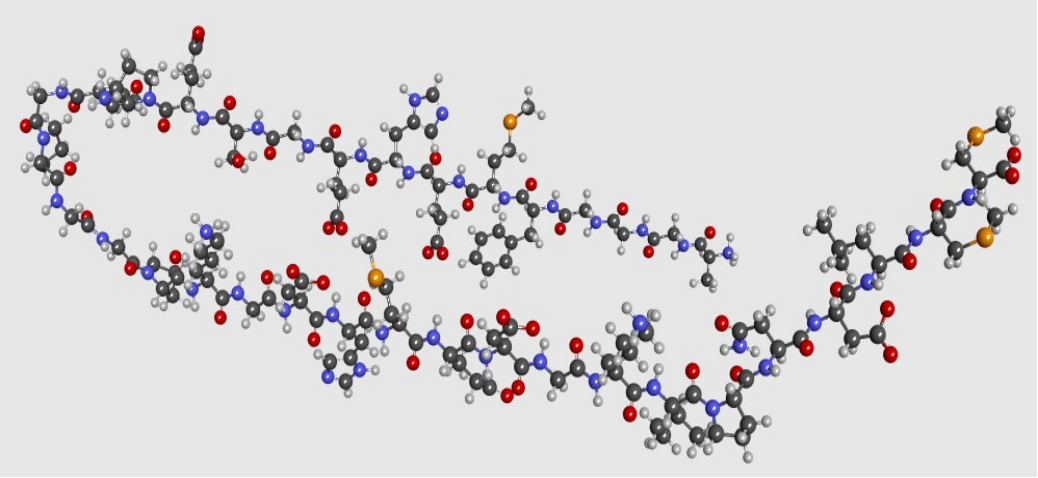


Fig 5.23 Primary structure of BpSl14. Blue indicates Nitrogen atoms, black balls indicate Carbon atoms, red balls indicate Oxygen atoms, orange balls indicate Sulphur atoms, and small grey balls indicate hydrogen atoms.

Fragment Ion Calculator Results

Sequence: **GNHDCCMGQLP**, pI: **5.07632**

Fragment Ion Table, monoisotopic masses

Seq	#	B	Y	# (+1)
G	1	58.02879	1174.44386	11
N	2	172.07171	1117.42239	10
H	3	309.13062	1003.37946	9
D	4	424.15757	866.32055	8
C	5	527.16675	751.29361	7
C	6	630.17594	648.28442	6
M	7	761.21642	545.27524	5
G	8	818.23789	414.23475	4
Q	9	946.29646	357.21329	3
L	10	1059.38053	229.15471	2
P	11	1156.43329	116.07065	1

Fragment Ion Calculator Results

Sequence: **CMLGPGQGAATXQG**, pI: **5.51820**

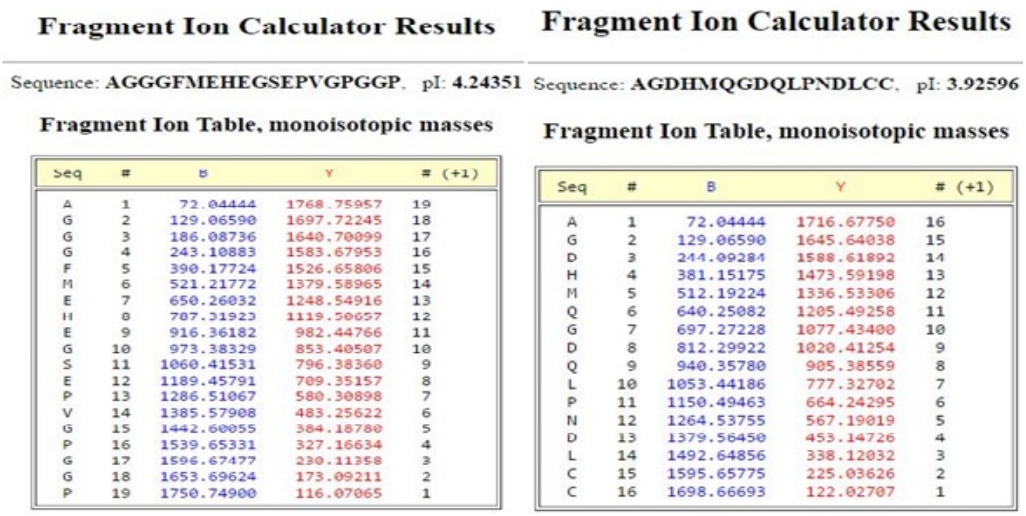
Fragment Ion Table, monoisotopic masses

Seq	#	B	Y	# (+1)
C	1	104.01651	1303.61336	14
M	2	235.05699	1200.60418	13
L	3	348.14106	1069.56369	12
G	4	405.16252	956.47963	11
P	5	502.21528	899.45816	10
G	6	559.23675	802.40540	9
Q	7	687.29532	745.38394	8
G	8	744.31679	617.32536	7
A	9	815.35390	560.30390	6
A	10	886.39101	489.26678	5
T	11	987.43869	418.22967	4
X	12	1100.52276	317.18199	3
Q	13	1228.58133	204.09793	2
G	14	1285.60280	76.03935	1

Fig 5.24 Screen shot of Fragment Ion Calculator for the derived sequence of BaCf3

While validating the BpSl14 sequence, the fragment ion calculator server gave the result as shown in fig 5.25 indicating that the MS/MS peaks and the fragments obtained from the calculator coincide and the derived sequence is of

least error. The slight change in the fragment ion values is due to the S-methylation of Cysteine. The presence of unusual residues like SMC (S-methylated Cysteine) adds to the presence of bioactive property.



Fragment Ion Calculator Results

Sequence: **AGGGFMEHEGSEPVGPGGP**, pI: **4.24351**

Fragment Ion Table, monoisotopic masses

Seq	#	B	Y	# (+1)
A	1	72.04444	1768.75957	19
G	2	129.06590	1697.72245	18
G	3	186.08736	1640.70099	17
G	4	243.10883	1583.67953	16
F	5	390.17724	1526.65806	15
M	6	521.21772	1379.58965	14
E	7	650.26032	1248.54916	13
H	8	787.31923	1119.50657	12
E	9	916.36182	982.44766	11
G	10	973.38329	853.40507	10
S	11	1060.41531	796.38360	9
E	12	1189.45791	709.35157	8
P	13	1286.51067	580.30898	7
V	14	1385.57908	483.25622	6
G	15	1442.60055	384.18780	5
P	16	1539.65331	327.16634	4
G	17	1596.67477	230.11358	3
G	18	1653.69624	173.09211	2
P	19	1750.74900	116.07065	1

Fragment Ion Calculator Results

Sequence: **AGDHMQGDQLPNDLCC**, pI: **3.92596**

Fragment Ion Table, monoisotopic masses

Seq	#	B	Y	# (+1)
A	1	72.04444	1716.67750	16
G	2	129.06590	1645.64038	15
D	3	244.09284	1588.61892	14
H	4	381.15175	1473.59198	13
M	5	512.19224	1336.53306	12
Q	6	640.25082	1205.49258	11
G	7	697.27228	1077.43400	10
D	8	812.29922	1020.41254	9
Q	9	940.35780	905.38559	8
L	10	1053.44186	777.32702	7
P	11	1150.49463	664.24295	6
N	12	1264.53755	567.19019	5
D	13	1379.56450	453.14726	4
L	14	1492.64856	338.12032	3
C	15	1595.65775	225.03626	2
C	16	1698.66693	122.02707	1

Fig 5.25 Screen shot of Fragment Ion Calculator for the derived sequence of BpSl14.

5.3.5 Multiple sequence alignment

The derived amino acid sequence of BaCf3 was aligned by Clustal w, with MEGA 6.0 to some of the known bacteriocin sequences and the alignment is shown in fig. 5.26, but sequence similarity could not be found. The derived sequence of BpSl14 was aligned with MEGA 6.0 to some of the known bacteriocin sequences and the alignment is shown in fig 5.27.

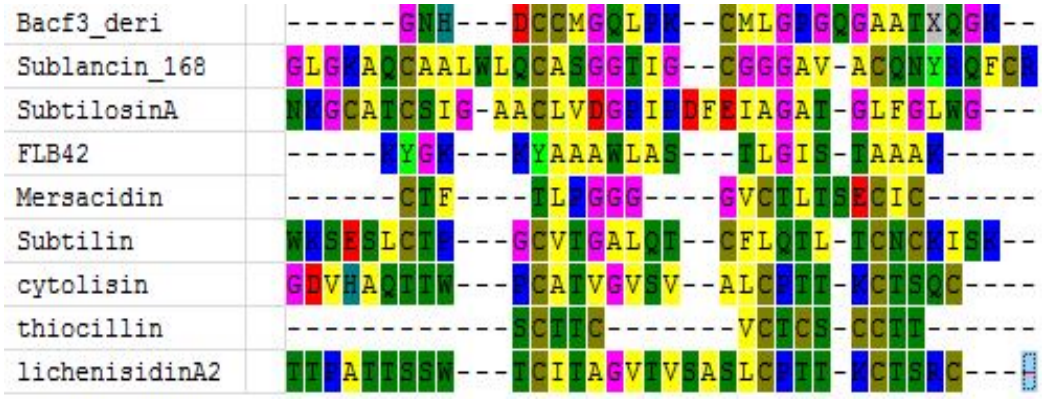


Fig 5.26 Alignment of BaCf3 sequence with some bacteriocin sequences.

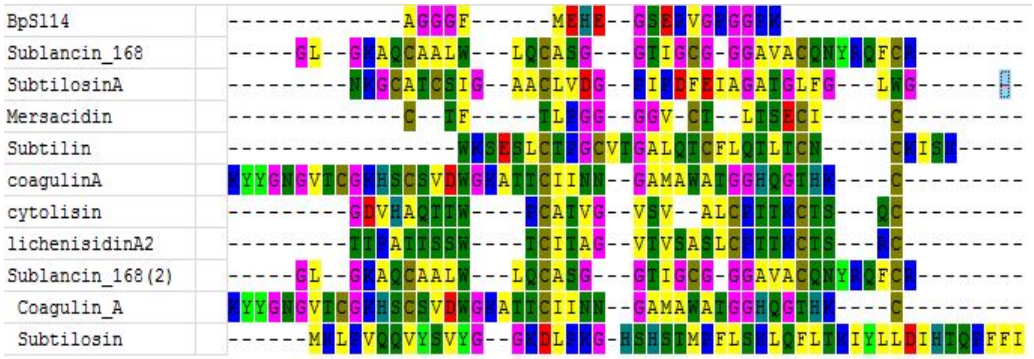


Fig 5.27 Alignment of BpSl14 sequence with some bacteriocin sequences.

5.3.6 Amino acid composition of bacteriocins

The analysis of sequence composition by ProtParam tool shows that the most prominent amino acid in both the bacteriocins was Glycine, 22.5 % in BaCf3 and 25.7 % in BpSl14. Cysteine and Glutamine form the next predominant amino acid of BaCf3 while Proline was the second predominant amino acid of BpSl14. The results of amino acid composition of bacteriocins are detailed in Table 5.6.

Table 5.6 Amino acid composition of bacteriocins as calculated by ProtParam tool

Amino Acid	BaCf3 (%)	BpSl14 (%)
Ala (A)	7.4	2.9
Arg (R)	0	0
Asn (N)	3.7	2.9
Asp (D)	3.7	8.6
Cys (C)	11.1	5.7
Gln (Q)	11.1	2.9
Glu (E)	0	8.6
Gly (G)	22.2	25.7
His (H)	3.7	5.7
Ile (I)	0	2.9
Leu (L)	7.4	2.9
Lys (K)	7.4	5.7
Met (M)	7.4	5.7
Phe (F)	0	2.9
Pro (P)	7.4	11.4
Ser (S)	0	2.9
Thr (T)	3.7	0
Trp (W)	0	0
Tyr (Y)	0	0
Val (V)	0	2.9
Pyl (O)	0	0
Sec (U)	0	0
X	3.7	0

Most of the AMPs are made up of hydrophilic and hydrophobic regions. The N terminal eighteen (from A1 to G18) amino acids of BpSl14 form the hydrophilic region of bacteriocin while the C terminal seventeen residues (from P19 to C35) is neutral or slightly hydrophobic in nature. The hydrophilic N-terminal region interacts with the negatively charged phospholipids of the target membrane and the hydrophobic region helps in the pore formation in cytoplasmic membrane (Oscariz and Pisabarro, 2010).

5.3.7 Secondary and tertiary structure prediction from the derived partial sequence

The secondary structure of BaCf3 as predicted by I-TASSER shows that the sequence contain helix, coils and strands. The high score shows more confidence in the secondary structure prediction (Fig 5.28).

```
                          20
                          |
Sequence    GNHDCCMGQLPKCMLGPGQGAATCQGK
Prediction  CCCCCCCCCCCCHHSSCCCCCCCCCCCC
Conf.Score  984431554741110588774321579
            H:Helix; S:Strand; C:Coil
```

Fig 5.28 Secondary structure of BaCf3

The secondary structure of BpSl14 as predicted by I-TASSER shows that the sequence contain only coils. The high score shows more confidence in the secondary structure prediction (Fig 5.29).

```
                         20
                         |
Sequence    AGGGFMEHEGSEPVGPGGPKGDHMQDGKIPNDLCC
Prediction  CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
Conf.Score  986543224777668888876533247687645 59
            H:Helix; S:Strand; C:Coil
```

Fig 5.29 Secondary structure of BpSl14

The tertiary structures of both the bacteriocins were predicted using I-TASSER. For each target, I-TASSER simulations generate a large ensemble of structural conformations, called decoys. To select the final models, I-TASSER uses the SPICKER program to cluster all the decoys based on the pair-wise structure similarity, and reports up to five models which corresponds to the five largest structure clusters. The confidence of each model is quantitatively measured by C-score that is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of [-5, 2], where a C-score of a higher value signifies a model with a higher confidence and vice-versa. The predicted models of BaCf3 are shown in Fig 5.30.

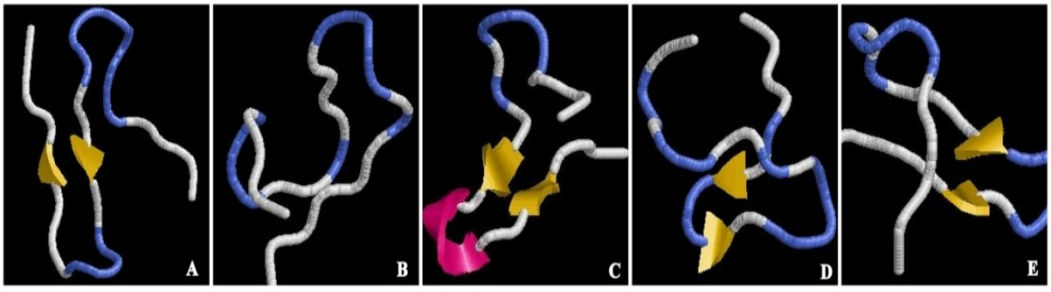


Fig 5.30 Models of BaCf3 as predicted by I-TASSER. A) Model 1, B) Model 2, C) Model 3, D) Model 4, E) Model 5. The C-score of the models 1-5 were -1.78, -2.56, -2.90, -2.64, -3.26

The predicted tertiary structures of bacteriocin BpSl14 is shown in fig 5.31. Tertiary structure of BpSl14 consists of α-helix and extended backbone. Among the predicted models of the bacteriocins, Model 1 is having the highest C-score value and hence high quality.

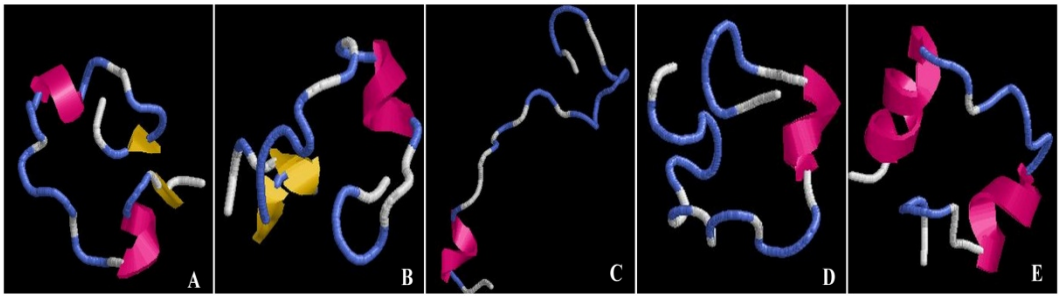


Fig 5.31 Models of BpSl14 as predicted by I-TASSER. A) Model 1, B) Model 2, C) Model 3, D) Model 4, E) Model 5. The C-score of the models 1-5 were -2.35, -2.72, -4.25, -3.23, -2.91

β- sheeted polyphemusin (PDB code 1RKK) like structure (Jenssen *et al*., 2006) of BaCf3 can be clearly visualized in the models predicted by I-TASSER. Polyphemusin is a horse shoe crab peptide, with antimicrobial property. Polyphemusin is a membrane partitioning bioactive molecule and the resemblance in the tertiary structure sheds light to the mechanism of action of the bacteriocin BaCf3 (Powers *et al*., 2005).

This structure is a combination of that of α-helical megainin-2 (PDB code 2MAG) and extended indolicidin (PDB code 1G89) (Rozek *et al*., 2000). Both these bacteriocins act on cytoplasmic membrane thus illuminates the mechanism of action of the bacteriocin.

5.3.8 Prediction of disulphide bridge

DiANNA 1.1 predicted the possibility of disulphide formation in BaCf3 between C6 and C13 (Fig 5.32), but there is no chance for disulphide formation in BpSl14 since it is having a methylated sulphur. The score for disulphide bridge formation between C6 and C13 is 0.45667.

Many of the biologically active peptides contain one or multiple disulphide bridges, which are believed to contribute to the peptides' stability and

activity. In BaCf3, a disulphide linkage can be formed between 6-13 Cysteine as in model 1. Disulphide bonds or the ability to form dimmers by forming disulphide bond accounts for the heat stability of bacteriocins. Disulphide bonds help in the stabilization of tertiary structures by reducing the number of possible unfolded structures (entropic effect).

Disulfide bond scores

Cysteine sequence position	Distance	Bond	Score
5 - 6	1	XGNHDCCMGQL-GNHDCCMGQLP	0.01074
5 - 13	8	XGNHDCCMGQL-GQLPKCMLGPG	0.01076
6 - 13	7	GNHDCCMGQLP-GQLPKCMLGPG	0.45666

Fig 5.32 Disulphide bond prediction of BaCf3 by DiANNA 1.1.

5.4 Summary

The bacteriocins were purified to homogeneity by ammonium sulphate precipitation, dialysis and gel filtration chromatography. Yield for BaCf3 and BpSl14 was 1.6 % and 4 % respectively. Both bacteriocins proved to be highly thermostable and pH tolerant. BpSl14 which was more stable than BaCf3 on enzyme treatment, action of oxidizing and reducing agents and 0.5mM metal ions, may be a glycol-peptide. Both bacteriocins have bactericidal mode of action on *B. circulans, S. aureus,* and *P. vulgaris*, as confirmed by confocal laser microscopy, scanning electron microscopy and transmission electron microscopy. The action of bacteriocins on the membranes of target cells could be visualized from the electron micrographs and confocal laser microscopy.

The sequences of the bacteriocins BaCf3 and BpSl14 were derived from MS/MS data. Their primary, secondary and tertiary structures were scrutinized using web servers and softwares. From the observations we found out that even

though the sequences are novel, both the bacteriocins had structural similarities to some identified antimicrobial peptides (AMPs). The presence of disulphide linkages and the presence of unusual amino acids were desirable properties of bioactive peptides which can be attributed to these bacteriocins too.

Chapter 6

Antibiofilm and anticancer action of bacteriocins BaCf3 and BpSl14

6.1 Introduction

In addition to antibacterial activity, bacteriocins show antibiofilm property as well as anticancer action; several have been studied for their application in food preservation.

Food borne diseases and food contamination is always an issue for humanity. Though the type and severity of food borne diseases have changed through the ages, it is still a concern in all nations. According to WHO, almost 1 in 10 people in the world fall ill eating contaminated food and 420000 die every year (WHO, 2017). One of the major causes of food contamination is bacteria and bacterial biofilms. In recent years it was noticed that the drinking water distribution system is a habitat for biofilm forming pathogenic bacteria (Wingender and Flemming, 2011). Biofilm formation imparts antibiotic resistance to the pathogens leading to complications. This difficulty of successful treatment of biofilm associated infections call for the hunt of novel compounds and technologies for biofilm eradication. Many natural products like antimicrobial peptides and bacteriocins have been successfully used as antibiofim agents in food preservation and medicine (Spizek *et al*., 2010).

Bacteriocins as well as other antimicrobial peptides (AMPs) had anticancer potential against different cancer cell types, including colon and breast cancer. The antiproliferative effect of these bacteriocins is attributed to their bactericidal mode of action despite their narrow spectrum of action. Many peptide antibiotics act as lead compound for an effective cure for cancer. Hence study of anticancer activity of bacteriocins is vital.

In this chapter the antibiofilm actions and anticancer actions of both the bacteriocins were studied. Anticancer studies include MTT assay, differential staining and microscopic analysis. The docking studies of the peptide models on some cancer marker proteins were also attempted.

6.2 Materials and Methods

6.2.1 Antibiofilm assay

The purified bacteriocins (BaCf3 and BpSl14) after gel filtration (Section 5.2.1.2) were used for the assay.

6.2.1.1 Standard strains used for antibiofilm assay

The biofilm producers from NCIM culture collection, Pune were used for the assay. The cultures used were *Escherichia coli*, *Bacillus pumilus*, *Clostridium perfringens*, *Bacillus coagulans* and *Salmonella* Typhimurium.

6.2.1.2 Antibiofilm assay on standard strains

The antibiofilm assay was done by the modified method of Mohanty *et al*. (2012) and Jackson *et al*. (2002) using microtitre plates. The wells were filled with 200 µL of tryptone soy broth and 30 µL of bacteriocin (1mg/mL). The wells were then seeded with 20 µL culture OD_{600} =1 of the biofim producers in their log phase. The plate was incubated at 37 °C for 24 h. Tryptone soya broth served as negative control and microorganisms in tryptone soya broth without bacteriocin as positive control. After the incubation, contents were poured off, washed thrice with 0.01 M phosphate buffer of pH 7.4, attached biofilm fixed with methanol for 15 minutes and air dried. Wells were then stained with 1 % crystal violet; excess stain was removed by rinsing under running tap water and air dried. The dye

bound to the adsorbed biofilm was extracted using 33 % (v/v) glacial acetic acid and absorbance was measured at 570 nm in BioSpectrometer (Eppendorf, India).

6.2.1.3 Antibiofilm assay on food isolates

The biofilm producers from different food sources were used for the assay. The biofilm formation of these nine isolates was previously studied (Laxmi and Bhat, 2014). The strains used were *B. altitudinus* BTMW1, *Pseudomonas aeruginosa* BTRY1, *Bacillus* sp. SD1, *B. licheniformis* SD2, *Staphylococcus warneri* DF2, *Micrococcus luteus* FF, *Geobacillus stearothermophilus* FF2, *B. niacini* DP3 and *Brevibacterium casei* DF1.

The % reduction of biofilm formation was calculated using the formula (Chaieb *et al*., 2011)

$$\% \text{ Biofilm reduction} = \left[\frac{\text{OD of Control} - \text{OD of Test}}{\text{OD of Control}}\right] \times 100$$

6.2.2 Biofilm inhibitory concentration

Biofilm inhibitory concentration (BIC) can be defined as the lowest concentration of the compound which inhibits biofilm formation (Laxmi & Bhat, 2016). Bacteriocins with initial concentration 100 µg/mL was serially diluted in microtitre plate and the biofilm producers at OD_{600} =1were added. The dilution without visible biofilm formation was considered as its BIC.

6.2.3 Cytotoxicity Test

3T3-L1, (from National Centre for Cell Science, Pune, India) a cell line derived from mouse 3T3 cells with a fibroblast-like morphology was used in cytotoxicity studies using different concentrations of bacteriocins (40, 80, 120,

160, 200 µg/mL) for 24 h, 48 h and 72 h. Cell death was then tested by MTT assay (Alley *et al*., 1988).

Cytotoxicity of the compound on the cells was calculated as cell growth inhibition (IR).

$$IR = 100 - PR$$

Where,

$$PR = \left[\frac{\text{Absorbance of Test} - \text{Absorbance of Blank}}{\text{Absorbance of Control} - \text{Absorbance of Blank}}\right] \times 100$$

6.2.4 Anticancer activity of bacteriocins

6.2.4.1 Cell line

A549 (adenocarcenomic human alveolar epithelial cell line from National Centre for Cell Science, Pune, India) was used for the experiments. Cell line was propagated in DMEM (Invitrogen, Carlsbad, CA, USA) and supplemented with 10 % heat inactivated foetal calf serum (PAN-Biotech GmbH, Aidenbach, Germany) and antibiotics (100 IU/mL penicillin,100 µg/mL streptomycin), and maintained at 37 °C in 5 % CO_2 atmosphere.

6.2.4.2 Anticancer Activity of bacteriocins in cell culture by MTT assay

The effect of bacteriocins on the proliferation of A549 cells was determined using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay (Alley *et al*., 1988). Briefly, cells were seeded (5000 cells/well) in 96-well, flat-bottom microtiter plates along with different concentrations of bacteriocins (40, 80, 120, 160, 200 µg/mL) and incubated for 24, 48 and 72 hours at 37 °C in 5 % CO_2 atmosphere. After incubation the medium was removed, wells washed with PBS, 100 µL of the working MTT dye in DMEM media was added and incubated for 2 hours. MTT lysis buffer (100 µL) was then added and

incubation continued for 4h. Absorbance was measured at 570 nm and the proliferation rate (PR) was calculated.

6.2.4.3 Morphological evaluation of apoptosis

The cell lines were treated with bacteriocins BaCf3 and BpSl14 and checked for apoptosis. Morphological assessments of apoptosis were done using phase contrast microscopy, acridine orange-ethidium bromide and Hoechst 33258 staining following standard protocols (Pitti *et al*., 1996; Wang *et al*., 2009).

Cells were seeded in a 96-well plate, different concentrations of bacteriocins (100 µg/mL) were added and incubated for 48h at 37 ºC in a 5 % CO_2 incubator (McGahon *et al*., 1995). After incubation, medium was discarded and 25 µL acridine orange (7.5 µg) / ethidium bromide (25 µg) stain was added, mixed well and observed under fluorescent microscope with FITC filter. At least 300 cells were examined in a fluorescence phase contrast microscope (Olympus IX51) using a fluorescein filter and a 40X objective.

A549 cells were seeded in a 96-well plate and different concentrations of bacteriocins were added and incubated for 48 h at 37 ºC in a 5 % CO_2 incubator. After incubation, medium was discarded and 50 µL Hoechst stain was added, mixed well and observed under fluorescent microscope with UV filter.

Morphological assessment of the treated cells was also studied by fluorescence phase contrast microscope (Olympus IX51).

6.2.4.4 *In silico* analysis of anticancer activity

Four target proteins, which could be considered as the possible binding site of the bacteriocins were selected randomly from literature and are listed in table 6.1. PDB IDs of the four cancer target proteins (Table 6.1) were obtained

and these were docked against the models of the bacteriocins predicted from I-TASSER (Section 5.3.7). The PDB Ids and the peptide models were loaded to the ClusPro 2.0 server and the results were obtained (Kozakov *et al*., 2017). ClusPro 2.0 (http://nrc.bu.edu/cluster) uses a multi-stage approach to protein docking. First, a rigid body docking program based on the Fast Fourier Transform (FFT) correlation approach, PIPER extended to pair wise interaction potentials was used and then the 1000 best energy conformations were clustered with retained structures using pair wise Root Mean Square Deviation (RMSD) as the distance measure and the 30 largest clusters were retained for refinement (Fig. 6.1). A cluster is considered stable if a strong attraction exists and contained several low energy structures.

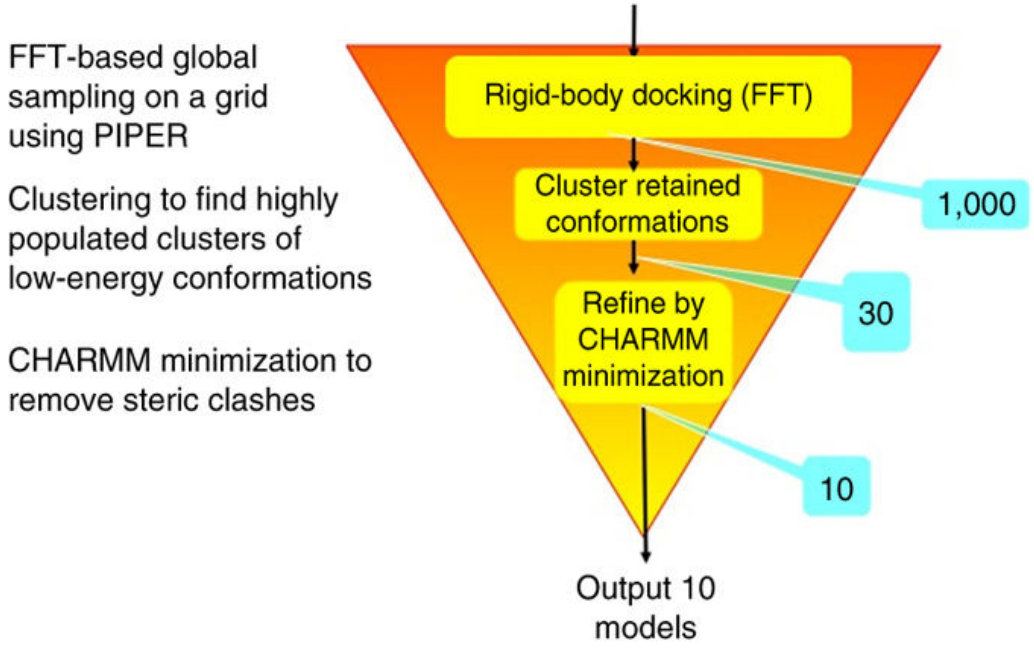


Fig 6.1 Outline of ClusPro algorithm adapted from Kozakov *et al*., 2017.

Discovery Studio® 2016 software (BIOVIA, San Diego, CA, USA) was used for visual representation, assessing the complex interaction, and measuring distances between the interacting amino acid residues.

Table 6.1 PDB ID's of the cancer cell markers and their role in cancer

PDB ID	Name of the receptor	Role in Cancer	Reference
1D0G	Death Receptor 5 (DR5)	The tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor, also called death receptor 5	Valley *et al*., 2012
1Suk	Glucose transporter 1	Glucose uptake of tumour cells is mainly mediated by GLUT1	Hauptmann *et al*., 2005
3DKC	MET receptor tyrosine kinase	MET is a player in so many aspects of cancer development and progression, it is a strong candidate for targeted therapy	Stellrecht & Gandhi, 2009
5E8T	TGF-Beta Receptor Type 1 Kinase Domain	Transforming growth factor-beta (TGF-beta) is a growth regulator which affects multiple cellular functions through the TGF-beta type I and type II receptor (TGF-beta RI and TGF-beta RII) serine/threonine kinases	Heldin, 2008

6.2.5 Statistical analysis

All the experiments were repeated thrice for each test organism/ cell line. The results were expressed as mean ± standard deviation. Statistical analysis was done by ANOVA, Kruskal-Wallis test in GraphPad Prism 6.0. P-values less than 0.05 were considered significant.

6.3 Results and discussion

6.3.1 Antibiofilm activity

The antibiofilm activities of the two bacteriocins, BaCf3 and BpSl14 were tested on NCIM cultures as well as food pathogens (lab isolates from previous work). The graphs (Fig. 6.2a and 6.2b) show percentage of biofilm reduction caused by 100µg/mL of each bacteriocin.

It was noted that while biofilm formation by *Escherichia coli* was unaffected by BaCf3, that by *Bacillus coagulans* was unaffected by BpSl14 (Fig.6.2a). It was also noted that *Salmonella* Typhimurium biofilm is least reduced by BpSl14. The antibiofilm action of BpSl14 against food pathogens was more than that of BaCf3, though both bacteriocins caused >50 % reduction in biofilm formation (Fig.6.2b). The tests were statistically significant with $p<0.05$ (Appendix-V).

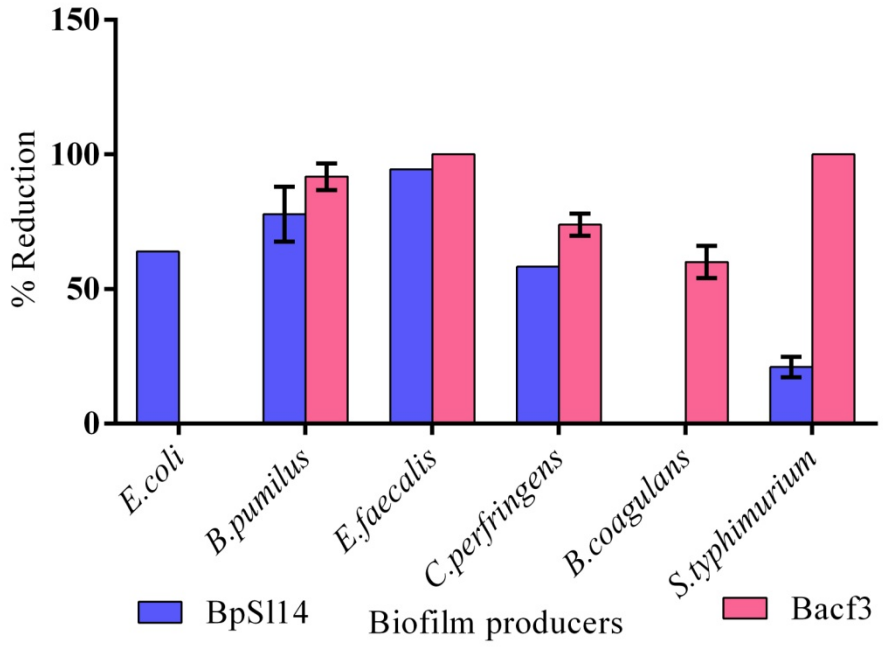


Fig. 6.2a Antibiofilm action of BaCf3 and BpSl14 on NCIM cultures

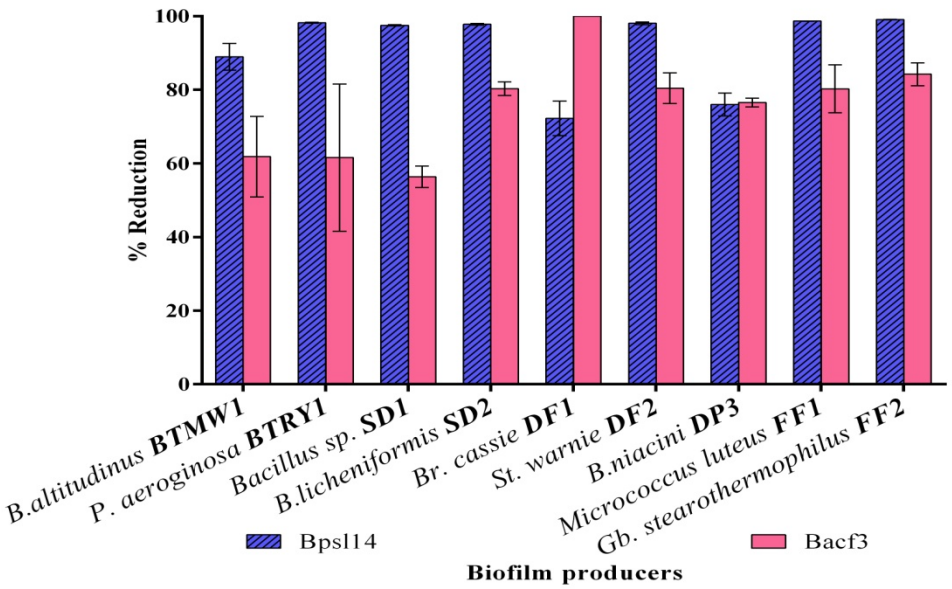


Fig. 6.2b Antibiofilm action of BaCf3 and BpSl14 on food pathogens

The biofilm inhibitory concentrations (BIC) of both bacteriocins against the food pathogens were determined. A graph was plotted with bacteriocin concentration on Y axis and biofilm producers on X-axis (Fig.6.3). The BIC at 0.0304±0.0118 µg/mL of BaCf3 was required for eradication of biofilm production by *Micrococcus luteus* FF1 and 0.486±0.189 µg/mL was required for *Bacillus licheniformis* SD2 and *Gb. stearothermophilus* FF2. It is also evident that higher concentrations of BpSl14 were required for the suppression of biofilms by all the food pathogens tested. A minimum concentration (0.497±0.085 µg/mL) of BpSl14 was required for the abolition of biofilm by *Brevibacterium cassie* DF1 where as a minimum concentration of 3.977±0.7 µg/mL was required for *Bacillus licheniformis* SD2 biofilm. The tests were statistically significant with $p<0.05$ (Appendix V).

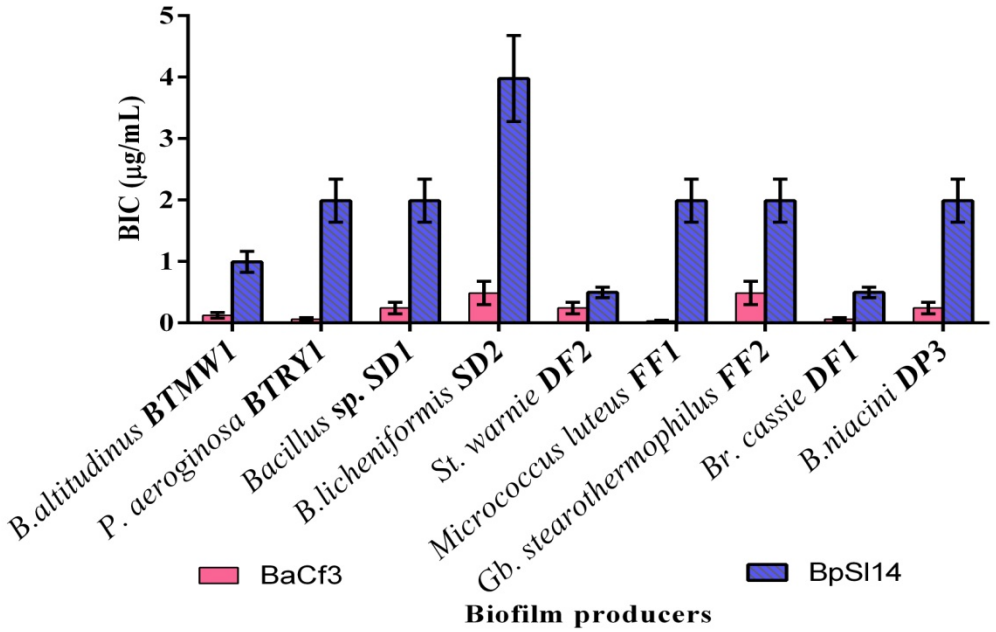


Fig. 6.3 Biofilm inhibitory concentration (BIC) of BaCf3 and BpSl14 on food pathogens

Bacteriocins were added directly to cheese to prevent *Clostridium* and *Listeria*. AMS produced by *B. firmus* H_2O-1 and *B. licheniformis* T6-5 strains

added to prevent colonization, removed existing biofilms and impacted biofilm structure (Korenblum *et al*., 2008). Sonorensin from marine isolate *Bacillus sonorensis* inhibited *S. aureus* biofilm, preventing development of resistance by targeting both multiplying and non-multiplying bacteria (Chopra *et al*., 2015). Lipopeptides from *B. subtilis* also reduced biofilm formation by 88 % and dispersion of mature biofilm by 81 % (Moryl *et al*., 2015). The inhibition of biofilms of *Pseudomonas aeroginosa* and *Brevibacterium casei* by both BaCf3 and BpSl14 is an added advantage since these bacteria are major contaminants of food sources. The biofilms by these bacteria were resistant to most antibiotics (Laxmi & Bhat, 2014) and other conventional treatments, hence their eradication pose a challenge for food industries.

6.3.2 Cytotoxicity Test

Cytotoxicity assay using MTT showed that 3T3-L1 cell growth was not much affected by the two bacteriocins, BaCf3 and BpSl14 (Fig.6.4). Even though there was growth inhibition, higher concentrations of bacteriocins (200 μg/mL) were required to cause 50 % or more inhibition of 3T3-L1 cells. Comparatively, BpSl14 was less cytotoxic than BaCf3. The growth inhibition was <40 % for all the concentrations tested even after 48 h of incubation. A significant growth inhibition is visible only after 72 h of incubation, pointing out its least cytotoxic effect on 3T3-L1 cells. The tests were statistically significant with $p<0.05$ (Appendix V).

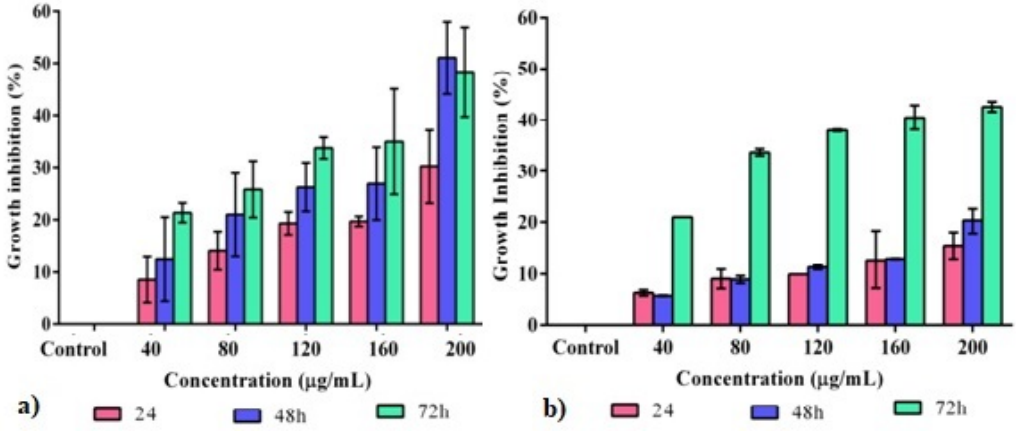


Fig. 6.4 Cytotoxicity assay of a) BaCf3 b) BpSl14 on 3T3-L1 cells

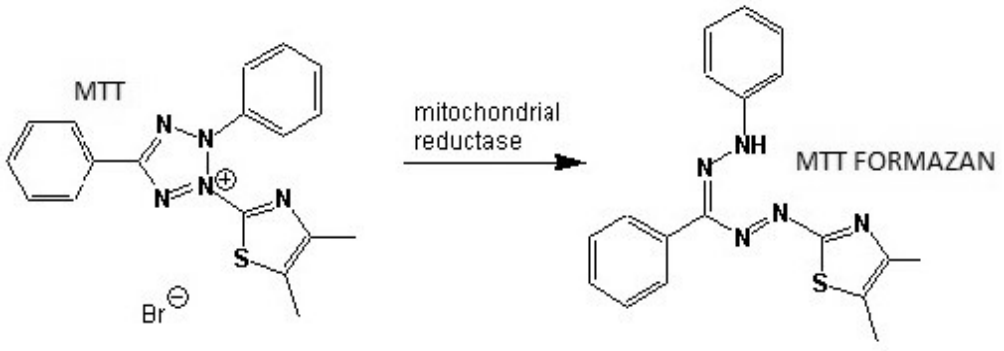
While using bacteriocins for human or animal use, it is mandatory to study their effects on normal cells. 3T3-L1 cells with their fibroblast like morphology stand for normal cells. This rat primary cultured hepatocytes 3T3-L1 represents a valuable tool for screening of bioactive compounds (Wang *et al*., 2002). From the results we can notice that the bacteriocins do not show significant growth inhibition on 3T3-L1 cells indicating its applicability in humans/animal use and food preservation. Chumchalova and Smarda (2003) studied the inhibitory effects of four pure colicins - A, E1, U, and E3 on normal human fibroblast cell line MRC5 by MTT (tetrazolium bromide) assay; wherein Colicin A caused 36 % inhibition of MRC5 whereas less sensitivity to Colicin E1 was.

6.3.3 Anticancer activity of bacteriocins in cell culture

6.3.3.1 MTT assay

MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, dark purple coloured formazan product. The cells are then solubilised with an organic solvent and the

released, solubilized formazan reagent is measured spectrophotometrically at 570 nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.



MTT assay showed that proliferation of A549 cells was inhibited by increasing concentration of both BaCf3 and BpSl14, indicative their anti-proliferation ability (Fig.6.5). The proliferation rate of BaCf3 treated cells was more significant after 48 h of incubation. The proliferation rate was less than 50 % for all the concentrations after 72 h of incubation showing that it is a slow acting anticancer compound (Fig.6.5a). The tests were statistically significant with $p<0.05$ (Appendix-V).

The proliferation rate of bacteriocin BpSl14 treated cells was very slow right from the onset of incubation, with a decline in proliferation with increased bacteriocin concentration and time. From the study, the bacteriocins dosage to be applied ranged from 80 µg/mL to 200 µg/mL of BpSl14.

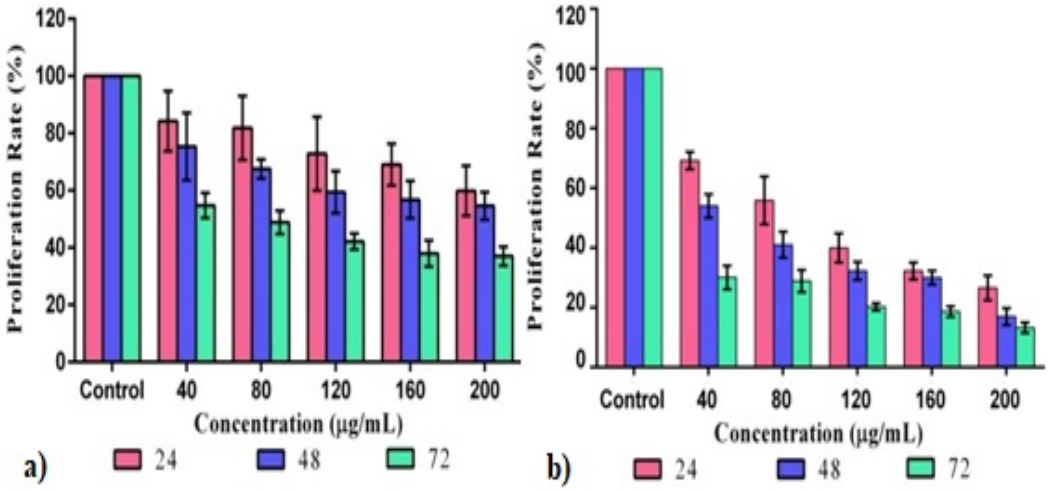


Fig 6.5 Anticancer activity of bacteriocins by MTT assay a) BaCf3 and b) BpSl14 on A549 cell line.

Colicins showed anticancer activities against several human tumour cell lines like breast cancer, colon cancer, bone cancer and uteri cell line HeLa (Kaur and Kaur, 2015). Pediocin PA-1 produced by *P. acidilactici* PAC1.0 and its recombinant product inhibited the growth of cell lines A-549 (Beaulieu, 2005). The dose and temperature dependent *in vitro* cytotoxicity of artificially synthesized plantaricin A (plnA) on Jurkat, human T cell leukaemia cell line was reported by Zhao *et al*. (2006). Chumchalova and Smarda (2003) observed that colicin E1 and A had 17- 40 % inhibition of 11 human tumour cell lines with small differences among individual cell lines. The fibrosarcoma HS913T was most sensitive to colicin A, E1 and U showing 50 % inhibition with colicin E1 treatment. Colicin A had strong inhibitory effects varying from 16 to 56 % inhibition of tumour cell lines. Breast carcinoma cell lines; BT474, ZR75, and SKBR3 were found resistant to all tested colicins and the inhibition ranged from 15 to 25 %.

6.3.3.2 Morphological evaluation of apoptosis

There were several significant morphological changes in the cancer cells that were bacteriocins induced; including cell shrinkage and apoptotic body formation. Untreated cells showed green fluorescence indicating no apoptosis. The intact nuclei of control cells could be visualized in Fig 6.6 (A, B, C) and 6.7 (A, B, C). Treatment with bacteriocins led to the appearance of red/orange fluorescence indicating onset of apoptosis. The condensed body inside the nucleus was clearly visible (Fig 6.6 & 6.7 D, E, F). The dead cells were visible as orange colour in the fluorescent microscopic image (6.6 & 6.7 D). All these indicated that there were some bacteriocin induced apoptotic pathways in A549 cells.

Acridine orange/ethidium bromide dual staining is commonly used to detect apoptosis based on the differential uptake of the two fluorescent DNA binding dyes to determine the viable and non viable cells in a population. Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost their membrane integrity. Live cells appear uniformly green. Early apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will incorporate ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, without condensed chromatin.

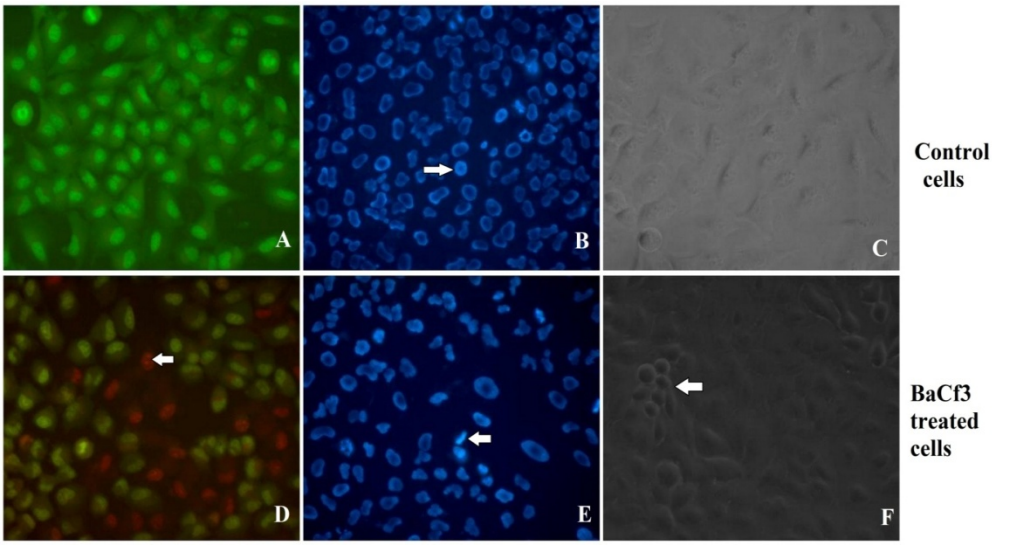


Fig.6.6 Cytochemical staining of cancer cells treated with BaCf3 using fluorescent dyes reveal characteristic features of apoptosis. Cells were treated with BaCf3 (100 µg/mL) for 48 h and changes associated with apoptosis such as nuclear condensation and cell shrinkage were noted as obtained by the acridine-orange/ethidium bromide dual staining (A-Control; D-Treated cells) B-Control cells after Hoechst 33342 staining; E-Treated cells after Hoechst 33342 staining. Bright fluorescence is evident in treated cells. C- Phase contrast image of control cells; F-Phase contrast image of treated cells, Rounding of affected cells is clearly visible. (Original magnification 40× for all the images)

Moreover, Hoechst 33342 staining also indicated signs of apoptosis in bacteriocin treated cells. Figure 6.6 & 6.7 (B & E), revealed the apoptotic effects induced by BpSl14 on A549 human lung carcinoma cells. As compared to control cells which exhibited normal cell morphology, bacteriocin-treated cells showed chromatin condensation, DNA fragmentation, and membrane blubbing all characteristic of significant levels of apoptosis. As compared to control cells, treated cells showed bright fluorescence resulting from cleaved DNA.

The Hoechst 33342 is a fluorescent stain for labelling DNA in fluorescence microscopy or flow cytometry as the dye label DNA and excited by UV light emitting blue/cyan fluorescence (Lizard *et al*., 1997). A cell that is

undergoing apoptosis demonstrates nuclear condensation and DNA fragmentation, which can be detected by staining with Hoechst 33342 and fluorescence microscopy.

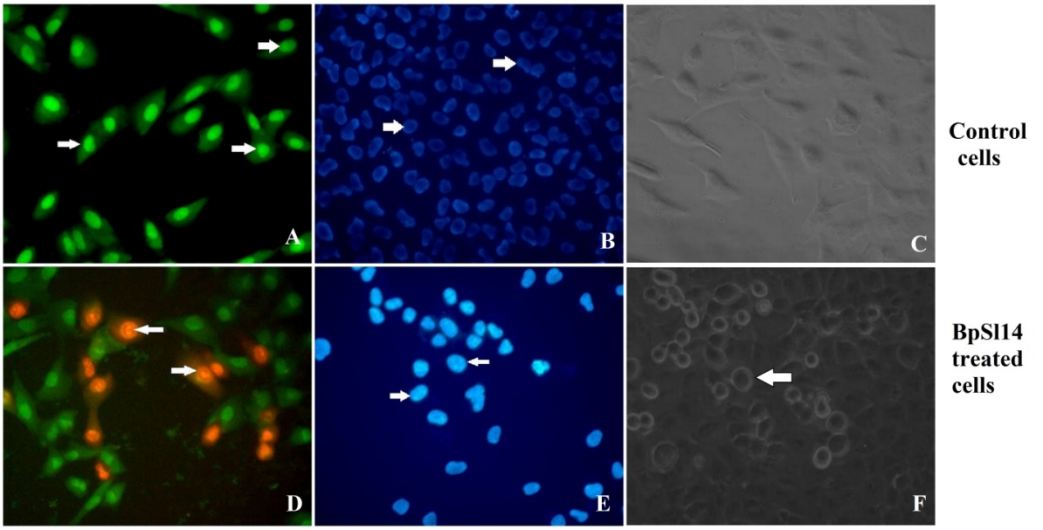


Fig.6.7 Cytochemical staining of cancer cells treated with BpSl14 using fluorescent dyes reveal characteristic features of apoptosis. Cells were treated with BpSl14 (100 µg/mL) for 48h and changes associated with apoptosis such as nuclear condensation and cell shrinkage were noted as obtained by the acridine-orange/ethidium bromide dual staining (A-Control; D-Treated cells) B-Control cells after Hoechst 33342 staining; E-Treated cells after Hoechst 33342 staining. Bright fluorescence is evident in treated cells. C- Phase contrast image of control cells; F-Phase contrast image of treated cells, Rounding of affected cells is clearly visible. (Original magnification 40× for all the images)

Several researchers have studied anticancer activity of bacteriocins. The design and research on AMPs with antitumor properties have high medical value as tumour cells are selectively killed. The cytotoxicity of bacteriocins and their ability to differentially target cancer cells depend on their structural properties like number of positively charged amino acids, hydrophobicity and ability to form amphipathic structures or oligomerization as in case of other antimicrobial peptides (Gaspar *et al*., 2013). From section 5.3.6 we can observe that both the bacteriocins BaCf3 and BpSl14 are slightly hydrophobic which could allow them

to interact with the cell surface molecules which are negatively charged and are superiorly expressed on the cancer cells (Zhao *et al*., 2006).

The mechanisms of cytotoxicity of bacteriocins may include induction of apoptosis and/or depolarisation of the cell membrane leading to permeability changes. Sometimes bacteriocins also cause necrosis, another cell destructive process (Kaur & Kaur, 2015). When cells undergo apoptosis or programmed death, a dramatic change in morphology and interior contents occur like ruptured nucleus, broken chromosomal DNA, flipped phosphatidyl serine on the cell membrane, etc (Martelli *et al*., 2001). During apoptosis, there is no inflammatory response and hence no damage to surrounding cells, where as necrosis is damaging to both cancer cells and the surrounding normal cells (Majno & Joris, 1995).

6.3.3.3 *In silico* analysis of anticancer activity

Docking studies with BaCf3 and BpSl14 used Death receptor DR5 (PDB ID - 1D0G), Glucose transporter GLUT1 (PDB ID - 1SUK), Tyrosine Kinase (PDB ID- 3DKC) and Transforming Growth Factor-β TGF-β (PDB ID - 5E8T) as a targets for anticancer activity.

ClusPro 2.0 generated hundreds of conformational models by minimizing their energy with at least one near native model and was clustered. These clusters were ranked based on their size. All clusters that were unstable or having high energy after optimization were discarded. The Fig 6.8 and Fig 6.9 show the optimized complexes. They were then scored for interaction of ligand with receptor like balanced, hydrophobic, electrostatic and Vander Waals (vdw) scores (Table 6.2). The scores were expressed in terms of energy (kcal/mol). For template based docking without specified binding sites, the balanced score is more important. Lower the scores and energy, greater the interaction.

The primary function of DR5 is to induce apoptosis. Oligomerization of death domains generates homophilic interaction surfaces for death domain containing adaptor proteins like FADD, which in turn engage initiator caspases such as caspase 8, leading to subsequent activation of effector caspases that execute apoptotic death of the cell (Thornberry & Lazebnik, 1998). Because of the selectivity of TRAIL towards cancer cells, there has been significant interest in developing agents targeting TRAIL or DR5 for cancer therapy, including recombinant protein, agonistic antibodies and small molecules (Yang *et al*., 2010; Wang *et al*., 2013).

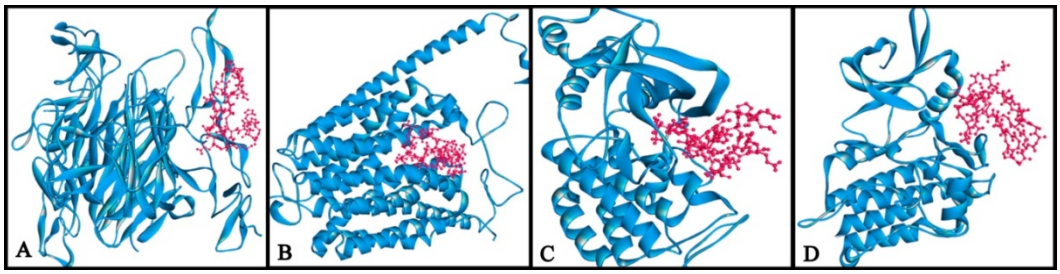


Fig 6.8 Docked models of apoptotic receptors with BaCf3 A) 3DKC B) 1SUK C) 1D0G D) 5E8T. Figures generated by BIOVIA Discovery Studio 2016. Ribbon structures indicate receptors, while the ball and stick models represent bacteriocin BaCf3.

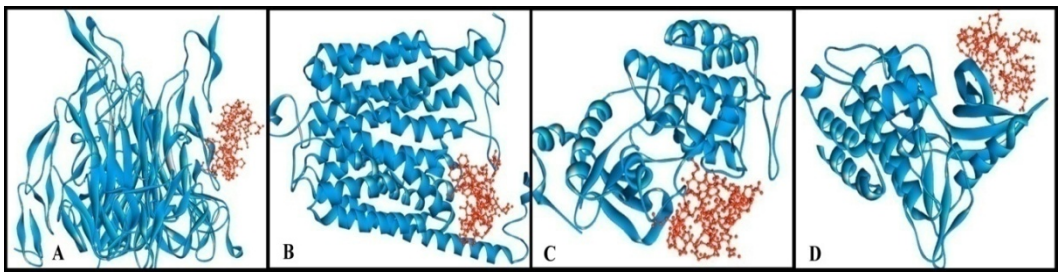


Fig 6.9 Docked models of apoptotic receptors with BpSl14 A) 3DKC B) 1SUK C) 1D0G D) 5E8T. Figures generated by BIOVIA Discovery Studio 2016. Ribbon structures indicate receptors, while the ball and stick models represent bacteriocin BpSl14.

Some studies with human tissue specimens indicate that DR5 is over expressed in several cancer types and significantly correlated with more

aggressive tumour behavior and poor survival of cancer patients (e.g., with breast, lung or renal cell cancer) (Spierings *et al*., 2003; Ganten *et al*., 2009). Knockdown of DR5 expression of human cancer cell line A549 significantly enhanced their invasive abilities and metastasis (Oh *et al*., 2015).

GLUT1 cell surface marker is over expressed in many tumour cells such as hepatic, pancreatic, breast, esophageal, brain, renal, lung, cutaneous, colorectal, head and neck, endometrial, ovarian and cervical cancers (Yamamoto *et al*., 1990; Rudlowski *et al*., 2003). The expression of GLUT1 thus appears to be a potential marker for malignant transformation. The increased expression of GLUT1 indicates an increased intake of glucose there by supplying the energy necessary for tumor cell proliferation. Positron emission tomography (PET) scans evaluated glucose uptake by cancer cells (Labak *et al*., 2016). Thus in order to properly target glucose transporters, it is crucial that GLUT inhibitors are identified.

MET receptor Tyrosine kinases are frequently deregulated in cancer and promotes cell proliferation, progression, metastasis and therapeutic resistance. Activation of MET by mutation or gene amplification has been linked to kidney, gastric, and lung cancers, while autocrine activation was demonstrated in glioblastoma. Since MET is a target in so many aspects of cancer development and progression, the MET receptor tyrosine kinase has emerged as an important target for the development of novel cancer therapeutics. Numerous agents have been developed that are able to target MET expression and/or function.

Table 6. 2 Docking Scores for BaCf3 and BpSl14

Receptor	Score (Balanced)		Score (Electrostatic)		Score (Hydrophobicity)		Score (vdw)	
	BaCf3	**BpSl14**	**BaCf3**	**BpSl14**	**BaCf3**	**BpSl14**	**BaCf3**	**BpSl14**
1SUK	-903.1	-749	-899.1	-762	-1197.9	-1339.1	-137.5	-159.9
5E8t	-605.1	-580	-601.6	-604.7	-681.6	-816.5	-115.5	-162.6
3DKC	-703.6	-588	-594.9	-602.4	-744.8	-784.6	-115.3	-165.8
1D0G	-753.7	-578.5	-805.6	-756.5	-849.3	-841.6	-145	-184.1

The biological and physiological function of TGF-β and its receptors are of prime importance in human diseases. They regulate cell growth, death and immortalization. In normal and early carcinomas TGF-β signalling pathways exerts tumour suppressor effects but as the tumours develop and progress the protective effect is often lost. The deregulation of signalling by TGF-β expression plays a role in tumour development and metastasis. Several in vivo studies demonstrated that the inhibitors of TGF-β and its receptors could reduce the invasive properties of experimental cancers (Dumont & Arteaga, 2003; Ge *et al*., 2004; Zavadil & Böttinger, 2005).

6.3.3.4 Docking interactions of bacteriocins

The interactions of the ligands (Bacteriocins) with the receptor molecules were identified in BIOVIA Discovery studio 2016. The interacting amino acids, distance of the amino acids and the type of interactions were spotted out.

These interactions included hydrogen bonds, hydrophobic interactions, electrostatic interactions and some others. The inhibition of a receptor by docking experiments is illustrated by the binding position, strength of interaction and also stearic hindrance caused by the binding of ligand. A table showing detailed list of

interactions, the atoms involved and distance in Angstrom is given in Appendix. VI.

Docking studies showed that BaCf3 interacts with many amino acids of death receptor 5 (PDB ID - 1D0G). The interacting amino acids of the receptor include Glu124, Glu94, Trp120, Leu147, Glu144, GLn85, Leu73, Ser74, Asn81, Arg80 and Tyr211 (Fig 6. 10).

The interaction study of BpSl14 with DR5 shows that the amino acids Glu94, Trp120, Glu144, Lys145, Cys84, Val83, Ser74, Thr64, Asn81, Arg80, Thr77 and Thr79 interacts with the ligand (Fig 6.11).

Fig 6.10 Docking interaction of death receptor 5 (PDB ID - 1D0G) with BaCf3. A) Docked model, B) Interaction with the receptor C) Interacting amino acids of the ligand, showing the bacteriocin BaCf3 in stick model.

Complex formation between TRAIL (TNF-related apoptosis-inducing ligand) and DR5 triggers apoptosis by oligomerization of intracellular death domain of DR5. Monoclonal antibodies targeting DR5 have been developed by Ichikawa *et al*. (2001) and are under clinical trials. Arg130, Gly131, Arg132, Lys145, Leu147, Gly148, Arg149, Lys150, Glu155 are some of the binding sites for DR5 activation. In the docked models we can see that BaCf3 interacts with Glu144, Tyr211, and Glu124 while BpSl14 interacts with Trp120, Glu144 and Glu145 which are proximal to the binding site of the monoclonal antibodies.

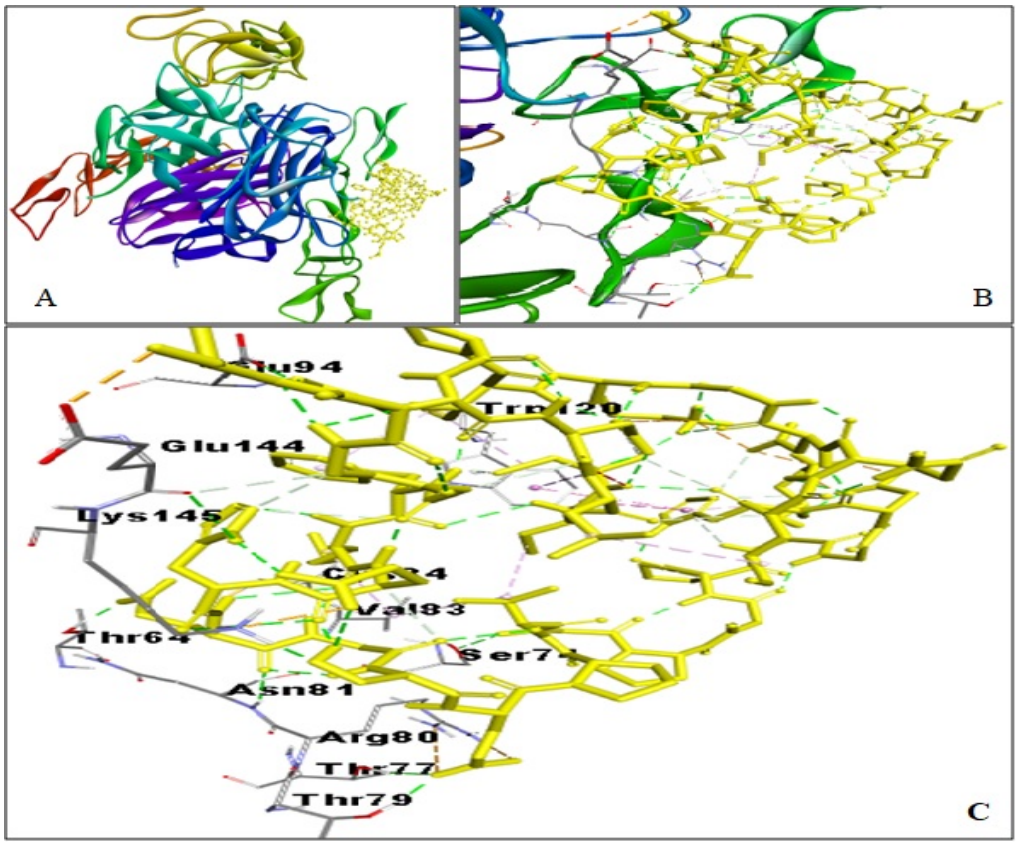


Fig 6.11 Docking interaction of death receptor 5 (PDB ID - 1D0G) with BpSl14. A) Docked model, B) Interaction with the receptor C) Interacting amino acids of the ligand, showing the bacteriocin BpSl14 in stick model.

Docking studies revealed that BaCf3 interacts with many amino acids of glucose transporter protein, GLUT1 (PDB ID - 1SUK). The interacting amino

acids of receptor are Ala405, Ala402, Pro401, Arg400, Gln397, Leu262, Glu261, Val257, Glu254, Arg253, Met252, Met251, Gln250, Lys225, His160, Phe152, Met142, Pro141, Gly138, Val87, Leu85 and Phe81 (Fig.6.12) some of which are involved in glucose transport.

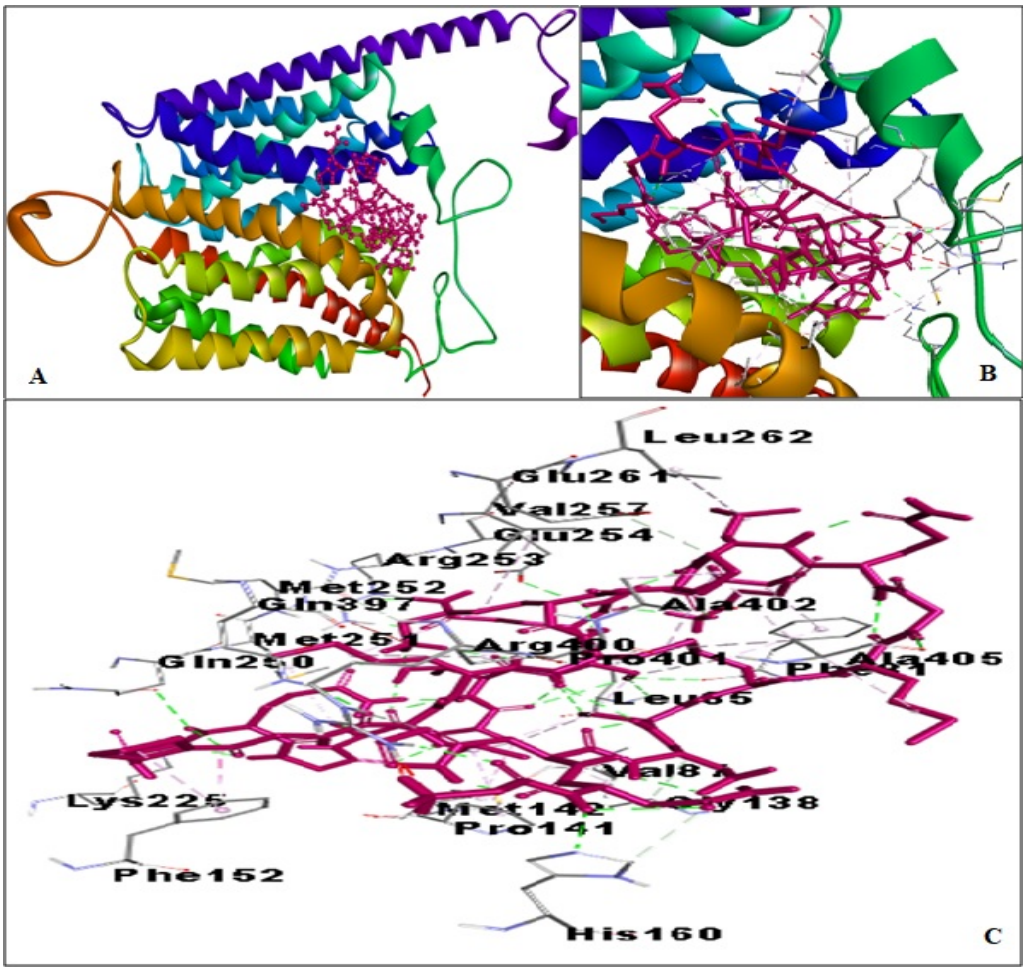


Fig 6.12 Docking interaction of Glucose transporter, GLUT1 (PDB ID-1SUK) with BaCf3. A) Docked model, B) Interaction with the receptor C) Interacting amino acids of the ligand, showing the bacteriocin BaCf3 in stick model.

The docking study showed that the amino acids Arg153, His239, Leu241, Gln242, Glu243, Glu329, Arg330, Arg334, Arg333, Phe389, Ile390, Leu394 and Glu462 of GLUT1 receptor interacts with the ligand BpSl14 (Fig. 6.13). Increased glucose uptake by cancer cells has been studied for decades. Alo *et al*. (2001)

observed an increase in the expression of GLUT1 receptors in breast cancer cells. Both the bacteriocins showed many favourable interactions in the glucose transporting regions of the receptor though we can't find any common interacting amino acid residues. From Fig 6.12A and Fig 6.12B it is evident that BaCf3 could block the glucose transport channel of GLUT1 receptor, while the interaction of BpSl14 with Arg153 could hinder the glucose transport (Fig 6.13C).

Quercetin, a GLUT1 inhibitor, binds to Glu254 and Gln397 by hydrogen bonding (Cunningham *et al*., 2006). The docking study with BaCf3 also gives an idea about the involvement of these amino acids in the non-bond interaction. This indicates that BaCf3 could inhibit glucose transport to the cells there by reducing the metabolic rate of cancer tissue. Docking of D-Glucose and quercetin on GLUT1 receptor revealed that uncharged quercetin also bind one of the glucose binding sites in the inner vestibule of GLUT1, which is negatively charged (Cunningham *et al*., 2006). Studies show that this site likely involved Glu254 and Lys256 that hydrogen-bond to quercetin and glucose. The residues R330-R334 of GLUT1 are part of conserved motif (Sato and Mueckler, 1999). In patients with the glucose transporter-1 deficiency syndrome (De Vivo *et al*., 1991), S66, T310, G91, R126, E247, K256, E146 and R333 are the proposed eight crucial residues for glucose transport in both helical and loop regions (Klepper *et al*., 1999; Klepper and Voit, 2002; Pascual *et al*., 2002; Brockmann *et al*., 2001; Wang *et al*., 2000). BpSl14 shows non-bond interaction with R330-R333 residues, showing its ability to block glucose transport.

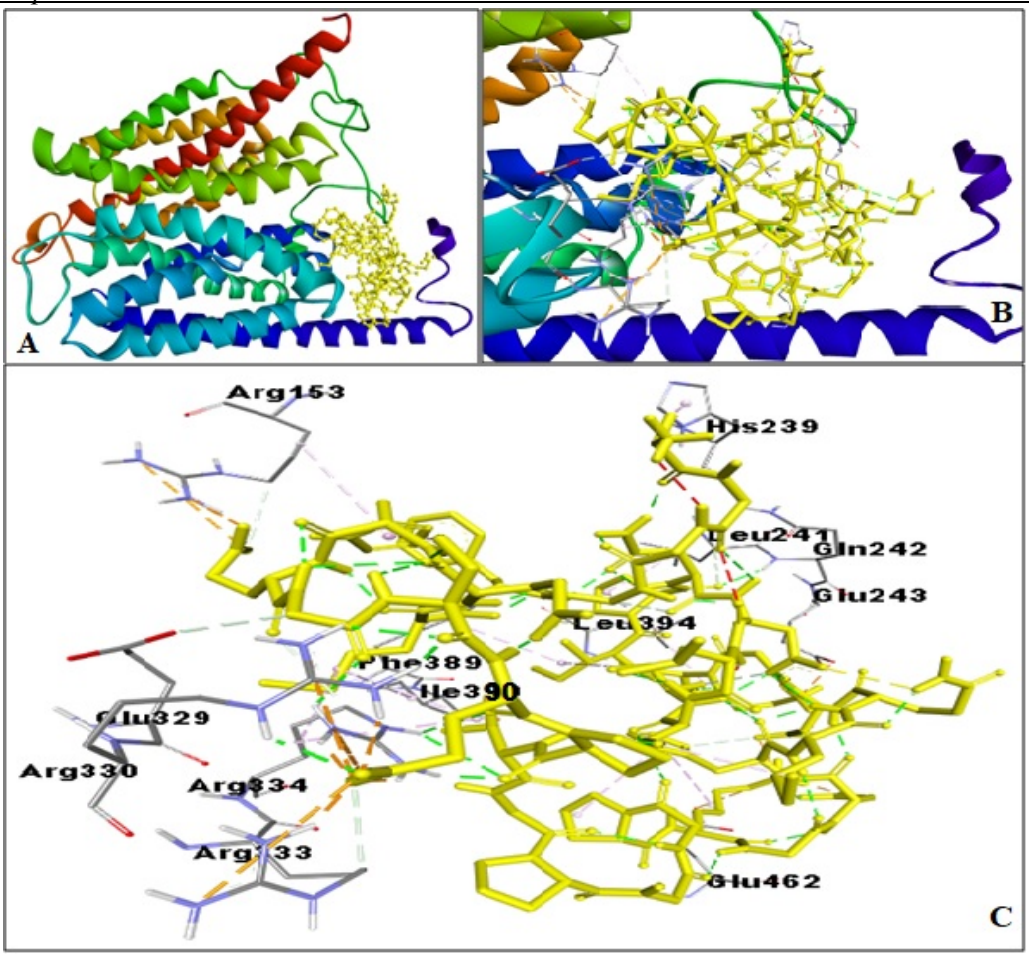


Fig 6.13 Docking interaction of Glucose transporter, GLUT1 (PDB ID-1SUK) with BpSl14. A) Docked model, B) Interaction with the receptor C) Interacting amino acids of the ligand, showing the bacteriocin BpSl14 in stick model.

The docking sites of BaCf3 on tyrosine kinase (PDB ID-3DKC) included 14 residues such as Pro1158, Asp1222, Tyr1159, Lys1110, Met1211, Met1160, Arg1208, His1088, Val1092, His1094, Arg1166, Asn1167, Val1083 and Arg1170 (Fig. 6.14).

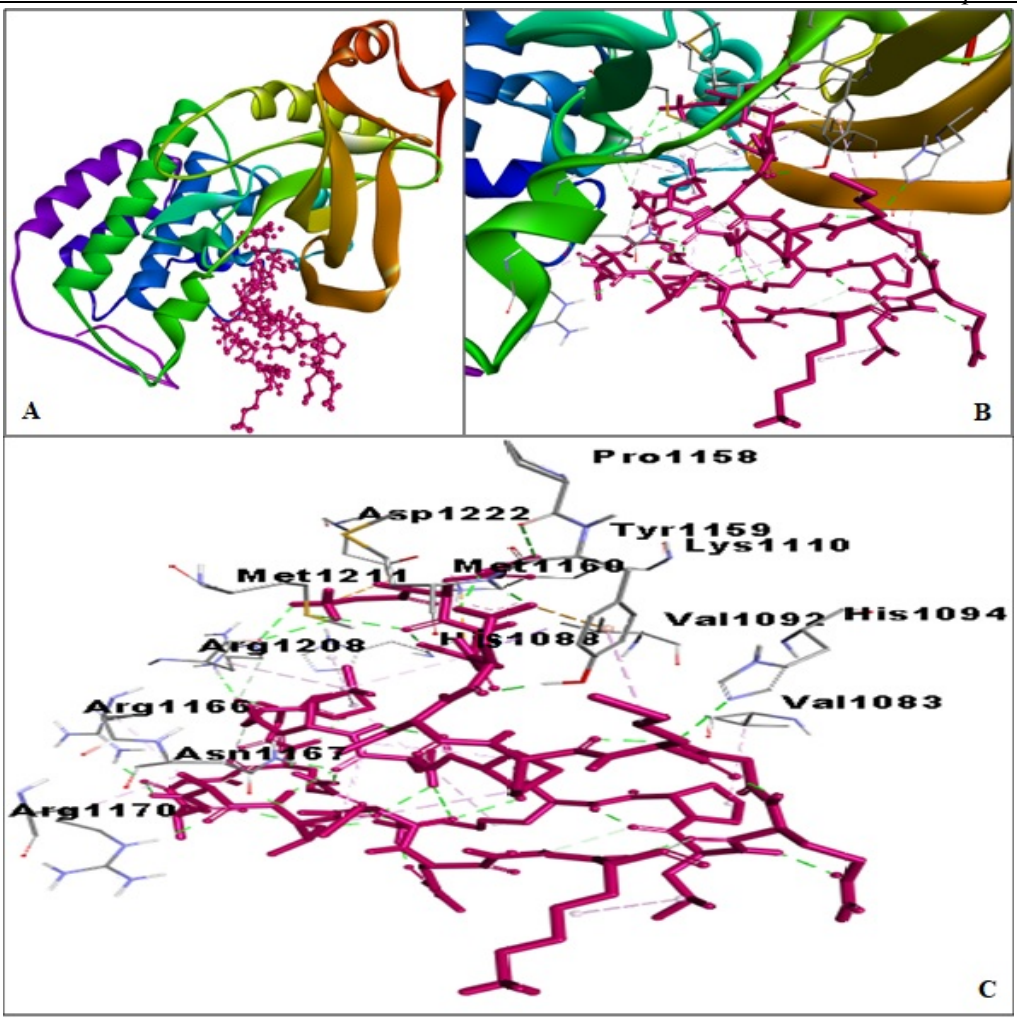


Fig 6.14 Docking interaction of tyrosine kinase (PDB ID-3DKC) with BaCf3. A) Docked model, B) Interaction with the receptor C) Interacting amino acids of the ligand, showing the bacteriocin BaCf3 in stick model.

Docking of BpSl14 with Tyrosine kinase receptor also shows interactions with 14 residues such as Arg1114, Val1083, Arg1086, His1088, Lys1244, Ala1213, Asn1288, Asp1286, Pro1285, Lys1248, Arg1208, Arg1166, Arg1170 and Ala1354 (Fig.6.15).

From the interactions (Fig. 6.14C and Fig. 6.15C) we can see that Arg1208, His1088, Arg1166, Val1083 and Arg1170 are the five amino acids of the Tyrosine kinase receptor that both bacteriocins interact with, showing some similarity in the binding region on the receptor with the bacteriocins. .

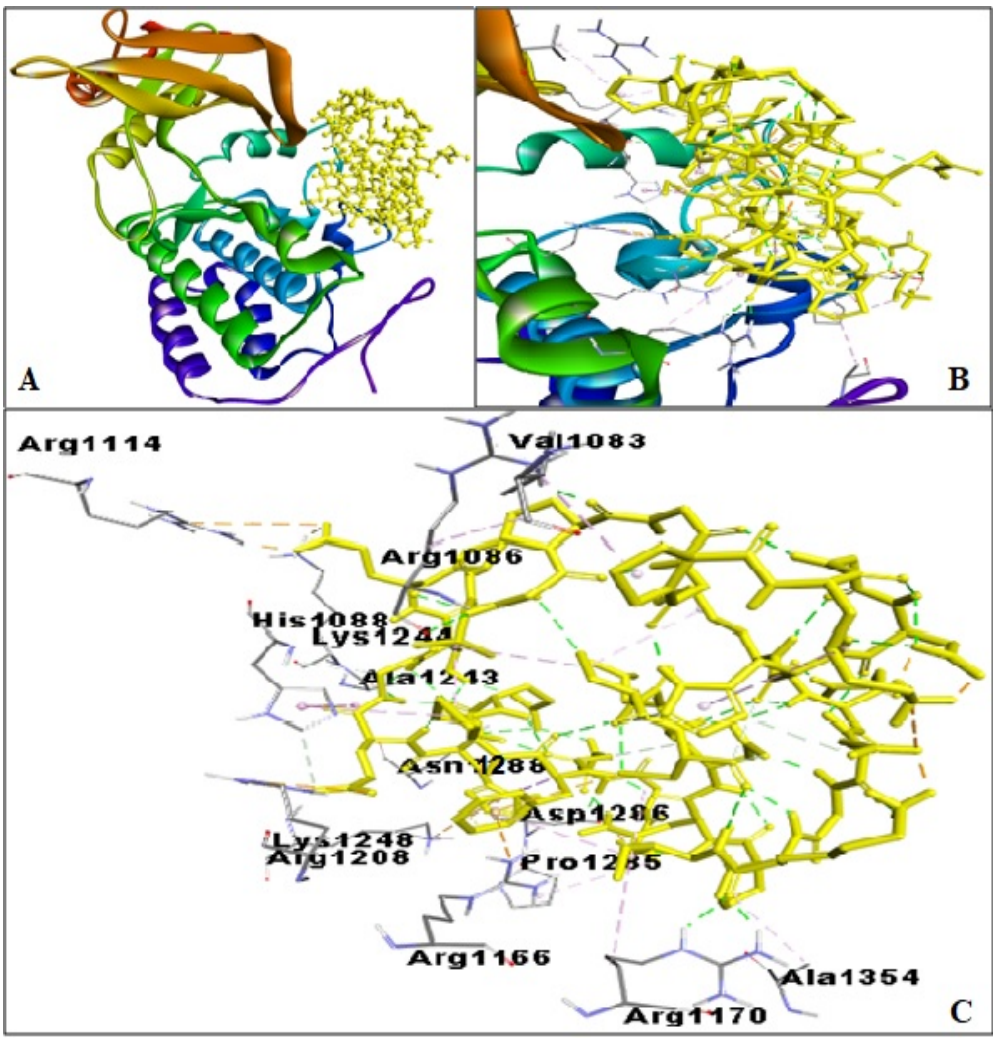


Fig 6.15 Docking interaction of tyrosine kinase (PDB ID-3DKC) with BpSl14. A) Docked model, B) Interaction with the receptor C) Interacting amino acids of the ligand, showing the bacteriocin BaCf3 in stick model.

Tyrosine kinases are frequently deregulated in cancer and this promotes tumor formation, progression and metastasis. The inhibition of ATP binding site

of the tyrosine kinases may inhibit it and hence prevent cancer metastasis. Thus many ATP analogues can act as cancer therapeutics (Buchanan *et al*., 2009). His1088, Lys1110, Asp1204, Pro1158, Asn1209, Arg1208, Met1160, Met1211, Val1092 are the ATP binding sites on MET receptor tyrosine kinase (Buchanan *et al*., 2009) According to the docking study, both bacteriocins bind to the ATP binding sites on tyrosine kinase; Arg1208 and His1088 in the case of BpSl14 and Arg1208, Pro1158, Asp1222 and His1088 for BaCf3.

Docking BaCf3 and Transforming Growth Factor-β (TGF-β, PDB ID - 5E8t) showed BaCf3 interacting with 11 amino acids residues of the receptor like Arg215, Phe216, Glu239, Arg240, Ser241, Ile367, Ala368, Asn370, His371, Val373, Lys376 (6.16).

The docking sites of BpSl14 on TGF-β was found to be the following 10 amino acids viz., Arg203, Val206, Leu207, Gln208, Glu209, Ser210, Trp220, Lys268, Asn270, Gln275 (Fig 6.17). TGF-β signalling simultaneously triggers several responses in cancer cells.

Studies show that staurosporine, an apoptosis inducer, binding to TGF-β receptor could interact with the amino acids 212 to 216 at the tip of P-loop of the receptor and Gln275 in the crystal structure (Tebben *et al*., 2016). When we look at the interacting sites of BaCf3 we can notice that it could form hydrogen bonds with Arg215 and Phe216; and BpSl14 could interact with Gln275, thereby probably having the ability to induce apoptosis.

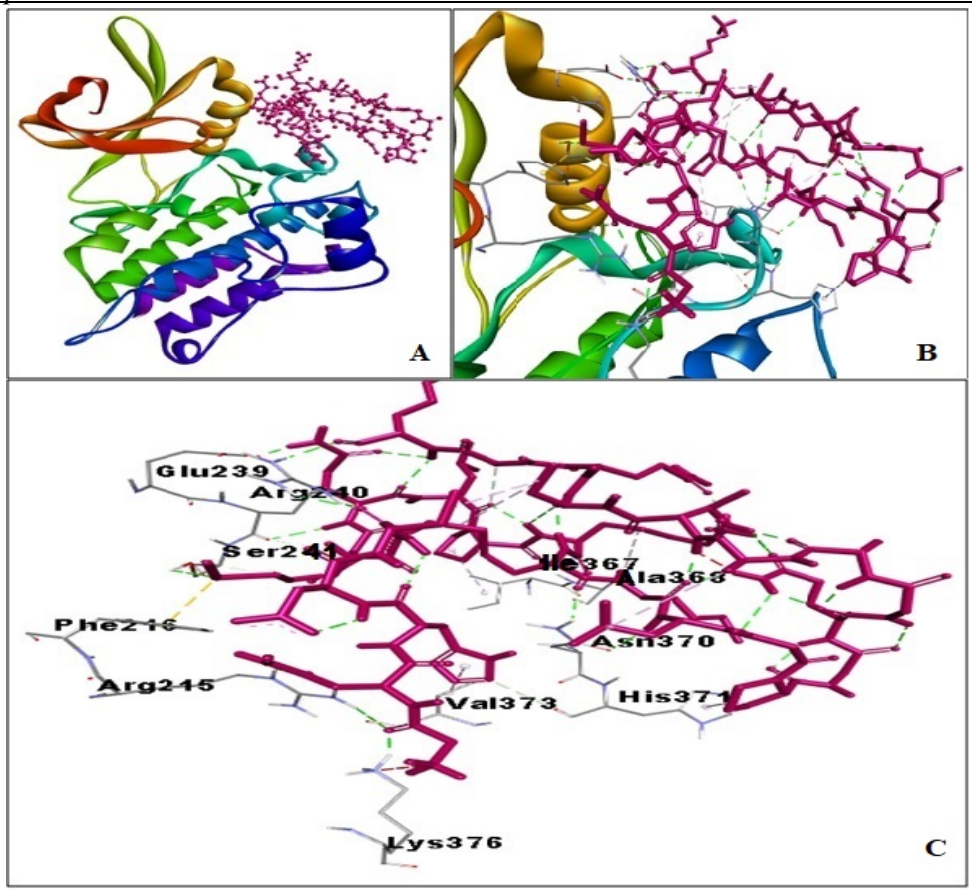


Fig 6.16 Docking interaction of Transforming Growth Factor-β (TGF-β, PDB ID - 5E8t) receptor with BaCf3. A) Docked model, B) Interaction with the receptor C) Interacting amino acids of the ligand, showing the bacteriocin BaCf3 in stick model

The morphological analysis studies using fluorescent microscopy described in section 6.3.3.2 is also an evidence of induction of apoptosis by BaCf3. TGF-β signalling is regulated by hundreds of factors which form a complex web, and the collapse of such networks leads to a crash of the signalling pathway, leading to the development and progression of malignant tumours. TGF-β also regulates cytokine and chemokine secretion resulting in development of the inflammatory tumour microenvironment. TGF-β can induce a pro-angiogenic environment and stimulates tumour angiogenesis, and increased TGF-β expression has been linked to increased microvessel density in certain tumour

types (Derynck *et al*., 2001). Inhibition of TGF-β signalling inhibits tumour viability, migration, malignancy in breast cancer, melanoma and prostate cancer models (Kaminska *et al*., 2005).

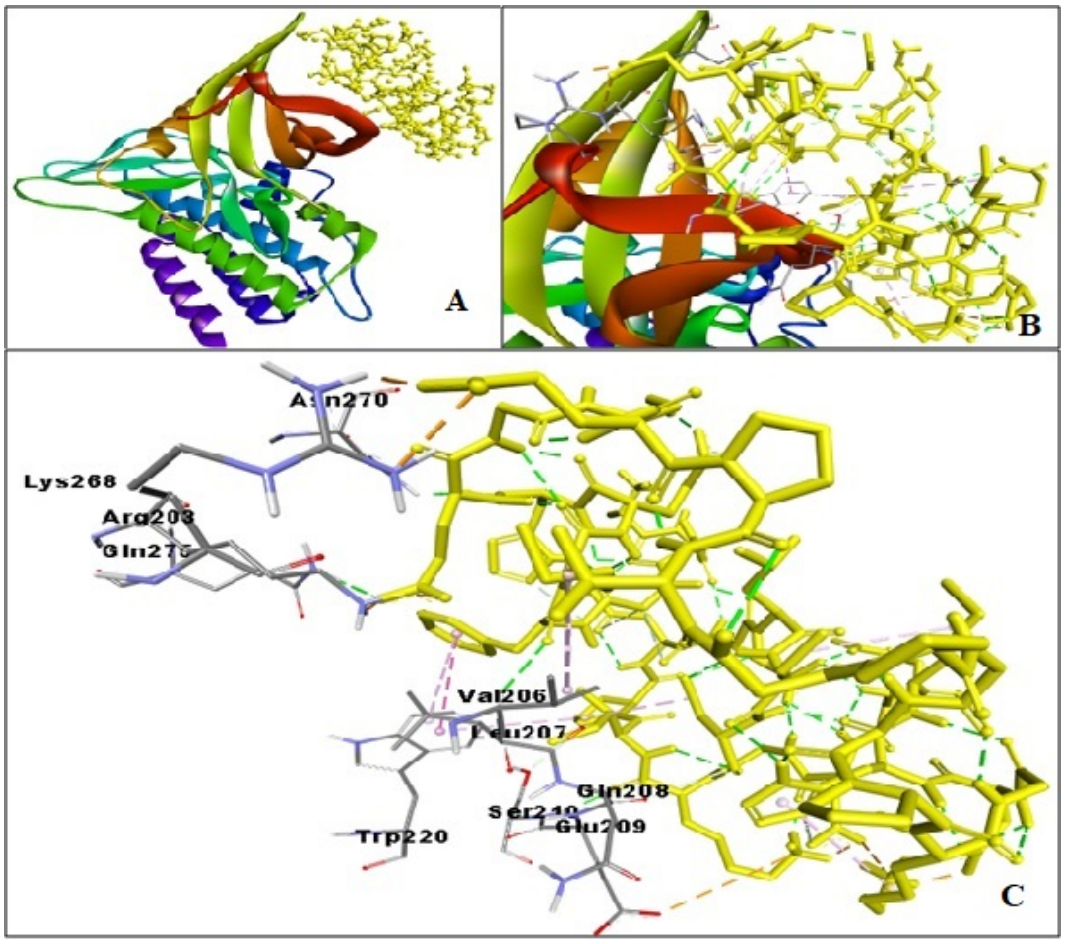


Fig 6.17 Docking interaction of Transforming Growth Factor-β (TGF-β, PDB ID - 5E8t) receptor with BpSl14. A) Docked model, B) Interaction with the receptor C) Interacting amino acids of the ligand, showing the bacteriocin BpSl14 in stick model

The docking parameters suggest that both the bacteriocins could bind to the tyrosine kinase (3DKC) effectively thereby possessing the capability of inhibiting the ATP binding and hence malignancy.

6.4 Summary

In essence we can conclude that both the bacteriocins exhibited pronounced antibiofilm and anticancer action, with bacteriocin BpSl14 having enhanced antibiofilm and anticancer property. Both are required in very small concentrations for biofilm eradication. The anticancer assay along with microscopic studies and docking studies proved that both bacteriocins could induce apoptosis in cancerous cells and can help prevent malignancy. The docking studies also prove that both the bacteriocins could interact with the cell surface markers studied.

Chapter 7

Characterization of bacteriocin producers Bacillus amyloliquefaciens BTSS3 and Bacillus pumilus SDG14 for their probiotic potential

7.1 Introduction

Probiotics benefit human and animal health by enhancing the gut microflora balance, representing a potential strategy to influence host immune system by modulating immune response. Administered as stand-alone or into food or feed systems (Fuller, 1989), they may exhibit antagonist action due to antimicrobial substances (Dunne *et al*., 1999). Immunomodulation by stimulating Gut-associated Lymphoid Tissue (GALT) cause cytokine production. Cell wall components of probiotic *Bacillus* strain (*Bacillus coagulans* GBI-30) reportedly had immunomodulatory properties (Fitzpatrick *et al*., 2012). Membrane Lipoteichoic acid (LTA) of *Bacillus* strains weakly induce Nitric oxide (NO) production, suggestive of immune system stimulation, but not to pathologic extent (Jones *et al*., 2005). *Bacillus* sp. possesses adhesion abilities, produce bacteriocins, and provide immuno-stimulation (Ravi *et al*., 2007) and hence often used as probiotic in aquaculture systems (Nakayama *et al*., 2009).

Dysbiosis, an imbalance of the gut microbiota, is associated with several diseases (Martin *et al*., 2014), thus promulgating the use of probiotics in maintenance of gut microbial balance. Many bacteriocin producing bacteria find use as probiotics. The primary aim of this chapter was to characterize the bacteriocin producer strains *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14, for their probiotic potential.

Certain physiological criteria, especially tolerance to low pH and bile salt, self aggregation and co-aggregation, as well as the capacity to adhere to intestinal mucus must be fulfilled by bacteria to qualify as probiotic (Table 7.1).

Table 7.1 *In vitro* assays employed during screening for novel probiotic strains

Probiotic Property	Assays	References
Surviving stress within the host	Low pH and bile salt	Marteau *et al.,* 1997
Safety assays	Antibiotic resistance	Delgado *et al.,* 2007
	Hemolytic activity	Pisano *et al.,* 2014
	Adhesion to mammalian cells	Cravioto *et al.,* 1979
Colonization of the host	Cell surface hydrophobicity	Ouwehand *et al.,* 1995
	Auto-aggregation screening	Del Re., 2000
	Biofilm formation	Jones & Versolovic., 2009
Antimicrobial assays	Production of antimicrobial substances such as organic acids and bacteriocins;	Tagg *et al.,* 1976
	Coaggregation with pathogens	Yuki *et al*., 2000
Additional health benefits	Cardiovascular effects	De Smet *et al*., 1998
	Anticancer action	Castro *et al*., 2010

7.2 Materials and methods

7.2.1 Low pH tolerance and bile salt tolerance

The bacteria (*Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14) were grown in Zobell marine broth at pH ranging from 3 to 9.

Bile salt tolerance was tested on Nutrient agar with 0.5 % bile salt (Sodium taurocholate). The organisms were streak plated on the agar and incubated at 37 °C for 24 h (Marteau *et al.,* 1997).

7.2.2 Haemolytic activity

Haemolytic activity was determined by streaking on Blood agar plates with 5% defibrinated blood, after incubating at 37 °C for 24 h (Pisano *et al.,* 2014).

7.2.3 Aggregation and co-aggregation assay

Aggregation assay was conducted according to Del Re (2000), wherein bacteria were cultured at 37 °C for 24 h in Zobell broth, cells harvested by centrifugation and suspended in PBS to get $O.D_{600}$ = 0.5. After incubation for 2 h at 37 °C, 1 mL of the upper suspension was transferred to another tube and absorbance (λ=600 nm) was measured. Aggregation was expressed as

$$\text{Aggregation (\%)} = 1 - (A_{time} / A_{initial}) \times 100$$

Where:

A_{time} = Absorbance (λ=600 nm) at different incubation times (2 and 24 h).

$A_{initial}$ = Absorbance (λ=600 nm) before incubation period.

For co-aggregation assay (Yuki *et al*., 2000**)** *B. amyloliquefaciens* BTSS3 and *B. pumilus* SDG14 strains were inoculated into Zobell broth, while the test pathogenic strains *Escherichia coli, Pseudomonas aeruginosa, Vibrio harveyi, Salmonella* Typhimurium *and Klebsiella pneumoniae* inoculated into Luria broth, and were incubated at 35 ± 2 °C for 24 h. The bacterial suspensions were then centrifuged at 7000X g for 10 min at 4 °C, cells washed three times with 0.1 M

PBS and resuspended in equal volume of buffer. Optical density (OD_{600}) was adjusted to 1.0 for probiotic strains & pathogens. Mixtures were then made of BTSS3 and SDG14 with each test pathogen at 1 :1 ratio (total volume = 10 mL) and incubated at 35 ± 2 °C for 4 h. Absorbance (λ=600 nm) was measured for the mixtures, as well as for the individual strains.

$$\text{Coaggregation (\%)} = \left[\left[\frac{\text{Ap} + \text{Ab}}{2}\right] - \text{Amix}\right] \div \left[\frac{\text{Ap} + \text{Ab}}{2}\right] \times 100$$

Where A_p and A_b are the absorption of pathogen and *Bacillus* sp respectively. A_{mix} is the absorption of the mixture.

Aggregation and co-aggregation was statistically evaluated by one way ANOVA assuming that variances are equal across groups or samples. Thus aggregation and co aggregation were tested for homogeneity by Brown and Forsythe test and Bartlett's test. Aggregation was evaluated for correlation with time by Pearson's correlation coefficient r.

7.2.4 Biofilm formation assay

Biofilms or adherent structured microbial communities of the gastrointestinal beneficial bacteria may have a protective role. Quantification of biofilm formation by the bacteriocin producers were performed by modified micro-titre plate method (Rode *et al.,* 2007).

Sterile 96 well polystyrene microtitre plates were filled with 230 µL Tryptone soy broth (TSB), and added 20 µL of bacterial cultures (OD=1) to each well (triplicates for each organism), and incubated without shaking at 37 °C for 24 h. TSB alone was the negative control. The contents were poured off, wells washed three times with 0.01 M phosphate buffer of pH 7.2 and the attached biofilms were fixed with methanol for 15 minutes. The plates were decanted, air

dried and stained with 1 % crystal violet for 5 minutes. Excess stain was rinsed under tap water and air dried. The bound crystal violet was extracted from each well with 33 % glacial acetic acid and absorbance at 570 nm was measured using UV-Visible Spectrophotometer (Schimadzu, Japan). Based on the absorbance, the biofilm producers were graded as A=Ac= Non Biofilm producers, Ac< A = Weak biofilm producers; 2Ac<A= Moderate biofilm producers; 4Ac<A= Strong biofilm producers; where cut off absorbance Ac was the mean absorbance of the negative control.

All tests were conducted thrice independently and statistically analyzed (Christensen *et al.,* 1985; Stepanovic *et al.,* 2007). All data from biofilm assays were expressed as mean ± SD with each assay conducted in triplicates.

7.2.5 Cell adhesion assay

Adherence was evaluated following Cravioto *et al*., (1979) using HEp-2 cell monolayer at 80 % confluence in minimum essential medium (MEM). HEp-2 (Human Epithelial type 2) cell line is valid for evaluating the adherence and colonization of bacteria in the GI tract. Cells were seeded onto a 24-well tissue culture plate at a concentration of 5 x 10^5 cells mL^{-1}. After 24 h of incubation, a monolayer was obtained.

The probiotic strains were inoculated into tubes with 5 mL Zobell marine broth and incubated at 30 ± 2 °C for 2 or 18 h. The cultures were centrifuged and resuspended in Zobell marine broth. The cell suspensions were mixed with equal volume of MEM (without antibiotic) to a final concentration of 5 x 10^7 cells/ mL, and 1 mL of each suspension was added to appropriate wells of the culture plate. After incubation at 37 °C, the monolayers were washed five times with phosphate-buffered saline (PBS) to remove bacteria unfastened to the HEp-2 cells. The plates were stained by Gram staining and observed under inverted

microscope (Leica, Germany). The result of the adhesion assay is expressed as adhesion index for each strain. The adhesion index is defined as the number of adherent bacterial cells per 100 epithelial cells. The minimum adherence criterion is that at least ten bacteria should adhere per HEp-2 epithelial cell.

The ANOVA and column statistics for cell adhesion were calculated.

7.2.6 Statistical analysis

All the calculations were done in Microsoft Excel 2007 and statistical analysis mentioned in above sections were carried out using GraphPad Prism v 6.0.

7.3 Results and discussion

7.3.1 Low pH tolerance and bile salt tolerance

B. amyloliquefaciens BTSS3 and *B. pumilus* SDG14 could survive in pH 3 to 8 (Fig 7.1). Their survival at low pH is considered a desirable probiotic trait as it helps the organisms to withstand the low stomach pH due to HCl. Though stomach pH is 1.5, survival even at pH 3 is desirable to consider an organism as probiotic. *B. subtilis* E20 isolated from natto, a *B. subtilis* fermented food, was considered as probiotic even though it could tolerate only a pH range of 5-10. This organism was studied as a probiotic on white shrimp, *Litopenaeus vannamei* and could enhance immunity and disease resistance (Liu *et al*., 2009).

In addition, the lactic acid bacteria*, Leuconostoc mesenteroides* isolated from the intestine of snakehead fish (*Channa striatus*) showing growth at pH 3-7 has also been considered as probiotic (Allameh *et al.,* 2012).

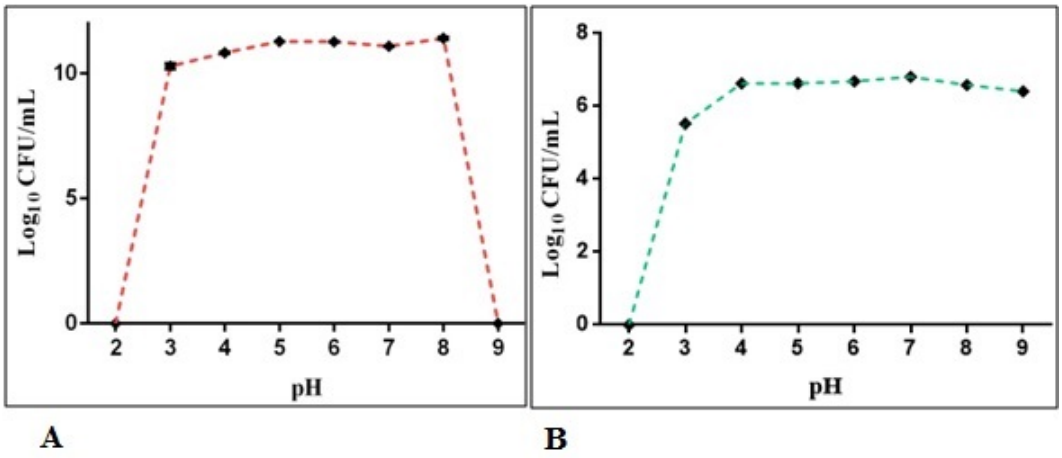


Fig 7.1. pH tolerance of A) *Bacillus amyloliquefaciens* BTSS3 and B) *Bacillus pumilus* SDG14

While studying their bile salt tolerance, it was noted that both BTSS3 and SDG14 could grow in nutrient agar with 0.5 % bile salt (Fig 7. 2).

In vitro studies have demonstrated the cytotoxic and bacteriostatic effects of bile salts on biliary organisms (Sung *et al*., 1993). Thus bile salt tolerance is an indispensable feature for probiotic stains to colonize in small intestine. The five probiotic strains from small intestine *L. plantarum* (G95a and G96a) and *L. rhamnosus* (G92, G99c, and G119b) were pH tolerant and could grow on varying concentrations of bile salt (0.5 % to 2.0 %) (Tambekar and Bhutada, 2010). Three strains *Bacillus amyloliquefaciens*, *Bacillus megaterium* and *Bacillus subtilis* tolerated up to 0.037 % of bile salt for 24h and were considered as Direct-fed microbials (DFM) (Galarza-Seeber *et al*., 2015). Following oral administration, only few spores of *B. cereus* var. *toyoi* were observed in pigs' stomach, where as there was a rapid increase in spore numbers in the duodenum and jejunum (Jadamus *et al.*, 2001). *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. clausii*, *B. megaterium*, *B. firmus* and species of the *B. cereus* group, isolated from broiler

gastrointestinal tract were all resistant to bile salt and acidic pH (Barbosa *et al*., 2005).

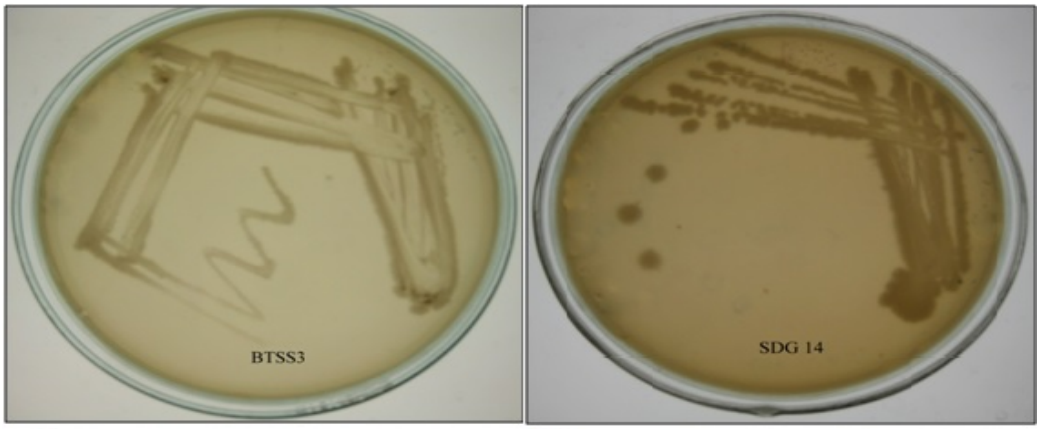


Fig 7.2. Bile salt tolerance plate assay A) *Bacillus amyloliquefaciens* BTSS3 and B) *Bacillus pumilus* SDG14

7.3.2 Haemolytic activity

Haemolytic activity is one of the safety assessment methods for probiotic bacteria. The bacteria *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14 were non-haemolytic on blood agar plates (Fig 7.3). Non-haemolytic bacteria are preferred as probiotic organisms; but certain *Lactobacillus* sp. that are haemolytic due to acid production in the medium are considered potential probiotics (Gómez *et al*., 2016).

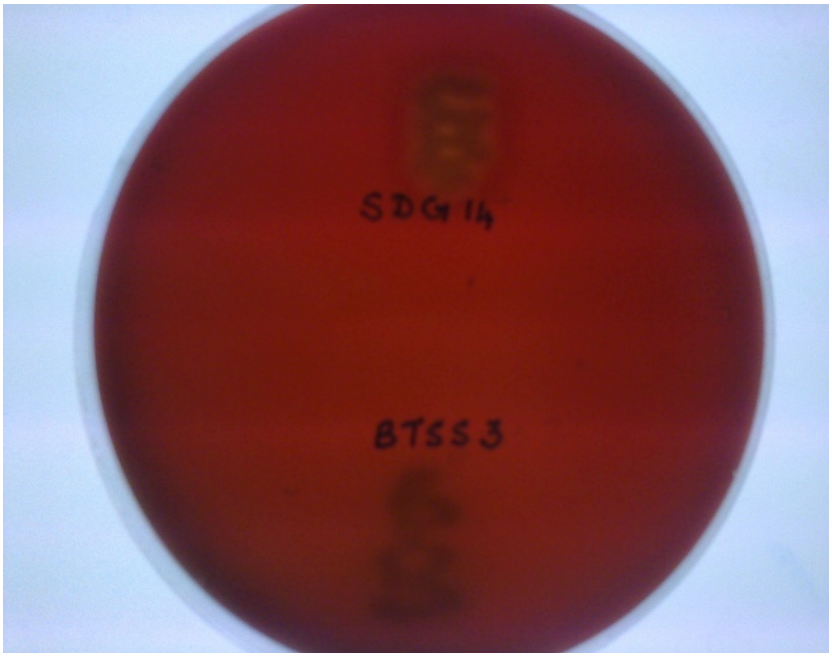


Fig 7.3 Non haemolytic action of BTSS3 and SDG14 on blood agar plate

Haemolytic reaction may be a clear zone of hydrolysis around the colonies (β- haemolysis), a partial hydrolysis and greenish zone (α- haemolysis), or no reaction (γ - haemolysis). α - haemolytic species cause oxidization of iron in haemoglobin molecules within red blood cells, giving it a greenish colour on blood agar. β - haemolytic species cause complete rupture of red blood cells.

7.3.3 Aggregation and co-aggregation assay

Autoaggregation is an important attribute in the attachment of bacteria to various substrata, which can also explain the probiotic nature of the microorganism. An aggregation percentage of 27.22±2.01 % and 18.4±0.48 % after 3 h was observed for *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14 respectively (Fig 7.4). The aggregation was statistically significant for both the organisms and was in correlation with time (Appendix-V).

Autoaggregation is a strain dependent specific trait. *Lactobacillus plantarum* ST8Sh had 51.32 % autoaggregation capacity (Todorov *et al*., 2017), while Collado *et al*., (2008) reported that *L. rhamnosus* GG, *L. rhamnosus* LC-705 and *L. fermentum* ME-3 showed a highest autoaggregation percentage of 20 % at 37 °C after 2 h of incubation and the autoaggregation percentage increased with time. *Lactobacillus acidophilus* M92 autoaggregation was not lost even after washing and suspending of the cells in PBS (Kos *et al*., 2003).

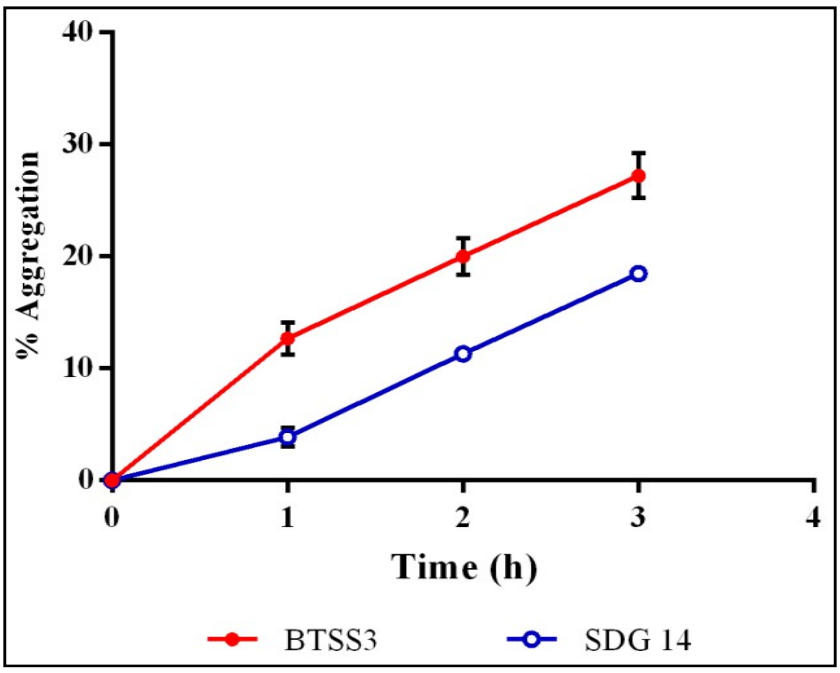


Fig 7. 4. Aggregation of *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14

Both the organisms showed significant co-aggregation with pathogens (Fig 7.5 and &. Fig 7.6). *Bacillus amyloliquefaciens* BTSS3 aggregated more with *Salmonella* Typhimurium. The coaggregation pattern was similar for *Vibrio harveyi*, *Klebsiella pneumoniae* and *E. coli*. Least co-aggregation was for *Pseudomonas aeruginosa* (Fig 7.5). *Bacillus pumilus* SDG14 co-aggregated the

most with *E. coli*, also showing good co-aggregation pattern with *Salmonella* Typhimurium and *Pseudomonas aeruginosa*. Least co-aggregation was with *Vibrio harveyi* and *Klebsiella pneumoniae* (Fig 7.6). Statistical analysis by ANOVA shows that the p value is < 0.0001 was significant (Appendix- V).

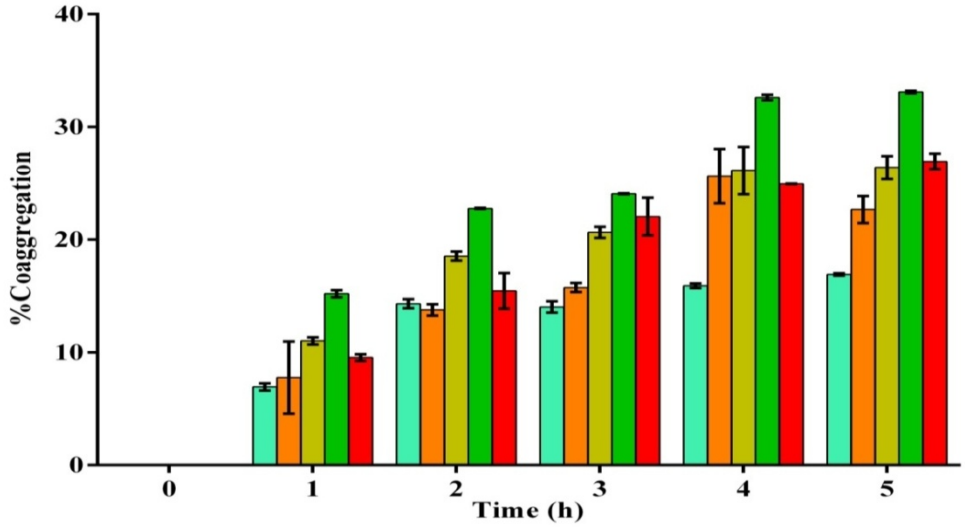


Fig 7.5 Co-aggregation of pathogens by *Bacillus amyloliquefaciens* BTSS3

Lactobacillus acidophilus M92 demonstrated marked co-aggregation with *Enterococcus faecium* L3 (19·46±2·63 %), *E. coli* 3014 (15·11±2·07 %) and *S.* Typhimurium (15·70±2·98 %) (Kos *et al*., 2003). *Lactobacillus plantarum* S1 best coaggregated with enterohaemorrhagic *Escherichia coli* (EHEC), at 41.5 % while *Lactobacillus plantarum* A co-aggregation with *Salmonella* Typhimurium was 40.5 % and *Lactobacillus plantarum* B had good result with *L. mononocytogenes* (37.4 %) (Jankovic *et al*., 2012). These reports strongly suggest that the coaggregation properties shown by *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14 contribute to their use as probiotics.

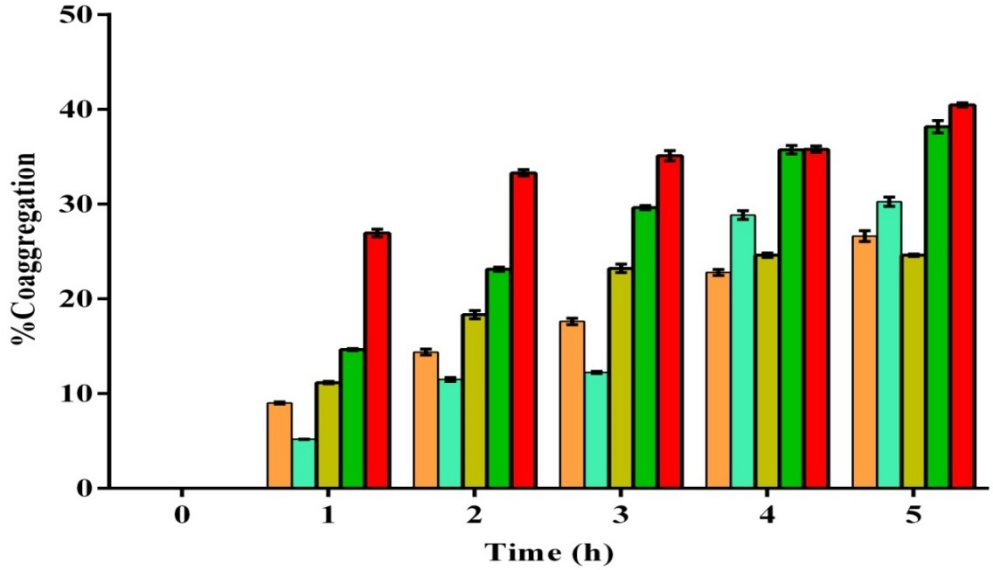


Fig 7.6 Co-aggregation of pathogens by *Bacillus pumilus* SDG14 , *E.coli*, *Vibrio*, *K. pneumoniae*, *P. aeruginosa*, S.Typhimurium

Likewise, many volatile compounds such as volatile sulfur compounds (VSCs) and H_2O_2 produced by oral bacteria *Fusobacterium nucleatum* cause harmful effects on human health. An in vitro test developed by Kang *et al*., (2006) showed that inhibition of volatile compounds production was related to co-aggregation of the LAB strains with *F. nucleatum*.

7.3.4 Biofilm formation assay

Bacillus amyloliquefaciens BTSS3 proved to be a moderate biofilm producer (2Ac<A, A=0.2535±0.0898), while *Bacillus pumilus* SDG14 showed biofilm forming capacity of 4Ac<A (A= 0.5220±0.0622), indicating strong biofilm production (Fig 7.7). The relevance of the assay is that biofilms on either the gastrointestinal tract surface or food particles provide protection against some of the harshest physical and chemical stresses found in the gut, thereby promoting their survival and persistence. Thus biofilm formation is an advantage for probiotic bacteria. Biofilms also have a role in protecting gut epithelium from

adherence of pathogenic agents. Lebeer *et al*., (2007) studied and reported *in vitro* biofilm forming capacity of *Lactobacillus rhamnosus* GG (ATCC53103). Many natural isolates of *B. subtilis* could form complex biofilms, where spore formation occurs preferentially (Branda *et al*., 2001). Probiotic bacilli mostly occur in gut as spores, hence sporulation may occur in the gut (Hoa *et al*., 2001; Rhee *et al*., 2004), with biofilms as the preferred site in this environment.

The biofilm like communities of gastrointestinal and female urogenital tracts contain beneficial microorganisms considered to have a protective role. Biofilm formed by strains of probiotic *Lactobacillus reuteri* modulated human TNF production and the relative abilities to suppress TNF in monocytoid cells were directly correlated to their abilities to aggregate and form biofilms on polystyrene surfaces (Jones and Versalovic, 2009).

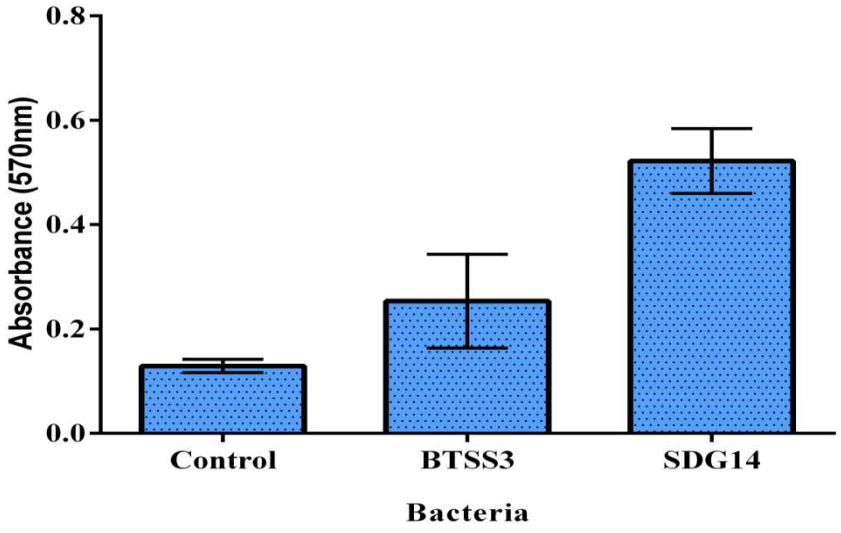


Fig 7.7 Biofilm formation by *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14

The biofilms of bacteriocinogenic microbes which are beneficiary organisms, offer a means to counter the start of pathogenic biofilms based on

competitive exclusion principle. *Lactobacilli* with biofilm-forming aptitudes controlled *Listeria monocytogenes* on abiotic surfaces (Ibarreche *et al*., 2014).

7.3.5 Cell adhesion assay

Adhesion of probiotics to pathogenic organisms facilitate their elimination from the body and their ability to self-aggregate are advantageous in gut their distribution.

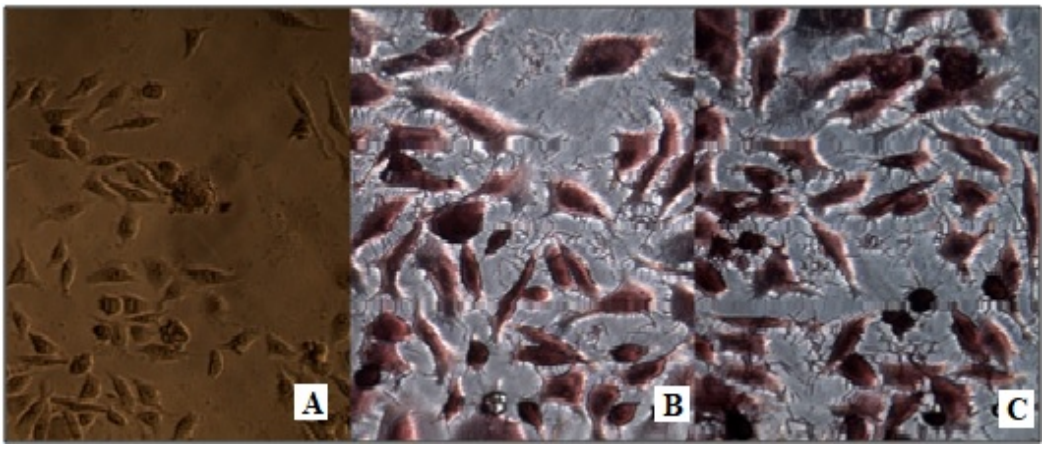


Fig 7.8 Bacterial cell adhesion to HEp-2 cell line. Adhesion of *Bacillus amyloliquefaciens* BTSS3 to HEp-2 cell line after incubation. A) Control B) 60 minutes and C) 90 minutes. The cells were viewed in inverted microscope at 40x magnification.

The bacteria, *Bacillus amyloliquefaciens* BTSS3 showed pronounced adherence to HEp-2 cells even after 60 min (Fig 7.8). The adhesion index was 14.91±3.45 after 60 minutes and 32±10.38 after 90 minutes.

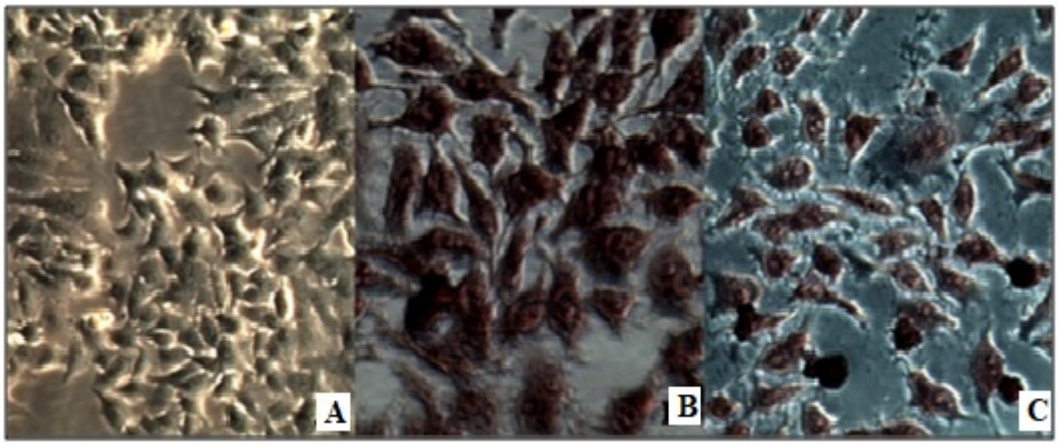


Fig 7.9 Bacterial cell adhesion to HEp-2 cell line. Adhesion of *Bacillus pumilus* SDG14 to HEp-2 cell line after incubation. A) Control B) After 60 minutes and C) After 90 minutes. The cells were viewed in inverted microscope at 40x magnification.

Bacillus pumilus SDG14 also showed very prominent adherence even after 1 h (Fig 7.9). The adhesion index was 36.82±5.93 after 60 minutes and 45.54±9.55 after 90 minutes. ANOVA showed that the P values were significant (< 0.05) with significantly different standard deviations (Appendix-V).

The intestinal mucus is a classic model for testing *in vitro* adherence since different receptors can be located in the small and large intestine mucus using the specific adherence. Bacteria have pili which adhere to intestinal epithelial cells and colonize the small intestine (Smith & Longgood, 1971). Several *Bacillus* sp. isolated from probiotic preparations had different adhesion properties. Biochemical analysis suggested the role of surface associated proteins in adhesion (Sa´nchez *et al.,* 2009). The probiotic potential of 10 bacillus isolates from chick intestine was studied against *Salmonella* Enteritidis infection (Thirabunyanon and Thongwittaya, 2012). In 2004 Duc *et al.,* reported Biosubtyl, a strain of *Bacillus pumilus*, a prospective probiotic agent producing antimicrobial agents. Several studies on adherence in potential probiotic LAB using the HEp-2 and Caco-2 cell

lines are reported (Forestier *et al*., 2001; Thornley *et al*., 1996). 112 LAB strains evaluated as probiotics showed significant autoaggregation and coaggregation, as well as strong adherence to HEp-2 cells (Ehrmann *et al.,* 2002). Tuomola and Salminen (1998) demonstrated variable adherence among twelve *Lactobacilllus* strains using the Caco-2 cell line and attributed this to strain source or surface properties.

7.4 Summary

From this study we conclude that autoaggregation capacity ability to coaggregate with pathogenic strains, together with 'low pH' and bile salt tolerance, are important criteria for preliminary selection and identification of probiotic bacteria with potential applications in human and animal systems. Most *Bacillus* species are harmless and are in fact beneficial to mammals. Their aggregation, co-aggregation properties and ability to adhere on animal cell line make *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14 promising probiotic organisms. Between the probiotic characteristics of the bacterium and bacteriocin producing capability, greater emphasis should be on the antimicrobial property, an added advantage in probiotics.

Chapter 8

Summary and Conclusion

Bacteriocins are ribosomally synthesized antagonistic peptides produced by bacteria against similar species. They are the latest additions to drugs and food preservatives using microbial technology. WHO encouraged research in the development of new peptide antibiotics not only due to increased antibiotic resistance in bacteria and the advent of new bacterial and viral diseases, but also due to increase in cancer patients.

In this study the gut contents of two marine fishes were screened for bacteriocin producers. The fishes selected for the study included the deep sea shark and an Indian oil sardine. Table 8.1 depicts the summary of the screening and identification of fish and bacteriocin producers.

Table 8.1 Summary of the screening and identification of fish and bacteriocin producers

Out put	Deep Sea fish	Indian oil Sardine
Identification of fish	*Centroscyllium fabricii* (SSDSS1)	*Sardinella longiceps* (SFF)
Number of bacterial isolates	72	25
Identification of selected bacteriocin producing bacteria	*Bacillus amyloliquefaciens* BTSS3	*Bacillus pumilus* SDG14
MCC Catalogue No.	MCC2981	MCC3070
Bacteriocin	BaCf3	BpSl14

Media was optimized for the production of BaCf3 by *Bacillus amyloliquefaciens* BTSS3 and BpSl14 by *Bacillus pumilus* SDG14. Time course study with the optimized media recalibrated the production time. The comparison

of bacteriocin production in optimized production media is summarized in table 8.2.

Table 8.2 Production of bacteriocins after media optimization

Component	BaCf3	BpSl14
Activity (AU/mL)	6400	12800
Specific activity (AU/mg)	4514.307±96.417	9339.276±88.445
Fold of increase in activity (AU/mL)	8	4
Fold of increase in specific activity (AU/mg)	36	5.8

Both bacteriocins were purified by ammonium sulphate precipitation, dialysis and gel filtration chromatography final yield of 6.4% and 8% for BaCf3 and BpSl14 respectively. The outcome of the characterization studies is summarized in table 8.3.

The amino acid composition of the bacteriocin peptide by ProtParm tool revealed Glycine to be the most predominant amino acid in both bacteriocins, followed by Cysteine and Glutamine in BaCf3 and Proline in BpSl14. The presence of Glycine indicates the flexibility of the molecules while other amino acids help in their action on bacterial cell wall. The secondary and tertiary structures of the two bacteriocins were derived from the *de novo* sequences using I-TASSER server. DiANNA 1.1 server predicted the possibility of disulphide bridge formation between C6 and C13 of BaCf3.

Table 8.3 Summary of characterization studies

Characterization	BaCf3	BpSl14
Temperature stability	30 to 100 °C for 1 h and 121 °C, 15 lbs for 15 minutes	30 to 100 °C for 1 h and 121 °C, 15 lbs for 15 minutes
pH stability**	2-13	2-13
Action of hydrolytic enzyme	Complete reduction in activity by 5 mg/mL of enzymes	50 % reduction in activity by 5 mg/mL of enzymes
Effect of metal ions*	Affected by Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+} and Al^{3+}	Cu^{2+}, Mn^{2+} and Al^{3+}
Effect of DTT	12.5 % residual activity in 50 mM and no activity in 100 mM	100 % residual activity
Effect of DMSO	12.5 % residual activity	100 % residual activity
Effect of β-mercaptoethanol	12.5 % residual activity	100 % residual activity
Molecular weight	3028.422 Da	6061.2 Da
Sequence	GNHDCCMGQLPKCML GPGQGAATXQGK	AGGGFMEHEGSEPVGP GGPKGDHMQDGKIPND LCC
Mode of action	Cell wall acting	Cell wall acting

* Activity of BaCf3 was nullified by the addition of Ca^{2+}, Mg^{2+}, Mn^{2+} and reduced by Ba^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}
* Activity of BpSl14 was reduced by Cu^{2+}, Mn^{2+} and Al^{3+}
** 50 % reduction in activity of BaCf3 in pH 11 to 13

The bacteriocins had antibiofilm action on some of the standard NCIM cultures as well as some food spoilage organisms. The organisms included *Escherichia coli*, *Bacillus pumilus*, *Clostridium perfringens*, *Bacillus coagulans*

and *Salmonella* Typhimurium from NCIM and *Bacillus altitudinus* BTMW1, *Pseudomonas aeruginosa* BTRY1, *Bacillus* sp. SD1, *Bacillus licheniformis* SD2, *Staphylococcus warneri* DF2, *Micrococcus luteus* FF, *Geobacillus stearothermophilus* FF2, *Bacillus niacini* DP3 and *Brevibacterium casei* DF1which were lab isolates from different food sources. The biofilm inhibitory concentrations of both the bacteriocins were very low; <5 μg/mL of bacteriocin completely inhibited biofilm formation by all the biofilm producers studied.

In order to ensure the broad application of the bacteriocins, their cytotoxicity was tested on normal mouse fibroblast cell line 3T3-L1. High concentration (>200 μg/mL) of bacteriocins was required for significant cytotoxicity on 3T3-L1 cells. Since the bacteriocins were non-cytotoxic, their anticancer activity was tested on A549 cell line by MTT assay, phase contrast and fluorescent microscopy. MTT assay revealed that cell proliferation of A549 was inhibited by both BaCf3 and BpSl14 in a concentration and time dependent manner. Fluorescent microscopy and phase contrast microscopy showed that bacteriocins induced morphological changes in the cancerous cells and apoptosis. Thick orange coloured apoptotic bodies were visible in the orange coloured dead cells.

The bacteriocin model suggested by I-TASSER was docked to selected apoptotic receptors like Death receptor, DR5 (PDB ID - 1D0G), Glucose transporter, GLUT1 (PDB ID - 1SUK), Tyrosine kinase (PDB ID- 3DKC) and Transforming Growth Factor-β, TGF-β (PDB ID - 5E8T). The docking scores (balanced score) showed lowest with DR5 receptor for BpSl14 where as for BaCf3, docked models with TGF-β had lowest energy. When the interactions were studied both the ligands were predicted to interact with the amino acids in the active site of the receptors.

Bacteria with antagonistic potential are generally used as probiotics. Anticancer activity of the bacteriocins along with their antibiofilm potential against pathogens is an added advantage of a probiotic. Both *Bacillus amyloliquefaciens* BTSS3 and *Bacillus pumilus* SDG14 were sensitive to several antibiotics tested, showing their safety in use. Probiotic characterization of the organisms are summarized in table 8.4

Table 8.4 Summary of probiotic characterization of the organisms

Probiotic trait	***Bacillus amyloliquefaciens* BTSS3**	***Bacillus pumilus* SDG14**
Bacteriocin production	+	-
pH tolerance	pH 3	pH 3
Bile Salt tolerance	+	+
Heamolytic activity	non-haemolytic	non-haemolytic
Aggregation (%)	27.22±2.01	18.4±0.48
Co-aggregation	+	+
Biofilm formation	2Ac <A	4Ac <A
Adherence capacity (after 90 min)	32±10.38	45.54±9.55

In a future perspective the bacteriocins could be studied for additional biological applications such as anti-inflammatory, enzyme inhibition and so on. The anticancer activity can be extensively studied for future clinical applications.

The bacteriocins can be used to develop antibiofilm surfaces and can be incorporated in polymeric substances for biofilm prevention in food preservation and biomedical applications. The bacteriocins may be checked for their compatibility may then be used as medical catheter material. Medical catheters are considered as a general source of infection in immunologically weak individuals such as cancer and kidney patients. Researchers are in constant search

for innovative methods to combat such secondary infections, thus the use of modified polymers is a solution in itself than other methods of antibiotic administration. Bacteriocins could also be used for making antibacterial polyurethane films used for food preservation.

The probiotic bacteria may be studied for feed application in aquaculture, poultry and others, to enhance their economic values. Probiotic feed development can be considered as one of the outcomes of the present study. A continuous study can be designed to develop an aquaculture feed supplement and its implications on the cultured fish. The study can be continued till the development of marketable probiotic feed.

REFERENCES

Aasen, I. M., Møretrø, T., Katla, T., Axelsson, L., & Storrø, I. (2000). Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. *Applied Microbiology and Biotechnology*, *53*(2), 159-166.

Abdi-Ali, A., Worobec, E. A., Deezagi, A., & Malekzadeh, F. (2004). Cytotoxic effects of pyocin S2 produced by *Pseudomonas aeruginosa* on the growth of three human cell lines. *Canadian journal of microbiology*, *50*(5), 375-381.

Abriouel, H., Franz, C. M., Omar, N. B., & Gálvez, A. (2011). Diversity and applications of *Bacillus* bacteriocins. *FEMS microbiology reviews*, *35*(1), 201-232.

Abriouel, H., Valdivia, E., Gálvez, A., & Maqueda, M. (2001). Influence of physico-chemical factors on the oligomerization and biological activity of bacteriocin AS-48. *Current microbiology*, *42*(2), 89-95.

Acuña, L., Picariello, G., Sesma, F., Morero, R. D., & Bellomio, A. (2012). A new hybrid bacteriocin, Ent35–MccV, displays antimicrobial activity against pathogenic Gram-positive and Gram-negative bacteria. *FEBS Open Bio*, *2*(1), 12-19.

Albano, H., Todorov, S. D., van Reenen, C. A., Hogg, T., Dicks, L. M., & Teixeira, P. (2007). Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. *International Journal of Food Microbiology*, *116*(2), 239-247.

Alisky, J., Iczkowski, K., Rapoport, A., & Troitsky, N. (1998). Bacteriophages show promise as antimicrobial agents. *Journal of Infection*, *36*(1), 5-15.

Allameh, S. K., Daud, H., Yusoff, F. M., Saad, C. R., & Ideris, A. (2012). Isolation, identification and characterization of *Leuconostoc mesenteroides* as a new probiotic from intestine of snakehead fish (*Channa striatus*). *African Journal of Biotechnology, 11*(16), 3810-3816.

Alley, M. C., Scudiero, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., ... & Boyd, M. R. (1988). Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer research*, *48*(3), 589-601.

Alò, P. L., Visca, P., Botti, C., Galati, G. M., Sebastiani, V., Andreano, T., ... & Pizer, E. S. (2001). Immunohistochemical expression of human erythrocyte glucose transporter and fatty acid synthase in infiltrating breast carcinomas and adjacent typical/atypical hyperplastic or normal breast tissue. *American journal of clinical pathology*, *116*(1), 129-134.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of molecular biology*, *215*(3), 403-410.

Al-Wendawi, S. A., & Al-Saady, A. A. (2012). Screening for bacteriocins production in Enteric Bifidobacterium isolates and study of some production affecting factors. *Medical journal of Babylon*, *9*(2-P), 386-396.

Anacarso, I., Messi, P., Condò, C., Iseppi, R., Bondi, M., Sabia, C., & de Niederhäusern, S. (2014). A bacteriocin-like substance produced from *Lactobacillus pentosus* 39 is a natural antagonist for the control of *Aeromonas hydrophila* and *Listeria monocytogenes* in fresh salmon fillets. *LWT-Food Science and Technology*, *55*(2), 604-611.

Anju, T. R., Ajayan, M. S., & Paulose, C. S. (2013). Disruption of cerebellar cholinergic system in hypoxic neonatal rats and its regulation with glucose, oxygen and epinephrine resuscitations. *Neuroscience*, *236*, 253-261.

Ansari, A., Aman, A., Siddiqui, N. N., & Iqbal, S. (2012). Bacteriocin (BAC-IB17): screening, isolation and production from *Bacillus subtilis* KIBGE IB-17. *Pakistan journal of pharmaceutical sciences*, *25*(1).

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*(2), 872-877.

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*(4), 308-313.

Austin, B. (1988). *Marine microbiology*. CUP Archive.

Austin, B. (2006). The bacterial microflora of fish, revised. *The Scientific World Journal*, *6*, 931-945.

Austin, B., Stuckey, L. F., Robertson, P. A. W., Effendi, I., & Griffith, D. R. W. (1995). A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *Journal of Fish Diseases*, *18*(1), 93-96.

Ausubel, F. M., Brent, R., Kingston, R. E., Morre, D., Seidman, J. G., & Struhl, K. (1987). Current protocols in molecular biology (New York: Greene and Wiley-interscience).

Aymerich, T., Artigas, M. G., Garriga, M., Monfort, J. M., & 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*(4), 686-694.

Bai, Y., Zhou, X., & Smith, D. L. (2003). Enhanced soybean plant growth resulting from co- inoculation of strains with. *Crop science*, *43*(5), 1774-1781.

Balasubramanyam, B. V., & Varadaraj, M. C. (1998). Cultural conditions for the production of bacteriocin by a native isolate of *Lactobacillus delbruecki* ssp. *bulgaricus* CFR 2028 in milk medium. *Journal of applied microbiology*, *84*(1), 97-102.

Balciunas, E. M., Martinez, F. A. C., Todorov, S. D., de Melo Franco, B. D. G., Converti, A., & de Souza Oliveira, R. P. (2013). Novel biotechnological applications of bacteriocins: a review. *Food Control*, *32*(1), 134-142.

Barbosa, T. M., Serra, C. R., La Ragione, R. M., Woodward, M. J., & Henriques, A. O. (2005). Screening for *Bacillus* Isolates in the Broiler Gastrointestinal Tract. *Applied and Environmental Microbiology*, *71*(2), 968–978. http://doi.org/10.1128/AEM.71.2.968-978.2005.

Barboza-Corona, J. E., de la Fuente-Salcido, N., Alva-Murillo, N., Ochoa-Zarzosa, A., & López-Meza, J. E. (2009). Activity of bacteriocins synthesized by

Bacillus thuringiensis against *Staphylococcus aureus* isolates associated to bovine mastitis. *Veterinary microbiology*, *138*(1), 179-183.

Bárcena, J. B., Sineriz, F., de Llano, D. G., Rodríguez, A., & Suárez, J. E. (1998). Chemostat production of Plantaricin C by *Lactobacillus plantarum* LL441. *Applied and environmental microbiology*, *64*(9), 3512-3514.

Barefoot, S. F., & Klaenhammer, T. R. (1984). Purification and characterization of the *Lactobacillus acidophilus* bacteriocin lactacin B. *Antimicrobial Agents and Chemotherapy*, *26*(3), 328-334.

Barton, M. D., & Hart, W. S. (2001). Public health risks: Antibiotic resistance. *Asian Australasian journal of animal sciences*, *14*(3), 414-422.

Beaulieu, L. (2005). Production, purification and characterization of natural pediocine PA-1 and its recombinant forms: Contribution to the discovery of a new biological activity

Bergh, Ø. (1995). Bacteria associated with early life stages of halibut, *Hippoglossus hippoglossus* L., inhibit growth of a pathogenic *Vibrio* sp. *Journal of Fish Diseases*, *18*(1), 31-40.

Bhardwaj, A., Gupta, H., Kapila, S., Kaur, G., Vij, S., & Malik, R. K. (2010). Safety assessment and evaluation of probiotic potential of bacteriocinogenic *Enterococcus faecium* KH 24 strain under in vitro and in vivo conditions. *International journal of food microbiology*, *141*(3), 156-164.

Bhugaloo-Vial, P., Dousset, X., Metivier, A., Sorokine, O., Anglade, P., Boyaval, P., & Marion, D. (1996). Purification and amino acid sequences of piscicocins V1a and V1b, two class IIa bacteriocins secreted by *Carnobacterium piscicola* V1 that display significantly different levels of specific inhibitory activity. *Applied and Environmental Microbiology*, *62*(12), 4410-4416.

Bhunia, A. K., Johnson, M. C., & Ray, B. (1988). Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. *Journal of Applied Microbiology*, *65*(4), 261-268.

Biéler, S., Silva, F., & Belin, D. (2010). The polypeptide core of Microcin E492 stably associates with the mannose permease and interferes with mannose metabolism. *Research in microbiology*, *161*(8), 706-710.

Bierbaum, G., & Sahl, H. G. (1985). Induction of autolysis of staphylococci by the basic peptide antibiotics Pep 5 and nisin and their influence on the activity of autolytic enzymes. *Archives of microbiology*, *141*(3), 249-254.

Bierbaum, G., & Sahl, H. G. (1987). Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of N-acetylmuramoyl-L-alanine amidase. *Journal of Bacteriology*, *169*(12), 5452-5458.

Bierbaum, G., Brötz, H., Koller, K. P., & Sahl, H. G. (1995). Cloning, sequencing and production of the lantibiotic mersacidin. *FEMS microbiology letters*, *127*(1-2), 121-126.

Bindiya, E. S., & Bhat, S. G. (2016). Marine Bacteriocins: A Review. *J Bacteriol Mycol Open Access*, *2*(5), 00040.

Bindiya, E. S., Tina, K. J., Raghul, S. S., & Bhat, S. G. (2015). Characterization of Deep Sea Fish Gut Bacteria with Antagonistic Potential, from *Centroscyllium fabricii* (Deep Sea Shark).*Probiotics and antimicrobial proteins*, *7*(2), 157-163.

Biswas, S. R., Ray, P., Johnson, M. C., & Ray, B. (1991). Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. *Applied and Environmental Microbiology*, *57*(4), 1265-1267.

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*(2), 158-162.

Bizani, D., Motta, A. S., Morrissy, J. A., Terra, R., Souto, A. A., & Brandelli, A. (2005). Antibacterial activity of cerein 8A, a bacteriocin-like peptide produced by *Bacillus cereus*. *International Microbiology*, *8*(2), 125-131.

Blum, H., Beier, H., & Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, *8*(2), 93-99.

Bosshard, P. P., Abels, S., Zbinden, R., Böttger, E. C., & Altwegg, M. (2003). Ribosomal DNA sequencing for identification of aerobic gram-positive rods in

the clinical laboratory (an 18-month evaluation). *Journal of clinical microbiology*, *41*(9), 4134-4140.

Bradley, D. E. (1967). Ultrastructure of bacteriophage and bacteriocins. *Bacteriological reviews*, *31*(4), 230.

Branda, S. S., González-Pastor, J. E., Ben-Yehuda, S., Losick, R., & Kolter, R. (2001). Fruiting body formation by *Bacillus subtilis*. *Proceedings of the National Academy of Sciences*, *98*(20), 11621-11626.

Breukink, E., Wiedemann, I., Van Kraaij, C., Kuipers, O. P., Sahl, H. G., & De Kruijff, B. (1999). Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science*, *286*(5448), 2361-2364.

Brockmann, K., Wang, D., Korenke, C. G., Von Moers, A., Ho, Y. Y., Pascual, J. M., ... & Fischbarg, J. (2001). Autosomal dominant Glut-1 deficiency syndrome and familial epilepsy. *Annals of neurology*, *50*(4), 476-485.

Bryers, J. D., & Ratner, B. D. (2004). Bioinspired implant materials befuddle bacteria. *ASM News-American Society for Microbiology*, *70*(5), 232-232.

Buchanan, S. G., Hendle, J., Lee, P. S., Smith, C. R., Bounaud, P. Y., Jessen, K. A., ... & Peterman, M. C. (2009). SGX523 is an exquisitely selective, ATP-competitive inhibitor of the MET receptor tyrosine kinase with antitumor activity in vivo. *Molecular cancer therapeutics*, *8*(12), 3181-3190.

Bucklin, A., Guarnieri, M., Hill, R. S., Bentley, A. M., & Kaartvedt, S. (1999). Taxonomic and systematic assessment of planktonic copepods using mitochondrial COI sequence variation and competitive, species-specific PCR. *Hydrobiologia*, *401*, 239-254.

Cao, L. T., Wu, J. Q., Xie, F., Hu, S. H., & Mo, Y. (2007). Efficacy of nisin in treatment of clinical mastitis in lactating dairy cows. *Journal of dairy science*, *90*(8), 3980-3985.

Carolissen-Mackay, V., Arendse, G., & Hastings, J. W. (1997). Purification of bacteriocins of lactic acid bacteria: problems and pointers. *International Journal of Food Microbiology*, *34*(1), 1-16.

Cascales, E., Buchanan, S. K., Duché, D., Kleanthous, C., Lloubes, R., Postle, K., ... & Cavard, D. (2007). Colicin biology. *Microbiology and Molecular Biology Reviews*, *71*(1), 158-229.

Castelli, P., Caronno, R., Ferrarese, S., Mantovani, V., Piffaretti, G., Tozzi, M., ... & Sala, A. (2006). New trends in prosthesis infection in cardiovascular surgery. *Surgical infections*, *7*(Supplement 2), s-45.

Castro, M. S., Molina, M. A., Di Sciullo, P., Azpiroz, M. B., Leocata Nieto, F., Sterín-Speziale, N. B., ... & Manghi, M. A. (2010). Beneficial activity of *Enterococcus faecalis* CECT7121 in the anti-lymphoma protective response. *Journal of applied microbiology*, *109*(4), 1234-1243.

Chaieb, K., Kouidhi, B., Jrah, H., Mahdouani, K., & Bakhrouf, A. (2011). Antibacterial activity of Thymoquinone, an active principle of Nigella sativa and its potency to prevent bacterial biofilm formation. *BMC complementary and alternative medicine*, *11*(1), 29.

Champomier-Vergès, M. C., Chaillou, S., Cornet, M., & Zagorec, M. (2001). *Lactobacillus sakei*: recent developments and future prospects. *Research in Microbiology*, *152*(10), 839-848.

Chavan, M., & Riley, M. (2007). Molecular evolution of bacteriocins in gram-negative bacteria. *Bacteriocins*, 19-43.

Chen, P., Novak, J., Kirk, M., Barnes, S., Qi, F., & Caufield, P. W. (1998). Structure-activity study of the lantibiotic mutacin II from *Streptococcus mutans* T8 by a gene replacement strategy. *Applied and environmental microbiology*, *64*(7), 2335-2340.

Chen, Y., Shapira, R., Eisenstein, M., & Montville, T. J. (1997). 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*(2), 524-531.

Cherif, A., Chehimi, S., Limem, F., Hansen, B. M., Hendriksen, N. B., Daffonchio, D., & Boudabous, A. (2003). Detection and characterization of the novel bacteriocin entomocin 9, and safety evaluation of its producer, *Bacillus*

thuringiensis ssp. *entomocidus* HD9. *Journal of Applied Microbiology*, *95*(5), 990-1000.

Chopra, L., Singh, G., Kumar Jena, K., & Sahoo, D. K. (2015). Sonorensin: A new bacteriocin with potential of an anti-biofilm agent and a food biopreservative. *Scientific Reports*, *5*, 13412. http://doi.org/10.1038/srep13412

Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, D. M., & Beachey, E. H. (1985). Adherence of coagulase-negative *Staphylococci* to plastic tissue culture plates: a quantitative model for the adherence of *Staphylococci* to medical devices. *Journal of clinical microbiology*, *22*(6), 996-1006.

Chumchalová, J., & Šmarda, J. (2003). Human tumor cells are selectively inhibited by colicins. *Folia microbiologica*, *48*(1), 111-115.

Cheung, J., Danna, K. J., O'Connor, E. M., Price, L. B., & Shand, R. F. (1997). Isolation, sequence, and expression of the gene encoding halocin H4, a bacteriocin from the halophilic archaeon *Haloferax mediterranei* R4. *Journal of bacteriology*, *179*(2), 548-551.

Cladera-Olivera, F., Caron, G. R., & Brandelli, A. (2004a). Bacteriocin production by *Bacillus licheniformis* strain P40 in cheese whey using response surface methodology. *Biochemical Engineering Journal*, *21*(1), 53-58.

Cladera-Olivera, F., Caron, G. R., & Brandelli, A. (2004b). Bacteriocin-like substance production by *Bacillus licheniformis* strain P40. *Letters in Applied Microbiology*, *38*(4), 251-256.

Clarke, D. J., Robson, R. M., & Morris, J. G. (1975). Purification of two Clostridium bacteriocins by procedures appropriate to hydrophobic proteins. *Antimicrobial agents and chemotherapy*, *7*(3), 256-264.

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International journal of food microbiology*, *71*(1), 1-20.

Coley, W.B. (1891). Contribution to the knowledge of sarcoma. *Annals of Surgery*. ;14:199–220.

Collado, M. C., Meriluoto, J., & Salminen, S. (2008). Adhesion and aggregation properties of probiotic and pathogen strains. *European Food Research and Technology*, *226*(5), 1065-1073.

Collins, B., Cotter, P. D., Hill, C., & Ross, R. P. (2010). Applications of lactic acid bacteria-produced bacteriocins. *Biotechnology of lactic acid bacteria: Novel applications*, 89-109.

Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., & Marrie, T. J. (1987). Bacterial biofilms in nature and disease. *Annual Reviews in Microbiology*, *41*(1), 435-464.

Coughlan, L. M., Cotter, P. D., Hill, C., & Alvarez-Ordóñez, A. (2016). New Weapons to Fight Old Enemies: Novel Strategies for the (Bio)control of Bacterial Biofilms in the Food Industry. *Frontiers in Microbiology*, *7*, 1641. http://doi.org/10.3389/fmicb.2016.01641

Cravioto, A., Gross, R. J., Scotland, S. M., & Rowe, B. (1979). An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. *Current Microbiology*, *3*(2), 95-99.

Cunningham, P., Afzal-Ahmed, I., & Naftalin, R. J. (2006). Docking studies show that D-glucose and quercetin slide through the transporter GLUT1. *Journal of Biological Chemistry*, *281*(9), 5797-5803.

Czárán, T. L., Hoekstra, R. F., & Pagie, L. (2002). Chemical warfare between microbes promotes biodiversity. *Proceedings of the National Academy of Sciences*, *99*(2), 786-790.

Dalmau, M., Maier, E., Mulet, N., Vinas, M., & Benz, R. (2002). Bacterial membrane injuries induced by lactacin F and nisin. *International Microbiology*, *5*(2), 73-80.

Pimentel-Filho, N.J., de Freitas Martins, M. C., Nogueira, G. B., Mantovani, H. C., & Vanetti, M. C. D. (2014). Bovicin HC5 and nisin reduce *Staphylococcus aureus* adhesion to polystyrene and change the hydrophobicity profile and Gibbs free energy of adhesion. *International journal of food microbiology*, *190*, 1-8.

De Lorenzo, V., & Pugsley, A. P. (1985). Microcin E492, a low-molecular-weight peptide antibiotic which causes depolarization of the *Escherichia coli* cytoplasmic membrane. *Antimicrobial agents and chemotherapy*, *27*(4), 666-669.

De Vivo, D. C., Trifiletti, R. R., Jacobson, R. I., Ronen, G. M., Behmand, R. A., & Harik, S. I. (1991). Defective glucose transport across the blood-brain barrier as a cause of persistent hypoglycorrhachia, seizures, and developmental delay. *New England Journal of Medicine*, *325*(10), 703-709.

De Vos, W. M., Jung, G., & Sahl, H. G. (1991). Definitions and nomenclature of lantibiotics. *Nisin and novel lantibiotics. ESCOM Science Pub-lishers, Leiden, The Netherlands*, 457-463.

De Vuyst, L., & Vandamme, E. J. (1992). Influence of the carbon source on nisin production in *Lactococcus lactis* subsp. *lactis* batch fermentations. *Microbiology*, *138*(3), 571-578.

Del Re, B., Sgorbati, B., Miglioli, M., & Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Letters in applied microbiology*, *31*(6), 438-442.

Delgado, A., Brito, D., Peres, C., Noe-Arroyo, F., & Garrido-Fernández, A. (2005). Bacteriocin production by *Lactobacillus pentosus* B96 can be expressed as a function of temperature and NaCl concentration. *Food Microbiology*, *22*(6), 521-528.

Delgado, S., O'sullivan, E., Fitzgerald, G., & Mayo, B. (2007). Subtractive screening for probiotic properties of *Lactobacillus* species from the human gastrointestinal tract in the search for new probiotics. *Journal of food science*, *72*(8).

Deraz, S. F., Karlsson, E. N., Hedström, M., Andersson, M. M., & Mattiasson, B. (2005). Purification and characterisation of acidocin D20079, a bacteriocin produced by *Lactobacillus acidophilus* DSM 20079. *Journal of biotechnology*, *117*(4), 343-354.

Derynck, R., Akhurst, R. J., & Balmain, A. (2001). TGF-β signaling in tumor suppression and cancer progression. *Nature genetics*, *29*(2), 117-129.

De Smet, I., De Boever, P., & Verstraete, W. (1998). Cholesterol lowering in pigs through enhanced bacterial bile salt hydrolase activity. *British Journal of Nutrition*, *79*(2), 185-194.

Diaz, D. (2007). Effect of Bacillus amyloliquefaciens CECT-5940 spores on broiler performance and digestibility. *Published online: http://en. engormix. com/MA-poultry-industry/articles/effect-bacillus-amyloliquefaciens-cect5940-t795/p0. htm*.

Diep, D. B., & Nes, I. F. (1995). A bacteriocin-like peptide induces bacteriocin synthesis in *Lactobacillus plantarum* C11. *Molecular microbiology*, *18*(4), 631-639.

Diop, M. B., Dubois-Dauphin, R., Destain, J., Tine, E., & Thonart, P. (2009). Use of a nisin-producing starter culture of *Lactococcus lactis* subsp. *lactis* to improve traditional fish fermentation in Senegal. *Journal of food protection*, *72*(9), 1930-1934.

Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin production: a probiotic trait?. *Applied and environmental microbiology*, *78*(1), 1-6.

Dodd, H. M., Horn, N., Giffard, C. J., & Gasson, M. J. (1996). A gene replacement strategy for engineering nisin. *Microbiology*, *142*(1), 47-55.

Dominguez, A. P. M., Bizani, D., Cladera-Olivera, F., & Brandelli, A. (2007). Cerein 8A production in soybean protein using response surface methodology. *Biochemical Engineering Journal*, *35*(2), 238-243.

Dong, X. Z., & Cai, M. Y. (2001). Determinative manual for routine bacteriology, pp 370-390.

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical microbiology reviews*, *15*(2), 167-193.

Drosinos, E. H., Mataragas, M., Nasis, P., Galiotou, M., & Metaxopoulos, J. (2005). Growth and bacteriocin production kinetics of *Leuconostoc mesenteroides* E131. *Journal of applied microbiology*, *99*(6), 1314-1323.

Driessen, A. J., van den Hooven, H. W., Kuiper, W., Van de Camp, M., Sahl, H. G., Konings, R. N., & Konings, W. N. (1995). Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. *Biochemistry*, *34*(5), 1606-1614.

Duc, L. H., Hong, H. A., Barbosa, T. M., Henriques, A. O., & Cutting, S. M. (2004). Characterization of *Bacillus* probiotics available for human use. *Applied and environmental microbiology*, *70*(4), 2161-2171.

Dufour, A., Hindré, T., Haras, D., & Le Pennec, J. P. (2006). The biology of lantibiotics from the lacticin 481 group is coming of age. *FEMS microbiology reviews*, *31*(2), 134-167.

Duitman, E. H., Hamoen, L. W., Rembold, M., Venema, G., Seitz, H., Saenger, W., ... & Stein, T. (1999). The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. *Proceedings of the National Academy of Sciences*, *96*(23), 13294-13299.

Duitman, E. H., Wyczawski, D., Boven, L. G., Venema, G., Kuipers, O. P., & Hamoen, L. W. (2007). Novel methods for genetic transformation of natural Bacillus subtilis isolates used to study the regulation of the mycosubtilin and surfactin synthetases. *Applied and environmental microbiology*, *73*(11), 3490-3496.

Dumont, N., & Arteaga, C. L. (2003). Targeting the TGFβ signaling network in human neoplasia. *Cancer cell*, *3*(6), 531-536.

Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O'Halloran, S., Feeney, M., ... & Kiely, B. (1999). Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. In *Lactic Acid Bacteria: Genetics, Metabolism and Applications* (pp. 279-292). Springer Netherlands.

Duquesne, S., Destoumieux-Garzón, D., Peduzzi, J., & Rebuffat, S. (2007). Microcins, gene-encoded antibacterial peptides from enterobacteria. *Natural product reports*, *24*(4), 708-734.

Ehrmann, M. A., Kurzak, P., Bauer, J., & Vogel, R. F. (2002). Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. *Journal of applied microbiology*, *92*(5), 966-975.

Eijsink, V. G., Brurberg, M. B., Middelhoven, P. H., & Nes, I. F. (1996). Induction of bacteriocin production in *Lactobacillus sake* by a secreted peptide. *Journal of bacteriology*, *178*(8), 2232-2237.

Elayaraja, S., Annamalai, N., Mayavu, P., & Balasubramanian, T. (2014). Production, purification and characterization of bacteriocin from *Lactobacillus murinus* AU06 and its broad antibacterial spectrum. *Asian Pacific Journal of Tropical Biomedicine*, *4*(Suppl 1), S305–S311. http://doi.org/10.12980/APJTB.4.2014C537

Ellen, A. F., Rohulya, O. V., Fusetti, F., Wagner, M., Albers, S. V., & Driessen, A. J. (2011). The Sulfolobicin Genes of *Sulfolobus acidocaldarius* encode novel antimicrobial proteins. *Journal of bacteriology*, *193*(17), 4380-4387.

Ellison, J. S., & Kautter, J. A. (1970). Purification and some properties of two boticins. *Journal of bacteriology*, *104*(1), 19-26.

Enan, G., El-Essawy, A. A., Uyttendaele, M., & Debevere, J. (1996). Antibacterial activity of Lactobacillus plantarum UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1. *International Journal of Food Microbiology*, *30*(3), 189-215.

FAO. 2016. The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp

Farías, M. E., Farías, R. N., Holgado, A. P., & Sesma, F. (1996). Purification and N-terminal amino acid sequence of Enterocin CRL 35, a 'pediocin-like'bacteriocin produced by *Enterococcus faecium* CRL 35. *Letters in applied microbiology*, *22*(6), 417-419.

Farkas-Himsley, H., & Cheung, R. (1976). Bacterial proteinaceous products (bacteriocins) as cytotoxic agents of neoplasia. *Cancer research*, *36*(10), 3561-3567.

Felgner, S., Kocijancic, D., Frahm, M., & Weiss, S. (2016). Bacteria in cancer therapy: renaissance of an old concept. *International journal of microbiology*, *2016*.

Ferrè, F., & Clote, P. (2006). DiANNA 1.1: an extension of the DiANNA web server for ternary cysteine classification. *Nucleic acids research*, *34*(suppl_2), W182-W185.

Field, D., Gaudin, N., Lyons, F., O'Connor, P. M., Cotter, P. D., Hill, C., & Ross, R. P. (2015). A bioengineered nisin derivative to control biofilms of *Staphylococcus pseudintermedius*. *PLoS One*, *10*(3), e0119684.

Finn, T. E., Nunez, A. C., Sunde, M., & Easterbrook-Smith, S. B. (2012). Serum albumin prevents protein aggregation and amyloid formation and retains chaperone-like activity in the presence of physiological ligands. *Journal of Biological Chemistry*, *287*(25), 21530-21540.

Fitzpatrick, L. R., Small, J. S., Greene, W. H., Karpa, K. D., Farmer, S., & Keller, D. (2012). *Bacillus coagulans* GBI-30, 6086 limits the recurrence of *Clostridium difficile*-Induced colitis following vancomycin withdrawal in mice. *Gut pathogens*, *4*(1), 13.

Flaherty, R. A., Freed, S. D., & Lee, S. W. (2014). The wide world of ribosomally encoded bacterial peptides. *PLoS pathogens*, *10*(7), e1004221.

Forestier, C., De Champs, C., Vatoux, C., & Joly, B. (2001). Probiotic activities of *Lactobacillus caseirhamnosus*: in vitro adherence to intestinal cells and antimicrobial properties. *Research in Microbiology*, *152*(2), 167-173.

Frederico, P. (1957). Colicins. *Annual Reviews in Microbiology*, *11*(1), 7-22.

Fuller, R. (1989). A review. *Journal of Applied Bacteriology, 66*, 365-378.

Galarza-Seeber, R., Latorre, J. D., Hernandez-Velasco, X., Wolfenden, A. D., Bielke, L. R., Menconi, A., ... & Tellez, G. (2015). Isolation, screening and identification of *Bacillus* spp. as direct-fed microbial candidates for aflatoxin B1 biodegradation. *Asian Pacific Journal of Tropical Biomedicine*, *5*(9), 702-706.

Gálvez, A., López, R. L., Abriouel, H., Valdivia, E., & Omar, N. B. (2008). Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. *Critical reviews in biotechnology*, *28*(2), 125-152.

Ganten, T. M., Sykora, J., Koschny, R., Batke, E., Aulmann, S., Mansmann, U., ... & Walczak, H. (2009). Prognostic significance of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor expression in patients with breast cancer. *Journal of molecular medicine*, *87*(10), 995.

Garcia, P., Rodriguez, L., Rodriguez, A., & Martinez, B. (2010). Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. *Trends in Food Science & Technology*, *21*(8), 373-382.

García-Almendárez, B. E., Cann, I. K., Martin, S. E., Guerrero-Legarreta, I., & Regalado, C. (2008). Effect of *Lactococcus lactis* UQ2 and its bacteriocin on *Listeria monocytogenes* biofilms. *Food Control*, *19*(7), 670-680.

Gardner, J. F. (1950). Some antibiotics formed by Bacterium coli. British journal of experimental pathology, 31(1), 102.

Gaspar, D., Veiga, A. S., & Castanho, M. A. (2013). From antimicrobial to anticancer peptides. A review. *Frontiers in microbiology*, *4*.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S. E., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). *Protein identification and analysis tools on the ExPASy server* (pp. 571-607). Humana Press.

Ge, J., Sun, Y., Xin, X., Wang, Y., & Ping, W. (2016). Purification and partial characterization of a novel bacteriocin synthesized by *Lactobacillus paracasei* HD1-7 isolated from Chinese sauerkraut juice. *Scientific reports*, *6*.

Ge, R., Rajeev, V., Subramanian, G., Reiss, K. A., Liu, D., Higgins, L., ... & McEnroe, G. (2004). Selective inhibitors of type I receptor kinase block cellular transforming growth factor-β signaling. *Biochemical pharmacology*, *68*(1), 41-50.

Gebhardt, K., Schimana, J., Müller, J., Fiedler, H. P., Kallenborn, H. G., Holzenkämpfer, M., ... & Schmid, D. G. (2002). Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. *FEMS microbiology letters*, *217*(2), 199-205.

Georgalaki, M. D., Van den Berghe, E., Kritikos, D., Devreese, B., Van Beeumen, J., Kalantzopoulos, G., ... & Tsakalidou, E. (2002). Macedocin, a food-grade lantibiotic produced by *Streptococcus macedonicus* ACA-DC 198. *Applied and Environmental Microbiology*, *68*(12), 5891-5903.

Gérard, F., Pradel, N., & Wu, L. F. (2005). Bactericidal activity of colicin V is mediated by an inner membrane protein, SdaC, of *Escherichia coli*. *Journal of bacteriology*, *187*(6), 1945-1950.

Gibson, L. F., Woodworth, J., & George, A. M. (1998). Probiotic activity of *Aeromonas media* on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii*. *Aquaculture*, *169*(1), 111-120.

Gibson, L.F. (1999) Bacteriocin activity and probiotic activity of *Aeromonas media*. *Journal of Applied Microbiology 85,* S243–S248.

Gillor, O., Kirkup, B. C., & Riley, M. A. (2004). Colicins and microcins: the next generation antimicrobials. *Advances in applied microbiology*, *54*, 129-146.

Glöckner, F. O., Gasol, J. M., McDonough, N., & Calewaert, J. B. (2012). Marine microbial diversity and its role in ecosystem functioning and environmental change.

Goebel, W. F., Barry, G. T., & Shedlovsky, T. (1956). COLICINE K: I. The production of colicine K in media maintained at constant pH. *The Journal of experimental medicine*, *103*(5), 577.

Goh, H. F., & Philip, K. (2015). Purification and characterization of bacteriocin produced by *Weissella confusa* A3 of dairy origin. *PloS one*, *10*(10), e0140434.

Gómez, N. C., Ramiro, J. M., Quecan, B. X., & de Melo Franco, B. D. (2016). Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes*, *Salmonella* Typhimurium, and *Escherichia coli* O157: H7 biofilms formation. *Frontiers in microbiology*, *7*.

Gong, X., Martin-Visscher, L. A., Nahirney, D., Vederas, J. C., & Duszyk, M. (2009). The circular bacteriocin, carnocyclin A, forms anion-selective channels in lipid bilayers. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1788*(9), 1797-1803.

Gratia, A. (1925). On a remarkable example of antagonism between two stocks of colibacillus. *CR Soc. Biol*, *93*, 1040-1041.

Gray, E. J., Lee, K. D., Souleimanov, A. M., Di Falco, M. R., Zhou, X., Ly, A., ... & Smith, D. L. (2006). A novel bacteriocin, thuricin 17, produced by plant growth promoting rhizobacteria strain *Bacillus thuringiensis* NEB17: isolation and classification. *Journal of Applied Microbiology*, *100*(3), 545-554.

Hale, E. M., & Hinsdili, R. D. (1975). Biological Activity of Staphylococcin 462: Bacteriocin from *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *7*(1), 74–81.

Hammami, I., Jaouadi, B., Bacha, A. B., Rebai, A., Bejar, S., Nesme, X., & Rhouma, A. (2012). Bacillus subtilis bacteriocin Bac 14B with a broad inhibitory spectrum: purification, amino acid sequence analysis, and physicochemical characterization. *Biotechnology and bioprocess engineering*, *17*(1), 41-49.

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*(2), 253-260.

Hammami, R., Zouhir, A., Le Lay, C., Hamida, J. B., & Fliss, I. (2010). BACTIBASE second release: a database and tool platform for bacteriocin characterization. *Bmc Microbiology*, *10*(1), 22.

Hamon, Y., & Péron, Y. (1963, January). Study of the bacteriocinogenic power in the genus Cloaca. In *Annales de l'Institut Pasteur* (Vol. 104, pp. 127-131).

Hastings, J. W., Sailer, M., Johnson, K., Roy, K. L., Vederas, J. C., & Stiles, M. E. (1991). Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. *Journal of Bacteriology*, *173*(23), 7491-7500.

Hauge, H. H., Mantzilas, D., Moll, G. N., Konings, W. N., Driessen, A. J., Eijsink, V. G., & Nissen-Meyer, J. (1998). Plantaricin A is an amphiphilic α-helical bacteriocin-like pheromone which exerts antimicrobial and pheromone activities through different mechanisms. *Biochemistry*, *37*(46), 16026-16032.

Hauptmann, S., Grünewald, V., Molls, D., Schmitt, W. D., Köbel, M., Kriese, K., & Schürmann, A. (2005). Glucose transporter GLUT1 in colorectal adenocarcinoma cell lines is inversely correlated with tumour cell proliferation. *Anticancer research*, *25*(5), 3431-3436.

He, X. (2009). *Effects of Class IId Bacteriocins: Thuricin 17 and Bacthuricin F4 on Crops Growth* (Doctoral dissertation, McGill University, Montreal Quebec, Canada).

Hebert, P. D., Cywinska, A., & Ball, S. L. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B: Biological Sciences*, *270*(1512), 313-321.

Héchard, Y., & Sahl, H. G. (2002). Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie*, *84*(5), 545-557.

Heldin, C. H. (2008). *Transforming Growth Factor-Beta in Cancer Therapy, Volume II: Cancer Treatment and Therapy*. Springer Science & Business Media.

Heng, N. C., Wescombe, P. A., Burton, J. P., Jack, R. W., & Tagg, J. R. (2007). The diversity of bacteriocins in Gram-positive bacteria. In *Bacteriocins* (pp. 45-92). Springer Berlin Heidelberg.

Heo, S., Lee, S. K., Lee, C. H., Min, S. G., Park, J. S., & Kim, H. Y. (2007). Morphological changes induced in *Listeria monocytogenes* V7 by a bacteriocin produced by *Pediococcus acidilactici*. *Journal of microbiology and biotechnology*, *17*(4), 663-667.

Herranz, C., Martinez, J., Rodriguez, J., Hernandez, P., & Cintas, L. (2001). Optimization of enterocin P production by batch fermentation of *Enterococcus faecium* P13 at constant pH. *Applied Microbiology and Biotechnology*, *56*(3-4), 378-383.

Hill, C., Rea, M., & Ross, P. (2014). *U.S. Patent No. 8,852,917*. Washington, DC: U.S. Patent and Trademark Office.

Hoa, T. T., Isticato, R., Baccigalupi, L., Ricca, E., Van, P. H., & Cutting, S. M. (2001). Fate and dissemination of *Bacillus subtilis* spores in a murine model. *Applied and environmental microbiology*, 67(9), 3819-3823.

http://db.systemsbiology.net:8080/proteomicsToolkit/

http://web.expasy.org/protparam/

https://zhanglab.ccmb.med.umich.edu/I-TASSER/

Hunt, D. F., Henderson, R. A., Shabanowttz, J., Sakaguchi, K., Michel, H., Sevilir, N., ... & Engelhard, V. H. (2007). Characterization of peptides bound to the class I MHC molecule HLA-A2. 1 by mass spectrometry. *Journal of immunology (Baltimore, Md.: 1950)*, *179*(5), 2669.

Hwanhlem, N., Jaffrès, E., Dousset, X., Pillot, G., Choiset, Y., Haertlé, T., ... & Chobert, J. M. (2015). Application of a nisin Z-producing *Lactococcus lactis* subsp. *lactis* KT2W2L isolated from brackish water for biopreservation in cooked, peeled and ionized tropical shrimps during storage at 8°C under modified atmosphere packaging. *European Food Research and Technology*, *240*(6), 1259-1269.

Iandolo, J. J., & Crupper, S. (1997). *U.S. Patent No. 5,703,040*. Washington, DC: U.S. Patent and Trademark Office.

Ibarreche, M. P., Castellano, P., & Vignolo, G. (2014). Evaluation of anti-Listeria meat borne *Lactobacillus* for biofilm formation on selected abiotic surfaces. *Meat science*, *96*(1), 295-303.

Ichikawa, K., Liu, W. M., Wang, Z., Liu, D., Zhao, L. M., Zhang, H. G., ... & Zhou, T. (2001). Tumoricidal activity in the absence of hepatocyte cytotoxicity of a novel anti-human DR5 monoclonal antibody. In *faseb journal* 15(5), A1198-A1198. 9650 Rockville Pike, Bethesda, MD 20814-3998 USA: Federation Amer Soc Exp Biol.

ICMR - News, Over 17 lakh new cancer cases in India by 2020: http://icmr.nic.in/icmrsql/archive/2016/7.pdf

Ivanova, E. P., Vysotskii, M. V., Svetashev, V. I., Nedashkovskaya, O. I., Gorshkova, N. M., Mikhailov, V. V., ... & Yoshikawa, S. (2010). Characterization of Bacillus strains of marine origin.*International microbiology*, *2*(4), 267-271.

Ivánovics, G., & Alföldi, L. (1954). A new antibacterial principle: megacine. *Nature*, *174*(4427), 465-465.

Jack, R. W., & Tagg, J. R. (1992). Factors affecting production of the group A streptococcus bacteriocin SA-FF22. *Journal of medical microbiology*, *36*(2), 132-138.

Jack, R. W., Tagg, J. R., & Ray, B. (1995). Bacteriocins of gram-positive bacteria. *Microbiological reviews*, *59*(2), 171-200.

Jack, R. W., Wan, J., Gordon, J., Harmark, K., Davidson, B. E., Hillier, A. J., ... & Coventry, M. J. (1996). Characterization of the chemical and antimicrobial properties of piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126. *Applied and environmental microbiology*, *62*(8), 2897-2903.

Jackson, D. W., Suzuki, K., Oakford, L., Simecka, J. W., Hart, M. E., & Romeo, T. (2002). Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli*. *Journal of bacteriology*, *184*(1), 290-301.

Jacob, F., & Wollman, E. L. (1953, January). Induction of phage development in lysogenic bacteria. In *Cold Spring Harbor symposia on quantitative biology* (Vol. 18, pp. 101-121). Cold Spring Harbor Laboratory Press.

Jadamus, A., Vahjen, W., & Simon, O. (2001). Growth behaviour of a spore forming probiotic strain in the gastrointestinal tract of broiler chicken and piglets. *Archives of Animal Nutrition*, *54*(1), 1-17.

Janković, T., Frece, J., Abram, M., & Gobin, I. (2012). Aggregation ability of potential probiotic *Lactobacillus plantarum* strains. *International Journal of Sanitary Engineering Research*, *6*(1), 17-22.

Jedrychowski, W., Perera, F., Maugeri, U., Mroz, E., Flak, E., Perzanowski, M., & Majewska, R. (2011). Wheezing and asthma may be enhanced by broad spectrum antibiotics used in early childhood. Concept and results of a pharmaco epidemiology study. Journal of physiology and pharmacology: an official journal of the Polish Physiological Society, 62(2), 189.

Jetten, A. M., & Vogels, G. D. (1972). Nature and properties of a *Staphylococcus epidermidis* bacteriocin. *Journal of bacteriology*, *112*(1), 243-250.

Jetten, A. M., Vogels, G. D., & De Windt, F. (1972). Production and purification of a *Staphylococcus epidermidis* bacteriocin. *Journal of bacteriology*, *112*(1), 235-242.

Joerger, M. C., & Klaenhammer, T. R. (1986). Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. *Journal of bacteriology*, *167*(2), 439-446.

Joerger, R. D. (2003). Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poultry science*, *82*(4), 640-647.

Jones, K. J., Perris, A. D., Vernallis, A. B., Worthington, T., Lambert, P. A., & Elliott, T. S. (2005). Induction of inflammatory cytokines and nitric oxide in J774. 2 cells and murine macrophages by lipoteichoic acid and related cell wall antigens from *Staphylococcus epidermidis*. *Journal of medical microbiology*, *54*(4), 315-321.

Jones, S. E., & Versalovic, J. (2009). Probiotic *Lactobacillus reuteri* biofilms produce antimicrobial and anti-inflammatory factors. *BMC microbiology*, *9*(1), 35.

Joo, N. E., Ritchie, K., Kamarajan, P., Miao, D., & Kapila, Y. L. (2012). Nisin, an apoptogenic bacteriocin and food preservative, attenuates HNSCC tumorigenesis via CHAC1. *Cancer medicine*, *1*(3), 295-305.

Kalchayanand, N., Dunne, P., Sikes, A., & Ray, B. (2004). Viability loss and morphology change of foodborne pathogens following exposure to hydrostatic pressures in the presence and absence of bacteriocins. *International journal of food microbiology*, *91*(1), 91-98.

Kaminska, B., Wesolowska, A., & Danilkiewicz, M. (2005). TGF beta signalling and its role in tumour pathogenesis. *ACTA BIOCHIMICA POLONICA-ENGLISH EDITION-*, *52*(2), 329.

Kang, M. S., Kim, B. G., Chung, J., Lee, H. C., & Oh, J. S. (2006). Inhibitory effect of *Weissella cibaria* isolates on the production of volatile sulphur compounds. *Journal of clinical periodontology*, *33*(3), 226-232.

Karthik, R., Gobalakrishnan, S., Hussain, A. J., & Muthezhilan, R. (2013). Efficacy of Bacteriocin from *Lactobacillus*Sp.(AMET 1506) as a Biopreservative for Seafood's Under Different Storage Temperature Conditions. *Journal of Modern Biotechnology*, *2*(3), 59-65.

Karthikeyan, P., Bhat, S. G., & Chandrasekaran, M. (2013). Halocin SH10 production by an extreme haloarchaeon *Natrinema* sp. BTSH10 isolated from salt pans of South India. *Saudi journal of biological sciences*, *20*(2), 205-212.

Kaur, S., & Kaur, S. (2015). Bacteriocins as Potential Anticancer Agents. *Frontiers in Pharmacology*, *6*, 272. http://doi.org/10.3389/fphar. 2015.00272

Kawulka, K. E., Sprules, T., Diaper, C. M., Whittal, R. M., McKay, R. T., Mercier, P., ... & Vederas, J. C. (2004). Structure of subtilosin A, a cyclic antimicrobial peptide from *Bacillus subtilis* with unusual sulfur to α-carbon cross-links: formation and reduction of α-thio-α-amino acid derivatives. *Biochemistry*, *43*(12), 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*(6), 600-607.

Kazazic, M., Nissen-Meyer, J., & Fimland, G. (2002). Mutational analysis of the role of charged residues in target-cell binding, potency and specificity of the pediocin-like bacteriocin sakacin P. *Microbiology*, *148*(7), 2019-2027.

Kelstrup, J., & Gibbons, R. J. (1969). Bacteriocins from human and rodent streptococci. *Archives of oral biology*, *14*(3), 251IN1-258IN2.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M. J., & Gibson, L. (2010). *Alteromonas macleodii* 0444 and *Neptunomonas* sp. 0536, two novel probiotics for hatchery-reared Greenshell™ mussel larvae, *Perna canaliculus*. *Aquaculture*, *309*(1), 49-55.

Kesarcodi-Watson, A., Miner, P., Nicolas, J. L., & Robert, R. (2012). Protective effect of four potential probiotics against pathogen-challenge of the larvae of three bivalves: Pacific oyster (*Crassostrea gigas*), flat oyster (*Ostrea edulis*) and scallop (*Pecten maximus*). *Aquaculture*, *344*, 29-34.

Ki, J. S., Zhang, W., & Qian, P. Y. (2009). Discovery of marine Bacillus species by 16S rRNA and rpoB comparisons and their usefulness for species identification. *Journal of microbiological methods*, *77*(1), 48-57.

Kim, M. H., Kong, Y. J., Baek, H., & Hyun, H. H. (2006). Optimization of culture conditions and medium composition for the production of micrococcin GO5 by Micrococcus sp. GO5. *Journal of biotechnology*, *121*(1), 54-61.

Kimura, H., Matsusaki, H., Sashihara, T., Sonomoto, K., & Ishizaki, A. (1998). Purification and partial identification of bacteriocin ISK-1, a new lantibiotic produced by *Pediococcus* sp. ISK-1. *Bioscience, biotechnology, and biochemistry*, *62*(12), 2341-2345.

Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS microbiology reviews*, *12*(1-3), 39-85.

Klaenhammer, T. R., & Kullen, M. J. (1999). Selection and design of probiotics. *International journal of food microbiology*, *50*(1), 45-57.

Klepper, J. (2008). Glucose transporter deficiency syndrome (GLUT1DS) and the ketogenic diet. *Epilepsia*, *49*(s8), 46-49.

Klepper, J., & Voit, T. (2002). Facilitated glucose transporter protein type 1 (GLUT1) deficiency syndrome: impaired glucose transport into brain-a review. *European journal of pediatrics*, *161*(6), 295-304.

Kluskens, L. D., Kuipers, A., Rink, R., de Boef, E., Fekken, S., Driessen, A. J., ... & Moll, G. N. (2005). Post-translational modification of therapeutic peptides by NisB, the dehydratase of the lantibiotic nisin. *Biochemistry*, *44*(38), 12827-12834.

Knowles, D. J. (1997). New strategies for antibacterial drug design. *Trends in microbiology*, *5*(10), 379-383.

Korenblum, E., de Araujo, L. V., Guimarães, C. R., De Souza, L. M., Sassaki, G., Abreu, F., ... & Seldin, L. (2012). Purification and characterization of a surfactin-like molecule produced by Bacillus sp. H2O-1 and its antagonistic effect against sulfate reducing bacteria. *BMC microbiology*, *12*(1), 252.

Korenblum, E., Der Weid, I., Santos, A. L. S., Rosado, A. S., Sebastian, G. V., Coutinho, C. M. L. M., ... & Seldin, L. (2005). Production of antimicrobial

substances by *Bacillus subtilis* LFE-1, *B. firmus* H2O-1 and *B. licheniformis* T6-5 isolated from an oil reservoir in Brazil. *Journal of Applied Microbiology*, *98*(3), 667-675.

Korenblum, E., Sebastián, G. V., Paiva, M. M., Coutinho, C. M. L. M., Magalhães, F. C. M., Peyton, B. M., & Seldin, L. (2008). Action of antimicrobial substances produced by different oil reservoir *Bacillus* strains against biofilm formation. *Applied microbiology and biotechnology*, *79*(1), 97-103.

Kos, B. V. Z. E., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., & Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of applied microbiology*, *94*(6), 981-987.

Kouakou, P., Dortu, C., Dubois-Dauphin, R., Vandenbol, M., & Thonart, P. (2010). Plasmid-associated bacteriocin production by *Lactobacillus* LMG21688 suppresses *Listeria monocytogenes* growth rebound in a food system. *FEMS microbiology letters*, *306*(1), 37-44.

Kozakov D, Hall DR, Xia B, Porter KA, Padhorny D, Yueh C, Beglov D, Vajda S. The ClusPro web server for protein-protein docking. *Nature Protocols.* 2017 Feb;12(2):255-278

Krier, F., Revol-Junelles, A. M., & 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*(3), 359-363.

Kruszewska, D., Sahl, H. G., Bierbaum, G., Pag, U., Hynes, S. O., & Ljungh, Å. (2004). Mersacidin eradicates methicillin-resistant *Staphylococcus aureus* (MRSA) in a mouse rhinitis model. *Journal of Antimicrobial Chemotherapy*, *54*(3), 648-653.

Kugler, M., Loeffler, W., Rapp, C., Kern, A., & Jung, G. (1990). Rhizocticin A, an antifungal phosphono-oligopeptide of *Bacillus subtilis* ATCC 6633: biological properties. *Archives of microbiology*, *153*(3), 276-281.

La Rosa-García, D., Muñoz-García, A. A., Barahona-Pérez, L. F., & Gamboa-Angulo, M. M. (2007). Antimicrobial properties of moderately

halotolerant bacteria from cenotes of the Yucatan peninsula. *Letters in applied microbiology*, *45*(3), 289-294.

Labak, C. M., Wang, P. Y., Arora, R., Guda, M. R., Asuthkar, S., Tsung, A. J., & Velpula, K. K. (2016). Glucose transport: meeting the metabolic demands of cancer, and applications in glioblastoma treatment. *American journal of cancer research*, *6*(8), 1599.

Lachowicz T (1965). Investigations on staphylococcins. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A 196:340-351.

Lachowicz, T., & Brodzicki, S. (1973). Stimulation of staphylococcin A: production and some properties. *Contributions to microbiology and immunology*, *1*, 450.

Lade, H. S., Chitanand, M. P., Gyananath, G., & Kadam, T. A. (2006). Studies on some properties of bacteriocins produced by *Lactobacillus* species isolated from agro-based waste. *Internet Journal of Microbiology*, *2*(1).

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

Law, D. J., & Dajani, A. S. (1978). Interactions between *Neisseria sicca* and viridin B, a bacteriocin produced by *Streptococcus mitis*. *Antimicrobial Agents and Chemotherapy*, *13*(3), 473–478.

Lawton, E. M., Ross, R. P., Hill, C., & Cotter, P. D. (2007). Two-peptide lantibiotics: a medical perspective. *Mini reviews in medicinal chemistry*, *7*(12), 1236-1247.

Laxmi, M., Kurian, N. K., Smitha, S., & Bhat, S. G. (2016). Melanin and bacteriocin from marine bacteria inhibit biofilms of food-borne pathogens. *Indian Journal of Biotechnology, 15*(3), 392-399.

Laxmi, M., Sarita, G. B. (2014). Diversity characterization of biofilm forming microorganisms in food sampled from local markets in Kochi, Kerala, India, *International Journal of Recent Scientific Research*, 5(6), 1070-1075.

Lebeer, S., De Keersmaecker, S. C., Verhoeven, T. L., Fadda, A. A., Marchal, K., & Vanderleyden, J. (2007). Functional analysis of luxS in the probiotic strain

Lactobacillus rhamnosus GG reveals a central metabolic role important for growth and biofilm formation. *Journal of bacteriology*, *189*(3), 860-871.

Lee, H. J. (2011). Review: Lantibiotics, Class I bacteriocins from the genus *Bacillus*. *Journal of microbiology and biotechnology*, *21*(3), 229-235.

Lee, H., & Kim, H. Y. (2011). Lantibiotics, class I bacteriocins from the genus Bacillus. *Journal of microbiology and biotechnology*, *21*(3), 229-235.

Lee, K. D., Gray, E. J., Mabood, F., Jung, W. J., Charles, T., Clark, S. R., ... & Smith, D. L. (2009). The class IId bacteriocin thuricin-17 increases plant growth. *Planta*, *229*(4), 747-755.

Lee, K. H., Jun, K. D., Kim, W. S., & Paik, H. D. (2001). Partial characterization of polyfermenticin SCD, a newly identified bacteriocin of *Bacillus polyfermenticus*. *Letters in Applied Microbiology*, *32*(3), 146-151.

Lejeune, R., Callewaert, R., Crabbé, K., & De Vuyst, L. (1998). Modelling the growth and bacteriocin production by *Lactobacillus amylovorus* DCE 471 in batch cultivation. *Journal of Applied Microbiology*, *84*(2), 159-168.

Levin, B. R., Antia, R., Berllner, E., Bloland, P., Bonhoeffer, S., Cohen, M., ... & Lipsitch, M. (1998). Resistance to antimicrobial chemotherapy: a prescription for research and action. *The American journal of the medical sciences*, *315*(2), 87-94.

Lewus, C. B., Kaiser, A., & Montville, T. J. (1991). Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Applied and Environmental Microbiology*, *57*(6), 1683-1688.

Lewus, C. B., Sun, S., & Montville, T. J. (1992). Production of an amylase-sensitive bacteriocin by an atypical *Leuconostoc paramesenteroides* strain. *Applied and Environmental Microbiology*, *58*(1), 143-149.

Lien, S., & Lowman, H. B. (2003). Therapeutic peptides. *Trends in biotechnology*, *21*(12), 556-562.

Lisboa, M. P., Bonatto, D., Bizani, D., Henriques, J. A., & Brandelli, A. (2006). Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic forest. *International microbiology*, *9*(2), 111-118.

Liu, C. H., Chiu, C. S., Ho, P. L., & Wang, S. W. (2009). Improvement in the growth performance of white shrimp, *Litopenaeus vannamei*, by a protease-producing probiotic, *Bacillus subtilis* E20, from natto. *Journal of applied microbiology*, *107*(3), 1031-1041.

Liu, W., & Hansen, J. N. (1992). Enhancement of the chemical and antimicrobial properties of subtilin by site-directed mutagenesis. *Journal of Biological Chemistry*, *267*(35), 25078-25085.

Lizard, G., Moisant, M., Cordelet, C., Monier, S., Gambert, P., & Lagrost, L. (1997). Induction of similar features of apoptosis in human and bovine vascular endothelial cells treated by 7-ketocholesterol. *The Journal of pathology*, *183*(3), 330-338.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of biological chemistry*, *193*(1), 265-275.

Lowy, F. D. (2003). Antimicrobial resistance: the example of *Staphylococcus aureus*. *Journal of Clinical Investigation*, *111*(9), 1265.

Lutz, G., Chavarría, M., Arias, M. L., & Mata-Segreda, J. F. (2006). Microbial degradation of palm (*Elaeis guineensis*) biodiesel. *Revista de biología tropical*, *54*(1), 59-63.

Macfarlane, G. T., & Cummings, J. H. (2002). Probiotics, infection and immunity. *Current opinion in infectious diseases*, *15*(5), 501-506.

Maftah, A., Renault, D., Vignoles, C., Héchard, Y., Bressollier, P., Ratinaud, M. H., ... & Julien, R. (1993). Membrane permeabilization of *Listeria monocytogenes* and mitochondria by the bacteriocin mesentericin Y105. *Journal of bacteriology*, *175*(10), 3232-3235.

Mageshwaran, V., Inmann, F., & Holmes, L. (2014). Growth kinetics of *Bacillus subtilis* in lignocellulosic carbon sources.*International journal of microbiology research*, 6(2), 570-574.

Mahony, D. E., & Butler, M. E. (1971). Bacteriocins of Clostridium perfringens. 1. Isolation and preliminary studies. *Canadian journal of microbiology*, *17*(1), 1-6.

Majno, G., & Joris, I. (1995). Apoptosis, oncosis, and necrosis. An overview of cell death. *The American journal of pathology*, *146*(1), 3.

Makridis, P., Martins, S., Tsalavouta, M., Dionisio, L. C., Kotoulas, G., Magoulas, A., & Dinis, M. T. (2005). Antimicrobial activity in bacteria isolated from Senegalese sole, *Solea senegalensis*, fed with natural prey. *Aquaculture Research*, *36*(16), 1619-1627.

Maqueda, M., Gálvez, A., Bueno, M. M., Sanchez-Barrena, M. J., González, C., Albert, A., ... & Valdivia, E. (2004). Peptide AS-48: prototype of a new class of cyclic bacteriocins. *Current Protein and Peptide Science*, *5*(5), 399-416.

Marjari, E. H., & Ivanovic, G. (1962). A second bacteriocin-like principle of *Bacillus megaterium*. 1. Characteristics of bactericidal principle. *Acta microbiologica academiae scientiarum hungaricae*, *9*(3), 285.

Marteau, P., Minekus, M., Havenaar, R., & Huis, J. H. J. (1997). Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. *Journal of dairy science*, *80*(6), 1031-1037.

Martelli, A. M., Zweyer, M., Ochs, R. L., Tazzari, P. L., Tabellini, G., Narducci, P., & Bortul, R. (2001). Nuclear apoptotic changes: an overview. *Journal of cellular biochemistry*, *82*(4), 634-646.

Martin, H. M., Campbell, B. J., Hart, C. A., Mpofu, C., Nayar, M., Singh, R., ... & Rhodes, J. M. (2004). Enhanced *Escherichia coli* adherence and invasion in Crohn's disease and colon cancer. *Gastroenterology*, *127*(1), 80-93.

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), 1.

Mayr-Harting, A., Hedges, A. J., & Berkeley, R. C. W. (1972). Chapter VII methods for studying bacteriocins. *Methods in microbiology*, *7*, 315-422.

McCafferty, D. G., Cudic, P., Michael, K. Y., Behenna, D. C., & Kruger, R. (1999). Synergy and duality in peptide antibiotic mechanisms. *Current opinion in chemical biology*, *3*(6), 672-680.

McCall, J. O., & Sizemore, R. K. (1979). Description of a bacteriocinogenic plasmid in *Beneckea harveyi*. *Applied and environmental microbiology*, *38*(5), 974-979.

McGahon, A. J., Martin, S. J., Bissonnette, R. P., Mahboubi, A., Shi, Y., Mogil, R. J., ... & Green, D. R. (1995). The end of the (cell) line: methods for the study of apoptosis in vitro. *Methods in cell biology*, *46*, 153-185.

McManus, P. S., Stockwell, V. O., Sundin, G. W., & Jones, A. L. (2002). Antibiotic use in plant agriculture. *Annual review of phytopathology*, *40*(1), 443-465.

Meijerink, M., Van Hemert, S., Taverne, N., Wels, M., De Vos, P., Bron, P. A., ... & Wells, J. M. (2010). Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridization. *PloS one*, *5*(5), e10632.

Meindl, K., Schmiederer, T., Schneider, K., Reicke, A., Butz, D., Keller, S., ... & Brönstrup, M. (2010). Labyrinthopeptins: a new class of carbacyclic lantibiotics. *Angewandte Chemie International Edition*, *49*(6), 1151-1154.

Meitert, E. (1969). On bacteriocins of *Corynebacterium diphtheriae*. II. Study of the bacteriocins produced by *C. diphtheriae*, *C. ulcerans*, *C. atypique*, *C. hoffmani* and *C. xerose*. *Archives roumaines de pathologie experimentales et de microbiologie*, *28*(4), 1086-1097.

Messens, W., Verluyten, J., Leroy, F., & De Vuyst, L. (2003). Modelling growth and bacteriocin production by *Lactobacillus curvatus* LTH 1174 in response to temperature and pH values used for European sausage fermentation processes. *International journal of food microbiology*, *81*(1), 41-52.

Metivier, A., Pilet, M. F., Dousset, X., Sorokine, O., Anglade, P., Zagorec, M., ... & Fremaux, C. (1998). Divercin V41, a new bacteriocin with two disulphide bonds produced by *Carnobacterium divergens* V41: primary structure and genomic organization. *Microbiology*, *144*(10), 2837-2844.

Michel-Briand, Y., & Baysse, C. (2002). The pyocins of *Pseudomonas aeruginosa*. *Biochimie*, *84*(5), 499-510.

Miller, J. H. (1992). *A] short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria* (No. QH434 M64).

Miller, M. B., & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annual Reviews in Microbiology*, *55*(1), 165-199.

Mohanty, S., Mishra, S., Jena, P., Jacob, B., Sarkar, B., & Sonawane, A. (2012). An investigation on the antibacterial, cytotoxic, and antibiofilm efficacy of starch-stabilized silver nanoparticles. *Nanomedicine: Nanotechnology, Biology and Medicine*, *8*(6), 916-924.

Molitor, E., Kluczny, C., Brötz, H., Bierbaum, G., Jack, R., & Sahl, H. G. (1996). Effects of the lantibiotic mersacidin on the morphology of *Staphylococci*. *Zentralblatt für Bakteriologie*, *284*(2-3), 318-328.

Moll, G. N., van den Akker, E., Hauge, H. H., Nissen-Meyer, J., Nes, I. F., Konings, W. N., & Driessen, A. J. (1999). Complementary and overlapping selectivity of the two-peptide bacteriocins plantaricin EF and JK. *Journal of bacteriology*, *181*(16), 4848-4852.

Moreno, I., Lerayer, A. L., Baldini, V. L., & Leitão, M. F. D. F. (2000). Characterization of bacteriocins produced by *Lactococcus lactis* strains. *Brazilian Journal of Microbiology*, *31*(3), 183-191.

Moryl, M., Spętana, M., Dziubek, K., Paraszkiewicz, K., Różalska, S., Płaza, G. A., & Różalski, A. (2015). Antimicrobial, antiadhesive and antibiofilm potential of lipopeptides synthesised by *Bacillus subtilis*, on uropathogenic bacteria. *Acta Biochimica Polonica*, *62*(4), 725-732.

Motta, A. S., & Brandelli, A. (2008). Evaluation of environmental conditions for production of bacteriocin-like substance by *Bacillus* sp. strain P34. *World Journal of Microbiology and Biotechnology*, *24*(5), 641-646.

Motta, A. S., Flores, F. S., Souto, A. A., & 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*(3), 275-284.

Motta, A. S., Lorenzini, D. M., & Brandelli, A. (2007). Purification and partial characterization of an antimicrobial peptide produced by a novel *Bacillus* sp. isolated from the Amazon Basin. *Current microbiology*, *54*(4), 282-286.

Mukherjee, S., Das, P., Sivapathasekaran, C., & Sen, R. (2009). Antimicrobial biosurfactants from marine *Bacillus circulans*: extracellular synthesis and purification. *Letters in applied microbiology*, *48*(3), 281-288.

Muriana, P. M., & Klaenhammer, T. R. (1991). Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. *Applied and Environmental Microbiology*, *57*(1), 114-121.

Mutuş, R., Kocabağli, N., Alp, M., Acar, N., Eren, M., & Gezen, Ş. Ş. (2006). The effect of dietary probiotic supplementation on tibial bone characteristics and strength in broilers. *Poultry science*, *85*(9), 1621-1625.

Naclerio, G., Ricca, E., Sacco, M., & De Felice, M. (1993). Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. *Applied and Environmental Microbiology*, *59*(12), 4313-4316.

Nakano, M., Kishimoto, Y., & Saito, K. (1968). Spot method in agar for the quantitative assay of anti-Salmonella antibody. *Japanese journal of microbiology*, *12*(3), 364-366.

Nakayama, K., Takashima, K., Ishihara, H., Shinomiya, T., Kageyama, M., Kanaya, S., ... & Hayashi, T. (2000). The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage. *Molecular microbiology*, *38*(2), 213-231.

Nakayama, T., Lu, H., & Nomura, N. (2009). Inhibitory effects of Bacillus probionts on growth and toxin production of *Vibrio harveyi* pathogens of shrimp. *Letters in applied microbiology*, *49*(6), 679-684.

National Center for Biotechnology Information. PubChem Compound Database, https://pubchem.ncbi.nlm.nih.gov/compound/

Neetoo, H., Ye, M., Chen, H., Joerger, R. D., Hicks, D. T., & Hoover, D. G. (2008). Use of nisin-coated plastic films to control Listeria monocytogenes on

vacuum-packaged cold-smoked salmon. *International journal of food microbiology*, *122*(1), 8-15.

Nel, H. A., Bauer, R., Vandamme, E. J., & Dicks, L. M. T. (2001). Growth optimization of *Pediococcus damnosus* NCFB 1832 and the influence of pH and nutrients on the production of pediocin PD-1. *Journal of applied microbiology*, *91*(6), 1131-1138.

Nguyen, V. D., Pham, T. T., Nguyen, T. H. T., Nguyen, T. T. X., & Hoj, L. (2014). Screening of marine bacteria with bacteriocin-like activities and probiotic potential for ornate spiny lobster (*Panulirus ornatus*) juveniles. *Fish & shellfish immunology*, *40*(1), 49-60.

Niedermeyer, T. H., & Strohalm, M. (2012). mMass as a software tool for the annotation of cyclic peptide tandem mass spectra. *PloS one*, *7*(9), e44913.

Nikapitiya, C. (2013). Marine bacteria as probiotics and their applications in aquaculture. *Marine Microbiology: Bioactive Compounds and Biotechnological Applications*, 97-126.

Nilsen, T., Nes, I. F., & Holo, H. (2003). Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. *Applied and environmental microbiology*, *69*(5), 2975-2984.

Nilsson, L., Huss, H. H., & Gram, L. (1997). Inhibition of Listeria monocytogenes on cold-smoked salmon by nisin and carbon dioxide atmosphere. *International journal of food microbiology*, *38*(2), 217-227.

Nissen-Meyer, J., Holo, H., Håvarstein, L. S., Sletten, K., & Nes, I. F. (1992). A novel lactococcal bacteriocin whose activity depends on the complementary action of two peptides. *Journal of bacteriology*, *174*(17), 5686-5692.

O'connor, E., & Shand, R. F. (2002). Halocins and sulfolobicins: the emerging story of archaeal protein and peptide antibiotics. *Journal of industrial microbiology & biotechnology*, *28*(1), 23-31.

Oguntoyinbo, F. A. (2007). Monitoring of marine Bacillus diversity among the bacteria community of sea water. *African journal of Biotechnology*, *6*(2).

Oh, Y. T., Yue, P., Wang, D., Tong, J. S., Chen, Z. G., Khuri, F. R., & Sun, S. Y. (2015). Suppression of death receptor 5 enhances cancer cell invasion and metastasis through activation of caspase-8/TRAF2-mediated signaling. *Oncotarget*, *6*(38), 41324.

Olafsen, J. A. (2001). Interactions between fish larvae and bacteria in marine aquaculture.*Aquaculture*, *200*(1), 223-247.

Oman, T. J., & van der Donk, W. A. (2009). Insights into the mode of action of the two-peptide lantibiotic haloduracin. *ACS chemical biology*, *4*(10), 865-874.

Oscáriz, J. C., & Pisabarro, A. G. (2010). Classification and mode of action of membrane-active bacteriocins produced by gram-positive bacteria. *International Microbiology*, *4*(1), 13-19.

Oscáriz, J. C., Lasa, I., & Pisabarro, A. G. (1999). Detection and characterization of cerein 7, a new bacteriocin produced by *Bacillus cereus* with a broad spectrum of activity. *FEMS Microbiology Letters*, *178*(2), 337-341.

O'sullivan, L., Ross, R. P., & Hill, C. (2003). A lacticin 481-producing adjunct culture increases starter lysis while inhibiting nonstarter lactic acid bacteria proliferation during Cheddar cheese ripening. *Journal of applied microbiology*, *95*(6), 1235-1241.

Ottenwälder, B. I. R. G. I. T., Kupke, T., Brecht, S., Gnau, V., Metzger, J., Jung, G., & Götz, F. (1995). Isolation and characterization of genetically engineered gallidermin and epidermin analogs. *Applied and environmental microbiology*, *61*(11), 3894-3903.

Ouwehand, A. C., Conway, P. L., & Salminen, S. J. (1995). Inhibition of S-fimbria-mediated adhesion to human ileostomy glycoproteins by a protein isolated from bovine colostrum. *Infection and immunity*, *63*(12), 4917-4920.

Paik, H. D., Bae, S. S., Park, S. H., & Pan, J. G. (1997). Identification and partial characterization of tochicin, a bacteriocin produced by *Bacillus thuringiensis* subsp *tochigiensis*. *Journal of industrial microbiology & biotechnology*, *19*(4), 294-298.

Paik, S. H., Chakicherla, A., & Hansen, J. N. (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 Chemistry*, *273*(36), 23134-23142.

Parente, E., & Ricciardi, A. (1999). Production, recovery and purification of bacteriocins from lactic acid bacteria. *Applied Microbiology and Biotechnology*, *52*(5), 628-638.

Parente, E., Brienza, C., Ricciardi, A., & Addario, G. (1997). Growth and bacteriocin production by Enterococcus faecium DPC1146 in batch and continuous culture. *Journal of industrial microbiology & biotechnology*, *18*(1), 62-67.

Parkes, R. J., Cragg, B. A., Bale, S. J., Getlifff, J. M., Goodman, K., Rochelle, P. A., ... & Harvey, S. M. (1994). Deep bacterial biosphere in Pacific Ocean sediments. *Nature*, *371*(6496), 410-413.

Pascual, J. M., van Heertum, R. L., Wang, D., Engelstad, K., & De Vivo, D. C. (2002). Imaging the metabolic footprint of Glut1 deficiency on the brain. *Annals of neurology*, *52*(4), 458-464.

Pattnaik, P., Grover, S., & Batish, V. K. (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*(2), 213-218.

Pattnaik, P., Kaushik, J. K., Grover, S., & Batish, V. K. (2001). Purification and characterization of a bacteriocin-like compound (Lichenin) produced anaerobically by *Bacillus licheniformis* isolated from water buffalo. *Journal of applied microbiology*, *91*(4), 636-645.

Pilet, M. F., & Leroi, F. (2011). Applications of protective cultures, bacteriocins and bacteriophages in fresh seafood and seafood products.

Pineiro, M., & Stanton, C. (2007). Probiotic bacteria: legislative framework—requirements to evidence basis. *The Journal of nutrition*, *137*(3), 850S-853S.

Pingitore, E. V., Salvucci, E., Sesma, F., & Nader-Macias, M. E. (2007). Different strategies for purification of antimicrobial peptides from lactic acid bacteria (LAB). *Communicating current research and educational topics and trends in applied microbiology*, *1*, 557-568.

Pinto, A. L., Fernandes, M., Pinto, C., Albano, H., Castilho, F., Teixeira, P., & Gibbs, P. A. (2009). Characterization of anti-Listeria bacteriocins isolated from shellfish: potential antimicrobials to control non-fermented seafood. *International journal of food microbiology*, *129*(1), 50-58.

Pisano, M. B., Viale, S., Conti, S., Fadda, M. E., Deplano, M., Melis, M. P., ... & Cosentino, S. (2014). Preliminary evaluation of probiotic properties of *Lactobacillus* strains isolated from Sardinian dairy products. *BioMed research international*, *2014*.

Pitti, R. M., Marsters, S. A., Ruppert, S., Donahue, C. J., Moore, A., & Ashkenazi, A. (1996). Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *Journal of Biological Chemistry*, *271*(22), 12687-12690.

Pons, A. M., Lanneluc, I., Cottenceau, G., & Sable, S. (2002). New developments in non-post translationally modified microcins. *Biochimie*, *84*(5), 531-537.

Powers, J. P. S., Rozek, A., & Hancock, R. E. (2004). Structure–activity relationships for the β-hairpin cationic antimicrobial peptide polyphemusin I. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1698*(2), 239-250.

Prangishvili, D., Holz, I., Stieger, E., Nickell, S., Kristjansson, J. K., & Zillig, W. (2000). Sulfolobicins, specific proteinaceous toxins produced by strains of the extremely thermophilic archaeal genus Sulfolobus. *Journal of bacteriology*, *182*(10), 2985-2988.

Preetha, R., Jayaprakash, N. S., Philip, R., & Singh, I. B. (2007). Optimization of medium for the production of a novel aquaculture probiotic, *Micrococcus* MCCB 104 using central composite design. *Biotechnology and Bioprocess Engineering*, *12*(5), 548-555.

Price, L. B., & Shand, R. F. (2000). Halocin S8: a 36-amino-acid microhalocin from the haloarchaeal strain S8a. *Journal of bacteriology*, *182*(17), 4951-4958.

Priest, F. G. (1993). Systematics and ecology of *Bacillus*. In *Bacillus subtilis and other Gram-positive bacteria* (pp. 3-16). American Society of Microbiology.

Prieto, M. L., O'Sullivan, L., Tan, S. P., McLoughlin, P., Hughes, H., O'Connor, P. M., ... & Gardiner, G. E. (2012). Assessment of the bacteriocinogenic potential of marine bacteria reveals lichenicidin production by seaweed-derived *Bacillus* spp. *Marine drugs*, *10*(10), 2280-2299.

Pringsulaka, O., Thongngam, N., Suwannasai, N., Atthakor, W., Pothivejkul, K., & Rangsiruji, A. (2012). Partial characterisation of bacteriocins produced by lactic acid bacteria isolated from Thai fermented meat and fish products. *Food control*, *23*(2), 547-551.

Pugsley, A. P., & Oudega, B. (1987). Methods for studying colicins and their plasmids. ., 105-161.

Rajaram, G., Manivasagan, P., Thilagavathi, B., & Saravanakumar, A. (2010). Purification and characterization of a bacteriocin produced by *Lactobacillus lactis* isolated from marine environment. *Adv J Food Sci Technol*, *2*(2), 138-144.

Ramachandran, R., Chalasani, A. G., Lal, R., & Roy, U. (2014). A broad-spectrum antimicrobial activity of *Bacillus subtilis* RLID 12.1. *The Scientific World Journal*, *2014*.

Ratnasingham, S., & Hebert, P. D. (2007). BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). *Molecular Ecology Resources*, *7*(3), 355-364.

Ravi, A. V., Musthafa, K. S., Jegathammbal, G., Kathiresan, K., & Pandian, S. K. (2007). Screening and evaluation of probiotics as a biocontrol agent against pathogenic Vibrios in marine aquaculture. *Letters in Applied Microbiology*, *45*(2), 219-223.

Reeves, P. (2012). *The bacteriocins* (Vol. 11). Springer Science & Business Media.

Reiss, R., Ihssen, J., & Thöny-Meyer, L. (2011). *Bacillus pumilus* laccase: a heat stable enzyme with a wide substrate spectrum. *BMC biotechnology*, *11*(1), 9.

Rhee, K. J., Sethupathi, P., Driks, A., Lanning, D. K., & Knight, K. L. (2004). Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. *The Journal of Immunology*, *172*(2), 1118-1124.

Riley, M. A., & Gordon, D. M. (1992). A survey of Col plasmids in natural isolates of *Escherichia coli* and an investigation into the stability of Col-plasmid lineages. *Microbiology*, *138*(7), 1345-1352.

Riley, M. A., & Gordon, D. M. (1999). The ecological role of bacteriocins in bacterial competition. *Trends in microbiology*, *7*(3), 129-133.

Riley, M. A., & Wertz, J. E. (2002a). Bacteriocin diversity: ecological and evolutionary perspectives.*Biochimie*, *84*(5), 357-364.

Riley, M. A., & Wertz, J. E. (2002b). Bacteriocins: evolution, ecology, and application. *Annual Reviews in Microbiology*, *56*(1), 117-137.

Ringø, E., Bendiksen, H. R., Wesmajervi, M. S., Olsen, R. E., Jansen, P. A., & Mikkelsen, H. (2000). Lactic acid bacteria associated with the digestive tract of Atlantic salmon (*Salmo salar* L.).*Journal of Applied Microbiology*, *89*(2), 317-322.

Rink, R., Kluskens, L. D., Kuipers, A., Driessen, A. J., Kuipers, O. P., & Moll, G. N. (2007). NisC, the cyclase of the lantibiotic nisin, can catalyze cyclization of designed nonlantibiotic peptides. *Biochemistry*, *46*(45), 13179-13189.

Rogers, A. H. (1972). Effect of the medium on bacteriocin production among strains of *Streptococcus mutans*. *Applied microbiology*, *24*(2), 294-295.

Rollema, H. S., Kuipers, O. P., Both, P., De Vos, W. M., & Siezen, R. J. (1995). Improvement of solubility and stability of the antimicrobial peptide nisin by protein engineering. *Applied and environmental microbiology*, *61*(8), 2873-2878.

Rozek, A., Friedrich, C. L., & Hancock, R. E. (2000). Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles. *Biochemistry*, *39*(51), 15765-15774.

Rudlowski, C., Becker, A. J., Schroder, W., Rath, W., Büttner, R., & Moser, M. (2003). GLUT1 messenger RNA and protein induction relates to the malignant transformation of cervical cancer. *American journal of clinical pathology*, *120*(5), 691-698.

Ryan, M. P., McAuliffe, O., Ross, R. P., & Hill, C. (2001). Heterologous expression of lacticin 3147 in Enterococcus faecalis: comparison of biological activity with cytolysin. *Letters in applied microbiology*, *32*(2), 71-77.

Sadekuzzaman, M., Yang, S., Mizan, M. F. R., & Ha, S. D. (2015). Current and recent advanced strategies for combating biofilms. *Comprehensive Reviews in Food Science and Food Safety*, *14*(4), 491-509.

Sahl, Hans-Georg. "Pore formation in bacterial membranes by cationic." *Nisin and novel lantibiotics* (1991): 347.

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution*, *4*(4), 406-425.

Sambrook, J., & Russell, D. W. (2006). Purification of nucleic acids by extraction with phenol: chloroform. *Cold Spring Harbor Protocols*, *2006*(1), pdb-prot4455.

Sand, S. L., Haug, T. M., Nissen-Meyer, J., & Sand, O. (2007). The bacterial peptide pheromone plantaricin A permeabilizes cancerous, but not normal, rat pituitary cells and differentiates between the outer and inner membrane leaflet. *Journal of Membrane Biology*, *216*(2-3), 61-71.

Sand, S. L., Oppegård, C., Ohara, S., Iijima, T., Naderi, S., Blomhoff, H. K., ... & Sand, O. (2010). Plantaricin A, a peptide pheromone produced by *Lactobacillus plantarum*, permeabilizes the cell membrane of both normal and cancerous lymphocytes and neuronal cells. *Peptides*, *31*(7), 1237-1244.

Sandiford, S., & Upton, M. (2012). Identification, characterization, and recombinant expression of epidermicin NI01, a novel unmodified bacteriocin produced by *Staphylococcus epidermidis* that displays potent activity against staphylococci. *Antimicrobial agents and chemotherapy*, *56*(3), 1539-1547.

Sarker, S. D., Nahar, L., & Kumarasamy, Y. (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*(4), 321-324.

Sass, P., Jansen, A., Szekat, C., Sass, V., Sahl, H. G., & Bierbaum, G. (2008). The lantibiotic mersacidin is a strong inducer of the cell wall stress response of *Staphylococcus aureus*. *BMC microbiology*, *8*(1), 186.

Sato, M., & Mueckler, M. (1999). A conserved amino acid motif (RXGRR) in the Glut1 glucose transporter is an important determinant of membrane topology. *Journal of Biological Chemistry*, *274*(35), 24721-24725.

Schlegel, R., & Slade, H. D. (1973). Properties of a *Streptococcus sanguis* (group H) bacteriocin and its separation from the competence factor of transformation. *Journal of bacteriology*, *115*(2), 655-661.

Sears, P. M., Smith, B. S., Stewart, W. K., Gonzalez, R. N., Rubino, S. D., Gusik, S. A., ... & Blackburn, P. (1992). Evaluation of a nisin-based germicidal formulation on teat skin of live cows. *Journal of dairy science*, *75*(11), 3185-3190.

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*(5), 1621-1631.

Shand, R. F., & Leyva, K. J. (2007). Peptide and protein antibiotics from the domain Archaea: halocins and sulfolobicins. In *Bacteriocins* (pp. 93-109). Springer Berlin Heidelberg.

Shayesteh, F., Ahmad, A., & Usup, G. (2014). Bacteriocin production by a marine strain of *Bacillus* sp. Sh10: Isolation, screening and optimization of culture condition. *Biotechnology*, *13*(6), 273-281.

Sherley, M., Gordon, D. M., & Collignon, P. J. (2000). Variations in antibiotic resistance profile in Enterobacteriaceae isolated from wild Australian mammals. *Environmental microbiology*, *2*(6), 620-631.

Shevchenko, A., Tomas, H., Havli, J., Olsen, J. V., & Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature protocols*, *1*(6), 2856-2860.

Shivaji, S., Bhanu, N. V., & Aggarwal, R. K. (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*(2), 247-252.

Shukla, R., & Goyal, A. (2014). Probiotic potential of *Pediococcus pentosaceus* CRAG3: a new isolate from fermented cucumber. *Probiotics and antimicrobial proteins*, *6*(1), 11-21.

Siefert, J. L., Larios-Sanz, M., Nakamura, L. K., Slepecky, R. A., Paul, J. H., Moore, E. R., ... & Jurtshuk Jr, P. (2000). Phylogeny of marine Bacillus isolates from the Gulf of Mexico. *Current microbiology*, *41*(2), 84-88.

Singh, P. K., Chittpurna, Ashish, Sharma, V., Patil, P. B., & Korpole, S. (2012). Identification, Purification and Characterization of Laterosporulin, a Novel Bacteriocin Produced by *Brevibacillus* sp. Strain GI-9. *PLoS ONE*, *7*(3), e31498. http://doi.org/10.1371/journal.pone.0031498

Skerman, V. B. D. (1967). A Guide to the Identification of the Genera of Bacteria. 2nd ed. *Baltimore: William and Wilkins,* pp.-81-99.

Slepecky, R. A., & Hemphill, H. E. (2006). The genus *Bacillus*—nonmedical. In *The prokaryotes* (pp. 530-562). Springer US.

Smith, D., Lee, K. D., Gray, E., Souleimanov, A., & Zhou, X. (2006). *U.S. Patent Application No. 12/093,779*.

Smith, H. W., & Linggood, M. A. (1971). Observations on the pathogenic properties of the K88, Hly and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. *Journal of medical microbiology*, *4*(4), 467-485.

Smith, P., & Davey, S. (1993). Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. *Journal of Fish Diseases*, *16*(5), 521-524.

Smitha, S., & Bhat, S. G. (2013). Thermostable bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment. *Journal of applied microbiology*, *114*(3), 688-694.

Spierings, D. C., de Vries, E. G., Timens, W., Groen, H. J., Boezen, H. M., & de Jong, S. (2003). Expression of TRAIL and TRAIL death receptors in stage III non-small cell lung cancer tumors. *Clinical Cancer Research*, *9*(9), 3397-3405.

Spížek, J., Novotná, J., Řezanka, T., & Demain, A. L. (2010). Do we need new antibiotics? The search for new targets and new compounds. *Journal of industrial microbiology & biotechnology*, *37*(12), 1241-1248.

Stanbury, P. F., Whitaker, A., & Hall, S. J. (2013). *Principles of fermentation technology*. Elsevier.

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular microbiology*, *56*(4), 845-857.

Stein, T., Heinzmann, S., Düsterhus, 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*(3), 822-828.

Stellrecht, C. M., & Gandhi, V. (2009). MET receptor tyrosine kinase as a therapeutic anticancer target. *Cancer letters*, *280*(1), 1-14.

Stentz, R., Osborne, S., Horn, N., Li, A. W., Hautefort, I., Bongaerts, R., ... & Brearley, C. A. (2014). A bacterial homolog of a eukaryotic inositol phosphate signaling enzyme mediates cross-kingdom dialog in the mammalian gut. *Cell reports*, *6*(4), 646-656.

Stepanović, S., Vuković, D., Hola, V., Bonaventura, G. D., Djukić, S., Ćirković, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *Staphylococci*. *Apmis*, *115*(8), 891-899.

Stern, N. J., Svetoch, E. A., Eruslanov, B. V., Kovalev, Y. N., Volodina, L. I., Perelygin, V. V., ... & Levchuk, V. P. (2005). *Paenibacillus polymyxa* purified

bacteriocin to control *Campylobacter jejuni* in chickens. *Journal of food protection*, *68*(7), 1450-1453.

Stoffels, G., Nes, I. F., & Guomundsdóttir, A. (1992). Isolation and properties of a bacteriocin-producing *Carnobacterium piscicola* isolated from fish. *Journal of Applied Bacteriology*, *73*(4), 309-316.

Suda, S., Westerbeek, A., O'Connor, P. M., Ross, R. P., Hill, C., & Cotter, P. D. (2010). Effect of bioengineering lacticin 3147 lanthionine bridges on specific activity and resistance to heat and proteases. *Chemistry & biology*, *17*(10), 1151-1160.

Suganthi, V., & Mohanasrinivasan, V. (2015). Optimization studies for enhanced bacteriocin production by *Pediococcus pentosaceus* KC692718 using response surface methodology. *Journal of food science and technology*, *52*(6), 3773-3783.

Sugita, H., Hirose, Y., Matsuo, N., & Deguchi, Y. (1998). Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. *Aquaculture*, *165*(3), 269-280.

Sugita, H., Matsuo, N., Hirose, Y., Iwato, M., & Deguchi, Y. (1997a). *Vibrio* sp. strain NM 10, isolated from the intestine of a Japanese coastal fish, has an inhibitory effect against *Pasteurella piscicida*. *Applied and Environmental Microbiology*, *63*(12), 4986-4989.

Sugita, H., Shibuya, K., Hanada, H., & Deguchi, Y. (1997b). Antibacterial abilities of intestinal microflora of the river fish. *Fisheries science*, *63*(3), 378-383.

Sung, J. Y., Shaffer, E. A., & Costerton, J. W. (1993). Antibacterial activity of bile salts against common biliary pathogens. *Digestive diseases and sciences*, *38*(11), 2104-2112.

Sutyak, K. E., Anderson, R. A., Dover, S. E., Feathergill, K. A., Aroutcheva, A. A., Faro, S., & Chikindas, M. L. (2008a). Spermicidal activity of the safe natural antimicrobial peptide subtilosin. *Infectious diseases in obstetrics and gynecology*, *2008*.

Sutyak, K. E., Wirawan, R. E., Aroutcheva, A. A., & Chikindas, M. L. (2008b). Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*. *Journal of applied microbiology*, *104*(4), 1067-1074.

Suzuki, M., Yamamoto, T., Kawai, Y., Inoue, N., & Yamazaki, K. (2005). Mode of action of piscicocin CS526 produced by *Carnobacterium piscicola* CS526. *Journal of applied microbiology*, *98*(5), 1146-1151.

Svetoch, E. A., Stern, N. J., Eruslanov, B. V., Kovalev, Y. N., Volodina, L. I., Perelygin, V. V., ... & Levchuk, V. P. (2005). Isolation of *Bacillus circulans* and *Paenibacillus polymyxa* strains inhibitory to *Campylobacter jejuni* and characterization of associated bacteriocins. *Journal of food protection*, *68*(1), 11-17.

Tagg, J. R., Dajani, A. S., & Wannamaker, L. W. (1975). Bacteriocin of a group B streptococcus: partial purification and characterization. *Antimicrobial agents and chemotherapy*, *7*(6), 764-772.

Tagg, J. R., Dajani, A. S., & Wannamaker, L. W. (1976). Bacteriocins of gram-positive bacteria. *Bacteriological reviews*, *40*(3), 722.

Tahiri, I., Desbiens, M., Benech, R., Kheadr, E., Lacroix, C., Thibault, S., ... & Fliss, I. (2004). Purification, characterization and amino acid sequencing of divergicin M35: a novel class IIa bacteriocin produced by *Carnobacterium divergens* M35. *International journal of food microbiology*, *97*(2), 123-136.

Tambekar, D. H., & Bhutada, S. A. (2010). Acid and bile tolerance, antibacterial activity, antibiotic resistance and bacteriocins activity of probiotic *Lactobacillus* species. *Recent Research in Science and Technology*, 2(4).

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular biology and evolution*, *24*(8), 1596-1599.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, *30*(12), 2725-2729.

Tebben, A. J., Ruzanov, M., Gao, M., Xie, D., Kiefer, S. E., Yan, C., ... & Kopcho, L. M. (2016). Crystal structures of apo and inhibitor-bound TGFβR2 kinase domain: insights into TGFβR isoform selectivity. *Acta Crystallographica Section D: Structural Biology*, *72*(5), 658-674.

Thirabunyanon, M. and Thongwittaya, N., 2012. Protection activity of a novel probiotic strain of *Bacillus subtilis* against *Salmonella Enteritidis* infection. *Research in veterinary science*, *93*(1), pp.74-81.

Thornberry, N. A., & Lazebnik, Y. (1998). Caspases: enemies within. *Science*, *281*(5381), 1312-1316.

Thornley, J. P., Shaw, J. G., Gryllos, I. A., & Eley, A. (1996). Adherence of *Aeromonas caviae* to human cell lines HEp-2 and Caco-2. *Journal of medical microbiology*, *45*(6), 445-451.

Todorov, S. D., & Dicks, L. M. T. (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*(6), 643-650.

Todorov, S. D., & Dicks, L. M. T. (2006). Effect of medium components on bacteriocin production by *Lactobacillus plantarum* strains ST23LD and ST341LD, isolated from spoiled olive brine. *Microbiological Research*, *161*(2), 102-108.

Todorov, S. D., Holzapfel, W., & Nero, L. A. (2017). *In Vitro* evaluation of beneficial properties of bacteriocinogenic *Lactobacillus plantarum* ST8Sh. *Probiotics and antimicrobial proteins*, *9*(2), 194-203.

Torreblanca, M., Meseguer, I., & Ventosa, A. (1994). Production of halocin is a practically universal feature of archaeal halophilic rods. Letters in applied microbiology, 19(4), 201-205.

Torrent, M., Badia, M., Moussaoui, M., Sanchez, D., Nogués, M., & Boix, E. (2010). Comparison of human RNase 3 and RNase 7 bactericidal action at the Gram-negative and Gram-positive bacterial cell wall. *The FEBS journal*, *277*(7), 1713-1725.

Tsai, T. L., Li, A. C., Chen, Y. C., Liao, Y. S., & Lin, T. H. (2015). Antimicrobial peptide m2163 or m2386 identified from *Lactobacillus casei* ATCC 334 can trigger apoptosis in the human colorectal cancer cell line SW480. *Tumor Biology*, *36*(5), 3775-3789.

Tubylewicz, H. (1966). Experimental studies on bacteriocinogeneity in *Clostridium perfringens* type A. Isolation of bacteriocines and their antibacterial spectrum. *Bulletin of polish academy of science (Biol.)14*, 31-36.

Tuomola, E. M., & Salminen, S. J. (1998). Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *International journal of food microbiology*, *41*(1), 45-51.

Twomey, D., Ross, R. P., Ryan, M., Meaney, B., & Hill, C. (2002). Lantibiotics produced by lactic acid bacteria: structure, function and applications. *Antonie Van Leeuwenhoek*, *82*(1-4), 165-185.

Urdaci, M. C., Bressollier, P., & Pinchuk, I. (2004). *Bacillus clausii* probiotic strains: antimicrobial and immunomodulatory activities. *Journal of clinical gastroenterology*, *38*, S86-S90.

Vaerewijck, M. J. M., 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*(6), 1074-1084.

Valdés-Stauber, N., & Scherer, S. (1994). Isolation and characterization of Linocin M18, a bacteriocin produced by *Brevibacterium linens*. *Applied and environmental microbiology*, *60*(10), 3809-3814.

Valenzuela, A. S., Benomar, N., Abriouel, H., Cañamero, M. M., & Gálvez, A. (2010). Isolation and identification of *Enterococcus faecium* from seafoods: antimicrobial resistance and production of bacteriocin-like substances. *Food microbiology*, *27*(7), 955-961.

Valley, C. C., Lewis, A. K., Mudaliar, D. J., Perlmutter, J. D., Braun, A. R., Karim, C. B., ... & Sachs, J. N. (2012). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces death receptor 5 networks that are highly organized. *Journal of Biological Chemistry*, *287*(25), 21265-21278.

van den Hooven, H. W., Lagerwerf, F. M., Heerma, W., Haverkamp, J., Piard, J. C., Hilbers, C. W., ... & Rollema, H. S. (1996). The structure of the lantibiotic lacticin 481 produced by *Lactococcus lactis*: location of the thioether bridges. *FEBS letters*, *391*(3), 317-322.

Van Houten, M. A., Luinge, K., Laseur, M., & Kimpen, J. L. (1998). Antibiotic utilisation for hospitalised paediatric patients. *International journal of antimicrobial agents*, *10*(2), 161-164.

Van Reenen, C. A., Dicks, L. M. T., & Chikindas, M. L. (1998). Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology*, *84*(6), 1131-1137.

Verellen, T. L., Bruggeman, G., Van Reenen, C. A., Dicks, L. M., & Vandamme, E. J. (1998). Fermentation optimization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423. *Journal of Fermentation and Bioengineering*, *86*(2), 174-179.

Verluyten, J., Messens, W., & De Vuyst, L. (2004). Sodium chloride reduces production of curvacin A, a bacteriocin produced by *Lactobacillus curvatus* strain LTH 1174, originating from fermented sausage. *Applied and Environmental Microbiology*, *70*(4), 2271-2278.

Vignolo, G. M., Kairuz, M. N., Holgado, A. A., & Oliver, G. (1995). Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705. *Journal of Applied Microbiology*, *78*(1), 5-10.

Vijayalakshmi, K.., Premalatha, A., & Suseela, R. (2011). 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(4), 243-249.

Vincent, S. S., John, S. B., 2009. Buffers: principles and practice. In Richard, RB., Murray, PD. (ed) *Methods in Enzymology*, Elsevier Science & Technology Books, Massachusetts, pp 43–56.

Vuyst, L. D. (1995). Nutritional factors affecting nisin production by *Lactococcus lactis* subsp. *actis* NIZO 22186 in a synthetic medium. *Journal of Applied Bacteriology*, *78*(1), 28-33.

Wang, D., Kranz-Eble, P., & De Vivo, D. C. (2000). Mutational analysis of GLUT1 (SLC2A1) in Glut-1 deficiency syndrome. *Human mutation*, *16*(3), 224.

Wang, G., Wang, X., Yu, H., Wei, S., Williams, N., Holmes, D. L., ... & Chen, S. (2013). Small-molecule activation of the TRAIL receptor DR5 in human cancer cells. *Nature chemical biology*, *9*(2), 84-89.

Wang, J. B., Qi, L. L., & Wu, T. X. (2009). Curcumin induces apoptosis through the mitochondria-mediated apoptotic pathway in HT-29 cells. *Journal of Zhejiang University Science B*, *10*(2), 93-102.

Wang, K., Shindoh, H., Inoue, T., & Horii, I. (2002). Advantages of in vitro cytotoxicity testing by using primary rat hepatocytes in comparison with established cell lines. *The Journal of toxicological sciences*, *27*(3), 229-237.

Wang, X. J., & Hsiao, K. C. (1995). Sugar degradation during autoclaving: effects of duration and solution volume on breakdown of glucose. *Physiologia Plantarum*, *94*(3), 415-418.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., & Hebert, P. D. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, *360*(1462), 1847-1857.

Wester, C. W., Durairaj, L., Evans, A. T., Schwartz, D. N., Husain, S., & Martinez, E. (2002). Antibiotic resistance: a survey of physician perceptions. *Archives of internal medicine*, *162*(19), 2210-2216.

Westerdahl, A. L. L. A. N., Olsson, J. C., Kjelleberg, S. T. A. F. F. A. N., & Conway, P. L. (1991). Isolation and characterization of turbot (*Scophtalmus maximus*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. *Applied and Environmental Microbiology*, *57*(8), 2223-2228.

Wilson, G. S., Raftos, D. A., Corrigan, S. L., & Nair, S. V. (2010). Diversity and antimicrobial activities of surface-attached marine bacteria from Sydney Harbour, Australia. *Microbiological research*, *165*(4), 300-311.

Wilson, K., & Walker, J. (2000). *Principles and techniques of practical biochemistry*. Cambridge University Press.

Wingender, J., & Flemming, H. C. (2011). Biofilms in drinking water and their role as reservoir for pathogens. *International journal of hygiene and environmental health*, *214*(6), 417-423.

World Health Organization. (2017). Global action plan on antimicrobial resistance. 2015. *ISBN*, *978*(924), 150976.

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*(20).

Yamamoto, T., Seino, Y., Fukumoto, H., Koh, G., Yano, H., Inagaki, N., ... & Imura, H. (1990). Over-expression of facilitative glucose transporter genes in human cancer. *Biochemical and biophysical research communications*, *170*(1), 223-230.

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*(10), 5746-5753.

Yamazaki, K., Suzuki, M., Kawai, Y., Inoue, N., & Montville, T. J. (2005). Purification and characterization of a novel class IIa bacteriocin, piscicocin CS526, from surimi-associated *Carnobacterium piscicola* CS526. *Applied and environmental microbiology*, *71*(1), 554-557.

Yang, A., Wilson, N. S., & Ashkenazi, A. (2010). Proapoptotic DR4 and DR5 signaling in cancer cells: toward clinical translation. *Current opinion in cell biology*, *22*(6), 837-844.

Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., & Zhang, Y. (2015). The I-TASSER Suite: protein structure and function prediction. *Nature methods*, *12*(1), 7-8.

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*(2), 181-187.

Yuki, N., Shimazaki, T., Kushiro, A., Watanabe, K., Uchida, K., Yuyama, T., & Morotomi, M. (2000). Colonization of the stratified squamous epithelium of the nonsecreting area of horse stomach by lactobacilli. *Applied and environmental microbiology*, *66*(11), 5030-5034.

Zavadil, J., & Böttinger, E. P. (2005). TGF-[beta] and epithelial-to-mesenchymal transitions. *Oncogene*, *24*(37), 5764.

Zhang, Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC bioinformatics*, *9*(1), 40.

Zhao, H., Sood, R., Jutila, A., Bose, S., Fimland, G., Nissen-Meyer, J., & Kinnunen, P. K. (2006). Interaction of the antimicrobial peptide pheromone Plantaricin A with model membranes: implications for a novel mechanism of action. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1758*(9), 1461-1474.

APPENDICES

APPENDIX – 1

NUTRIENT BROTH

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.

LURIA BERTANI BROTH

Ingredients		g/L
Casein enzymic hydrolysate	-	10
Yeast extract	-	5
Sodium chloride	-	10

Suspended 25g 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 40.25g 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.

MINIMAL MEDIA (M9G)

Ingredients		**g/ 100 mL**
Sodium phosphate	-	1.28
Dipotassium phosphate	-	0.3
Sodium chloride	-	0.5
Tryptone	-	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 sterilized		
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.

STARCH AGAR

Suspended 1.3 g of Nutrient Broth (Sigma Aldrich) in 1000 mL of distilled water. 2.0 % agar (w/v) was added to the medium for agar plate preparation. Starch (Sigma Aldrich) (0.1 %) was made ready as an aqueous solution and sterilized (121°C for 15 min), separate from the rest of the medium components and supplemented to the agar when the temperature reached 55°C.

GRAM'S IODINE SOLUTION

Ingredients		**g/L**
Iodine	-	1.0
Potassium iodide	-	2.0

Ingredients were dissolved in 1000 mL distilled water and stored in amber bottles.

SKIMMED MILK AGAR

Suspended 1.3 g of Nutrient Broth (HiMedia) in 1000 mL of distilled water. 2.0 % agar (w/v) was added to the medium for agar plate preparation. Skimmed milk agar powder (HiMedia) (10 %) was made ready as aqueous solution and sterilized (121°C for 15 min) separate from the rest of the medium components and supplemented to the agar when the temperature reached 55°C.

TRIBUTYRIN AGAR

Suspended 1.3 g of Nutrient Broth (HiMedia) in 1000 mL of distilled water. 2.0 % agar (w/v) was added to the medium for agar plate preparation. Tributyrin (HiMedia) (10 %) was made ready as an aqueous solution and sterilized (121°C for 15 min) separate from the rest of the medium components and supplemented to the agar when the temperature reached 55°C.

APPENDIX – II

0.01 M Phosphate buffer (pH 7.5)

Solution A: 0.2 M NaH2PO4
Solution B: 0.2 M Na2HPO4
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.

3 M 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 (10 mg/mL)

Ethidium bromide - 10 mg
Distilled water - 1mL
Dissolved well and stored by wrapping in aluminium foil (to be kept in dark)

TE buffer

1M Tris-Cl (pH 8) - 10mL
500mM EDTA (pH 8.0) - 2mL

1 M Tris-HCl (pH 8)

Tris base - 60.57 g
Deionised water - 500mL
Adjusted to desired pH using concentrated HCl

0.5 M EDTA (pH 8)

EDTA	-	18.6 g
Deionised water	-	100mL

Adjust the pH to 8.0 with NaOH

50 X 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.

1 X 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.

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 NaH2PO4
Solution B: 0.2 M Na2HPO4
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 (hydroxymethyl) amino 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: 0.2 M 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- III

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).

COOMASSIE STAINING SOLUTIONS

Protein staining solution

Coomassie brilliant	-	100 mg blue (0.1%)
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 SDSPAGE.

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 100oC. After a quick microcentrifuge spin, loaded directly on to a gel. The composition of the marker mix is as given below.

Components	**MW (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- IV

Peaks Patterns of MS'MS fragments

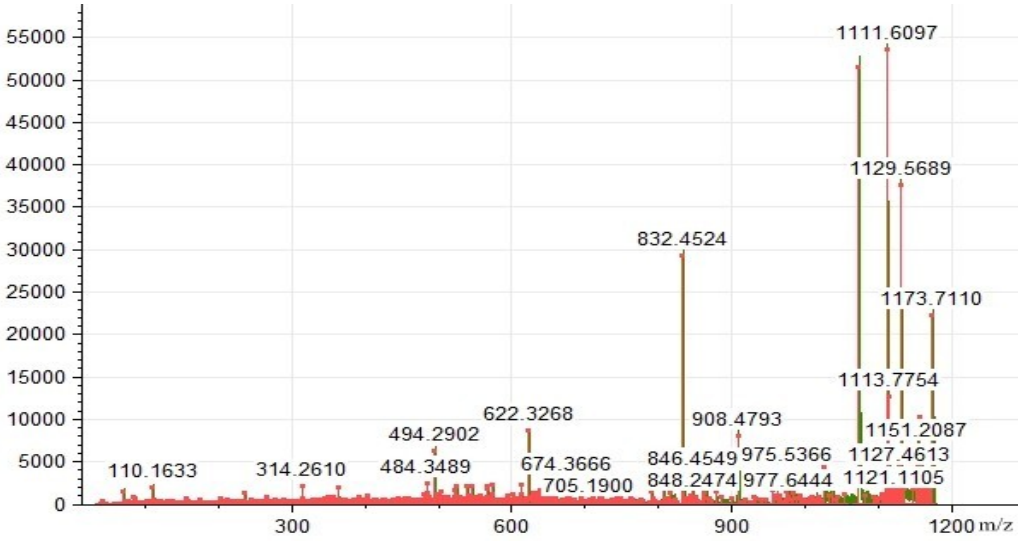


Fig A.1 Pattern of peaks distribution of 1173 fragment of MS/MS of BaCf3

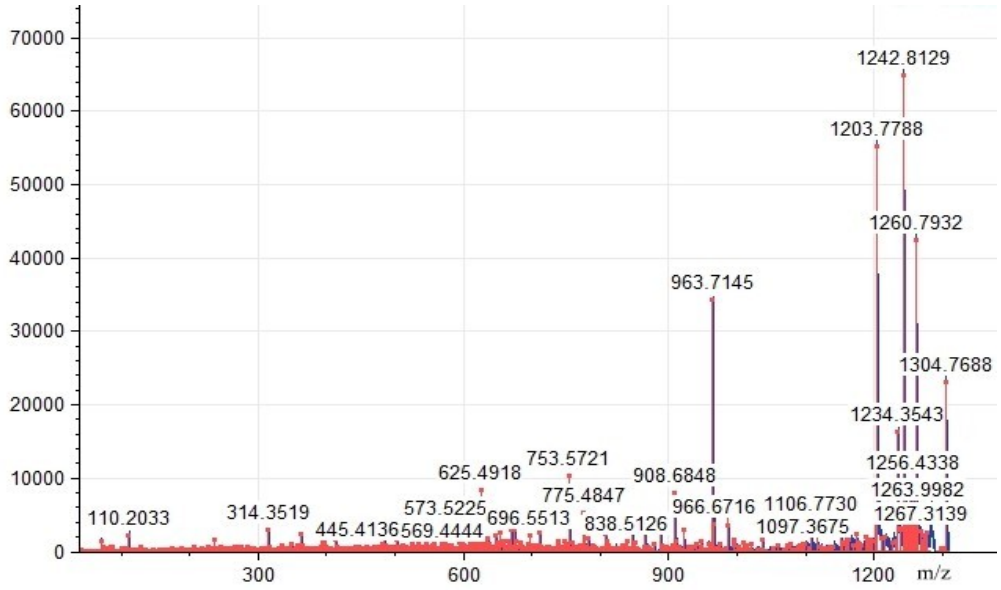


Fig A. 2 Pattern of peaks distribution of 1304 fragment of MS/MS of BaCf3

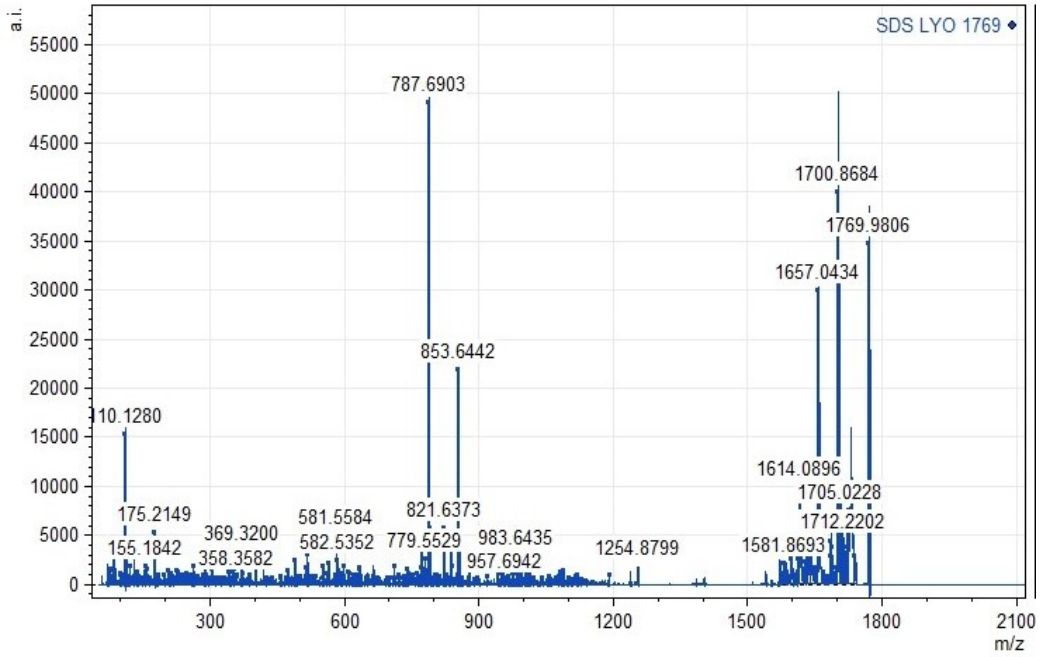


Fig A. 3 Pattern of peaks distribution of 1769 fragment of MS/MS of BpSl14

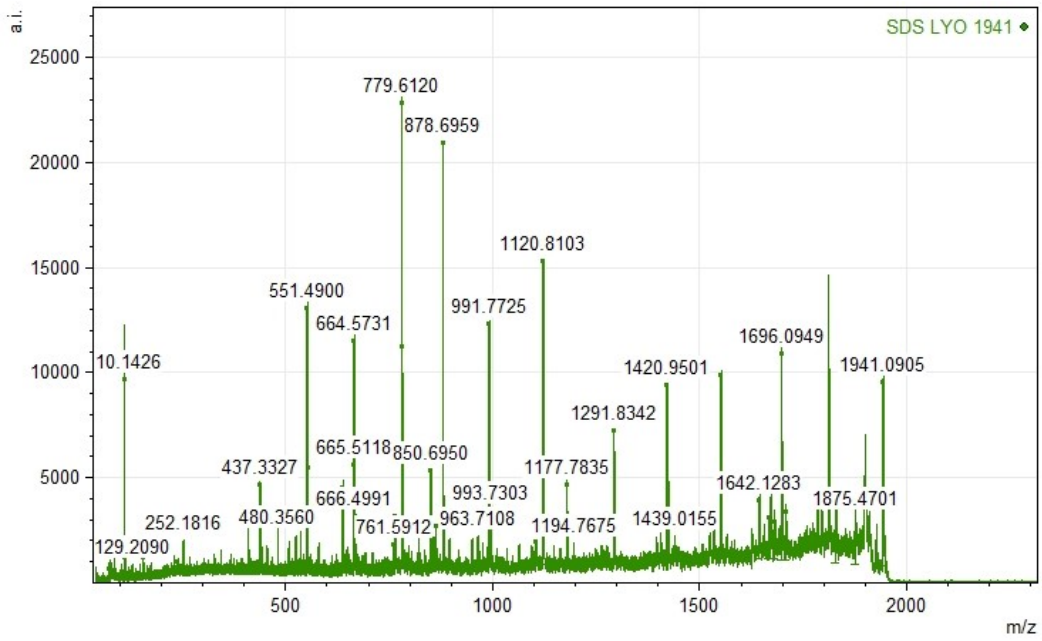


Fig A. 4 Pattern of peaks distribution of 1941 fragment of MS/MS of BpSl14

APPENDIX- V

Table A.1 ANOVA table of Aggregation Assay

Table Analyzed	**ANOVA Aggregation**				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	5.013				
P value	0.0869				
P value summary	ns				
Statistically significant (P < 0.05)?	No				
Geisser-Greenhouse's epsilon	0.5124				
R square	0.5562				
Was the matching effective?					
F	10.56				
P value	0.0028				
P value summary	**				
Is there significant matching (P < 0.05)?	Yes				
R square	0.7009				
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	1293	2	646.7	F (1.025, 4.099) = 5.013	P = 0.0869
Individual (between rows)	5450	4	1362	F (4, 8) = 10.56	P = 0.0028
Residual (random)	1032	8	129.0		
Total	7775	14			

Correlation analysis of Aggregation with time

Pearson r	**Time (h) vs. % Aggregation (A_a%) BTSS3**	**Time (h) vs. % Aggregation (A_a%) SDG14**
r	0.9786	0.9750
95% confidence interval	0.7051 to 0.9986	0.6634 to 0.9984
R square	0.9577	0.9507
P value		
P (two-tailed)	0.0037	0.0047
P value summary	**	**
Significant? (alpha = 0.05)	Yes	Yes

Table A.2 One-way ANOVA adhesion BTSS3

Table Analyzed	**One-way ANOVA adhesion BTSS3**
Column B	1.5 h (BTSS3)
vs.	vs.
Column A	1h(BTSS3)
Unpaired t test	
P value	< 0.0001
P value summary	****
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.181 df=20
How big is the difference?	
Mean ± SEM of column A	14.91 ± 1.040 N=11
Mean ± SEM of column B	32.00 ± 3.130 N=11
Difference between means	17.09 ± 3.299
95% confidence interval	10.21 to 23.97
R square	0.5731
F test to compare variances	
F,DFn, Dfd	9.066, 10, 10
P value	0.0017
P value summary	**
Significantly different? (P < 0.05)	Yes

Table A.3 One-way ANOVA adhesion SDG14

Table Analyzed	**One-way ANOVA adhesion SDG14**
Column B	1.5h (SDG14)
vs.	vs.
Column A	1h (SDG14)
Unpaired t test	
P value	0.0181
P value summary	*
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.574 df=20
How big is the difference?	
Mean ± SEM of column A	36.82 ± 1.788 N=11
Mean ± SEM of column B	45.55 ± 2.881 N=11
Difference between means	8.727 ± 3.390
95% confidence interval	1.655 to 15.80
R square	0.2489
F test to compare variances	
F,DFn, Dfd	2.596, 10, 10
P value	0.1484
P value summary	ns
Significantly different? (P < 0.05)	No

Table A.4 ANOVA table for co-aggregation assay of *Bacillus amyloliquefaciens* BTSS3

Table Analyzed	**coaggr BTSS3**
Repeated measures ANOVA summary	
Assume sphericity?	No
F	12.43
P value	0.0037
P value summary	**
Statistically significant (P < 0.05)?	Yes
Geisser-Greenhouse's epsilon	0.4239
R square	0.7131
Was the matching effective?	
F	71.97
P value	< 0.0001
P value summary	****
Is there significant matching (P < 0.05)?	Yes
R square	0.8377

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	324.0	4	81.00	F (1.696, 8.478) = 12.43	P = 0.0037
Individual (between rows)	2345	5	469.1	F (5, 20) = 71.97	P < 0.0001
Residual (random)	130.4	20	6.518		
Total	2800	29			

Table A.5 ANOVA table for co-aggregation assay of *Bacillus pumilus* SDG14

Table Analyzed	**coaggr SDG14**				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	12.37				
P value	0.0024				
P value summary	**				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.4760				
R square	0.7122				
Was the matching effective?					
F	38.53				
P value	< 0.0001				
P value summary	****				
Is there significant matching (P < 0.05)?	Yes				
R square	0.7350				
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	887.9	4	222.0	F (1.904, 9.519) = 12.37	P = 0.0024
Individual (between rows)	3457	5	691.4	F (5, 20) = 38.53	P < 0.0001
Residual (random)	358.9	20	17.94		
Total	4704	29			

Table A.6 ANOVA table of MTT assay for anticancer activity

Table Analyzed	**Anticancer**
Repeated measures ANOVA summary	
Assume sphericity?	No
F	11.17
P value	0.0160
P value summary	*
Statistically significant (P < 0.05)?	Yes
Geisser-Greenhouse's epsilon	0.5609
R square	0.6908
Was the matching effective?	
F	53.81
P value	< 0.0001
P value summary	****
Is there significant matching (P < 0.05)?	Yes
R square	0.8927

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	1065	2	532.6	F (1.122, 5.609) = 11.17	P = 0.0160
Individual (between rows)	12829	5	2566	F (5, 10) = 53.81	P < 0.0001
Residual (random)	476.8	10	47.68		
Total	14371	17			

Table A.6 ANOVA table of MTT assay for cytotoxicity

Table Analyzed	**Cytotoxicity**
Repeated measures ANOVA summary	
Assume sphericity?	No
F	19.83
P value	0.0058
P value summary	**
Statistically significant (P < 0.05)?	Yes
Geisser-Greenhouse's epsilon	0.5224
R square	0.7986
Was the matching effective?	
F	6.571
P value	0.0059
P value summary	**
Is there significant matching (P < 0.05)?	Yes
R square	0.3982

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	1579	2	789.6	F (1.045, 5.224) = 19.83	P = 0.0058
Individual (between rows)	1309	5	261.7	F (5, 10) = 6.571	P = 0.0059
Residual (random)	398.3	10	39.83		
Total	3286	17			

Table A.7 Two-way ANOVA table of antibiofilm action

Table Analyzed	**std strains**
Two-way ANOVA	Ordinary
Alpha	0.05

Source of Variation	**% of total variation**	**P value**	**P value summary**	**Significant?**
Interaction	25.71	0.0015	**	Yes
Biofilm producers	22.88	0.0036	**	Yes
% reduction by Bacteriocin	7.561	0.0060	**	Yes

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	950184	7	135741	F (7, 48) = 4.020	P = 0.0015
Biofilm producers	845731	7	120819	F (7, 48) = 3.578	P = 0.0036
% reduction by Bacteriocin	279455	1	279455	F (1, 48) = 8.276	P = 0.0060
Residual	1.621e+006	48	33767		

Table A.8 Two-way ANOVA table of antibiofilm action

Table Analyzed	**Food pathogens**				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	37.32	< 0.0001	****	Yes	
Biofilm Producers	16.92	< 0.0001	****	Yes	
% Biofilm Reduction	28.50	< 0.0001	****	Yes	
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	7004	8	875.5	F (8, 61) = 17.38	P < 0.0001
Biofilm Producers	3176	8	397.0	F (8, 61) = 7.883	P < 0.0001
% Biofilm Reduction	5350	1	5350	F (1, 61) = 106.2	P < 0.0001
Residual	3072	61	50.36		

APPENDIX-VI

Interaction Table of BaCf3 with 1D0G

Name	Distance	category	Types	from chem	To chemistry
A:GLY18:HN - S:GLU124:OE1	2.70091	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN25:HE21 - S:GLU94:OE2	2.01709	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:LEU15:HN - S:TRP120	3.22253	Hydrogen Bond	Pi-Donor Hydrogen Bond	H-Donor	Pi-Orbitals
A:LEU147 - A:LEU10	5.36119	Hydrophobic	Alkyl	Alkyl	Alkyl
A:LYS27:HZ2 - A:GLU144:OE2	1.69923	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:LYS27:HZ3 - A:GLU144:OE1	1.74505	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:LYS27:HN - A:GLU144:O	2.24985	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
S:GLN85:HE22 - A:GLY1:O	2.02822	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:HIS3:CD2 - S:GLN85:OE1	3.32962	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:ASN2:HD22 - S:LEU73:O	2.13671	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
S:SER74:HG -	1.95963	Hydrogen Bond	Conventional	H-Donor	H-Acceptor

A:ASP4:OD1			Hydrogen Bond		
A:GLY8:CA - S:ASN81:OD1	3.42947	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
S:ARG80 - A:MET7	4.99598	Hydrophobic	Alkyl	Alkyl	Alkyl
S:ARG80:HH11 - A:ASP4:OD1	1.92585	Hydrogen Bond;Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:GLN9:HE22 - A:TYR211:OH	2.03408	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BpSl14 with 1D0G

Name	**Distance**	**category**	**Types**	**from chem**	**To chemistry**
A:HIS8:HD1 - B:GLU144:O	2.96561	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
B:LYS145:NZ - A:GLU7:OE1	2.58222	Electrostatic	Attractive Charge	Positive	Negative
B:LYS145:HZ1 - A:GLU7:O	2.65585	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
B:LYS145:HZ1 - A:HIS8:O	1.74955	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
B:LYS145:HZ3 - A:SER11:OG	1.73601	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:MET6:SD - T:CYS84:O	3.27953	Other	Sulfur-X	Sulfur	O,N,S
T:VAL83 - A:MET6	4.83281	Hydrophobic	Alkyl	Alkyl	Alkyl
T:VAL83 - A:VAL14	4.66628	Hydrophobic	Alkyl	Alkyl	Alkyl

Interaction Table of BpSl14 with 1D0G

A:GLY15:HN - T:SER74:OG	2.43164	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
T:THR64:HG1 - A:GLU9:OE2	1.89811	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
T:ASN81:HN - A:GLU9:O	2.44338	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
T:ARG80:HH11 - A:GLU12:OE1	2.41588	Hydrogen Bond; Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
T:ARG80:NH2 - A:GLU12:OE2	5.04634	Electrostatic	Attractive Charge	Positive	Negative
T:THR77:HG1 - A:GLU12:OE2	2.17749	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
T:THR79:HG1 - A:GLU12:OE2	1.92165	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN31:HD21 - T:GLU94:OE2	2.13485	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:CYS35:SG - T:TRP120	3.50572	Hydrogen Bond;Other	Pi-Donor Hydrogen Bond;Pi-Sulfur	H-Donor; Sulfur	Pi-Orbitals;Pi-Orbitals
A:CYS35:CA - T:TRP120	3.96747	Hydrophobic	Pi-Sigma	C-H	Pi-Orbitals
A:ALA1:HT1 - B:GLU144:OE2	2.68281	Hydrogen Bond; Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative

Interaction Table of BaCf3 with 1SUK

Name	Distance	Category	Types	from chem	To chemistry
A:LEU85:HN - A:CYS24:SG	2.74741	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:LYS225:HZ1 - A:GLY18:O	1.66753	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG253:HH22 - A:GLN25:OE1	1.69203	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN397:HE22 - A:LEU15:O	2.29124	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG400:HH22 - A:GLY1:O	2.13858	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG400:HH22 - A:HIS3:NE2	2.16162	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN2:HN - A:HIS160:NE2	2.18033	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY8:HN - A:ALA405:O	2.20693	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN19:HN - A:GLN250:OE1	2.81476	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:CYS24:SG - A:PHE81:O	2.68771	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN25:HE22 - A:MET252:O	1.97279	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BaCf3 with 1Suk

Name	Distance	Category	Types	from chem	To chemistry
A:GLY26:HN - A:GLU254:OE2	2.85014	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY138:CA - A:ASP4:O	3.1782	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:HIS160:CE1 - A:ASN2:OD1	3.28494	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:PRO11:CD - A:GLU261:OE1	3.63828	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:LYS12:CE - A:MET252:O	3.11793	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:PRO141 - A:LEU15	4.27452	Hydrophobic	Alkyl	Alkyl	Alkyl
A:VAL257 - A:LYS12	5.38401	Hydrophobic	Alkyl	Alkyl	Alkyl
A:LEU262 - A:LEU10	5.44374	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PRO401 - A:MET14	5.38128	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA402 - A:PRO11	4.08387	Hydrophobic	Alkyl	Alkyl	Alkyl

Interaction Table of BaCf3 with 1Suk

Name	Distance	Category	Types	from chem	To chemistry
A:ALA405 - A:MET7	5.26735	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA405 - A:PRO11	4.73364	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA405 - A:CYS13	4.38401	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA21 - A:MET251	4.54021	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA22 - A:VAL87	5.26049	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA22 - A:MET142	4.41994	Hydrophobic	Alkyl	Alkyl	Alkyl
A:CYS24 - A:LEU85	4.63144	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PHE81 - A:CYS6	4.17266	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:PHE81 - A:LYS27	4.88927	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl

Interaction Table of BaCf3 with 1Suk

Name	Distance	Category	Types	from chem	To chemistry
A:PHE152 - A:PRO17	4.77991	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:LEU85:HN - A:CYS24:SG	2.74741	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:LYS225:HZ1 - A:GLY18:O	1.66753	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG253:HH22 - A:GLN25:OE1	1.69203	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN397:HE22 - A:LEU15:O	2.29124	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG400:HH22 - A:GLY1:O	2.13858	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG400:HH22 - A:HIS3:NE2	2.16162	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN2:HN - A:HIS160:NE2	2.18033	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY8:HN - A:ALA405:O	2.20693	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BaCf3 with 1Suk

Name	Distance	Category	Types	from chem	To chemistry
A:GLN19:HN - A:GLN250:OE1	2.81476	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:CYS24:SG - A:PHE81:O	2.68771	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN25:HE22 - A:MET252:O	1.97279	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY26:HN - A:GLU254:OE2	2.85014	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY138:CA - A:ASP4:O	3.1782	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:HIS160:CE1 - A:ASN2:OD1	3.28494	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:PRO11:CD - A:GLU261:OE1	3.63828	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:LYS12:CE - A:MET252:O	3.11793	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:VAL87 - A:MET142	4.62653	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PRO141 - A:LEU15	4.27452	Hydrophobic	Alkyl	Alkyl	Alkyl

Interaction Table of BaCf3 with 1Suk

Name	Distance	category	Types	from chemistry	To chemistry
A:VAL257 - A:LYS12	5.38401	Hydrophobic	Alkyl	Alkyl	Alkyl
A:LEU262 - A:LEU10	5.44374	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PRO401 - A:MET14	5.38128	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA402 - A:PRO11	4.08387	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA405 - A:MET7	5.26735	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA405 - A:PRO11	4.73364	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA405 - A:CYS13	4.38401	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA21 - A:MET251	4.54021	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA22 - A:VAL87	5.26049	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA22 - A:MET142	4.41994	Hydrophobic	Alkyl	Alkyl	Alkyl

Interaction Table of BpSl14 with 1Suk

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG330:HH22 - A:GLU12:OE1	2.0588	Hydrogen Bond; Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:ARG333:HH12 - A:GLU12:OE2	1.82669	Hydrogen Bond; Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:ARG334:HH11 - A:GLU12:OE2	1.94661	Hydrogen Bond; Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:ARG334:HH21 - A:GLU12:OE2	1.84013	Hydrogen Bond; Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:ARG153:NH1 - A:GLU9:OE2	2.63543	Electrostatic	Attractive Charge	Positive	Negative
A:ARG153:NH2 - A:GLU9:OE1	5.0217	Electrostatic	Attractive Charge	Positive	Negative
A:ARG330:NH1 - A:GLU12:OE2	5.0286	Electrostatic	Attractive Charge	Positive	Negative
A:ARG333:NH2 - A:GLU12:OE1	5.29813	Electrostatic	Attractive Charge	Positive	Negative
A:ARG334:NH1 - A:GLU12:OE1	4.43949	Electrostatic	Attractive Charge	Positive	Negative
A:LYS20:NZ - A:GLU462:OE1	4.79	Electrostatic	Attractive Charge	Positive	Negative
A:LYS28:NZ - A:GLU243:OE1	2.69541	Electrostatic	Attractive Charge	Positive	Negative

Interaction Table of BpSl14 with 1Suk

Name	Distance	Category	Types	from chemistry	To chemistry
A:GLN242:HN - A:PRO30:O	2.09601	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG330:HE - A:GLU12:OE1	2.12445	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG330:HH11 - A:SER11:O	2.12281	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG330:HH21 - A:SER11:O	1.86451	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:HE - A:GLU12:O	2.64005	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:HE - A:PRO13:O	2.96152	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:HH12 - A:GLY10:O	2.25314	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:HH22 - A:PRO13:O	1.75797	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG153:CD - A:GLU9:OE1	3.70627	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:LEU241:CA - A:PRO30:O	3.25136	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BpSl14 with 1Suk

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG333:CD - A:GLU12:OE2	3.27028	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:CD - A:GLU9:O	3.61801	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:GLY10:CA - A:GLU329:OE2	3.01308	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334 - A:MET6	4.56361	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ARG334 - A:VAL14	4.98904	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ILE390 - A:MET6	4.94935	Hydrophobic	Alkyl	Alkyl	Alkyl
A:LYS28 - A:LEU394	5.12518	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PRO30 - A:LEU241	4.59686	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PRO30 - A:LEU394	4.89467	Hydrophobic	Alkyl	Alkyl	Alkyl
A:HIS239 - A:ALA1	4.49233	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl

Interaction Table of BpSl14 with 1Suk

Name	Distance	Category	Types	from chemistry	To chemistry
A:PHE389 - A:ILE29	5.10214	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:HIS8 - A:ARG153	5.17644	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:ARG330:HH22 - A:GLU12:OE1	2.0588	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor;Positive	H-Acceptor; Negative
A:ARG333:HH12 - A:GLU12:OE2	1.82669	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor;Positive	H-Acceptor; Negative
A:ARG334:HH11 - A:GLU12:OE2	1.94661	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor;Positive	H-Acceptor; Negative
A:ARG334:HH21 - A:GLU12:OE2	1.84013	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor;Positive	H-Acceptor; Negative
A:ARG153:NH1 - A:GLU9:OE2	2.63543	Electrostatic	Attractive Charge	Positive	Negative
A:ARG153:NH2 - A:GLU9:OE1	5.0217	Electrostatic	Attractive Charge	Positive	Negative
A:ARG330:NH1 - A:GLU12:OE2	5.0286	Electrostatic	Attractive Charge	Positive	Negative

Interaction Table of BpSl14 with 1Suk

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG333:NH2 - A:GLU12:OE1	5.29813	Electrostatic	Attractive Charge	Positive	Negative
A:ARG334:NH1 - A:GLU12:OE1	4.43949	Electrostatic	Attractive Charge	Positive	Negative
A:LYS20:NZ - A:GLU462:OE1	4.79	Electrostatic	Attractive Charge	Positive	Negative
A:LYS28:NZ - A:GLU243:OE1	2.69541	Electrostatic	Attractive Charge	Positive	Negative
A:GLN242:HN - A:PRO30:O	2.09601	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG330:HE - A:GLU12:OE1	2.12445	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG330:HH11 - A:SER11:O	2.12281	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG330:HH21 - A:SER11:O	1.86451	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:HE - A:GLU12:O	2.64005	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:HE - A:PRO13:O	2.96152	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BpSl14 with 1Suk

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG334:HH12 - A:GLY10:O	2.25314	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:HH22 - A:PRO13:O	1.75797	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG153:CD - A:GLU9:OE1	3.70627	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:LEU241:CA - A:PRO30:O	3.25136	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:ARG333:CD - A:GLU12:OE2	3.27028	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:CD - A:GLU9:O	3.61801	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:GLY10:CA - A:GLU329:OE2	3.01308	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334 - A:MET6	4.56361	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ARG334 - A:VAL14	4.98904	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ILE390 - A:MET6	4.94935	Hydrophobic	Alkyl	Alkyl	Alkyl

Interaction Table of BpSl14 with 1Suk

Name	**Distance**	**Category**	**Types**	**from chemistry**	**To chemistry**
A:LYS28 - A:LEU394	5.12518	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PRO30 - A:LEU241	4.59686	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PRO30 - A:LEU394	4.89467	Hydrophobic	Alkyl	Alkyl	Alkyl
A:HIS239 - A:ALA1	4.49233	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:PHE389 - A:ILE29	5.10214	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:HIS8 - A:ARG153	5.17644	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:ARG330:HH22 - A:GLU12:OE1	2.0588	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor;Positive	H-Acceptor; Negative
A:ARG333:HH12 - A:GLU12:OE2	1.82669	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor;Positive	H-Acceptor; Negative
A:ARG334:HH11 - A:GLU12:OE2	1.94661	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor;Positive	H-Acceptor; Negative
A:ARG153:NH1 - A:GLU9:OE2	2.63543	Electrostatic	Attractive Charge	Positive	Negative

Interaction Table of BpSl14 with 1Suk

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG153:NH2 - A:GLU9:OE1	5.0217	Electrostatic	Attractive Charge	Positive	Negative
A:ARG330:NH1 - A:GLU12:OE2	5.0286	Electrostatic	Attractive Charge	Positive	Negative
A:ARG333:NH2 - A:GLU12:OE1	5.29813	Electrostatic	Attractive Charge	Positive	Negative
A:ARG334:NH1 - A:GLU12:OE1	4.43949	Electrostatic	Attractive Charge	Positive	Negative
A:LYS20:NZ - A:GLU462:OE1	4.79	Electrostatic	Attractive Charge	Positive	Negative
A:LYS28:NZ - A:GLU243:OE1	2.69541	Electrostatic	Attractive Charge	Positive	Negative
A:GLN242:HN - A:PRO30:O	2.09601	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG330:HE - A:GLU12:OE1	2.12445	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG330:HH11 - A:SER11:O	2.12281	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG330:HH21 - A:SER11:O	1.86451	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BpSl14 with 1Suk

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG334:HE - A:GLU12:O	2.64005	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:HE - A:PRO13:O	2.96152	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:HH12 - A:GLY10:O	2.25314	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:HH22 - A:PRO13:O	1.75797	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG153:CD - A:GLU9:OE1	3.70627	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:LEU241:CA - A:PRO30:O	3.25136	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:ARG333:CD - A:GLU12:OE2	3.27028	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:ARG334:CD - A:GLU9:O	3.61801	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:GLY10:CA - A:GLU329:OE2	3.01308	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BpSl14 with 1Suk

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG334 - A:MET6	4.56361	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ARG334 - A:VAL14	4.98904	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ILE390 - A:MET6	4.94935	Hydrophobic	Alkyl	Alkyl	Alkyl
A:LYS28 - A:LEU394	5.12518	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PRO30 - A:LEU241	4.59686	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PRO30 - A:LEU394	4.89467	Hydrophobic	Alkyl	Alkyl	Alkyl
A:HIS239 - A:ALA1	4.49233	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:PHE389 - A:ILE29	5.10214	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:HIS8 - A:ARG153	5.17644	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl

Interaction Table of BaCf3 with 3DKC

Name	Distance	Category	Types	from chemistry	To chemistry
A:GLY1:N - A:ASP1222:OD2	4.77538	Electrostatic	Attractive Charge	Positive	Negative
A:LYS1110:HZ2 - A:GLY1:O	1.65507	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:TYR1159:HH - A:ASP4:O	1.89269	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:MET1160:HN - A:ASP4:OD1	1.99733	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG1166:HH11 - A:GLY20:O	1.84663	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN1167:HD22 - A:MET14:O	1.99758	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG1170:HE - A:ALA22:O	1.98832	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG1208:HH12 - A:GLY16:O	2.01346	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY1:HT1 - A:ARG1208:O	2.5904	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY1:HT3 - A:ARG1208:O	2.6753	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BaCf3 with 3DKC

Name	Distance	Category	Types	from chemistry	To chemistry
A:GLY1:HT3 - A:MET1211:SD	2.84219	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN2:HD22 - A:PRO1158:O	2.79951	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY8:HN - A:HIS1094:NE2	2.76022	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:CYS24:HN - A:ASN1167:OD1	1.92629	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG1208:CD - A:GLY16:O	3.21796	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:LYS1110:NZ - A:HIS3	4.79493	Electrostatic	Pi-Cation	Positive	Pi-Orbitals
A:ASP4:OD1 - A:TYR1159	3.52768	Electrostatic	Pi-Anion	Negative	Pi-Orbitals
A:VAL1092:CG2 - A:HIS3	3.80476	Hydrophobic	Pi-Sigma	C-H	Pi-Orbitals
A:VAL1083 - A:PRO11	5.43489	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ARG1208 - A:LEU15	4.88084	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA22 - A:ARG1166	4.06894	Hydrophobic	Alkyl	Alkyl	Alkyl

Interaction Table of BaCf3 with 3DKC

Name	Distance	Category	Types	from chemistry	To chemistry
A:ALA22 - A:ARG1170	4.38351	Hydrophobic	Alkyl	Alkyl	Alkyl
A:HIS1088 - A:PRO17	5.37463	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:TYR1159 - A:MET7	5.10853	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:LYS1110:HZ1 - A:ASP1222:OD2	1.77636	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:LYS1110:HZ3 - A:ASP1222:OD1	1.69744	Hydrogen Bond;Electrostatic	Salt Bridge;Attractive Charge	H-Donor;Positive	H-Acceptor; Negative
A:GLY1:N - A:ASP1222:OD2	4.77538	Electrostatic	Attractive Charge	Positive	Negative
A:LYS1110:HZ2 - A:GLY1:O	1.65507	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:TYR1159:HH - A:ASP4:O	1.89269	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:MET1160:HN - A:ASP4:OD1	1.99733	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BaCf3 with 3DKC

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG1166:HH11 - A:GLY20:O	1.84663	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN1167:HD22 - A:MET14:O	1.99758	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG1170:HE - A:ALA22:O	1.98832	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG1208:HH12 - A:GLY16:O	2.01346	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY1:HT1 - A:ARG1208:O	2.5904	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY1:HT3 - A:ARG1208:O	2.6753	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY1:HT3 - A:MET1211:SD	2.84219	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN2:HD22 - A:PRO1158:O	2.79951	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLY8:HN - A:HIS1094:NE2	2.76022	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:CYS24:HN - A:ASN1167:OD1	1.92629	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BaCf3 with 3DKC

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG1208:CD - A:GLY16:O	3.21796	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:LYS1110:NZ - A:HIS3	4.79493	Electrostatic	Pi-Cation	Positive	Pi-Orbitals
A:ASP4:OD1 - A:TYR1159	3.52768	Electrostatic	Pi-Anion	Negative	Pi-Orbitals
A:VAL1092:CG2 - A:HIS3	3.80476	Hydrophobic	Pi-Sigma	C-H	Pi-Orbitals
A:VAL1083 - A:PRO11	5.43489	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ARG1208 - A:LEU15	4.88084	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA22 - A:ARG1166	4.06894	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA22 - A:ARG1170	4.38351	Hydrophobic	Alkyl	Alkyl	Alkyl
A:HIS1088 - A:PRO17	5.37463	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:TYR1159 - A:MET7	5.10853	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl

Interaction Table of BpSl14 with 3DKC

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG1114:HH21 - A:GLU12:OE2	1.95723	Hydrogen Bond;Electrostatic	Salt Bridge; Attractive Charge	H-Donor;Positive	H-Acceptor;Negative
A:ARG1208:HH21 - A:GLU9:OE2	2.5165	Hydrogen Bond;Electrostatic	Salt Bridge; Attractive Charge	H-Donor;Positive	H-Acceptor;Negative
A:LYS1244:HZ1 - A:GLU12:OE1	1.95043	Hydrogen Bond;Electrostatic	Salt Bridge; Attractive Charge	H-Donor;Positive	H-Acceptor;Negative
A:ARG1114:NH1 - A:GLU12:OE1	5.34765	Electrostatic	Attractive Charge	Positive	Negative
A:ARG1208:NH1 - A:GLU9:OE2	2.70558	Electrostatic	Attractive Charge	Positive	Negative
A:ARG1208:NH2 - A:GLU9:OE1	4.50715	Electrostatic	Attractive Charge	Positive	Negative
A:ARG1086:HH22 - A:PRO16:O	1.87377	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG1170:HE - A:LYS28:O	2.04012	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG1170:HH22 - A:LYS28:O	1.80494	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN1288:HD21 - A:GLU7:OE2	2.8391	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BpSl14 with 3DKC

Name	Distance	Category	Types	from chemistry	To chemistry
A:HIS1088:CE1 - A:GLU9:OE2	3.5173	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:GLY10:CA - A:LYS1244:O	3.23421	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:ARG1166:NH1 - A:PHE5	3.26847	Electrostatic	Pi-Cation	Positive	Pi-Orbitals
A:LYS1248:NZ - A:HIS8	4.93842	Electrostatic	Pi-Cation	Positive	Pi-Orbitals
A:GLU9:C,O;GLY10:N - A:HIS1088	4.3237	Hydrophobic	Amide-Pi Stacked	Amide	Pi-Orbitals
A:VAL1083 - A:PRO19	4.82325	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ARG1086 - A:PRO16	4.79523	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ARG1170 - A:ILE29	5.4775	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA1354 - A:PRO30	4.21004	Hydrophobic	Alkyl	Alkyl	Alkyl
A:ALA1 - A:PRO1285	5.25798	Hydrophobic	Alkyl	Alkyl	Alkyl
A:HIS1088 - A:MET6	5.40475	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl

Interaction Table of BaCf3 with 5E8T

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG215:HE - A:ASN2:OD1	2.06259	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG215:HH11 - A:GLY1:O	3.03306	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG215:HH12 - A:ASN2:OD1	1.75958	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG240:HE - A:CYS5:O	1.93284	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG240:HH12 - A:CYS5:O	2.03863	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ARG240:HH22 - A:LYS27:O	2.14162	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:SER241:HG - A:MET7:SD	2.48056	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ALA368:HN - A:PRO11:O	1.98892	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN370:HD22 - A:CYS13:O	2.12574	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:LYS376:HZ1 - A:GLY1:O	1.76502	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BaCf3 with 5E8T

Name	Distance	Category	Types	from chemistry	To chemistry
A:ASP4:HN - A:ASP4:OD2	2.02652	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN9:HN - A:ARG240:O	2.31757	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN9:HE21 - A:GLU239:OE2	1.99496	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:LEU15:HN - A:ASN370:OD1	2.0409	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:SER241:CA - A:MET7:O	3.27753	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:HIS3:CE1 - A:HIS371:O	3.3416	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:GLY8:CA - A:ARG240:O	3.18192	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:MET7:SD - A:PHE216	3.29862	Other	Pi-Sulfur	Sulfur	Pi-Orbitals
A:ALA368 - A:LYS12	4.25136	Hydrophobic	Alkyl	Alkyl	Alkyl
A:PRO11 - A:ILE367	4.12716	Hydrophobic	Alkyl	Alkyl	Alkyl
A:HIS371 - A:PRO17	5.02751	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:HIS3 - A:VAL373	4.77271	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl

Interaction Table of BpSl14 with 5E8T

Name	Distance	Category	Types	from chemistry	To chemistry
A:ARG203:HH11 - A:GLU12:OE2	1.83449	Hydrogen Bond;Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:LYS268:HZ1 - A:GLU9:OE1	1.83883	Hydrogen Bond;Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:LYS268:HZ3 - A:GLU9:OE2	1.80226	Hydrogen Bond;Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:ARG203:NH2 - A:GLU12:OE1	2.74568	Electrostatic	Attractive Charge	Positive	Negative
A:LYS28:NZ - A:GLU209:OE1	5.47863	Electrostatic	Attractive Charge	Positive	Negative
A:LEU207:HN - A:PHE5:O	2.76943	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:SER210:HN - A:LYS28:O	1.92671	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN270:HD21 - A:HIS8:O	1.90186	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN275:HE22 - A:GLU9:OE2	2.02713	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:PRO30:CD - A:SER210:OG	3.22257	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:CYS35:C - A:GLN208:O	3.12744	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BpSl14 with 5E8T

Name	Distance	Category	Types	from chemistry	To chemistry
A:TRP220 - A:PHE5	4.88304	Hydrophobic	Pi-Pi T-shaped	Pi-Orbitals	Pi-Orbitals
A:VAL206 - A:MET6	4.4587	Hydrophobic	Alkyl	Alkyl	Alkyl
A:VAL206 - A:VAL14	5.08216	Hydrophobic	Alkyl	Alkyl	Alkyl
A:TRP220 - A:ILE29	5.30398	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:PHE5 - A:LEU207	5.32961	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:ARG203:HH11 - A:GLU12:OE2	1.83449	Hydrogen Bond;Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:LYS268:HZ1 - A:GLU9:OE1	1.83883	Hydrogen Bond;Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:LYS268:HZ3 - A:GLU9:OE2	1.80226	Hydrogen Bond;Electrostatic	Salt Bridge; Attractive Charge	H-Donor; Positive	H-Acceptor; Negative
A:LYS28:NZ - A:GLU209:OE1	5.47863	Electrostatic	Attractive Charge	Positive	Negative
A:ARG203:HH22 - A:ARG203:O	2.19709	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:LEU207:HN - A:PHE5:O	2.76943	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor

Interaction Table of BpSl14 with 5E8T

Name	Distance	Category	Types	from chemistry	To chemistry
A:SER210:HN - A:LYS28:O	1.92671	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:LYS268:HN - A:GLN275:O	1.92878	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:ASN270:HD21 - A:HIS8:O	1.90186	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN275:HN - A:LYS268:O	1.87634	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:GLN275:HE22 - A:GLU9:OE2	2.02713	Hydrogen Bond	Conventional Hydrogen Bond	H-Donor	H-Acceptor
A:PRO30:CD - A:SER210:OG	3.22257	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:CYS35:C - A:GLN208:O	3.12744	Hydrogen Bond	Carbon Hydrogen Bond	H-Donor	H-Acceptor
A:TRP220 - A:PHE5	4.88304	Hydrophobic	Pi-Pi T-shaped	Pi-Orbitals	Pi-Orbitals
A:VAL206 - A:MET6	4.4587	Hydrophobic	Alkyl	Alkyl	Alkyl
A:VAL206 - A:VAL14	5.08216	Hydrophobic	Alkyl	Alkyl	Alkyl
A:TRP220 - A:ILE29	5.30398	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl
A:PHE5 - A:LEU207	5.32961	Hydrophobic	Pi-Alkyl	Pi-Orbitals	Alkyl

LIST OF PUBLICATIONS

Full paper in peer-reviewed journals

Bindiya, E. S., Tina, K. J., Raghul, S. S., & Bhat, S. G. (2015). Characterization of Deep Sea Fish Gut Bacteria with Antagonistic Potential, from *Centroscyllium fabricii* (Deep Sea Shark). *Probiotics and antimicrobial proteins*, *7*(2), 157-163.

Bindiya, E. S., Bhat, S.G. (2016). Marine Bacteriocins: A Review. *Journal of Bacteriology and Mycology Open Access* 2(5): 00040. DOI: 10.15406/jbmoa. 2016.02.00040

Posters/Abstracts in International/National Seminars and Conferences

Bindiya ES, Sarita G Bhat. Production Optimization of bacteriocin by *Bacillus amyloliquefaciens* BTSS3 from deep sea fish gut", World Ocean Science Congress 2015

Bindiya ES, Tina KJ, Raghul Subin S, Sarita G Bhat and M Chandrasekaran. Antimicrobial peptide from *Bacillus* sp. BTSS3- a gut bacterium from deep sea fish. Conference abstract in the Proceedings of National Symposium on Emerging Trends in Biotechnology 2014.

Accession Numbers Obtained

Bindiya, E. S., Tina,K.J., Raghul,S.S., Zaira,E. and Sarita,G.B.(2016) KT905423- *Centroscyllium fabricii* isolate T6.3 cytochrome oxidase subunit 1(COX1) gene, partial sequence

Bindiya, E. S., Tina,K.J., Raghul,S.S., Cikesh,P.C., Karthikeyan,P, Sarita,G.B. and Chandrasekaran,M (2013) KF151867- *Sardinella longiceps* voucher BT-MOES025 cytochrome oxidase subunit 1 (COI) gene, partial sequence

Bindiya,E.S., Tina,K.J., Raghul,S.S., Cikesh,P.C., Karthikeyan,P, Sarita,G.B. and Chandrasekaran,M (2014) KF018921- *Bacillus amyloliquefaciens* strain BTSS3 16S ribosomal RNA gene, partial sequence.

Bindiya,E.S., Tina,K.J., Zaira,E. and Sarita,G.B (2015) *Bacillus pumilus* strain SDG14 16S ribosomal RNA gene, partial sequence.

Probiotics & Antimicro. Prot. (2015) 7:157–163
DOI 10.1007/s12602-015-9190-x

Characterization of Deep Sea Fish Gut Bacteria with Antagonistic Potential, from *Centroscyllium fabricii* (Deep Sea Shark)

E. S. Bindiya · K. J. Tina · Subin S. Raghul · Sarita G. Bhat

Published online: 6 March 2015
© Springer Science+Business Media New York 2015

Abstract The bacterial isolates from *Centroscyllium fabricii* (deep sea shark) gut were screened for antagonistic activity by cross-streak method and disc diffusion assay. This study focuses on strain BTSS-3, which showed antimicrobial activity against pathogenic bacteria including *Salmonella Typhimurium, Proteus vulgaris, Clostridium perfringens, Staphylococcus aureus, Bacillus cereus, Bacillus circulans, Bacillus macerans* and *Bacillus pumilus*. BTSS3 was subjected to phenotypic characterization using biochemical tests, SEM imaging, exoenzyme profiling and antibiotic susceptibility tests. Comparative 16S rDNA gene sequence analysis indicated that this strain belonged to the genus *Bacillus*, with high (98 %) similarity to 16S rDNA sequences of *Bacillus amyloliquefaciens*. The chemical nature of the antibacterial substance was identified by treatment with proteolytic enzymes. The antibacterial activity was reduced by the action of these enzymes pointing out its peptide nature. It was observed from the growth and production kinetics that the bacteriocin was produced in the eighth hour of incubation, i.e., during the mid-log growth phase of the bacteria.

E. S. Bindiya · K. J. Tina · S. S. Raghul · S. G. Bhat (✉)
Department of Biotechnology, Cochin University of Science and Technology, Cochin 682 022, India
e-mail: saritagbhat@gmail.com

E. S. Bindiya
e-mail: bindiya79@yahoo.co.in

K. J. Tina
e-mail: tina.kj05@gmail.com

S. S. Raghul
e-mail: raghulzubin@gmail.com

Keywords *Bacillus amyloliquefaciens* · Deep sea shark · Bacteriocin · Phenotypic identification · Genotypic identification

Introduction

Many bacteria elucidate antibacterial properties. The antibacterial effect of bacteria is due to antibiotics, bacteriocins, lysozymes, siderophores, proteases, and/or hydrogen peroxide and the alteration of pH values by organic acids produced either singly or in combination [1, 2]. Bacteriocins are ribosomally synthesized peptides which kill bacteria that are often closely related to the producer strain [3]. They are heterogeneous compounds that display variable molecular weights, biochemical properties, inhibitory spectra and mechanisms of action [4]. They are found in almost every bacterial species examined till date, and within a species, different kinds of bacteriocins are produced [5, 6].

In fish, colonization of the microorganisms may start at the egg and/or larval stage, and go on with the development of the fish [7]. Thus, the numbers and range of microorganisms present in the larval stage, on food and in water, will influence the microbiota of the developing fish. Several functions can be assigned to the resident symbiotic gut microbiota. These include digestion of algal cells, provision of amino acids, and possibly prevention of colonization by bacterial pathogens [8]. Bacteria particularly those in the digestive tract, produce inhibitory compounds responsible for controlling the colonization of potential pathogens in fish [9, 10]. Many studies have isolated bacteriocin and bacteriocin-like substances from fish associated bacteria. Vibrionaceae representatives, coliforms, *Bacillus* sp. [11], *Vibrio* sp. [12] are a few among them.

The present study aims at the screening and identification of bacteriocin-producing culture-dependent bacteria associated with deep sea fish gut. This study focuses on the identification of the deep sea fish and the most potent isolate, designated strain BTSS-3. The study also includes monitoring of bacteriocin production kinetics.

Materials and Methods

Sampling of Deep Sea Organisms

The deep sea shark, *Centroscyllium fabricii* (KJ888145.1) caught during the cruise no. 305 of FORV Sagar Sampada using HSDT (High Speed Demersal Trawl) net from station number 17 (8°11.4″N and 75°54.9″E) at a depth of 1000 m was used. The gut contents of the deep sea fish were isolated aseptically and diluted in a tenfold series, and a 100 µL sample of each dilution was spread onto Nutrient Agar (Supplemented with 2 % NaCl) (HiMedia, Mumbai, India). The plates were incubated at 28 °C for 1 week, and all the isolated colonies were purified and maintained at 4 °C. The glycerol stocks were also made.

Bacterial Isolation and Screening for Antibacterial Activity

The isolates were screened for antibacterial activity by cross-streak method [13]. Also, the cell-free supernatants of the cultures in Zobell marine broth (HiMedia), obtained by filtering through 0.22-µm filter, were used to confirm the antibacterial activity by disk diffusion method [14]. Twelve hour old culture of test organisms (Table 1) grown in nutrient broth (HiMedia) were swab-inoculated on Mueller–Hinton agar (HiMedia) plates. Sterile filter paper discs (4 mm diameter) loaded with 20 µL (total protein concentration = 3.88 mg/mL) of culture filtrate were placed on the plates. Plates were incubated for 24 h at 37 °C, and the inhibition zones were measured. Un-inoculated Zobell marine broth was used as control. Protein concentration was estimated by Lowry's method [15].

Ammonium Sulphate Precipitation and Action of Proteolytic Enzymes

The cell-free supernatant was fractionated using ammonium sulphate (0–30, 30–60 and 60–90 %) [16]. The precipitates obtained were dissolved in milliQ water and dialysed using a 2-kDa benzoylated dialysis tubing (Sigma-Aldrich, USA) against milliQ water at 4 °C for 36 h with six changes of water. The activity of each fraction was analysed. To confirm the presence of active peptide, the effect of different enzymes such as proteinase K, trypsin, and pepsin were mixed with the active fraction from ammonium sulphate precipitation at final concentrations of 1 and 5 mg/mL. The mixture was incubated for 1 h at 37 °C and used to check the activity.

Quantitative Estimation of Antibacterial Titres by Critical Dilution Assay

Serial twofold dilutions were made from cell-free supernatants (CFS) of the culture. From each dilution, 5 µL was spotted on an indicator strain, swab-inoculated on Mueller–Hinton Agar plate. The plates were incubated at the optimum growth temperature of the indicator strain. The bacteriocin activity was expressed in activity units per mL (AU/mL). One arbitrary unit (AU) of bacteriocin was defined as 5 µL of the highest dilution yielding a zone of growth inhibition on the indicator lawn [17]. The reciprocal of the highest dilution was multiplied by 200 (1 mL/5 µL) to obtain activity units per mL (AU/mL) [18].

Bacterial Characterization

Phenotypic Characterization

Gram staining was performed using a Gram-stain kit (Himedia, Mumbai) according to the manufacturer's

Table 1 Antibacterial activity of BTSS3

Test organisms	NCIM no.	Activity (AU/mL)	Sp. activity (AU/mg)
Pseudomonas aeruginosa	2863	–	–
Salmonella Typhimurium	2501	400	103.1918
Escherichia coli	2343	–	–
Proteus vulgaris	2027	400	103.1918
Clostridium perfringens	2677	800	206.3836
Staphylococcus aureus	2127	800	206.3836
Bacillus cereus	2155	400	103.1918
Bacillus circulans	2107	800	206.3836
Bacillus macerans	2131	400	103.1918
Bacillus pumilus	2189	200	51.5959

instructions. Cell motility was observed by the hanging-drop method [19]. Different Biochemical tests were carried out according to standard methods [20]. The optimal growth temperature and pH of the media was determined over a temperature range of 4–55 °C and pH 2–12, at 1 pH unit intervals in Zobell Marine Broth. Tolerance to NaCl was tested in minimal salt broth with varying NaCl concentrations of 0, 1, 2, 3, 4 and 5 % (w/v). Growth curve was constructed. Antibiotic susceptibility tests were performed using Combi 69 and G VIII plus octo-discs (Himedia, Mumbai). Carbohydrate utilization was tested using HiIMVIC^{TM} (Himedia, Mumbai) kit according to the manufacturer's instructions. Growth of the organism was studied by turbidimetry assay.

The cell morphology of the selected isolate was examined by field emission scanning electron microscopy (JEOL/EO) operated at 20.0 kV and a magnification of 7000×. One millilitre sample from 12-h old culture was centrifuged at 8000×*g* for 10 min. The pellet containing cells was dissolved in 1 ml of sterile saline solution (0.85 %, w/v) and fixed with equal volume of glutaraldehyde (2.5 %, v/v) for 4–5 h. Glutaraldehyde was removed by centrifuging at 8000×*g* for 10 min, and cells were dehydrated using different concentrations of acetone and finally dried in vacuum desiccator.

Genotypic Identification

Bacterial DNA was isolated according to the protocol described by Ausubel et al. [21]. The 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 [22]. PCR was carried out with an initial denaturation at 94 °C for 1.5 min, followed by 35 cycles of [94 °C/30 s, 56 °C/30 s, 72 °C/2′] and a final extension at 72 °C for 10 min.

Average Nucleotide Identity (ANI)

The ANI between the most similar sequences was calculated using J Species (V1.2.1) [23].

Agarose Gel Electrophoresis and Sequencing

PCR products were visualized on 1.5 % agarose gels, and the most intense products were selected for sequencing by ABI Prism 310 genetic analyser (Applied Biosystems, Carlsbad, CA, USA) using big dye terminator kit. The identity of the sequences was determined by comparing the sequences in the NCBI database using Basic Local Alignment Search Tool (BLAST) software [24]. The sequence was submitted to GenBank, and accession number was secured.

Phylogenetic analysis was performed using MEGA version 6.0 [25]. Clustering using the neighbour-joining [26] were determined using bootstrap values based on 1,000 replicates.

Growth and Production Kinetics

Zobell marine broth was inoculated with *Bacillus* sp. BTSS3 (inoculum 1 %, v/v) and incubated at 37 °C for 24 h in a rotary shaker. Growth was assessed by measuring the optical density at 600 nm at 2 h intervals. Antibacterial activity of each sample was assayed by the twofold dilution method as described above. Protein concentration was estimated [15]. The specific activity was calculated as the activity units per milligram of protein in the cell-free supernatant (CFS).

Results and Discussion

Bacterial Isolation and Screening for Antibacterial Activity

Among the one hundred and thirty-seven isolates obtained by culture-dependent methods from fish gut, several were observed to exhibit antibacterial potential. The cell-free supernatant was subjected to ammonium sulphate precipitation and action of proteolytic enzymes.

Ammonium Sulphate Precipitation and Action of Proteolytic Enzymes

The antibacterial substance was precipitated in the 0–30 % ammonium sulphate fraction. The active fraction was found to be affected by all the proteolytic enzymes used. The activity was totally lost when the active fraction was treated with trypsin and proteinase K, while 1 mg/mL of pepsin could reduce the activity to 50 %. This leads to the confirmation that the active substance is a peptide antibiotic generally termed as bacteriocin. The organism labelled BTSS3 was identified as a bacteriocin producer and selected for further studies. The antibacterial spectrum of BTSS3 was quantified by twofold dilution method and is represented in Table 1.

Phenotypic Characteristics

Strain BTSS3 was observed as Gram-stain positive, spore-forming, rod-shaped motile bacteria, which could grow at varying concentrations (0–5 %) of NaCl and 8–42 °C, (optimum 30–42 °C). Strain BTSS3 could tolerate pH of 4–9. The detailed biochemical characteristics are mounted in Table 2. The organism was found to be sensitive to most

of the antibiotics tested. The scanning electron microscopic analysis showed that the isolate was phenotypically homogeneous with rod-shaped cells with an average length of 1.41 µm and an average breadth of 673.6 µm (Fig. 1).

From the growth curve (Fig. 2), it was observed that the exponential growth started after two hours of incubation and the generation time of the bacteria was 120 min.

The antibiotic susceptibility test showed that the organism is sensitive to all the antibiotics tested, except bacitracin (Table 3). Kinetic studies of bacteriocin showed that the production starts in the mid-log phase with maximum production after 8 h of incubation. The activity declined as the culture reached stationary phase.

Genotypic Identification

Comparative 16S rDNA gene sequence analysis indicated that strain BTSS3 belongs to the genus *Bacillus*, with high (98 %) similarity to 16S rDNA sequences of *Bacillus amyloliquefaciens*. The sequence was submitted to GenBank, and the accession number is KF018921.1. The phenotypic characterization also confirmed the identification of strain BTSS3 as KJ870199.1 *B. amyloliquefaciens* B9. The phylogeny based on partial 16S rDNA sequences of strain BTSS3 and related *Bacillus* sp. is shown in Fig. 3. The optimal tree with the sum of branch length = 5.61153123 is shown. The analysis involved six nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 894

Table 2 Biochemical characteristics of the strain BTSS3

Biochemical characteristics of the organism	
Gram-staining	+
Shape	Short rod
Indole production	–
Methyl red	–
Voges Proskauer	–
Citrate utilization	–
Triple sugar iron test	Alkaline
MoF test	Fermentative
Catalase	+
Urea utilization	+
Starch utilization	+
Casein hydrolysis	+
Lipid hydrolysis	+

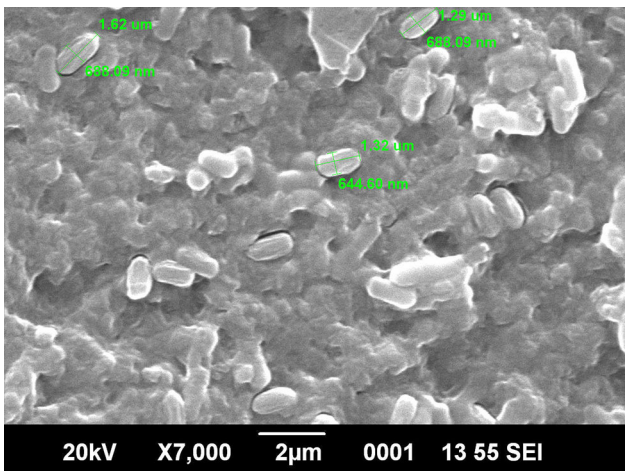


Fig. 1 SEM image of *Bacillus amyloliquefaciens* BTSS3

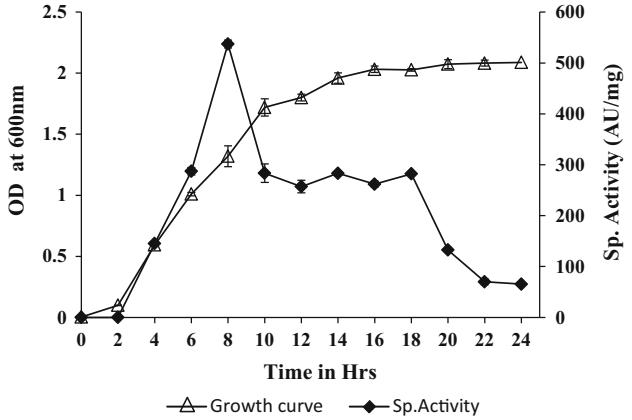


Fig. 2 Growth of *Bacillus amyloliquefaciens* BTSS3 and corresponding production rate. *Triangles* indicate growth curve and *rhombus* indicate bacteriocin production

Table 3 Antibiogram of the isolate BTSS3

Antibiotic	Concentration	Activity
Combi 69		
Ciprofloxacin (CIP)	5 µg	Sensitive
Ofloxacin (OF)	5 µg	Sensitive
Sparfloxacin (SPX)	5 µg	Sensitive
Gatifloxacin (GAT)	5 µg	Sensitive
Aztreonam (AT)	30 µg	Sensitive
Azithromycin (AZM)	15 µg	Sensitive
Vancomycin (VA)	30 µg	Sensitive
Doxycycline Hydrochloride (DO)	30 µg	Sensitive
G VIII Plus		
Bacitracin (B)	10 Unit	Resistant
Chloramphenicol (C)	30 µg	Sensitive
Co-Trimoxazole (COT)	25 µg	Sensitive
Penicillin-G (P)	10 Unit	Sensitive
Polymyxin-B (PB)	300 Unit	Sensitive
Gentamicin (GEN)	10 µg	Sensitive
Neomycin (N)	30 µg	Sensitive
Tetracycline (TE)	30 µg	Sensitive

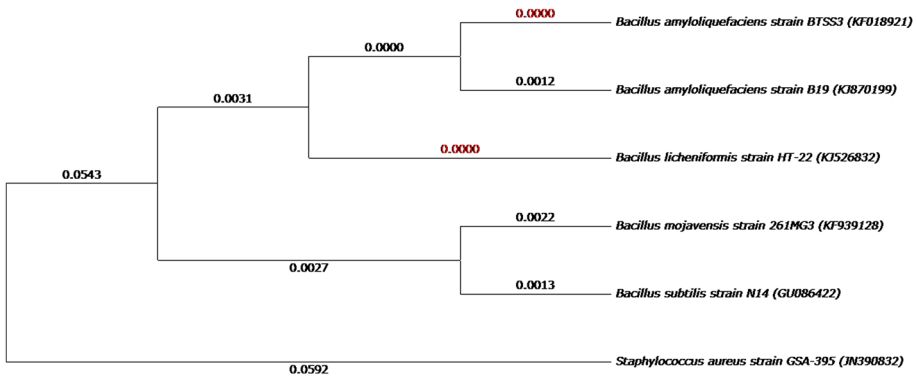


Fig. 3 Phylogenetic tree analysis of BTSS3. The optimal tree with the sum of branch length = 5.61153123 is shown. The organism shows more similarity to *Bacillus amyloliquefaciens* B19

positions in the final dataset. It shows that the bacteria come under *B. amyloliquefaciens*.

Discussion

Marine environment represents a largely untapped source for isolation of new microorganisms with potential to produce bioactive compounds. The host organism synthesizes these compounds as primary or secondary metabolites, to protect themselves and to maintain homeostasis in their environment. Marine bacteria constitute approximately 10 % of the living biomass of carbon in the biosphere [27]. However, only a small percentage of marine bacteria have been studied for their bioactive potential.

The fish gut acts a microenvironment for bacteria since it facilitates the growth of a mixture of microorganisms both pathogenic and probiotic. Deep sea fishes are the least explored area for research. The environments in which they survive are also less explored. The screening has pointed towards the presence of several bacteria with antimicrobial activity in the deep sea fish gut. Moreover, the strain BTSS3 showed activity against almost all the Gram-positive bacteria tested.

According to Sugita et al. [28], 11.2 % of the bacteria isolated from fish intestine could inhibit fish pathogens. Smith and Davey [29] reported that *Pseudomonas fluorescence* isolated from fish, reduced diseases caused by *Aeromonas salmonicida*. Austin et al. [30] too observed that *Vibrio alginolyticus*, when used as a probiotic strain, reduced diseases caused by *A. salmonicida*, *V. anguillarum* and *Vibrio ordalii*. These reports strongly suggest the effective control of microbiota in the fish intestine by using antibiotic-producing bacteria. The results suggest that *B. amyloliquefaciens* BTSS3 may be a suitable candidate as a biocontrol agent in fish intestines and culture water.

The action of proteolytic enzymes on the antibacterial compound strongly points out the presence of peptide antibacterial substance, indicating that the compound is a bacteriocin. The antibacterial substance produced by *Bacillus licheniformis* P40 was identified as bacteriocin-like substance by the action of proteolytic enzymes [31]. In 1982, Hoyt and Sizemore [32] showed that harveyicin, an antibiotic produced by *Vibrio harveyi*, was a proteinaceous substance sensitive to trypsin and papain.

Bacillus species are found in diverse environments such as soil and clays, rocks, dust, aquatic environments, vegetation, food and the gastrointestinal tracts of various insects and animals [33]. *Bacillus* species produce a large number of bacteriocins, subtilosin by *B. amyloliquifaciens* [34], haloduracin by *B. halodurans* [35], thuricin by *B. thuringiensis* [36], bacillocin 490 by *B. licheniformis* [37], cerein by *B. cereus* [38], and subtilin by *B. subtilis* [39], all of which are mostly active against Gram-positive organisms. They are widely used in industrial and agricultural production because of their ability to produce various enzymes [40], antimicrobial compounds [41], and so on.

The kinetic study shows that the bacteriocin production is growth related. Maximum production occured after 8 h of incubation; during the mid-log phase of the growth curve. Thus, the bacteriocin can be considered as the primary metabolite of the organism like the BLS and bacteriocins produced by *Bacillus licheniformis* P40 [31], *Enterococcus faecium* [42] and *Lactobacillus amylovorus* [43] BL8 by *B. licheniformis* [44].

The activity reduced after the tenth hour. In a study by Naclerio et al. [45], the bactericidal activity of bacteriocin produced by *Bacillus cereus* appeared at the beginning of stationary growth and declined after 2–3 h of its appearance. The loss of activity may be due to the action of proteolytic enzyme produced by the bacteria as indicated in Table 1. The activity of the bacteriocin produced by BTSS3 was susceptible to proteolytic enzymes like trypsin, pepsin and proteinase K. Such a reduction can be overcome by cloning and over expression of the bacteriocin gene, which will be attempted.

These results suggest that *B. amyloliquefaciens* BTSS3 may be a suitable candidate as a biocontrol agent and its

potential as a probiotic in fish intestines and culture water needs to be evaluated.

Acknowledgments This study was supported by project Grants from Centre for Marine Living Resources and Ecology- Ministry of Earth Sciences, Government of India (MOES/10-MLR/2/2007 and MOES/10-MLR-TD/03/2013) given to Dr. Sarita G. Bhat, Department of Biotechnology, Cochin University of Science and Technology, Kochi, India.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard This article does not contain any studies with animals or human participants performed by any of the authors.

References

1. De Vuyst L, Vandamme EJ (1994) Bacteriocins of lactic acid bacteria: microbiology, genetics and applications. London, Blackie Academic & Professional, Genetics and Applications
2. De Vuyst L, Leroy F (2007) Bacteriocins from lactic acid bacteria: production, purification, and food applications. J Mol Microbiol Biotechnol 13:194–199
3. 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. Int Microbiol 9:111–118
4. O'Sullivan L, Ross RP, Hill C (2002) Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie 84:593–604
5. Riley MA, Gordon DM (1992) A survey of col plasmids in natural isolates of *Escherichia coli* and an investigation into the stability of col-plasmid lineages. J Microbiol 138:1345–1352
6. James R, Lazdunski C, Pattus F (eds) (1991) Bacteriocins, microcins and lantibiotics, vol 65. Springer, New York
7. Olafsen JA (2001) Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture 200:223–247
8. Westerdahl A, Olsson JC, Kjelleberg S, Conway P (1991) Isolation and Characterization of Turbot (*Scophtalmus maximus*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl Environ Microbiol 57:2223–2228
9. Ringø E, Bendiksen HR, Wesmajervi MS, Olsen RE, Jansen PA, Mikkelsen H (2000) Lactic acid bacteria associated with the digestive tract of Atlantic salmon (*Salmo salar* L.). J Appl Microbiol 89:317–322
10. Makridis P, Martins S, Tsalavouta M, Dionisio LC, Kotoulas G, Magoulas A, Dinis MT (2005) Antimicrobial activity in bacteria isolated from Senegalese sole, *Solea senegalensis,* fed with natural prey. Aquacult Res 36:1619–1627
11. Sugita H, Shibuya K, Hanada H, Deguchi Y (1997) Antibacterial abilities of intestinal microflora of the river fish. Fish Sci 63:378–383
12. Bergh Ø (1995) Bacteria associated with early-life stages of halibut, *Hippoglossus hippoglossus*, inhibit growth of a pathogenic *Vibrio* sp. J Fish Dis 18:31–40
13. Gardner JF (1950) Some antibiotics formed by *Bacterium coli*. Br J Exp Pathol 31:102–111
14. Bauer AW, Kirby WMM, Sherris JC, Truck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45:493–496
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
16. Heyns W, De Moor P (1971) The binding of 17β-hydroxy-5α-androstan-3-one to the steroid-binding β-globulin in human plasma, as studied by means of ammonium sulphate precipitation. Steroids 18:709–730
17. Mayr-Harting A, Hedges AJ, Berkeley CW (1972) Methods for studying bacteriocins. Methods Microbiol 7:315–412
18. Enan G, El-Essway AA, Uyttendaele M, Debevere J (1996) Antibacterial activity of *Lactobacillus plantarum* UG1 isolated from dry sausage: characterization, production and bactericidal action of plantaricin UG1. Int J Food Microbiol 30:189–215
19. Skerman VBD (1967) A guide to the identification of the genera of bacteria, 2nd edn. Williams & Wilkins, Baltimore
20. Dong XZ, Cai MY (2001) Determinative manual for routine bacteriology. Scientific Press (English translation), Beijing
21. 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
22. Shivaji S, Bhanu NV, Aggarwal RK (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
23. Richter M, Rossello-Mora R (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci USA 106:19126–19131
24. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
25. 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. Mol Biol Evol 28:2731–2739
26. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425
27. Parkes RJ, Cragg BA, Bale SJ, Getlifff JM, Goodman K, Rochelle PA, Fry JC, Weightman AJ, Harvey SM (1994) Deep bacterial biosphere in Pacific Ocean sediments. Nature 371:410–413. doi:10.1038/371410a0
28. Sugita H, Ushioka S, Kihara D, Deguchi Y (1985) Changes in the bacterial composition of water in a carp rearing tank. Aquaculture 44:243–247
29. Smith P, Davey S (1993) Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. J Fish Dis 16:521–524
30. Austin B, Stuckey LF, Robertson PAW, Effendi I, Griffith DRW (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. J Fish Dis 18:93–96
31. Cladera-Olivera F, Caron GR, Brandelli A (2004) Bacteriocin-like substance production by *Bacillus licheniformis* strain P40. Lett Appl Microbiol 38:251–256
32. Hoyt PR, Sizemore RK (1982) Competitive dominance by a bacteriocin-producing *Vibrio harveyi* strain. Appl Environ Microbiol 44:653–658
33. Nicholson WL (2002) Roles of *Bacillus* endospores in the environment. Cell Mol Life Sci 59:410–416
34. Sutyak KE, Wirawan RE, Aroutcheva AA, Chikindas ML (2008) Isolation of the *Bacillus subtilis* antimicrobial peptide subtilosin from the dairy product-derived *Bacillus amyloliquefaciens*. J Appl Microbiol 104:1067–1074
35. Lawton EM, Paul D, Cotter CH, Ross PR (2006) Identification of a novel two-peptide lantibiotic, Haloduracin, produced by the alkaliphile *Bacillus halodurans* C-125. FEMS Microbiol Lett 267:64–71
36. Gray EJ, Lee KD, Souleimanov AM, Di Falco MR, Zhou X, Ly A, Charles TC, Driscoll BT 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
37. Martirani L, Varcamonti M, Naclerio G, Felice MD (2002) Purification and partial characterization of bacillocin 490, a novel bacteriocin produced by a thermophilic strain of *Bacillus licheniformis*. Microb Cell Fact. doi:10.1186/1475-2859-1-1
38. Oscariz JC, Lasa I, Pissabarro AG (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
39. Banerjee S, Hansen JN (1988) Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. J Biol Chem 263:9508–9514
40. Reiss R, Ihssen J, Thöny-Meyer L (2011) *Bacillus pumilus* laccase: a heat stable enzyme with a wide substrate spectrum. BMC Biotechnol 11:9. doi:10.1186/1472-6750-11-9
41. Mukherjee S, Das P, Sivapathasekaran C, Sen R (2009) Antimicrobial biosurfactants from marine *Bacillus circulans*: extracellular synthesis and purification. Lett Appl Microbiol 48:281–288
42. Parente E, Ricciardi A (1994) Influence of pH on the production of enterocin 1146 during batch fermentation. Lett Appl Microbiol 19:12–15
43. De Vuyst L, Callewaert R, Crabbe K (1996) Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiology 142:817–827
44. Smitha S, Bhat SG (2013) Thermostable Bacteriocin BL8 from *Bacillus licheniformis* isolated from marine sediment. J Appl Microbiol 114:688–694
45. Naclerio G, Ricca E, Sacco M, De Felice M (1993) Antimicrobial activity of a newly identified bacteriocin of *Bacillus cereus*. Appl Environ Microbiol 59:4313–4316

MedCrave
Step into the World of Research

Journal of Bacteriology & Mycology: Open Access

Marine Bacteriocins: A Review

Abstract

Marine environment is entirely different from terrestrial environment and exploration of resources of marine origin is of perpetual interest to scientists. In this review we have attempted to concisely present a general idea of bacteriocins. This also includes a review on bacteriocins of marine origin, their use in marine aquaculture and sea food industry.

Keywords: Bacteriocins; Marine; Probiotics; Classification

Mini Review

Volume 2 Issue 5 - 2016

Bindiya ES and Sarita G Bhat*

Department of Biotechnology, Cochin University of Science and Technology, India

*Corresponding author: Sarita G Bhat, Department of Biotechnology, Cochin University of Science and Technology, India, Email: saritagbhat@gmail.com

Received: July 20, 2016 | Published: October 25, 2016

Introduction

Like all organisms in nature, bacteria too have their own immune system and defense mechanisms. The antagonistic factors like antibiotics, bacteriocins, lysozymes, siderophores, proteases, and/or hydrogen peroxide and the alteration of pH values by organic acids produced either singly or in combination act as defense substances. Bacteriocins are potent antimicrobial peptides and proteins, found in almost every bacterial species examined till date, and within a species tens or even hundreds of different kinds of bacteriocins are produced [1].

The three types of cells in a microbial community are, bacteriocinogenic (produce bacteriocin), sensitive, or resistant to each bacteriocin. Thus in marine environments, all three cell types compete with each other for limited resources, with only a small percentage of bacteriocinogenic cells induced to produce and release bacteriocin. While some sensitive cells are killed immediately by the bacteriocin, others harbor mutations that impart resistance. These resistant cells rapidly displace the producer cells. In contrast to traditional antibiotics that are used in human health applications, bacteriocins mostly target members of the producer species and their closest relatives [2]. Hence they are classically considered to be narrow spectrum antibiotics. *Halobacteria* and *archaea* too produce their own version of bacteriocins, the halocins [3]. Some bacteriocins are capable of inhibiting archaea [4], but there is no confirmed inhibition of bacteria by a halocin, although there are reports that halophilic archaea are capable of inhibiting halophilic bacteria.

Bacteriocin was first discovered by Gratia in 1925 [5], during his search for ways to kill bacteria. He named it a colicine because it killed *E. coli*. The term bacteriocin was coined by Jacob and coworkers in 1953 [6], which paved the way for the development of microbial antibiotics and the discovery of bacteriophages, all within the span of a few years. High-throughput sequencing technologies reveal that bacterial diversity is larger than expected in marine microbial ecology and contains an extremely large number of microbial genes of unknown function [7]. Nevertheless, only a few bacteriocins and bacteriocin- like- substances have been described from marine bacteria. In the limited knowledge of marine bacterial biodiversity and the urgent requirement for antibiotic alternatives, the marine bacteriocin research is an open alternative in the near future.

Discussion

Bacteriocin definition

Bacteriocins are ribosomally synthesized proteinaceous compounds, lethal to closely related species of producing bacteria, the latter being protected by self immunity. These toxins play a critical role in mediating microbial population or community interactions. Bacteriocins may serve as anti-competitors enabling the invasion of a strain into an established microbial community or act as communication molecules in bacterial consortia like biofilms. i.e., they play a defensive role and act to prohibit the invasion of other strains or species into an occupied niche or limit the advance of neighboring cells [8]. An additional role proposed by Miller & Bassler [9] for Gram-positive bacteriocins is in quorum sensing. Some bacterial species produce toxins which exhibit numerous bacteriocin-like features, but they are yet not fully characterized; such toxins are referred to as bacteriocin-like inhibitory substances, or BLIS. This review focuses on bacteriocins [10-14] and bacteriocin like substances [15-18] isolated from marine environment and marine food products [19,20].

A precise definition of the bacteriocins is obscure and futile. Conventional criteria for definition of bacteriocins were based on the characteristics of colicins. These criteria have been used in varying combinations and applied with different degrees of consistency and proof in defining other bacteriocins: (i) A narrow inhibitory spectrum of activity centered about the homologous species; (ii) a bactericidal mode of action; (iii) the presence of an essential, biologically active protein moiety; (iv) attachment to specific cell receptors; (v) plasmid-borne genetic determinants of bacteriocin production and of host cell bacteriocin immunity; (vi) production by lethal biosynthesis (i.e., commitment of the bacterium to produce a bacteriocin will ultimately lead to cell death) [21].