Strategies for control of bacterial biofilms using bioagents

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LAXMI M
Reg no: 4439

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

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DEPARTMENT OF BIOTECHNOLOGY
COCHIN UNIVERSITY OF SCIENCE AND TECHNOLOGY
COCHIN - 682 022, KERALA, INDIA.
Ph: 0484 – 257667 | Email: saritagbhat@gmail.com | Fax: 91-484-2576267, 2577595

Dr. Sarita G. Bhat
Professor

Date: 01/10/2015

CERTIFICATE

This is to certify that the research work presented in the thesis entitled **"Strategies for control of bacterial biofilms using bioagents"** is based on the original research work carried out by Ms. Laxmi M under my guidance and supervision at the Department of Biotechnology, Cochin University of Science and Technology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any degree.

DR. SARITA G BHAT
(Supervising Guide)

DECLARATION

I hereby declare that the thesis entitled **"Strategies for control of bacterial biofilms using bioagents"** 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, associateship or other similar titles or recognition.

Cochin-22 **Laxmi M**

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ABBREVIATIONS

%	-	Percentage
°C	-	Degree Celsius
A_{570}	-	Absorbance at 570 nm
ABC complex	-	ATP Binding Cassette protein complex
Acyl – HSL	-	Acyl-Homoserinelactones
AI	-	AutoInducer
AMPs	-	Antimicrobial peptides
ANOVA	-	Analysis of Variance
AOC	-	Assimilable Organic Carbon
APS	-	Ammonium persulfate
BCA	-	Bio Control Agent
BHI	-	Brain Heart Infusion broth
BIC	-	Biofilm Inhibitory Concentration
BLAST	-	Basic Local Alignment Search Tool
Bp/bp	-	Base pair
CAD	-	Canadian Dollars
CDC	-	Centre for Disease Control
CFU	-	Colony Forming Units
CGA	-	Chlorogenic acids
CIP	-	Clean- In - Place
CLSM	-	Confocal Laser Scanning Microscopy
Cm/cm	-	Centimeter
CRA	-	Congo Red Agar
CTAB	-	Cetyl-trimethyl ammonium bromide
Da	-	Dalton
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotide triphosphate
DPPH-2	-	2-diphenyl-1-picrylhydrazyl
DTT	-	Dithiothreitol
DW	-	Distilled water
EDTA	-	Ethylene diamine tetra acetic acid
EFSA	-	European Food Safety Authority

EGTA	-	N,N,N9,N9-tetracetic acid
EPA	-	Environment Protection Agency
EPS	-	Extracellular Polymeric Substances
ESEM	-	Environmental Scanning Electron Microscopy
EtBr	-	Ethidium bromide
Fig	-	Figure
FTIR	-	Fourier Transform Infra Red
g	-	Grams
GRAS	-	Generally Recognized As Safe
h	-	Hours
HCl	-	Hydrochloric acid
H_2O_2	-	Hydrogen peroxide
ICTV	-	International Committee on Taxonomy of Viruses
i.e.	-	that is
kb	-	Kilobase
kDa	-	Kilo Dalton
L	-	Litre
LAB	-	Lactic acid bacteria
LB	-	Luria Bertani
Log	-	Logarithm
M	-	Molar
m	-	Meter
MAR	-	Multiple Antibiotic Resistance
MDR	-	Multi Drug Resistant
mg	-	Milligram
MIC	-	Minimum Inhibitory Concentration
Min	-	Minutes
mL	-	Millilitre
mm	-	Millimeter
mM	-	Millimolar
MRSA	-	Methicillin resistant *Staphylococcus aureus*
MSM	-	Minimal Salts Medium
MTT-3	-	(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
N	-	Normality

NA	-	Nutrient agar
NaCl	-	Sodium chloride
NaClO	-	Sodium hypochlorite
NaOH	-	Sodium hydroxide
NB	-	Nutrient Broth
NCBI	-	National Center for Biotechnology Information
NCCH	-	National Centre for Cell Science
NCIM	-	National Collection of Industrial Microorganisms
ng	-	Nanogram
NMR	-	Nuclear Magnetic Resonance
No.	-	Number
OD	-	Optical density
OD_{600}	-	Absorbance at 600nm
PACs	-	Pro AnthoCyanidins
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
PEG	-	Polyethylene glycol
PFU	-	Plaque Forming Units
pH	-	Power of Hydrogen
PPAS	-	Proteose peptone ammonium salt medium
QPS	-	Qualified presumption of safety
QS	-	Quorum Sensing
RGB	-	Red Green Blue
RLs	-	Rhamnolipids
RNA	-	Ribonucleic acid
Rpm	-	Revolutions per minute
rRNA	-	Ribosomal RNA
RTE	-	Ready -to -Eat
s	-	Seconds
SEM	-	Scanning Electron Microscopy
SDS	-	Sodium dodecyl sulphate
sp. /spp	-	Species
STAMPS	-	Specifically targeted Antimicrobial Peptides

TAE	-	Tris-acetate-EDTA
TCP	-	Tissue Culture Plate
TE	-	Tris-EDTA
TEM	-	Transmission Electron Microscopy
TEMED	-	N-N-N'-N'-Tetramethyl ethylene diamine
TSB	-	Tryptone soya broth
TTC	-	2,3,5-triphenyltetrazolium chloride
US EPA	-	United States Environment Protection Agency
UV	-	Ultraviolet
UV-VIS	-	Ultraviolet-Visible
V	-	Volts
v/v	-	Volume/volume
viz.	-	Namely
WHO	-	World Health Organization
w/v	-	Weight/volume
µg	-	Microgram
µL	-	Microlitre
µM	-	Micromole
µm	-	Micrometer

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CHAPTER 1

INTRODUCTION

Microorganisms are often viewed as simple life forms when compared with "higher" organisms. The study of microbial development however, has shown that microorganisms are capable of complex differentiation and behaviors; mostly working as communities rather than as individuals. Biofilms are defined simply and broadly as communities of microorganisms attached to a surface.

The discovery of biofilms was credited to Anton van Leeuwenhoek who discovered microbial attachment on his own tooth surface (Kokare *et al.,* 2009). Biofilms as they occur in nature consist primarily of viable and nonviable microorganisms embedded in polyanionic extracellular polymeric substances anchored to a surface (Wimpenny, 2000). Extracellular polymeric substances (EPS) may contain polysaccharides, proteins, phospholipids, teichoic and nucleic acids, and other polymeric substances hydrated to 85 to 95% water (Sutherland,1999). EPS provide protection to the biofilm inhabitants by concentrating nutrients, preventing access of biocides, sequestering metals and toxins, and preventing desiccation (Carpentier and Cerf, 1993). The ability of many bacteria to adhere to surfaces and to form biofilms has major implications in a diversity of industries including the food industry, where biofilms create a persistent source of contamination. Food industry biofilms in addition may also have high food residue and mineral content originating from product and process water. These constituents also provide protection to microorganisms held within the biofilm (Chmielewski and Frank, 2003).

Biofilms may also be considered as 'The city of microbes'. There are several steps that must be taken to optimize lives in a city. The first is to choose the city in which to live, select the neighborhood in the city that best suits our needs, and finally make our home amongst the homes of many others. Occasionally, when life in the city sours, we leave. The same steps occur in the formation of a bacterial biofilm. First, the bacterium approaches the surface so closely that motility is slowed. The bacterium may then form a transient

association with the surface and other microbes previously attached to the surface. This transient association allows it to search for a place to settle down. When the bacterium forms a stable association as a member of a microcolony, it has chosen the neighborhood to live in. Finally, the buildings go up and a three-dimensional biofilm is erected. Occasionally, the biofilm-associated bacteria detach from the biofilm matrix. Thus, in addition to fixed cells, there are motile cells that maintain their association with the biofilm for long period of time, swimming between pillars of biofilm-associated bacteria. The biofilm, therefore, demonstrates a level of activity similar to that of a bustling city (Watnick and Kotler, 2000).

Biofilms comprising of single or multiple microbial species can form on a range of biotic and abiotic surfaces. Although mixed-species biofilms predominate in most environments, single-species biofilms exist in a variety of infections and on the surface of medical implants (Dickinson and Bisno, 1993). These single species biofilms are the focus of most current research. *Pseudomonas aeruginosa* has emerged as the most studied single-species biofilm-forming Gram negative bacterium. The Gram positive biofilm forming bacteria that have been mostly studied include *Staphylococcus epidermidis*, *Staphylococcus aureus*, and the enterococci.

Biofilms consist of microorganisms and their self-produced extracellular polymeric substances (EPS). A fully developed biofilm contains many layers including a matrix of EPS with vertical structures, and a conditioning film. Vertical structures of microorganisms sometimes take the form of towers or mushrooms which are separated by interstitial spaces. Interstitial spaces allow the bulk of the biofilm to easily and rapidly take in nutrients from the surrounding liquid and move byproducts away from the biofilm (Percival *et al.,* 2011). Formation of biofilms are rather complex, but can be generalized in four basic steps: deposition of the conditioning film, microbial (planktonic) attachment to the conditioning film, growth and bacterial colonization and finally biofilm formation followed by dispersion (Deb *et al.,* 2014).

Multiple studies have shown that during the course of time when a biofilm is being created, the pathogens inside can communicate with each other,

by a phenomenon called quorum sensing. Although the mechanisms behind quorum sensing are not fully understood, the phenomenon allows a single celled bacterium to perceive how many other bacteria are in close proximity. If a bacterium can sense that it is surrounded by a dense population of other pathogens, it is more inclined to join them and contribute to the formation of a biofilm (Singh *et al.,* 2000). Bacteria which engage in quorum sensing communicate their presence by emitting chemical messages that their fellow infectious agents are able to recognize. When the messages grow strong enough, the bacteria respond en masse. Quorum sensing can occur within a single bacterial species as well as between diverse species. It can regulate a host of different processes, essentially serving as a simple communication network. A variety of different molecules can act as signals (Hall-Stoodley *et al.,* 2004). Biofilm bacteria can move in numerous ways: Collectively, by rippling or rolling across a surface, or by detaching in clumps. Individually, through a "swarming and seeding" dispersal whereby a biofilm colony differentiates to form an outer "wall" of stationary bacteria, while the inner region of the biofilm "liquefies", allowing planktonic cells to "swim" out of the biofilm and leave behind a hollow mound (Hall-Stoodley *et al.,* 2005).

The beneficial aspects of biofilms are many: (1) They can act as pollutant monitors-biodegrade toxic compounds, (2) They are natural forms of immobilization, that increase the ability of fermentation, (3) There is application in the field of industrial production. e.g.: acetic acid, ethanol, polysaccharides, (4) they are part of gut flora and thus have probiotic effect. The adverse effects are: (1) They are harmful if found in water distribution systems (2)Reduces permeability of membranes in filtration units, (3) Cause corrosion of metal surfaces eg: sulphate reducing or acid producing bacteria (4) Biofouling has an economic impact on the marine and naval transport and eventually the food industry (Kokare *et al.,* 2009)..

Different pathogenic mechanisms of the biofilms have been proposed (Hall-Stoodley *et al.,* 2006; Ward *et al.,* 1992; Sritharan and Sritharan, 2004). These include: attachment to a solid surface; "Division of labor" thereby increasing metabolic efficiency of the community; evading host defenses such as phagocytosis; a repository of high density of microorganisms; exchange of genes

that can result in emergence of more virulent strains of microorganisms; production of large concentration of toxins; protection from antimicrobial agents; detachment of microbial aggregates thereby transmitting microorganisms to other sites.

Biofilms are found to have immense impact in the infections through medical devices, deterioration of water quality and in the contamination of food industry. Bacterial biofilms are now commonly recognized as problematic for food industry. Recent years have showed that the scientific interest in biofilms has undoubtedly elevated bringing valuable information about the biofilm mode of existence by bacteria. The more we learn about the biofilm formation, the more we understand about the forces that holds the biofilm cells than planktonic cells. Certainly, microscopic techniques can be incorporated in most of experimental conditions concerning biofilms. Microscopic approaches are very useful not just to understand biofilm formation, but to study the efficacy of antimicrobials against biofilms. The understanding of bacterial attachment to solid surfaces and factors which influence this process, such as stainless steel, may help in the development of surfaces with reduced attachment for cells. Besides the development of effective sanitation procedures in food processing units also helps in reducing the potential contamination of foods, can be also achieved. Microscopic techniques can also allow locating viable cells in respect to different physiological functions within a food tissue, in order to assess the risk of food contamination and indicate factors which influence bacterial attachment to products. Further development of different techniques can support inspections for biofilm contaminants occurring on food processing surfaces in order to ensure food quality and safety (Olszewska, 2013).

Bacterial biofilms are problematic for several food industry branches, including dairy processing, poultry and red meat processing, brewing, fresh produce (Simoes *et al.,* 2010), as they may pose a risk of food contamination and transmission of foodborne pathogens (Lindsay and von Holy, 2006; Shi and Zhu, 2009). A consequence of biofilm existence is that it may lead to food process perturbations and technological problems that are difficult to control (De Araujo *et*

al., 2011). Bacterial biofilms are difficult to eliminate from food processing environments, which makes biofilm control a big challenge in this industry (Simoes *et al.,* 2010). The emergence of resistant cells within the biofilm clearly shows the need of novel approaches for bio control. In order to select suitable antimicrobials and adjust the dosing, it is crucial to examine and compare the antimicrobials' behavior on biofilms, primarily on carefully selected 'persister' cells. The combination of several antimicrobials may be a strategy to improve biofilm control efficiency and this strategy has to be comprehensively studied. In this background, attention should be focused on better understanding of the interaction between different antimicrobial agents and persisting biofilm cells. The susceptibility of these elusive cells in biofilms to antimicrobials, especially to non-antibiotic agents is not well-understood and has yet to be resolved (Olszewska, 2013). The recognition of spoilage or pathogenic bacteria on food-contact surfaces as they build up and thereby form biofilms is an important area of focus towards their elimination from food processing environments.

The presence and persistence of biofilm on food processing surfaces may pose a risk of food spoilage or food poisoning that has been a cause for great public concern . A better understanding of bacterial adhesion and resistance of biofilms is needed to ensure microbiological quality and safety of food products. The discovery of new biofilm control strategies, following the specifications required by the food industry, the use of biologicals-based solutions with high antimicrobial activity and specificity, seems to be a rational step ahead in overcoming the biofilm resistance issue (Chari *et al.,* 2014).

The present study was focused on applying different strategies for the biocontrol of bacterial biofilms. These involve use of bioactive antimicrobial compounds, namely pyocyanin, rhamnolipids, melanin and bacteriocin, and the use of bacteriophages in controlling biofilm produced by food borne pathogens originating from certain common foods. The foods were sourced from the local markets in and around Kochi, Kerala.

Objectives of the study:

1. To screen for food pathogens originating from certain common foods, sourced from the local markets and to test their biofilm forming capability.
2. Characterization of the strong biofilm producers based on
    - 16S rRNA based identification
    - Antibiogram.
    - Exoenzyme profile
3. Isolation, purification and partial characterization of pyocyanin and rhamnolipids from *Pseudomonas aeruginosa* BTRY1.
4. Biocontrol of biofilm by different biomolecules - pyocyanin, rhamnolipids, melanin and bacteriocin
5. Isolation, purification and characterization of bacteriophages.
6. Biofilm mitigation using bacteriophages.

Chapter 2

Review of Literature

Biofilms are microbially derived sessile communities characterized by numerous cells attached to an abiotic or living surface, and embedded in matrix of extracellular polymeric substances. Biofilm formation has been reported in the fossil record (~3.25 billion years ago) (Maric and Vranes, 2007). The first published report of biofilms in 1943 was made by Zobell, using buried slide culture method to obtain an attachment of microorganisms (Kokare *et al,* 2009).

Until the 1920s, the concept of bacterial biofilms was not formulated. Angst (1923) showed that marine bacteria on hull surfaces of ships was higher in number than surrounding floating cells; and proposed that bacterial biofilms led to serious corrosion of these hulls. By 1980s, bacteria were observed on solid surfaces in many ecological environments including the waste water treatment systems, equipments used to manufacture vinegar, industrial water systems, tooth decay, urinary tract and also on other implanted medical devices (Zottola and Sasahara, 1994). These observations led to the development of new electronic techniques including scanning electron microscopy, transmission electron microscopy and laser scanning confocal microscopy.

Recent advances show that biofilms are structurally complex, dynamic systems with attributes of both multicellular organisms and are multifaceted ecosystems. The formation of biofilm represents a protected mode of growth allowing cells to survive in hostile environments, to disperse and colonize new niches (Stoodley *et al,* 2004). The most important feature of every biofilm formed is that they are highly resistant to antibiotics.

Biofilms are responsible for chronic bacterial infections, infections on the medical devices, deterioration of the water quality and the contamination of food. *This study is focused on the importance of biocontrol of the biofilms in food industry.*

The formation of a biofilm is a complex and dynamic process involving different steps (Costerton *et al.,* 1987; Melo *et al.,* 1992)

2.1. Steps in biofilm formation

2.1.1 Conditioning of a surface

Biofilm formation usually occurs on submerged surfaces in any environment, where bacteria are present. In food processing environments, bacteria along with organic and inorganic molecules including proteins from milk and meat etc gets adsorbed to the surface forming a conditioning film. The conditioning also alters the physico-chemical properties of the surface viz., surface free energy, changes in hydrophobicity and electrostatic charges affecting the subsequent sequence of microbial events.

2.1.2 Adhesion of cells

The second step in the biofilm formation is the attachment of microorganisms to the conditioned surface. This process may be active or passive, depending on the bacterial motility, transport diffusion or fluid dynamic forces from the surrounding environment. The initial attachment of microorganisms is reversible in nature, due to the weak interactions like Vander Waals forces, electrostatic forces and hydrophobic interactions. The next crucial step is the irreversible attachment. In irreversible adhesion, the various short-range forces involved include the dipole-dipole interactions, hydrogen, ionic and covalent bonding, as well as hydrophobic interactions. In some cases, mechanical methods like scrubbing or scrapping can remove the attached cells at this point of time. The pH and temperature of the surface also influence the attachment in case of certain organisms. For eg: *Pseudomonas fragi* grows well at pH 7-8 on stainless steel surface and another pathogen *Yersinia enterocolitica* adhere firmly to the steel surface at 21°C (Herald and Zottola, 1988a).

2.1.3 Formation of microcolony

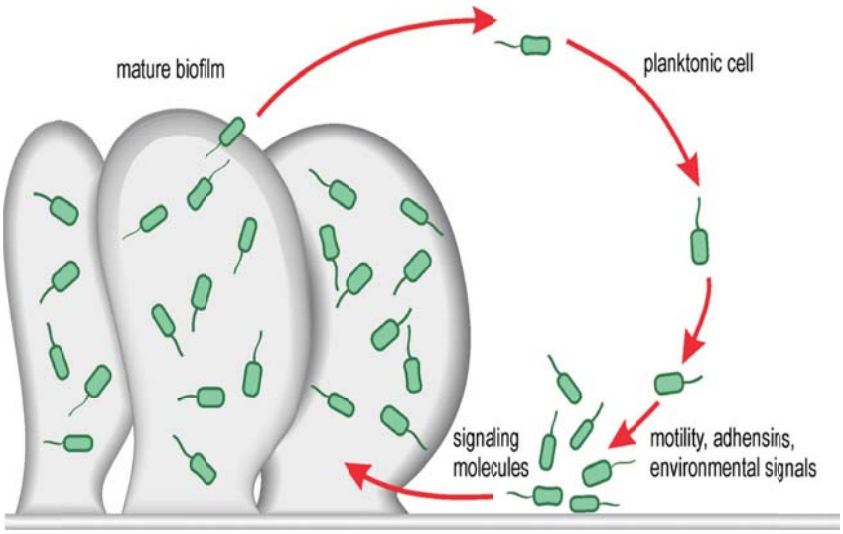
The irreversibly attached bacterial cells grow and divide by obtaining the nutrients present in the conditioned film and the surrounding environment. This can lead to the formation of microcolonies that enlarge and coalesce to form a layer of cells covering the surface. During this period, they also produce additional polymers called extracellular polymeric substances (EPS), which help anchoring the cells to the surface, thereby stabilizing the colony from the environmental fluctuations (Characklis and Marshall, 1990).

Extracellular Polymeric Substances (EPS) - After the initial contact, the microbes start producing thin fibers that become thicker with time, leading to a biofilm matrix. It is also reported that within the biofilm matrix many other organic, inorganic substances, and particulate matter get entrapped along with the microbial products and other microorganisms, linking together to form a consortium protected by the glycocalyx (Bryers, 1984). Glycocalyx is an integral element of the outer membrane of the Gram negative cells and the peptidoglycan of the Gram positive cells. This is known either as slime or capsule, and is composed of fibrous polysaccharides or globular glycoproteins (Costerton *et al*., 1978)

Terms like glycocalyx, slime, capsule and sheath have often been used to refer to the EPS associated with the biofilms (Geesey, 1982; Characklis and Cooksey, 1983). In case of *P. aeruginosa,* alginate forms the major constituent of the glycocalyx and is important for the development of monospecies biofilms (Boyd and Chakrabarty, 1995). The EPS produced by the microorganisms play an important role in initial adhesion, as well as firm anchorage of bacteria to solid surfaces (Marshall, 1992). It can protect bacteria from dehydration, as it can retain water several times its own mass and only slowly become desiccated (Roberson and Firestone, 1992; Ophir and Gutnick, 1994). For example, in *P.aeruginosa,* the presence of acetylated uronic acids in the bacterial alginate increases its hydration capacity. In addition, the biofilm polysaccharides are critical for the persistence and survival in hostile environments (Rinker and Kelly, 1996). This also helps in trapping and retaining nutrients for biofilm growth and protecting cells from antimicrobial agents.

2.1.4 Biofilm formation

The continuous attachment of bacterial cells to the surface and its subsequent growth along with associated EPS production forms biofilm. It is a slow process and the composition of biofilms is highly heterogenous due to the colonization of different microorganisms possessing different nutritional requirements (Fig.2.1)



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2.1.4.1. Effect of interspecies microbial interactions

The interactions of various microbes during the initial stages of adhesion play a significant role in both structure and physiology of the biofilm. In nature, microbial interactions are complex and are often of mixed type, wherein more than one type of interaction occurs between species (Bull and Slater, 1982). In addition, the initial colonizing species may potentially encourage colonization of physiologically compatible species. While inhibiting attachment of others. The biofilm formed by the microbial communities are mostly mixed species biofilms, which are often thicker and more stable than mono species biofilms. The EPS mainly helps in the colonization of other organisms to surfaces. It is presumed that in mixed species biofilm, the EPS produced by one species may enhance the stability of other species within a biofilm and enable stable interactions between polymers of different species (McEldowney and Fletcher, 1987).

In a study, Sasahara and Zottola (1993) observed an extended biofilm formation by *Listeria monocytogenes* in association with a primary a colonizing organism, *Pseudomonas fragi*, than with when either is grown individually.

Intercellular communication of bacterial cells is provided by extracellular signalling molecules called autoinductors. Accumulation of signalling molecules in the medium enables each and every single bacterial cell to estimate the total

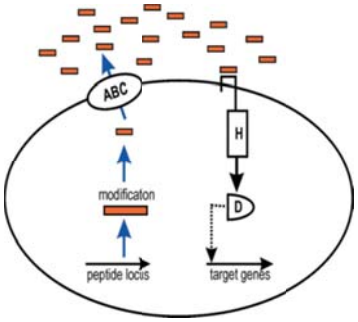
number of bacteria, i.e., cell density.this phenomenon is known as *quorum sensing* (Waters and Bassler, 2005).

2.1.4.2. Molecular basis of Biofilm formation

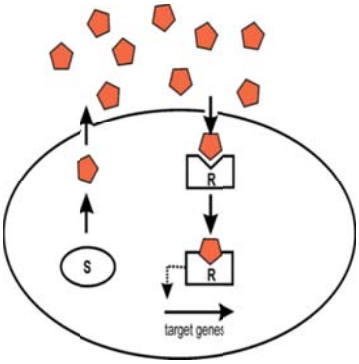
Signalling molecules in Gram-negative bacteria are non-essential amino acids called acyl-homoserine lactones (acyl-HSL) Synthetized acyl-HSL produce acyl-HSL molecules that diffuse through the cell membrane and gradually get accumulated in the medium. When the concentration of signalling molecules in the medium becomes high, they enter the cell and bind to the HSL receptor. A complex consisting of a signalling molecule and a receptor bind to suitable target genes and activates transcription. Gram-positive bacteria commonly use oligopeptides as their signalling molecules. Protein complex ABC transports the oligopeptides out of the cell into intercellular space. At sufficiently high concentrations of autoinducers in the medium, the signal is sensed by a protein system consisting of protein kinase and a regulatory protein. After binding the signalling molecules, this kinase becomes activated and phosphorylates. The activated protein kinase activates the regulatory protein which thereafter binds to specific target genes and activates their transcription (Faqua and Winans, 1996) (Fig 2.2 (a).

Davies *et al*., (1998) first reported the role for quorum sensing in the formation of biofilms, and also launched a period of active research of cell-to-cell signaling in biofilms. He showed that *lasI*- mutant cells of *Pseudomonas aeruginosa* that were unable to synthesize 3OC12-HSL (3-oxododecanoylhomoserine lactone) were able to attach and initiate the biofilm formation similar to that of wild type cells, but the mature biofilms were continuous sheets lacking the differentiated architecture with microcolonies and water channels. The biofilms were also sensitive to SDS in contrast to the wild type biofilms. When 3OC12-HSL was added the mutant cell formed biofilms that resisted detergent wash, the architecture was noted to be similar to that of wild type biofilms. Similarly, biofilm development of *Aeromonas hydrophila* and *Burkholderia cepacia* also involved AHL-mediated signalling (Lynch *et al*., 2002). The addition of 7,8-cis-tetradecenoyl-HSL to aggregates of *Rhodobacter sphaeroides* mutant cells caused the cells to disperse and grow as individual cells in suspension (Greenberg, 1999). Similarly, AHLs and/or another factor present in stationary-phase culture

supernatants mediated a reduction of *P. fluorescens* biofilm and loss of EPS (O'Toole *et al*., 2000). These studies suggest that AHL signals may be involved in biofilm dispersal. In mixed-species biofilms AHLs have been shown to mediate interspecies communication (Riedel *et al*., 2001) (2. 2 (b))



2.2 (a) Mechanism of quorum sensing in Gram-positive bacteria. ***ABC*** *= transporter protein complex,* ***H*** *= histidin kinase,* ***D*** *=regulator protein*



2.2 (b) Mechanism of quorum sensing in Gram-negative bacteria. *pentagons = acylated homoserine lactone (acyl-HSL),* ***S*** *= acyl- HSL synthases,* ***R****= acyl-HSL binding protein*

Fig .2.2. (a) & (b): Fig adapted from Maric and Vranes, 2007

In addition to quorum-sensing molecules, a variety of other signals also trigger biofilm formation. These include secondary metabolites like antibiotics, pigments, and siderophores. For eg: It is found that sub-inhibitory concentrations of the antibiotic Imipenem induced expression of the polysaccharide alginate in *Pseudomonas aeruginosa* biofilms (Bagge *et al*., 2004). Similarly, sub-inhibitory concentrations of the aminoglycoside antibiotic tobramycin, induced biofilm formation not only in *P. aeruginosa* but also in *E. coli* (Hoffman *et al*., 2005). However, the mechanism of this signaling process is not understood

In the case of *S. aureus,* the activation of quorum-sensing system inhibits biofilm formation. Thus, small molecules that inhibit quorum sensing also favor biofilm formation. This was recently described for the furanones, which are natural products derived from marine algae (de Nys *et al*., 2006), and able to inhibit the quorum-sensing systems of many Gram-negative bacteria (Wu *et al*., 2004) Australian macroalga called *Delisea pulchra* produced halogenated

furanone compounds that interfere with the AHL-mediated quorum sensing, and in this way protect the macroalga from bacterial fouling (Hentzer *et al*., 2002). Also, addition of a synthetic furanone compound made *P. aeruginosa* biofilms thinner and less virulent, and enhanced bacterial detachment. Thus furanones are attractive candidates for biofilm control in the future.

A large number of bacteria have a common quorum sensing system mediated by autoinducer 2 (AI-2), which is present in both Gram-negative and Gram-positive bacteria. AI-2 is a product of the enzyme, LuxS, which is involved in the activated methyl cycle or AMC pathway and generates S-adenosyl methionine, the major methyl donor (Vendeville *et al.,* 2005). Toxic S-ribosyl homocysteine is produced as part of the AMC pathway. One of the roles of LuxS is to detoxify *S-ribosylhomocysteine* by forming 4, 5−*dihydroxy*-2, 3-*pentanedione* (DPD) and *homocysteine*. The DPD cyclizes with boron to form AI-2. AI-2 can be considered as a byproduct of the AMC cycle (McDougald *et al.,* 2007). LuxS therefore has a role in quorum sensing as well as in cellular metabolism. Boron-containing AI-2 was reported to be involved in bioluminescence of *Vibrio harveyi*. On the other hand, AI-2 in *S. enterica* serovar Typhimurium and *E. coli* do not contain boron.

The dual role of LuxS makes it necessary to separate the metabolic role of the enzyme from the quorum sensing activity of AI-2, a product of LuxS action (Doherty *et al.,* 2006). Certain bacterial phenotypes may be due to metabolic defects owing to the loss of LuxS function in the activated methyl cycle, rather than due to a defect in signaling. Thus for a proper study of the effects of LuxS and AI-2, experiments must include complementation with both luxS gene and purified AI-2. Such procedures will separate the effects of AI-2 as a quorum sensing compound from metabolic effects under the control of the luxS gene (Hardie *et al.,* 2003).

Certain foodborne enteric pathogens such as *E. coli, Shigella, Salmonella, Yersinia*, and other Gram-negative bacterial species have the autoinducer-3/epinephrine/norepinephrine (AI-3/epi/norepi) signaling system (Walters and Sperandio, 2006). Epinephrine and norepinephrine which are both mammalian hormones, cross talk with AI-3 and are recognized by the same receptor(s). So there may be quorum sensing systems through which host cells communicate with bacteria (Sperandio *et al.,* 2002). AI-3 is chemically distinct from AI-2, and AI-3

synthesis is not dependent on luxS (Walters *et al.,* 2006). The AI-3/epi/norepi system has an important role in the virulence of *E. coli*O157:H7 (Sperandio *et al.,* 2002). AI-3 activates transcription of the genes found on the locus of enterocyte effacement (LEE) chromosomal pathogenicity island in enterohemorrhagic *E. coli*. Other types of signaling molecules have also been described, and these include indole; 3,4-dihydroxy-2-heptylquinolone (PQS); butyrolactones; 3-hydroxy palmytic acid methyl ester (3OH PAME); and cyclic dipeptides (Yang *et al.,* 2007).

Salmonella, E. coli, Shigella, and *Klebsiella* do not possess members of the luxI family and thus do not produce AHLs. These organisms carry sdiA (suppressor of cell division inhibition), a LuxR homologue; thus they can detect AHLs produced by other bacterial species (Michael *et al.,* 2001). Several genes are regulated by sdiA in *Salmonella*, including rck found on the virulence plasmid and involved in resistance to human complement (Ahmer, 2004). In *E. coli,* the sdiA gene cloned on a multicopy plasmid upregulated expression of genes involved in cell division, ftsQAZ, and in enterohemorrhagic *E. coli*, overexpression of sdiA caused abnormal cell division and reduced adherence to epithelial cells and expression of the intimin adherence protein (Karnetova *et al.,* 2000). The 5- to 13-fold upregulation of ftsQAZ was noted when SdiA was overexpressed on a multicopy plasmid, but sdiA was only slightly activated when expressed as a single copy on the chromosome compared to an sdiA mutant. The sdiA mutant did not show notable defects in cell division. Overexpressed SdiA positively regulated the multidrug resistance pump AcrAB, and it was suggested that AcrAB may play a role in the export of quorum sensing molecules (Rahmati *et al*., 2002). The amino acid identity shared by *E. coli* and *Salmonella* sdiA is only 69%. Indole is formed from tryptophan by the tryptophanase enzyme and is secreted in large quantities by *E. coli* during growth in rich medium. It can also act as a signaling molecule in *E. coli* and *Salmonella*, regulating the expression of a number of genes. It may have a role in adaptation of bacterial cells to nutrient-poor environment in which amino acid catabolism is an important energy source. Using *E. coli* with mutations in genes that control indole synthesis, Lee *et al.,* (2007) showed that indole controls biofilm formation by repressing motility, inducing SdiA, and influencing acid resistance. They found that indole signaling decreased biofilm formation in *E. coli* while it was increased in Pseudomonads.

Indole and AHLs are signals in *E. coli* biofilm formation, and the mechanism of inhibition of motility and biofilm formation in *E. coli* was through SdiA.

There is evidence that in many bacteria biofilm formation is a carefully orchestrated process controlled by quorum sensing. The use of bacterial strains with mutations in genes involved in the production of signaling molecules and the analysis of temporal differential gene expression in biofilms are revealing information on the molecular mechanisms of biofilm formation and the role of quorum sensing. While most research supports the role of quorum sensing in biofilm formation and in the resulting characteristics of the biofilm community, other studies indicate that it does not affect biofilms formation. Moreover, knowledge of the chemical structures of different types of signaling molecules allows identification of compounds that can modulate quorum sensing-related processes, including biofilm formation. Additional research is needed to understand how quorum sensing works mechanistically in biofilms and how cell-to-cell signaling may influence the virulence and antimicrobial resistance of biofilm communities. This information is important to identify possible targets and to design strategies that control biofilm formation on industrial, medical, and food and food processing surfaces.

2.1.5 Detachment and dispersal of biofilms

As the biofilm ages, the attached bacteria for its survival and colonization of new niches, must be able to detach and disperse from the biofilm. Sloughing is a discrete process where the periodic detachment of relatively large particles of biomass from the biofilm occurs. This can be due to various factors including the stress responses, altered physicochemical properties of the surface and environmental fluctuations. The released bacteria may be transported to newer locations and enable restart of the biofilm formation. (Marshall, 1992).These are the important steps in biofilm formation.

2.2 Regulation of biofilm by genetic and environmental factors

Biofilm formation is regulated by different genetic and environmental factors. In the context of evolution and adaptation, it is likely that biofilms provide homeostasis in the face of fluctuating, harsh conditions of primitive earth (extreme temperatures, pH and exposure to ultraviolet (UV) light), thereby facilitating the

development of complex interactions between individual cells and providing an environment sufficient for the development of signalling pathways and chemotactic motility. Significant roles in biofilm formation are played by bacteria mobility, cell membrane proteins, extracellular polysaccharides and signalling molecules.

Bacterial mobility is enabled by two types of protein growths on the cell surface, flagella and fimbriae**.** In *Escherichia coli* and *Pseudomonas aeruginosa* both kinds of bacterial mobility are necessary for biofilm formation (Pratt and Kolter, 1998; O'Toole and Kolter, 1998). Stable connection between bacteria and substrate surface is maintained by specific cell membrane proteins called adhesins**.** If this activity is inhibited, there is no biofilm formation, which was proved in *E. coli* and *Vibrio cholerae* (Watnik and Kolter, 1999). In *Pseudomonas aeruginosa* activation of genes necessary for extracellular polysaccharide synthesis took place after establishing stable connection between bacteria and substrate surface (Davies *et al.*,1998) In *Staphylococcus epidermidis,* the bacteria lose ability to form biofilm if the genes responsible for EPS matrix synthesis are inactivated. Different signals from environment, such as availability of certain nutrients, presence of oxygen, temperature and pH, take part in regulation of a biofilm formation. Phosphates and sugars like mannose & trehalose effected biofilm formation in *Listeria monocytogenes* (Kim *et al*., 1995); while environmental pH was important for biofilm formation by*Vibrio cholera* (Hommais *et al*., 2002).

2.3. Roles of Biofilm in microbial community

In microbial communities biofilms play different roles (Kokare *et al.,* 2009):

1. Protection of microbes from the harsh environmental conditions.
2. Increase nutrient availability,
3. Acquisition of new genetic traits through horizontal gene transfer mechanisms inside the biofilm microbial communities,
4. Provision of barriers for the penetration of antimicrobial agent.

Different factors affect the biofilm formation, including substratum effect, conditioning of the surface, hydrodynamics, characteristics of aqueous medium, horizontal gene transfer and quorum sensing signals.

2.4. Biofilm resistance to antibiotics:

Biofilms are notoriously difficult to eradicate, are a source of many recalcitrant infections and therefore the cause of numerous chronic diseases (Lewis 2001).Three hypothesis described to date justify this type of resistance (Decho, 1990; Evans *et al.,* 1991)

1. Slow or incomplete diffusion of antibiotics into biofilm inner layers.
2. Based on changes that occur in biofilm microenvironment.
3. There is a subpopulation of cells within the biofilm whose differentiation resembles the process of spore formation.

The protective mechanisms at work in biofilms appear to be distinct from those responsible for conventional antibiotic resistance. In biofilms, poor antibiotic penetration, nutrient limitation, slow growth, adaptive stress responses, and formation of persister cells are hypothesized to constitute a multi-layered defense. The genetic and biochemical details of these biofilm defenses are only now beginning to emerge. Each gene and gene product contributing to this resistance may be a target for the development of new chemotherapeutic agents. Disabling biofilm resistance may enhance the ability of existing antibiotics to clear infections involving biofilms that are refractory to current treatments (Stewart 2002). The mechanisms of resistance in biofilms are different from the now familiar plasmids, transposons, and mutations that confer innate resistance to individual bacterial cells. In biofilms, resistance seems to depend on multicellular strategies (Stewart and Costerton, 2001).

Restricted penetration- Biofilms are enclosed within an exopolymeric matrix which restricts diffusion of substances and bind antimicrobials. This provides an effective resistance for biofilm cells against large molecules such as antimicrobial proteins. The diffusion barrier is probably effective against smaller peptides like the numerous defensins and their analogs. The negatively charged exopolysaccharide is very efficient in protecting cells from positively charged aminoglycoside antibiotics by restricting their permeation, mostly through binding (Ishida *et al.,* 1998). In most cases involving small antimicrobial molecules, the barrier of the polysaccharide matrix could only postpone the death of cells rather than afford useful protection. A case in point is fluoroquinolone antibiotics, which readily equilibrate across the biofilm (Watnik and Kolter, 1999) proving effective

in inhibiting biofilm. At the same time, restricted diffusion can protect the biofilm from a degradable antimicrobial agent. Retarded diffusion will decrease the concentration or the amount of the antibiotic entering the biofilm, helping an enzyme like β-lactamase to destroy the incoming antibiotic. This synergy between retarded diffusion and degradation provides a very effective resistance to *Pseudomonas aeruginosa* biofilms expressing a β-lactamase (Brooun *et al.,* 2000).

Decreased growth rate- Some antibiotics have an absolute requirement for cell growth in order to kill. Penicillin and ampicillin do not kill nongrowing cells at all, and the rate of killing is proportional to the rate of growth. Some of the advanced β-lactams, cephalosporins, aminoglycosides, and fluoroquinolones kill nongrowing cells, but they are distinctly more effective in killing rapidly dividing cells. Slow growth undoubtedly contributes to biofilm resistance (Hoyle and Costerton, 1991). Similarly, slow growth can be a major factor in the increased resistance of stationary planktonic cells.

Expression of possible biofilm-specific resistance genes- Multiple Drug Resistance (MDR) pumps play a role in biofilm resistance at low antibiotic concentrations and there is reason to believe that unknown MDR pumps might be overexpressed in certain biofilms (Maira *et al.,*2000). It can be hypothesized that a certain mechanism is specifically over expressed in a biofilm, until a broad range of conditions that planktonic cells grow under has been examined. The majority of cells in a biofilm may not necessarily be more resistant than planktonic cells, and hence die rapidly when treated with a cidal antibiotic that kill slow-growing cells. Persisters survive and are actually preserved by the presence of an antibiotic that inhibits their growth. Paradoxically, the antibiotic helps the persisters to persevere. The role of persisters in biofilms' resistance to killing has not been much studied, but numerous reports over the years show similar biphasic dose-dependent or time-dependent killing of planktonic microbial cells (Lewis, 2001). In *E. coli*, increasing concentrations of ciprofloxacin or imipenem caused an initial decrease in live cell number, while the remaining small population was essentially insensitive to further increase in drug concentration (Ashby *et al.,* 1994). This pattern was also observed with amoxicillin and clindamycin in *Lactobacillus acidophilus;* with erythromycin and metronidazole in *Gardnerella vaginalis* biofilms, in which initial rapid killing was followed by a plateau of resistant cells

(Stewart and Franklin, 2008). It is possible that biofilms produce more persisters than the planktonic populations. Increased number of persisters however is not the main factor responsible for the vastly better survival of biofilms than planktonic cells *in vivo.*

If the concentration of the antibiotic temporarily drops or if symptoms disappear due to the eradication of planktonic cells and therapy is discontinued, the persisters will reform the biofilm, which begin to shed off new planktonic cells. This dynamics explains the relapsing nature of biofilm infections and the need for a lengthy antibiotic therapy. This view of biofilm infection suggests, somewhat counter intuitively, that the recalcitrance of biofilms does not necessarily depend on their higher levels of intrinsic resistance to killing by antibiotics, rather than the level of intrinsic resistance of planktonic cells. Indeed, if a biofilm of a particular species under given conditions *in vivo* happens to be just as sensitive or even more sensitive to killing by the antibiotics than a planktonic population, it will still survive better than planktonic cells, since it is invulnerable to immune attack (Lewis, 2001).

2.5 Biofilms and pathogenesis

2.5.1 Biofilms, human body and device associated infections

According to the recent public announcement from the National Institutes of Health, more than 80% of all microbial infections are by biofilms. This seems high in such common infections as urinary tract infections (caused by *E. coli* and other pathogens), catheter infections (caused by *Staphylococcus aureus* and other gram-positive pathogens), child middle-ear infections (by *Haemophilus influenza, etc*), common dental plaque formation and gingivitis; all of which are caused by biofilms which are very hard to treat or that are frequently relapsing. The less common but certainly more threatening is the biofilm infections that cause serious morbidity and mortality. These include endocarditis due to *S. aureus*; infections due to permanent in- dwelling devices, such as joint prostheses and heart valves, also caused by *S. aureus*; and infections in cystic fibrosis patients caused by *P. aeruginosa* (Otto, 2008).

There are many microorganisms forming biofilms on the in-dwelling medical devices and include both Gram positive and Gram negative organisms.

Table 2.1. Common biofilms forming microorganisms on in-dwelling medical devices

Medical devices	**Causative organisms**
1. Urinary catheter, Intrauterine devices, prosthetic heart valves, central venous catheter	Coagulase negative staphylococci
2. Urinary catheter, central venous catheter	*Klebsiella pneumoniae* *Pseudomonas aeruginosa*
3. Artificial hip prosthesis, Urinary catheter, central venous catheter	*Candida albicans*
4. Artificial voice prosthesis, Urinary catheter, Intrauterine devices	*Staphylococcus aureus*
5. Artificial hip prosthesis, central venous catheter, Intrauterine devices, prosthetic heart valves	*Enterococcus spp*
6. Artificial hip prosthesis, Urinary catheter, prosthetic heart valves	

Table 2.1. Table adapted from Kokare *et al.,* 2009

Primary infections also occur in the presence of intravenous catheters, urinary catheters and implantable devices. Secondary infections from a biofilm source may affect brain, kidneys, joints and inter vertebral spaces. In cystic fibrosis, excess mucus production in the airways, hosts bacteria such as *Pseudomonas aeruginosa,* which mop up dead white blood cells from the immune system, enabling them to construct their protective biofilm coat.

There are different implants in different systems in human body where the infections are due to biofilm formation. There are different primary sites and secondary sites of infections. The primary sites include artificial hip implant, and the subvenous catheter, while the secondary sites of infections include brain, kidneys, hip, intervertebral spaces, to name a few ((Fig.2.3(a) and Fig 2.3 (b)) demonstrates the infections caused in humans due to formation of biofilms.

Biofilms and human body

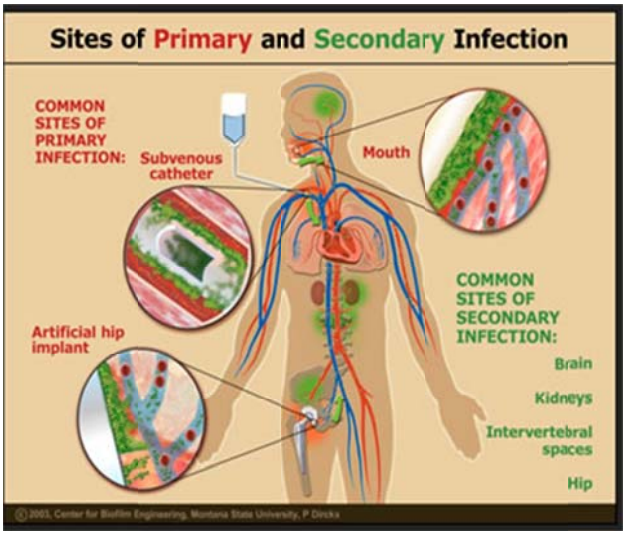
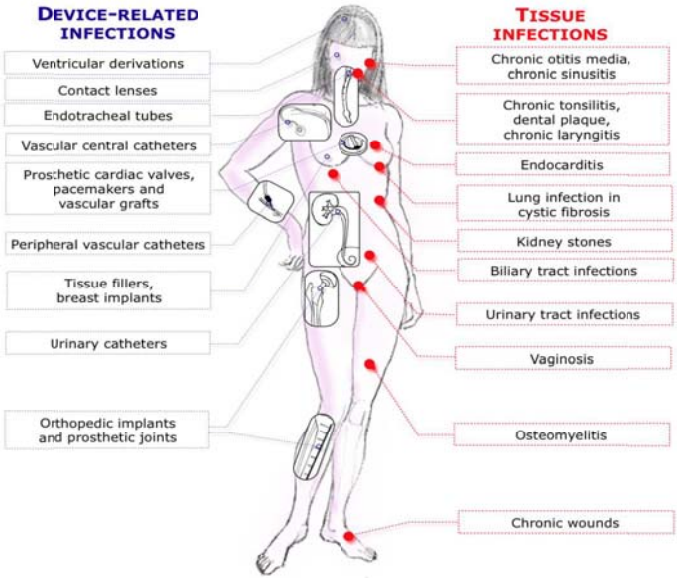


Fig 2.3.(a). Schematic showing three examples of possible points of entry into the body for infectious biofilms; catheter, hip replacement, and periodontal disease. Fig adapted from Stoodley *et al.,* 2004



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Biofilms are mostly involved in endocarditis, a rare but serious heart disease, in which one of the four heart valves, the heart lining, or heart muscle are infected by bacteria, usually the streptococci and become inflamed. The formation of an endocarditic plaque is unique, involving bacteria, platelets, coagulation

factors and leucocytes, and is considered a special kind of biofilm. Since the biofilm is much resistant to antibiotics and the immune system's white blood cells, often the only option is surgery to replace a damaged valve. Greater knowledge may allow new strategies to be developed that break up the biofilm.Chronic ear infections and tonsillitis in children have been mainly linked to biofilm formation as the causative agent. Other chronic biofilm infections include prostatitis, Legionnaire's disease and peritonitis (Lebeaux *et al.,* 2012).

A new endoscope does not contain biofilm but shortly after the first use, a conditioning film is created on the biomaterials of the endoscope. The film may be composed of the bodily fluids proteins, polysaccharides and other components. This alteration of the surface characteristic allows bacteria to commence growth and colonization.The initial stages of biofilm formation, i.e. surface conditioning from patient secretions, microbial attachment, growth and colonization, are very much similar to natural biofilm buildup. However, medical devices including gastrointestinal endoscopes are repeatedly used daily, with cyclic exposure to high levels of microbes, due to contact with the mucosal surface of the gut. In addition, each procedure reprocessing cycle involves scope exposure to hydrated phases, post patient cleaning and disinfection, as well as drying phases between procedures and during storage.

The data in different studies showed that a combination of an organic matrix and aldehyde (fixative) disinfection quickly produced a protective buildup biofilm that facilitated high levels of organism survival. A key finding was that once established, the microbial load in a buildup biofilm formed by glutaraldehyde exposure had a faster rate of accumulation than in a natural biofilm formation. However, if an oxidizing agent such as peracetic acid or AHP (accelerated hydrogen peroxide) was used for disinfection and if the organic levels were kept low, organism survival did not occur (Muscarella, 2010).

If initial biofilm is not removed, repeated instrument use can facilitate biofilm formation over time, with different layers of dried organic material with embedded microorganisms. Deep within the biofilm structure, organisms are protected from the disinfectant challenge, particularly from glutaraldehyde (Grobe and Stewart, 2000). This supports the current concerns regarding the exposure of low concentrations or activities of biocides to organisms embedded within biofilm and the selection of tolerant bacteria.

The results of buildup biofilm models indicate that high disinfection level is effective in killing bioburden within young biofilm, but not within a mature one. It also highlights the value of analysing biofilm formation in reprocessed scopes over extended periods of time. The buildup biofilm model demonstrated for the first time that although a longer time was needed for organisms to be detected within the biofilm, outgrowth of surviving bioburden was faster and the ultimate level achieved was greater. This provide a possible explanation for the published reports describing the persistence of residual levels of organisms in scope channels even when proper reprocessing is followed.

The findings suggest that biofilm is difficult to eliminate during endoscopic reprocessing. They also stress the importance of reducing bioburden during pre-cleaning and the imperative to maintain a contaminant-free, dry scope during storage. In an Australian study, the channels of thirteen endoscopes were examined using endoscope. Biofilm was present on the suction/biopsy channels of five out of the thirteen scopes. Biofilm was also present on the air/water channels of twelve scopes, with a level of contamination determined to be extensive on nine (Pajkos *et al.,* 2004). A better method to remove bioburden from these channels, either with more effective detergents or through changes in scope design and channel accessibility, would help to eliminate this risk factor.

Using proper procedures, an initial biofilm should be removed with manual pre-cleaning, brushing accessible channels, followed by high level disinfection and thorough drying. The drying step must take place between cases as well as at the end of the day. If the biofilm is not completely removed, it will continue to grow and develop through repeated cycles of use and cleaning. Research has shown that under minimal growth conditions, 67% of adherent *Pseudomonas aeruginosa* strains remained metabolically active (Lazar and Chifiriuc, 2010).

2.5.2. Impact of Biofilms on deterioration of water quality

The old misconception of free floating microbes is invalidated by a different knowledge pattern: the great majority of terrestrial microorganisms live in communities associated to surfaces termed to be biofilms (Costerton *et al.,* 1987; Wingender and Flemming, 2008). This organization mode is associated with all surfaces in contact with water in drinking water processing, storage and its

distribution. Such biofilms are mostly represented by structured consortia of sessile microorganisms characterized by surface attachment, self-produced exopolymeric matrix, structural, metabolic and functional heterogeneity, capable of intercellular communication by quorum-sensing and plurispecific composition. Biofouling in drinking and industrial water systems has many detrimental effects such as microbiological and chemical deterioration in water quality, corrosion induction, drinking water treatment yield loss, efficiency reductionin cooling and heat exchange and transport, as well as in membrane processes (Le Chevallier *et al.,*2004; Coetser and Cloete, 2005).

There are two important factors responsible for deterioration of water quality-

1. Introduction of bacteria from external sources (through open reservoirs or breakage of pipelines)
2. Bacterial number may increase due to internal regrowth(occurs due to the use of biodegradable compounds)

Formation of biofilm in water distribution system may also depend on piping material, temperature, type of disinfectants and the resistance of bacteria to the disinfectants. The resistance is mainly due to indiscriminate use of disinfectants-genes acquired by Horizontal gene transfer mechanisms (Momba *et al*., 2000). Pathogenic microorganisms can also emerge in drinking water systems by intrusion, due to external contamination in different steps of water treatment, storage and transportation like cross connections, backflow events, pipe breaks, negative pressure and because of improper flushing and disinfection procedures.

The most alarming consequences of biofouling in drinking water distribution systems is the presence, multiplication and dispersion into water of bacterial pathogens, opportunistic pathogens, parasitic protozoa, viruses and toxins releasing fungi and algae. They can also appear as primary colonizers promoting the adhesion at the interface and subsequent biofilm formation (Costerton *et al.,* 1994), but more often found as secondary colonizers in ecological microniches offered by the existent attached community.

Emerging pathogens are those that have appeared in a human population for the first time, or had occurred previously but are increasing in incidence or expanding to areas, where they have not previously been reported over the last 20 years. They include bacteria (pathogenic *E. coli, Helicobacter pylori, Campylobacter jejuni and Mycobacterium avium* complex), parasitic protozoa

(*Cryptosporidium spp., Cyclospora cayetanensis, Toxoplasma gonidii*), viruses (noroviruses, hepatitis E) and toxic cyanobacteria (Hunter, 2003). Opportunistic pathogens are commonly members of water microbiota that are normally harmless to healthy individuals but which can infect a compromised host (US EPA, 2002). In drinking water carefully treated and distributed at high standards, pathogenic contamination and disease outbreaks might occur (Wimpenny *et al.,* 2000; Wingender & Flemming, 2011) demonstrating the imperative requirement for comprehensive water safety plans implementation.

Some of the recommended strategies in drinking water associated biofilm control include source waters protection, appropriate treatment, infrastructure contamination prevention, reservoirs and pipes maintenance, corrosion control, appropriate disinfection practices, nutrient levels reducing, water quality monitoring, personnel training, water safety plans implementation. Having in mind the virtual idea of self-cleaning surfaces, researchers in nanotechnology field are targeting innovative repellent materials with a wide range of applications, for the biofouling control in water distribution systems. The super hydrophobicity models such as "the lotus effect" characterizing the lotus (*Nelumbo nucifera*) leaf, offered by natural patterns are investigated at a nanoscale. The interdependence between surface roughness, reduced particle adhesion and water repellence has been proved to be the keystone in the self-cleaning mechanism of many biological surfaces (Barthlott and Neinhuis, 1997).

Microbial communities in water networks and biofilms represent complex ecosystems; their ecology is influenced by a series of abiotic and biotic factors such as raw water sources quality, temperature, flow rate and system hydraulics, nutrient concentration, pipe material, particles accumulation, ingress and intrusion, water treatment, water disinfection and microbial interactions. Further research is required to understand attached microbial consortia for biofouling prevention and control in drinking water industry, as a matter of public security.

As with most areas, opportunities exist for research on the health impacts associated with drinking water distribution systems. For the better control of pathogen survival and growth in the biofilm and other public health problems associated with the biofilm in the distribution system, research in the link between organisms in distribution system biofilms and human health impacts, the effectiveness of potential indicators of extensive biofilm growth, including loss of

disinfectant residual, high AOC levels, pipe corrosion, and the presence of red or black water, identifying the potential problems created by cleaning deteriorated pipes, study on the level of public health protection provided by adding disinfectant residuals to the distribution system, are all necessary. Some specific research opportunities related to drinking water distribution systems are outlined in two reports being prepared for EPA as part of Comprehensive Drinking Water Research Strategy and the Microbial/Disinfection Byproducts (M/DBP) Research Council.

2.5.3Biofilms and food industry

Biofilms food-processing environments are of special importance since they have the potential to act as a persistent source of microbial contamination leading to food spoilage or transmission of diseases. Poor sanitation of food contact surfaces, equipment, and processing environments has been a contributing factor in many foodborne disease outbreaks, especially those involving *Listeria monocytogenes* and *Salmonella*. Improperly cleaned surfaces promotes oil buildup, and in the presence of water, contribute to the development of bacterial biofilms containing pathogenic microorganisms (Boulange-Peterman *et al.,* 1997).Cross contamination occurs when food passes over contaminated surfaces or via exposure to aerosols or condensate that originate from contaminated surfaces (Boulange-Peterman 1996; Bower and Daeschel, 1999). Type of food contact surface and topography plays a very significant role in the inability to decontaminate a surface (Frank and Chmielewski, 1997; Holah *et al.,* 1990). Abraded surfaces accumulate soil and are more difficult to clean than smoother surfaces. Surface defects provide protection against the removal of soil and bacteria (Mafu *et al.,* 1990), with the result that surviving bacteria can regrow and produce a biofilm.

Bacteria within a biofilm are more resistant to disinfectants, thereby assisting the survival of *Listeria spp.* and other food borne pathogens in food processing environment (Bower and Daeschel, 1999). Direct evidence that pathogen-containing biofilms play a role in the spread of foodborne illness is lacking, as identification and characterization of biofilms has not been included in foodborne illness investigations.

The formation of microbial biofilm is a very complex process. Firstly, organic molecules from food are deposited on equipment surfaces. Secondly, biologically active microorganisms are attracted to the conditioned surfaces. Thirdly, some of the microbial cells remain even after cleaning and sanitizing, and initiate growth. Lastly, larger biofilms are formed with the help of gene expression and quorum sensing. In the process of biofilm formation, properties of substrata and cell surfaces, surrounding environmental factors and genetic regulation of bacteria play an important role in reversible or irreversible attachment, micro-colony formation to a large biofilm.

Physical properties of the substratum- The physical characteristics of solid surfaces in the food-processing industry are much important for biofilm formation because they influence initial cell attachment. Bryers (1987) indicated that bacterial attachment depends on the critical surface tension of a solid surface. High free energy and wet surfaces promote bacterial adhesion (Boulange-Petermann *et al.,* 1997). More cells attach to hydrophilic surfaces (like stainless steel, glass etc.) than hydrophobic surfaces (like Buna-N rubber and other plastics) (Bendinger *et al.,* 1993). In contrast, Baker (1984) found no difference between the hydrophilic glass slides and polystyrene petri plates in cellular adhesion of freshwater bacteria. In addition, Busscher and Mei, (2000) reported that bacterial colonization happened at the hydrophilic region of the hydrophobic interface of the stainless steel surface. Even though contradictory observations have been reported, hydrophobic interaction apparently occurs between the cell surface and the substratum.

Stainless steel type 304, used in the food-processing industry is an ideal material for fabricating equipments due to its physico-chemical stability and high resistance to corrosion. Teflon and other plastics are mainly used for gaskets and accessories of instruments. These surfaces become rough or creviced with continuous reuse and form a harborage to protect bacteria from shear forces in the food fluid. Marshall (1990) observed that the extent of microbial attachment correlates to the surface roughness. Additional studies by Jones *et al.,* (1999) also demonstrated that surface defects were associated with a significant increase in bacterial adhesion.

The conditioning of substratum also plays a key role in the rate of bacterial attachment. The substratum would be covered by a film of organic molecules such as proteins from milk, pork, beef and even EPS produced by bacteria .Numerous food-contact surfaces namely stainless steel and Teflon are known to attract milk proteins and form conditioned substrata (Mcguire and Swartzel, 1989; Speers and Gilmour, 1985), which may encourage or inhibit bacterial attachment according to the concentration of milk. The substratum conditioned by diluted milk was better for attachment of pathogens than that of whole milk (Hood and Zottola, 1997). It was assumed that some proteins like bovine serum album (BSA) inhibited bacterial attachment to various surfaces. In summary, initiation of bacterial attachment depends on the surface properties of the conditioned substrata (Bryers, 1987).

Physiochemical properties of bacterial cells- The physiochemical properties of cell surfaces are an important aspect in the active bacterial adhesion. The surfaces of most bacterial cells are negatively charged, and the extent of this charge varies with growth environments. The net negative charge of the cell surface is adverse to the bacterial adhesion due to electrostatic repulsive forces. This keeps cells a short distance away from the surface. However, the bacterial cell-surface possesses hydrophobicity due to fimbriae, flagella and lipopolysaccharide (LPS). The importance of a hydrophobic surface is to reduce the repulsive force of interaction between two surfaces.

Environmental factors- Environmental factors including pH, temperature, nutrient composition and population characteristics of bacteria play an important role in the phenotypic changes from planktonic cells to the sessile form. It was shown that maximum adhesion to stainless steel surfaces at 3°C occurred at pH 7 for *L. monocytogenes* and pH8-9 for *Y. enterocolitia* (Herald and Zoottola, 1988a; 1988b). Kim and Frank (1995) have suggested that the low levels of phosphates initially stimulated *Listeria* biofilm formation. The presence of NaCl in the food matrix (Weigel *et al.,* 2007), the use of alcohol as a disinfecting agent (Gravesen *et al.,* 2005), or the presence of other bacteria (Carpentier and Chassaing, 2004) may also enhance the adhesion and biofilm maturation.

In food industry, the term biofouling is used and it causes serious problems such as impeding the flow of heat across the surface, increase in the fluid frictional resistance at the surface and increase in the corrosion rate at the surface leading to energy and product losses. The biofilms due to spoilage and pathogenic microflora formed on food surfaces such as poultry, other meat surfaces and in the processing environments, cause considerable problems of cross contamination and post-processing contamination.

Biofilms have been of considerable interest in the context of food hygiene. The attachment of the bacteria to the food product or the product contact surfaces leads to serious hygienic problems and economic losses due to food spoilage. In food systems, the attachment of microorganisms leading to the formation of biofilms may be undesirable and also detrimental. The majority of data generated to date indicate the attachment of bacteria to food contact surfaces are under simulated conditions. The following tables (Table 2.2 (a) & (b)) show food borne pathogens and the spoilage organisms in the biofilm:

Table 2.2 (a): Food pathogens on different growing surfaces. Table adapted from Kokare *et al.,* 2009

Food borne pathogen	**Growing surface**
Listeria monocytogens	Dairy processing plant, conveyor belt
Bacillus sp	Pipeline, Food processing environments
Salmonella sp	Poultry processing environment
Pseudomonas sp	Vegetables and meat surfaces

Table 2.2 (b): Microbial species in biofilms in various food environments. Table adapted from Shi & Zu, 2009.

Place	**Biofilm forming isolates**
Dairy processing plant, Pasteurization lines	*Bacillus cereus, E.coli, Shigella sp, Staphylococcus aureus*
Ice cream plant, conveyor belt, feeding unit	*Listeria monocytogens, Shigella*
Fish industry	*Neisseriaecae, Pseudomonas,Vibrio sp, Listeria*
Shrimp factory	*Pseudomonas fluorescens, Pseudomonas putida*

These said investigations revealed that biofilms were often established by various microorganisms on equipment surfaces of production lines. It was also indicated that biofilms containing pathogens like *L. monocytogenes* became one of the major causes of contamination of food products or transmission of diseases. Thus it is very important to develop cleaning and disinfection methods, and control systems in food-processing plants and environments.

The problems induced by the biofilms mainly affect many of the food industries listed below, which lead to the screening of the food items in the present study for food pathogens.

2.5.3.1. Produce industry (Caron, 2011; Fransisca *et al.,* 2011)

Currently, microbial control strategies are not efficient to provide complete eradication of hazardous microorganisms without affecting product qualities. Trimming, cutting, washing, rinsing, dewatering and packaging are all used in produce industry and are considered to be the primary source of cross-contamination. In 2011, there was an outbreak linked to whole cantaloupe contaminated with *L. monocytogenes.* It is speculated that the root cause of the outbreaks was the unsanitary condition of the packing shed. Moreover, the microorganisms were also found in other places including the conveyor belt, drying area and floor drain.

2.5.3.2. Dairy industry

Milk and milk products are perishable products and is truly vulnerable to contamination from improperly cleaned and sanitized equipment. It is speculated that type of bacteria in milk samples may show biofilm formation. For instance, the larger amount of thermoduric Streptococci and *Bacillus* sp.in pasteurized milk compared to raw milk could due to contamination by dispersion of biofilm. Dairy products are very susceptible to contamination by biofilms and it is challenging to eliminate those microorganisms. (Lattore *et al.,* 2010; Sharma and Anand, 2010).

2.5.3.3. Fish processing industry

In the fish processing industry, both equipment and water quality are major concerns. Many types of fish-contaminating-bacteria are reported to be biofilm-forming. Many genera including *Vibrio spp., L. monocytogenes, Salmonella spp., Bacillus spp., Aeromonas, and Pseudomonas spp.,* are known biofilm formers in fish and seafood processing. It also indicated that the level of biofilm formation can be affected by environmental factors and natural microflora (Rajkowski, 2009; Shikongo-Nambabi *et al.,* 2010)

2.5.3.4. Poultry industry

Many studies have been carried out on the biofilm formation in the poultry processing industry. Under many investigations, it has been identified that dust, surfaces, feces, poultry feed, and transportation of live poultry between production and processing units are the important risk factors. *Salmonella spp.* and *Campylobacter spp.* are the most commonly found pathogens in poultry and poultry processing. (Marin *et al.,* 2009; Park *et al.,* 2011).

2.5.3.5. Meat industry

Organic residues in food processing could be a niche for microorganism accumulation and biofilm formation as it is a source of cross-contamination, and has become quite a concern for numerous researchers. It is now well documented that multispecies biofilms may increase opportunities for pathogens to thrive in the food industry (Dourou *et al.,* 2011; Simoes, 2010).

2.5.3.6. Ready-to-eat (RTE) industry

Due to lifestyle changes, RTE foods have become very popular. However, RTE foods can be considered as relatively high risk foods, since the products will be consumed directly without any bactericidal processes. Even though RTE foods have been well processed, the chances of contamination are relatively high.

Furthermore, the storage times and conditions are known to be important factors affecting RTE foods quality. RTE foods could potentially be susceptible to cross contamination during processing and handling. The surveys conducted show that there is more chance of contamination for unpackaged or repackaged RTE foods; furthermore, raw meat sausages were postulated to be potentially contaminated. (Osaili *et al.,* 2011; Antunes *et al.,* 2010).

2.6. Methods to study biofilms

A single standard method for the study of biofilm susceptibility is not available, and this is impeding progress in this field. It is very difficult, if not impossible to compare results obtained with biofilms of even the same species cultured and assayed under vastly different conditions. It is hoped that a unified method will emerge.

The enumeration of biofilms helps in confirming the source and extent of contamination and the types of microorganisms involved as contaminating agents. The different methods employed for sampling and enumeration of biofilms are swabbing, rinsing, agar flooding and agar contact method (Kumar and Anand, 1997).

A popular method used to study biofilms is the Robbins device (Tyler Instruments, Calgary, Alberta, Canada), that is mainly based on passing a bacterial suspension through a flow cell that has 24 detachable coupons to which cells adhere and grow into a biofilm (Kharazmi *et al.,*1999). Once a biofilm is formed, the feeding liquid can be switched to a culture medium that contains the test compounds. After incubation period, the device is taken apart and the cells are dislodged by sonication and plated. This method enables reproducible biofilm formation and the observation of biofilm dynamics. The coupons can be then used for microscopic observations of biofilm structure. The strengths of this approach are in the well-controlled conditions that emulate *in vivo* biofilm formation and in the ability to characterize the formed biofilm by a variety of techniques. However, this method is ill suited for susceptibility studies, which require hundreds and often thousands of samples to be examined.

Tube method is a qualitative method for biofilm detection (Christensen *et al.,* 1982). A loopful of test organisms was inoculated in 10 mL of trypticase soy broth (TSB) with 1% glucose in test tubes. The tubes were incubated at 37ºC for

24 h. After incubation, the tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times. But the method was not much validated.

A simple qualitative method to detect biofilm production by using Congo Red Agar (CRA) medium was also described (Freeman *et al.,* 1989). CRA medium was prepared with brain heart infusion broth (Oxoid, UK) and Congo Red indicator (Oxoid, UK). First Congo red stain was prepared as a concentrated aqueous solution and autoclaved (121°C for 15 min) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55°C. CRA plates were inoculated with test organisms and incubated at 37°C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production (Reid, 1999). The experiment was performed in triplicate and repeated three times.

Several reports suggested that Tissue culture plate (TCP) method was a quantitative and reliable method to detect biofilm forming microorganisms (Mathur *et al.,*2006; Hassan *et al.*,2011), since the other two qualitative assay probably gave many false positive results, for example in coagulase negative staphylococci (Oliveira and Maria, 2010). When compared to TM and CRA methods, and TCP can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

The microtiter plate method or the tissue culture plate (TCP) method has been introduced for the study of biofilm development. It was successfully used to search for genes participating in the biofilm development of several Gram-negative species (Genevaux *et al.,* 1996; O'Toole *et al.,* 2000). Wells of microtiter plates are inoculated with a bacterial suspension, following which biofilms form on the well surfaces. After 24 to 48 h incubation, the planktonic cells are removed by rinsing the wells. A solution of crystal violet is then added to stain the cells. The wells are then rinsed, and the bound dye is extracted with acetone-ethanol and

quantified spectrophotometrically. This provides a quantitative measure of the mass of biofilm cells. It would be very useful to adapt this simple method to antimicrobial susceptibility measurements.

A promising apparatus for susceptibility testing is the Calgary-Biofilm Device (Ceri *et al.,* 1999). This disposable apparatus ingeniously combines a shearing force that makes a robust biofilm with the microtiter plate capability. The device looks like a 96-prong replicator with plastic pins. It inserts into a grooved tray that is filled with growth medium inoculated with cells. The apparatus is then placed on a tilting shaker platform, and the growing cell suspension washes the pins, on which biofilms grow. Importantly, any cell or cell mass that is not clinging well to the pin is washed away. As a result, one can form a robust biofilm that can be rinsed without loosing its integrity. After the biofilm is formed, the lid with pins can be placed into a microtiter plate for susceptibility testing. After a period of incubation with antibiotics, the cells can be dislodged from the pins by mild sonication and plated for determination of colony counts. But the challenge is that the round pins do not make it easy to perform microscopic observations of the biofilms and thus the most reliable method for quantification of biofilm formation is the standard microtiter assay (Rode *et al.,* 2007).

Different methods in microscopy including Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Atomic Force microscopy (AF), Confocal Laser Scanning Microscopy (CLSM) etc can be used, of which, SEM of surfaces has gained considerable attention in the study of biofilms. For analysing microstructure and metabolism of biofilms, Flourescent in Situ Hybridisation (FISH) can be used (Donlan and Costerton, 2002).

In the recent past, environmental scanning electron microscopy (ESEM) has also been widely used which helps in visualizing samples without the need of conventional microscopic procedures like dehydration, fixation and staining. Very recently, cellular automation models have also found application for the study of biofilms. The most commonly used method is direct viable count method. There are some limitations that arise during sampling of biofilms.

There are certain limitations while studying the biofilms. Grooves, crevices, dead ends, corrosion patches, etc. are some of the areas where the biofilms can grow and are very hard to access. Some of the bacteria present in biofilms on the surfaces in food and dairy environments are subjected to various

stresses including starvation, chemicals, heat, cold and desiccation that injure the cells, rendering them non-culturable. There is a chance that a small proportion of bacteria may also escape counting by the usual conventional culturing techniques, for which appropriate media and culture methods should be adopted (Wong and Cerf, 1995).

2.7 Control and removal strategies

The control of biofilms poses one of the most persistent challenges within food and industrial environments. Since biofilms are a great concern in the food sectors, many studies are being done to gain a better understanding of their development and spread. Consequently, many studies have also come up with different countermeasures. The first and the foremost is to prevent biofilm formation by regular cleaning and disinfecting, disallowing cells to attach firmly to contact surfaces (Midelet and Carpentier, 2004; Simoes *et al.,* 2006). Three different strategies were suggested: (i) disinfection "in time", before biofilm develops, (ii) disinfection of biofilms using harsh disinfectants, and (iii) inhibition of the microbial attachment by selecting surface materials that do not promote attachment or by supplementing with nutrients (Meyer, 2003).

Many other researchers have accounted for the incorporation of antimicrobial products in the surface materials themselves (Knetsch and Koole, 2011; Park *et al.,* 2004) by coating surfaces with antimicrobials (Thouvenin *et al,* 2003) or by modifying the physiochemical properties of the surfaces (Chandra *et al.,* 2005; Rosmaninho *et al.,* 2007). In a study on biofilm control, microparticles (eg: $CaCO_3$) coated with benzyldimethyldodecyl ammonium chloride were effectively in inactivating biofilm formation (Ferreira *et al.,* 2013). Many others had reported inhibition of biofilm formation by silver coating surfaces (Hashimoto, 2001). Pre-conditioning the surface with any surfactant has also been reported to prevent bacterial adhesion (Chen, 2012; Choi *et al.,* 2011). The research of Zeraik and Nitschke (2010) demonstrated that after conditioning with a surfactant, the surface became more hydrophilic. The data illustrated the decrease in hydrophobicity on the treated surfaces and thus showed a significant decrease in bacterial attachment. However, other factors are still considered for contributing to the reduction of bacterial attachment.

2.7.1. Cleaning and disinfection

In the food industry, there is debris everywhere that promotes the accumulation of microorganisms and thereby encourage biofilm formation. Therefore, regular cleaning is essential to prevent the contamination of food products. A good cleansing process that can remove food residues and other compounds that promote bacteria proliferation and biofilm formation is particularly effective (Simoes *et al.,* 2010). Many different chemical products can be used in cleansing, including surfactants or alkali products, or used to suspend and dissolve food debris by decreasing its surface tension, emulsifying fats, and denaturing proteins (Forsythe and Hayes, 1998). Cleaning should be carried out in away that can dissolve the EPS matrix associated with the biofilms so that disinfectants can gain access to the bacterial cells (Simoes *et al.,* 2006). It is evident that the use of high temperatures can reduce physical force such as water turbulence or scrubbing required inactivating biofilm cells (Chmielewski and Frank, 2006). Besides, cleaning only allows the removal of approximately 90% of bacteria from any of the surfaces and does not kill. They might later re-attach to other surfaces and thus form a biofilm, there by disinfection is indispensable with the intention of eliminating those (Graham *et al.,* 2002). Antimicrobial agents are used in the disinfection process so as to kill the microorganisms and to reduce surface population along with microbial growth. However, the effectiveness of disinfectants is limited by the presence of organic material like fat, carbohydrates and protein-based materials. Other than these, pH, temperature, water hardness, chemical inhibitors, concentration, and the contact time are also important factors influencing effectiveness of disinfectants (Bremer *et al.,* 2002; Cloete *et al.,* 2003; Kuda *et al.,* 2008). There are many types of disinfectants including chlorine, hydrogen peroxide, iodine, ozone, peracetic acid (Chmielewski and Frank, 2007).

2.7.2. Clean-in-Place (CIP)

Clean-in-Place (CIP) is a process allowing a complete system to be cleaned without dismantling it or without the manual involvement of the operator. It includes jetting and spraying on the surfaces or the circulation of cleaning solutions throughout the plant with an increased turbulence and flow velocity (Romney, 1990). There are so many factors that can influence CIP efficacy, including the nature of the biofilm layer, cleaning chemical composition and

concentration, time (Jullien *et al.,* 2004), cleaning temperature (Lelievre *et al.,*2001), cleaning flow rate and hydrodynamic (Benezech *et al.,* 2002), as well as the cleaning surface characteristics (Blel *et al.,* 2009). Relatively, Walton (2008) also summarized the basic principles of cleaning (i) to consider the physical nature and construction of the equipment to be cleaned, (ii) to assess the nature of the soil to be removed; (iii) to select a detergent appropriate to the removal of that soil, (iv) to bring the soil and the detergent together, (v) to rinse away all traces of detergent and soil, with the objective of achieving the standard of cleanliness appropriate to the duty for which the equipment is destined to be used, (vi) to always undertake cleaning as soon as possible after completion of the production operation, and (vii) when necessary, undertake a disinfection or sterilization process immediately before the equipment is returned to processing or production duties in order to reduce the level of contamination to one consistent with the hygienic standard required for that duty.

It was found that the CIP methods with small volumes and low temperatures, like enzyme-based cleaning and one-phase alkaline cleaning, were the most highly recommended alternative methods (Eide *et al.,* 2003).The study on biofilm removal of bacterial isolates sampled in the food industry by enzymes proposed that the implementation of enzymatic control of bacterial biofilms in the food industry would present a noteworthy alternative, while the conventional CIP using chemical agents is not providing any satisfactory hygienic results (Lequette *et al.,* 2010).

The different strategies currently used for biofilm control can be divided as physical, chemical and biological methods.

Chemical methods

The conventional control strategies are chemical-based, however, it is possible that microorganisms hold a certain degree of resistance to such strategies, or may acquire it later through mutation or genetic exchange. In the study on the effect of mechanical stress on biofilms challenged by different chemicals, it was stated that most of the chemical agents would react with the EPS complex which would enhance the mechanical biofilm removal. The removal rate was significantly improved after treating the biofilm with chemical agents (Simoes *et al.,* 2005). However, in another study, bacterial cells were destroyed after being

subjected to chemical agents, while these matrix was left unaffected. The best result was attained by applying both chemical and mechanical treatment (Exner *et al.,* 1987). Accordingly, it was suggested that mechanical treatment cannot remove bacterial cells (Jessen and Lammert, 2003). As such, it can be postulated that chemical and mechanical treatment has a synergistic effect and both play an important roles in biofilm and bacterial cell removal.

Detergents containing chelating agents like EDTA and ethylene glycol-bis (b-aminoethyl ether) N,N,N9,N9-tetracetic acid (EGTA) helped in removal of biofilms (Wirtanen and Mattila-Sandholm, 1996, 1994). Some detergents are bactericidal and some disinfectants may even depolymerize EPS, thus enabling the detachment of biofilms from surfaces, e.g. oxidants such as peracetic acid, chlorine, iodine etc. (Oh and Marshall, 1996).The impregnation of materials with biocides have been shown to play a major role in resisting bacterial colonization for as long as the antibacterial agents are released from the surfaces.eg:
Antifoulant paints containing silver against *Legionella pneumophila* (Rogers *et al*., 1995).

Sodium hypochlorite (NaClO) was reported to be a potential biofilm antimicrobial agent against *Staphylococcus aureus* (Cos *et al.,* 2010), *Prevotella intermedia, Peptostreptococcus miros, Streptococcus intermedius, Fusobacterium nucleatum,* and *Enterococcus faecalis* when compared to other disinfectants used in a study (Spratt *et al.,* 2001). Hydrogen peroxide (H_2O_2) is one of the most widely used disinfectants due to its highly oxidizing capacity based on the production of free radicals which can affect the biofilm matrix and has been efficient against biofilms (de Carvalho, 2007). It was used against four strains of *Vibrio spp*. in seawater. It was observed to be very effective in inhibiting biofilm formation at a concentration of 0.05% (500 mg/L). It can also kill mature biofilms at concentrations between 0.08% and 0.2% (Shikongo- Nambabi *et al.,* 2010).It is a potent antimicrobial agent against bacteria, fungi, viruses, protozoa, and bacterial and fungal spores (Khardre *et al.,* 2001). The microorganisms are eradicated by the disruption or breakdown of the cell envelope, which in turn leads to the leakage of the cell contents. Cell lysis is always a faster inactivation mechanism than that of other antimicrobial agents where permeation through the cell membrane is indispensable in order to effectively inactivate the microbe. Due to its mechanism, it is speculated that it cannot lead to microorganism resistance

(Rodriguez-Martinez *et al.,* 2007). Many researchers demonstrated the efficiency of ozone against bacterial cells and biofilms (Dosti *et al.,* 2005). A study by Tachikawa *et al.,*(2009) on the disinfection and removal of biofilms by ozone water on *P. fluorescens* and *P. aeruginosa* biofilms showed that by forming biofilms, the resistibility of the microorganisms against ozone was increased by 3000 and 10 times, respectively. The reason behind the resistance could be the reaction between ozone and the biofilm matrix introduced into the environment by the bacteria. Peracetic acid is known as an ideal antimicrobial agent according to its extreme oxidizing capacity. Furthermore, it cannot be deactivated by catalase and peroxidase enzymes that degrade H_2O_2. This agent also decomposes into safe and environmental friendly residues in food (acetic acid and hydrogen peroxide), hence it can be applied without rinsing and its efficacy is not affected by protein residues. A study showed that peracetic acids can reduce *L. monocytogenes* biofilm adhered for 24 h by 5 log with a concentration of 0.50% w/v (Cabeca *et al.,*2008).Others studies also showed the effectiveness of peracetic acid against various microorganisms (Salvi *et al.,* 2014). There are also several other studies which showed that peracetic acid is inefficient or less effective than other disinfectants against biofilms (Krolasik *et al.,* 2010; Rossoni and Gaylarde, 2000). It is suggested that aldehydes do not degrade biofilm matrix, and instead improve the stability. The biofilm complex needs to be eradicated before chemical agents can be used effectively (Exner *et al.,* 1987).

Physical methods

The application of super-high magnetic fields, ultrasound treatment and high pulsed electrical fields can eliminate the formation of biofilms on different surfaces. Recently, low electrical currents in combination with antibiotics were successfully employed for biofilm control (Qian *et al*., 1997).

Ultrasonication is a very well-known technique used in various food industry processes namely freezing, cutting, drying, tempering, bleaching, sterilization, and extraction (Chemat, 2011). It was reported to be used also as an efficient biofilm removal method (Oulahal *et al.,* 2000a). Oulahal *et al.,* (2000b) investigated the use of an ultrasonic apparatus on biofilm removal from stainless steel and polypropylene surfaces. The apparatus was demonstrated to remove twice as much of the industrial milk biofilm as the swabbing method on

polypropylene sheets. However, it has been well documented that even though lower frequency in sonation is remarkably more efficient for reducing biofilm cells viability, bacteria in food industries cannot be solely eliminated using the present ultrasonic technologies. Thus combining techniques of ultrasound with other treatment techniques were recommended (Piyasena *et al.,* 2003; Qian et al., 1997). Accordingly, the combination of ultrasound and ethylene diamine tetra acetic acid (EDTA), and ultrasound and enzymes showed a higher efficacy in removing biofilms. The results were promising against *S. aureus* than *E. coli* biofilms, and was in agreement with an industrial control method, i.e. a combined treatment of ultrasound generation in enzymes preparation restricted to an active chamber area with fast and good reproducible recovery compared to other approaches (Oulahal *et al.,* 2007).Ultrasound was also reported to increase the effectiveness of antibiotics against biofilm cells (Peterson and Pitt, 2000). Two processes were observed to be account for the increased efficiency: i) the ultrasound improves the diffusion of oxygen into the biofilm matrix which allows biofilm cells to become active, and therefore affected by antibiotics and ii) the enhanced transport by ultrasound of antibiotics into the complex may destroy the bacteria before they gain resistance to the agents (Carmen *et al.,* 2004). Baumann *et al.,* 2009, also showed a significant effect on biofilm removal on stainless steel food contact surfaces by combining the use of ozonation and sonication.

Biological means/Green strategies

Enzymes are effective in cleaning the extracellular polymers forming the biofilm matrix, and in removal of biofilms. Efficiency of biofilm removal by enzymes may vary according to the species of bacteria, and it can also be enhanced by combining with surfactants (Lequette *et al.,* 2010); this suggests that proteins also contribute to the adhesion of biofilms as proposed by Hinsa and O'Toole (2006). There search of Molobela *et al.,* (2010) indicated that protease enzymes were very effective in the degradation of *P. fluorescens* biofilm's EPS, while amylase enzymes were less effective. It was also suggested that the structural composition of EPS varies even amongst bacteria of the same species, with the way they were formulated and their mode of action, are the reasons for the inefficiency of enzymes. It is well known that a mixture of proteases and amylases is commonly used to respond to the variety present in a single biofilm.

While proteases hydrolyze the proteins, amylases can break the bonds of carbohydrates associated with the complex.

Among all 1,4-glycosidic bond cleaving amylases, α-Amylases are the frequently used because of their thermostability; however, they will not stay active for long as they are calcium metalloenzymes (Craigen *et al.,* 2011). Similarly, a combination of polysaccharide-hydrolyzing enzymes and oxidoreductases were recommended for bacteria biofilm removal due to a wide range of polysaccharide hydrolyzing enzymes activities which make it useful for the degradation of biofilms matrix; the bactericidal effect of oxidoreductases (Johansen *et al.,* 1997). In another study individual biofilm cleaning efficiencies of pectin esterase, pectin lyase, and cellulase were shown to be not effective against *P. fluorescens* mature biofilms. However, the efficacy was enhanced when pronase was used in the treatment process (Orgaz *et al.,* 2007). As can be seen, enzymatic control against biofilms could be used as a new and improved environmental friendly alternative strategy according to its nontoxic characteristics and instability. But different studies reported that hurdle technology which includes the action of enzymes, showed more efficiency in control of biofilm than using them alone for biocontrol (Srey *at al.,* 2013)

2.8 Biocontrol of biofilms using different bioactive compounds

A bioactive compound can be defined as a compound that has an effect on a living organism, tissue or cell (MedicineNet 14, June, 2012). In the field of nutrition, bioactive compounds are distinguished from essential nutrients. While nutrients are essential for the sustainability of a body, the bioactive compounds are not essential since the body can function properly without them, or because nutrients fulfil the same function. Bioactive compounds can influence health. They are found in both plant and animal products or can be synthetically produced. Examples for plant bioactive compounds are carotenoids and polyphenols (from fruits and vegetables), or phytosterols (from oils). Example in animal products is fatty acids, found in milk and fish. Some examples of bioactive compounds are flavonoids, caffeine, carotenoids, carnitine, choline, coenzyme Q, creatine, dithiolthiones, phytosterols, phytoestrogens, glucosinolates, polyphenols, anthocyanins, prebiotics, andtaurine (Zhang *et al.,* 2014).

Recently, new anti-biofilm agents have been developed as adjuncts or alternatives to classical antibiotic treatment. Many of these novel agents show "resistance" to the emergence of antimicrobial resistance, and even enhance the activity of conventional antibiotics. Anti-biofilm substances may be synergistic with other antimicrobials to overcome persistent infections (Wu *et al.,* 2004).

Quorum-Sensing (QS) is a form of communication bacteria use to cooperatively build biofilm communities. Most of the bacteria produce QS signals, as well as QS inhibitors. usnic acid, which is a lichen metabolite, possesses inhibitory activity against bacterial and fungal biofilms via QS interference. QS Inhibitors can increase the susceptibility of biofilms to antibiotics. QS Inhibitors are generally regarded as safe in humans (Sun *et al.,* 2013). Garlic inhibits the expression of several genes that control bacterial QS. The star in garlic's arsenal is ajoene, the sulfur-containing compound produced when garlic is crushed. Ajoene inhibits production of rhamnolipids in *Pseudomonas aeruginosa*, which shields biofilms from white blood cells. Over 90% of biofilm bacteria were killed with a combination of ajoene and the antibiotic tobramycin. Garlic also has anti-viral, anti-fungal, and anti-protozoal properties, and benefits the cardiovascular and immune systems (Jakobsen *et al.,* 2012). These sulfur compounds from garlic quickly lose their activity upon exposure to oxygen. A willow bark extract, hamamelitannin also inhibits quorum sensing (Morgan, 2013).

The anticancer, antioxidant, and anti-inflammatory effects of different flavonoids are very well established. However, their biofilm disrupting function is practically unknown. Flavonoids appear to suppress the formation of biofilms through a non-specific QS inhibition (Vikram *et al.,* 2010). The flavonoid phloretin inhibited biofilm formation in strain *E. coli* O157:H7, and ameliorated colon inflammation in rats without harming the beneficial biofilms (Lee *et al.,* 2011). Cranberry has the reputation for keeping bacteria from sticking to surfaces. The red pigments in cranberries, the proanthocyanidins (PACs)were reported to inhibit biofilm formation and are known to possess many properties such as antimicrobial, anti-adhesion, antioxidant, and anti-inflammatory (Bodet *et al.,* 2006). They prevent the attachment of many pathogens to host tissues, and can inhibit the formation of biofilms in the mouth and urinary tract (Labrecque *et al.,* 2006). Cranberry PACs were reported to stop the gum disease pathogen *Porphyromonas gingivitis* from adhering and forming biofilm, whereby its

invasiveness was markedly reduced. These unique PACs also prevented adherence and biofilm formation by *Candida albicans*, the causative agent of thrush and yeast infections (Iwashkiw *et al.,* 2012). Cranberry juice extract at a low micromolar levels inhibited tissue-destroying enzymes made by bacteria and humans (Bodet *et al.,* 2007) Cranberry PACs also prevented dental plaque by inhibiting biofilm-forming enzymes (Steinberg *et al.,* 2004) and keeping bacteria from aggregating themselves (Yamanaka *et al.,* 2004).

Chlorogenic acids (CGA) from coffee are cinnamic acid derivatives with important antioxidant and anti-inflammatory activities (Farah *et al.,* 2008). *In vitro* antibacterial and anti-biofilm activities of chlorogenic acid against many clinical isolates of *Stenotrophomonas maltophilia* resistant to trimethoprim/sulfamethoxazole (TMP/SMX) were investigated. The Minimum Inhibitory Concentration (MIC) values ranged from 8 to 32 µg/mL. *In vitro* antibiofilm testing showed a 4-fold reduction in biofilm viability at 4x MIC (Karunanidhi *et al.,* 2012). Boswellic acids are pentacyclic triterpenes produced in plants of the genus *Boswellia*, have potent anti-biofilm properties. Acetyl-11-keto-β-boswellic acid inhibited biofilms formed by *S. aureus and S. epidermidis*, and could also disrupt preexisting biofilms. Disruption of bacterial membranes is the likely the mode of action (Raja *et al.,* 2011).

Five Indonesian medicinal plant extracts were shown to inhibit *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilm formation at concentrations as low as 0.12 mg/mL (Pratiwi *et al.,* 2015a). Wheat bran extract exhibited anti-biofilm activity, and destroyed pre-formed *S. aureus* biofilm in dairy cows with mastitis (Pratiwi *et al.,* 2015b).

Farnesol and xylitol are two compounds which were also reported to possess antibiofilm and antibacterial effects when used as root canal irrigants (Alves *et al.,* 2013). Xylitol is actually a low-carb sweetener found in toothpaste and diet sodas. When bacteria incorporate xylitol into the biofilm, it makes for a flimsy structure. (Morgan, 2013). Pro-oxidants would also be effective against biofilms. Oxidative agents are microbicidal, and offer possibilities for reducing the pathogenic activities of biofilms, especially those with an anaerobic component.

Not surprisingly, bacteria compete with one another for turf. Certain substances on the surface of one bacteria work to inhibit biofilms from another. The extracellular polysaccharides (EPS) are the essential building blocks for the

biofilm matrix of most microorganisms. Although EPS is the stuff of biofilms, but it can also inhibit their neighbors' biofilms, from initial adhesion, dispersion, cell to cell communication, to matrix degradation (Rendueles *et al.,* 2013). One example of this EPS anti-biofilm activity was in *Actinobacillus pleuropneumoniae* serotype 5. The EPS from these bacteria inhibited cell-to-cell and cell-to-surface interactions of other bacteria, preventing them from forming or maintaining biofilms. This is one of a growing number of natural bacterial polysaccharides that exhibit broad-spectrum, non-biocidal anti-biofilm activity (Karwacki *et al.,* 2013).

Numerous bacteria produce anti-biofilm agents. Extracts of a coral associated bacteria induced a reduction in *S. aureus* and *Serratia marcescens* biofilm formation. A novel natural product, 4-phenylbutanoic acid, from the marine bacterium *Bacillus pumilus,* showed inhibitory activity against biofilms for a broad range of bacteria (Nithya *et al.,* 2011). Ethyl acetate extracts of the bacterium, *Bacillus firmus* which is a coral-associated bacterium showed antibiofilm activity against biofilms formed by multidrug resistant *S. aureus* (Gowrishankar *et al.,* 2012).

Streptococcus salivarius, a non-biofilm, harmless inhabitant of the human mouth, uses two enzymes to inhibit the formation of dental biofilms, otherwise known as dental plaques. These enzymes were identified as fructosyltransferase (FTF) and exo-beta-d-fructosidase (FruA), which affected a decrease in EPS production. Large quantities of FruA that *S. salivarius* produces may play an important role in microbial interactions for sucrose-dependent biofilm formation in the mouth (Ogawa *et al.,* 2011).

Antimicrobial Peptides (AMPs) are cationic, amphipathic substances that are part of the innate immunity in animals, plants, and some microbes. AMPs bind to and disrupt the bacterial membranes and thus efficiently kill biofilms. AMPs from sea urchins, sea cucumbers and echinoderms have all been shown to disrupt biofilms. Their drawbacks are their sensitivity to salt, ionic strength, pH and proteolytic activity in body fluids. Synthetic AMPs have recently emerged as attractive anti-biofilm agents. Specifically targeted AMPs (STAMPs) are fusion peptides that can target single pathogens, and are relatively stable under a range of physiological conditions. STAMPs can selectively eliminate the biofilm-forming, tooth-decaying pathogen *Streptococcus mutans* from a mixed-species environment (Sun, 2013).

The biocontrol of biofilms can also be achieved by several other bioactive compounds isolated from microorganisms. Several bioactive agents other than mentioned and different compounds from fungi were also reported to be efficient in controlling biofilm rather than only their antimicrobial properties. Out of these, the most unexplored biofilm controlling bioactive compounds are discussed in the sections below.

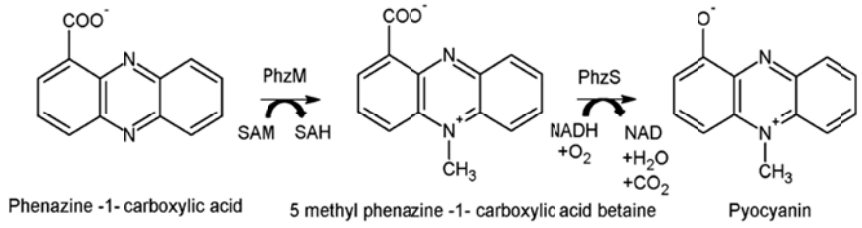
2.8.1 Pyocyanin

Pseudomonas aeruginosa is a member of the Gamma Proteobacteria class among bacteria. Based on the revisionist taxonomy done by analysis of conserved macromolecules (e.g. 16S ribosomal RNA), the genus *Pseudomonas* was included in the bacterial family *Pseudomonadaceae*. Other members in the genus include *P. alcaligenes, P. anguilliseptica, P. citronellolis, P. flavescens, P. jinjuensis, P. mendocina, P. nitroreducens, P. oleovorans, P. pseudoalcaligenes, P. resinovorans* and *P. straminae* (Moore *et al.,* 2006).

P. aeruginosa is a Gram-negative, aerobic rod-shaped bacterium. Almost all strains are motile by means of a single polar flagellum (Palleroni, 2005). It thrives not only in normal atmospheric conditions but also in most of the hypoxic atmospheres, and has colonised many natural and artificial surroundings (Suthar *et al.,* 2009). Its habitat is thus widespread and it is found in soil, water and many other environments. *Pseudomonas aeruginosa* attracts attention because of its colour and pigment production. One of the most recognizable signs of an unknown colony being *P. aeruginosa*is the characteristic fruity, grape-like odour derived from the production of 2-aminoacetophenone by the organism.

No other species in Gram-negative on-fermenting bacteria produce pyocyanin, making its presence helpful in identification. Pigments secreted by *P. aeruginosa* include pyocyanin (blue-green in colour), pyoverdin (yellow, green and fluorescent), pyomelanin (light-brown) and pyorubrin (red-brown) (Reyes *et al.,* 1981; Meyer 2000).Pyocyanin is actually a phenazine which is anitrogen-containing heterocyclic compound. It is a redox active secondary metabolite and is soluble in chloroform. This metabolite, 1-hydroxy-N-methyl phenazine, contributes to bacterium survival. Little is known about the two enzymes designated PhzM and PhzS that function in the synthesis of pyocyanin from the precursor phenazine-1-carboxylic acid.

Pyocyanin is composed of 2 subunits of N-methyl-1-hydroxyphenazine. It has been crystallized in pure form and classified as a phenazine-type of molecule (Norman *et al.,* 2004). The biosynthesis of phenazine has been extensively studied in *Pseudomonas* (Hollstein and McCamey, 1973; Herbert *et al.*, 1974, 1976). Phenazine is a heterocyclic, compound produced naturally as the deep red,5-methly-7-amino-1-carboxyphenazium betaine, which is further converted to the lemon yellow coloured phenazine-1-carboxylic acid (PCA), and finally to the bright blue1-hydroxy-5-methyl phenazine (pyocyanin) (Gohain *et al.,*2006). The shikimic acid pathway was found to be the primary metabolic pathway which gets branched into phenazine biosynthesis. Shikimic acid pathway was the precursor for phenazine which gives a branching point for chorismic acid for the synthesis of phenazine (Millican, 1962). In the synthesis of pyocyanin by *P. aeruginosa*, seven genes have been identified, namely *phz C, D, E, F, G, M* and *S*. The phenazine biosynthetic loci were found in all *Pseudomonas* species (Mavrodi *et al.,* 2001; Ahuja, 2006). Of these genes, *phzM* and *phzS* were the main genes responsible for converting phenazine-1-carboxylic acid to pyocyanin (Fig. 2.4). Two steps are mainly suggested to be involved in the synthesis of pyocyanin from PCA, which is the common precursor for many different species-specific phenazines. In the first step, it is catalyzed by the enzyme PhzM, an S-adenosylmethionine (SAM) dependent methyltransferase, where PCA is converted to 5-methylphenazine-1-carboxylic acid betaine by transfer of a methylgroup to the nitrogen atom of phenazine-ring moiety. The second step is catalyzed by the enzyme PhzS, a FAD dependent monooxygenase involved in the hydroxylativedecarboxylation of 5-methylphenazine-1-carboxylic acid betaine to pyocyanin (Parsons *et al.,* 2007).



Previously, pyocyanin production was carried out using King's Medium (King *et al.,* 1954). Based on King's Medium, Pseudomonas agar medium was formulated which is recommended for pyocyanin production by *Pseudomonas* species. This medium enhances elaboration of pyocyanin but inhibits the formation of other pigments. There are many reports that determined the cultural characteristics and conditions which favour the production of pyocyanin (Burton *et al.,* 1948; Karpagam *et al.,* 2013; Sudhakar *et al.,* 2013). Media formulations which produce optimal levels of pyocyanin contain glycerol, alanine, sulphur, and iron. Presence of alanine and glycerol as joint substrates were highly effective and acted as a precursor for pyocyaninproduction. This joint substrate medium is the Frank and De Moss medium, recommended for the rapid diagnosis of *P. aeruginosa* and demonstration of pyocyanin (Frank and De Moss, 1958). Formerly, liquid glycerol–peptone–phosphate medium was used for enhancing pyocyanin production from *P. aeruginosa*. The pyocyanin pigment which is diffusible into the medium can be solvent-extracted by chloroform and purity was checked by both UV/visible and IR spectroscopy (Saosoong *et al.,* 2009). Porter (2009) also reported the production of pyocyanin from *P. aeruginosa* and it was extracted using chloroform. The pigment was produced by the inner part of the cells which was lysed by addition of chloroform. If required, the purity was confirmed by passing the aqueous solution into a Sephadex G-10 column or ion-exchange chromatography or high pressure thin layer chromatography (Fontoura *et al.,* 2009).

2.8.1.1 Antibacterial activity of pyocyanin

Little attempts have been made to determine the relation between pyocyanin and its inhibitory action. Anti-bacterial activity of pyocyanin has been reported since 1940 (Waksman and Woodruff, 1942). The pigment inhibited the growth of *Escherichia coli* and was named as Colicin. The protein fraction which was liberated uponlysis of bacteria exhibited the properties of pyocyanin (Young, 1947). The purified form of pyocyanin showed antibacterial activity and depending on the pyocyanin concentration, the bactericidal effect varied (Baron and Rowe, 1981). The mechanism by which pyocyanin inhibits bacterial growth was investigated and it was concluded that, pyocyanin interacts with the cell membrane respiratory chain resulting in the inability of the bacterial cells to perform their active metabolic transport process (Baron *et al.,* 1981). Exposure of

E. coli cultures to pyocyanin causes depletion of oxygen supply to the cells produces H_2O_2 and also diverts the electron flow, causing toxicity (Hassan and Fridorich, 1980). Pyocyanin negatively influences the active transport mechanism of several organisms (Baron *et al.,* 1989).

There are several reports on anti-staphylococcal activity by *P. aeruginosa.* In cystic fibrosis (CF) patients, *P. aeruginosa* present in the sputum inhibited the growth of *Staphylococcus aureus*. The antagonistic effects were proved by cross-streak test, well plate assay and growth of mixed culture (Machan *et al.*, 1992). Nearly 90–95 % of antimicrobial inhibitions of *P. aeruginosa*strains were due to production of the pyocyanin. It showed antagonistic activity against pathogenic bacteria like *Salmonella paratyphi, E. coli* and *Klebsiella pneumonia* (Saha *et al.,* 2008). Pyocyanin isolated from *P. aeruginosa 4B* strain showed antibiotic activities against various pathogens and food spoilage bacteria like *Listeria monocytogens* and *Bacillus cereus*. The secondary metabolite along with various enzymes like haemolysin and hydrolytic enzymes played a key role in their antimicrobial activities (Fontoura *et al.,* 2009).

Gram-positive microorganisms *(*like *B. subtilis, S. aureus, S. epidermis and M. luteus)* were more susceptible to pyocyanin than Gram-negative bacteria (like *P. teessidea, P. clemancea etc*) and the eukaryotes (*A. niger* and *Sacc. cerevisiae)*. According to previous studies, it was proved that Gram-positive organisms are more susceptible to pyocyanin than Gram negative organisms (Waksman and Woodruff, 1942; Baron and Rowe, 1981). The resistance to pyocyanin by the Gram-negative bacteria may due to the presence of an outer membrane composed mainly of lipopolysaccharides (Ferguson *et al.,* 2007). Previous findings have demonstrated that the outer membrane confers resistance to antibiotics, detergents and disinfectants by the Gram-negative species. Reports by Fridovich *et al.,* (1995) suggested that organisms, which has high levels of superoxide dismutase enzyme are resistant to action of pyocyanin.

2.8.1.2. Anti-fungal activity of pyocyanin

The compound also inhibited growth of fungi like *Aspergillus fumigatus* and *Candida albicans* isolated from the sputum of CF patients (Kerr *et al.,* 1999). Sudhakar *et al.,* (2013) reported the production of pyocyanin from *P. aeruginosa* WS1 and its antagonistic activity against commonly encountered phytopathogens. The extracted pyocyanin had 210.23 kDa molecular weight. The MIC of

pyocyanin against phyto pathogens was 64 mg/mL against *Aspergillus flavus* and *Aspergillus fumigatus*, and128 mg/mL against *Candida* species.

The inhibition of pathogenic bacteria and fungi suggests that pyocyanin could be used as an effective antibiotic as well as a biological control agent in agriculture and food industry. The report by Vukomanovic *et al.,* (1997) has shown that pyocyanin have a variety of pharmacological effects on both eukaryotic and prokaryotic cells.

2.8.1.3 Bio-control activity of pyocyanin

In pathology, the term biocontrol applies to the use of microbial antagonists to suppress diseases as well as the use of host specific pathogens to control infection agents. The microbial agent that suppresses the pathogen is referred to as the biological control agent (BCA).The interaction of *P. aeruginosa* with plants as a beneficial association is quite common. Recent studies have provided an insight into this complex regulatory network. *P. aeruginosa* produces pyocyanin in the rhizosphere soil where it promotes direct plant growth and protects plants from phyto pathogens (Cook, 1988; Glick, 1995; Bashan and Holguin, 1998). Despite their importance in biological control, little is known about the genes involved in bio-control activity. Natural products have recently become a promising source for deriving molecules that can potentially inhibit QS related anti-virulent activities.

Pyocyanin is a natural product which has the ability to act as a bio-control agent, thus helping to create an eco-friendly solution for the replacement of chemical pesticides. Further new insights about pyocyanin are being contributed by new research.

2.8.2 Rhamnolipids

Rhamnolipids (RLs) are a class of glycolipids produced by *Pseudomonas aeuroginosa,* having several potential industrial and environmental applications including the production of fine chemicals, the characterization of surfaces and surface coatings, as additives for environmental remediation, and as a biological control agent (Maier and Chavez ,2000).

The discovery of RLs dates back to 1946 when Bergstrom *et al.,*(1946a) reported an oily glycolipid produced by *Pseudomonas pyocyanea* (now *P. aeruginosa*) grown on glucose. This substance was named pyolipic acid and its structural units were identified as L-rhamnose and b-hydroxydecanoic acid (Bergstrom *et al.,* 1946 b; Hauser and Karnovsky, 1954; Jarvis and Johnson, 1949). The exact chemical nature of these biomolecules was unraveled by Jarvis and Johnson (1949) which was followed by Edwards and Hayashi (1965). Since then, extensive investigations have been conducted that covers various aspects of RL research.

Studies about the interactions of RL with other biological systems are numerous. The antibacterial (Abalos *et al.,* 2001; Shen *et al.,* 2009), antifungal (Kim *et al.,* 2000; Yoo *et al.,* 2005), antiviral (Cosson *et al.,* 2002; Remichkova *et al.,* 2008), antiphyto pathogenic (De Jonghe *et al.,* 2005; Nielsen *et al.,* 2006), and algicidal (Wang *et al.,* 2005) properties of RLs have been extensively investigated.

RLs released by *P. aeruginosa* have long been known as the heat-stable extracellular hemolysin (Fujita *et al.,* 1988; Johnson and Marrazzo, 1980) and more recently, a RL congener produced by *Burkholderia pseudomallei* was shown to display hemolytic and cytotoxic activities (Haussler *et al.,*2003). Due to their excellent surface activity, the physicochemical properties of RLs have received considerable interest (Abdel-Mawgoud *et al.,* 2011; Cohen and Exerowa, 2007). Due to their hydrocarbon solubilizing properties, they also have been used in the fields of bioremediation and biodegradation (Avramova *et al.,* 2008; Cameotra and Singh, 2009).

The potential industrial and biotechnological applications of RLs are therefore quite diverse (Singh *et al.,* 2007). RLs have been used for the synthesis and stabilization of nanoparticles (Palanisamy and Raichur, 2009), the preparation of micro emulsion (Xie *et al.,* 2007), as an anti-agglomeration agent (York and Firoozabadi, 2008), as dispersing agent (Tripathy and Raichur, 2008), in cleaning

soap mixtures (Ecover™ products) and as a source of rhamnose (Linhardt *et al.,* 1989).

Clinical testing of RLs as pharmaco-active compounds has been performed. Some successful trials proved their potential applications for the treatment of ulcers (Piljac *et al.,* 2008) and of full-thickness wounds (Stipcevic *et al.,* 2006). These promising properties and potential application of RLs have encouraged researchers to improve the production of RLs, using industrially safe and more affordable processes in order to reduce the production costs, which currently restrict the competitiveness of RLs with petroleum-derived surfactants. This goal has been sought through different approaches. First, many attempts have been made to isolate RL producers other than the opportunistic pathogen *P. aeruginosa* (Abouseoud *et al.,* 2008; Rooney *et al.,*2009) or to transfer the genes responsible for RL production into more industrially safe heterologous hosts, such as *E. coli* (Cabrera *et al.,* 2006). Second efforts have been dedicated to the identification of low-cost and renewable raw material as production substrates, such as agro industrial wastes (Nitschke *et al.,* 2005; Rahman *et al.,* 2002). Finally, an even production of rhamnolipids through pure chemical synthesis was also reported (Howe *et al.,* 2006).

The structure of the first identified rhamnolipids is shown in figure 2.5. These compounds are predominantly constructed from the union of one or two rhamnose sugar molecules and 1/2 – hydroxyl (3-hydroxy) fatty acids (Lang and Wullbrandt, 1999). Rhamnolipids with one sugar molecule are commonly referred to as mono-rhamnolipids, while those with two sugar molecules are di-rhamnolipids. The length of the carbon chains found on the hydroxyacyl portion of the rhamnolipids can vary significantly. However, in the case of *P. aeruginosa* 10 carbon molecule chains are the predominant form (Deziel *et al.,* 2000). Primary rhamnolipids production by *P. aeruginosa* occurs during stationary growth phase in rapidly agitated liquid medium with limiting concentrations of nitrogen or iron (Guerra-Santos *et al.,* 1986). *P. aeruginosa* is capable of growth and the rhamnolipid production using a range of different carbon sources; however, the highest levels of rhamnolipid production can result from using vegetable-based oils as carbon sources, including soybean oil, corn oil, canola oil, and olive oil (Sim *et al.,*1997). *Pseudomonas aeruginosa* synthesizes mixture of mono- and di-RLs with hydroxyacyl moieties mostly from C8 up to C12.

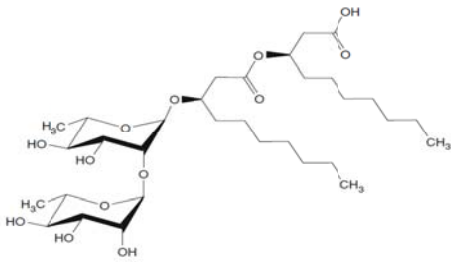


Fig 2.5. General Structure of rhamnolipids. Adapted from Mawgoud *et al.,* 2011

For the enhanced production of rhamnolipids, several media were optimized; the maximum production of rhamnolipids was reported in the successive cultivation of the producer strains in Kay's minimal medium, proteose peptone ammonium salt (PPAS) medium and a mineral salts medium (MSM) (Gunther *et al.,* 2005).

The most widely used method for qualitative, high throughput screening of rhamnolipid producing bacterial strains is the cetyltrimethylammonium bromide (CTAB) agar test (Pinzon and Ju, 2009; Siegmund and Wagner, 1991). In this method, the anionicRLs form an insoluble complex with this cationic bromide salt, and the complex is clearly revealed using methylene blue present in the agar. The RL-producing strains are revealed by a dark blue halo around the colony, allowing the facile identification of the presence of RLs. Another indirect way to detect RLs is based on their hemolytic properties. This approach can be performed in solution using an erythrocyte suspension (8%) to which the RL containing solution is added. After a predetermined time, the residual erythrocytes are removed by centrifugation and the hemoglobin released can be measured at 540 nm (Johnson and Marrazzo, 1980).

The drop collapsing test (Jain *et al.,* 1991) is a very sensitive method for the rapid screening of RL production by various isolates. This assay consists of applying a drop of a bacterial culture supernatant to be tested over a polystyrene plate which contain shallow wells covered with oil. The droplet will spread over the oil only if the culture supernatant sample contains RLs. The quantitative analysis was done by orcinol method (Rikalovic *et al.,* 2012) and the concentration of the rhamnolipids were measured as per Wilhem *et al.,* 2001; Wang *et al.,* 2007). Further characterization can be done using infra red and Nuclear magnetic resonance spectroscopic techniques (Rikalovic *et al.,* 2012).

Three enzymatic reactions are required in the final steps of RL biosynthesis in *P. aeruginosa* (Soberon-Chávez *et al.,* 2005): (1) RhlA is involved in the synthesis of the HAAs, the fatty acid dimers, from two 3-hydroxyfatty acid precursors; (2) the membrane-bound RhlB rhamnosyltransferase uses dTDP-L-rhamnose and an HAA molecule as precursors, yielding mono-RL; (3) these mono-RLs are in turn the substrates, together with dTDP-L-rhamnose of the RhlC rhamnosyltransferase to produce di-RLs. Unfortunately, few works have characterized these three enzymes.

The Genetic regulation of rhamnolipid (RL) biosynthesis is complex and less understood in *P. aeruginosa*. Multiple systems of quorum sensing (QS) participate in the control of RL synthesis genes (rhlA, rhlB, rhlC). Two QSsystems, LasR/I and RhlR/I, depend on acyl homoserine lactones (AHL) ligands, N-3-oxododecanoyl-HSL and N-butanoyl-HSL, respectively, that bind to their cognate transcriptional regulators, LasR and RhlR, respectively, for regulation of expression of several genes, among which are RL biosynthesis genes. LasR/I and RhlR/I activate the expression of their own autoinducer synthase genes, *lasI* and *rhlI,* respectively, as a positive feedback. The transcription of *lasI* and *rhlI* is also controlled by other regulators. RhlR/C4-HSL complex is positively regulating expression of the operon rhlAB as well as the operon encoding the *rhlC* gene. These last three genes encode the three enzymes responsible for biosynthesis of RLs. LasR/oxo-C12-HSL activates the other QS system in which the transcriptional regulator MvfR (PqsR) binds to its co-inducers 4-hydroxy-2-heptylquinoline (HHQ) and 3,4-dihydroxy-2-heptylquinoline (*Pseudomonas* Quinolone Signal; PQS). LasR/oxo-C12-HSL activates the expression of *mvfR* which is finally responsible for the production of rhamnolipids.

Despite of improving fermentation strategy, an efficient and economical product recovery technique is needed for maximum product recovery. It is essential to recover and purify the biosurfactants in a cost-effective manner to lower down the whole cost of production as the industrial demand for biosurfactants is constantly growing. Three different recovery techniques including solvent extraction, ammonium sulphate precipitation and acid precipitation were mostly used. Even though solvent extraction gives the

maximum yield, the purity was mostly achieved using acid precipitation test (Salleh *et al.,* 2011).

Biosurfactants are reported to inhibit the adhesion of microorganisms to solid interfaces and infection sites (Rodrigues & Teixeira, 2010). When biosurfactants adsorb to a surface, its characteristics will change. The surface becomes more hydrophobic, which can control microbial adhesion (Rufino *et al.,* 2011). The primary adhesion of microorganisms to a surface is a vital target to prevent its colonization and biofilm formation. By precoating the surfaces with a biosurfactant solution of the right concentration, a strong inhibition of both adhesion of microorganisms and biofilm formation has been found (Rodrigues *et al.,* 2006).

Anti-biofilm potential of a glycolipid surfactant produced by a tropical marine strain of *Serratia marcescens* was reported in 2011, where the glycolipid prevented adhesion of *Candida albicans* BH, *P. aeruginosa* PAO1 and *Bacillus pumilus* TiO1. The glycolipid also disrupted preformed biofilms of these cultures in microtitre polystyrene plates (Dusane *et al.,* 2011) Another study, reported that biosurfactants from two types of Lactobacilli displayed anti-adhesive and anti-biofilm abilities against *Acinetobacter baumannii, Escherichia coli* and *Staphylococcus aureus* (Sambanthamoorthy *et al.,* 2014).

Thus rhamnolipids have several antimicrobial and antibiofilm effects along with immense biotechnological and industrial applications.

2.8.3 Melanins

Melanin is a very well-known, universal pigment in living organisms. It brings many benefits to human beings and especially plays a key role to protect internal tissues from the harmful effects of ultraviolet rays (Romero-Martinez *et al.,* 2000). The most commonly seen form of biological melanin is a complex polymer of either or both of 5, 6-indolequinone and 5, 6- dihydroxyindole carboxylic acid. Melanins are negatively charged hydrophobic (Butler and Day, 1998), high-molecular-weight compounds. It is commercially used as a component of photo productive creams for anti-melanoma therapy and also reported to possess several immuno-pharmacological properties (Montefiori and Zhou, 1991). Recent studies have shown that melanins are highly immunogenic and have anti-inflammatory properties (Nosanchuk *et al.,* 1998). It has been shown to protect micro-organisms against UV-radiation (Selvameenal *et al.,*

2009), microbial lysis (Casadevall *et al.,* 2000), oxidants killing by alveolar macrophages (Jacobson, 2000) and defense responses of host plants and animals against fungal infection (Jacobson et al., 1994). Several types of melanin have been described in bacteria, fungi and animals; eumelanins, phaeomelanins, allomelanins and pyomelanins. Eumelanins are formed from Quinines and free radicals. Fungal melanins are usually complex pigments which are produced by two different synthetic pathways, known asthe DHN (1,8-dihydroxynaphthalene) and L-DOPA (L-3,4-*dihydroxyphenyl-alanine*) pathways, depending on the species (Wheeler and Bell, 1988). The DHN pathway of melanin biosynthesis is found to be very common in the fungal kingdom (Howard and Ferrari, 1989). When compared to terrestrial fungi the marine fungi are expected to produce novel and potentially active metabolites and there are scarce reports regarding melanin from marine habitats especially hypersaline environments. The *Hortaea werneckii* melanin showed antibacterial activity against life threatening bacterial pathogens like *Vibrio parahaemolyticus, Klebsiella pneumoniae* and *Salmonella typhi* (Rani *et al.,* 2013).

Melanin mostly offers protection from UV light and ionizing gradiations, and resistance to heat or cold, and activity of inorganic antimicrobial compounds, such as silver nitrate (Garcia *et al.,* 2001). Such a phenomenon was also discovered in an arctic lichene *Cetraria islandica*, where fungal melanin produced in the sun greatly lowered the cortical transmittance for UV-B (Nybakken *et al*., 2004). It was reported that crude melanin from *Streptomyces* sp. showed antibacterial activity against *Escherichia coli* and *Lactobacillus vulgaris* (Vasanthabharathi *et al*., 2011). Apart from the antimicrobial activity, the antibiofilm activity of melanin was also reported (Bin *et al.,* 2012). *Auricularia auricula*, which is commonly known as 'tree-ear', is a species of edible mushroom found worldwide. From ancient times, this mushroom has been used widely in Chinese cuisine and is well known for its pharmaceutical effects in folk medicine. It has been reported to have many biological activities including antitumor (Mizuno *et al.,* 1995), hypocholesterolemia (Cheung, 1996), hypoglycemic (Takeujchi *et al.,* 2004), antioxidant (Acharya *et al.,* 2004) and anticoagulant (Yoon *et al.,* 2003) activities. These potent medicinal properties are mediated mostly by non-starch polysaccharide components, especially beta-glucans (Zhang *et al.,* 1995). Nowadays, there is considerable interest in the exploitation of this

type of fungi. Most recently, Zhu *et al.,* (2011) reported that the extracted pigments could effectively inhibit the production of violacein, a quorum-sensing (QS)-regulated behavior in *Chromobacterium violaceum* CV026.

Thus the melanins from marine bacteria could be more explored for its antibiofilm activity against the bacterial food pathogens which will pave the way for the future use of melanins in the food industry in a large scale.

2.8.4 Bacteriocins

Bacteriocins are antibacterial proteins or peptides produced by bacteria that kill or inhibit the growth of other bacteria. Though these bacteriocins are produced by Lactic acid bacilli found in numerous fermented and non-fermented foods, nosing is currently the only bacteriocin widely used as a food preservative. Many bacteriocins have been characterized both biochemically and genetically. Although there is a basic understanding of their structure, function, biosynthesis, and mode of action, many aspects of these compounds are still unknown (Cleveland *et al.,* 2001).The bacteriocins were first characterized in Gram-negative bacteria. The colicins of *E.coli* are the mostly studied bacteriocins (Lazdunski, 1988). Colicins constitute a diverse group of antibacterial proteins which can kill closely related bacteria by various mechanisms such as either inhibiting cell wall synthesis, or permeabilizing the target cell membrane, or by inhibiting RNase or DNase activity. They are ribosomal synthesized and kill closely related bacteria (Klaenhammer, 1993).Among the Gram-positive bacteria, the lactic acid bacteria have been comprehensively exploited as reservoir for antimicrobial peptides with food applications (Miteva *et al.,* 1998; Cai *et al.,* 1997; Hechard *et al.,* 1992). Bacteriocins are classified into class I (Ia & Ib), class II (IIa & IIb) and class III; several of these are used in food industry since they are regarded as safe, and have potential as effective natural food preservatives.

Since bacteriocins are isolated from foods such as meat and dairy products, which normally contain lactic acid bacteria, they have been consumed unknowingly for centuries. A study of 40 wild-type strains of *Lactococcus lactis* showed that 35 produced nisin (Hurst, 1981). Nisin is approved for use in over forty countries and has been in use as a food preservative for over fifty years. It is however not considered 'natural' when it is applied in concentrations that exceed what is found in foods naturally fermented with a nisin producing starter culture.

The term 'natural' is compromised when the bacteriocin is produced by genetically modified bacteria. Though nisin is currently the only bacteriocin approved for use in the United States, many bacteriocins produced by members of the LAB have potential application in food products like pediocin (Cutter and Siragusa, 1998; Schlyter *et al.,* 1993).

The chemical composition and the physical conditions of food can have a significant influence on the activity of bacteriocin. For example, Nisin is 228 times more soluble at pH 2 than at pH 8 (Liu and Hansen, 1990).Since lactic acid bacteria are commonly used as starter cultures in food fermentations, investigators have explored the use of bacteriocin producers as starter cultures. In some cases, natural bacteriocin producers, such as *Lactobacillus plantarum*, *Pediococcus acidilactici* and *Enterococcus faecalis*, are used in such studies. Similarly, the surviving number of *L.monocytogenes* found in a naturally contaminated salami sausage decreased when the product was inoculated with the bacteriocin producer *Lactob. Plantarum* MCS1 (Campanini *et al.,* 1993). Most commercial starter cultures do not produce bacteriocins; however, a few bacteriocin-producing meat starter cultures are sold today.

Bacteriocins have been directly added to foods such as cheese to prevent *Clostridium* and *Listeria*. Nisin inhibits the outgrowth of *C. botulinum* spores in cheese spreads (Wessels *et al.,* 1998) and is approved as a food additive in the United States for this purpose (U.S. Food and Drug Administration, 1988).Since there are difficulties using nisin in raw meat applications, the use of other bacteriocins have been examined. Leucocin A, enterocins, sakacins and the carnobactericins A and B prolong the shelf life of fresh meat. The most promising results in meats were obtained using pediocin PA-1 (which has an amino acid sequence identical to AcH). Produced by *P. acidilactici*, pediocin PA-1 suddenly reduces the number of target organisms (Nielsen *et al.,* 1990) but is not yet an approved food additive in the United States. Used alone (Coventry *et al.,* 1995) orin combination with diacetate (Schlyter *et al.,* 1993), pediocin PA-1 is active against the foodborne pathogen *L. monocytogenes* and *Lactob. curatus*, a spoilage organism.

In the *Lactob.curatus* study, however, pediocin PA-1 is less active than nisin in the model meat system, and neither preservative is effective when used in a commercially manufactured meat product (Coventry *et al.,* 1995). Pediocin

AcHPA-1 had successfully controlled the growth of *L.monocytogenes* in raw chicken in another study (Goff *et al.,* 1996). Pediocin binds to raw chicken, but not to be cooked. However, when raw chicken with applied pediocin was cooked, activity was retained. It is suggested that pediocin should be applied to chicken before cooking for maximum effectiveness.

A comprehensive literature search shows that most of the information regarding the safety of nisin was collected over 20 years ago (Fowler, 1973). It is likely that more information regarding nisin safety exists, but is not available to the public. Patents claiming nisin as an antibacterial agent in food, personal care products or for medical applications do not provide new data, and instead rely on previously published information (Blackburn *et al.,* 1998). When patents for new bacteriocins are submitted, often full toxicological data is not complete (Vedamuthu *et al.,* 1992).Their synthesis and mode of action can distinguish them from clinical antibiotics. Additionally, organisms that show resistance to antibiotics may not generally be cross-resistant with bacteriocins, and unlike antibiotic resistance, bacteriocin resistance usually cannot be determined genetically. Thus bacteriocins are not only effective, but are also proved to be safe for use in the food supply.

2.9 Biocontrol of biofilms using bacteriophages

Bacteriophages, which are commonly known as phages are the natural viral pathogens of bacteria. The first application of phages was in the early 20th century for treatment of bacterial infections in Eastern Europe, and have been shown to decrease biofilm formation (Curtin and Donlan, 2006; Merril *et al.,* 2003). The existence of bacteriophages was first reported in India. Ernest Hankin, a British bacteriologist working in India, reported the existence of unidentified substance (which passed through fine porcelain filters and was heat labile. responsible for marked activity against the bacterial pathogen *Vibrio cholerae* in the waters of the Ganges and Yamuna rivers in 1896 (Hankin, 1896). After almost 20 years, Frederick Twort, a medically trained bacteriologist from England reported a similar phenomenon and put forth a hypothesis that it may have been due to among other possibilities, a virus (Twort, 1915). But the final credit of officially discovering phages goes to Felix d'Herelle, a French-Canadian microbiologist at the Institute Pasteur in Paris (d'Herelle, 1917). The term

"bacteriophage" was proposed by d'Herelle that comes from the words "bacteria" and "phagein" which in Greek means to eat or devour.

Initial classification of bacteriophages was based only on host specificity (Nelson, 2004). The advent of transmission electron microscopy (TEM) enabled scientists to classify phages based on their morphology (Luria *et al*., 1943). Molecular characteristics of phages like size and type of nucleic acid (single stranded DNA, double-stranded DNA and single-stranded RNA) were also taken into account with the advances in the field of molecular biology (Thomas and Abelson**,** 1966). Present day classification by International Committee on Taxonomy of Viruses (ICTV) is derived from the scheme proposed by Bradley (1967) using gross morphology and nature of their nucleic acid. Over the years many new families were added and currently includes one order, 17 families and three "floating" groups (Ackermann, 2007, 2009). The ICTV classification method as is currently being re-evaluated, since it ignores the vast amount of available genome sequence data which can occasionally cause a contradiction in the classification criteria adopted (Ceyssens, 2009) and also because new phages are discovered daily and the ICTV lags in its classification schedule (Ackermann, 2007). The overview of the phage families is given in the table 2.3 and various phage morphotypes are listed in figure 2.6.

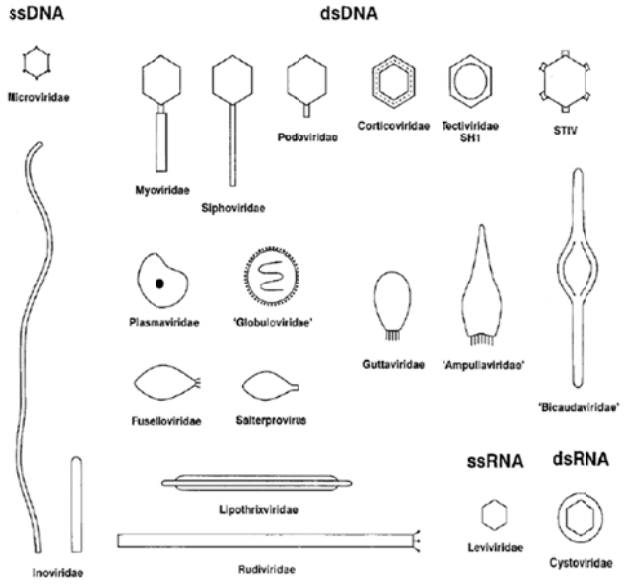


Fig.2.6. Phage morphotypes (Adapted from Ackermann, 2009)

Table 2.3. Overview of phage families (ICTV Classification)

Order	Family	Morphology	Nucleic acid	Examples
Caudovirales	*Myoviridae*	Nonenveloped, contractile tail	Linear dsDNA	T4 phage, Mu, PBSX etc
	Siphoviridae	Nonenveloped, noncontractile tail (long)	Linear dsDNA	λ phage, T5 phage, phi etc
	Podoviridae	Nonenveloped, noncontractile tail (short)	Linear dsDNA	T7 phage, T3 phage, P22 etc
Ligamenvirales	*Lipothrixviridae*	Enveloped, rod-shaped	Linear dsDNA	Acidianus filamentous virus 1
	Rudiviridae	Nonenveloped, rod-shaped	Linear dsDNA	Sulfolobus islandicus rod-shaped virus 1
Unassigned	*Ampullaviridae*	Enveloped, bottle-shaped	Linear dsDNA	ABV
	Bicaudaviridae	Nonenveloped, lemon-shaped	Circular dsDNA	ATV
	Corticoviridae	Nonenveloped, isometric	Circular dsDNA	PM2
	Cystoviridae	Enveloped, spherical	Segmented dsRNA	φ6
	Fuselloviridae	Nonenveloped, lemon-shaped	Circular dsDNA	SSV1
	Globuloviridae	Enveloped, isometric	Linear dsDNA	PSV
	Guttaviridae	Nonenveloped, ovoid	Circular dsDNA	SNDV
	Inoviridae	Nonenveloped, filamentous	Circular ssDNA	M13
	Leviviridae	Nonenveloped, isometric	Linear ssRNA	MS2, Qβ
	Microviridae	Nonenveloped, isometric	Circular ssDNA	ΦX174
	Plasmaviridae	Enveloped, pleomorphic	Circular dsDNA	L2
	Tectiviridae	Nonenveloped, isometric	Linear dsDNA	PRD1

Lytic and lysogenic cycles are the two different methods of viral replication. While they are different, they can be interchangeable or the replication can involve both methods in separate phases.In short, in the lytic cycle, the virus hijacks the infected cell and finally destroys it. The lytic cycle mostly occurs in virulent viruses. The symptoms from a viral infection occur when the virus is in a lytic state.

In the lysogenic cycle, the viral DNA or RNA enters the cell and integrates into the host DNA as a new set of genes called prophage. That is, the viral DNA becomes part of the cell's genetic material. No progeny particles, like in the lytic phase, will be produced. Each time the host cell DNA chromosome replicates during cell division, the passive and non-virulent prophage replicates too. This may alter the cell's characteristics, but it does not destroy it. There are no viral symptoms in the lysogenic cycle; it occurs after the viral infection is over. But the viral DNA or RNA remains in the cell and it would remain there permanently. However, if the prophage undergoes any stress or mutation or is exposed to UV radiation, the viral lysogenic cycle can change into the viral lytic cycle. In which case, there will be symptoms of a new viral infection.Some viruses first replicate by the lysogenic cycle and then switch to the lytic cycle. The lytic phages are found to have more applications in the food industry as biocontrol agents since both the host and phage cells are finally destroyed ensuring the safety as additives in the common foods to prevent bacterial spoilage (Hagens and Loessner, 2010).

Control using the phages is an area which can be explored for effective biocontrol. In recent years, there has been an increase of bacterial resistance to one or more antimicrobial agents. This has drawn attention to phage therapy as a therapeutic alternative for killing pathogenic bacteria. The procedure for using phages as therapeutic agents is very simple asphages have many advantages over antimicrobial agents such as: specificity against a host or host range not affecting any normal microflora; self-replication capability at the site of infection, as long as the host bacteria is present; no serious side effects have ever been reported till present, the production is simple and inexpensive and phages are environmentally friendly (Hughes *et al.,* 1998).

Unlike chemical-based antimicrobial agents that cause corrosion, phages are a suitable substitute (Goldman *et al.,* 2009), thus it has also been proposed as a

biofilm control method (Curtin and Donlan, 2006).Bacteriophage spray treatment was suggested to be an alternative to dipping, brushing, or sponging of the spinach harvester blade on a chlorine solution according to its compatibility with harvest sanitation practices (Patel *et al.,* 2011).

The high specificity of phage may make them particularly useful tools in the selective removal of a potentially pathogenic species within a mixed species biofilms. The action of combined treatments of disinfectant and phage enzyme as a potentially effective control strategy was also investigated. The use of phage enzymes in conjunction with disinfectants could provide an effective means of biofilm removal. The polysaccharide depolymerase affords better access for disinfection, and consequently, better removal and eradication. This may be used as a semi-specific treatment in the control of biofilm formation (Hughes *et al.,* 1998).

It was documented that the phage components and their assembly synthesis vary according to the host bacterial growth rate and the amount of protein-synthesized during the time of infection; however, it was also reported that even under a glucose limited chemostat, T4 phage could affect *E. coli* biofilms (Corbin *et al.,* 2001). In a study on the effect of the phagephilBB-PF7A on *P. fluorescens* biofilms, the important role of convection mechanism was discussed. It was said that the biofilm cellslysis is more efficient under static conditions than the dynamic conditions. It was also reported that flBB-PF7A was remarkable biological agent according to its biofilm cells lysing capability in a markedly rapid time (Sillankorva *et al.,* 2008).The same phage was also used to control the dual species biofilm of *P. fluorescens* and *Staphylococcus lentus,* and accounted for a dramatic decrease in the target bacteria cells *(P. fluorescens*). Surprisingly, it was proved that phages can be used to efficiently reach and lyse their target bacterium in both single and dual species biofilms notwithstanding the presence of a non-susceptible host (Sillankorva *et al.,*2010).

Cerca *et al.,* (2007) studied the susceptibility of *S. epidermidis* planktonic cells and biofilms according to the lytic action of *Staphylococcus* bacteriophage K. The phage K lysis efficiency depends on the bacterial growth phase. This was also found to be true in the research of Sillankorva *et al.,* (2004 a). Fluorescence Correlation Spectroscopy was also used to study the diffusion and reaction of bacteriophages inside biofilms; the results indicated that bacteriophages can

infiltrate different biofilm complexes, and that generally, they are immobilized, amplified, and released by a lytic cycle in the biofilm and interact with their own specific binding sites on the hosts, although the lytic activity was not observed (Briandet *et al.,* 2008).According to Tait *et al.*,(2002), phages and bacteria can steadily co-exist in biofilms, thus a mixture of phages and polysaccharide depolymerases and disinfectant was suggested for a better biofilm control. To address this challenge, bacteriophages can be engineered to express specific biofilm-degrading enzyme, which would be a good asset in overcoming the challenges in controlling biofilms. The engineered phages were reported to noticeably mitigate bacterial cells in biofilms (approximately 99.99% removal), as well as the biofilm complex (Lu and Collins, 2007).

The use of phage or phage products in food production has recently become an option for the food industry and accepted as a novel method for biocontrol of unwanted pathogens, enhancing the safety of especially fresh and ready-to-eat food products (Hagens and Loessner, 2010). There are so many evidences supporting the use of bacteriophages as food additives or preservatives thereby controlling the rate of biofilm formation:

In accordance with the regulatory issues associated with the use of phages for treatment of bacteria in foods, a mixed *Listeria* phage preparation (www.intralytix.com) received the approval for use as a food additive in the production of ready-to-eat meat and poultry products, while another phage preparation comprising a virulent single *Listeria* phage received the highly desirable GRAS (generally recognized as safe) status for its use in all food products (Gerner–Smidt *et al*., 1993).

Phage preparations active against *E. coli* and *Salmonella* are also available while some have approval for being sprayed, showered, or nebulized on cattle and chickens respectively, prior to the slaughter of the animals (Johnson *et al* 2008;). Phage preparations which are active against tomato and pepper pathovars of *Pseudomonas putida,* developed for treatment of plants against bacterial spot diseases, have been approved for use by the US Environmental Protection Agency (EPA) (Sulakvelidze *et al* .,2005).

These recent developments in the field highlight the fact that, besides the use of phage for direct addition to food, much effort has been done for phage-based control of pathogens that can colonize plants or animals used in food

production. It can be expected that many more phage products would appear on the market in the near to mid-term future. The safety of using bacteriophages in preserving the food products is assured because:

Phages are Non-Toxic:

Phages are found to be highly specific and can infect only a very limited range of host bacteria. All available evidences indicate that their oral consumption (even at high levels) is entirely harmless to humans. Safety studies have been performed for example with the *Listeria*-phage P100, in which rats were fed with high doses of phages with no measurable effects compared to the control group (Carlton *et al*., 2005) A study with *E. coli* phages both in mice and also in human volunteers also showed no significant effects on the test subjects (Chibani *et al*., 2004; Bruttin *et al*., 2005)

Phages are Ubiquitous in Foods

Apart from the environmental sources, we are constantly exposed to contact with phages through their food. Phages are associated with bacteria and any foodstuff that has not undergone much extensive processing, mostly in fermented food which are having especially high phages numbers infecting the fermentation flora. Fresh vegetables can be also a rich source of bacteriophages. Several examples can be cited for its proof. Some of them are listed below.

Fermented cabbage (also called Sauerkraut) is a good source of phages, with one study describing 26 different phages isolated from commercial Sauerkraut fermentation units (Yoon *et al*., 2003; Lu *et al*., 2003). Swiss Emmental cheese yielded phages against *Propionibacterium freudenreichii* at levels of up to 7×10^5 PFU/g (Gautier *et al*., 1995). In Argentina, phages against thermophilic lactic acid bacteria had been isolated from dairy plant samples at numbers of up to 10^9 PFU/mL, though these were from batches that failed to achieve the desired fermentation levels (Suarez *et al.*, 2002).

Phages were also being isolated from non-fermented foods. *E. coli* phages had been recovered from fresh chicken, pork, ground beef, mushrooms, lettuce, raw vegetables, chicken pie, and other delicatessen foods, with counts as high as 10^4 phages per gram (Allwood *et al.*, 2004). *Campylobacter* phages had been

isolated at levels of 4 x 10^6PFU from chicken (Atterbury *et al*., 2003). *Brochothrix thermosphacta* phages were isolated from beef (Greer, 2005).

Desirable Properties of Food-Applied Phages (Hagens and Loessner, 2010)

In general, phages suitable for biocontrol of pathogens in food should have the properties like broader host range (infecting members of the target species and/or genus), strictly lytic (virulent), could propagate on non-pathogenic host, their complete genome sequences must be known, must be non transducing, absence of any genes encoding pathogenicity associated or potentially allergenic proteins, oral feeding studies should show no adverse effects, GRAS approval for use in foods, sufficiently stable for storage and application and also amendable to scale up for commercial production.

There are different mechanisms by which the bacteria may resist phages thereby creating hindrance to the control of biofilm in foods using phages. These include (i) Adsorption resistance, which results in reduced interaction between phage and bacterium or "restriction," where bacteria live but phages die (ii) abortive infections, where both phage and bacterium die.

But the phages also evolve different mechanisms to survive and escape the host immune system. Adsorption resistance can result from phage-encounter blocks or barriers (e.g., capsules) as well as receptor modification or loss, with the latter also referred to as envelope resistance or surface exclusion Restriction mechanisms include phage-genome uptake blocks, superinfection immunity, restriction modification, and CRISPR(Clustered Regularly Interspaced Short Palindromic sequences), all of which function post phage adsorption but prior to terminal phage takeover of host metabolism.

2.10 Hurdle technology

Hurdle technology is a combination of two or more different control techniques which are proved effective. However, in order to achieve an effective treatment, the right combination is required. It was shown that the combination treatment of NaClO with UV irradiation had a better reduction of foodborne pathogens in food than single treatment (Ha and Ha, 2011). DeQueiroz and Day (2007) studied the antimicrobial activity and effectiveness of a combination of NaClO and hydrogen peroxide (H_2O_2) in killing and removing *P. aeruginosa*

biofilms from the surfaces, with increased reduction in the cell number with short exposure time. The synergistic effect of a combined treatment of biofilms with H_2O_2 and UV was 10 -fold more effective than the single use of H_2O_2 (Vankerckhoven *et al.,* 2011). Schurman *et al.,* (2001) tested the effectiveness of H_2O_2 and demonstrated that its efficacy was enhanced when there was an increase in the temperature. Moreover, an increase in organic acid concentration can also increase its efficiency. Additionally, the synergistic effect of ozone and ultrasound was also proved to be efficient for biofilm cell reduction. The amount of cells eradicated was only slightly more compared to that when ozone was used alone. (Patil, 2010).Moreover, the efficiency of antibiotics alone and synergizing with lytic bacteriophage in the removal of old *Klebsiella pneumonia* biofilms was examined. The observations revealed that phages can be surprisingly capable in the removal of older biofilms on account of their depolymerase. However, the eradication of biofilms was improved when a bacteriophage was used with an antibiotic (Verma *et al.,* 2010). Hurdle technology is thus a very promising new approach in controlling biofilms in the food industry.

Pathogenic microorganisms in biofilms formed in different food- industry settings are a source of food contamination. As the demand for fresh, RTE and processed foods increases, many studies are required to address the biofilm removal and disinfectant efficacy in food industries. Even though conventional control strategies are still used and developed, a more economical and ecofriendly control strategy is indispensable to satisfy the need of industrial food safety. It should also be taken into account that disinfection method shall provide a desirable cost effective result and not cause any adverse effect on human health as well as the environment. Therefore, it is highly recommended to use hurdle technology since the synergistic effect helps to reduce materials and energy consumption. Nevertheless, inhibiting biofilms and quorum sensing by natural antimicrobials would also be an alternative to combat the biofilms.

Chapter 3

Screening and characterization of food pathogens with biofilm forming capability from various food samples

3.1 Introduction

Biofouling in the food industry is cause for grave problems; impeding heat flow across surfaces, increasing fluid frictional resistance and corrosion rates at the surfaces, all leading to energy and product losses. Biofilms due to spoilage and pathogenic microflora on surfaces of food like poultry, meat and in processing environments also pose considerable problems of cross contamination and post-processing contamination. Therefore in the context of food hygiene, biofilms are of considerable interest, as they increase contamination and risk to public health (Zottola and Sasahara, 1994).

The microbes involved in biofilm formation and health risks include bacteria belonging to the genera *Vibrio, Salmonella, Pseudomonas, Listeria, Bacillus, Escherichia, Clostridium,* to name a few. With the emergence of resistance in pathogenic bacteria to traditional antibiotics, development of alternative control measures gained momentum. In addition, microorganisms produce saccharolytic, proteolytic, pectinolytic and lipolytic enzymes, whose metabolic end products are associated with food spoilage and poisoning. Thus the food industry faces multitude of challenges to keep products safe and free of pathogenic microorganisms for the consumers and also to augment product shelf life (Costerton *et al*., 1994; Melo *et al*., 1992).

The resistance of biofilm producers to the commonly used antibiotics is a great challenge that stands as an unfavourable factor for their biocontrol. Most reported to be multiple drug resistant due to the persisters, which are mostly survivors of extreme conditions found in every biofilm. The usual suspects of the biofilm resistance include different factors like restricted penetration of antimicrobials into a biofilm, decreased growth rate, and expression of possible resistance genes. Alone or in combination, these factors are useful in explaining biofilm survival in a number of cases (Gilbert *et al.*, 1997).

This chapter deals with the screening of strong biofilm producing bacteria from the foods available in local markets at Kochi, Kerala and their further characterization by molecular identification, exoenzyme profiling and antibiotic resistance profile.

3.2 Materials and Methods

3.2.1. Screening for bacterial food borne pathogens from different food items

The food samples like beef, cheese, raw milk, pasteurized milk, curd, chilly powder, turmeric powder, coriander powder, soft drinks, fresh fish, dried fish and dried prawn collected from the local stores and markets in Kochi, Kerala were analyzed using standard plate assay. 1 g of sample was serially diluted in10 mL of sterile distilled water, 0.1 mL of each dilution was plated on nutrient agar (HiMedia, Mumbai, India) plates using spread plate technique. The isolated bacterial colonies were picked and preserved in nutrient slants at 4^{O}C. These bacterial isolates were tested for their biofilm forming ability using Congo red assay and microtiter plate assay

3.2.2. Qualitative analysis for the biofilm producers by Congo red assay

The qualitative analysis for biofilm producers was by Congo red assay method (Freeman *et al.,* 1989). The isolates obtained were spotted on to Congo red agar (Appendix- 1) and incubated at 37°C for 24 h. After incubation, the plates were observed for black precipitated colonies produced by reaction of the strong slime with the media components; and the indicator congo red will be a sign of the colour variation due to spotted colonies. Strong biofilm producers tend to show dark black coloured colonies, while the intensity of the colour reduces with the decrease in strength of biofilm production. The method cannot be stated as reliable since there is possibility of false positive results (Oliveira and Maria, 2010) and hence need to be confirmed by quantification by microtiter plate assay.

3.2.3. Quantification of biofilm formation by pathogens using microtiter plate assay

Quantification of biofilm formation by pathogens was by using microtiter plate assay (Rode *et al.*, 2007).

The wells of a sterile 96 well polystyrene microtiter plates were filled with 230 µL of tryptone soy broth (TSB) (HiMedia, Mumbai, India). 20 µL bacterial cultures (OD_{600} =1) were added into each well separately, with triplicates for each bacterial culture; and incubated aerobically for 24 h at 37°C. Negative control included only TSB. The contents of the plates were poured off, wells washed 3 times with phosphate buffer (0.01 M, pH 7.2) and the attached bacteria were fixed with methanol. After 15 min, the plates were decanted, air dried and stained with of 1% crystal violet for 5 min. The excess stain was rinsed under running tap water. After air drying, the dye bound to adherent cells was extracted with 33% (V/V) glacial acetic acid per well and the absorbance was measured at 570 nm using a UV-VIS spectrophotometer (Schimadzu, Japan). Based on the absorbance (A_{570}) they were graded A=Ac= No biofilm producers; Ac< A= Weak biofilm producers; 2Ac<A= Moderate biofilm producers; 4Ac<A= Strong biofilm producers; where cutoff absorbance Ac was the mean absorbance of the negative control.

All tests were conducted and interpreted thrice independently and statistically analysed (Christensen *et al*., 1988; Stepanovic *et al*., 2007). All data from biofilm quantitative assays were expressed as mean± SD with each assay conducted in triplicates. The statistical significance of associations between variables in different categories of isolates (Strong, moderate and weak) was calculated using Kruskal- Wallis test one way analysis of variance test, which is an extension of Mann Whitney *U* test, for more than two groups using StatsDirect statistical software (version 3.0, Cheshire, UK) computer program (Appendix-5).

Finally, to plot the graphs, the percentage of reduction in biofilm formation was calculated as:

% in biofilm reduction = (OD of Control - OD of Test/ OD of Control) X 100

3.2.4. Molecular characterization of biofilm producers

Genomic DNA was isolated and purified (Ausubel *et al*., 1987). A portion of the 16S rDNA was amplified using a primer pair for16S rDNA (Shivaji *et al*., 2000).

The sequences for the primer pair is as follows:

Forward primer - 5' AGAGTTTGATCCTGGCTCAG 3'
Reverse primer - 5'ACGGCTACCTTGTTACGACTT 3'
The conditions used for PCR amplification are listed in Table 3.1

Table 3.1 The conditions used for PCR amplification of the 16S rDNA gene

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

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

The identity of the sequences was determined by comparing the 16S rDNA sequence with the sequences available in the NCBI nucleotide databases using BLAST (Basic Local Alignment Search Tool) algorithm (Altschul *et al*., 1990). A phylogenetic tree was also constructed for the biofilm producers by neighbor joining method (Saitou and Nei, 1987) using the MEGA 6 software (Tamura *et al*., 2007).

3.2.5. Antibiotic sensitivity tests

All strong biofilm producers were tested for antibiotic sensitivity in accordance with the Kirby- Bauer method (Bauer *et al*., 1966), with 12 antibiotics (HiMedia, Mumbai) belonging to different classes, namely ampicillin (5 µg/disc), azithromycin (15µg/disc), cefixime (5 µg/disc), cefuroxime (30 µg/disc), ceftriazone (15 µg/disc), chloramphenicol (30 µg/disc), ciprofloxacin (5 µg/disc),

gentamicin (10 µg/disc), nalidixic acid (30 µg/disc), norfloxacin (5 µg/disc), tetracycline (30 µg/disc), and trimethoprim (5 µg/disc). The results were interpreted as per the manufacturers' instructions shown in table 3.2.

Table 3.2 Zone Size Interpretative Chart for antibiotics from HiMedia as per CLSI

Antibiotic	**Concentration per disc (µg/disc)**	***Range (**indicate zone of growth inhibition in mm**)**		
		R	**I**	**S**
Ampicillin	5	≤11	12-14	≥15
Azithromycin	15	≤13	14-17	≥18
Chloramphenicol	30	≤12	13-17	≥18
Cefixime	5	≤15	15-17	≥18
Cefuroxime	30	≤14	15-17	≥18
Ceftriaxone	15	≤13	14-20	≥21
Ciprofloxacin	5	≤15	16-20	≥21
Gentamicin	10	≤12	13-14	≥15
Nalidixic acid	30	≤13	14-18	≥19
Tetracyclin	30	≤14	15-18	≥19
Trimethoprim	5	≤10	11-15	≥16

From the 20 biofilm producers, the strongest biofilm producers with high antibiotic resistance profile were selected for further study. Their multiple antibiotic resistance (MAR) Indices were calculated.

MAR index for each isolate is calculated as: - No. of antibiotics to which the isolate was resistant/ Total no. of antibiotics to which the isolate was subjected (Subramani & Vignesh, 2012).

3.2.6. Enzyme profiling of the biofilm producers

The qualitative assessment of enzyme activities including amylases, proteases, cellulases and lipases was using starch agar, skimmed milk agar, carboxymethyl cellulose agar and tributyrin agar respectively was done, as a part of characterization of the selected strong biofilm producers, and consequently for the determination of their ability to degrade the nutritional substances in the food samples.

3.2.6.1. Amylases activity

For detecting amylase activity, organisms were patched onto 0.5% starch agar plate (Appendix-1) and incubated for 24 h at room temperature. Gram's Iodine solution (Appendix-1) was flooded onto the inoculated plate. A clear zone around the colony indicates that amylase has hydrolysed the starch thereby giving no blue colour on reaction with iodine (Murray *et al*., 2007).

3.2.6.2. Proteases activity

The test organisms were patched on 10% skimmed milk agar plate (Appendix -1) and incubated overnight at room temperature. Clear zones produced around the colony indicate that the casein in the medium has been hydrolysed. No clearance of the medium is seen as the negative test (Sivakumar *et al*., 2012).

3.2.6.3. Lipases activity

The test organisms were patched onto 1% tributyrin agar plate (Appendix -1) and incubated the plates for 48 - 72 h at room temperature. Clear zones around the colony indicate the presence of lipases (Karnetova *et al*., 1984).

3.2.6.4. Cellulases activity

For cellulolytic activity, the test organisms were patched onto 0.5% carboxy methyl cellulose agar (Appendix -1) and incubated for 48 h at room temperature. The plates were flooded with 0.1% Congo red solution and kept for 20-30 min with intermittent shaking, drained flooded with 1N NaCl solution and kept for 15 min. A yellow colour around the colony leaving the other portion of the plate red, indicates positive reaction (Eggins & Pughg, 1962)

3.3 Results

3.3.1. Screening for bacterial food borne pathogens from different food items

Several food sample types were screened for bacterial food borne pathogens using standard plate count assay. This yielded thirty six isolates, which were screened for their ability to produce biofilms. The isolates were subjected to qualitative and quantitative assays for biofilm production. Annexure-3 lists all the 36 isolates from different food samples and the food sources from which they were isolated along with the number of isolates obtained from each source.

3.3.2. Qualitative analysis for the biofilm producers by congo red plate assay

Qualitative analysis for biofilm production involved Congo red plate assay, which helped to segregate the strong, moderate and weak biofilm producers. Twenty of the thirty six isolates showed intense black coloured colonies, while 10 produced lighter black coloured colonies. Only one showed very light black precipitate, while 5 others did not produce any black colour. According to the intensity of the black colour, the isolates were categorized as strong, moderate and weak. The figure 3.1 shows the appearance of biofilm formers on Congo red agar. The result was confirmed by the quantification assay by microtiter plate.

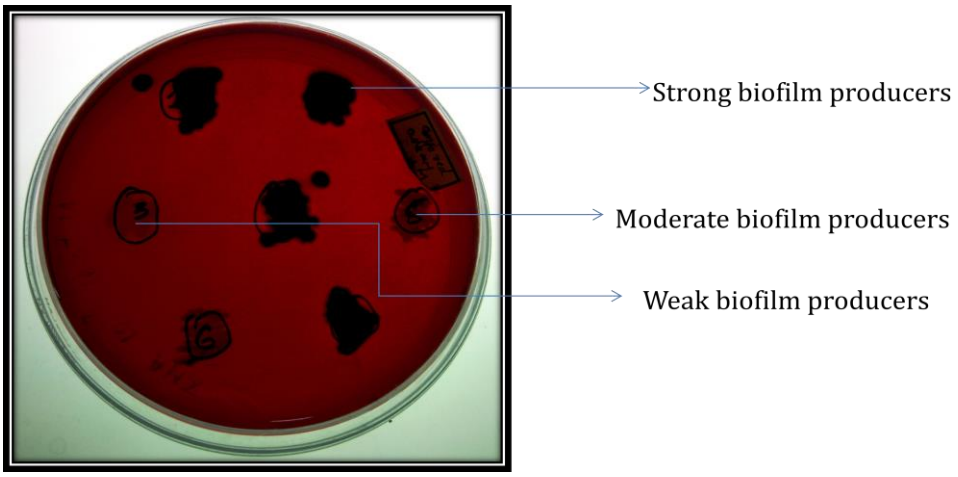


Figure 3.1 Congo red agar plate showing black coloured colonies of biofilm producers

3.3.3. Quantification of biofilm forming pathogens by microtiter plate assay

After quantification by microtiter palte assay, it was observed that thirty one (86.11%) of the thirty six isolates obtained were biofilm producers. Figure 3.2 shows the microtiter plate for quantification of biofilm producers after the crystal violet staining. In the microplate assay, statistical analysis of the mean crystal violet staining (i.e. optical density values) caused segregation of three significantly distinguishable dissimilar groups (with a confidence level of 99%) with strong, moderate and weak levels of crystal violet staining. Thus based on statistically significant difference between three groups at $P<0.0001$ (seen in the figure 3.2), they were classified as strong, moderate and weak biofilm producers. Figure 3.3 shows the classification or strength of the biofilm production in all the 36 isolates obtained in the form of a pie chart. The figure 3.2 shows varying intensities of

crystal violet, which is indicative of the strength of the biofilm formed. The more intense the color, stronger the biofilm formed. From the figure, the levels of biofilm formed by different food pathogens is evident and can be clearly utilized to differentiate the strong, moderate and weak biofilm producers.

Of the thirty six isolates 55% (n=20) were strong biofilm producers, 28% (n=10) moderate producers while 3 % (n=1) were weak producers. 14% (n=5) did not form biofilm. This is depicted in figure 3.3. The biofilm production at OD_{570} nm by the 20 strong biofilm producers as per microtiter assay is shown in figure 3.4. These 20 strong biofilm producers were selected for further study.

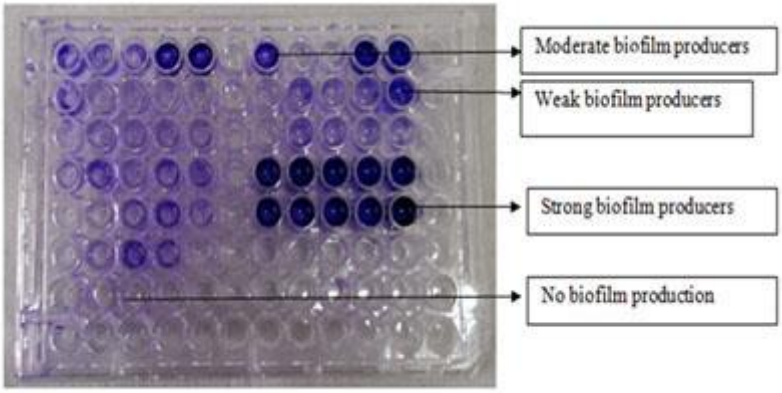


Figure 3.2 Microtiter plate for quantification of biofilm producers after the crystal violet staining (Laxmi and Bhat, 2014)

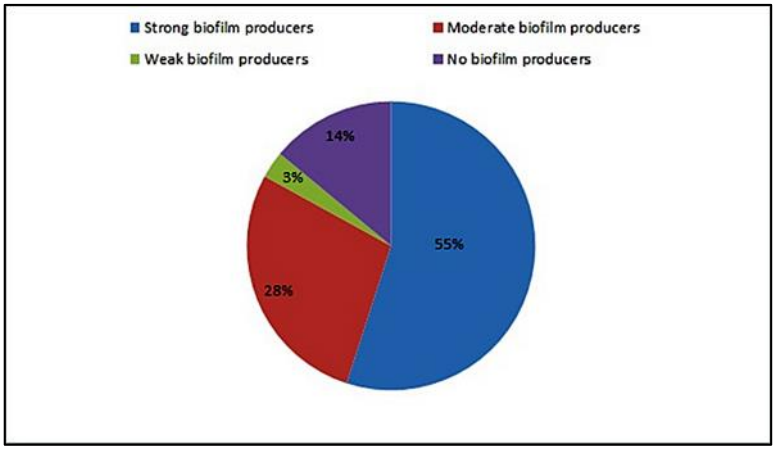


Figure 3.3 Classification of biofilm producers (Laxmi and Bhat, 2014)

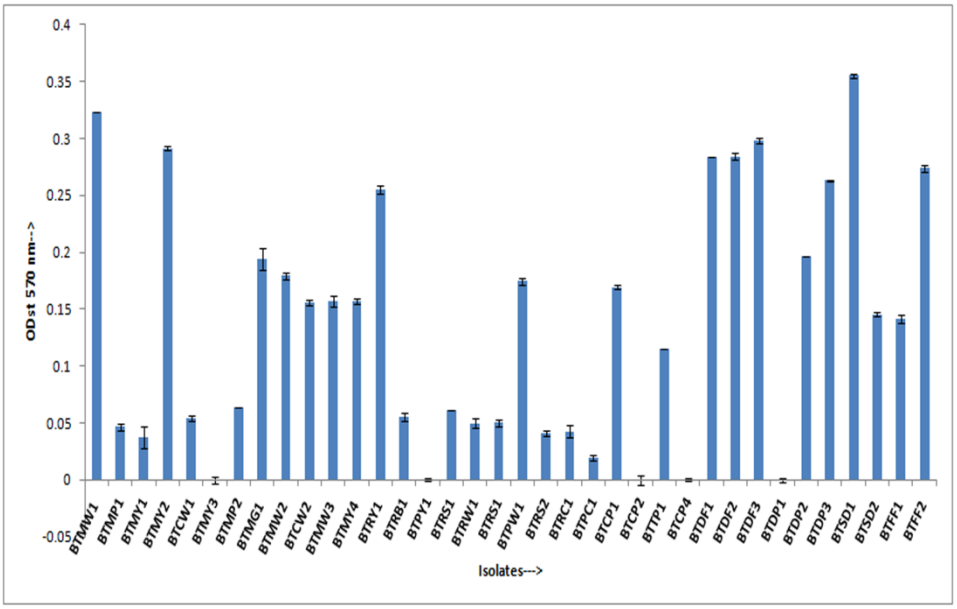


Figure 3.4 Biofilm production by 36 food isolates. Ac- 0.019, 2Ac- 0.039, 4Ac- 0.078. Values < 0.019 do not indicate biofilm producers, values > 0.019 are weak producers. While values between 0.039 and 0.078 are moderate biofilm producers. Values > 0.078 are strong biofilm producers where cutoff absorbance A_c was the mean absorbance of the negative control.

3.3.4. Molecular characterization of biofilm producers using 16S rDNA sequence analysis

The 20 strong biofilm producers were identified by using 16S rDNA sequence analysis.

Genomic DNA was isolated, purified and quantified. The agarose gel electrophoresis of genomic DNA isolated from the twenty isolates was performed is represented in fig.3.5.

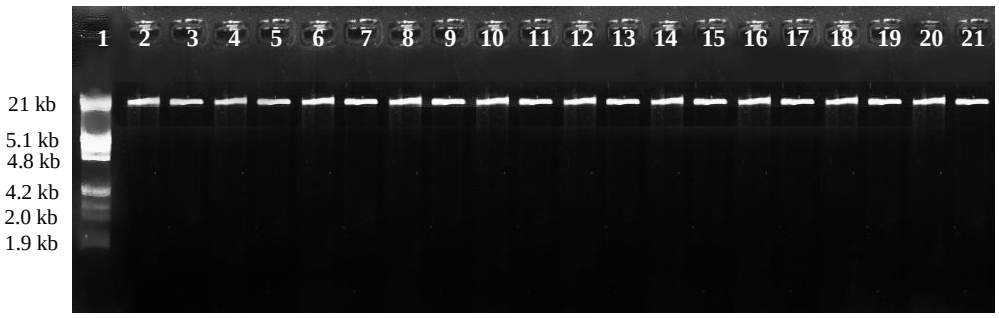


Fig 3.5 Agarose gel electrophoresis of genomic DNA isolated from 20 biofilm producing isolates Lanes 1- Lambda DNA / EcoR1/Hind III/ Double digest,(2-21-DNA) 2-BTMW1, 3-BTMY2, 4-BTMG1, 5-BTMW2, 6-BTCW2, 7-BTMW3,8-BTMY4, 9-BTRY1, 10-BTPW1, 11-BTCP1, 12-BTTP1, 13-BTDF1, 14-BTDF2, 15-BTDF3, 16-BTDP2, 17-BTDP3, 18-BTSD1, 19-BTSD2, 20-BTFF1, 21-BTFF2

PCR based 16S rDNA amplification and sequence analysis thereafter, was used for molecular characterization of the biofilm formers. The agarose gel containing amplified 16S rDNA gene of the biofilm producing isolates is represented in fig. 3.6.

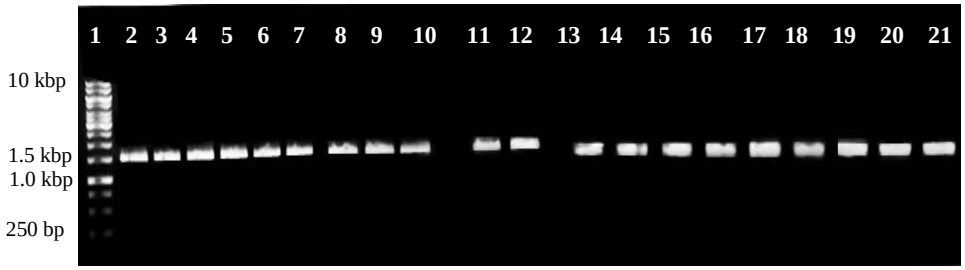


Fig 3.6 Agarose gel showing amplified 16S rDNA gene from biofilm producing isolates Lanes 1- 1kb ladder, 2-21 (16 S rRNA gene amplicons)2-BTMW1, 3-BTMY2, 4-BTMG1, 5-BTMW2, 6-BTCW2, 7-BTMW3,8-BTMY4, 9-BTRY1, 10-BTPW1, 11-BTCP1, 12-BTTP1, 13-BTDF1, 14-BTDF2, 15-BTDF3, 16-BTDP2, 17-BTDP3, 18-BTSD1, 19-BTSD2, 20-BTFF1, 21-BTFF2

Following BLAST the identity of the biofilm formers was determined and the sequence data was submitted to the NCBI database and accession numbers were obtained. Table 3.3 shows the identity of the twenty biofilm producers based on NCBI BLAST analysis. The 16S rDNA analysis revealed that 14 of the biofilm formers were *Bacillus* species, 4 were lactic acid bacteria and one each *Brevibacterium* and *Pseudomonas* species.

Table 3.3 Identity of isolates with biofilm forming ability (Laxmi and Bhat, 2014)

Isolate	Organism	Genbank accession number	Isolate	Organism	Genbank accession number
BTMW1	***Bacillus altitudinis***	KF460551	BTTP1	***Bacillus altitudinis***	KF460561
BTMY2	***Bacillus pumilus***	KF460552	BTDF1	***Brevibacterium casei***	KF573739
BTMG1	***Bacillus altitudinis***	KF460553	BTDF2	***Staphylococcus warneri***	KF573740
BTMW2	***Bacillus pumilus***	KF460554	BTDF3	***Micrococcus luteus***	KF573741
BTCW2	***Bacillus altitudinis***	KF460555	BTDP2	***Micrococcus sp***	KF573742
BTMW3	***Bacillus altitudinis***	KF460556	BTDP3	***Bacillus niacini***	KF573743
BTMY4	***Bacillus pumilus***	KF460557	BTSD1	***Bacillus sp***	KF573744
BTRY1	***Pseudomonas aeruginosa***	KF460558	BTSD2	***Bacillus licheniformis***	KF573745
BTPW1	***Bacillus altitudinis***	KF460559	BTFF1	***Micrococcus luteus***	KF573746
BTCP1	***Bacillus pumilus***	KF460560	BTFF2	***Geobacillus stearothermophilus***	KF573747

This furthermore revealed that most of these strong biofilm producers were also food pathogens. Phylogenetic analysis of the biofilm strains obtained in the study was done to understand their interrelatedness and this is depicted in figure 3.7. The number at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on the Neighbour-Joining analysis of 1000 resampled data sets using MEGA 6 software.

It was observed that the *B.altitudinis* and *B. pumilus* strains grouped together in a single clade as did the three *Micrococcus* sp. The organisms, *Bacillus altitudinis* BTMW1, *B. pumilus* BTMY2, *B. altitudinis* BTMG1, *B. altitudinis* BTMW2, *B. altitudinis* BTMW3, *B. altitudinis* BTPW1 and *B. pumilus* BTMY4

were isolated from meat (beef) sample, *B. altitudinis* BTCW2 *and B. pumilus* BTCP1 were isolated from chicken, *Pseudomonas aeruginosa* BTRY1 from milk, *B. altitudinis* BTTP1 from turmeric powder, *Brevibacterium casei* BTDF1, *Staphylococcus warneri BTDF2 and Micrococcus luteus* BTDF3 from dried fish, *Micrococcus sp* BTDP2 *and B. niacini* BTDP3 from dried prawn sample, *Bacillus sp and B. licheniformis* from soft drink and *M. luteus* BTFF1 and *Geobacillus staerothermophilus* BTFF2 from fresh fish sample.

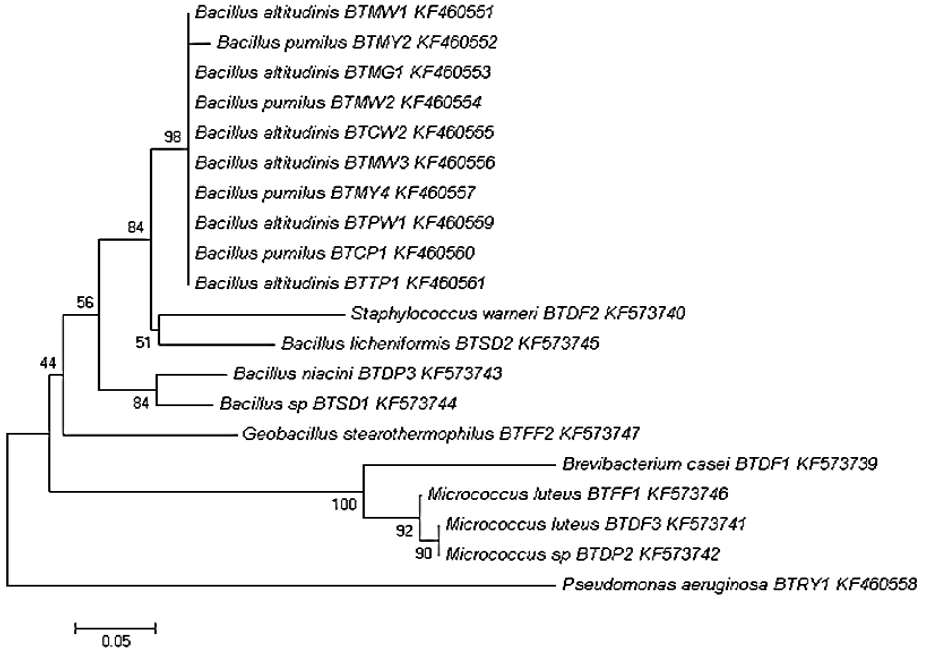


Figure 3.7 Phylogenetic analysis of the biofilm strains (N=20) (Laxmi and Bhat, 2014)

3.3.5. Antibiogram of the strong biofilm producers

Antibiotic Sensitivity Test using 12 antibiotics was done and the antibiogram of the strong biofilm producers is given in table 3.4 (a) & (b).

Table 3.4 (a) Antibiotic sensitivity of isolates 1-10.

Name of the antibiotic	Isolates									
	1	2	3	4	5	6	7	8	9	10
Ampicillin	R	R	R	S	S	S	I	R	S	S
Azithromycin	R	S	R	S	S	S	R	S	S	S
Cefixime	R	R	I	I	R	I	R	R	R	R
Cefuroxime	I	R	S	S	S	S	R	R	R	R
Ceftriazone	R	I	R	S	I	I	I	I	S	S
Chloramphenicol	R	S	R	S	S	S	R	S	I	S
Ciprofloxacin	R	S	R	S	S	S	R	S	S	I
Gentamicin	R	S	R	S	S	S	R	R	I	S
Nalidixic acid	R	I	I	I	S	S	R	R	S	R
Norfloxacin	R	S	I	S	S	S	R	S	S	R
Tetracycline	R	S	R	S	S	S	R	R	S	S
Trimethoprim	I	R	S	S	S	S	R	R	S	I

***S:-Sensitive, I:-Intermediate, R:-Resistant**

*Isolates---1-BTMW1, 2- BTMY2, 3-BTMG1, 4-BTMW2, 5-BTCW2, 6- BTMW3, 7- BTMY4, 8- BTRY1, 9-BTPW1, 10-BTCP1

Table 3.4 (b) Antibiotic sensitivity of isolates 11-20.

Name of the antibiotic	Isolates									
	11	12	13	14	15	16	17	18	19	20
Ampicillin	R	I	I	S	S	R	I	S	S	S
Azithromycin	R	R	R	R	S	S	S	R	S	S
Cefixime	R	R	R	R	R	R	S	R	R	R
Cefuroxime	I	R	S	S	S	R	R	S	S	R
Ceftriazone	S	I	I	I	I	I	I	I	I	S
Chloramphenicol	R	R	S	S	S	R	R	S	S	I
Ciprofloxacin	R	R	I	S	S	R	R	S	S	S
Gentamicin	S	R	I	S	S	I	I	R	S	S
Nalidixic acid	S	I	R	R	I	S	S	S	S	S
Norfloxacin	I	S	R	R	S	R	R	S	S	R
Tetracycline	S	R	R	S	S	R	R	S	R	S
Trimethoprim	S	R	R	S	R	S	S	R	S	S

***S:-Sensitive, I:-Intermediate, R:-Resistant**

*Isolates---11-BTDF1, 12-BTDF2, 13-BTDF3, 14-BTDP2, 15-BTDP3, 16-BTSD1, 17-BTSD2, 18-BTFF1, 19-BTFF2, 20- - BTTP1.

The antibiotic sensitivity profile (%) of the twenty strong producers is given in the figure 3.8. It was observed that percentage of biofilm producers resistant or intermediately resistant to most of the antibiotics was greater. From the figure 3.8, it was observed that even though the percentage sensitivity was more, the sum total of intermediate resistance and total resistance was higher. This unveiled the increased risks of food poisonings and food related deaths due to these potent pathogens.

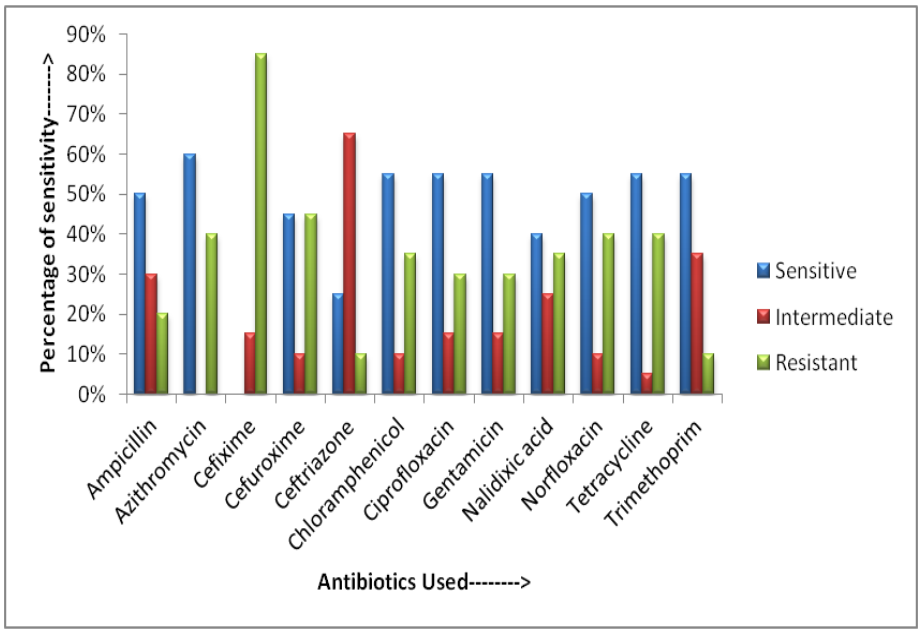


Fig 3.8 The antibiotic profile (%) of the twenty strong producers (Laxmi and Bhat, 2014)

Out of the twenty strong producers, nine were selected based on their strong biofilm production (Fig 3.4) and greater resistance profile (Fig. 3.8). MAR indices were calculated and is as shown in table 3.5. All nine organisms showed greater MAR indices implying their potential as highly antibiotic resistant strong biofilm producers.

Table 3.5 Multiple Antibiotic Resistance (MAR) Index of the nine strong biofilm producers

Isolate	(a)	(b)	MAR Index(a/b)
Bacillus altitudinis (BTMW1)	10	12	0.83
Bacillus pumilus (BTMY1)	4	12	0.33
Pseudomonas aeruginosa (BTRY1)	10	12	0.83
Brevibacterium casei (BTDF1)	5	12	0.42
Staphylococcus warneri (BTDF2)	8	12	0.66
Micrococcus luteus (BTDF3)	6	12	0.50
Bacillus niacini (BTDP2)	4	12	0.42
Bacillus sp (BTSD1)	5	12	0.33
Geobacillus staerothermophilus (BTFF2)	2	12	0.17

***(a) - No. of antibiotics to which the isolate was resistant**
***(b) - Total no. of antibiotics to which the isolate was subjected**

3.3.6. Exoenzyme profile of biofilm producers

The enzyme profile showed the hydrolytic capabilities of the strong biofilm producers. It was observed that all were capable of producing more than one enzyme. The enzyme profile is as reported in the table 3.6. This characteristic feature pointed out that these isolates, in addition to biofilm formation, can also reduce the nutritional value of the food they contaminate.

Table 3.6 Exoenzyme profile of the biofilm producers

Strain	Amylase	Protease	Cellulase	Lipase
Bacillus altitudinis (BTMW1)	-	+	+	+
Bacillus pumilus (BTMY2)	-	+	+	+
Pseudomonas aeruginosa (BTRY1)	-	+	-	+
Brevibacterium casei (BTDF1)	-	+	-	+
Staphylococcus warneri (BTDF2)	-	+	-	+
Micrococcus luteus (BTDF3)	-	+	+	-
Bacillus niacini (BTDP3)	+	-	+	+
Bacillus sp (BTSD1)	+	+	+	-
Geobacillus stearothermophilus (BTFF2)	+	+	-	-

*+ - >Presence of enzyme, - ->Absence of enzyme

The figures 3.9- 3.12 depict the different qualitative enzymatic assays using special media mentioned in the section 3.2.6 (Laxmi and Bhat, 2014).

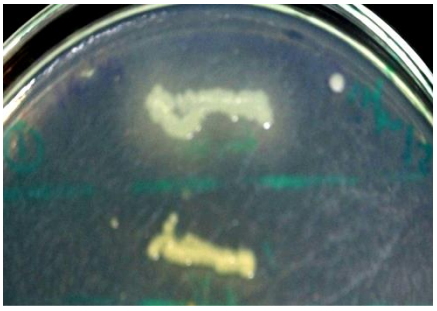


Fig 3.9 Lipase detection on tributyrin agar

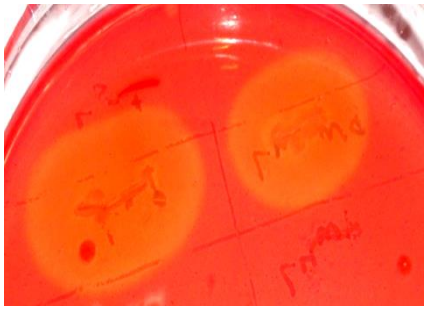


Fig 3.10 Cellulase detection on Carboxymethyl cellulose agar

Fig 3.11 Protease detection on skimmed milk agar

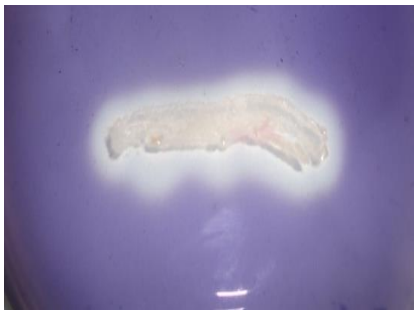


Fig 3.12 Amylase detection on starch agar

3.4 Discussion

An extensive multiplicity of microorganisms are equipped for shaping biofilms; and subsequently biofilms exist in an assortment of situations. Some biofilms play a beneficial part in nature by serving as support for bigger living beings in the evolved way of life (Agarwal *et al.*, 2011). However, those included as human and foodborne pathogens represent a huge danger to food security. Late flare-ups of foodborne ailment can be credited to biofilms. The capacity of biofilm microorganisms to act on the whole to make a microbial province more grounded and more impervious to traditional sanitation and nourishment wellbeing strategies is overwhelming. Likewise, a refined system of cell-to-cell communication—majority detecting—upgrades biofilms' entrance to supplements and good ecological specialties, for example, new leafy foods (Costerton *et al.*, 1995). To decrease the dangers that biofilms stance to the nourishment business,

further research is required not just to comprehend biofilm development in pathogenic life forms additionally to focus compelling systems for blocking majority detecting and inactivating biofilms on foods (Trachoo, 2003).

Several reports have been published on screening of food borne pathogens from different foods. There are incidence of *Pseudomonas* sp in food items like beef, milk, anchovy and chicken (Keskin and Ekmekci, 2007). Biofilm forming ability of different *Salmonella* serotypes evaluated using the microtiter plate assay with the crystal violet staining, showed biofilm formation on plastic surfaces by most strains in the study, which also categorized the isolated pathogens as strong, moderate and weak biofilm producers (Agarwal *et al*., 2011).

Food samples from outbreaks of Salmonellosis were molecularly characterized. Their antimicrobial susceptibility was tested to 12 different antibiotics, however only low frequency antimicrobial resistance was observed (Murmann *et al*., 2008). Bacteria in biofilms are reported to have intrinsic mechanisms that protect them from most aggressive environmental conditions, including exposure to antimicrobials (Davies, 2003).

Different species of microorganisms may possess diverse ability to attach or form biofilm on different surfaces. In a study that compared attachment of *Listeria monocytogenes*, *E. coli* O157:H7, and *Pseudomonas fluorescens* on iceburg lettuce, it was seen that *L. monocytogenes* and *E. coli* O157:H7 attached preferentially to cut edges while *P. fluorescens* attached to the intact surfaces (Takeuchi *et al.,* 2000). Biofilm can be formed by all types of microorganisms, including spoilage and pathogenic microorganisms, under suitable conditions. Some bacteria have a higher tendency to form a biofilm (Nivens *et al.,* 1995). The most common of these are *Pseudomonas, Enterobacter, Flavobacterium, Alcaligenes, Staphylococcus and Bacillus* (Wirtanen and Mattila-Sandalholm, 1992). *L. monocytogenes* and other human pathogens have been found in food processing industries working with meat, milk and other kinds of foods (Genigeorsis, 1995).

In food processing environments, bacteria in both biofilm and suspended structures encounters burdens such as dehydration, high heat, low temperature, and antimicrobial agents. Biofilm bacteria can be physically and morphologically unique from their planktonic counterparts. Biofilm bacteria are up to 500 times more resistant to antimicrobial agents (Costerton *et al.,* 1995). Biofilms formed on

food-processing equipment and other food contact surfaces act as a persistent source of contamination threatening the microbiological quality and safety of food products, and resulting in food-borne disease and economic losses (Van Houdt and Michiels, 2009).

Enzymes secreted from biofilms into raw milk during transportation can potentially reduce the quality of different dairy products and could lead to severe economic losses in the food industry (Teh *et al*., 2012). Enzymes secreted by the bacteria modify EPS composition in response to changes in nutrient availability (Sauer *et al.,* 2004; Gjermansen *et al.,* 2005), thereby tailoring biofilm architecture to the specific environment (Ma *et al.,* 2009). Biofilms may provide bacteria with a niche for both extracellular and cell associated enzyme production. Several investigations are carried out in the field of production of spoilage enzymes within biofilms and their effect on the product quality (Teh, 2013).

The undesirable effect of the extracellular enzymes like proteases, amylases, lipases, cellulases, etc produced by the microbial biofilms were reported to degrade the food quality (Schroeder *et al.,* 2009). The amount of enzymes produced is also greater within biofilm community compared to the planktonic cells (Oosthuizen *et al.,* 2001; Frolund *et al.,* 1995). Enzyme production by biofilms are most likely to have biggest impact since it occurs mainly during the transportation of food products in containers and thus in the start of manufacturing process. Some of the heat stable enzymes can remain active even after Hurdle heat treatments which are surely a reason for the degradation of such food products (Teh, 2013). Extracellular or cell free enzymes are most commonly produced than cell-associated enzymes (Wang and Chen, 2009). While quorum sensing may influence the production of cell free enzymes in biofilms, the correlation between the AHL molecules and the amount of enzymes produced have not been yet reported (Khajanchi *et al.,* 2009).

Thus biofilm production by food pathogens poses immense threat to the food industry. In the present study, 20 strong biofilm producers were characterized by 16S rDNA sequencing and their identity revealed. The strains belonged to the genera *Bacillus, Pseudomonas, Micrococcus, Staphylococcus, Brevibacterium and Geobacillus.* The enzyme profiling showed that the strongest biofilm producers produced most of the important starch, cellulose, protein and lipid hydrolyzing enzymes and were thereby capable of easily diminishing food quality. Multiple

antibiotic resistances were observed among the strong biofilm producers, which are also food pathogens.

According to the present study, most of the biofilm forming food pathogens were multiple antibiotic resistant and produced more than one enzyme responsible for food perishability. Several bioactive compounds find application against biofilm formation and their safety needs to be confirmed prior to application in the food industry. Since biofilm formation is a serious issue, their control must be considered since it directly affects public health.

Chapter 4

Isolation, purification and partial characterization of pyocyanin and rhamnolipids from *Pseudomonas aeruginosa* BTRY1

4.1 Introduction

Pseudomonas aeruginosa is a Gram-negative, aerobic rod shaped bacterium, motile by single polar flagellum (Moore *et al.*, 2006); attracting attention due to the different pigments produced such as pyocyanin (blue-green), pyoverdin (yellow, green and fluorescent), pyomelanin (light-brown) and pyorubrin (red-brown) (Meyer 2000). Nearly 90–95 % of *P. aeruginosa* isolates produce pyocyanin, normally referred to as ‘‘blue pus’’ (from pyocyaneus) (Ran *et al.*, 2003). Pyocyanin is a secondary metabolite with the ability to oxidise and reduce other molecules and therefore can kill microbes competing against *P. aeruginosa* (Sudhakar *et al.,* 2013).

Rhamnolipids are a class of glycolipids produced by *P. aeruginosa* (Jarvis and Johnson, 1949). It is a viscous sticky oily yellowish brown liquid with a fruity odour. *P. aeruginosa* produce a mixture of mono & di rhamnolipids with hydroxyacyl moieties mostly from C_8 up to C_{12} and they are one of the virulence factors contributing to the pathogenesis of *P. aeruginosa* infections. Biosurfactants produced by most microorganisms are used in various industries for their different activities and thus the rhamnolipids produced from the test strain was screened for various activities (Abdel-Mawgoud *et al.,* 2011). FTIR and NMR spectroscopic techniques were mainly used for the partial characterization of both compounds.

FTIR (Fourier Transform Infra Red) is the most preferred method of infrared spectroscopy. In an infrared spectroscopy, IR radiation is passed through a sample. Some of the radiation is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission which creates a molecular fingerprint of the sample. This is unique for every sample. This makes infrared spectroscopy useful for several types of analysis (Aziz *et al.,* 2012).

The structural elucidation of the pigment was determined using Nuclear Magnetic Resonance (NMR) Spectroscopy by dissolving the compound in Cadmium chloride. NMR relies on the phenomenon of nuclear magnetic resonance which can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecule under study and the technique is also used to confirm the identity of the compound (Sudhakar *et al*., 2013).

The peaks obtained for the FTIR and NMR spectra were analysed and further study was conducted. The present chapter deals with the isolation, purification and partial characterization of both compounds.

4.2 Materials and Methods

4.2.1. Isolation of pyocyanin from Pseudomonas aeruginosa strain BTRY1

For the extraction of pyocyanin, *P. aeruginosa* strain BTRY1 was inoculated in Pseudomonas Isolation High Veg^{TM} (HiMedia) (Appendix-1) broth to optimize production of pyocyanin. They were incubated at 37°C for 18-24 h and observed for color change. Pyocyanin was extracted (Hassani *et al.,* 2012), and concentration determined (Aziz *et al.,* 2012). Chloroform was added to the production culture in 1:2 ratio. The Blue layer of the chloroform was collected and washed with acidified (0.2 N HCl) water. The layer was then neutralized using 1 M Tris Base pH 11.0. Re- extraction was done by repeating steps 1-3 to extract large amounts of pyocyanin.

4.2.2. Quantification of the pyocyanin from Pseudomonas aeruginosa strain BTRY1

The concentration of pyocyanin was done with modifications (Frank and De Moss, 1959; Aziz *et al.,* 2012). For this, 2 mL of the culture sample was added to 2 mL 20% trichloroacetic acid, followed by heating in boiling water for 5-10 min, cooling to room temperature and centrifugation at 10,000 rpm (9168 x *g*) for 20 min at 30°C. 4 mL of 2 M tris (hydroxymethyl) amino methane was added and shaken vigorously to ensure complete autooxidation of any reduced pyocyanin. The absorbance was read 570 nm in spectrophotometer (Shimadzu).

The resultant value was multiplied by the molecular weight of pyocyanin and expressed in µg/mL. This is termed as luminar density. The concentration was obtained from the luminar density and the absorption coefficient of the pyocyanin compound as follows:

$$\text{Pyocyanin concentration} = \frac{\text{Luminar density of pyocyanin suspension at 690 nm}}{\text{Absorption coefficient}}$$

4.2.3. Determination of the UV-Vis absorption spectrum of pyocyanin from P. aeruginosa BTRY1

The pigment was further characterized using UV-visible spectrophotometer (Schimadzu, Japan) and the absorption maximum was observed. Red color pigment obtained by adding 0.2N HCl was separated and absorbance maxima determined by UV spectrophotometric analysis (Karpagam *et al.,* 2013).

4.2.4. Isolation and extraction of rhamnolipids from P. aeruginosa BTRY1

The micro-organism was grown in nutrient broth and successively transferred to Kay's minimal medium, proteose peptone ammonium salt (PPAS) medium and a Mineral Salts Medium (Appendix- 1). *Pseudomonas aeruginosa* strain was first grown in Kay's minimal medium for 24 h. then diluted 1:100 into PPAS medium and incubated for 24 h. In all cases, incubations were done at 37°C with orbital shaking at 250 rpm. The culture from PPAS medium was transferred to mineral salts medium (1:100) and incubated at 37°C for 48-72 h (Gunther *et al.,* 2005).

Extraction was as per Selim *et al.,* (2011). Cells removed from culture broth by centrifugation at 10,000 x *g*, 4°C for 10 min (Sigma 3K, Germany)**.** Supernatant was acidified with 2N HCl until pH =2, and incubated overnight at 4°C. This was followed by centrifugation at 10,000 x *g*, 4°C for 30 min and washed with acetone. The precipitate was collected, dried and was used for further analysis.

4.2.5. Qualitative analysis of rhamnolipids from P. aeruginosa BTRY1

4.2.5.1. CTAB Methylene blue agar test

The most widely used method for qualitative screening for RL production by bacterial strains is the Cetyl Trimethyl Ammonium Bromide (CTAB) Agar test. The composition is mentioned in Appendix- 1 .In this method, the anionic RLs form an insoluble complex with this cationic bromide salt and the complex is revealed using methylene blue present in the agar (Pinzon and Ju, 2009).

4.2.5.2. Drop collapsing test

Since rhamnolipids are biosurfactants, drop collapsing assay was conducted as a qualitative analysis. This assay consists of applying a drop of a bacterial culture supernatant to a polystrene plate containing shallow wells covered with oil. The droplet will spread over the oil only if the culture supernatant sample contains RLs (Abdel-Mawgoud *et al.*, 2011).

4.2.6. Quantitative analysis of rhamnolipids from P. aeruginosa BTRY1

After removal of the biomass from the growth medium, 0.25 mL of 500 mM glycine buffer, pH 2 (Appendix -2), was added to 0.25 mL of supernatant. The mixture was well stirred and centrifuged for 10 min at 10000 rpm (9168 x *g*) (Sigma 3K, Germany). The supernatant was discarded and precipitate resuspended in 0.5 mL of a mixture of chloroform/methanol (2:1), with intense agitation for 5 min. The suspension was then centrifuged for 5 min at 10000 rpm (9168 x *g*) and 0.25 mL of supernatant was transferred to a new eppendorf tube. After evaporation of the solvent mixture, the precipitate remaining was dissolved in water. The concentration of RL (cr_l) was determined spectrophotometrically by the orcinol reaction using rhamnose as a standard (Rahman *et al.,* 2002). The orcinol reagent (0.19 % orcinol in 53 % (v/v) sulfuric acid) was prepared immediately before use. The reaction mixture, composed of 150 µL of sample and 1350 µL of reagents, was well stirred, warmed for 30 min at 80°C, and kept for 15 min at room temperature. The standard used was L-rhamnose. The rhamnolipid concentration was quantified from the standard L-Rhamnose calibration curve.

The absorbance was measured at 421 nm spectophotometrically (Schimadzu, Japan). Concentration of RL (cr_l) was calculated based on the assumption that 1 µg of rhamnose corresponds to 2.5 µg of RL (Rikalovic *et al.,* 2012; Wang *et al.,* 2007; Wilhelm *et al.,* 2007).

4.2.7. Characterization of pyocyanin and rhamnolipids from P.aeruginosa BTRY1 by FTIR and Proton NMR spectroscopy

The two compounds, pyocyanin and rhamnolipids were quantified as described in the section 4.2.2 and 4.2.5, were lyophilized and dissolved in DMSO followed by conduct of FTIR (Thermo Nicolet, Avatar 370) & Proton NMR (Bruker Avance III, 400 MHz) analyses.

4.2.8. Free radical scavenging activity of pyocyanin and rhamnolipids from P.aeruginosa BTRY1

Free radical scavenging activity of pyocyanin and rhamnolipids was estimated by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Liyana and Shahidi, 2005). As per this protocol, pyocyanin was added to 0.135 mM DPPH in methanol to get concentrations ranging from 0.2 to 1 µg/mL, mixed well, and kept at room temperature in dark for 30 min. Similarly, rhamnolipids was added in different concentrations ranging from 20 to 75 µg/mL. Absorbance was measured spectrophotometrically (Schimadzu, Japan) at 517 nm. Ability to scavenge DPPH radical was calculated as follows:

Free radical scavenging activity (%) $= A_{control}\text{-}A_{sample} / A_{control} \times 100$

where, $A_{control}$ is absorbance of the DPPH + methanol, and A_{sample} is absorbance of free radical solution with pyocyanin/rhamnolipids/standard antioxidant. One microgram of ascorbic acid (standard antioxidant) was used as positive control. The results were analyzed after three independent repeats.

4.2.9. Cytotoxicity of pyocyanin and rhamnolipids from P.aeruginosa strain BTRY1

4.2.9.1. Assay of hemolytic activity

Human red blood cells were centrifuged 12,000 rpm (11,168 x *g*) for 10 min at 4°C; Thermo Scientific) and washed three times with phosphate-buffered saline (Appendix -2). The hemolytic activity of pyocyanin/rhamnolipids was evaluated by measuring the release of hemoglobin from fresh human erythrocytes. Aliquots (100 µL) of an 8% suspension of red blood cells were transferred to sterile 96-well plates, along with 50 µL of pyocyanin concentrations (0.2 – 1.0 µg/mL), rhamnolipids at concentrations from 20-75 µg/mL, positive control (Triton X-100) and negative control (PBS) in respective microtiter wells. Hemolysis was determined at 414 nm (Shimadzu). No hemolysis (0%) and full hemolysis (100%) were determined in the presence of PBS and 0.1% Triton X-100, respectively.

The percent hemolysis was calculated using the following equation: [(A_{414nm} with pyocyanin/rhamnolipids- A_{414nm} in PBS) / (A_{414nm} with 0.1% Triton-X 100 - A_{414nm} in PBS)] × 100 (Park *et al.*, 2004).

4.2.9.2. Determination of in vitro cytotoxic effect of pyocyanin and rhamnolipids on cultured L929 cell lines

L929 fibroblast cell lines (NCCS, Pune, India) was maintained in Dulbecco's modified Eagles medium (HiMedia) supplemented with 10% FBS (Invitrogen) and grown to confluence at 37°C in 5 % CO_2 in humidified atmosphere of CO_2 incubator (NBS, Eppendorf, Germany). The cells were trypsinized (500 µL of 0.025 % Trypsin in PBS/ 0.5mM EDTA solution (Himedia) for 2 min and passaged to conical flasks in complete aseptic conditions, followed by addition of 6.25 µg, 12.5 µg, 25 µg, 50 µg, 100 µg of sample and incubated for 24 h. The % difference in viability was determined by standard MTT assay after 24 h of incubation. The morphological characteristics of cells were imaged using inverted phase contrast microscope (Olympus CKX41) with Optika Pro5 CCD camera. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) Assay (Arung *et al.*, 2009) was used to measure cell viability. Wells with pyocyanin/rhamnolipids and cells were washed with 1 x PBS, added

50 µL of MTT (MTT -5 mg/mL dissolved in PBS) and incubated at 37°C for 3 h. MTT was removed by washing with 1 X PBS and formazan was eluted with 200 µL of isopropanol. Incubated at room temperature for 30 min, when the cell lysed and colour was obtained. This was followed by (2 min) centrifugation to precipitate cell debris.

Optical density was read at 540 nm with DMSO as blank using microplate reader (LISASCAN, Erba).

% viability = (OD of Test/ OD of Control) X 100

4.3 Result

4.3.1. Isolation of pyocyanin from Pseudomonas aeruginosa BTRY1

The extraction and concentration of the pyocyanin was done as mentioned in section 4.2.1. Pyocyanin production after 18 h of incubation was indicated by change in color to bluish green on addition of chloroform; it was soluble in chloroform. The blue coloured layer was then acidified with 0.2 N HCl to produce a deep pink to red colour. The red coloured layer was separated and was re-extracted by the same procedure improve purity of pyocyanin (Figure 4.1).

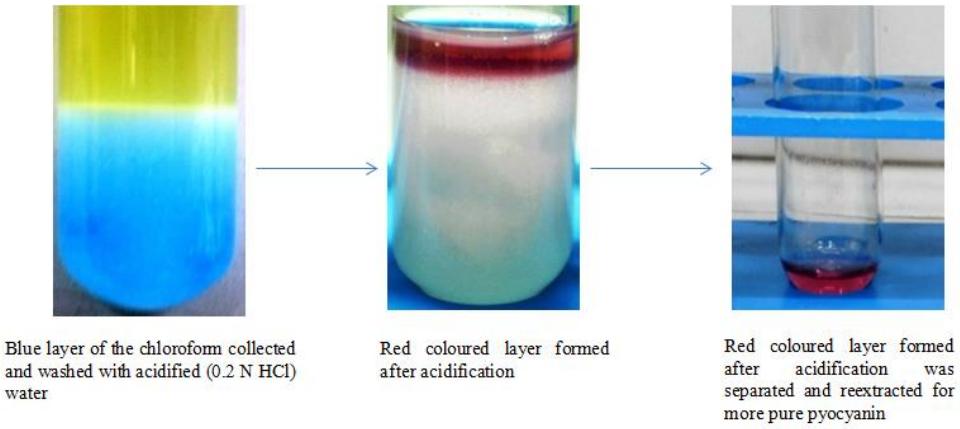


Fig.4.1. Extraction of pyocyanin from *P.aeruginosa* BTRY1

4.3.2. Quantification of the pyocyanin from Pseudomonas aeruginosa BTRY1

The quantification of the extracted pyocyanin was as per section 4.2.2. The concentration of pyocyanin produced by the strain *Pseudomonas aeruginosa BTRY1* was calculated to be 1.245 ± 0.001414 µg/mL.

4.3.3. Determination of UV-Vis absorption spectrum of pyocyanin from P. aeruginosa BTRY1

The absorption maximum of the extracted pyocyanin from *Pseudomonas aeruginosa BTRY1* at 270 nm was comparable to that of standard pyocyanin. The UV-Vis absorption spectrum is plotted in figure 4.2.

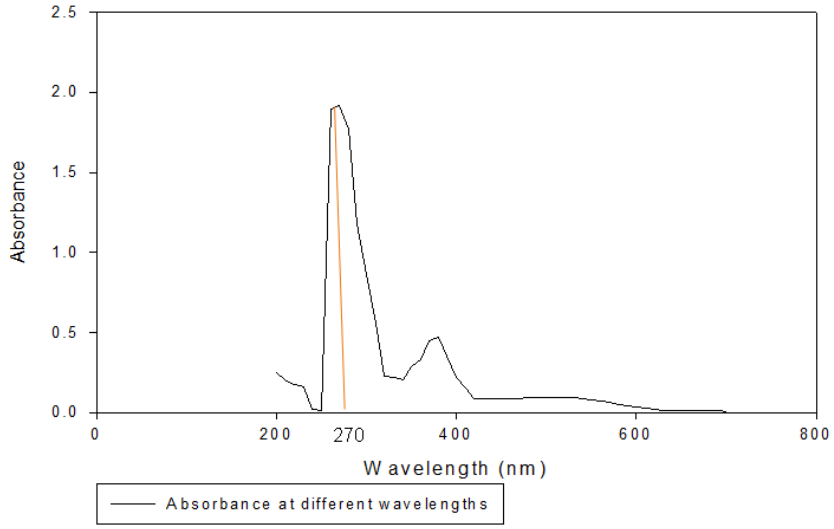


Fig. 4.2.UV Absorption spectra of *Pseudomonas aeruginosa* BTRY1 showing λ_{max} at 270 nm

4.3.4. Isolation and extraction of rhamnolipids from P. aeruginosa BTRY1

Brown coloured rhamnolipids was obtained from *Pseudomonas aeruginosa* BTRY1 after the 24 h of drying process and it was easily soluble in water. The compound obtained after the extraction process was used for further assays.

4.3.5. Qualitative analysis of rhamnolipids from P. aeruginosa (BTRY1)

4.3.5.1. CTAB Methylene blue agar test

CTAB Methylene blue agar test was used for qualitative analysis of rhamnolipids produced by *P.aeruginosa* BTRY1, where the bacteria produced colonies with blue halo around them, allowing for facile identification of the presence of rhamnolipids (Figure 4.3).

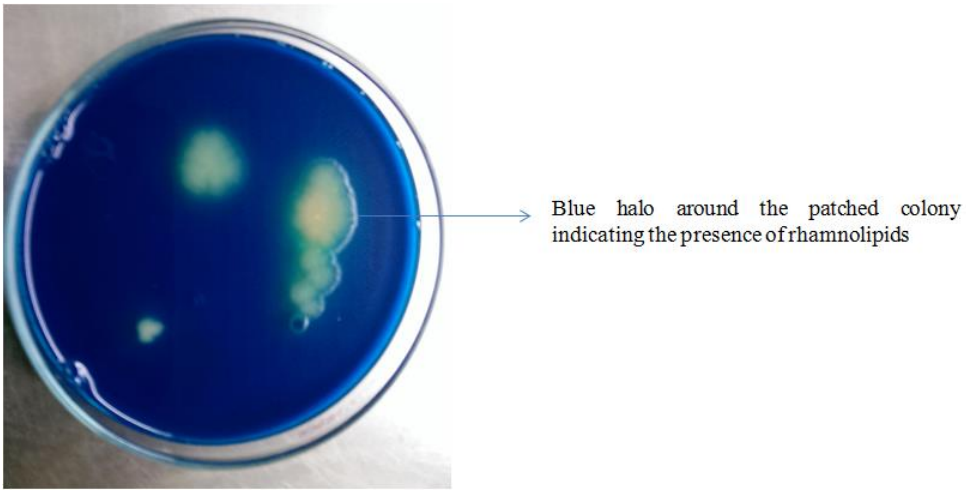


Fig. 4.3.CTAB Agar test for rhamnolipids

4.3.5.2. Drop collapsing test

The drop collapsing assay helps in proving the surfactant properties of rhamnolipids. This was carried as mentioned in section 4.2.5.2. On addition of the culture supernatant from *P.aeruginosa* BTRY1 over the oil drop in the polystyrene wells, the droplet was observed to spread over the oil, proving that the sample contained rhamnolipids (Figure 4.4).

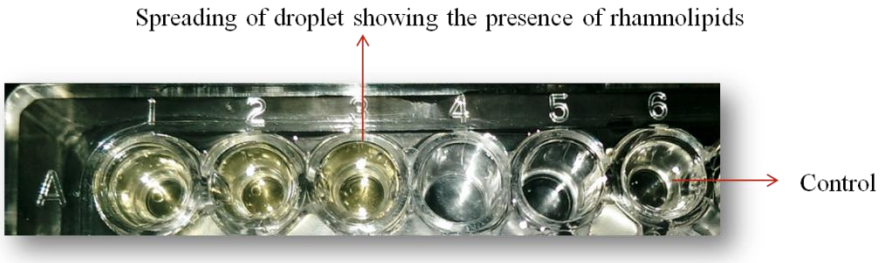


Fig 4.4. Drop collapsing test on microtiter plate

4.3.6. Quantitative analysis of rhamnolipids from P. aeruginosa BTRY1

The rhamnolipids obtained after the extraction was quantified as described in section 4.3.6. from the standard L-Rhamnose calibrated curve. 1 µg of L-Rhamnose corresponds to 2.5 µg of RL. Thus the concentration of the rhamnolipids was calculated as 75 ± 0.007171 µg/mL from the graph (Figure 4.5).

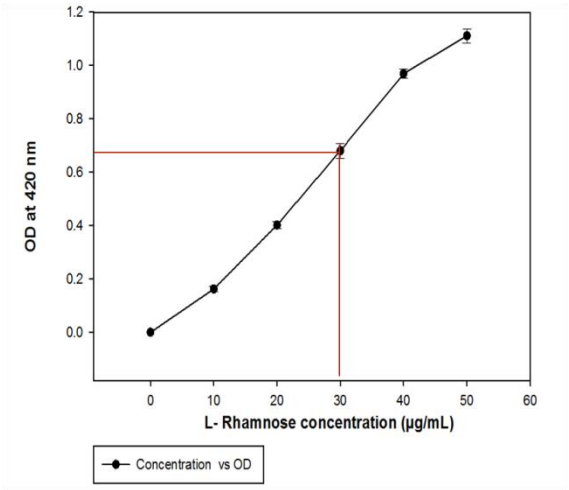


Fig 4.5. Determination of concentration of rhamnolipids

4.3.7. Characterization of pyocyanin and rhamnolipids from P.aeruginosa BTRY1 by FTIR and Proton NMR spectroscopy

The isolated pyocyanin was subjected to ^{1}H NMR analysis using cadmium chloride ($CdCl_3$) as solvent. In ^{1}H NMR (figure 4.6), the peak at δ 2.7 to 3.4 ppm indicated the presence of methyl group linked to aromatic nitrogen atom. The peak at δ 7.5 to 7.7 ppm represented the condensed aromatic nitrogen ring (Laxmi and Bhat, 2015). The ^{1}H NMR analysis of isolated rhamnolipids (figure 4.7) showed different peaks. The long hydrocarbon chain and rhamnose ring were indicated by the appearance of the peak at δ 3.33 to 3.47 ppm, respectively. ^{1}H NMR analysis

showed anomeric signal at δ 4.883, suggesting L-rhamnosyl-hydroxy fatty acid linkage. The rhamnosyl methyl protons were assigned to the two overlapping doublets at δ 1.44 ppm. The chemical shifts observed for hydrocarbon chains were 0.894 ppm (for $-CH_3$), 1.54 ppm (for $-(CH_2)-$ chain), 2.17 ppm (for $-CH_2-COO-$), and 3.66 ppm (for –O-CH-) which is comparable to standards.

Further characterization of pyocyanin and rhamnolipids was by analyzing their IR spectrum. The pyocyanin spectrum in figure 4.8 indicated the presence of phenazine as specified by side chains of the molecule. The peak at 3448.59 cm^{-1} shows the presence of O-H bond. The peak at 2951.18 cm^{-1} relates to the C-H-aromatic bond. The peak shown at 1637.34 cm^{-1} represents C=N bond and the peak at 130.7.02 cm^{-1} corresponds to C-O bond. The spectrum of rhamnolipids shown in figure 4.9 peaks at 3432.89 cm^{-1} (-OH free stretch due to hydrogen bonding), 1635.28 cm^{-1}(C=O stretch due to the ester functional group), 1384.36 – 1068.17 cm^{-1} (C–O–C stretching in the rhamnose), 612.28 cm^{-1} (pyranyl sorption band). This is comparable to the reports of standard FTIR spectra.

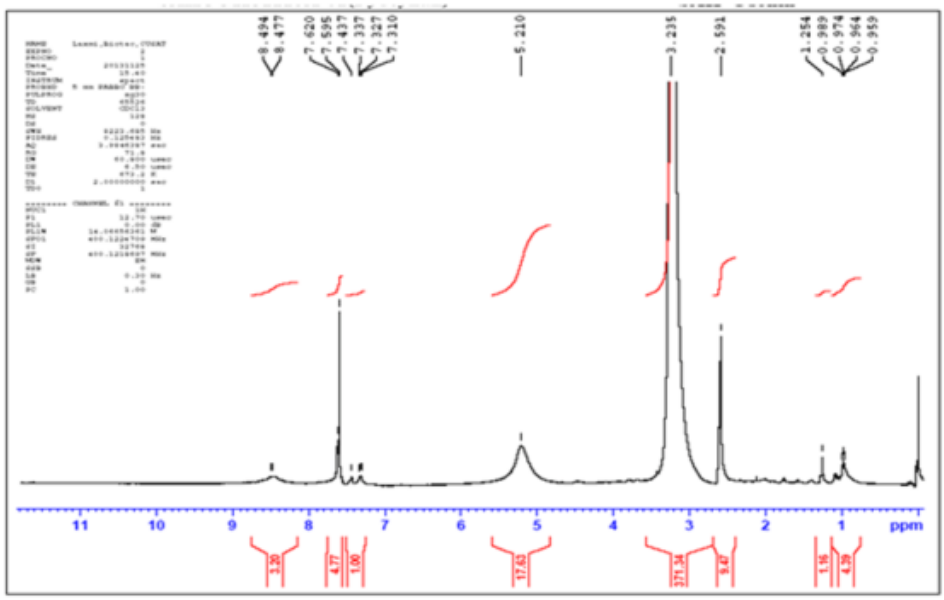


Fig.4.6 NMR spectrum of pyocyanin from *P.aeruginosa BTRY1* (Laxmi and Bhat, 2015)

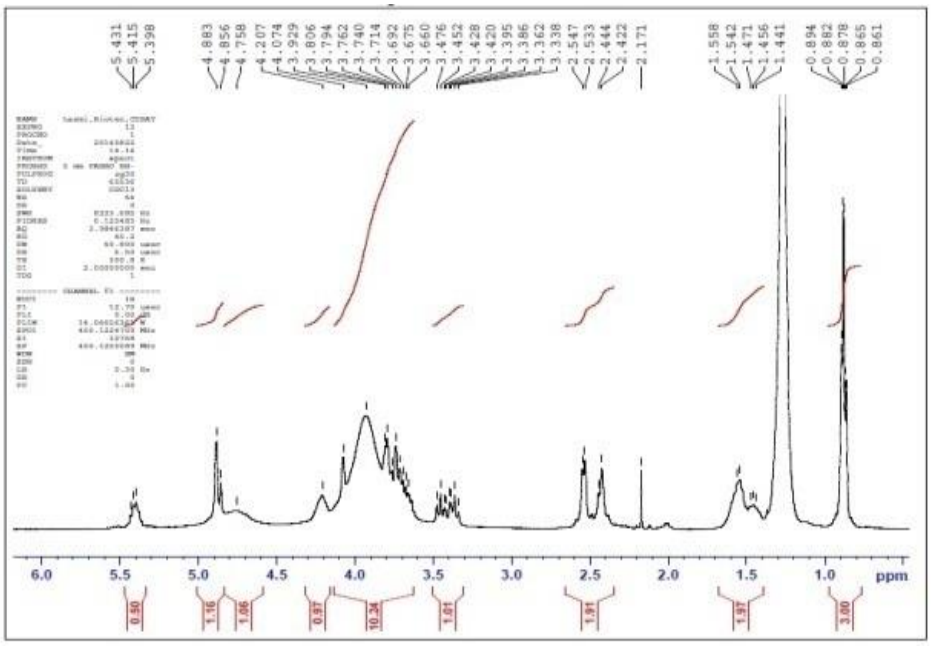


Fig.4.7 NMR spectrum of rhamnolipids from *P.aeruginosa BTRY1*

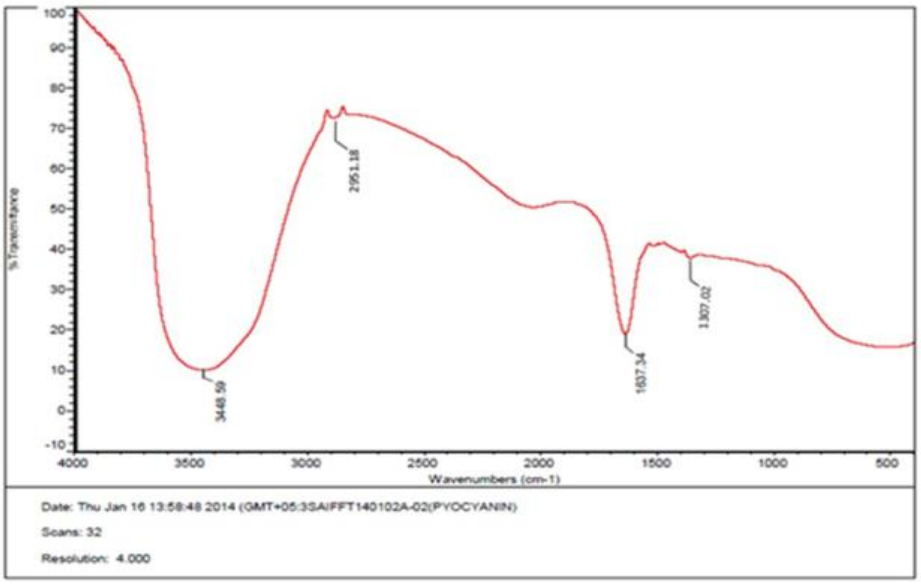


Fig.4.8 FTIR spectrum of pyocyanin from *P.aeruginosa BTRY1* (Laxmi and Bhat, 2015)

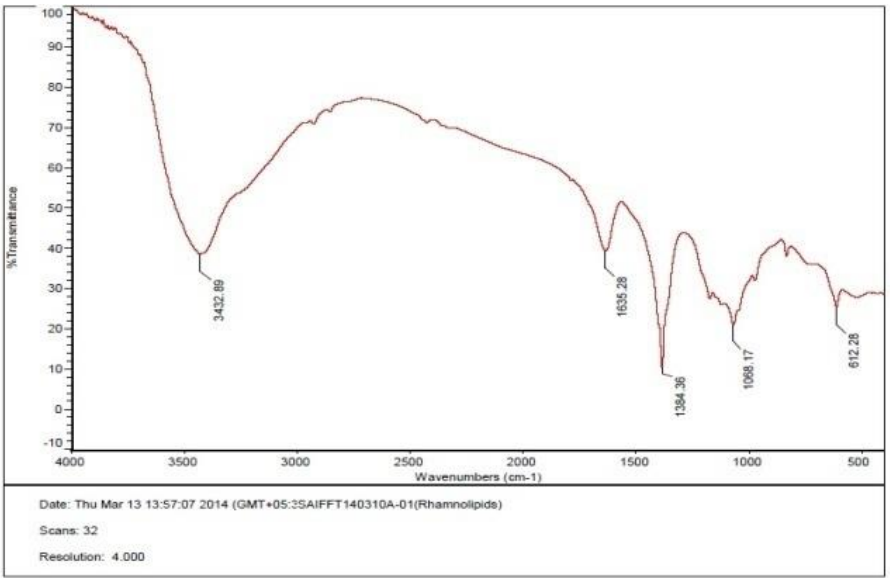


Fig.4.9 FTIR spectrum of rhamnolipids from *P.aeruginosa BTRY1*

The Proton NMR and FTIR spectra of both the compounds revealed that their peaks were comparable to standards reported and thus do not need further purification of the compounds for their other studies.

4.3.8. Free radical scavenging activity of pyocyanin and rhamnolipids from P.aeruginosa BTRY1

The scavenging activity of the isolated compounds was measured as per described in section 4.2.8. Ascorbic acid with appropriate concentrations was used as standard or the positive control. Free radical scavenging activity of pyocyanin was much higher than that of ascorbic acid (Figure 4.10), while for rhamnolipids, the activity was less compared to the positive control (Figure 4.11). The results are significant as higher radical scavenging activities were obtained for pyocyanin (0.2 µg/mL) even at concentration very much lower than that of the ascorbic acid (0.2 µg/mL). In the case of rhamnolipids, even at 20µg/mL, the scavenging activity was much higher than that of ascorbic acid.

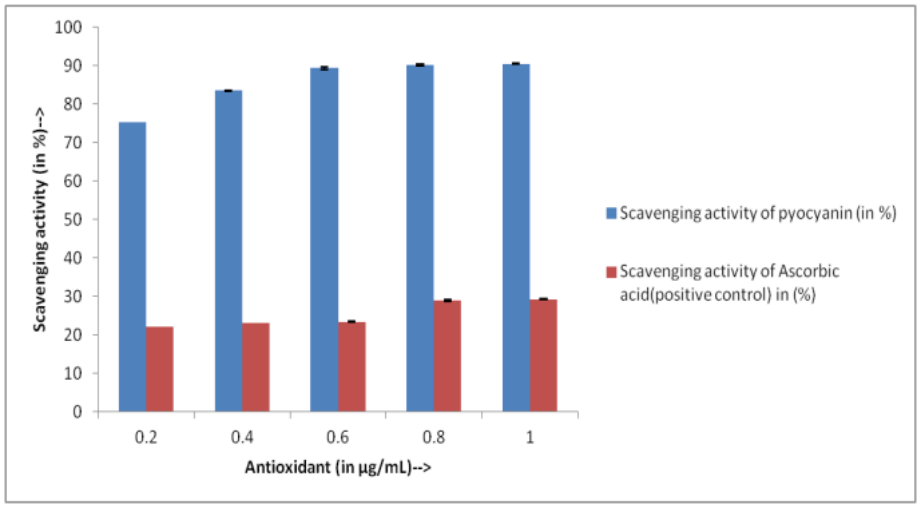


Fig.4.10 Radical Scavenging activity of pyocyanin from *P.aeruginosa BTRY1* (Laxmi and Bhat, 2015)

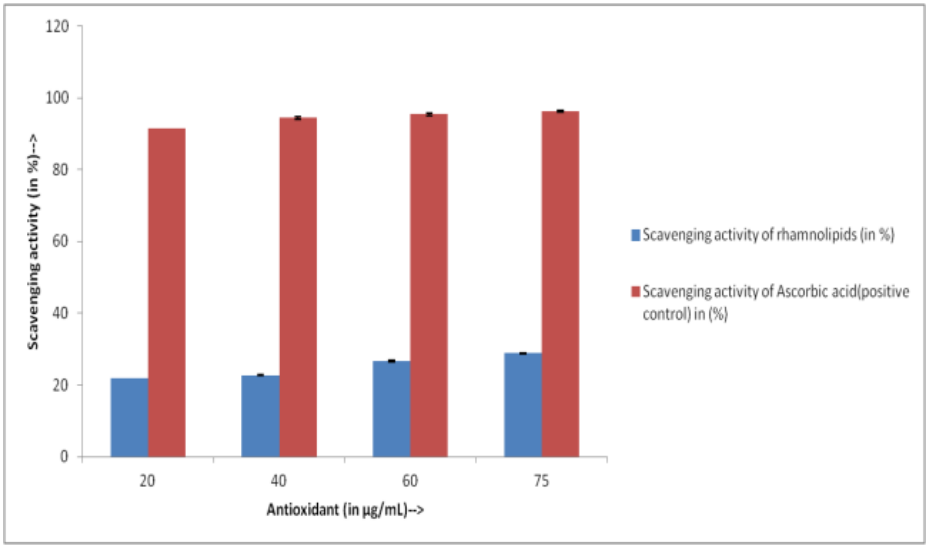


Fig.4.11 Radical Scavenging activity of rhamnolipids from *P.aeruginosa BTRY1*

4.3.9. Cytotoxicity assays of pyocyanin and rhamnolipids from P.aeruginosa strain BTRY1

4.3.9.1. Assay of hemolytic activity

Cytotoxicity of pyocyanin/rhamnolipids was measured by lysis of human erythrocytes. From figures 4.12 and 4.13 it was observed that pyocyanin and rhamnolipids had less hemolytic activity even at higher concentration. At 1 µg/mL for pyocyanin, the hemolytic activity was <16%, while at 75 µg/mL rhamnolipids, it was < 30%, but there was no hemolytic activity at its MIC concentrations. All the results were analyzed with respect to the positive control Triton 100 X, which was considered to be 100% hemolytic and thus the compounds were compared.

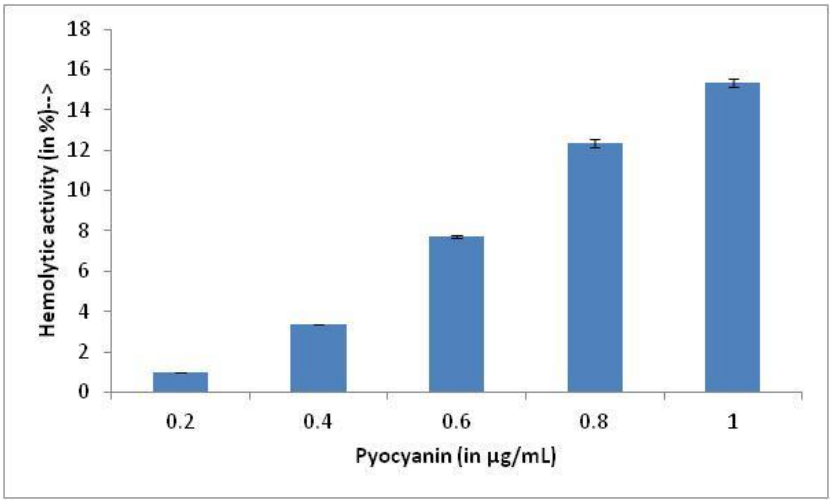


Fig 4.12. Hemolytic activity of pyocyanin from *P.aeruginosa BTRY1* (Laxmi and Bhat, 2015)

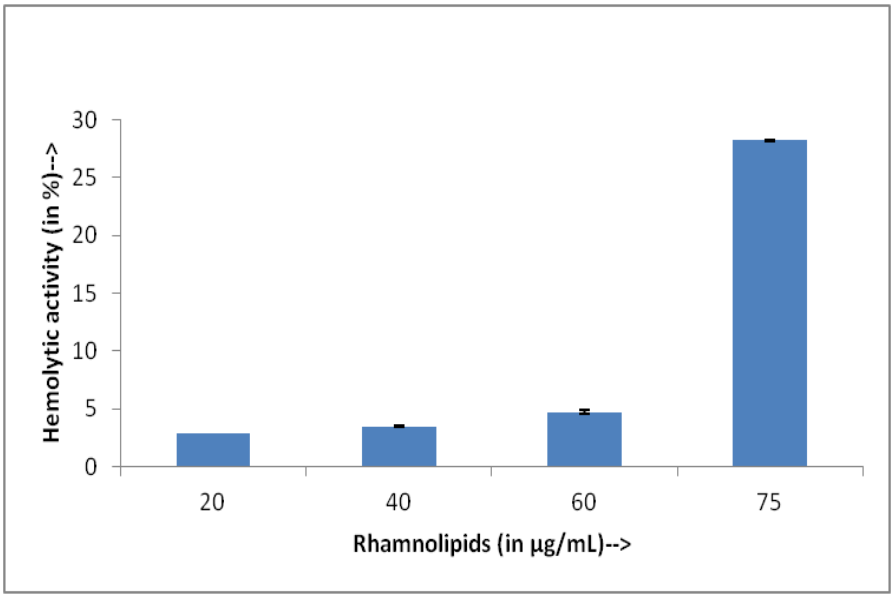


Fig 4.13. Hemolytic activity of rhmanolipids from *P.aeruginosa BTRY1*

4.3.9.2. Determination of in vitro cytotoxic effect of pyocyanin and rhamnolipids on cultured L929 cell lines

MTT assay for analyzing the cytotoxic effect of compounds on cell lines showed that they were not cytotoxic at the tested concentrations. It was observed that the cells showed almost 90% viability after pyocyanin/rhamnolipids treatment at 6.25 µg/mL. Figures 4.14 and 4.15 show the cell viability of around 80% even at high concentrations indicating its safety of use in food consumption for humans. The phase contrast micrographs show the sample treated with pyocyanin/rhamnolipids at the highest concentrations used in the experiment (100 µg/mL) and the untreated (control) sample after the MTT assay (Figures 4.16 & 4.17) (at a magnification of 20x).

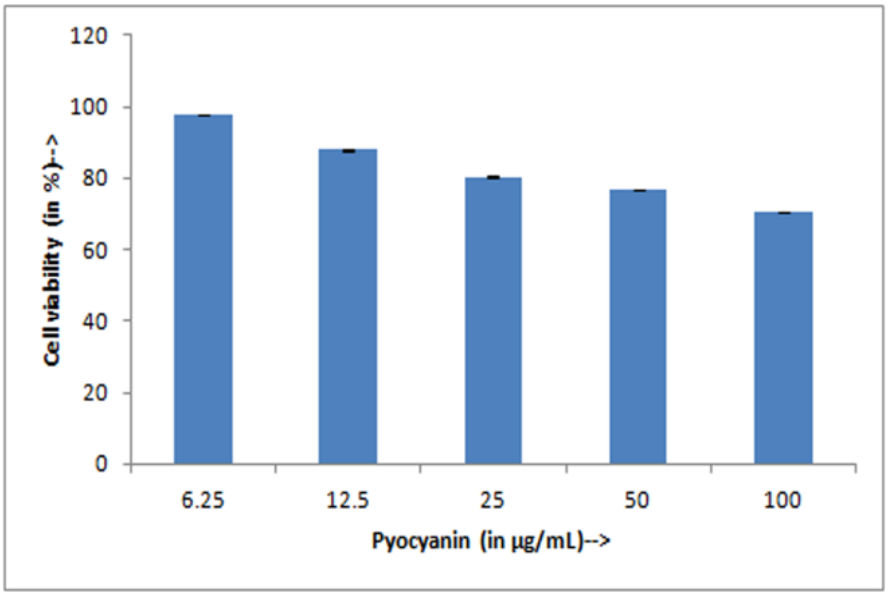


Fig 4.14. Cytotoxicity of pyocyanin on L929 cells (Laxmi and Bhat, 2015)

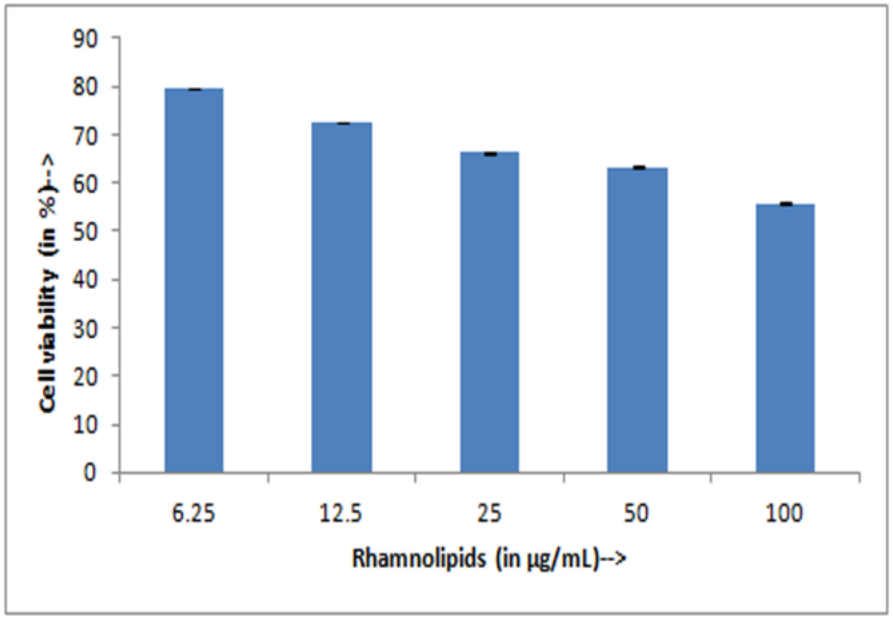


Fig 4.15. Cytotoxicity of rhamnolipids on L929 cells

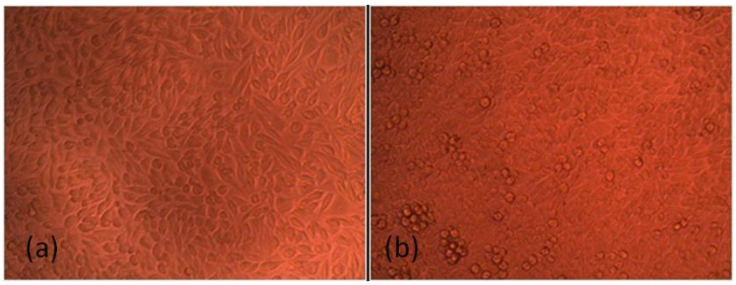


Fig 4.16 (a) - control **(b) -** Pyocyanin (100 µg/mL) treated (Laxmi and Bhat, 2015) (at a magnification of 20x)

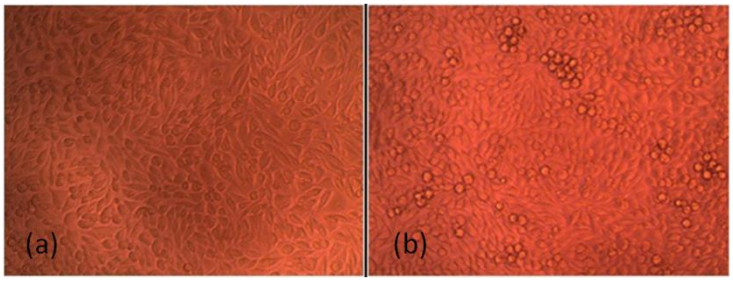


Fig 4.17 (a) – control **(b) -** Rhamnolipids (100 µg/mL) treated (at a magnification of 20x)

4.4 Discussion

In recent years, drug resistance of human pathogenic bacteria has been widely reported. In addition, persistent infections were also attributed to enhanced resistance of bacteria in biofilm (Davies and Davies 2010). This leads to huge economic losses and pressures the medical community to find alternative approaches for treatment of diseases related with biofilms. Consequently, efforts are been applied to discover efficient antimicrobial molecules not amenable to bacterial resistance mechanisms, including those in biofilms. Some natural products have distinctive properties that make them perfect candidates for these highly required niche- based therapeutics (Simoes *et al.*, 2009). This study revealed the significance of bioactive compounds as alternatives to the amplified use of antibiotics. The biofilm forming food pathogens in this study showed multiple antibiotic resistance (MAR), when tested against commonly used antibiotics (Laxmi and Bhat, 2014).

Among the several pigments of Pseudomonads, pyocyanin is the major antibacterial agent, with the inhibitory effect associated with the 1-hydroxy phenazine component (Karpagam *et al.*, 2013). Pyocyanin production is a widely accepted criteria for distinguishing *Pseudomonas aeruginosa* from other closely

related organisms. Pyocyanin produced by strain BTRY1 showed strong antibiofilm activity against the test biofilm forming bacteria.The properties of the pigment make it an important bioactive compound with the ability to arrest the electron transport chain of microbes and exhibit antibacterial activities towards *E.coli, Proteus spp. Staphylococcus aureus and Klebsiella spp.* (Sudhakar *et al.*, 2015).The growth of most of the Gram-positive bacteria and fungal cultures like *Candida* species was completely inhibited when cultivated on the agar plates containing this blue pigment. Whereas, Gram negative bacteria, *Klebsiella pneumoniae* and *P. aeruginosa* were resistant to pyocyanin. *Salmonella* Typhi and *Proteus mirabilis* were intermediately affected (El- Shouny *et al.,* 2011). In a conclusion, the Gram-positive bacteria having multidrug resistance were highly susceptible as a group to the antibiotic action of pyocyanin than the Gram negative bacteria. The antibacterial activity of the pigment is attractive for the topical treatment of wound infections (El-Shouny *et al.,* 2011).

From different studies (Sudhakar *et al.,* 2013; Karpagam *et al.,* 2013), pyocyanin was extracted using chloroform and the presence of secondary metabolite was confirmed by the addition of 0.2 N HCl. Partial purification of the Pigment was by column chromatography and subjected to UV-Vis spectrophotometer. A maximum absorption was observed at 277-278 nm (Sudhakar *et al.,* 2015). Structural elucidation of pyocyanin was done using Proton NMR and FTIR. In the present study, Pyocyanin produced by *Pseudomonas aeruginosa strain* BTRY1 was extracted from Pseudomonas broth by chloroform and the blue green compound was separated. Addition of 0.2 N HCl to obtain a pinkish red colored confirmed the presence of pyocyanin pigment (Raoof and Latif, 2010). The separated red color compound on UV-spectrophotometric analysis showed maximum absorption at 270 nm which is in accordance with the previously published reports (Kerr *et al.,* 1999). It also has the capacity to arrest the electron transport chain of the different microorganisms and to exhibit antimicrobial and antibiofilm activity (Kerr, 1994). The FTIR and Proton NMR spectrum of the BTRY1 pigment were characteristic of pyocyanin. There are only few published reports for the antibiofilm potential of pyocyanin (Bhattacharya *et al.,* 2013) and the present study demonstrated the ability of pyocyanin as an antibiofilm agent against food borne pathogens.

It was proved that the glycolipid biosurfactants was produced by *P. aeruginosa*. Rhamnolipids (RLs) are the most intensively studied biosurfactants. This is due to two contrasting facts. Firstly, they display relatively high surface activities and secondly, they are produced in relatively higher yields after shorter incubation periods. Since the rhamnolipids are known to have several antimicrobial effects (Abdel-Mawgoud *et al.,* 2011). Along with their immense biotechnological and industrial applications their antibiofilm activity adds to the importance of the rhamnolipids produced by the strain *P. aeruginosa*. Anti-biofilm potential of a glycolipid surfactant produced by a tropical marine strain of *Serratia marcescens* was reported, where the glycolipid prevented adhesion of *Candida albicans* BH, *P. aeruginosa* PAO1 and *Bacillus pumilus* TiO1 and the glycolipids were also reported to have disrupted the preformed biofilms of these cultures in microtiter polystyrene plates (Dusane *et al.*,2011) Another recent study, reported that biosurfactants from two types of Lactobacilli displayed anti-adhesive and anti-biofilm abilities against *Acinetobacter baumannii*, *Escherichia coli* and *Staphylococcus aureus* (Sambanthamoorthy *et al.,* 2014)

The presence of rhamnolpids were confirmed using orcinol method, CTAB agar method, oil spreading assay and the structural elucidation by NMR and FTIR spectra (Rooney *et al.,* 2009; Mohammed *et al.,* 2014; Kalyani *et al.,* 2014), In this study, the qualitative methods for screening RL production by bacterial strains was by CTAB agar test (Siegmund and Wagner, 1991) and drop collapsing assay (Jain *et al.,* 1991) which established the presence of rhamnolipids. The FTIR and Proton NMR spectra of rhamnolipids were as previously published (Rahman *et al.,* 2002), proving the structural characteristics of the compound. In addition to this, the compounds showed no cytotoxic effects on human red blood cells (Park *et al.,* 2004) and in cultured L929 cells (Arung *et al.,* 2009).

In 2013, it was reported that a yellow pigment produced from *Exiguobacterium profundum* showed maximum free radical scavenging activity (Arulselvi and Gurumayum, 2013). Production of yellow green fluorescent pigment by *Pseudomonas flourescens* was also found to have high free radical scavenging activity (Silva and Almeida, 2006). Microorganisms other than *Pseudomonas* like *Legionella pneumophila* (Liles *et al.,* 2000), *Halomonas*, *Marinobacter* (Martinez *et al.,* 2000), and *Rhizobium* (Roy *et al.,* 1994), can

produce iron-scavenging molecules. There are only few reports regarding the radical scavenging activity of glycolipids from microbes. MIF-A3, a peptidoglycolipid extracted from *Mycobacterium avium* was reported to have antioxidant activities (Scherer *et al.*, 1997). Biosurfactant obtained from *B. subtilis* RW-I has the antioxidant capacity to scavenge free radicals and that these results suggest that the biosurfactant could be used as alternative natural antioxidants after toxicological examination (Yalcin and Cavusoglu, 2010). In a study, RL from *P. aeruginosa CEMS077* has been tested for free radical scavenging activity and identified as a promising antioxidant in biological system (Singh *et al.*, 2014). As pigments/surfactants, both the compounds showed very high free radical scavenging activity at very minute concentrations, which is a positive indication for the safe use of compounds.

Chapter 5

Biocontrol of biofilm by different biomolecules - pyocyanin, rhamnolipids, melanin and bacteriocin

5.1 Introduction

Pseudomonas aeruginosa are Gram-negative, aerobic rod-like bacteria, motile by single polar flagellum (Palleroni *et al.,* 2006); thriving in normal atmospheric as well as hypoxic atmospheres, besides colonising many natural and artificial environs (Suthar *et al.,* 2009). Recent research indicates that secretions from *Pseudomonas aeruginosa* also inhibit biofilm formation by several fungi, as well as other bacterial pathogens (Holcombe *et al.,* 2010). There are several reports that *P. aeruginosa* itself can inhibit growth and biofilm formation (Bandara *et al.,* 2012). Bacterial pigments used for biofilm control are reported to have free radical scavenging activity along, which in addition to absence of cytotoxicity, make them useful in food industry for control of food borne infections.

Melanins have several biological functions including photo protection, thermoregulation, action as free radical sinks, cation chelators, and antibiotics. In plants, melanin is incorporated in the cell walls as strengtheners (Riley *et al.,* 1997), while in humans it not only determines skin color but most importantly also protects skin against UV light injury (Huang *et al.,* 2012). In the microbial world, melanins act as protective agents against environmental stresses. For example melanin producing bacteria are more resistant to antibiotics (Li Bin *et al.,* 2012) and in fungi too, melanins are involved in pathogenesis (Butler and Day, 1998). Crude melanin from *Streptomyces* showed antibacterial activity against *Escherichia coli* and *Lactobacillus vulgaris* (Vasanthabharathi *et al*., 2011). Melanin derived from *Auricularia auriculara,* an edible jelly mushroom significantly inhibited biofilm formation of *E. coli* K-12, *Pseudomonas aeruginosa* PAO1 and *P. fluorescens* P-3 (Bin *et al.,* 2012).

Bacteriocins are ribosomally synthesized by bacteria and are defined as proteins/small peptides which have antibacterial activity, that kill or inhibit the growth of closely related bacteria by various mechanisms like increasing cell membrane permeability, inhibiting cell wall synthesis or by inhibiting DNAse or

RNAse activity (Lazdunski, 1988). They were first characterized in Gram negative bacteria; are safe with potential for use as natural preservatives in food industry. The term 'natural' is compromised when bacteriocin is obtained from genetically modified microorganism. Though nisin is currently the only bacteriocin approved for use as food preservative, several other bacteria are reported to produce bacteriocins that can safely be used in food industry (Cleveland *et al.,* 2001). The genus *Bacillus* includes representatives such as *Bacillus subtilis* and *Bacillus licheniformis* that are 'generally recognized as safe' (GRAS) (Zheng and Slavik, 1999) and thus find application in the control of food pathogens and spoilage microorganisms in food processing environment. The qualification concerning qualified presumption of safety (QPS) for *Bacillus sp* is modified to *'absence of food poisoning toxins, absence of surfactant activities, absence of enterotoxic activities'* (EFSA, 2008).

This chapter deals with the evaluation of the ability of melanin, bacteriocin, pyocyanin and rhamnolipids for biocontrol of nine strong biofilm forming food pathogens. The four bioactive compounds were extracted and purified from various bacterial sources- melanin from *Providencia rettgeri* (BTKKS1), bacteriocin BL8 from *Bacillus licheniformis* (BTHT8) (Smitha and Bhat, 2013) and pyocyanin and rhamnolipids from *P.aeruginosa* (BTRY1). *In vitro* biofilm formation and inhibition was tested using microtiter plate assay with crystal violet staining. The antibiofilm activity was demonstrated using two techniques namely Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM). The quantification of the confocal images was by Image J software. Different application studies of the pyocyanin and rhamnolipids in food preservation are also discussed in the chapter.

5.2 Materials and Methods

5.2.1. *Antibiofilm activity of pyocyanin, rhamnolipids, melanin and bacteriocin BL8*

The compounds were analysed for their ability to control biofilm formation. Microtitre 96 well plates were used for antibiofilm assay (Rode *et al.*, 2007). Briefly, 230 µL of tryptone soy broth (TSB) (Appendix -1) was added to the wells, followed by 20 µL each of the bacterial culture (OD_{600} =1), in triplicates for each test organism and incubated aerobically for 24 h at 37°C. 10 µL of

pyocyanin (1.245 µg/mL)/ rhamnolpids (75 µg/mL) / bacteriocin (2380 µg/mL)/melanin (100 µg/mL) was added to respective wells and incubated for 24 h at 37°C. TSB served as negative control. Positive control is the well containing the microorganism alone. The contents of the plates were poured off; washed thrice with phosphate buffer (0.01 M, pH 7.2) and attached bacteria were fixed with methanol. After 15 min, plates were decanted, air dried and stained with 1% crystal violet for 5 min and excess stain rinsed under running tap water and air dried. The dye bound to adherent cells was extracted with 33% (v/v) glacial acetic acid, and measured at 570 nm using a UV-VIS spectrophotometer (Schimadzu, Japan). All tests were repeated thrice independently and statistically analysed (Christensen *et al.,* 1988; Stepanovic *et al.,* 2000).

Finally, the percentage reduction in biofilm formation was calculated as: % in biofilm reduction = (OD of Control - OD of Test/ OD of Control) X 100 Statistical evaluations were done by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) computer program (Appendix-5). If there are more positive as negative changes, then $p > 0.5$ which means the test is significant.

5.2.2. *Determination of biofilm inhibitory concentration (BIC) of pyocyanin, rhamnolipids, melanin and bacteriocin BL8 for antibiofilm activity*

Biofilm Inhibitory Concentration (BIC) is defined as the lowest concentration of the compound which inhibits biofilm formation. Pyocyanin (1.245 µg/mL) /rhamnolpids (75 µg/mL)/ bacteriocin (2380 µg/mL)/ melanin (100 µg/mL) was serially diluted and checked for antibiofilm activity as described in section 4.2.1. Azithromycin (15 µg/disc, Himedia) was used as the positive control. Minimum Inhibitory concentrations (MICs) for the antibacterial activity against the nine test pathogens were calculated for all the four compounds and were done by broth dilution assay (Jiang, 2011). The results are given in Annexure – I.

5.2.3. *Antibiofilm activity of different combinations of bioactive compounds*

The four compounds namely pyocyanin, rhamnolipids, bacteriocin and melanin in different combinations of their BIC concentrations was tested for their antibiofilm activity against nine strong biofilm producing food pathogens as discussed in 5.2.1. The combinations tested were as follows:

1. Bacteriocin + Rhamnolipids
2. Bacteriocin + Melanin
3. Bacteriocin + Pyocyanin
4. Melanin + Rhamnolipids
5. Melanin + Pyocyanin
6. Rhamnolipids + Pyocyanin
7. Bacteriocin + Rhamnolipids + Melanin
8. Bacteriocin + Pyocyanin + Melanin
9. Bacteriocin + Pyocyanin + Rhamnolipids
10. Melanin + Pyocyanin + Rhamnolipids
11. Pyocyanin + Rhamnolipids + Pyocyanin

All tests were repeated thrice independently and statistically analysed. Statistical evaluations were done by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) computer program (Appendix-5).

5.2.4. *Effect of the four bioactive compounds on Extracellular polymeric substances (EPS) production by the strong biofilm producers*

Extraction of EPS from strong biofilm producers

The biofilm forming test culture was centrifuged at 11,000 rpm for 10 min. The supernatant collected was mixed with an equal volume of ice-cold ethanol and incubated at 4°C for 24 h. The refrigerated solution was then centrifuged at 2500 rpm for 20 min. The obtained pellet was resuspended in distilled water along with an equal volume of ice-cold ethanol. The solution was then centrifuged again at 2500 rpm for 20 min. The final pellet obtained was dried at 60°C and weighed (Razack *et al.*, 2013).

Effect of the bioactive compounds on (EPS) production

The experiment was repeated thrice for each test organism and the extraction of EPS was done for each of the nine pathogens used before and after

treatment with all the four bioactive compounds- pyocyanin, rhamnolipids, melanin and bacteriocin BL8. This was done to analyse the difference in the quantity of EPS production after biocontrol of biofilms with the mentioned bioagents.

Statistical evaluations were done by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) (Appendix-5).

5.2.5. Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was used to visualize the antibiofilm effect of pyocyanin (1.245 µg/mL) /rhamnolipids (75 µg/mL) / bacteriocin BL8 (2380 µg/mL) / melanin (100 µg/mL) on microslides. Culture broth with micro-slide was kept for biofilm formation in the incubator for 24 h at 37°C, added pyocyanin/ rhamnolipids/ bacteriocin BL8/ melanin at their BIC concentrations and incubated under same conditions. Fixation was done with little modifications (Lembke *et al.,* 2006) as shown in the table 5.1, followed by SEM

Table 5.1 Fixation steps in Scanning electron microscopy

Steps	Solution	Time	Repetitions
1. Primary fixation	2.5% glutaraldehyde	1 h RT*	-
2. Wash	0.1 M Sodium phosphate buffer(pH 7.3)	5-10 min	3-5
3. Dehydration	25% ethanol	5 min	-
	50% ethanol	5 min	-
	75% ethanol	5 min	-
	90% ethanol	5 min	-
	100% ethanol	5-10 min	2

RT* =room temperature; (Laxmi *et al.,* 2015)

After fixation the cover slips were thereafter dried in a critical point dryer, mounted on studs and coated with gold plasma and examined using the scanning electron microscope (JEOL Model JSM - 6390LV).

5.2.6. Confocal Laser Scanning Microscopy (CLSM)

For Confocal Laser Scanning Microscopy, biofilms on micro-slide were fixed using Glutaraldehyde (2.5%), followed by To-pro-3 staining (diluted 1:1000 in PBS) for 10 min 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 live cells can be seen as red spots due to the far red fluorescence of the dye. Therefore the pixel intensity of the red coloured spots appearing in the images before (Control) and after (Treated) treatment with pyocyanin/ rhamnolipids /bacteriocin BL8 /melanin can be quantified.

The quantification of data for the confocal microscopy was by Image J software (Image J 4.8v/ Java 1.6.0_20, 64-bit) (Peter, 2014) and the graph plots were generated from the software in the form of Red, Green, Blue (RGB) plots. The variations seen in the RGB plots before and after treatment with pyocyanin, rhamnolipids, bacteriocin and melanin are directly related to the pixel intensity provided by the software.

5.2.7. Application studies of the bioactive compounds in the biocontrol of biofilms

5.2.7.1. Effect of pyocyanin and rhamnolipids on biofilm formation of different test pathogens from the culture collection of the laboratory.

Pyocyanin and the rhamnolipids extracted in the study was tested against the biofilm production of pathogenic strains from the culture collection of the laboratory. The pathogenic strains used were *Vibrio diabolicus (TVMS3), Vibrio alginolyticus (KK16), Vibrio harveyi (KKS4), Vibrio parahemolyticus (KK10), Salmonella Enteritidis (S37) and Salmonella Enteritidis (S49).*The microtiter assay was repeated thrice with triplicates and statistically analysed. Statistical evaluations were done by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) (Appendix-5).

5.2.7.2. Effect of bioactive compounds singly and in combination on multispecies biofilm formation.

Biofilms in most cases are not due to a single species. Multispecies biofilms exist as consortia. Different consortia of biofilm producers were made by

the study of antagonism. Antagonism is the property by which microorganisms produce metabolites or as such inhibit the growth of another organism. The antagonism was done using cross streak method (Madigan *et al.,* 1997). Antagonism was tested with the strong biofilm producers in the study to know which among them can be grown together make a biofilm. Thus antagonism was checked and the effect of bioactive compounds on these different biofilm forming consortia were statistically analysed. Statistical evaluations were done by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) computer program (Appendix-5).

Cross streak method:

Nutrient agar plates were prepared and inoculated with a test organism in a single streak in the center of the petri dish. After incubation at 37°C the plates were seeded with indicator bacteria by a single streak at a 90° angle to that of test organism. The microbial interactions were analyzed by the observation of the inhibition zone (Madigan *et al.*,1997).The antagonism was checked for the preparation of microbial consortia capable of forming biofilm; further an efficient biocontrol against these consortia of biofilm producers was developed using combinations of the four different bioactive compounds under study.

5.2.7.3. The application of the bioactive compounds in the preservation of common foods available in market

The application of the four bioactive compounds as preservatives/ additives in common food was tested. Four food samples were selected for the study which included chilly powder, milk, soft drink and dried fish. The samples were suspended in normal saline along with the compounds (in BIC concentrations) in respective boiling tubes and incubated at 37°C for 24 h. The experimental set up is depicted in table 5.2.

Table 5.2. Experimental design to study bioburden control by bioactive compounds

The experimental groups
1. Normal saline + sample (positive control)
2. Normal saline + sample+ 100 µL pyocyanin
3. Normal saline + sample+ 100 µL rhamnolipids
4. Normal saline + sample+ 100 µL melanin
5. Normal saline + sample+ 100 µL bacteriocin
6. Normal saline + sample+ 100 µL combination
7. Normal saline (negative control)

Every set was diluted to 10^{-4} dilution and 0.1 mL was spread onto nutrient agar plates. Experiment was conducted in triplicates and statistically analysed. Statistical evaluations were by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) computer program (Appendix-5).

5.3 Results

5.3.1. Antibiofilm activity of pyocyanin, rhamnolipids, melanin and bacteriocin BL8

The antibiofilm activity of the four bioactive compounds was tested. The graphs 5.1-5.4 show the percentage reduction in biofilm formation by the nine strong biofilm producing food pathogens on treatment with the pyocyanin, rhamnolipids, melanin and bacteriocin BL8.

It was clear that pyocyanin at 1.245 µg/mL had a profound inhibitory effect at $p>0.5$, on the biofilm forming capability of six of the nine isolates tested. It caused significant reduction of biofilm formation by *Bacillus altitudinis, Bacillus pumilus, Brevibacterium casei, Staphylococcus warneri, Bacillus niacin* and *Bacillus sp*. (Figure 5.1). However, biofilm formation by *Pseudomonas aeruginosa, Micrococcus luteus* and *Geobacillus stearothermophilus* could not be controlled.

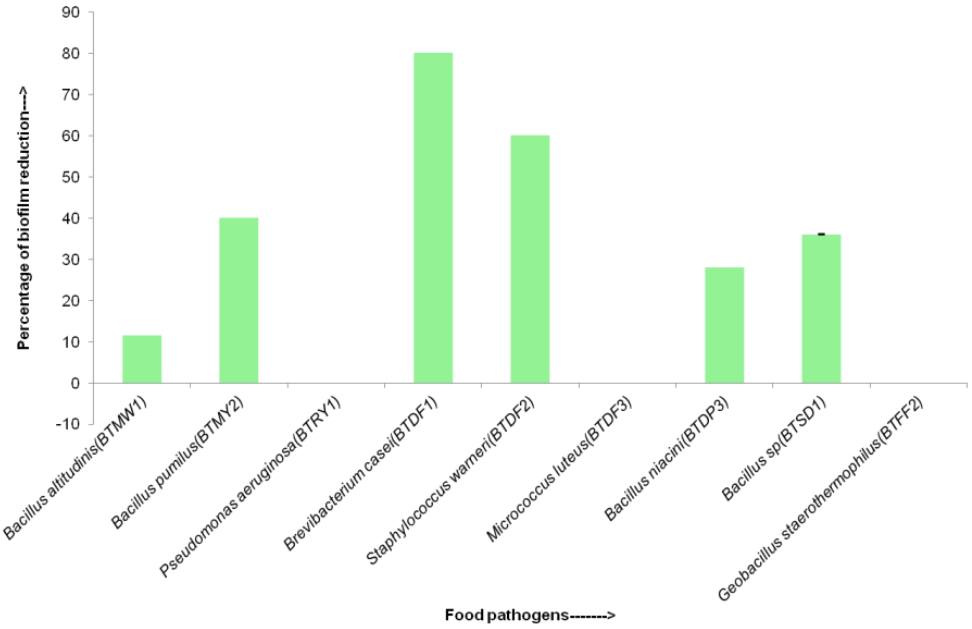


Fig. 5.1. Percentage reduction in biofilm formed by strong biofilm producers (N=9) on treatment with pyocyanin

Rhamnolipids (75 µg/mL) too, had also an inhibitory effect ($p>0.5$) on the biofilm forming capability of six out of the nine isolates tested, wherein it caused significant reduction of biofilm formation by *B. pumilus*, *B. casei*, *S. warneri*, *M. luteus*, *G.stearothermophilus* and *Bacillus sp*. (Figure 5.2). However, biofilm formation by *B. altitudinis*, *P.aeruginosa and B. niacin* could not be controlled by rhamnolipids at the concentration tested.

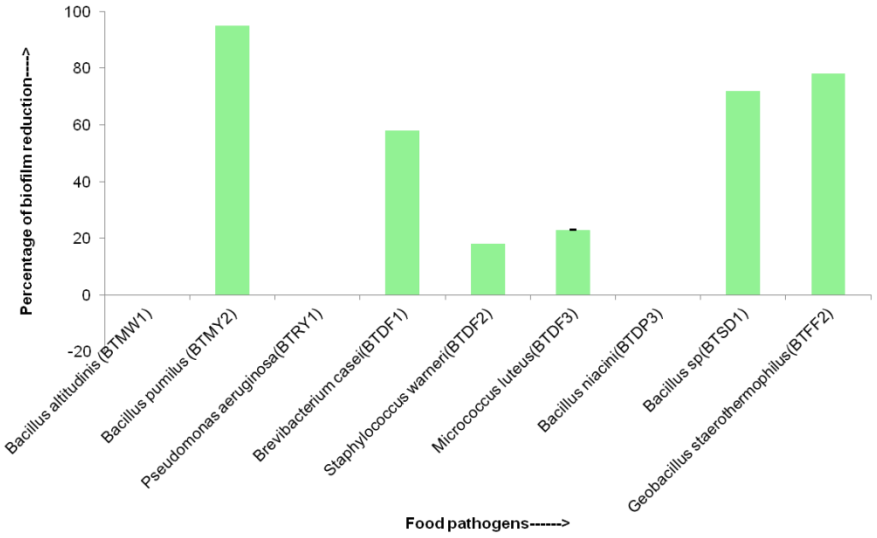


Fig. 5.2. Percentage reduction in biofilm formed by strong biofilm producers (N=9) on treatment with rhamnolipids

Treatment with melanin at 100 µg/mL caused inhibitory effect (p>0.5) on the biofilm forming capability of six of the nine isolates tested. It caused significant reduction of biofilm formed by *B. altitudinis, P.aeruginosa, B. casei, S. warneri, B.niacini* and *Bacillus sp*. (Figure 5.3). However, biofilm formation by *B.pumilus, M. luteus and G. stearothermophilus* could not be controlled.

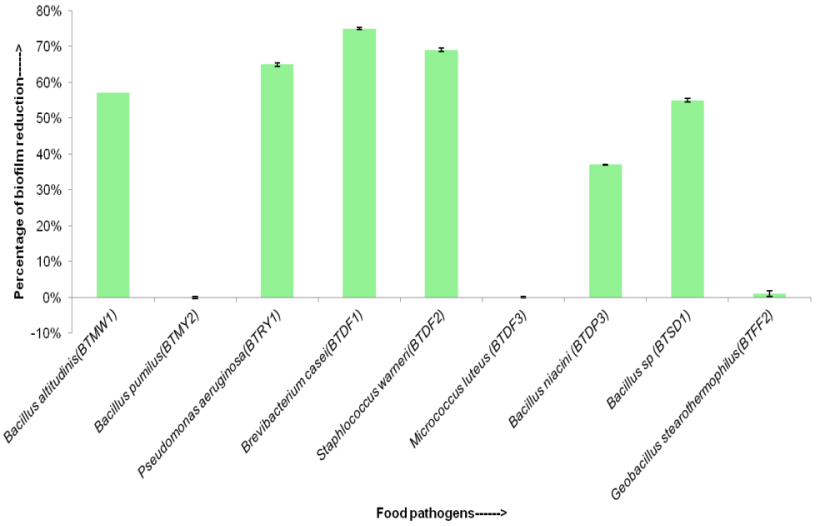


Fig. 5.3. Percentage of reduction in biofilm formed by strong biofilm (N=9) producers on treatment with melanin (published as in Laxmi *et al.*, 2015).

Bacteriocin BL8 at 2380 µg/mL showed the best activity of all four biomolecules tested; it had aninhibitory effect (p>0.5) on the biofilm forming capability of all nine isolates tested (Fig 5.4).

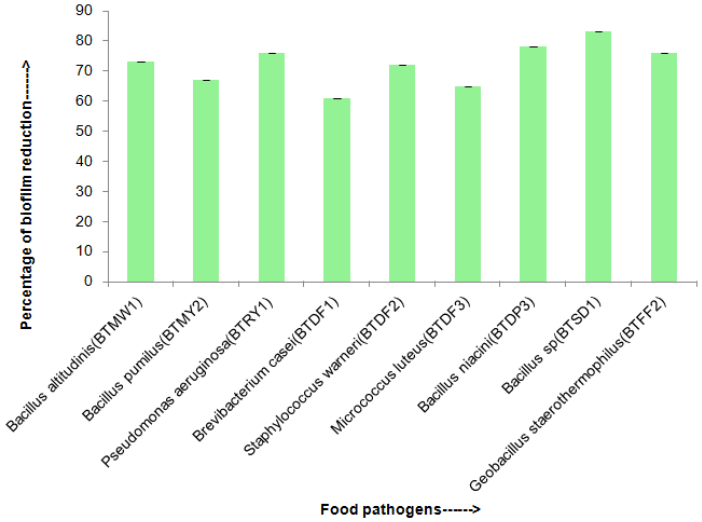


Fig. 5.4. Percentage reduction in biofilm formed by strong biofilm producers (N=9) on treatment with bacteriocin BL8 (published as in Laxmi *et al.*, 2015).

Table 5.3. Percentage Reduction of biofilm formation of the food pathogens on treatment with the test bioactive compounds

Organism	Pyocyanin treated (%inhibition)	Rhamnolipids treated (%inhibition)	Melanin treated (%inhibition)	Bacteriocin treated (%inhibition)
B.altitudinis (BTMW1)	11.6	0	57	73
B.pumilus (BTMY2)	40	95	0	67
P.aeruginosa (BTRY1)	0	0	65	76
Brevibacterium casei(BTDF1)	60	58	75	61
Staphylococcus warneri (BTDF2)	80	18	69	72
Micrococcus luteus (BTDF3)	0	23	0	65
Bacillus niacini (BTDP3)	28	0	37	78
Bacillus sp (BTSD1)	36	72	55	83
Geobacillus staerothermophilus (BTFF2)	0	78	1	76

From the graphs and table, it was clear that bacteriocin BL8 was more efficient in biocontrol of biofilm forming food pathogens than the other biomolecules. Further biofilm inhibitory concentration (BIC) of each of the biomolecules was estimated to study their biocontrol efficiency.

5.3.2. Determination of biofilm inhibitory concentration (BIC) of pyocyanin, rhamnolipids, melanin and bacteriocin BL8 for antibiofilm activity

The biofilm inhibitory concentration of all the four compounds on nine strong biofilm producers was tested. The initial concentrations for pyocyanin, rhamnolipids, bacteriocin and melanin was 1.245 µg/mL, 75 µg/mL, 2380 µg/mL

and 100 µg/mL respectively. The assay was conducted as mentioned in section 4.2.2 with serial dilutions starting from 10^{-1}(P1, R1, B1, M1) to 10^{-4}(P4, R4, B4, M4) with the initial concentrations as stated above. The graphs 5.5 - 5.8 and table 5.4 represent the BIC values of four compounds with appropriate serial dilutions. The X-axis in the graphs refers to nine pathogens tested while the Y-axis indicated the reduction in biofilm formation at different concentrations. The concentration at which the biofilm is greatly inhibited was considered to be Biofilm Inhibitory Concentration (BIC). It was found that 10^{-2} dilution was found to be most appropriate. Similarly BIC for all compounds on all tested pathogens and the values for BICs were calculated in accordance with it.

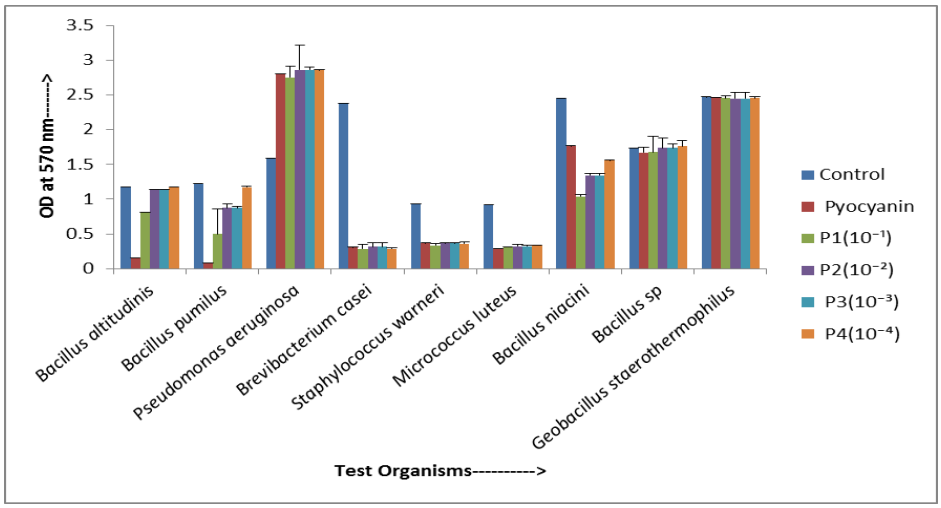


Fig 5.5. Demonstration of Biofilm Inhibitory Concentration of pyocyanin at different dilutions

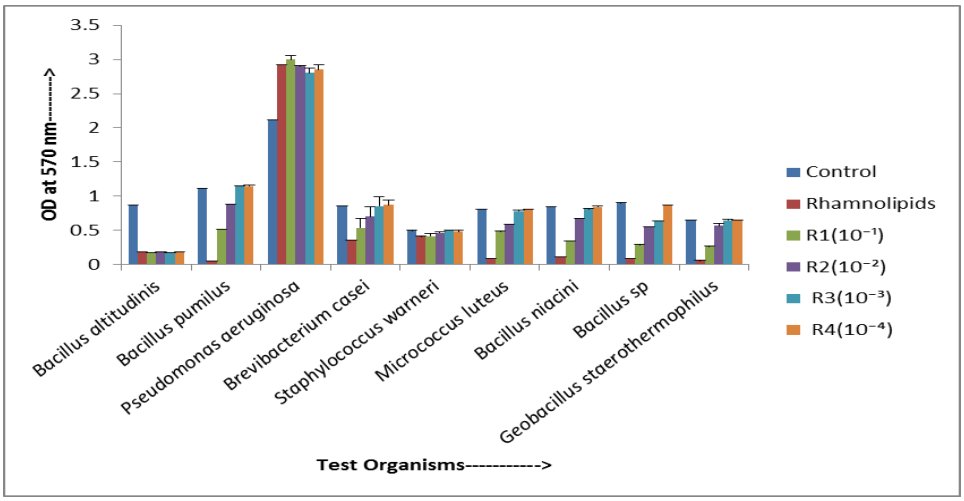


Fig 5.6. Demonstration of Biofilm Inhibitory Concentration of rhamnolipids at different dilutions

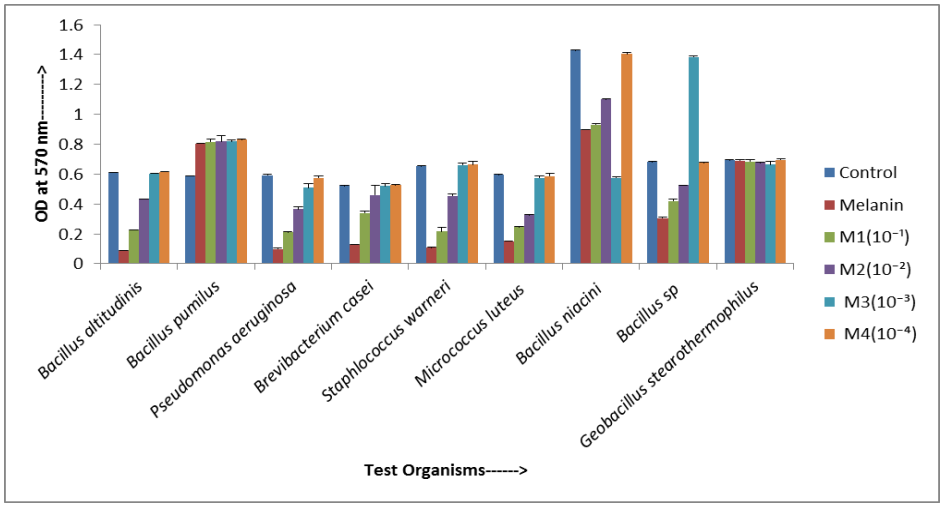


Fig 5.7. Demonstration of Biofilm Inhibitory Concentration of melanin at different dilutions

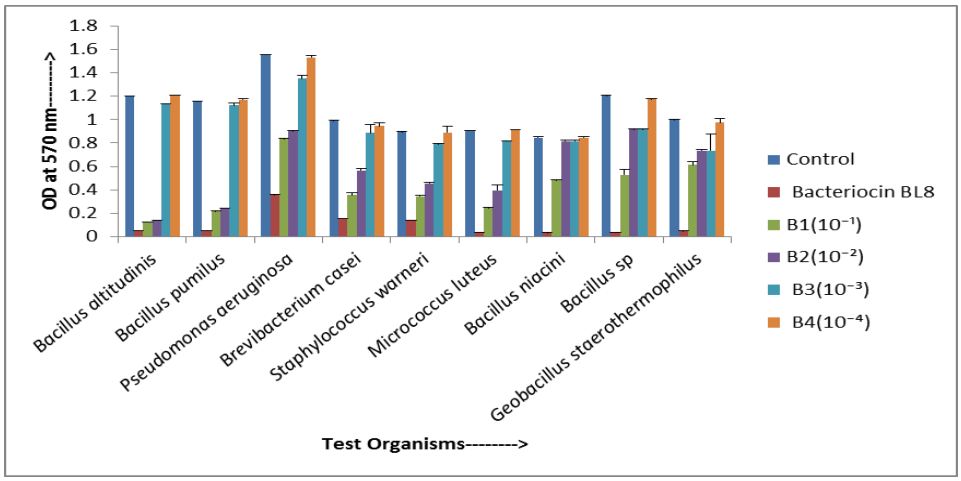


Fig 5.8. Demonstration of Biofilm Inhibitory Concentration of bacteriocin BL8 at different dilutions

The Biofilm Inhibitory Concentration of pyocyanin, rhamnolipids, melanin and bacteriocin BL8 was estimated and is tabulated in Table 5.4

Table 5.4. Biofilm Inhibitory Concentrations for the bioactive compounds

Bioactive compound	**Biofilm Inhibitory concentration (BIC)**
1. Pyocyanin	2 x 10^{-2}ng/µL
2. Rhamnolipids	1.2 ng/µL
3. Melanin	16 x 10^{-2}ng/µL
4. Bacteriocin BL8	3.8 ng/µL

Azithromycin was the test antibiotic to which most of the biofilm producers were sensitive and its concentration was 15 µg/disc. The BIC values of the bioactive compounds used in the study were in nanogram quantities against the tested food pathogens. This clearly indicates the immensely potent strength of the bioactive compounds in biofilm control compared to the current antibiofilm strategies like antibiotic treatments.

The comparison in reduction of biofilm formation (in %) of the food pathogens on treatment with the four bioactive compounds at their BIC concentrations is illustrated in the table 5.3.

5.3.3. Antibiofilm activity of different combinations of bioactive compounds

The antibiofilm activity of eleven different combinations made with the four bioactive compounds was done as discussed in 4.2.3. The compounds are present in their biofilm inhibitory concentrations (2 x 10^{-2}ng/µL for pyocyanin, 1.2 ng/mL for rhamnolipids, 16 x 10^{-2}ng /mL for melanin and 3.8 ng/mL for bacteriocin) in these combinations.The reduction in biofilm formation by the nine food borne pathogens are represented in percentage ($p>0.5$) The Figures 5.9– 5.19 represent the percentage reduction in biofilm formation due to combination of different bioactive compounds. The tables (Annexure-2) lists the percentage of reduction due to each of the eleven combinations to evaluate the most effective combination for each of the test pathogens used.

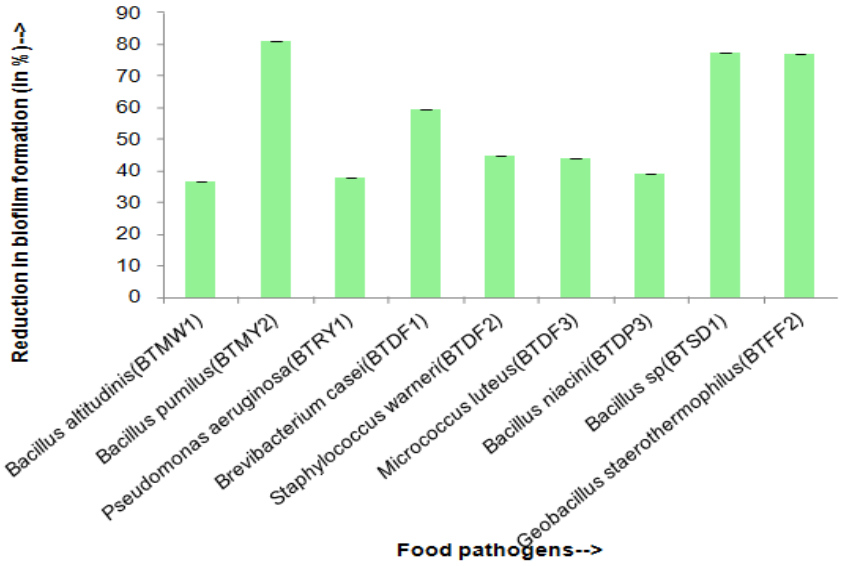


Fig 5.9.Bacteriocin + Rhamnolipids

From the figure 5.9, it is clear that almost 50% biofilm reduction is seen in all organisms. 80% reduction is seen in case of *B. pumilus* BTMY2, *Bacillus sp* BTSD1 and *G. staerothermophilus* BTFF2.

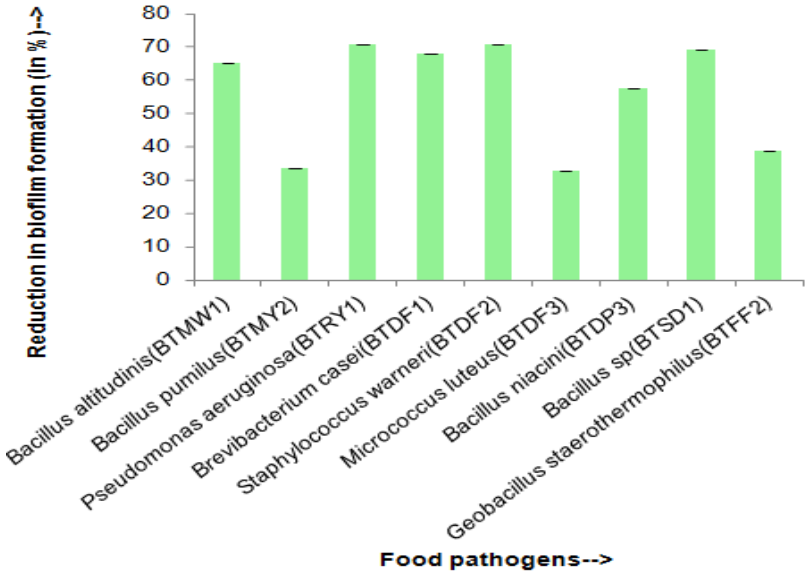


Fig 5.10. Bacteriocin + Melanin

From figure 5.10, it is visible that it is one of the most efficient combinations out of the eleven combinations tried showing the biofilm inhibition >60 for almost all pathogens.

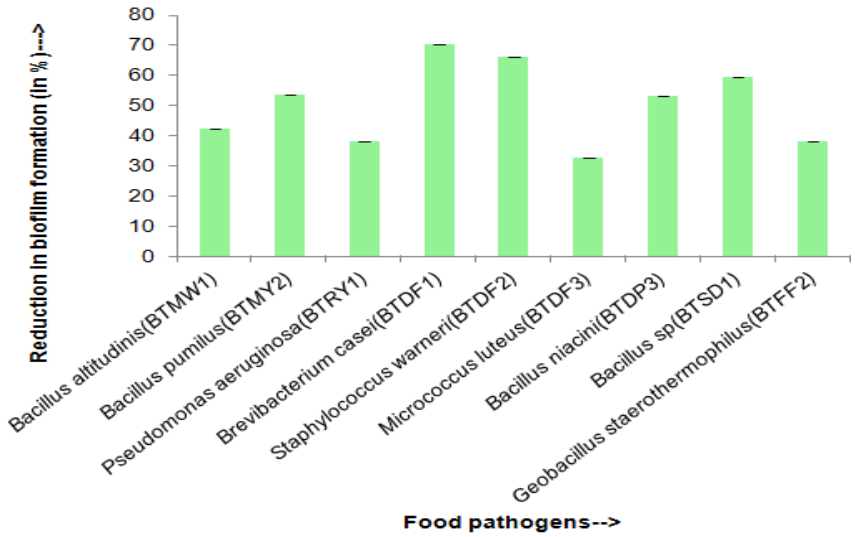


Fig 5.11.Bacteriocin +Pyocyanin

Figure 5.11 shows that around 70 % biofilm inhibition in case of *B. casei* BTDF1 and *S. warneri* BTDF2 along with an inhibition not less than 35% in other pathogens.

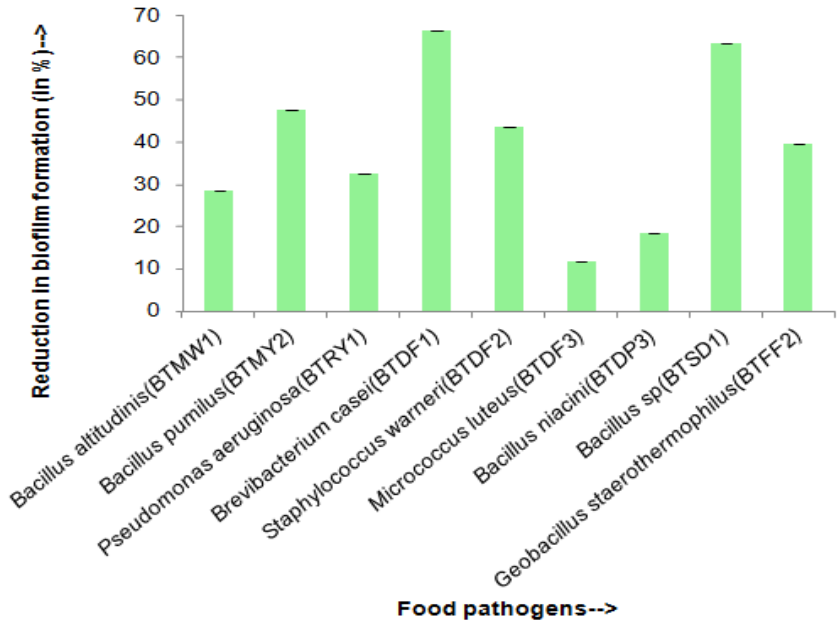


Fig 5.12.Melanin + Rhamnolipids

Figure 5.12 depicts the around 60% biofilm inhibition in *B.casei* BTDF1 and *Bacillus sp* BTSD1. But the combination was not found to be very effective in other tested pathogens.

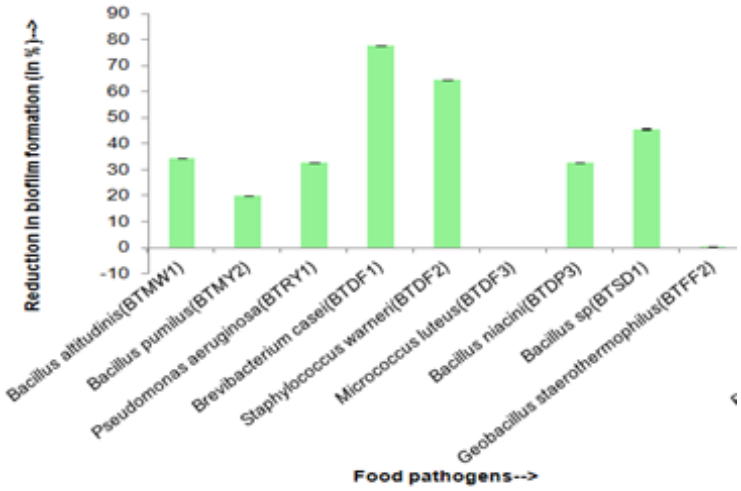


Fig 5.13 Melanin+ Pyocyanin

From figure 5.13, it is clear that both *B.casei* BTDF1 and *S. warneri* BTDF2 were inhibited to 70 %. But it is not inhibiting the biofilms in *B. niacini* BTDP3 and *G.staerothermophilus* BTFF2 and thus not a very effective combination.

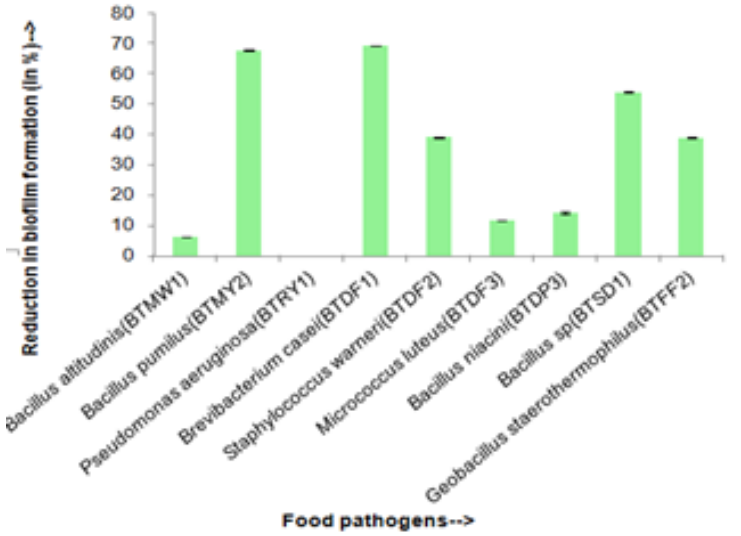


Fig 5.14.Rhamnolipids + Pyocyanin

From figure 5.14, it is clear that both *B.casei* BTDF1 and *B. pumilus* BTMY2 were inhibited to 70 %. But it does not inhibit the biofilms in *P. aeruginosa* BTRY1.

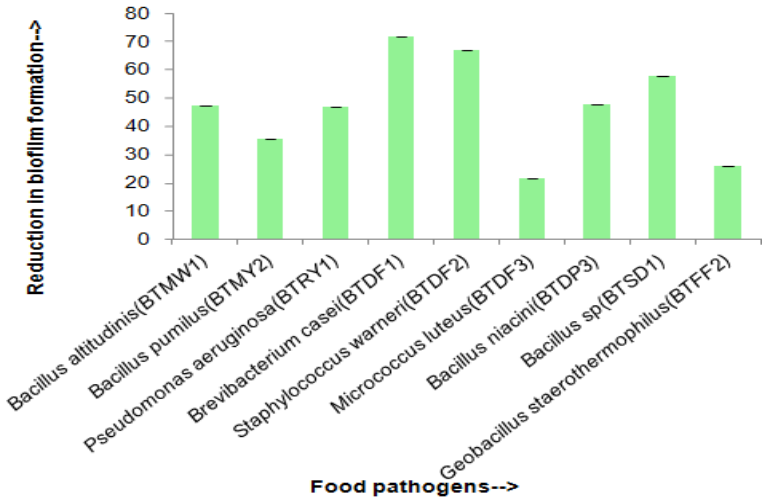


Fig 5.15. Bacteriocin+.Melanin+Pyocyanin

Figure 5.15 depicts the combination of three compounds which was found to inhibit almost 50 % in most of the pathogens with a 70 % reducion in biofilms formed by *B. casei* BTDF1 *and S. warneri* BTDF2.

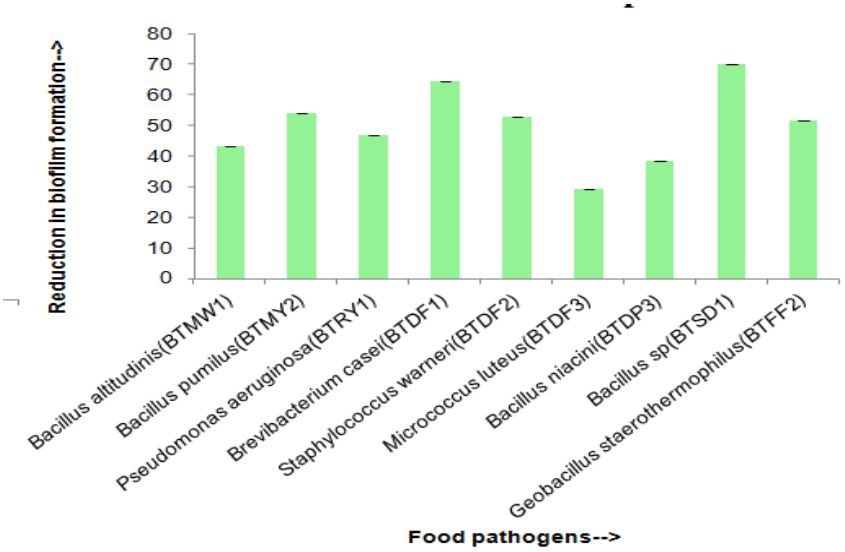


Fig 5.16.Bacteriocin+ Melanin+Rhamnolipids

The combination showed in the figure 5.16 is also found to be highly effective against all the tested pathogens with maximum reduction in *Bacillus sp* BTSD1 by 70%.

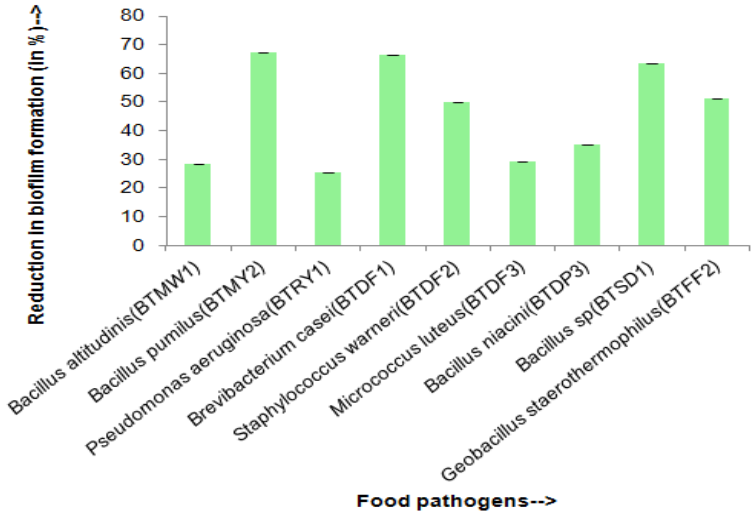


Fig 5.17. Bacteriocin+Rhamnolipids+Pyocyanin

From figure 5.17, greater than 65% reduction is observed in case of biofilm formation by *B. pumilus* BTMY2, *B. casei* BTDF1 *and Bacillus sp* BTSD1.

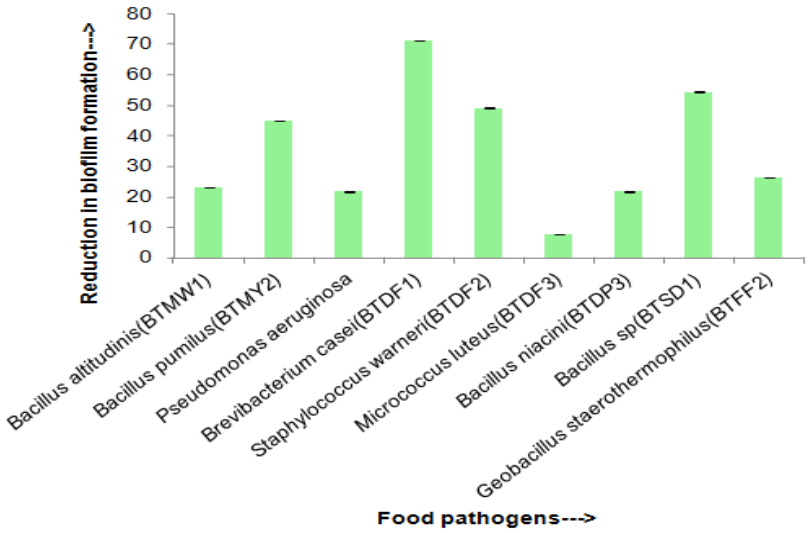


Fig 5.18. Rhamnolipids+Melanin+Pyocyanin

From figure 5.18, it is found that the combination used is not much effective and the synergism was not seen and hence very less inhibition in most of the pathogens except for a reduction by 70% in case of *B.casei* BTDF1.

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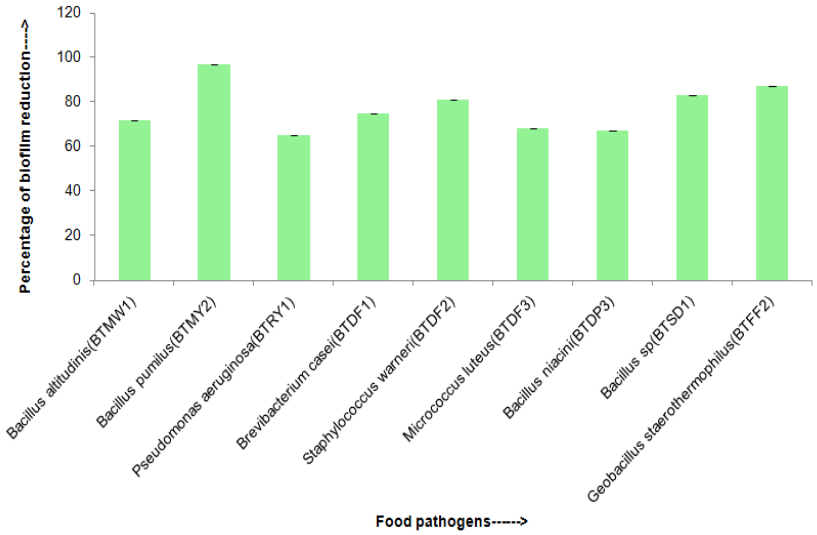


Fig 5.19. Bacteriocin+Rhamnolipids+Pyocyanin+ Melanin

From figure 5.19, it is undoubtedly proved that the combination of four compounds at their BIC values found to be highly effective against all the tested pathogens where almost 100% inhibition seen in most of the food borne pathogens under study. This synergism paves the way for hurdle technology in the field of biofilm mitigation.

The graphs clearly represent the synergistic effect of different combinations of the compounds together at their BIC concentrations for biocontrol. The most effective combination was undoubtedly the combination of all the four bioactive compounds together. This was followed by the combinations bacteriocin and rhamnolipids together and bacteriocin and melanin together. Bacteriocin and rhamnolipids together was able to inhibit greater than 50 % biofilm formation of *B.pumilus (*BTMY2), *Micrococcus luteus* (BTDF3), *Bacillus sp* (BTSD1) and *Geobacillus staerothermophilus*(BTFF2*).*Bacteriocin and melanin together inhibits biofilm formation of *Bacillus altitudinis* (BTMW1), *Pseudomonas aeruginosa* (BTRY1), *Staphylococcus aureus* (BTDF2), *Bacillus niacini* (BTDP3) by around 70%. Thus the different combinations evaluated the efficiency of combined effect of the bioactive compounds on test pathogens used in the study.

5.3.4. Effect of the four bioactive compounds on Extracellular polymeric substances (EPS) production by the strong biofilm producers

Extracellular Polymeric Substances (EPS) were isolated and extracted from the nine strong biofilm producers. The EPS obtained from each organism was quantified before and after treatment with the bioactive compounds individually and in combination as discussed in 4.2.4 (Figure 5.20).

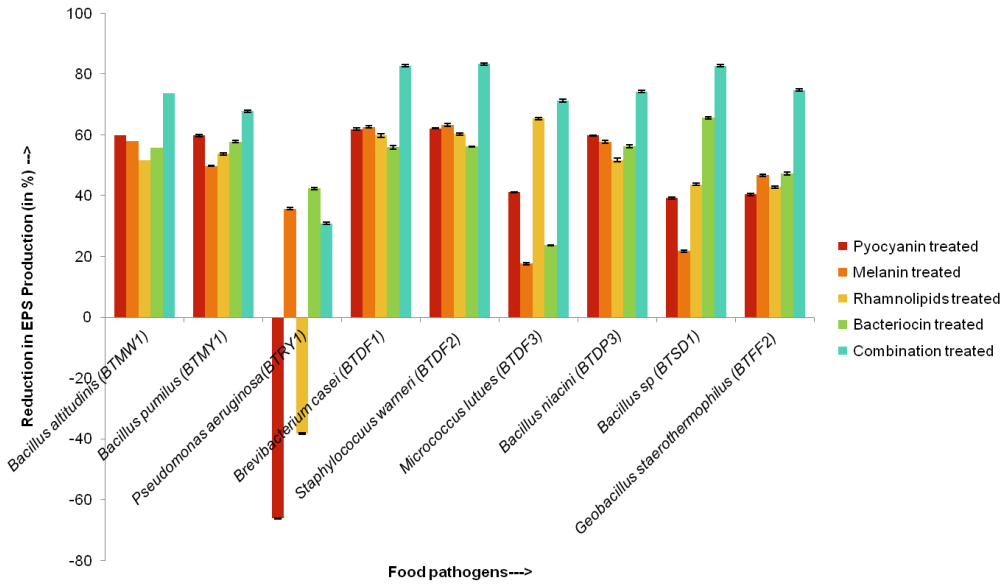


Fig. 5.20.EPS production by biofilm producers (N=9) and their percentage reduction on treatment with melanin, pyocyanin, rhamnolipids, bacteriocin and combination

From the graph, it clearly indicates that the EPS production was reduced to a greater extent on treatment with the compounds at their respective concentrations ($p>0.5$).

Pyocyanin(1.245 µg/mL) was able to bring about 62% inhibition in the EPS production by *B. casei* BTDF1 and *S. warneri* BTDF2, followed by 59% in case of *B.altitudinis* BTMW1 , *B.pumilus* BTMY2 and *B.niacini* BTDP3. Around 41% reduction was observed in the case of *M. luteus* BTDF3 and *G. staerothermophilus* BTFF2 followed by 40% reduction in *Bacillus sp* BTSD1.

Rhamnolipids (75 µg/mL) caused inhibition of 65% in EPS production by *M. luteus* BTDF3 followed by 60 % in *S.warneri* BTDF2. 59% inhibition was seen in *B.casei* BTDF1 followed by 53% in *B.pumilus* BTMY2 while 52% was observed in *B.altitudinis* BTMW1 and *B.niacini* BTDP3. Only 44 % reduction

observed in case of *Bacillus sp* BTSD1 followed by 43% in case of *G.staerothermophilus* BTFF2 on treatment with rhamnolipids.

In the case of *P.aeruginosa,* both pyocyanin and rhamnolipids caused an increase in the EPS content. Since they are quorum sensing molecules that are known to augment the biofilm formation in *P.aeruginosa species;* besides they are the quorum sensing molecules extracted from the same organism.

Melanin (100 µg/mL) was able to reduce the quantity of the EPS produced in most of the test pathogens. 63% EPS reduction was observed in *B.casei* BTDF1 and *S.warneri* BTDF2 followed by 57% reduction in *B.altitudinis* BTMW1 and *B.niacini* BTDP3. 49% reduction was noticed in the case of *B.pumilus* BTMY2 followed by 46% in *G. staerothermophilus* BTFF2. EPS production in *P.aeruginosa* BTRY1was inhibited by 35% followed by 21% in *Bacillus sp* BTSD1 and 17% in the case of *M.luteus* BTDF3.

Bacteriocin BL8 (2380 µg/mL) was able to reduce the intensity of EPS production by 65% in case of *Bacillus sp* BTSD1 followed by around 57% in case of *B. pumilus* BTMY2, *B. niacini* BTDP3 and *S. warneri* BTDF2. 55% was observed in case of *B. altitudinis* BTMW1 and *B. casei* BTDF1 followed by 47 % in the case of *G. staerothermophilus* BTFF2 and 42% in *P.aeruginosa*. Only 24% reduction in EPS production was observed in *M. luteus* BTDF3 on treatment with the bacteriocin

On treatment with combination of all the bioactive compounds almost all of the test pathogens showed very less EPS concentration on extraction ;and the inhibition was more than 80% except in the case of *P.aeruginosa* BTRY1 , which was observed to get inhibited by only 30% since pyocyanin and rhamnolipids are known boosters of its EPS production.

5.3.5. Scanning Electron Microscopy (SEM)

The scanning electron microscopy helped to illustrate the effect of pyocyanin (1.245 µg/mL), rhamnolipids (75 µg/mL), melanin (100 µg/mL) and bacteriocin BL8 (2380 µg/mL) on the biocontrol of biofilms of eight food borne pathogens. The micrographs (Figures 5.21 – 5.29) show the difference in the untreated and treated samples of the nine tested pathogens and clearly confirm the reduced microbial presence in the compound treated slide compared to control (untreated).

For **Figures 5.21-5.29**. (a)- Control, (b)-pyocyanin treated, (c) rhamnolipids treated, (d) melanin treated, (e) bacteriocin treated, (f) combination treated. The specifications regarding all SEM images (15kV, X6000, 5µM, 11 41 SEI) taken from JEOL Model JSM was the same.

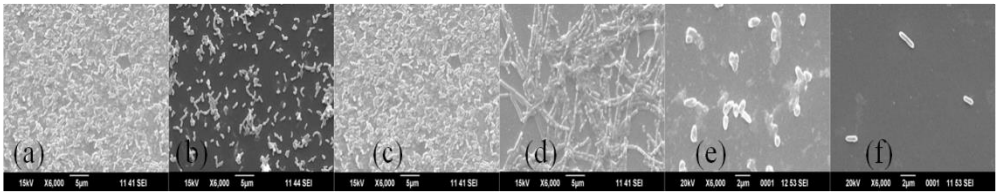


Fig 5.21. SEM images for biocontrol of biofilm of *Bacillus altitudinis* (BTMW1***)*.**

The reduction in biofilm formation is clearly visible on treatment, with Bacteriocin> Melanin>Pyocyanin. But there was no reduction with rhamnolipids treatment on the biofilm formation by *B. altitudinis* BTMW1.Combination of all the compounds is found to be very effective in the mitigation of biofilm formation on whole.

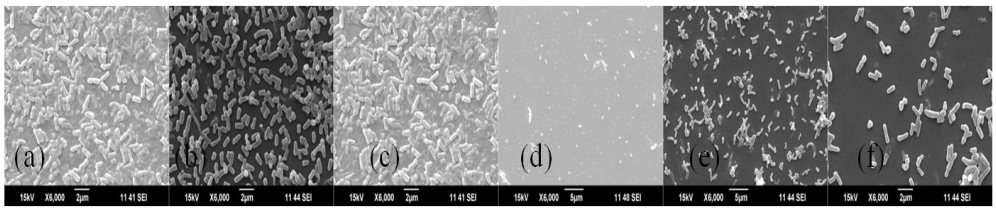


Fig 5.22. SEM images for biocontrol of biofilms of *Bacillus niacini* (BTDP3***)***

The reduction in biofilm formation is clearly visualized on treatment, with Bacteriocin> Melanin>Pyocyanin. But there was no reduction with rhamnolipids

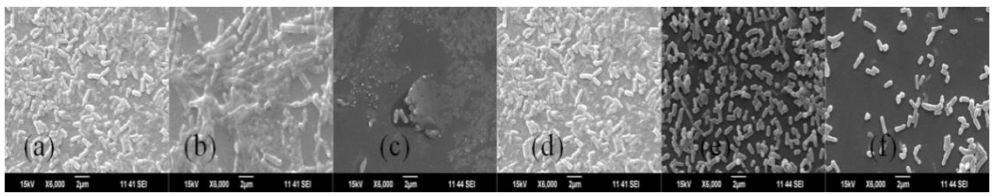


Fig 5.23.SEM images for biocontrol of biofilms of *Bacillus pumilus* (BTMY2***)***

Treatment on the biofilm formation by *B. niacini* BTMY2. Combination of all the compounds is found to be very effective in the mitigation of biofilm formation on whole.

The reduction in biofilm formation is noticeably seen on treatment, with Rhamnolipids>Bacteriocin>Pyocyanin. But there was no reduction with rhamnolipids treatment on the biofilm formation by *B. niacini* BTMY2. Combination of all the compounds is found to be very effective in the mitigation of biofilm formation on whole.

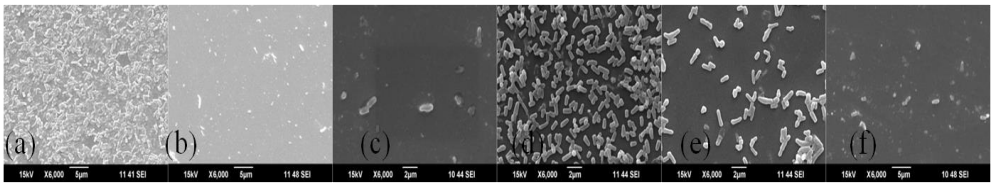


Fig 5.24.SEM images for biocontrol of biofilms of *Bacillus sp (*BTSD1*)*

In the case of *Bacillus sp* (BTSD1), the reduction in biofilm formation is evidently visualised on treatment, with Bacteriocin>Rhamnolipids> Melanin>Pyocyanin. Combination of all the compounds is found to be very effective in the mitigation of biofilm formation on whole.

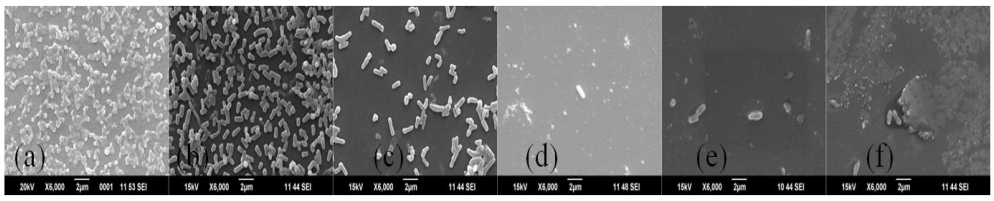


Fig 5.25.SEM images for biocontrol of biofilms of *Brevibacterium casei*

In the case of *B. casei* (BTDF1), the reduction in biofilm formation is seen on treatment with Melanin >Bacteriocin>Pyocyanin>Rhamnolipids. Combination of all the compounds is found to be very effective in the mitigation of biofilm formation on whole.

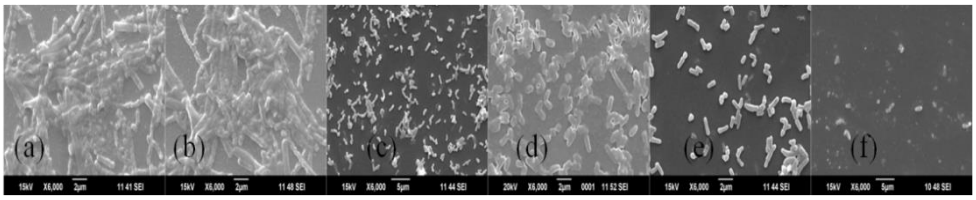


Fig 5.26.SEM images for biocontrol of biofilms of *Geobacillus staerothermophilus (*BTFF2*)*

The reduction in biofilm formation is clearly visualised on treatment, with Rhamnolipids>Bacteriocin>Melanin. But there was no reduction with pyocyanin

treatment on the biofilm formation by *G.staerothermophilus* BTFF2. Combination of all the compounds is found to be very effective in the mitigation of biofilm formation on whole.

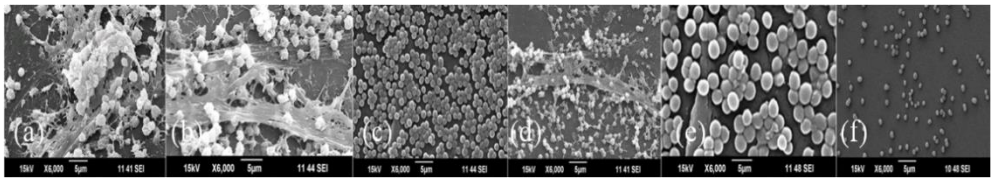


Fig 5.27.SEM images for biocontrol of biofilms of *Micrococcus luteus*

The reduction in biofilm formation is manifestly visualised on treatment with Bacteriocin>Rhamnolipids. But there was no reduction with melanin and pyocyanin treatments on the biofilm formation by *M. luteus* (BTDF3). Combination of all the compounds is found to be very effective in the mitigation of biofilm formation on whole.

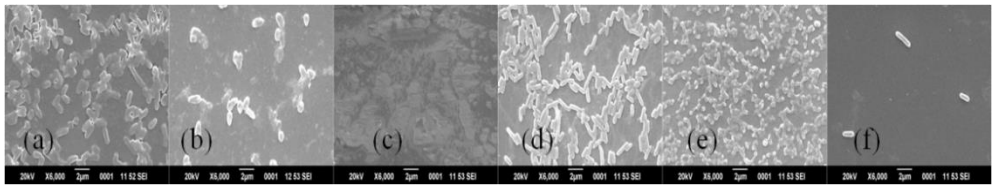


Fig 5.28.SEM images for biocontrol of biofilms of *Pseudomonas aeruginosa*

The case is different in *P.aeruginosa* where the molecules pyocyanin and rhamnolipids are boosting its biofilm formation since they are known to be the quorum sensing molecules involved in its biofilm formation. Both melanin and bacteriocin individually and in combination is effective for the vindication of the *P.aeruginosa* biofilms.

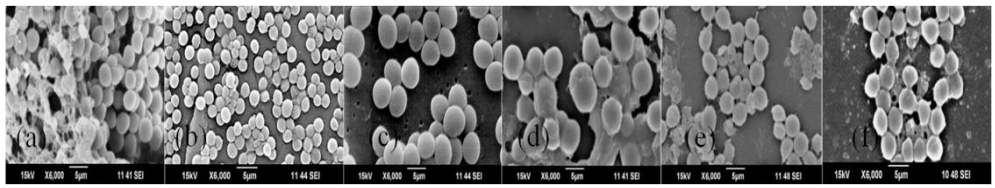


Fig 5.29.SEM images for biocontrol of biofilms of *Staphylococcus warneri* (BTDF2**)**

In the case of *S. warneri* (BTDF2), the reduction in biofilm formation is clearly visualised on treatment with Pyocyanin>Bacteriocin>Melanin>Rhamnolipids. Combination of all the compounds is found to be very effective in the mitigation of biofilm formation on whole.

5.3.6. Confocal Laser Scanning Microscopy (CLSM)

Similarly confocal microscopy was also evidence to support the biofilm reduction by the four compounds. The micrographs (Figures 5.30 – 5.38) clearly show not only a reduction of microorganisms, but also the biofilm formation by the test (treated) compared to control (untreated). Live cells were indicated by To-pro-3 stain. The reduced intensity due to the reduction in the live cells on treatment with pyocyanin/rhamnolipids/melanin/bacteriocin was easily visible, which signified the shrinking of biofilm formation.

For **figures 5.30-5.38.**Confocal images from (Leica TCS SP 5) (a) - control, (b)-pyocyanin treated, (c) rhamnolipids treated, (d) melanin treated, (e) bacteriocin treated, (f) combination treated. Bar: - 250 µm.

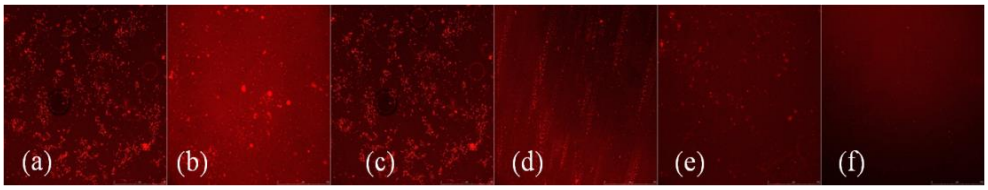


Fig 5.30.Confocal images of *Bacillus altitudinis (*BTMW1*).*

The same pattern of biofilm inhibition as observed in figure 5.21 were found in confocal micrographs on treatment with different bioactive compounds in *B. altitudinis* BTMW1 that reconfirms the results of SEM imaging.

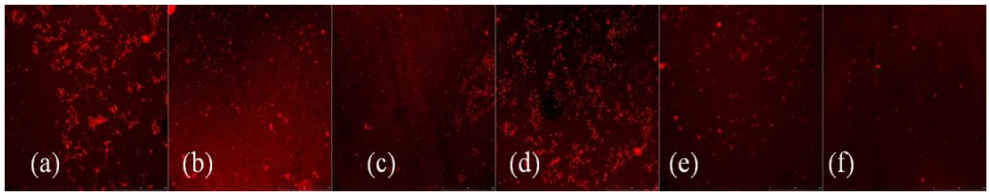


Fig 5.31.Confocal images for *Bacillus pumilus (*BTMY2*)*

The same pattern of biofilm inhibition as observed in figure 5.23 were found in confocal micrographs on treatment with different bioactive compounds in *B. pumilus* BTMY2 that reconfirms the results of SEM imaging.

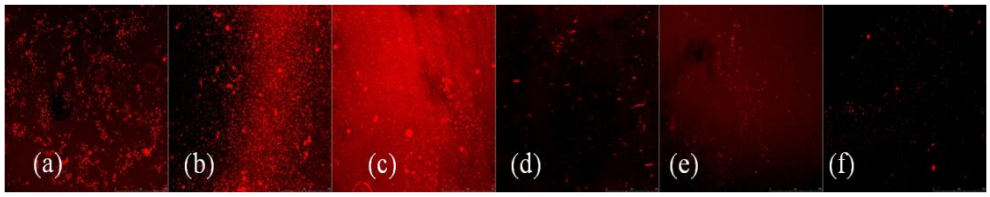


Fig 5.32.Confocal images for *Pseudomonas aeruginosa (*BTRY1*).*

The same pattern of biofilm inhibition as observed in figure 5.28 were found in confocal micrographs on treatment with different bioactive compounds in *P. aeruginosa* BTRY1 that reconfirms the results of SEM imaging.

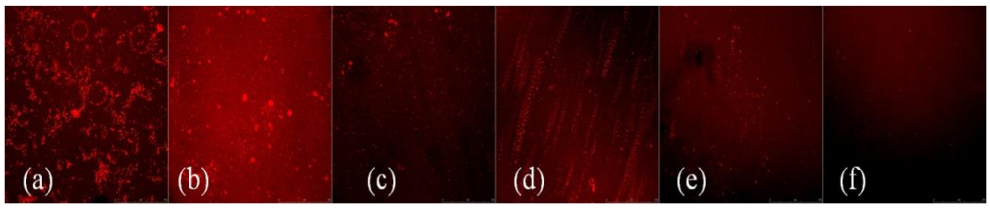


Fig 5.33.Confocal images for *Brevibacterium casei (*BTDF1*)*

The same pattern of biofilm inhibition as observed in figure 5.25 were found in confocal micrographs on treatment with different bioactive compounds in *B. casei* BTDF1 that reconfirms the results of SEM imaging.

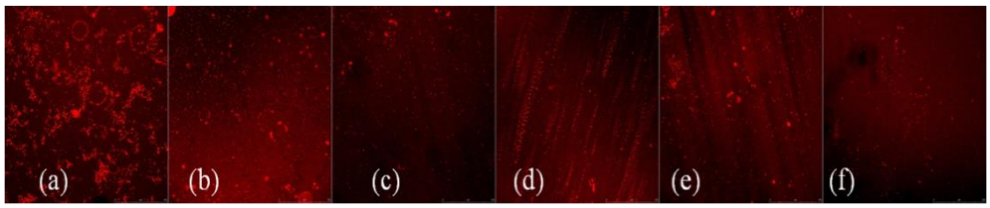


Fig 5.34.Confocal images for *Staphylococcus warneri (*BTDF2*)*

The same pattern of biofilm inhibition as observed in figure 5.29 were found in confocal micrographs on treatment with different bioactive compounds in *S. warneri* BTDF2 that reconfirms the results of SEM imaging.

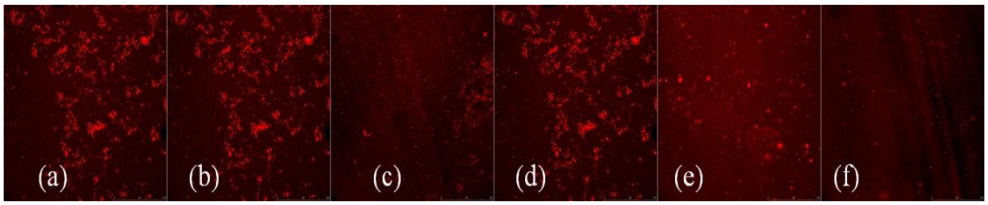


Fig 5.35.Confocal images for *Micrococcus luteus (*BTDF3*).*

The same pattern of biofilm inhibition as observed in figure 5.27 were found in confocal micrographs on treatment with different bioactive compounds in *M. luteus* BTDF3 that reconfirms the results of SEM imaging.

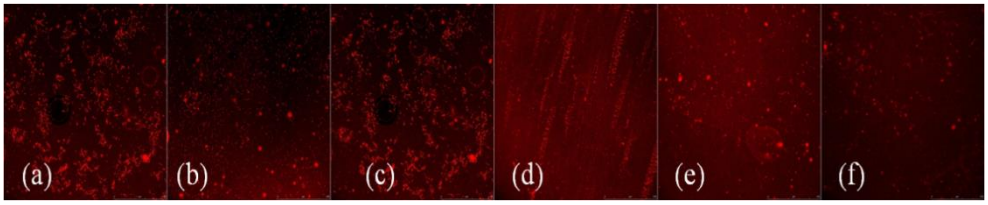


Fig 5.36.Confocal images for *Bacillus niacini (*BTDP3*).*

The same pattern of biofilm inhibition as observed in figure 5.22 were found in confocal micrographs on treatment with different bioactive compounds in *B. niacini* BTDP3 that reconfirms the observations found in SEM imaging.

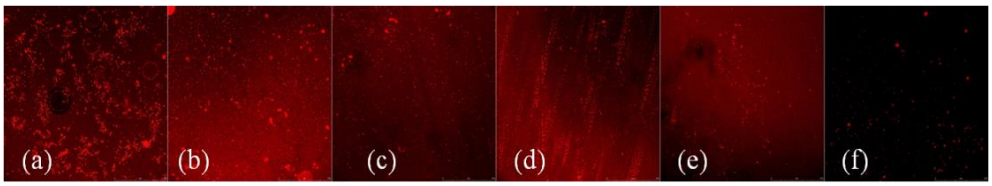


Fig 5.37.Confocal images for *Bacillus sp (*BTSD1*).*

The same pattern of biofilm inhibition as observed in figure 5.24 were found in confocal micrographs on treatment with different bioactive compounds in *Bacillus sp* BTSD1 that reconfirms the results of SEM imaging.

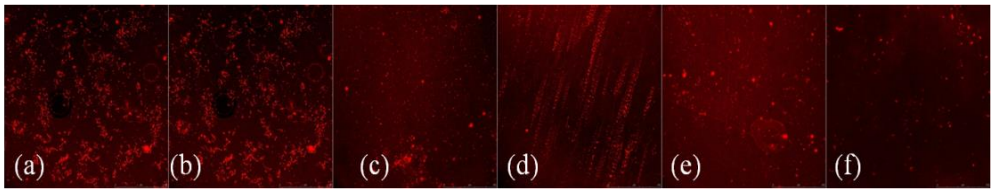


Fig 5.38.Confocal images for *Geobacillus staerothermophilus (*BTFF2*).*

The same pattern of biofilm inhibition as observed in figure 5.26 were found in confocal micrographs on treatment with different bioactive compounds in *G. staerothermophilus* BTFF2 that reconfirms the results of SEM imaging.

The intensity data of the confocal images were analyzed using Image J (Image J 4.8v/ Java 1.6.0_20, 64-bit) (Peter, 2014), which produced Red Green Blue (RGB) graphs for each image. The output data for pixel intensities consisted of 6 columns, consisting of the sample type, colour (red since topro is used as dye), Total pixels (1048576 which is same for all images), red particles detected (before treatment (control) and after each treatment and colour in percentage (depending on the number of the red spots counted before and after treatment with bioactive compounds. By comparing the data of red particles detected and colour percentage, before and after treatment with compounds on each test pathogen will paves the way to the proof of biocontrol of bacterial biofilms by four different bioactive compounds individually and in combination. The reduction is most clearly identified in most of the cases by analyzing reduced values for particles detected and colour percentage between the control sample and each treated sample for each and every pathogen. There will be no change in these data if there is no effect of inhibition by that particular compound on that pathogen.

The Red Green Blue plots (in this case it is red since the colour coded is Red) are also produced by the same software. The graphs add to the data produced as pixel intensities where there is a change in the peak formats **i.e** due to the change in the red colour spots'/ particles' intensities, before and after treatment, with all the compounds on each of the nine pathogens tested in the study.

Figure 5.39 represents the data of pixel intensities and figures 5.40-5.48 the corresponding RGB plots generated from the software for the nine food borne pathogens.

Figure 5.39. The reduction in pixel intensities before and after treatment with different bioactive compounds on nine test pathogens.

Quantification data of confocal microscopy for *Bacillus altitudinis (BTMW1)*:

Sample	Color	Color Pixels	Total Pixels	Particles	% Color ± SD
Control	Red	22030	1048576	104904.8	2.101 ± 0
Pyocyanin treated	Red	13388	1048576	63752.38	1.2755 ± 0.002121
Rhamnolipids treated	Red	22030	1048576	104904.8	2.101 ± 0
Melanin treated	Red	6720	1048576	32000	0.6405 ± 0.000707
Bacteriocin treated	Red	1042	1048576	4961.905	0.6405 ± 0.001414
Combination treated	Red	599	1048576	2852.381	0.098 ± 0.001414

Quantification data of confocal microscopy for *Bacillus niacini (BTDP3)*:

Sample	Color	Color Pixels	Total Pixels	Particles	% Color ± SD
Control	Red	22030	1048576	104904.8	2.101 ± 0
Pyocyanin treated	Red	8296	1048576	39504.76	0.79 ± 0.001414
Rhamnolipids treated	Red	22030	1048576	104904.8	2.101 ± 0
Melanin treated	Red	7670	1048576	36523.81	0.7295 ± 0.002121
Bacteriocin treated	Red	3790	1048576	18047.62	0.3625 ± 0.002121
Combination treated	Red	1042	1048576	4961.905	0.0965 ± 0.003536

Quantification data of confocal microscopy for *Bacillus pumilus(BTMY1)*:

Sample	Color	Color Pixels	Total Pixels	Particles	% Color ± SD
Control	Red	21972	1048576	104628.6	2.098 ± 0.004243
Pyocyanin treated	Red	8849	1048576	42138.1	0.842 ± 0.002828
Rhamnolipids treated	Red	5492	1048576	26152.38	0.5255 ± 0.002121
Melanin treated	Red	22030	1048576	104904.8	2.098± 0.004243
Bacteriocin treated	Red	1696	1048576	8076.19	0.163 ± 0.001414
Combination treated	Red	772	1048576	3676.19	0.076 ± 0.002828

Quantification data of confocal microscopy for *Micrococcus luteus(BTDF3)*:

Sample	Color	Color Pixels	Total Pixels	Particles	% Color ± SD
Control	Red	21972	1048576	104628.6	2.0975 ± 0.003536
Pyocyanin treated	Red	21972	1048576	104628.6	2.0975 ± 0.003536
Rhamnolipids treated	Red	5492	1048576	26152.38	0.5265 ± 0.003536
Melanin treated	Red	21972	1048576	104628.6	2.0975 ± 0.003536
Bacteriocin treated	Red	3790	1048576	18047.62	0.365 ± 0.005657
Combination treated	Red	844	1048576	4019.048	0.075 ± 0.007071

Quantification data of confocal microscopy for *Staphylococcus warneri(BTDF2)*:

Sample	Color	Color Pixels	Total Pixels	Particles	% Color ± SD
Control	Red	30067	1048576	143176.2	2.862 ± 0.007071
Pyocyanin treated	Red	8849	1048576	42138.1	0.8465 ± 0.003536
Rhamnolipids treated	Red	2556	1048576	12171.43	0.242 ± 0.002828
Melanin treated	Red	7472	1048576	35580.95	0.716 ± 0.004243
Bacteriocin treated	Red	7239	1048576	34471.43	0.695 ± 0.007071
Combination treated	Red	1724	1048576	8209.524	0.166 ± 0.002828

Quantification data of confocal microscopy for *Pseudomonas aeruginosa (BTRY1)*:

Sample	Color	Color Pixels	Total Pixels	Particles	% color ± SD
Control	Red	22030	1048576	104904.8	2.1155 ± 0.020506
Pyocyanin treated	Red	47350	1048576	225476.2	4.463 ± 0.074953
Rhamnolipids treated	Red	52551	1048576	250242.9	5.061 ± 0.069296
Melanin treated	Red	1642	1048576	7819.048	0.155 ± 0.002828
Bacteriocin treated	Red	1724	1048576	8209.524	0.1625 ± 0.002121
Combination treated	Red	2839	1048576	13519.05	0.273 ± 0.002828

Figures 5.40-5.48. RGB plots generated for the quantified data of confocal images using Image J software. (a)- control, (b)-pyocyanin treated, (c)-rhamnolipids treated, (d)- melanin treated, (e)- bacteriocin treated, (f)-combination treated.

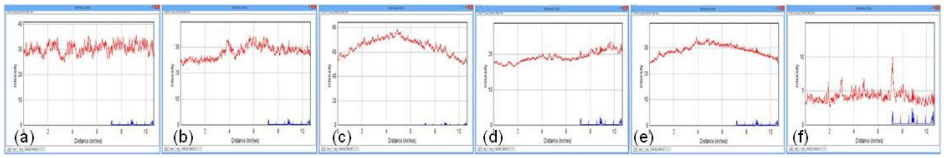


Fig 5.40 .RGB plots in accordance to pixel intensity for *Bacillus altitudinis (*BTMW1*)*

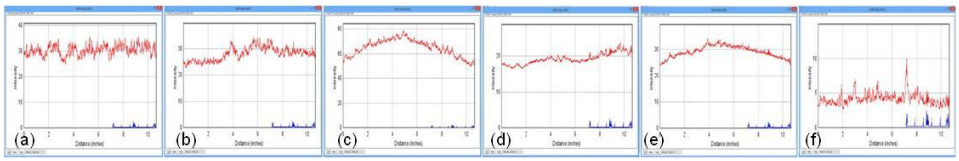


Fig 5.41.RGB plots in accordance to pixel intensity for *Bacillus pumilus (*BTMY2*)*

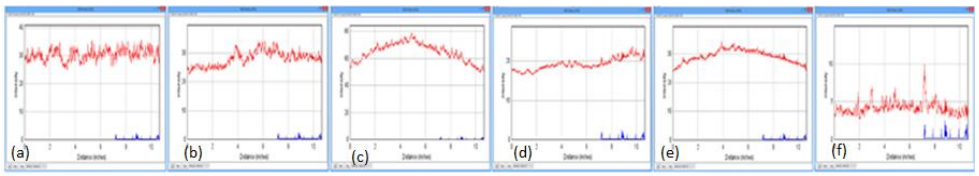


Fig 5.42.RGB plots in accordance to pixel intensity for *Pseudomonas aeruginosa (*BTRY1*)*

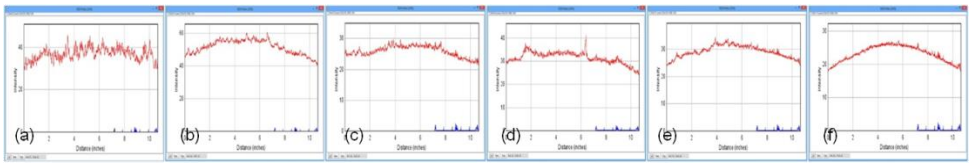


Fig 5.43.RGB plots in accordance to pixel intensity for *Brevibacterium casei (*BTDF1*)*

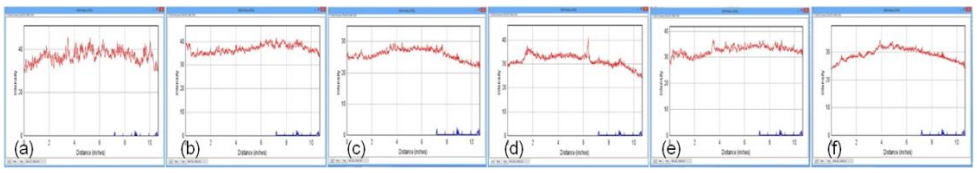


Fig 5.44.RGB plots in accordance to pixel intensity for *Staphylococcus warneri (*BTDF2*)*

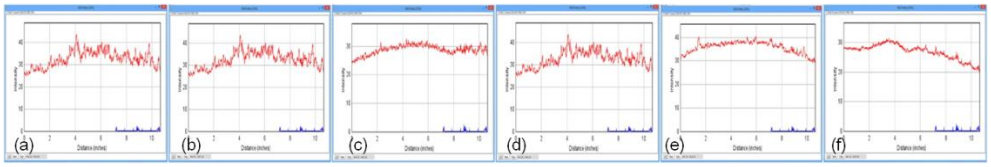


Fig 5.45.RGB plots in accordance to pixel intensity for *Micrococcus luteus (*BTDF3*)*

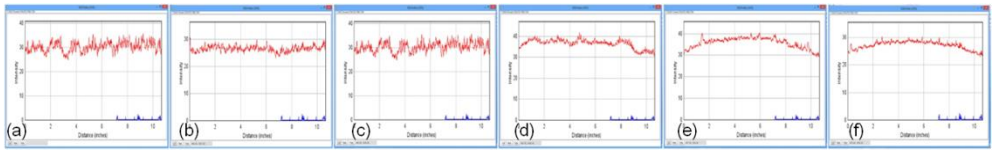


Fig 5.46.RGB plots in accordance to pixel intensity for *Bacillus niacini (*BTDP3*)*

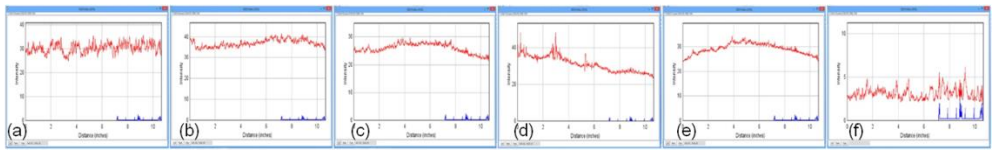


Fig 5.47.RGB plots in accordance to pixel intensity for *Bacillus sp (*BTSD1*)*

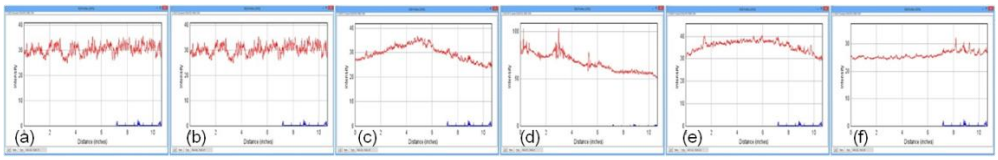


Fig 5.48.RGB plots in accordance to pixel intensity for *Geobacillus staerothermophilus (*BTFF2*)*

The variations in the RGB plots from control to the treated samples due to the change in the pixel intensity clearly indicated the biocontrol of bacterial biofilms on treatment with the above said bioactive molecules.

5.3.7. Application studies of the bioactive compounds in the biocontrol of biofilms

4.3.7.1. Effect of pyocyanin and rhamnolipids on biofilm formation of different test pathogens from the culture collection of the laboratory

The assay was carried out as discussed in section 4.2.7.1. The statistical analysis showed that even though all the six strains produce biofilm, only two of them *Vibrio diabolicus TVMS3* and *Salmonella Enteritidis S49*(Figure 5.49.a).were strong biofilm producers.

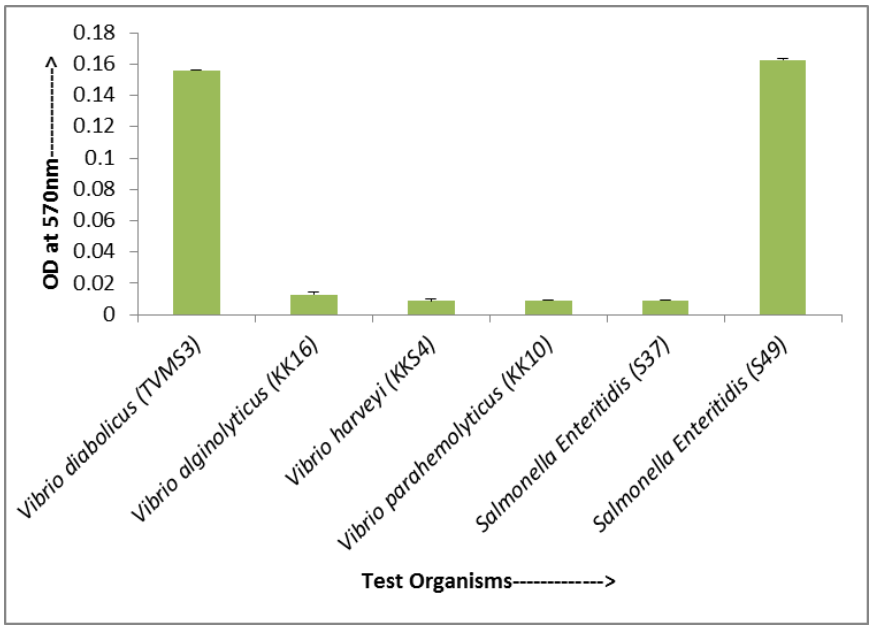


Fig 5.49.a. Biofilm formation by Gram negative pathogens

The antibiofilm activity was tested with pyocyanin at 1.245 µg/mL and rhamnolipids at 75 µg/mL with serial dilutions thereafter, and the biofilm inhibitory concentration (BIC) for pyocyanin was calculated to be 2 $x10^{-2}$ng/µL, while that for rhamnolipids was 1.2 ng/µL. Both the compounds caused reduction of biofilm formation ($p>0.5$) by *Vibrio diabolicus (TVMS3)* and *Salmonella Enteritidis(S49) with* > 80% and >40% reduction of biofilm formation respectively (Fig.5.49.b).

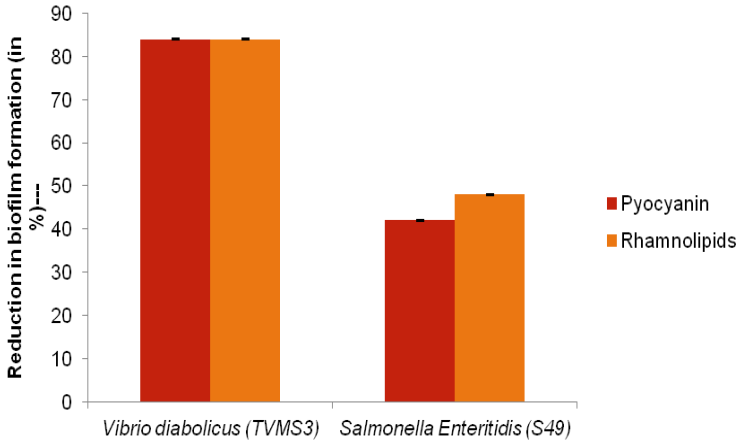


Fig 5.49.b. Percentage Reduction in Biofilm formation on treatment with pyocyanin and rhamnolipids

5.3.7.2. Effect of bioactive compounds individually and in combination on multispecies biofilm formation

The antagonism was checked with the test strains producing biofilms by cross streak method (Fig 5.50. and table 5.6.) as discussed in 4.2.7.2.

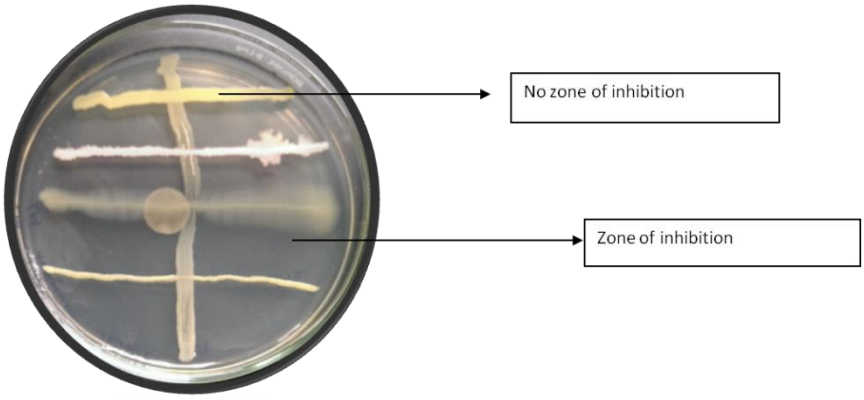


Fig 5.50. Antagonism by cross streak method

Table 5.5. Antagonism by cross streak method

Organism	BTMW1	BTMY2	BTRY1	BTDF1	BTDF2	BTDF3	BTDP2	BTDP3	BTSD1	BTSD2	BTFF2
BTMW1	-	-	+	-	-	-	-	-	-	-	-
BTMY2	-	-		-	-	-	-	-	-	-	-
BTRY1	-	-	+	-	-	-	-	-	-	-	-
BTDF1	-	-	+	-	-	-	-	+	-	-	-
BTDF2	+	+	+	-	-	-	-	+	-	-	-
BTDF3	+	+	+	-	-	-	-	+	-	+	+
BTDP2	+	+	+	-	-	-	-	+	+	+	-
BTDP3	+	+	+	-	-	-	-	-	-	+	-
BTSD1	+	+	+	-	-	-	-	-	-	+	-
BTSD2	-	-	+	-	-	-	-	-	-	-	-
BTFF2	+	+	+	-	-	-	-	-	-	+	-

*** (+):-Antagonism, (-):-No Antagonism**

***** BTMW1- *B. altitudinis,* BTMY2 – *B. pumilus,* BTRY1 – *P.aeruginosa,* BTDF1- *B. casei,* BTDF2 – *S.warneri,* BTDF3 – *M.luteus,* BTDP2 – *Micrococcus sp,* BTDP3- *B.niacini,* BTSD1- *Bacillus sp,* BTSD2- *B.licheniformis,* BTFF2- *G. staerothermophilus.*

Based on the antagonism data, the types of consortia formed included the following:

1. **Type 1**- *B.altitudinis, B.pumilus & B.licheniformis*
2. **Type2**- *B.casei, M.luteus, S.warneri, Micrococcus sp, Bacillus sp & G.staerothermophilus*
3. **Type 3**- *B.casei, M.luteus, S.warneri & Bacillus sp*

4. **Type 4**- *B.casei, M.luteus, S.warneri, Bacillus sp & Micrococcus sp*

Pseudomonas aeruginosa was observed to inhibit all other microorganisms and is thus not seen usually in consortia, but are powerful enough to form biofilm on its own in most of the food processing environments and in ready-to-eat foods.

Biofilm formation due to the multispecies biofilm as indicated in the four types of consortia was also tested as well as their response to treatment with bioactive compounds (Figure 5.51.).

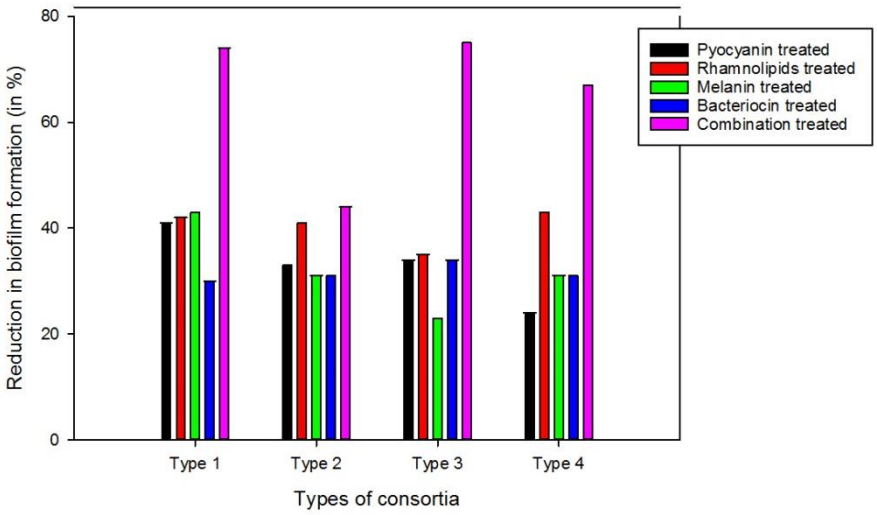


Fig.5.51. Effect of bioactive compounds on different types of consortia forming multispecies biofilms.

Type 1 consortia ((*B.altitudinis, B.pumilus & B.licheniformis*) was inhibited 41%, 42%, 43% and 30% on treatment with pyocyanin (1.245 µg/mL), rhamnolipids (75 µg/mL), melanin (100 µg/mL)and bacteriocin (2380 µg/mL) respectively. 74% reduction was seen after combined treatment of all the four compounds.

Type 2 consortia (*B.casei, M.luteus, S.warneri, Micrococcus sp, Bacillus sp & G.staerothermophilus*) was inhibited 33%, 41%, 31% and 31% on treatment with pyocyanin, rhamnolipids, melanin and bacteriocin respectively. 44% reduction was seen after combined treatment of all the four compounds.

Type 3 consortia (*B.casei, M.luteus, S.warneri & Bacillus sp*) was inhibited 34%, 35%, 23% and 34% on treatment with pyocyanin, rhamnolipids,

melanin and bacteriocin BL8 respectively. 75% reduction was seen after combined treatment of all the four compounds.

Type 4 consortia (*B.casei, M.luteus, S.warneri, Bacillus sp & Micrococcus sp)* was inhibited 24%, 43%, 31% and 31% on treatment with pyocyanin, rhamnolipids, melanin and bacteriocin respectively. 67% reduction was seen after combined treatment of all the four compounds.

Thus the graph clearly represents the reduction in the biofilm formation ($p>0.5$) of all types of consortia studied with the treatment of bioactive compounds at their respective BIC individually and in combination with each other.

5.3.7.3. The application of the bioactive compounds in the preservation of common foods available in market

The four bioactive compounds were assayed for their capability to act as preservatives/additives by testing their ability to reduce the bioburden in the common foods availability in markets (Fig.5.52.). It was done as discussed in 4.3.7.3.

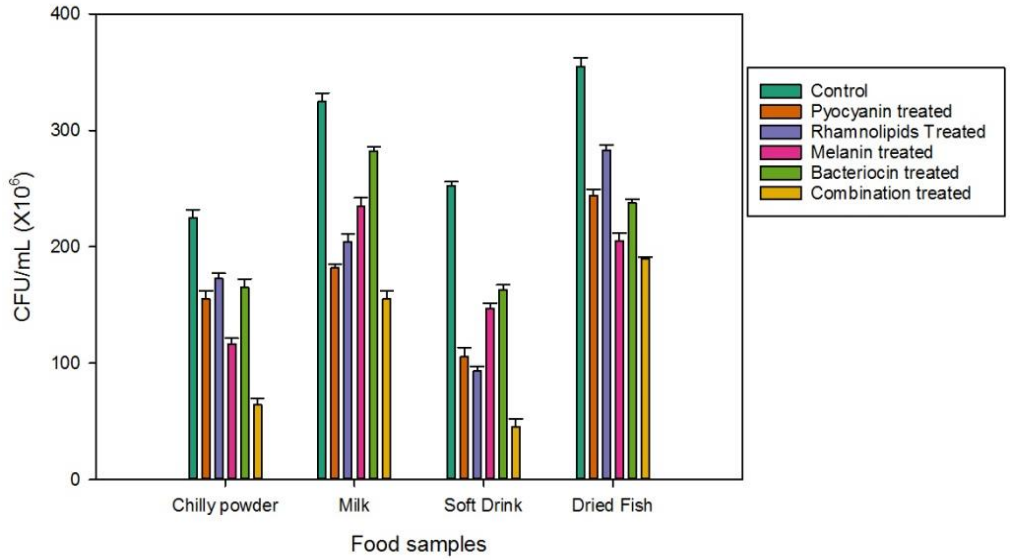


Fig.5.52. Reduction of microflora/burden in the common foods available in local markets on treatment with the four bioactive compounds

The graph (**Fig.5.52)** depicted the reduction of microflora /bioburden (in terms of CFU/mL) in the food samples ($p>0.5$) locally available for consumption on addition of the compounds used in the study.

In the case of chilly powder (control value before any treatment was 225 CFU/mL) on addition of pyocyanin, rhamnolpids, melanin, bacteriocin and combination of four, there was a reduction to 155 CFU/mL, 173 CFU/mL, 116.5 CFU/mL, 165 CFU/mL and 64 CFU/mL respectively.

In milk sample (control value before any treatment was 325 CFU/mL), on addition of pyocyanin, rhamnolpids, melanin, bacteriocin and combination of four, there was a reduction to 182 CFU/mL, 204.5 CFU/mL, 235 CFU/mL, 282.5 CFU/mL and 155 CFU/mL respectively.

In the soft drink (control value before any treatment was 252.5 CFU/mL), on addition of pyocyanin, rhamnolpids, melanin, bacteriocin and combination of four, there was a reduction to 105.5 CFU/mL, 93 CFU/mL, 147 CFU/mL, 163 CFU/mL and 43 CFU/mL respectively.

On addition of pyocyanin, rhamnolpids, melanin, bacteriocin and combination of four, in the case of dried fish sample (control value before any treatment was 355 CFU/mL), there was a reduction to 244 CFU/mL, 283 CFU/mL, 205 CFU/mL, 237.5 CFU/mL and 190 CFU/mL respectively.

This proves their ability to be used as preservatives/additives in the food industry.

5.4 Discussion

This study evaluated the antibiofilm activity of four bioactive compounds using *in vitro* inhibition assay in microtiter plates, against nine strong biofilm producers. Among the several pigments of Pseudomonads, pyocyanin is the major antibacterial agent, with the inhibitory effect associated with the 1-hydroxy phenazine component (Karpagam *et al.,* 2013). Its production is a widely accepted criteria for distinguishing *Pseudomonas aeruginosa* from other closely related organisms. In this study, pyocyanin produced by strain BTRY1 showed good antibiofilm activity against the test biofilm producers normally present in the food industry.

The anti-biofouling activity of a red pigment prodigiosin from *Serratia marcescens* against *Staphylococcus aureus* was previously reported (Sathish and Aparna, 2014). These reports substantiate the application of pigments and other bioactive compounds against biofilms produced by bacterial pathogens. The *Flavobacterium psychrophilum* biofilms were controlled *in vitro* by the fluorescein pigments released from *Pseudomonas flourescensFF48 in vitro* (de la Fuente-Núñez *et al.*, 2013). The anti-fouling capability of *P. tunicate* is important for their survival in the marine ecosystem and is likely required for their colonization of different substrate surfaces. Other bacterial strains such as *P. luteoviolacea*, *P. aurantia*, *P. citrae* and *P. ulvae* have also been reported to produce antibiotic compounds essential in preventing the settlement of fouling species. All of these strains have the advantage of being resistant to natural antibiotics, but no one strain produces a collection of compounds that allows them to become dominant over all the other organisms in their given econiche. To overcome this barrier, multiple strains of antibiotic producing bacteria form complex biofilm communities. Because the biofilm contains a diverse array of species producing a variety of antibiotics, it prevents itself from being overgrown by any one species and also reduces invasion by other species. Biofilm forming *Pseudoalteromonas* species have not only been found to inhibit settlement and germination of anti-fouling species but they have been found to directly lyse cells of various algal species. An example is *Pseudomonas. sp.* Strain Y, which produces a unidentified brominated antibiotic, low in molecular weight, that can completely lyse algal cells within a matter of h (Bowman, 2007).

Single-species biofilms are rare in natural environments, especially in agricultural food processing industry, where micro-communities exposed to plenty of organic matter have the potential to develop into multispecies biofilms with high bacterial density and diversity (Burmolle *et al.,* 2010). Different species, exhibiting different growth and survival properties, encased in an extracellular polymeric network may lead to the spatial and functional heterogeneity within biofilms. Even in a single-species biofilm, the physical, chemical (e.g. gradients of nutrients, waste products and signalling compounds) and biological (distinct metabolic pathways and stress responses) heterogeneity can develop. The interactions among these microorganisms in a multi species biofilm and with the external environment critically influence the development, structure and function of the biofilm and clearly have a dramatic effect on the communication between different biofilm components, allowing for the development of a complex multispecies community (Stewart and Franklin, 2008). Several observations in a study indicate that bacteria increase fitness from joining multispecies biofilms in food industry mainly in the food processing environments (Burmolle *et al.,* 2014). If this fitness advantage applies to all of the species present, the underlying interaction is categorized as being cooperative (West *et al.,* 2007). In this study, four different types of consortia were made after checking the antagonism between different food borne biofilm forming pathogens. There was a positive synergistic action found in the formation of strong biofilms.

Bacterial attachment and biofilm formation can lead to poor hygienic conditions in food processing environments. Furthermore, interactions between different bacteria may induce or promote biofilm formation. In most of the reported cases, biofilm formation of multispecies biofilm formation was enhanced when comparing to monospecies biofilms (Ren *et al.,* 2014). Even though there are only very few reports available on this research, most of them shed light into the ability of co-localized isolates to influence co-culture biofilm production with high relevance for food safety and food production facilities (Burmolle *et al.,* 2014).

Many chemical methods are being reported for the control of biofilms. Active chlorine concentrations as high as 1000 ppm are necessary for an extensive reduction in bacterial numbers in multispecies biofilms (formed by *L. monocytogenes, Ps. fragi and S. xylosus* compared to 10 ppm for planktonic cells

(Norwood and Gilmour 2000). Similarly peroxy acid disinfectants were more effective than chlorine for inactivating multispecies biofilms of *Pseudomonas sp*. and *L. monocytogenes* on stainless steel (Fatemi and Frank, 1999). The disinfectant most effective to planktonic cells could not necessarily the most active against the biofilm cells, and the most active disinfectant against pure culture biofilm is not necessarily the most active against multispecies biofilms in challenging (food-processing) environments (Van Houdt and Michiels, 2009). Further investigations in the area of biological control is improving on one side. Enzyme treatments were not found to be very effective for the multispecies biofilms. Besides these, bacteriophages are the main focus of biocontrol mechanisms (Zottola and Sahasara, 1994; Wong, 1998). Many imaging techniques have been done to analyse the multispecies biofilms like FISH and confocal microscopy (Fatemi and Frank, 1999).

The identification of several natural compounds that inhibited biofilm formation by clinical isolates of *Klebsiella pneumoniae* was done by Magesh and coworkers (2013), where out of the six compounds that inhibited biofilm formation, reserpine and linoleic acid were potent biofilm inhibitors and their BICs were in milligram quantities. It is very well known that the quorum sensing pathways are inhibited by very low concentrations of natural compounds (Saleem *et al.,* 2010). Natural compounds like embelin and piperine were required in milligram quantities to control biofilm formation by *Streptococcus mutans* SM06 (Dwivedi and Singh, 2014).The significance of diverse natural products has been perceived by humans because of their gainful properties. Comprehensively, numerous classes of plant auxiliary metabolites have shown their potential as antimicrobials or synergists of different items (Hemaiswarya *et al.,* 2008). Along these lines, phytochemicals are a major source of substance with assorted qualities and are important segments of the current pharmaceuticals (Dixon 2001; Saavedra *et al.,* 2010).

BIC of pyocyanin, rhamnolipids, melanin and bacteriocin was in ng/µL therefore promising efficient biocontrol of biofilms at low concentrations. Bacterial biofilms are imaged by various kinds of microscopy including confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). One limitation of CLSM is its restricted magnification, which is resolved by the use of SEM that provides high-magnification spatial images of how the single

bacteria are located and interact within the biofilm. New modifications like Cryo SEM, Focused Ion Beam (FIB)-SEM, environmental-SEM (ESEM) etc. that do not require dehydration (Alhede *et al.,* 2014).The Scanning electron micrographs and Confocal laser scanning micrographs confirmed the biofilm biocontrol capability of the four mentioned compounds in the present study.

In recent years, drug resistance of human pathogenic bacteria has been widely reported. In addition, persistent infections were also attributed to enhanced resistance of bacteria in biofilm (Davies & Davies 2010). This leads to huge economic losses and pressures the medical community to find alternative approaches for treatment of diseases related with biofilms. Consequently, efforts are been applied to discover efficient antimicrobial molecules not amenable to bacterial resistance mechanisms, including those in biofilms (Li *et al.,* 2011). Some natural products have distinctive properties that make them perfect candidates for these highly required niche- based therapeutics (Simoes *et al.,* 2009). The study revealed the significance of bioactive compounds as alternatives to the amplified use of antibiotics. The biofilm forming food pathogens in this study showed multiple antibiotic resistance (MAR), when tested against commonly used antibiotics.

Different application studies conducted and thereby the compounds proved an effective agent both in control of growth and *in vitro* biofilm formation, and can be considered too as another measure to counter current antibiofilm strategies. This could very well assist in the gradual reduction in multiple resistance of pathogens emerging from the food industry.

Chapter 6

Isolation, purification and characterization of bacteriophages

6.1 Introduction

Resistance of pathogenic bacteria to traditional antibiotics has become a serious problem, therefore initiating a keen interest in alternative methods of control (Hagens and Loessner, 2010). Phages can play a role in biocontrol of microbes in fresh foods, without compromising the viability of other normal flora or food quality for that matter. However, there is very little published material on biofilms-bacteriophage interactions.

Recent developments in the field highlight the fact that, besides the use of phage for direct addition to food, much effort has gone into phage-based control of pathogens that colonize plants or animals used in food production (Balogh *et al.*, 2007).The safety of using bacteriophages in preserving food products is assured because they are non-toxic and ubiquitous in foods (Bruttin *et al.*, 2005).Many of the phage preparations used in food are reported to be safe for use as preservatives and are GRAS (Gerner-Smidt *et al.*, 1993).

The present chapter deals with the isolation, purification and characterization of bacteriophages using different methods and techniques and proving their ability as biocontrol agents against biofilm.

6.2 Materials and methods

6.2.1. Bacteriophage isolation

All nine food pathogen isolates used in the study listed below (Table 6.1), which are strong biofilm producers were used as host cultures to screen for bacteriophages; appropriate food samples from which host were isolated were used in the concept that the phages and hosts co-exist in a sample.

Table 6.1. Host organisms and the source for their respective phage isolation.

Sl no:	Host Organism	Source for phage isolation
1	*Bacillus altitudinis* (BTMW1)	Meat
2	*Bacillus pumilus* (BTMY2)	Meat
3	*Pseudomonas aeruginosa* (BTRY1)	Milk
4	*Brevibacterium casei* (BTDF1)	Dried Fish
5	*Staphylococcus warneri* (BTDF2)	Dried Fish
6	*Micrococcus luteus* (BTDF3)	Dried Fish
7	*Bacillus niacini* (BTDP3)	Dried Prawn
8	*Bacillus sp* (BTSD1)	Soft Drink
9	*Geobacillus stearothermophilus* (BTFF2)	Fresh Fish

6.2.1.1 Sample preparation

6.2.1.1(a) Direct method

Food samples such as beef, cheese, raw milk, pasteurized milk, curd, chilly powder, turmeric powder, coriander powder, soft drink, fresh fish, dried fish and dried prawn were processed for phage isolation. Solid samples were homogenized in sterile physiological saline of pH 8.5 and thoroughly mixed. The liquid samples were taken as such and this homogenate was centrifuged at 4000 g for 10 min (Sigma, 3K30, Germany) at 4ºC, filtered through 0.22 µm membrane filter (Millipore, USA) to make them bacteria-free and this filtrate was screened for the presence of phages.

6.2.1.1(b) Enrichment method

As a part of the enrichment process, a portion of the crude lysate was mixed with the host bacteria (in log phase), incubated at 37°C for 12-15 h, after which it was made bacteria-free by centrifugation and filtration as described in section 6.2.1.1(a). This enrichment step was intended to amplify the phage counts that may otherwise be undetected due to low number.

6.2.1.2. Double agar overlay method

The lysate was then assayed according to the double-agar overlay method of Adams (1959) with modifications. The logarithmic phase cells (1 mL) of the host bacterial strains in nutrient broth were mixed with 1 mL of the serially diluted lysate and were incubated at 37°C in a water bath (Scigenics, Chennai, India) for 1 hour. After incubation, 3 mL of sterile soft agar (nutrient broth containing 0.8% agarose) was added to this, mixed well and was immediately overlaid on nutrient agar plates. The plates were incubated for 16-18 h at 37 °C. Phage-free cultures (containing only bacterial host) and host-free cultures (containing only phage) were used as controls. A sample was scored positive for phages when plaques were observed on the bacterial lawn in the plates.

6.2.1.3. Tetrazolium staining

Tetrazolium staining helps to improve phage plaque visibility against the backdrop of bacterial growth. Each plaque appears as a sharp, clear area against the intense red background produced by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to the insoluble formazan by the bacterial cells (Pattee, 1966). The petri plates with plaques were flooded with 10 mL of trypticase soy broth (HiMedia) containing 0.1% 2, 3, 5 -triphenyltetrazolium chloride (TTC) (HiMedia). After incubation at 37°C for 20-30 min, the broth was poured off and the plaques were observed.

6.2.2. Phage purification

A single plaque was picked from the test plate with a sterile tooth-pick, introduced into 3 mL of a log phase culture of the host in nutrient broth, and was incubated at 37°C in an environmental shaker (Orbitek, Scigenics, India) at 100 rpm for 12-15 h. This was then centrifuged at 10000 x *g* (Sigma, 3K30, Germany) followed by filtration through 0.22 μm membrane (Millipore, USA). The lysate obtained was used for double agar overlay. This procedure was repeated 5-6 times, until uniform sized plaques were obtained on the plate (Sambrook *et al.*, 2000).

6.2.3. Large scale production of phage lysate

The plates prepared as described in the section 6.2.2., showing infective centers at the rate of 10^{10} plaque forming units (PFU)/mL, were washed with SM buffer(Appendix - 2) using the following method. The plates with uniform sized plaques were overlaid with 10 mL of SM buffer and were incubated overnight at 4°C, with gentle rocking so that phages could easily diffuse into the buffer. The phage suspension was then recovered after incubation from all plates and pooled. Chloroform was added to this pooled mixture to a final concentration of 5 % (v/v), mixed well using a vortex mixer and then incubated at room temperature for 15 min. The cell debris was then removed by centrifugation at 5000 x *g* for 10 min (Sigma, 3K30, Germany) and the supernatant was transferred to sterile polypropylene tube. Chloroform was added to a final concentration of 0.3 % (v/v) and this was stored at 4°C until use. The titer of this lysate could be noted after serial dilution (Sambrook *et al.*, 2000).

6.2.4. Phage concentration

Phage was concentrated using Polyethylene glycol (PEG) 6000 (Sambrook *et al*., 2000). Briefly 1% (v/v) of an overnight culture of the host bacteria was transferred to 200 mL nutrient broth (Himedia, Mumbai, India), and incubated at 37°C for 3.5 h in an environmental shaker at 100 rpm (Orbitek, Scigenics, India). Phage was added at a multiplicity of infection (MOI) of 0.2 and the incubation at 37°C was continued at 100 rpm for 12-16 h. This broth was centrifuged at 10000 x *g* for 20 min (Sigma, 3K30, Germany), the supernatant was then collected and filtered through 0.22 μm membrane filter (Millipore, USA).

DNase I (Bangalore Genei) and RNase (Bangalore Genei) was added, to a final concentration of 1 μg/mL each, and incubated at room temperature for 30 min. Solid NaCl was added to a final concentration of 1 M and dissolved by stirring with a sterile glass rod. The mixture was then kept in ice for 1 hour, followed by centrifugation at 11000 x *g* for 10 min at 4°C. Solid PEG 6000 (SRL, India) was added to the supernatant at a final concentration of 10% (w/v), dissolved by slow stirring on a magnetic stirrer at room temperature. This was then kept in ice overnight, followed by centrifugation at 11000 x *g* for 10 min at 4°C. The supernatant was discarded completely, while the pellet was resuspended in 5 mL of Phosphate buffered saline (PBS) (Appendix-2). PEG and cell debris were removed from the phage suspension by the addition of an equal volume of chloroform, vortexing for 30 s, followed by centrifugation at 3000 x *g* for 15 min at 4°C. The aqueous phase containing the phage particles were recovered and stored at -20°C.

6.2.5. Maintenance and storage of phages

Phage lysate, for long term storage, was maintained as stock cultures employing 2 methods, viz. storage at 4°C as such and as glycerol stock.

Phage lysate obtained after large scale production (Section 6.2.3) was stored in sterile 40 mL polypropylene screw-cap tubes at 4°C until use. Nutrient broth containing 50% glycerol was mixed with filtered phage lysate in a sterile microfuge tube (2 mL capacity) and the mixture was frozen at -80°C, until use.

6.2.6. Characterization of phages

The host specific lytic phages that exhibited excellent and consistent bacterial cell lysis capacity were chosen for further characterization.

6.2.6.1. Morphological analysis by Transmission Electron Microscopy (TEM)

One drop of high titer phage sample was spotted onto a carbon-coated TEM grid, allowed to settle for 2-3 min and excess of sample was removed by blotting. A drop of 2 % phosphotungstic acid hydrate (Sigma- Aldrich) was used

for negative staining and the mechanism of the adsorption onto tissue has been proposed as being electrostatic rather than involving hydrogen bonding, as adsorption is not affected by pH (Quintarelli *et al.,* 1971). This was placed on the spot, allowed to react for 2-3 min and the excess stain drained off by touching a blotting paper strip to the edge of the grid. The grids were dried for 15 min, examined and photographed using a Transmission Electron Microscope (Model Jeol/JEM 2100 2000X) operated at 200 kV at Sophisticated Test and Instrumentation Centre, Kalamassery, Kerala. Phage morphology was observed from the micrographs.

6.2.6.2. Determination of optimal multiplicity of infection

Multiplicity of infection (MOI) is the ratio of phage particles to host bacteria. It is calculated by dividing the number of phage added (volume in mL x PFU/mL) by the number of bacteria added (volume in mL x colony forming units/mL). Optimal MOI was determined (Lu *et al*., 2003). Briefly bacteria were infected at different MOI (0.01, 0.1, 0.5, 1, 5 and 10 PFU/mL) and incubated at 37°C for one hour. At the end of the incubation period, the mixture was centrifuged (Sigma, 3K30, Germany) at 8000 x *g* for 10 min and supernatant was passed through 0.22 µm membrane filter (Millipore, USA). The lysate was then assayed to determine the phage titre employing the double agar overlay method described previously. Phage-free cultures (containing only bacterial host) and host-free cultures (containing only phage) were used as controls. All assays were performed in triplicates. The MOI giving maximum yield was considered as optimal MOI.

6.2.6.3. Phage adsorption

The first step in the growth of bacteriophage is its attachment to susceptible bacteria. This process is called as adsorption. The adsorption studies were carried out as per Lu *et al*.(2003). Log phase culture of host was infected using the optimal MOI of the phage and incubated at 37°C. Aliquots of 5 mL were sampled at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 min time intervals after infection. All the samples drawn were immediately filtered through 0.22 µm membrane filter (Millipore, USA). The phage titer was determined using double agar overlay method after appropriate dilutions. All plating's were done in

triplicates and appropriate controls were maintained. The percentage of phage adsorption was calculated as follows: [(control titer - residual titer)/control titer] X 100% (Durmaz, 1992). The phage titer observed at time zero was considered as the control titer.

6.2.6.4. One step growth curve

The construction of the one-step growth curve of a phage is very important as it helps in the calculation of the growth kinetics parameters like latent period, rise period and the burst size. Latent period is the time elapsed between the moments the host culture is infected with phage to the moment the first bacterial cells are lysed. The rise period is the time span starting from the end of latent period until all phages are extra cellular. Burst size is the average number of progeny phage particles produced per infected bacterium. It is calculated as follows: (final PFU- initial PFU)/ number of infected bacterial cells (Adams, 1959).

One step growth curve experiment was performed according to the protocol (Capra *et al*., 2006). Mid log phase culture of the host (200 mL) was harvested by centrifugation at 9000 x *g* for 10 min and resuspended in 1/5 of the initial volume (40 mL) of pre -warmed nutrient broth. The phage was then added at the optimal MOI, allowed to adsorb for 15 min at 37°C, followed by harvesting of phages by centrifugation at 10000 x *g* (Sigma, 3K30, Germany) for 5 min and resuspension in 200 mL nutrient broth. This was incubated at 37°C. Samples were taken at 10 min intervals (up to 2 h) and immediately titered by the double agar overlay method. Assays were carried out in triplicates and appropriate controls were maintained. The graph was plotted with log of PFU/mL against time. The latent period, the rise period and the burst size of the phage were calculated from the one step growth curve obtained.

6.2.6.5. Influence of physical and chemical parameters on phage viability

Physical and chemical parameters have a critical role in maintaining viability of phages. Hence the effect of different physical and chemical parameters like temperature, pH, NaCl and different sugars on phage viability was studied.

6.2.6.5.1 Effect of temperature on phage viability

The effect of temperature on phage viability/propagation was studied as per protocol (Lu *et al*., 2003). 900 µL of sterile distilled water was pre-heated to temperatures ranging from 50°C to 100°C, followed by the addition of 100 µL of phage sample (10^{10}PFU/mL) to these pre-heated tubes. The tubes were maintained at these temperatures for varying intervals, i.e., 15 s, 30 s, 60 s, 120 s and 180 s. After incubation, these phage containing tubes were immediately placed in ice. Samples were assayed using double agar overlay method to determine the number of surviving plaque PFU. All plate assays were done in triplicates and appropriate controls were maintained. The counts of surviving phage were expressed as PFU/ mL and plotted against temperature values.

6.2.6.5.2Effect of NaCl on phage viability

In order to study the effect of NaCl on phage viability, NaCl solutions of varying molar concentrations such as 0.1 M, 0.25 M, 0.5 M, 0.75 M, 1 M, 2 M and 3 M were prepared in sterilised deionised water. Phage sample was added (10^{10} PFU/mL), incubated for 30 min at 37°C (Capra *et al*., 2006), then diluted and assayed with the mid-log phase host for surviving phage particles by the double agar overlay method. The plaques obtained on the plates were counted. All plate assays were performed in triplicates and appropriate controls were maintained. The result was expressed as PFU/mL and plotted against concentration of NaCl.

6.2.6.5.3Effect of pH on phage viability

Effect of pH on phage viability was evaluated by incubating the phages in appropriate buffers of different pH, ranging from 2-13(Capra *et al*., 2006). Hydrochloric acid-potassium chloride buffer at pH 2, the citrate – phosphate for pH 3 to 6; Phosphate buffer for pH 7, Tris (hydroxymethylamino methane) buffer system for pH 8 and 9; carbonate – bicarbonate buffer for pH 10 and 11; Sodium hydroxide - Potassium chloride buffer (pH 12 and 13) (Appendix- 2) were used. The phage was added (10^{10} PFU/mL) to 10 mL of sterilized buffer solutions, incubated at 37°C for 30 min and assayed with the mid log phase host using double-layer agar plate method to determine the surviving PFU. The plate assay was done in triplicates and appropriate controls were maintained. The viable phage particles on the plates were counted. The results were expressed as PFU/ mL and plotted against the values of pH.

6.2.6.5.4Effect of sugars on phage viability

Influence of various sugars like sucrose, dextrose, galactose, fructose, maltose, mannitol, mannose, lactose, rhamnose, ribose and xylose on phage viability was studied (Capra *et al*., 2006). Sugars were added at a final concentration of 500 mM/1000 mL to each phage sample (10^{10} PFU/mL). The mixture was incubated at 37°C for 30 min, diluted adequately and assayed with the mid log host cells for plaques by the double agar overlay method to determine the surviving phage particles. The plate assay was done in triplicates and appropriate controls were maintained. The results were compared with titre of control samples without the sugars and then expressed as a percentage of phage inactivation.

6.2.6.6. Influence of physical and chemical parameters on phage adsorption

Phage adsorption is a critical step for causing phage infection in bacteria. All factors influencing phage adsorption, also affect phage infection. Hence the influence of various physical and chemical parameters on phage adsorption was studied.

6.2.6.6.1Effect of temperature on phage adsorption

The adsorption of phages on the hosts was determined at temperatures of 0, 10, 20, 30, 37, 40, 45 and 50°C. The methodology was adopted from Capra *et al*., 2006. Briefly, exponentially growing host culture ($O.D_{600}$ = 1) was centrifuged and resuspended (approximately 10^8 PFU/mL) in nutrient broth (pH 8). Phage was added at the optimal MOI and was incubated, each at the different temperatures mentioned above for 30 min. After centrifugation at 12000 x *g* for 5 min (Sigma) at 4°C, the supernatant after appropriate dilutions were assayed for unabsorbed phages employing double agar overlay method. All plating`s was done in triplicates and appropriate controls were maintained. The phage titre was compared with the control titre. The results were expressed as percentages of adsorption and plotted against temperature.

6.2.6.6.2Effect of NaCl on phage adsorption

Effect of different concentrations of NaCl on adsorption was investigated (Capra *et al.,* 2006). Exponentially growing host culture was added to nutrient broth with concentrations of NaCl ranging from 0.1, 0. 25, 0.5, 0.75 and 1 M NaCl, and infected with optimal MOI of phages and incubated at 37°C for 30 min for adsorption. The mixture was then centrifuged at 10000 x *g* for 5 min at 4°C (Sigma) to sediment the phage adsorbed bacteria. The supernatant was assayed for unabsorbed free phages employing double agar overlay method and the counts were compared with the titre of the control that contained no NaCl in nutrient broth. All platings were in triplicates and appropriate controls were maintained. The results were expressed as a percentage of adsorption and then plotted against NaCl concentration.

6.2.6.6.3Effect of pH on phage adsorption

In order to study the effect of pH, exponentially growing host culture was centrifuged at 10000g for 10 min (Sigma) at 4°C, and the cells resuspended in nutrient broth adjusted to the desired pH. Adsorption was determined at the pH values ranging from 2 to 13. Phage was added at an optimal MOI, incubated at 37°C for 30 min for adsorption, centrifuged at 10000 x *g* for 5 min, the supernatants assayed to determine surviving PFU employing double agar overlay method and was compared to control (Capra *et al* ., 2006). All experiments were performed in triplicates and appropriate controls were maintained. The results were expressed as a percentage of adsorption and then plotted against pH values.

6.2.6.6.4 Effect of calcium ions on phage adsorption and propagation

The effect of calcium ions on phage adsorption and propagation was determined (Lu *et al*., 2003). The protocol involved the addition of 10 mL of exponentially growing host culture to 100 mL nutrient broth, and incubation for 3.5 h at 120 rpm (Sigma). 10 mL each of this mid log phase host culture was added to five, 15 ml McCartney bottles. Appropriate volumes of filter sterilized 1M $CaCl_2$ (Millipore, USA) solution were added to the host aliquots to make 0, 1, 10, 20, and 30 mM concentrations. After the final volume was adjusted to 15 mL with sterile distilled water, each tube was infected with the phage at optimal MOI. All tubes were incubated at 37°C for 2 h. 1 mL aliquots were drawn and

centrifuged at 10000 x *g* for 10 min (Sigma) at 4°C. The supernatants were serially diluted and assayed using double agar overlay method to determine plaque formation. The phage titre was determined employing double agar overlay method for the medium with and without $CaCl_2$. All platings were in triplicates and appropriate controls were maintained. The results were expressed as PFU/ mL and plotted against $CaCl_2$ concentrations.

6.2.6.7. Effect of optimized physicochemical parameters on phage propagation

The cumulative effect of all the parameters optimized under section 6.2.6.2, 6.2.6.3, 6.2.6.5 and 6.2.6.6was studied. Phage lysate was added at its optimum MOI to mid log phase host cells grown in nutrient broth (pH adjusted to 8 with 10 mM $CaCl_2$and optimum NaCl concentration). The incubation temperature was set at 40°C. One step growth curve experiment was repeated as described in section 6.2.6.4. Aliquots were sampled at 10 min intervals, mixed with mid log phage host cells grown in nutrient broth (pH adjusted to 8 with 10 mM $CaCl_2$and 0.25 M NaCl), followed by incubation for 30 min and was immediately titered by double agar overlay method. All plating's were done in triplicates. Appropriate controls were maintained. The graph was plotted with log of PFU/mL against time. The latent period, the rise period and the burst size of the phage were calculated.

6.2.6.8 Propagation of phage under nutrient depleted states of the host cell

Phages intended for use as biocontrol agents have an added advantage when they have the capability to infect host under stationary as well as various nutrient deprived conditions. Thus the ability of the phages to infect host under different nutrient deprived conditions was studied.

6.2.6.8.1. Preparation of log- and stationary-phase, starved- and nutrient-depleted cultures

The host cells were grown as overnight cultures at 37°C were used for the stationary phase cell infection experiments. A fresh 6 hour culture was used for the exponential phase host cell infection experiments. The starved host cells were prepared by resuspending cell pellet obtained after centrifugation of a 6 hour old culture, in an equal volume of physiological saline and incubating it for 24 h at

37°C (Kadavy *et al.*, 2000). Nutrient depleted cultures were prepared by growing the bacteria to log phase in nutrient broth, and the cells harvested by centrifugation were resuspended in appropriate starvation suspension media, like minimal media (Appendix- 2) without carbon, minimal media without phosphate and minimal media without ammonium chloride (nitrogen source) (Nystrom *et al*., 1992). In all the cases except for stationary phase, the $O.D_{600}$ of cultures was adjusted to 0.5 ($1X10^5$ colony forming units (CFU)/mL) using respective medium prior to the addition of the phage. Phages were introduced into each of these cultures at a very low multiplicity of infection (MOI) of 0.1, incubated at 37°C for 24 hours and plated (double agar overlay method) to observe the efficacy of phagemultiplication under various nutrient deprived conditions. All plating`s was done in triplicates and appropriate controls were maintained.

6.2.6.8.2 Statistical analysis

Statistical evaluations were done by ANOVA, followed by Newman–Keuls Test and Tukey Test using StatsDirect statistical software (version 2.8.0, Cheshire, UK) computer program.

6.2.6.9 Bacteriophage genome analysis

6.2.6.9.1. Phage DNA isolation

Phage DNA extraction was carried as previously described (Sambrook *et al.*, 2000). Briefly, 1 mL of the PEG 6000 concentrated phage suspension was incubated at 56°C for one hour with proteinase K at a final concentration of 50 µg/mL and SDS at a final concentration of 0.5%. After incubation, the digestion mix was cooled to room temperature and extracted first by adding equal volume of phenol which is equilibrated with 50 mM Tris (pH 8.0). The digestion mix in the tube was gently inverted a few times until complete emulsion was formed. The phases were separated by centrifugation at 3000 x *g* for 5 min at room temperature (Sigma). The aqueous phase was then transferred to a clean tube using wide-bore pipette, and then extracted with 50:50 mixtures of equilibrated phenol: chloroform, followed by a final extraction with equal volume of chloroform. Double volume of ethanol and sodium acetate (pH 7) (Appendix- 3) to a final concentration of 0.3 M was added to the extract followed by incubation at room temperature for 30 min. After incubation, the precipitated DNA was collected by

centrifugation at 10000 x *g* for 5 min at 4°C. The supernatant was discarded and DNA was dissolved in Tris- EDTA (TE) buffer (pH 7.6) (Appendix-3). Finally the DNA was run on 1 % agarose gel and the gel was stained with ethidium bromide and visualized in UV light. The image of the gel was captured using gel documentation system (Syngene, UK).

6.2.6.9.2. Restriction analysis

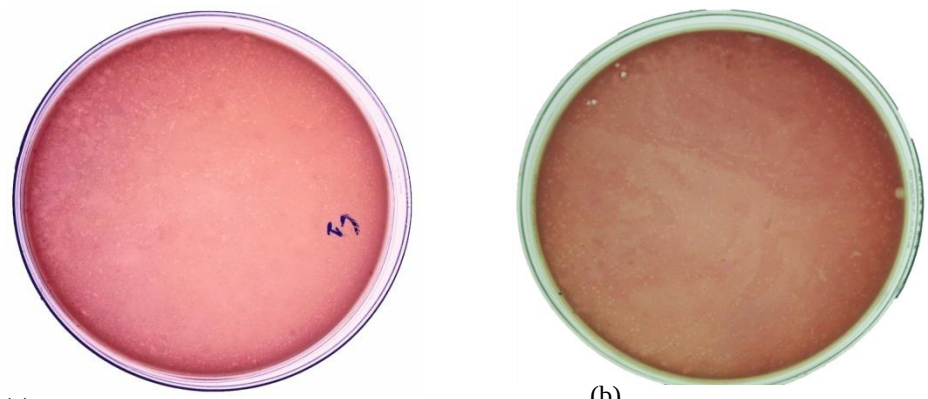
The restriction pattern of the phage DNA was studied using the enzyme, *Bam* HI. (Fermentas, USA). Enzyme digestions were performed as recommended by the manufacturer. For digestion, each 20μL digestion solution containing approximately 1 μg of bacteriophage DNA and 1U of the restriction enzyme in reaction buffer was incubated for 1 hour at reaction temperature as the protocol prescribed for the enzyme. Restricted fragments were separated by agarose (1.2%) gel electrophoresis. Gel was stained with ethidium bromide and visualized in UV light. The image of gel was captured using gel documentation system (Syngene, UK).

6.3 Results

6.3.1. Bacteriophage isolation

Different samples like beef, cheese, raw milk, pasteurized milk, curd, chilly powder, turmeric powder, coriander powder, soft drink, fresh fish, dried fish and dried prawn were screened for bacteriophage isolation. A lytic phage was isolated from meat sample from which the host strain *Bacillus altitudinis* (BTMW1) was also isolated and was named ΦBAP-1. Another lytic phage was obtained on the lawn of the strain *Pseudomonas aeruginosa*(BTRY1) from milk sample and named as ΦPAP-1. These two phages were selected for further study.

ΦBAP-1 and ΦPAP-1 were purified by repeated plating and picking of single isolated plaques from the lawns of *B. altitudinis (BTMWI)* and *P. aeruginosa* (BTRY1) respectively. Both produced large, clear plaques indicating their lytic nature. The tetrazolium stained plates showing plaques formed by phages ΦBAP-1 and ΦPAP-1 on bacterial lawn of respective hosts are as presented in Fig 6.1 a and b.



(a) (b)

Fig 6.1. Tetrazolium plates showing plaques formed by phage on bacterial lawn (a) ΦBAP-1 on *Bacillus altitudinis* (b) ΦPAP-1 on *Pseudomonas aeruginosa*.

6.3.2. Phage concentration

ΦBAP-1 and ΦPAP-1 were concentrated up to 1 X 10^{10} PFU/mL using PEG precipitation and these phage concentrates prepared in large quantities were used for all further studies.

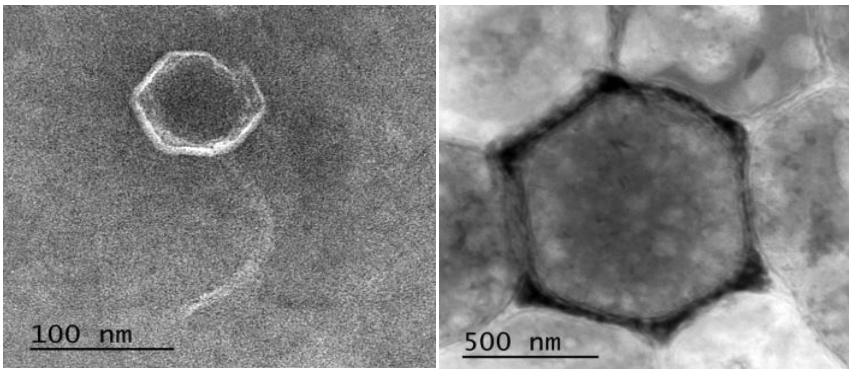
6.3.3. Maintenance and storage of phages

Glycerol stocks of both ΦBAP-1 and ΦPAP-1 were maintained at -80°C for further study.

6.3.4. Characterization of phages

6.3.4.1. Morphological analysis by Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy is routinely used in the morphological characterization of phage. The TEM elucidated morphology has great significance, as it forms the basis for the classification of bacteriophages (Figure 6.2. (a) & (b)).



(a) (b)

Fig 6.2 Transmission Electron micrograph image of phage stained with 1% phosphotungstic acid hydrate (a) ΦBAP-1 (bar represents 100 nm) (b) ΦPAP-1 (bar represents 500 nm).

The TEM image of ΦBAP-1 revealed isomeric head outline, indicating their icosahedral nature, along with a long contractile tail. The diameter of the head was measured to be 80.40 ± 1.1nm and the length of the tail was 168 ± 1.4 nm (Fig 6.2.a).

The electron micrograph of ΦPAP-1 clearly showed bacteriophage with a hexagonal head of size 264.47 ± 0.91nm without any tail (Fig 6.2.b). The phage sizes were determined from the average of 3 independent measurements (mean ± standard deviation). These morphological characteristics of the phages placed them under different families. ΦBAP-1 with non- enveloped icosahedral head and a long contractile tail belongs to family *Siphoviridae*, while ΦPAP-1 non-enveloped with icosahedral head and with no head-tail structure goes .under the family *Tectiviridae.*

6.3.4.2. Determination of optimal multiplicity of infection

Multiplicity of infection is defined as the ratio of virus particles to that of the host cells. This is a very important criterion for the large scale production of bacteriophages, due to its significant impact on phage titre. The optimal MOI of ΦBAP-1 with *B.altitudinis* strain BTMW1 as host, was one phage per bacterium, while MOI of PAP-1 was five phages per bacterium with *P.aeruginosa*s strain BTRY1 as host.. These optimal MOI resulting in the highest phage titre under standard conditions were used in all subsequent large scale phage production of ΦBAP-1 and ΦPAP-1 respectively, unless otherwise specified.

6.3.4.3. Phage adsorption

The adsorption curve of ΦBAP-1 and ΦPAP-1are shown in Fig 6.3. For ΦBAP-1 adsorption nearing 100% was achieved after 35 min of exposure to the host bacteria, whereas for ΦPAP-1 it took 40 min to achieve the same.

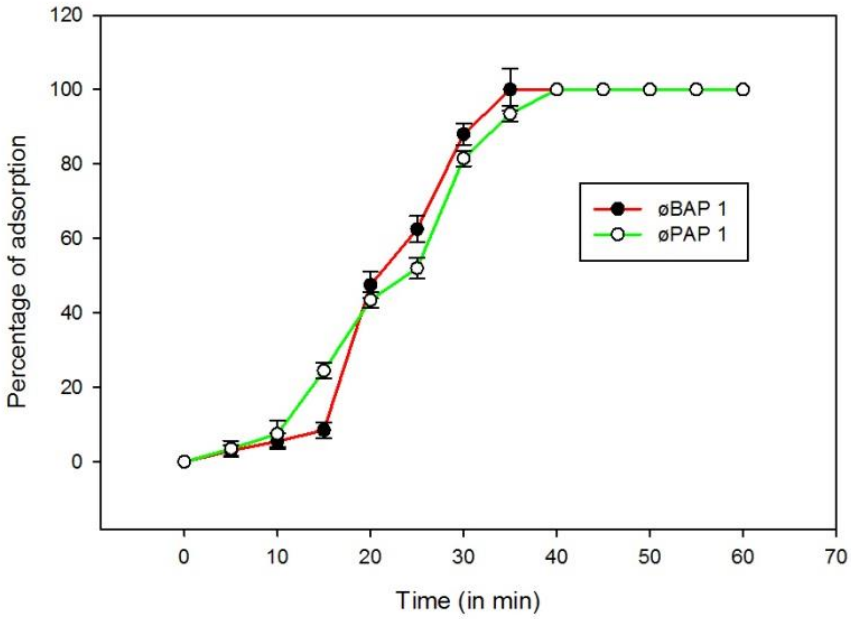


Fig 6.3. Adsorption curves of ΦBAP-1 and ΦPAP-1

6.3.4.4. One step growth curve

The one step growth curve helped in understanding the growth kinetics parameters like latent period, rise period and the burst size of the bacteriophages under study. The one step growth curve of ΦBAP-1 and ΦPAP- 1 is as given in Fig 6.4. These experiments were performed at 37°C, with an MOI of one for ΦBAP-1 and five for ΦPAP-1.

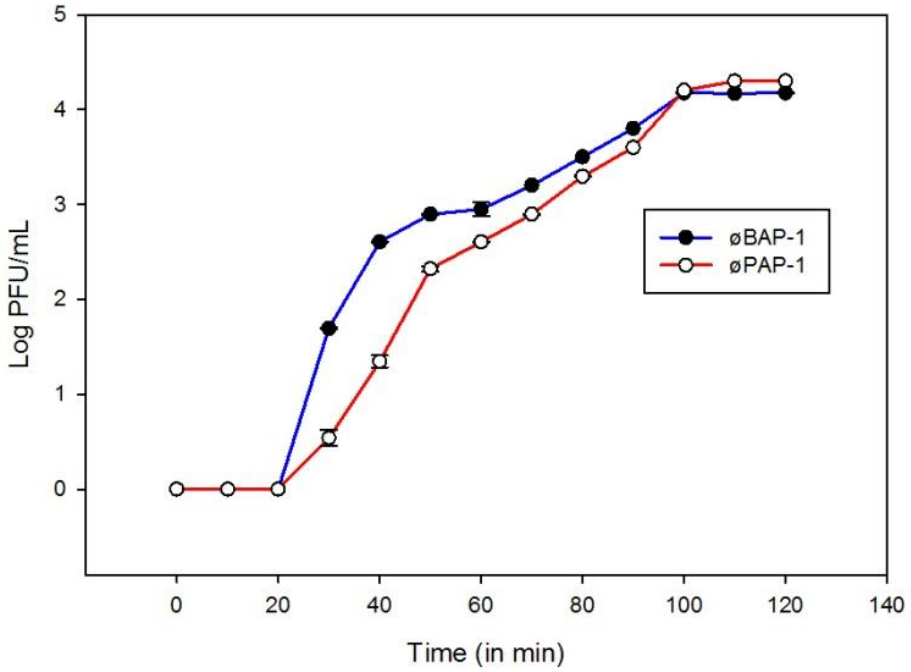


Fig 6.4. One step growth curve of ΦBAP-1 and ΦPAP-1

The calculated latent period of ΦBAP-1 was approximately 30 min, the rise period as 50 min, and the burst size was 56 phages per bacterial cell. The multiplication period reached a plateau at about 100 min after infection with ΦBAP-1 (Fig 6.4).

The one step growth curve of ΦPAP-1 (Fig 6.4) showed the latent period was about 30 min while rise period was 60 min. The calculated burst size was 60 phages per bacterium. It was noted from the results that ΦPAP1 has a larger burst size and a larger generation period than that of ΦBAP1.

6.3.4.5. Influence of physical and chemical parameters on phage viability

6.3.4.5.1 Effect of temperature on phage viability

The effect of temperature on the viability of ΦBAP-1, investigated by heat treatment at different temperatures, over varying time intervals is represented in Fig 6.5(a).

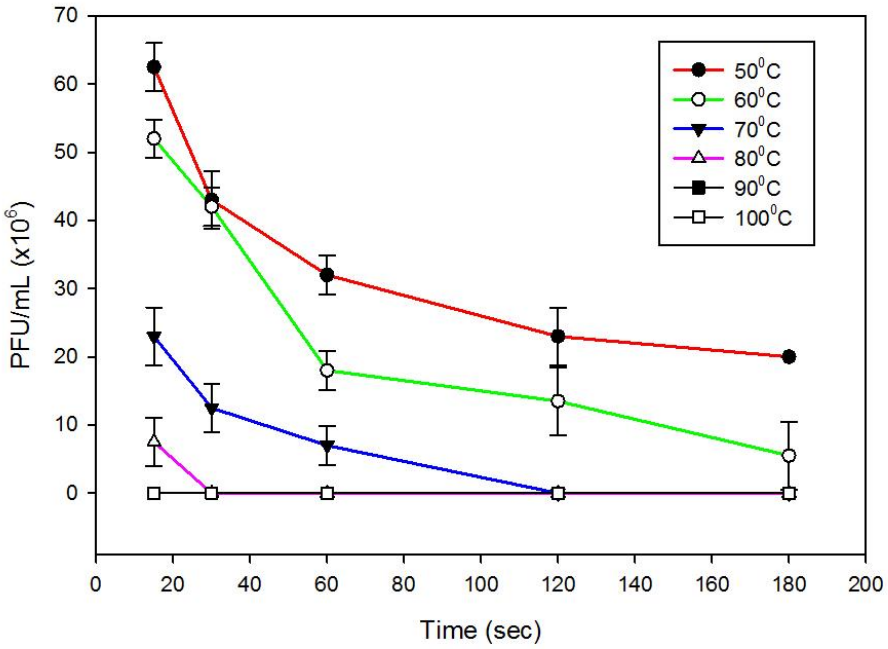


Fig 6.5 (a). Effect of temperature on viability of ΦBAP-1

It is evident that phage viability was drastically reduced by exposure to high temperatures. Viable PFU of ΦBAP-1 were highest when exposed to 50°C for 3 min. At 60°C viable PFU were reduced by more than 50% at the end of 3 min when compared to the count at 50°C, whereas complete viability was lost at the end of 2 min at 70°C. Although exposure to 80°C was fatal over an exposure period of 3 min, there were nevertheless a few survivors at the end of 15 s. ΦBAP-1 failed to survive when exposed to 90°C and 100°C, even for a few seconds. In all cases, phage count steadily decreased with increase in exposure time at different temperatures.

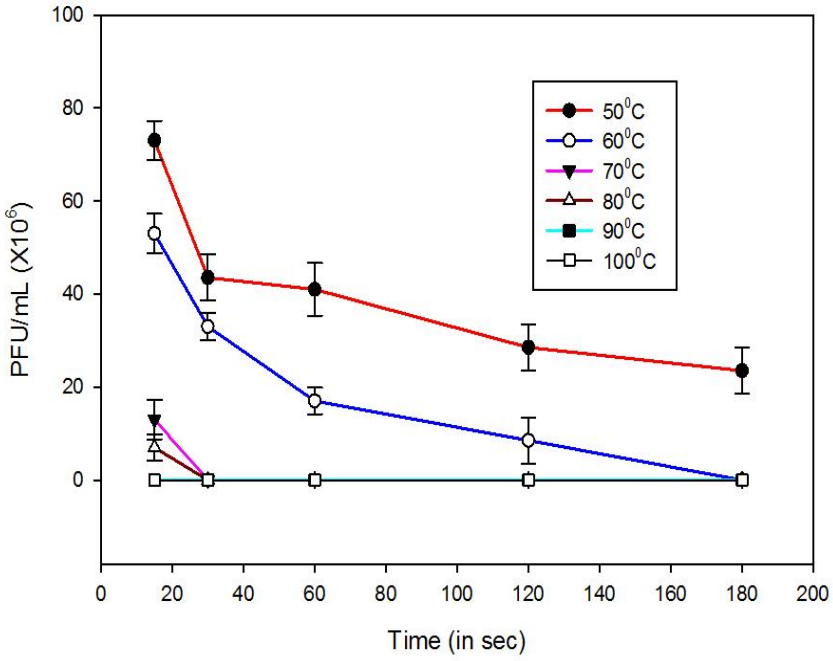


Fig 6.5 (b) Effect of temperature on viability of ΦPAP-1

The influence of temperature variation on viability of phage ΦPAP-1 is as depicted in Fig 6.5 (b). Ample viability was noted at 50°C and 60°C. Viability was drastically reduced to a few PFU/mL at 70°C, while exposure to temperatures above 70°C even for a few seconds, was fatal for phage ΦPAP-1 as there were no survivors.

It may be noted that ΦBAP-1 was more tolerant to higher temperatures than ΦPAP-1 under experimental conditions. However, both phages show reduced viability at temperatures below 20°C.

6.3.4.5.2 Effect of NaCl on phage viability

The effect of varying concentration of NaCl on the viability of the phages Φ BAP-1 and ΦPAP-1 is as shown in the Fig 6.6.

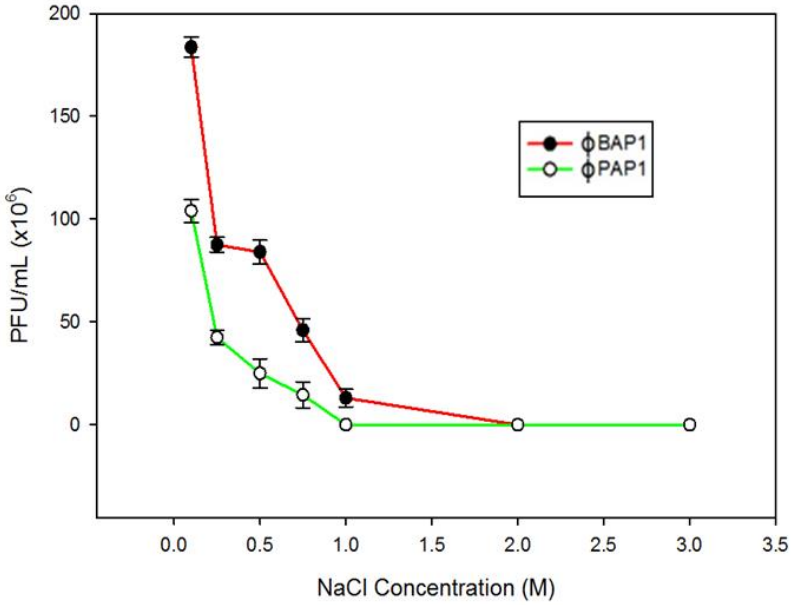


Fig 6.6 Effect of NaCl on viability of phage ΦBAP-1 and ΦPAP-1

It is evident from Fig 6.6 that for ΦBAP-1**,** the optimum concentration of NaCl for phage survival was 0.1M .There was significant reduction in viability of ΦBAP-1 at concentrations higher than 0.1M NaCl and the phages did not survived concentrations beyond 1M NaCl.

Meanwhile, study on viability of ΦPAP-1 in the presence of varying concentration of NaCl revealed 0.5M NaCl as optimal for phage survival. The phage showed higher viability even at 0.75 M and further higher concentrations of NaCl caused a decline in the viability of ΦPAP-1 as observed from Fig 6.6.

6.3.4.5.3 Effect of pH on phage viability

The viability of the phages was studied over a pH range of 2 – 13. Fig 6.7 elucidates the effect of pH on viability of ΦBAP-1 and ΦPAP-1**.**

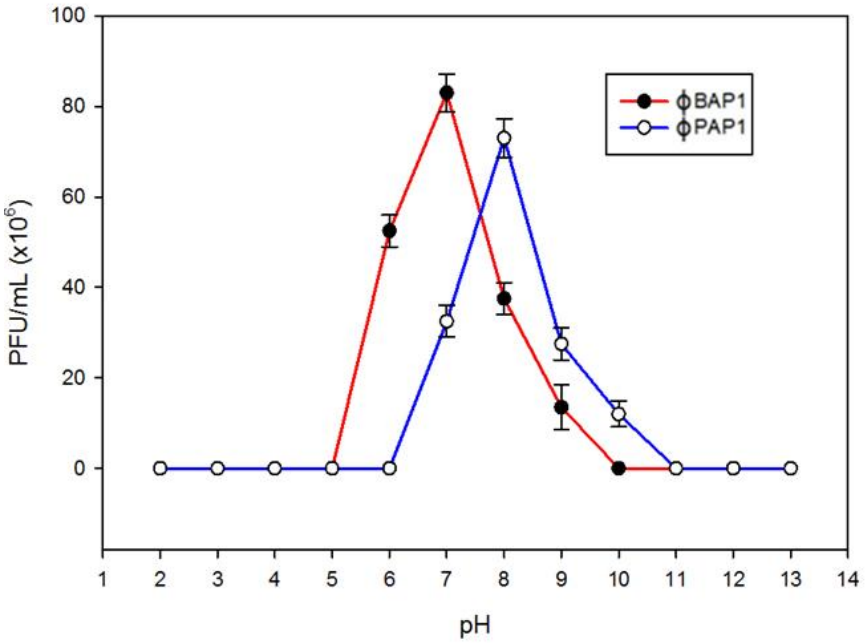


Fig 6.7 Effect of pH on viability of phage ΦBAP-1 and ΦPAP-1

From the figure, it is evident that pH 7 is optimum for survival of ΦBAP-1. Viability was observed even at pH as high as 10, although in small numbers. At the acidic pH ≤ 5 and at pH ≥10, ΦBAP-1 did not survive.

The optimum pH for ΦPAP-1 viability was found to be 8. It was clear from the figure that ΦPAP-1 could not survive at acidic pH ranging from 2 to 6 and from 10 -13. Both ΦBAP-1 and ΦPAP-1 had poor tolerance to acidic pH conditions, but showed greater survival at alkaline pH.

In short, ΦBAP-1 survived in the pH range 5-10 while ΦPAP-1 survived at the range 6-11 which indicated their similarity of survival at different pH.

6.3.4.5.4 Effect of sugars on phage viability

The effect of sugars on the viability of phages ΦBAP-1 and ΦPAP-1 was studied using sugars xylose, ribose, mannose, mannitol, dextrose, sucrose, fructose, galactose, rhamnose, maltose and lactose, each at a final concentration of 500 mM/L, is as shown in Fig 6.8 (a) and (b).

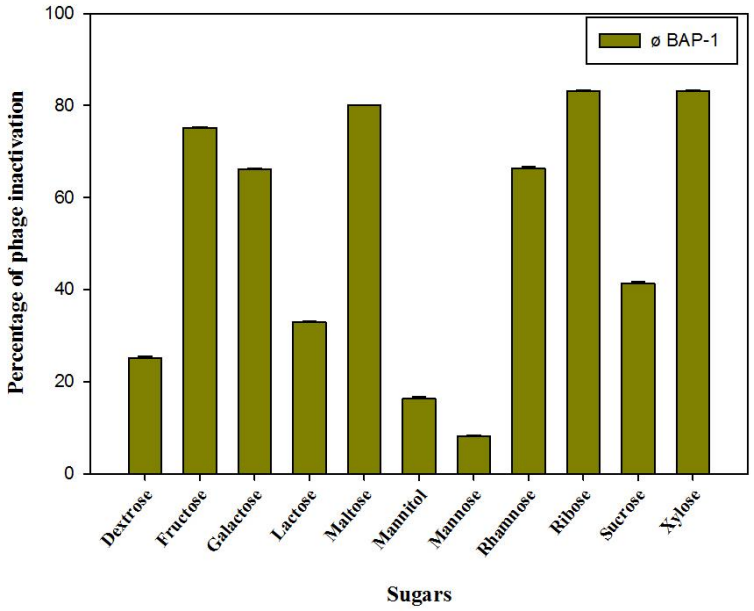


Fig 6.8 (a) Effect of sugars on viability of ΦBAP -1

The influence of 11 different sugars on viability of ΦBAP-1 is depicted in the figure 6.8 (a).Sugars like fructose, galactose, maltose &rhamnose resulted in drastic inactivation of ΦBAP-1by as much as 75%, 66%, 80% & 66% respectively. Ribose & xylose caused 83% inactivation while dextrose, lactose & sucrose caused 25%, 32% & 41% respectively. Mannitol caused only 16% inactivation while mannose caused the least inactivation of 8% compared to the control.

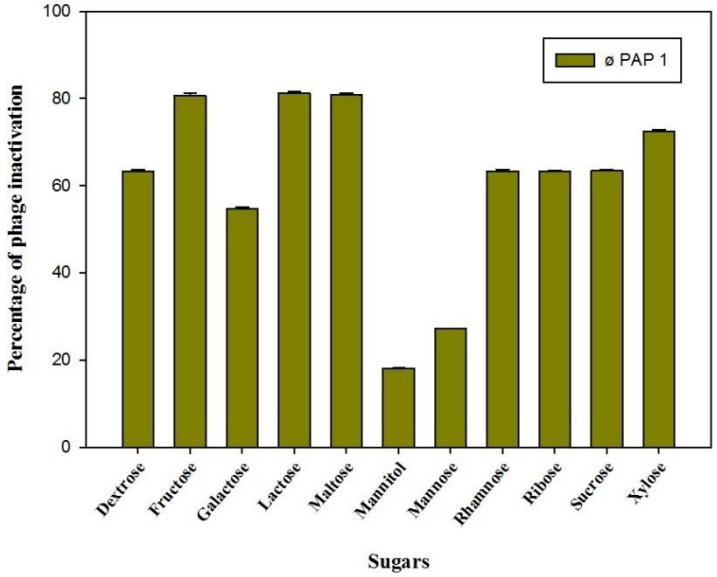


Fig 6.8 (b) Effect of sugars on viability of ΦPAP-1

The influence of 11 different sugars on viability of ΦPAP-1 is depicted in the figure 6.8 (b). Sugars like fructose, lactose &maltose resulted in drastic inactivation of ΦPAP-1 by as much as 80%.Dextrose, rhamnose, sucrose &ribose caused 63% inactivation while galactose& xylose caused 66% & 72% inactivation respectively. Mannose caused only 27% inactivation while mannitol caused the least inactivation of 18% compared to the control.

6.3.4.6. Influence of physical and chemical parameters on phage adsorption

6.3.4.6.1 Effect of temperature on phage adsorption

The effect of different temperatures ranging from 0°C to 50°C on the adsorption of ΦBAP-1 and ΦPAP-1 are as represented in Fig 6.9.

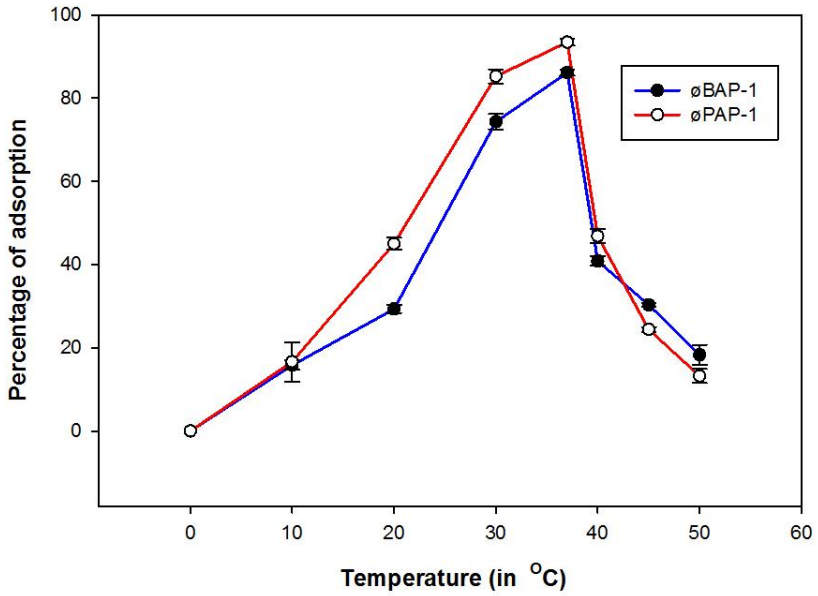


Fig 6.9 Effect of temperature on adsorption of ΦBAP-1 and ΦPAP-1

In case of ΦBAP-1, maximum adsorption was observed at 37°C, and this temperature was therefore considered optimal for ΦBAP-1. There was no observable adsorption at 0°C, but there was a steady increase in adsorption as the temperature was raised to 10°C. It was noted that little adsorption occurred at higher temperatures i.e., 30% adsorption at 45°C and 18% adsorption at 50°C

Similarly for ΦPAP-1,maximum adsorption was observed at 37°C, while 85% adsorption was observed at 30°C. At higher temperatures above 45°C, both phages could not adsorb well which resulted in low survival rate.

6.3.4.6.2 Effect of NaCl on phage adsorption

The influence of sodium chloride on adsorption by ΦBAP-1 and ΦPAP-1 is as represented in Fig 6.10.

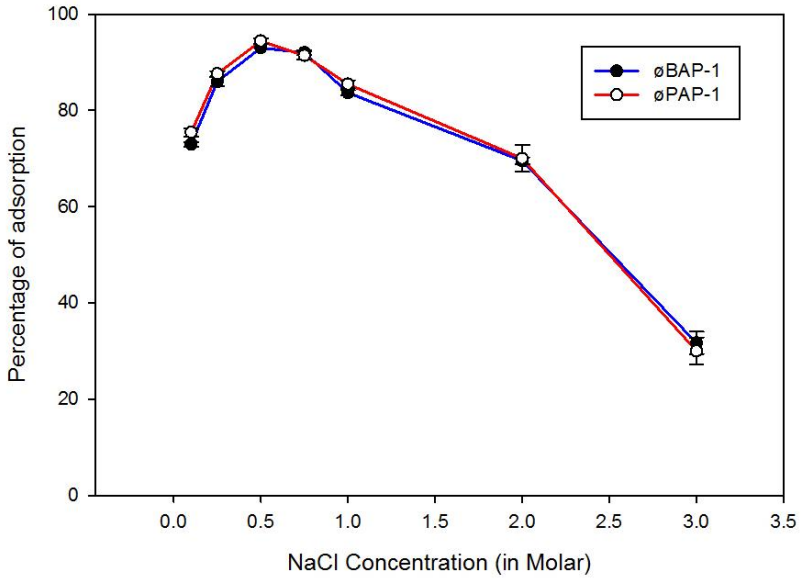


Fig 6.10 Effect of NaCl on adsorption of phage ΦBAP-1and ΦPAP-1

The influence of sodium chloride on adsorption by ΦBAP-1and ΦPAP-1 is as represented in Fig 6.10.

Optimal NaCl concentration for maximal adsorption was 0.5 M & 0.75 M for ΦBAP-1, beyond which the efficacy dropped. Adsorption was observed to take place even at a concentration of 3M NaCl, although at comparatively lower levels. The percentage of adsorption dropped to 83% at 1 M NaCl concentration. 72% adsorption was observed at a concentration of 0.1M NaCl.

In case of ΦPAP-1, the same pattern as that of ΦBAP-1 was observed. 0.25M concentrations of NaCl favoured 75% adsorption. Maximum adsorption of 94% was observed at 0.5M sodium chloride followed by 91% at 0.75 M. Adsorption continued to occur at higher concentration of NaCl i.e. 1 M and 2M (85 and 70% respectively). An adsorption of 30 % was found even at 3M NaCl concentration.

Both phages are thus proved to adsorb even at high concentration of NaCl, albeit at lower rates.

6.3.4.6.3 Effect of pH on phage adsorption

The influence of pH on the adsorption of ΦBAP-1 and ΦPAP-1 is as shown in Fig 6.11.

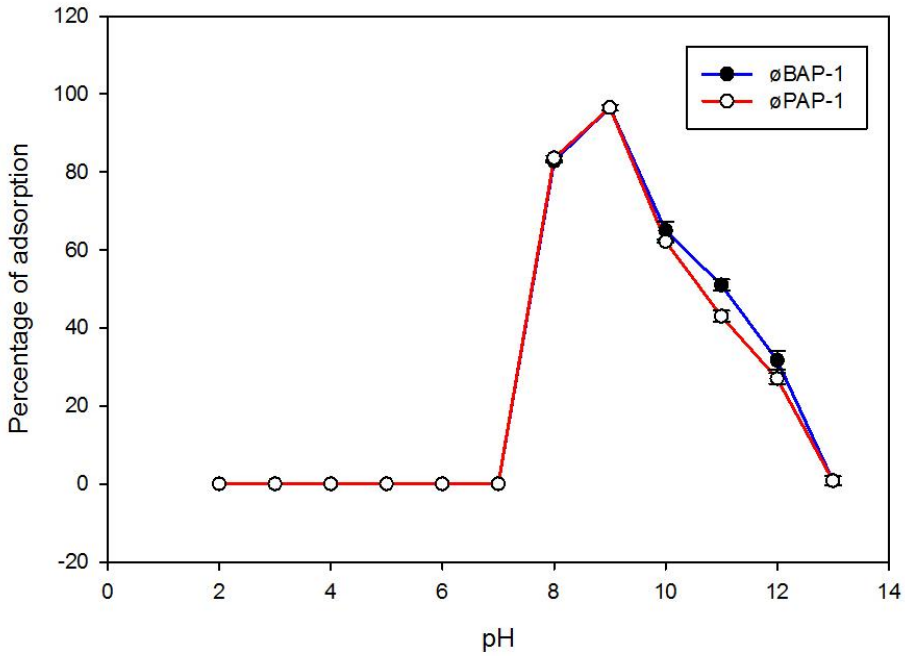


Fig 6.11. Effect of pH on adsorption of ΦBAP-1and ΦPAP-1

From the Fig 6.11, it is evident that 96% adsorption of ΦBAP-1 was recorded at pH 9, while adsorption greater than 83% was observed at pH 8, and 27 % at pH 11. Optimal pH for maximal adsorption was pH 9, above and below this pH the efficacy decreased and 0% adsorption was recorded below pH 7.

In the case of ΦPAP-1, pH 9 was observed to be optimum, giving maximum adsorption (96%), followed by pH 8, at which 83% of phages were successfully adsorbed on the bacterial host. Less than 45% of the phage ΦPAP-1 adsorbed at pH 11. Adsorption was not observed at pH ≤ 7. The pH optimum was pH 9 for both ΦBAP-1 and ΦPAP-1. pH ≤ 7 was detrimental in both cases, with no observed adsorption.

6.3.4.6.4 Effect of calcium ions on phage adsorption and propagation

The propagation of Φ BAP-1 and Φ PAP-1 in the presence of varying concentration of $CaCl_2$ is as depicted in Fig 6.12.

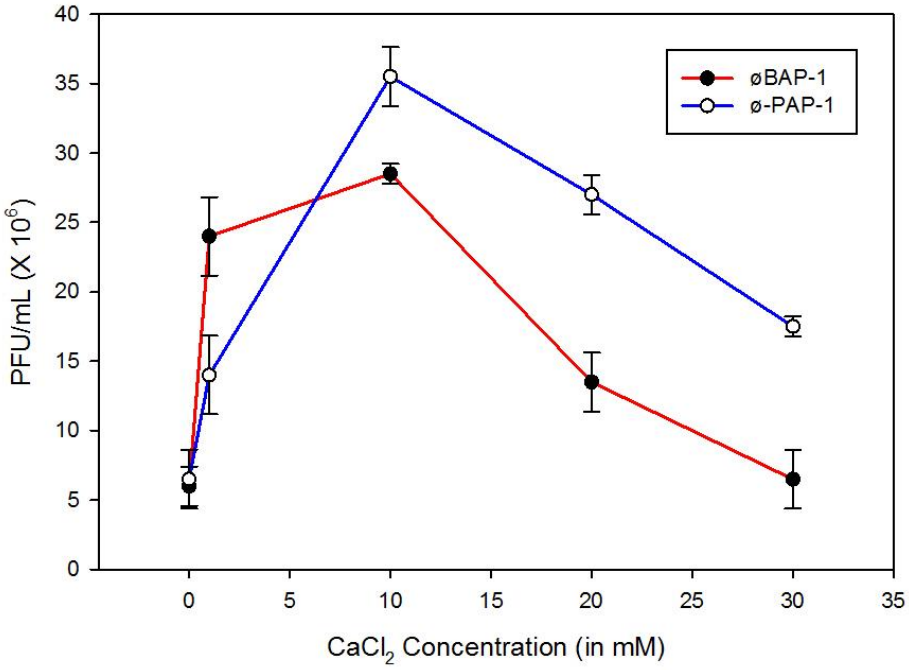


Fig 6.12 Effect of $CaCl_2$ on propagation of phage ΦBAP-1 and ΦPAP-1

In the case of ΦBAP-1, propagation was optimum at 10 mM $CaCl_2$, closely followed by 1mM $CaCl_2$ concentration.

In the case of ΦPAP-1, 10mM of $CaCl_2$was observed to be optimum and the decline in the number of viable viral particles was gradual beyond 10mM $CaCl_2$, unlike ΦBAP-1 where a drastic dip in viral count was observed.

6.3.4.7. Cumulative effect of optimized parameters on propagation of ΦBAP-1 and ΦPAP-1.

The phages were propagated under optimized parameters viz. section 6.2.6.2, 6.2.6.5, 6.2.6.6 and 6.2.6.7and this affected an increase in the phage titer of both ΦBAP-1 and ΦPAP-1, as evidenced from Fig. 6.13(a) and 6.13 (b) respectively.

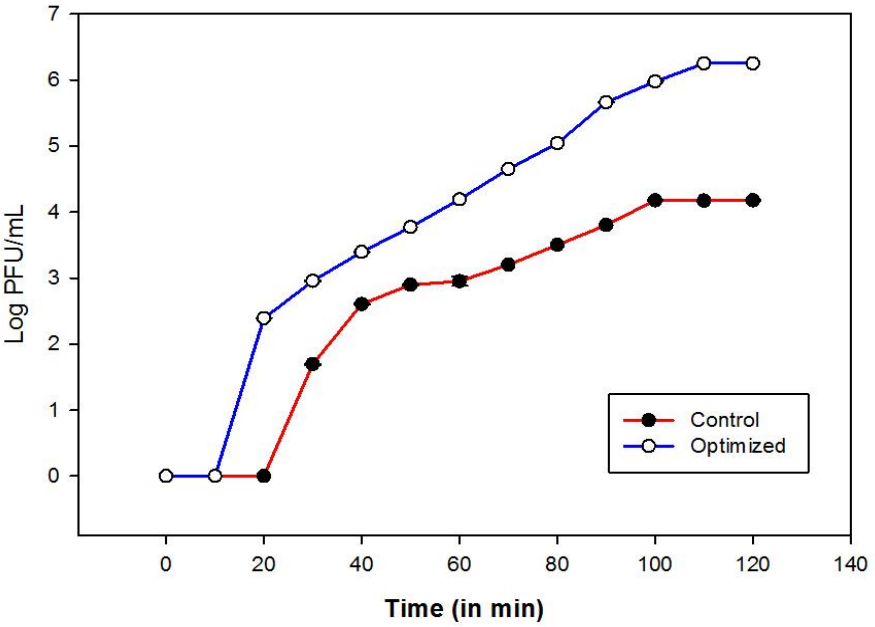


Fig 6.13 (a) Effect of optimized parameters on propagation of ΦBAP-1

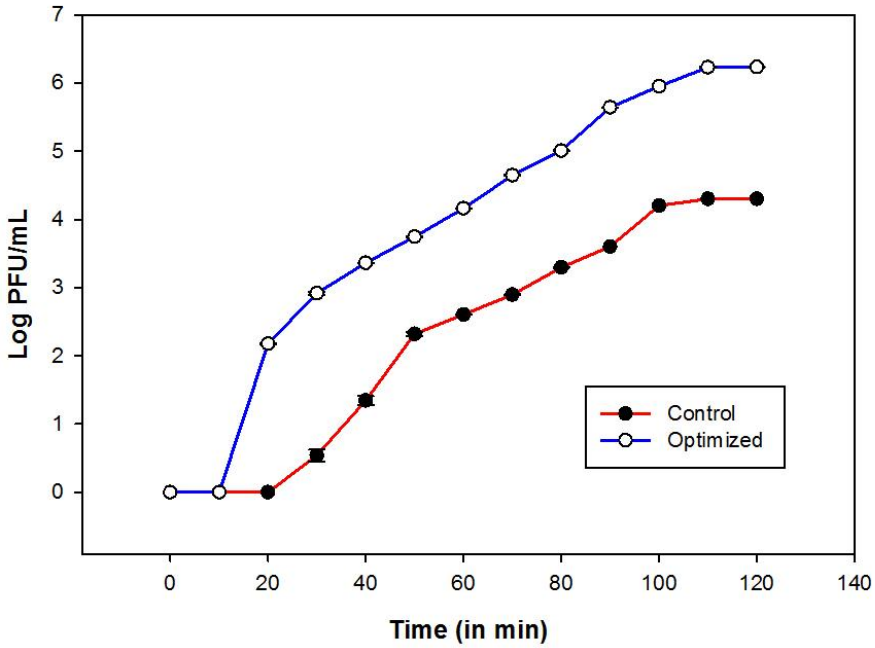


Fig 6.13 (b) Effect of optimized parameters on propagation of ΦPAP-1

Under optimized conditions, the latent period was minimised to 20 min for both ΦBAP-1 and ΦPAP-1. A longer rise period of 100 min was observed in case of ΦBAP-1 and the burst size increased from 56 to 71 phages per bacterial cell.

For ΦPAP-1, the rise period increased to 100 min. Burst size was hiked from 60 to 73 phages per bacterial cell.

6.3.4.8 Propagation of phage under nutrient depleted states of the host cell

The ability of ΦBAP-1 and ΦPAP-1 to infect host under different nutrient deprived conditions as depicted in Fig 6.14 (a) and 6.14 (b) respectively.

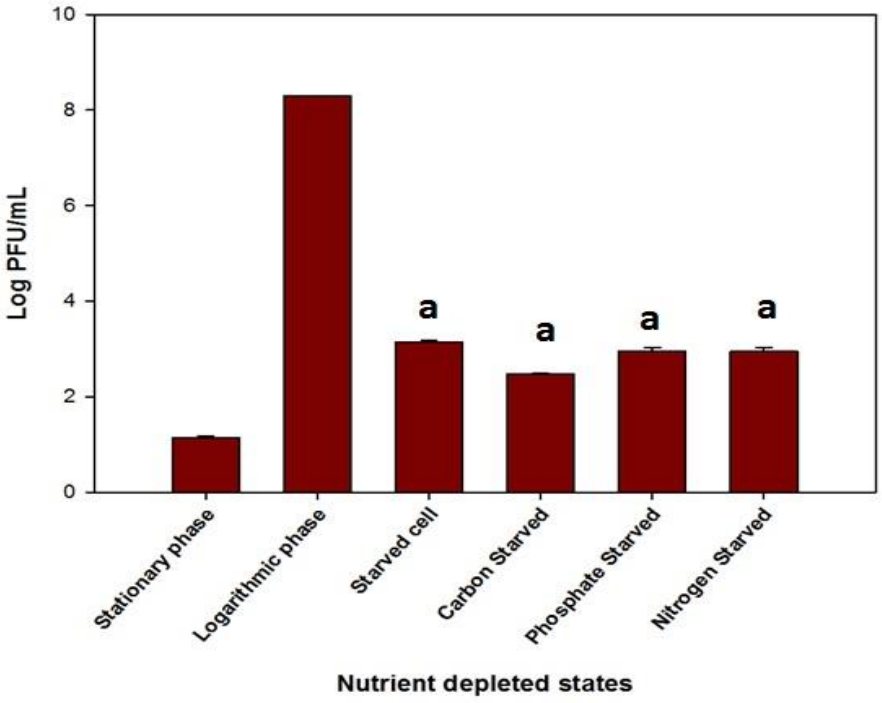


Fig 6.14 (a) ΦBAP-1 propagation in nutrient deprived conditions of the host cell BTMW1 (^{a} indicates $P < 0.0001$ when compared to stationary phase).

The propagation ΦBAP-1 on host bacterial strain *Bacillus altitudinis* BTMW1 under various nutrient limited conditions exhibited significant outcome (Fig 6.14.(a)). ΦBAP-1 multiplication was maximum when the host was in the logarithmic phase ($\log_{10}$ 8.3 ± 0^{a} PFU/mL) as depicted in Fig 6.14.(a). ΦBAP-1 also infected host under stationary phase, although in low numbers ($\log_{10}$1.14 ± 0.0424 PFU/mL) and was able to multiply even under multiple nutrient starved

states, as evidenced by a significant level of increase in phage titre, $\log_{10}3.14 \pm 0.15^{a}$PFU/mL. Under phosphate and nitrogen starved conditions, ΦBAP-1infected its host at same rate ($\log_{10} 2.95 \pm 0.0707^{a}$). Nevertheless, successful propagation of ΦBAP-1 in the host under carbon limiting conditions ($\log_{10}2.48 \pm 0.0141^{a}$ PFU/mL)

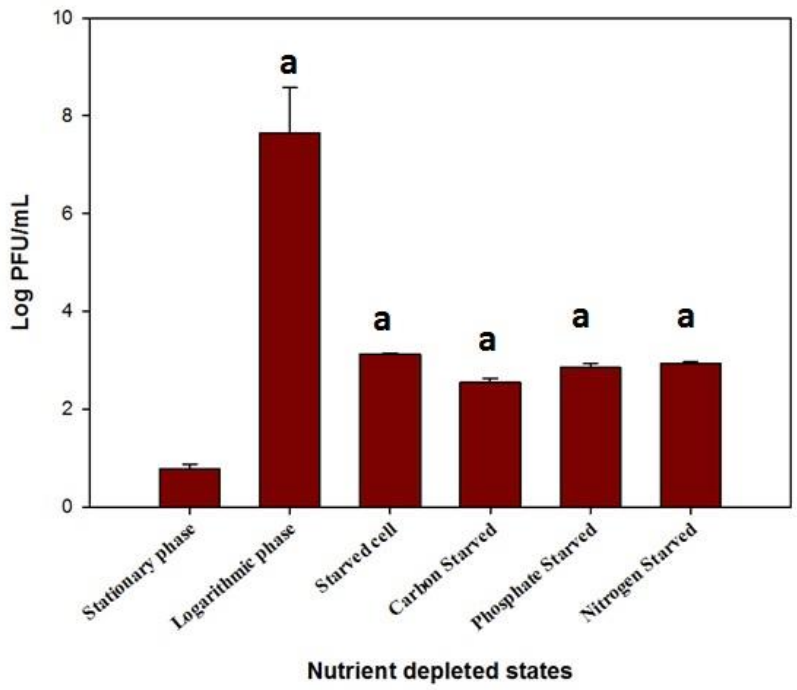


Fig 6.14(b) ΦPAP-1 propagation in nutrient deprived conditions of the host cell BTRY1 (**a** indicates $P < 0.0001$ when compared to stationary phase).

The propagation ΦPAP-1 on host bacterial strain *Pseudomonas aeruginisa*BTRY1 under various nutrient limited conditions exhibited significant outcome (Fig 6.14.(b)). ΦPAP-1 multiplication was maximum when the host was in the logarithmic phase ($\log_{10}$ 7.65 ± 0.9192[a] PFU/mL) as depicted in Fig 6.14.(b). ΦPAP-1 also infected host under stationary phase, although in very few numbers ($\log_{10}0.7650 \pm 0.1061$ PFU/mL) and was able to multiply even under multiple nutrient starved states, as evidenced by a significant level of increase in phage titre at the rate of$\log_{10}$ 3.12 ± 0.0283[a] PFU/mL. Successful propagation of ΦBAP-1 in the host observed under carbon limiting conditions ($\log_{10}2.48 \pm 0.0141^{a}$ PFU/mL), nitrogen limiting conditions ($\log_{10}2.925 \pm 0.0707^{a}$ PFU/mL) and phosphate limiting conditions ($\log_{10}2.85 \pm 0.0141^{a}$ PFU/mL).

6.3.4.9 Bacteriophage genome analysis

6.3.4.9.1. Phage DNA isolation

The genomic DNA of ΦBAP-1 and ΦPAP-1 was isolated and was visualized by agarose gel electrophoresis as single band (Fig 6.15).

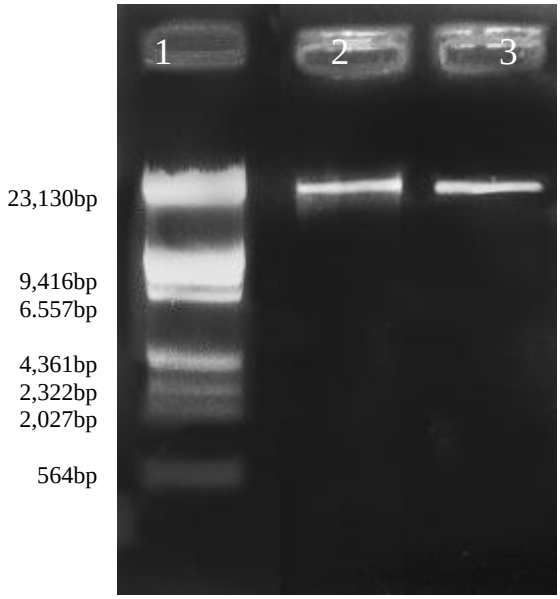


Fig 6.15. Agarose gel (1%) electrophoresis of phage DNA**.** Lane 1: Lambda DNA / *Hind* III Digest, Lane 2: ΦBAP-1 DNA and Lane 3: ΦPAP-1DNA.

6.3.4.9.2.Restriction analysis

The restriction pattern of the phage DNA revealed the susceptibility of phage genome to the restriction endonuclease *Bam* HI. The result is as shown in Fig. 6.16.

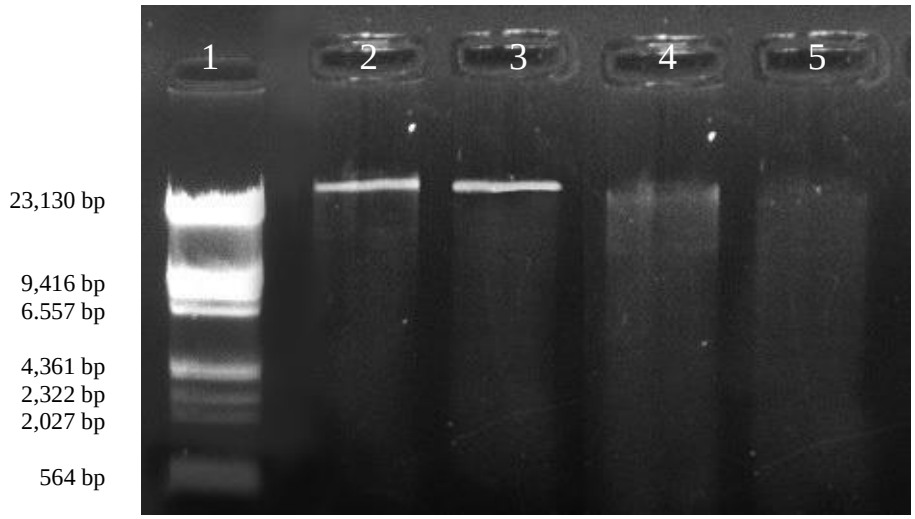


Fig.6.16. Restriction analysis of phage DNA. Lane 1: Lambda DNA/*Hin*d III Digest, Lane 2: uncut ΦBAP-1 DNA ; Lane 3:uncut ΦPAP-1 DNA, Lane 4: *Bam*HI digest of ΦBAP-1DNA and Lane 5:.*Bam*HI digest of ΦPAP-1DNA.

The nature of the genome of both the phages ΦBAP-1 and ΦPAP-1, was identified as double stranded DNA on the basis of its sensitivity to digestion by restriction endonuclease. The double stranded nature of the phage DNA places Φ BAP-1 under the order *Caudovirales* of Siphoviridae family while ΦPAP-1 under the family *Tectiviridae* whose order is still unassigned since they have different characteristics from the orders *Caudovirales* and *Ligamenvirales.*

6.4 Discussion

In the present study, two lytic bacteriophages, namely ΦBAP-1 and ΦPAP-1, were isolated from meat and milk samples respectively. It is very important to note that their respective hosts, i.e, *Bacillus altitudinis (*BTMW1*)* and *Pseudomonas aeruginosa* (BTRY1) were also isolated from the meat and milk samples respectively. This is supported by the fact that bacteriophages being natural viral pathogens of bacteria co-exist with their hosts, sharing the same ecological niches (Goyal, 1987; Heilmann *et al.*, 2010).

Phages were isolated employing double agar overlay method of Adams, (1959).The two phages ΦBAP-1 and ΦPAP-1 consistently produced large, clear and round plaques with well-defined edges. Since concentration and purification of virus particles are prerequisites for structural and functional characterization of phages (Boulanger, 2009), ΦBAP-1 and ΦPAP-1 were purified and concentrated before further characterization. Concentration was done employing PEG-NaCl precipitation method was as described by Sambrook *et al.*, 2000. The efficiency of this method is almost independent of phage concentration and is therefore useful in order to concentrate even phage lysates having very low titer (Yamamoto and Alberts, 1970). This mild, but fast procedure allows a 100-fold phage concentration, even after low speed centrifugation with negligible loss of infectivity (Boulanger, 2009).

The morphological features of bacteriophages greatly aid in their classification (Ackermann, 2009). Therefore, transmission electron microscopy was employed to aid in this morphological analysis.

The micrograph of ΦBAP-1 exhibited morphological traits, typical of family *Siphoviridae* according to ICTV (Mc Grath and van Sinderen, 2007). The *Caudovirales* are an order of viruses also known as the tailed bacteriophages. The *Caudovirales* are group I viruses as they have double stranded DNA (dsDNA) genomes, which can be anywhere from 18,000 base pairs to 500,000 base pairs in length.The virus particles have a distinct shape; where each virion has an icosohedral head that contains the viral genome, and is attached to a flexible tail by a connector protein (Mc Grath and van Sinderen, 2007). Out of the three families of this order, the *Siphoviridae* have long noncontractile tails (Maniloff and Ackermann, 1998). and constitute the majority of the known tailed viruses(Ackermann, 2003).They are ds DNA viruses around 50kb in length (Brüssow and Desiere, 2001)and currently 313 species in this family, divided among 47 genera (Niu *et al.*, 2014). The characteristic structural features of this family are a nonenveloped head and noncontractile tail (Maniloff and Ackermann, 1998). ΦBAP-1 had a hexagonal head 80.40 ± 1.1 nm in diameter with a 168 ± 1.4 nm long tail. In the study on phages used by laboratory of enteric pathogens (Health Protection Agency, London, UK), the reported average head diameter for phages of family *Siphoviridae* is 62.5 nm with 120 nm long tail (De Lappe *et al.*,2009).Other reported phages from family *Siphoviridae* with similar

morphological dimensions as that of ΦBAP-1 are ARл against *Bacillus subtilis* B3, which had icosahedral heads and non-contractile, long tails (Krasowska *et al.*, 2014), *B. cereus* phage Wβ which had an icosahedral head (61 nm×67 nm) and a long tail (204 nm×5.7 nm), Gammaphage (ɣ), Fah, F7, F9 against *B. Anthracis* (Jonczyk-Matysiak *et al.*, 2014).

The micrograph of ΦPAP-1 exhibited morphological traits, typical of family *Tectiviridae* according to ICTV (Mc Grath and van Sinderen, 2007). Tectivirus is a genus of viruses, and is currently the only genus in the family *Tectiviridae* and the name is derived from Latin *tectus* (meaning 'covered').Gram-negative bacteria usually serve as natural hosts. There are currently four species in this genus including the type species *Enterobacteria phage PRD1* (Caldentey *et al.*, 1994; Rydman and Bamford, 2003). Tectiviruses have no head-tail structure, but are capable of producing tail-like tubes. The virions of *Tectiviridae* species are non-enveloped, icosahedral and display a pseudo T=25 symmetry. The capsid has two layers. The outer layer is a protein structure of 240 capsid proteins trimers, and the inner one is a proteinaceous lipid membrane which envelopes the virus genome and the genome is a single molecule of linear double-stranded DNA of 15 kilobases in length (San Martin *et al.*, 2002). ΦPAP-1 had a hexagonal head 264.47 ± 0.91 nm and had no tail. Other reported bacteriophages from family *Tectiviridae* with similar morphological dimensions as that of ΦPAP-1 are PR3, PR4, PR5, P722 and PRD-1 against different *Pseudomonas sp* (Fraenkel-Conrat, 2012; Knezevic, 2011).

For a productive phage- host interaction, several critical factors need to come into play simultaneously. One of the first and vital factor is the careful deduction of the optimal MOI (Adams, 1959). Determination of optimal MOI is important as too many phages attaching to a single bacterial cell can cause cell lysis, even before the infection process can yield progeny (lysis from without). The optimal MOI for ΦBAP-1 was 1 phage/bacterium while that for ΦPAP-1was 5 phages/bacterium.

Phage adsorption to the susceptible host is the second significant factor affecting the booming phage- host interaction. Careful determination of the time taken by the phages to adsorb onto to the host cell is of supreme importance, as it may serve in later experiments for accurate characterization of the phage. While it

took 35 min for 100% adsorption by ΦBAP-1, the same was achieved by ΦPAP-1 in 40 min.

The details of intra cellular kinetics of virus growth have been modelled for several phages (Gaspar *et al*., 1980; Buchholtz and Schneider, 1987; Rabinovitch *et al*., 1999).The one step growth curve to study the growth kinetics of ΦBAP-1 and ΦPAP-1, used log phase host cells at 37°C. These two bacteriophages showed a comparable latent period of 30 min. The rise period of ΦPAP-1 was relatively longer at 60 min when compared to that of ΦBAP-1, where it was of 50 min. The burst size for ΦBAP-1 was calculated to be 56phages per bacterial cell, which was smaller than that for ΦPAP-1, which were 60 phage particles per bacterial cell. The results show similar patterns to the other bacillus phages like SP5, SP6, SP8, SP13- 168 B against different *Bacilli sp.,* where the average latent period and rise period were found to be 35-40 min and the burst size 50 to 110 (Brodetsky *et al.,* 1964; Aposhian, 1965; Lee *et al.,* 2011). Several reports on the lytic bacteriophages against *Pseudomonas sp* also showed the similar latent and rise periods along with the burst size as shown by ΦPAP-1 (Minor and Nordeen, 1996; Ceyssens, 2009; Di Lallo *et al.*, 2014).

A variety of environmental properties such as temperature and the chemical makeup of the phage-host ecology have a considerable influence (Schlesinger, 1932; Stent, 1963) not only on the phage viability, but also most importantly on phage adsorption, a very essential step in phage infection (Capra *et al.,* 2006). Best possible host and growth conditions must be carefully studied and selected for the production of each bacteriophage candidate for application as biocontrol agents (Sillankorva *et al*., 2010).

In the present work, the influence of both physical and chemical parameters on phage viability/propagation and phage adsorption was studied. The parameters studied include temperature, pH, salinity, presence of calcium ions and sugars.
The effect of each factor and the knowledge about the phage growth dynamics in varying ecological conditions can be exploited during their future intended use as a therapeutic agent. Furthermore it will also help in optimization of the large scale phage dissemination process in the laboratory conditions (Augustine *et al.,* 2013a).Capsular polysaccharides of most of the bacteria directly involve in phage host interaction (Deveau *et al*., 2002) and hence the influence of various sugars on ΦBAP-1 and ΦPAP-1 viability was studied. The optimum temperature for the

phage viability was found to be 50°C in case of both the phages. The optimum pH was 7 in case of ΦBAP-1 and 8 in case of ΦPAP-1. The optimum saline condition was 0.1 M NaCl for both while the sugars mannitol and mannose were favourable for the increased viability of phages. It can be subjective that these sugars and/or their analogues may have a key role as phage receptors on the host surface outer membrane, as their presence in the host phage medium during the adsorption stage effectively inhibited the process (Augustine *et al.*, 2013b). Rhamnose was reported to be a determinant of a phage receptor in *Lactobacillus casei* (Monteville *et al.*, 1994). Bacterial phage inactivation by free sugars like D-glucosamine, D-mannose and L-rhamnose has been previously demonstrated (Patel and Roa, 1983). The possibility of phage receptors in lipopolysaccharide containing L-rhamnose, D-glucosamine, and (or) D-glucose, or a structurally related molecule has also been suggested (Castillo and Bartell, 1974)**.**

The optimum temperature, pH, NaCl and $CaCl_2$ concentrations for the phage adsorption was 37°C, 9, 0.5 M and 10 mM for both the phages. Temperature dependant phage resistance has been reported earlier (Kim and Kathariou, 2009). The ionic environments in which the phage and host interact have a thoughtful influence on the irreversible phage adsorption on host surface (Adams, 1959). As early as in 1923, it was reported that bacteria grown in salt-free Witte's peptone resisted lysis by phage, but that the addition of sodium chloride or calcium chloride resulted in phage infection followed by bacterial cell lysis (da Costa, 1923). The presence of electrolytes in the phage- bacteria growth medium has an insightful effect on the adsorption of phage to host cell. It was demonstrated by Lisbonne and Carrere (1923) that phage and bacteria mixed in salt-free peptone could readily be separated by centrifugation, but in the presence of salts, the phage speedily became attached to the bacteria and were therefore not separated by centrifugation. The calcium necessity for successful phage-host interaction varies from phage to phage (Brodetsky and Romig, 1965). As phages usually require higher concentration of divalent cations like calcium, effect of varying concentration of $CaCl_2$ on phage adsorption was studied. Numerous studies have proved the affirmative influence of calcium on phage- host interaction (Shafia and Thompson, 1964; Watanabe and Takesue, 1972).

Under optimised conditions, the latent period of ΦBAP-1 decreased from 30 to 20 min, rise period increased from 30 to 100 min, while burst size increased

from 56 to 71 phages/bacterium. In the case of ΦPAP-1, latent period decreased from 30 to 20 min, generation period increased from 60 to 100 min and burst size increased from 60 to 73 phages/bacterium. Both burst size and the phage generation time are controlled by the phage latent period (Abedon *et al*., 2001; Augustine *et al.*, 2013(b) and in the present study , even though the latent period showed a dip, an overall increase in phage generation time was observed under optimized conditions, which ultimately resulted in an increase in burst size.

The physiological state of the host is an important factor for fruitful phage host interaction (Chibani-Chennoufi *et al.*, 2004; Capra *et al.*, 2006). The capability of *Bacillus sp and Pseudomonas sp.* to survive under diverse stress environments (Foster and Spector, 1995) makes it a difficult target for phages. The standard protocol followed for isolation of phages involved using host in exponential growth phase (Adams, 1959), and for this reason the isolated phages were capable of propagating only in fast growing host cells. Bacteria can be maintained in the log-phase only when there is no nutrient limitation, which can be achieved only under laboratory conditions (Robb and Hill, 2000). In natural environments though, bacteria exist as "long-term stationary-phase cultures" where a set of stress response genes and metabolic pathways are essential for survival (Finkel, 2006). These stress conditions are experienced in the laboratory set up when the culture reaches stationary phase (Chibani-Chennoufi *et al.*, 2004). Thus, it can be incidental that phages infecting stationary phase bacteria can well infect bacteria in the natural conditions. The ability to infect host under stationary as well as nutrient deprived conditions confer an added advantage on phages intended for use as a biocontrol agents. There are not many reports on this aspect. The first case of phage infection in stationary phase was reported in α 3, a phage infecting *Achromobacter* (Woods, 1976). Another was regarding a *Pseudomonas* phage that successfully infected host cells that were starved for 5 years (Schrader *et al.*, 1997). Infection of MS2 virus on glucose, sulphur and nitrogen starved cells of *Escherichia coli* resulted in production but no progeny release (Propst-Ricciuti, 1976).According to Augustine *et al.*, 2013(b), of the two *Salmonella* Entertidis phages, ΦSP-3 multiplication was maximum when host was in logarithmic phase (PFU: log 10 9.81± 0.10). Successful phage infection yielded progeny, when phages were added to the bacterial host growing in optimal conditions

Under various nutrient deprived conditions, both ΦBAP-1 and ΦPAP-1 produced excellent results; a not so infrequent situation in the natural environment (Lenski, 1988). Physiological state of the host, characterized by levels and activities of host cellular functions, plays a pivotal role in phage infection and propagation (You *et al*., 2002). The infection and propagation of a phage on a susceptible bacterial host can be modulated with alterations in growth medium under laboratory conditions (Hedén, 1951; Hadas *et al*., 1997). Bacterial cells entering into stationary phase undergo substantial changes in cell morphology, including metabolism and surface characteristics (Kjelleberg *et al*., 1987) that may negatively interfere with phage infection (Sillankorva *et al*., 2004).However, in this study, both phages were able to infect hosts under stationary phase, although in low numbers. Restrictions in nutritional factors are known to limit the phage propagation (Miller and Day, 2008), but the phages under study were able to multiply even under multiple nutrient starved states as evidenced by a significant level of increase in phage titre. Bacteria grown in carbon - starved condition are reported to defy phage infection (Marcin *et al*., 2007), but both phages under study, ΦBAP-1 and ΦPAP-1, were able to infect the host strains (p<0.0001) even under such a nutrient- deprived state. However, ΦBAP-1 against *Bacillus altitudinis* (BTMW1) and ΦPAP-1 against *Pseudomonas aeruginosa* (BTRY1) were able to successfully propagate in their respective hosts (p<0.0001) when compared to stationary phase) even under nitrogen limiting conditions. There are only few reports on phages competent to infect their host under both nutrient- rich and nutrient- deprived conditions (Chibani-Chennoufi *et al.*, 2004; Augustine *et al.*, 2013a). This characteristic of ΦBAP-1 and ΦPAP-1 is a distinctive quality required to be an effective, successful biocontrol agent.

The nature of the phage ΦBAP-1 and ΦPAP-1 genome was identified as double stranded DNA on the basis of its sensitivity to digestion by restriction endonuclease – *Bam* HI. The double stranded nature of their DNA places ΦBAP-1 under the family *Siphoviridae* of order *Caudovirales* and ΦPAP-1 under *Tectiviridae* of unassigned family of bacteriophages.

Thus to conclude, two double stranded DNA non enveloped iscosahedral phages,namely,ΦBAP-1 against *Bacillus altitudinis* (BTMW1) of *Siphoviridae* family and ΦPAP-1 against *Pseudomonas aeruginosa* (BTRY1) of *Tectiviridae* family were isolated from meat and fish samples respectively. Their

characterization was by Tetrazolium staining, TEM analysis and influence of different physicochemical parameters on their viability and propagation. Both the phages showed viability even under nutrient deprived states which is advantageous, especially when it comes down to its portended use in the food industry against food borne pathogens.

Chapter 7

Biofilm mitigation using bacteriophages

7.1 Introduction

The emergence of new antibiotic resistant bacterial strains, along with an aversion to chemical preservatives in food by common people, has highlighted the need for adoption of alternative and more natural approaches to mitigate the effect of these "super-bugs." Phages can infect and multiply within their specific hosts even if they are antibiotic resistant. Host specificity is generally observed at a strain level, species level, or, more rarely, at genus level. This specificity had led to the idea of using phages for directed targeting of dangerous bacteria (Hagens and Loessner, 2010). Phages have been currently employed in human and veterinary medicine to control bacterial infections after Felix d'Herelle proved their effectiveness in 1919.

Phage application is a novel approach in the food industry to control bacterial contamination in food, in a process called "biocontrol" (Hagens and Loessner, 2010). As mentioned above, lytic phages have the ability to attach to bacteria and integrate into their cellular machinery, while utilizing the host resources to reproduce. The release of new phages leads to destruction of the bacterial cell. Virulent (strictly lytic) phages are the obvious choice for food safety applications (Mahony *et al.,* 2011).

Over the past two decades, concentrated research efforts have been devoted to phage biology, to enhance our knowledge of these interesting organisms and their possible applications. Foodborne diseases and outbreaks are costly in any country, and recent estimates showed that they almost cost the U.S. economy from about $51.0 to $77.7 billion (Scharff, 2012) and cost Canada about $1.33 billion CAD a year (Snowdon *et al.,* 2002).The application of phages to reduce pathogenic bacteria during the pre and post-harvest stages of food production has shown promise (Strauch *et al.,* 2007). Moreover, the recent FDA approval of phage preparations as food additives for preservation has also triggered the search for new applications for these natural bacterial killers. Phages were considered as promising agents for the suppressing growth of spoilage

bacteria in different beverages and food matrices to extend shelf life of these products.

Biofilm formation is an important problem in the food industry because it represents an important source of contamination for food materials contacting biofilm-containing areas; and therefore cause of food spoilage or transmission of diseases (Bonaventura *et al.,* 2008). Once formed, biofilm allows pathogens to persist in the food environment for prolonged periods and to resist treatment with antimicrobial and sanitizing agents (Folsom and Frank, 2006). Several studies have described the use of phages for surface decontamination and to control formation of biofilms by various pathogens. The effectiveness of various phages to remove *Listeria* from stainless steel and polypropylene surfaces has been investigated (Roy *et al.,* 1993). A combination of phage and quaternary ammonium salts resulted in great reduction in levels of *Listeria* attached to surfaces. Hibma and coworkers (1997) isolated a phage specific for L-forms of *Listeria*, in which the cell wall structure is either deficient or absent, and used this phage to control biofilm formation. The phage was as successful as lactic acid at inactivating preformed L-form biofilms on stainless steel. In a more recent study, *Listeria* phage P100 could to control biofilm formation of *L. monocytogenes* on stainless steel surfaces (Montanez *et al.,* 2012).

There are many promising studies on the use of phages to control biofilm formation by bacteria such as *Pseudomonas sp.* (Knezevic *et al.,* 2011; Pires *et al.,* 2011), *Campylobacter jejuni* (Siringan *et al.,* 2011), and *Staphylococcus epidermidis* (Curtin and Donlan, 2006). Phage mixture BEC8 was investigated to control enterohemorrhagi*c E. coli* O157:H7 on some food-processing surfaces like stainless steel and ceramic tiles (Viazis *et al.,* 2011). *Enterococcus faecalis* specific lyticphage øSUT1 caused significant reduction in bacterial cell number on hard and porous surfaces contaminated with *enterococci* (McLean *et al.,* 2011).

Lysins are enzymes produced by lytic phages, which play a role in degradation of the bacterial cell wall by targeting various peptidoglycan bonds, allowing the newly formed progeny phages to release from the host cell (Borysowski *et al.,* 2006). As lysins attack the cell wall peptidoglycan, they are highly effective against Gram-positive bacteria when added externally and may be used as biocontrol agents to enhance food safety (Fischetti, 2008). Thus lysins could be added as a purified protein directly to food or feed, or via lysine secreting

recombinant bacteria (Borysowski *et al.,* 2006). The absence of bacterial resistance against lysin is considered as a major advantage of using phage lysins (Fischetti, 2010), as the bacterial cell would have to modify the structure of its cell wall to avoid enzymatic action. The production of lysin is expensive, moreover, they are relatively unstable, large proteins that are prone to proteolysis and lose activity in some foods (Coffey *et al.,* 2010). In brief, it can be concluded that phages and their lysin enzymes may be applied along the farm-to-fork continuum to enhance food safety.

The present chapter deals with the host range studies, extraction of phage proteins and antibiofilm activity of the two phages, ΦBAP-1 and ΦPAP-1 as well by the phage proteins.

7.2 Materials and methods

7.2.1. Host Range Studies

The host range of the phages ΦBAP-1 and ΦPAP-1 was assessed on the basis of their ability to form plaques on respective test strains. Both phages were tested against twenty strong biofilm producers used in the study. Host range studies was also conducted using NCIM cultures including *Pseudomonas aeruginosa (2863), Salmonella* Typhimurium*(2501), Escherichia coli (2343), Klebsiella pneumoniae (2957), Proteus vulgaris (2027), Clostridium perfringens (2677), Staphylococcus aureus (2127), Bacillus cereus (2155), Bacillus pumilus (2189), Bacillus circulans (2107)* and various isolates from the culture collection of Microbial Genetics laboratory, CUSAT that include *Vibrio diabolicus (TVMS3), Vibrio alginolyticus (KK16), Vibrio harveyi (KKS4), Vibrio parahemolyticus (KK10), Salmonella* Enteritidis *(S37) and Salmonella* Enteritidis *(S49).*The phage lysate was added to the cultures in their exponential phase, incubated for 1 hour and plated using the double agar overlay method. All platings was performed in triplicates. The plates were incubated at 37°C and were observed for plaques.

7.2.2. Anti biofilm activity of ΦBAP-1 and ΦPAP-1.

Both phages, ΦBAP-1 and ΦPAP-1, singly and in combination were tested for their antibiofilm activity against nine strong biofilm producing food pathogens as discussed in 5.2.1. 10 µL of the phage (10^{10} PFU/mL) was added in respective wells; while in the case of combination treatment, 5 µL of each phage was added. The assay was repeated in triplicate and statistically analysed. Statistical evaluation was by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) computer program. If there are more positive as negative changes, then $p > 0.5$; it means the test is significant.

7.2.3 Phage structural protein analysis

7.2.3.1 Non Reductive Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) of purified phage proteins was performed under non reducing conditions for evaluating the nature of the phage capsid protein using vertical slab electrophoresis (Genei, Bangalore, India) by the method of Laemmli as adopted by Sambrook *et al*.(2000). The protein marker from New England Biolabs, UK was used as standard and molecular weight was determined using Image J software (Image J 1.49v/ Java 1.6.0_24, 64-bit).

The gel plates were cleaned and assembled. Resolving gel (16 %) of 5 mL was prepared by mixing 2.65 mL of acrylamide: bis-acrylamide (30:0.8), 1.25 mL of resolving gel buffer stock, 100 µL of 10% SDS and 1.05 mL of water followed by 100µL of ammonium persulfate solution (10%) and TEMED (10 µL) (Appendix -4). The mixture was immediately poured into the cast and a layer of water was added over the gel and allowed to polymerize for at least one hour. Water layer was poured out after polymerization. The stacking gel (4 %) of 2.5 mL was prepared by combining 0.425 mL of 30:0.8 acrylamide: bis-acrylamide solution, 0.625 mL of stacking gel buffer stock, 25 µL 10% SDS and 1.425 mL of distilled water, followed by 25 µL of ammonium persulfate and 2.5 µL of TEMED (Appendix 4). The stacking gel was then poured into the gel assembly, above the resolving gel and the comb was immediately inserted. Gel was allowed to polymerize for 30 min, placed in the electrophoresis apparatus and upper and

lower reservoirs filled with reservoir buffer (Appendix-4) and was pre run for 1 hour at 80V.

7.2.3.2 Sample Preparation

Phage sample was prepared by mixing 15 µL of 1X sample buffer for non-reductive SDS-PAGE (Appendix-4) with concentrated phage lysate. This sample and 5µL low molecular weight marker mix was loaded on to the gel and run at 80V. The current was increased to 100V, when the dye front entered the resolving gel. The run was stopped when the dye front reached 1 cm from the lower end of the plate, the gel was removed and stained.

7.2.3.3. Silver staining

The gel was fixed for 30 min in fixing solution 1, followed by incubation in fixing solution 2 for 15 min. This gel was washed 5 times in water for duration of 5 min each**.** Sensitized the gel in freshly prepared sensitizer for 1 minute and washed in water twice for 2 min each. The gel was then incubated in staining solution for 25 min at 4°C, washed twice for duration of 1 min each and then incubated in developing solution until the bands appear. To prevent over staining, the gel was treated for 10 min in sodium EDTA (Appendix-4), washed in water twice for duration of 2 min each. The image of gel was captured using gel documentation system (BIORAD, USA).

7.2.3.4. Anti biofilm activity of proteins extracted from ΦBAP-1 and ΦPAP-1

The anti biofilm activity of proteins (100 µg/mL each) extracted from ΦBAP-1 and ΦPAP-1 was tested as 5.2.1. The microtiter assay was repeated thrice and statistically analysed.Statistical evaluations were done by ANOVA, followed by Sign Test using StatsDirect statistical software (version 3.0, Cheshire, UK) computer program.

7.3 Results

7.3.1. Host Range Studies

The host range of the phages ΦBAP-1 and ΦPAP-1 was assessed on the basis of their ability to form plaques on different strains. The results are detailed in the table below (Table 7.1).

Table 7.1 Host range studies of ΦBAP-1 and ΦPAP-1

Sl no:	Strain name/ NCIM isolate number	Identity	ΦBAP - 1	ΦPAP - 1
1	BTMW1	*Bacillus altitudinis*	✓	x
2	BTMY2	*Bacillus pumilus*	x	x
3	BTMG1	*Bacillus altitudinis*	✓	x
4	BTMW2	*Bacillus pumilus*	x	x
5	BTCW2	*Bacillus altitudinis*	✓	x
6	BTMW3	*Bacillus altitudinis*	✓	x
7	BTMY4	*Bacillus pumilus*	x	x
8	BTRY1	*Pseudomonas aeruginosa*	x	✓
9	BTPW1	*Bacillus altitudinis*	✓	x
10	BTCP1	*Bacillus pumilus*	x	x
11	BTTP1	*Bacillus altitudinis*	✓	x
12	BTDF1	*Brevibacterium casei*	x	x
13	BTDF2	*Staphylococcus warneri*	x	x
14	BTDF3	*Micrococcus luteus*	x	x
15	BTDP2	*Micrococcus sp*	x	x
16	BTDP3	*Bacillus niacini*	x	x
17	BTSD1	*Bacillus sp*	✓	x
18	BTSD2	*Bacillus licheniformis*	x	x
19	BTFF1	*Micrococcus luteus*	x	x
20	BTFF2	*Geobacillus stearothermophilus*	x	x
21	2863	*Pseudomonas aeruginosa*	x	✓
22	2501	*Salmonella Typhimurium*	x	x

23	2343	*Escherichia coli*	x	x
24	2957	*Klebsiella pneumoniae*	x	x
25	2027	*Proteus vulgaris*	x	x
26	2677	*Clostridium perfringens*	x	x
27	2127	*Staphylococcus aureus*	x	x
28	2155	*Bacillus cereus*	x	x
29	2189	*Bacillus pumilus*	x	x
30	2107	*Bacillus circulans*	x	x
31	TVMS3	*Vibrio diabolicus*	x	x
32	KK16	*Vibrio alginolyticus*	x	x
33	KKS4	*Vibrio harveyi*	x	x
34	KK10	*Vibrio parahemolyticus*	x	x
35	S37	*Salmonella* Enteritidis	x	x
36	S49	*Salmonella* Enteritidis	x	x

***✓ - plaque formation; x – no plaque formation**

Both phages were found to be strain specific. ΦBAP-1 formed plaques on lawn of the strains *Bacillus altitudinis* (BTMW1, BTMG1, BTCW2, BTMW2, BTPW1 and BTTP1) and *Bacillus sp* (BTSD1). ΦPAP-1 formed plaques on lawns of the strains *Pseudomonas aeruginosa* (BTRY1) and 2863 only. They did not infect the other organisms used in the study.

7.3.2. Anti biofilm activity of ΦBAP-1 and ΦPAP-1

Even though both phages were strain specific, the biofilm formed by the nine strong biofilm forming strains were considerably reduced ($p>0.5$) with the treatment of ΦBAP-1 andΦPAP-1 individually, as well as in combination to a greater extent.

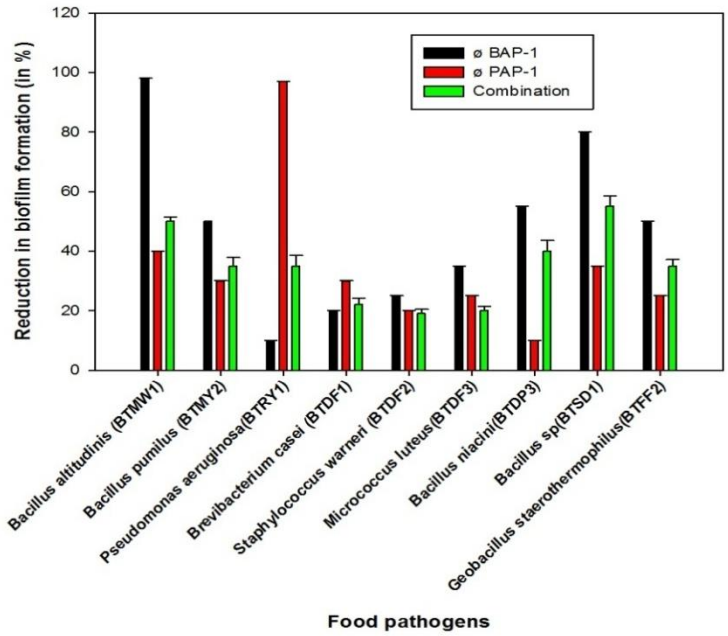


Fig.7.1. The percentage reduction in biofilm formation by test pathogens on treatment with whole phages ΦBAP-1 and ΦPAP-1

From figure 7.1, biofilm formation of all the nine strains was inhibited irrespective of the host specificity of both phages. ΦBAP-1 was able to inhibit biofilm formation of *Bacillus altitudinis* (BTMW1) by 98%, followed by that of *Bacillus* sp (BTSD1) by 80%. 55% reduction was observed in biofilm by *Bacillus niacini* (BTDP3). 50% in that by *Bacillus pumilus* (BTMY2) and *Geobacillus staerothermophilus* (BTFF2) on treatment with ΦBAP-1. The biofilm formation of *Micrococcus luteus* (BTDF3), *Staphylococcus warneri* (BTDF2), *Brevibacterium casei* (BTDF1) *and Pseudomonas aeruginosa* (BTRY1) was reduced by 35%, 25%, 20% and 10% respectively in the presence of ΦBAP-1.

ΦPAP-1 was able to inhibit biofilm formation of *P.aeruginosa* (BTRY1) by 97 %; 40% reduction in biofilm by *B. altitudinis* (BTMW1), 35% of *Bacillus sp* (BTSD1) while 30% reduction in biofilm formation was observed by *B. pumilus* (BTMY2*)* and *B. casei* (BTDF1). The biofilm formation of *M. luteus* (BTDF3) *and G. staerothermophilus* (BTFF2) was also inhibited by 25%.

On addition of ΦPAP-1, there was 25% biofilm inhibition in *S.warneri* (BTDF2) and 10% reduction *B.niacini* (BTDP3).

The effect of the combination of both ΦBAP-1 and ΦPAP-1, on biofilm reduction was intermediate in all nine test pathogens. 55% reduction was observed

in case of biofilm of *Bacillus sp* (BTSD1), and 50% in *B.altitudinis* (BTMW1); 35% reduction in biofilm formation of *B. pumilus (BTMY2), P.aeruginosa* (BTRY1) *and G.staerothermophilus* (BTFF2).40% reduction is seen in case of *B.niacini* (BTDP3) followed by 22 % in *B.casei* (BTDF1) and 20% and 19% in case of *M.luteus* (BTDF3) and *S.warneri* (BTDF2) respectively. The combination could not affect 100% inhibition of biofilm by the test organism.

7.3.3 Phage structural protein analysis

To compare the structural protein of the phages, the protein profile of ΦBAP-1 and ΦPAP-1 were analysed by SDS-PAGE under non reducing conditions (Fig 7.2). Molecular weights of the proteins were compared using the protein marker from New England Biolabs, UK and molecular weights were determined using Image J software (Image J 1.49v/ Java 1.6.0_24, 64-bit).

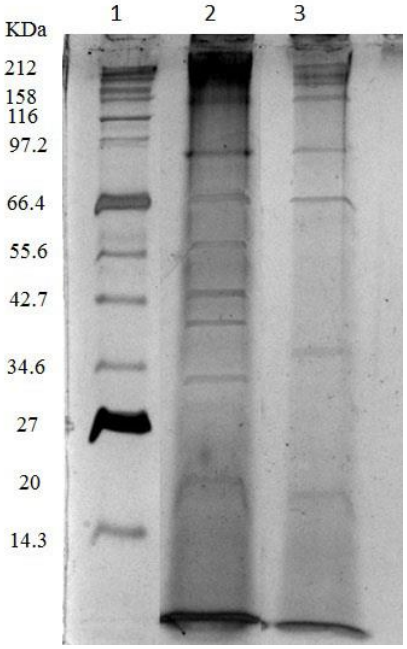


Fig.7.2. SDS PAGE of phage proteins of ΦBAP-1 and ΦPAP-1 under non reducing conditions lane 1: marker; lane 2: ΦBAP-1 and lane 3: ΦPAP-1.

Under non reducing conditions, ΦBAP-1 profile showed a total of eight clear bands on the gel, with four prominent bands, whose molecular size was calculated to be 158000 Da, 91372 Da, 68064 Da, 57593 Da, 44341 Da, 39936 Da, 32994 Da and 18298 Da (Fig 7.2, Lane 2)

For ΦPAP-1 under non-reducing conditions, seven protein bands were observed, of which the bands with molecular weights around 212000 Da, 191750 Da, 158000 Da, 91372 Da, 68064 Da, 36410 Da and 18298 Da, were prominently visualized on the gel (Fig 7.2, Lane 3).

7.3.4. Anti biofilm activity of proteins extracted from ΦBAP-1 and ΦPAP-1

The proteins extracted from the phages ΦBAP-1 and ΦPAP-1 were also tested for their antibiofilm capability.

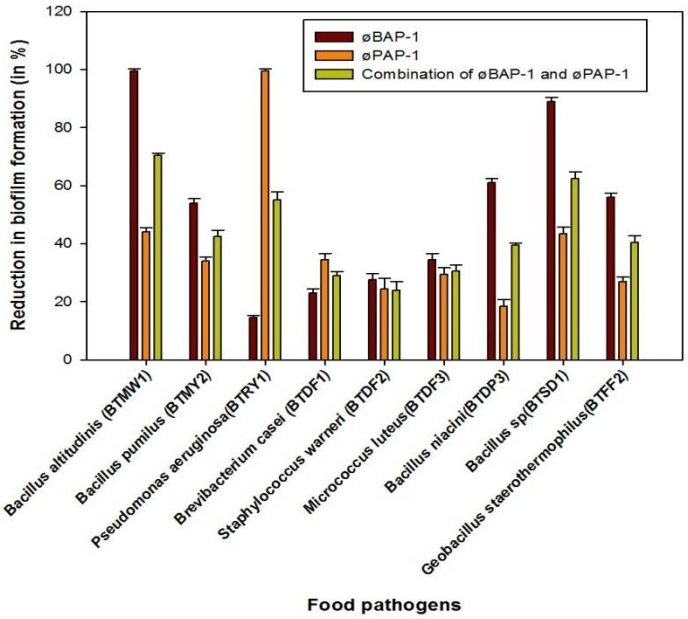


Fig.7.3. The percentage of reduction in biofilm formation by test pathogens on treatment with extracted proteinsΦBAP-1 and ΦPAP-1

The reduction in biofilm formation was enhanced to 99%, 89%, 61%, 56%, 54%, 34%, 27%, 23% and 14% in case of *B.altitudinis* (BTMW1), *Bacillus sp* (BTSD1), *B.niacini* (BTDP3), *G.staerothermophilus* (BTFF2), *B. pumilus* (BTMY2), *M. luteus* (BTDF2), *S. warneri* (BTDF2), *B. casei* (BTDF1) and *P. aeruginosa* (BTRY1) respectively on addition of ΦBAP-1proteins alone.

The reduction in biofilm formation was increased to 99%, 44%, 43%, 34%, 34%, 29%, 27%, 24% and 18% in case of *P.aeruginosa (BTRY1), B.altitudinis* (BTMW1), *Bacillus sp* (BTSD1), *B.pumilus* (BTMY2),*B. casei*

(BTDF1), *M.luteus* (BTDF2), *G.staerothermophilus* (BTFF2), *S.warneri* (BTDF2) and *B.niacini* (BTDP3) respectively on addition of ΦPAP-1 proteins alone.

On treatment with combination of proteins from both phages, the reduction in biofilm formation observed was only 70%, 62%, 55%, 42 %, 40%, 39%, 30%, 29% and 24% in case of *B. altitudinis* (BTMW1), *Bacillus sp* (BTSD1), *P. aeruginosa* (BTRY1), *B. pumilus* (BTMY2), *G. staerothermophilus* (BTFF2), *B. niacini* (BTDP3), *M. luteus* (BTDF2), *B. casei* (BTDF1) and *S. warneri* (BTDF2) respectively. The results were not enhanced further due to the combination.

The biofilm formation by the test strains was controlled ($p>0.5$) to a greater extent by the mixture of phage structural proteins from ΦBAP-1 and ΦPAP-1, as compared to the addition of whole phages.

7.4. Discussion

The control strategies adopted, including preventive measures and chemical treatments, have not proved resolute in the eradication of the biofilm formation.There is a growing interest in the use of bacteriophages for the prevention and treatment of food borne bacterial infectious diseases, mainly due to the emergence MAR bacteria (Matsuzaki *et al.,* 2005; Sulakvelidze *et al.,* 2001).

The lack of efficient bactericides pushed phage therapy as one of many promising approaches for the control of the biofilm producing bacteria (Merril *et al.,* 2003).The ability of the phages to eradicate biofilms has been demonstrated for biofilms of various pathogens including *P. aeruginosa, B. cereus, K. pneumoniae, E. coli, P. mirabilis* and *S. Epidermidis* (Doolittle *et al.,* 1995; Kudva *et al.,* 1999). Infection by phages is exceptionally conditional on their chemical composition and environmental factors, such as growth stage, media, temperature and phage concentration (Sillankorva *et al.,* 2004 b; Chaignon *et al.,* 2007). The main mechanism of action of phages on biofilm formation is the degradation of extra cellular polymeric substances or EPS. EPS probably may act as primary bacterial receptor for phage infection (Cornelissen *et al.,* 2011).

Application of bacteriophages in inhibiting mixed biofilms of *Pseudomonas flourescens* and *Staphylcoccus lentus* has also been reported (Sillankorva *et al.,* 2011). In that study, the biofilms were challenged with phage ΦBB-PF7A, specific for *P. flourescens,* and showed that the phage readily reached

the target host and caused a major population decrease. This phage was also capable of causing partial damage to the biofilms leading to the release of the non-susceptible host (*S. lentus*) from the dual species biofilms. In the present study, both phages, ΦBAP-1 and ΦPAP-1, irrespective of their hosts and narrow host range, were able to control the biofilm formation by different food pathogens, making them promising tools in the eradication of food industry biofilms.

Very recently, phages and phage-encoded proteins have been proposed as natural food preservatives and antimicrobial agents to battle bacterial infections in humans, animals, or crops of agricultural importance (Drulis-Kawa *et al.,* 2012; Glonti *et al.,* 2010). In our study, the phage proteins were precipitated using acetone and the crude protein extract was assayed for its antibiofilm activity. The study uncovered that the proteins alone could repress the bacterial adhesion to the polystyrene microtiter plates which highlights the importance of phage proteins in the antibiofilm action of phages.

The antibiofilm activity of phage endolysins, is available which includes reports on Φ11endolysin (Sass and Bierbaum, 2007) and lysostaphin (Kokai-Kun *et al.,* 2009) against staphylococcal biofilms. One regulatory difference between phages and lysins is that phages are natural while the endolysins are mostly purified from a recombinant expression system, thereby increasing the hurdles in the approval process for use in food safety. At present, there are no approved enzybiotics (endolysins) for use in foods for human consumption. The specific use of peptidoglycan hydrolases have also been reviewed recently but the safety of its use in foods has not yet been proved (Callewaert *et al.,* 2011; Garcia *et al.,* 2010). Recently, potential of bacteriophage derived peptidases, $CHAP_K$, for the fast disruption of biofilms was reported against staphylococci where the purified protein completely eliminated *S.aureus* DPC5246 biofilms within four h. Furthermore,there was rapid degradation of *S. Pyogenes* biofilms by PlyC, a bacteriophage- encoded endolysin (Shen *et al.,* 2013). The role of engineered phages and coded proteins are also on their way to succeed in their aim to inhibit mixed biofilms (Pei and Samanamud, 2014).

Progression into advanced trials is now under way and the combination of antibiotic resistance, clinical need and the availability of suitable bacteriophage combines to make this a potential ‘perfect storm’ with which to advance in this new (but also very old) therapeutic approach. Phage therapy is very effective in killing

drug resistant strains because of its specificity towards particular bacterial populations. Formation of a protected biofilm environment is one of the major causes of the increasing antibiotic resistance development. These certainties stress the need to create alternative antibacterial strategies, like phage therapy (Cornelissen *et al.,* 2011).

This study revealed a set of eight proteins derived from, ΦBAP-1 and seven from ΦPAP-1. The phage proteins were not particularly specific in inhibiting biofilms of their phage hosts; rather they were capable in restraining biofilms of different pathogens. Four proteins with similar molecular weights were observed in both phages. However these proteins were not further characterised as part of this study. In the future, detailed study on these proteins would throw more light on their amino acid sequence, their structure, function and their role in biofilm mitigation.

CHAPTER 8

SUMMARY AND CONCLUSION

In food industries, biofilms are a source of recalcitrant contaminations causing food spoilage, and possible cause of public health problems like outbreaks. Biofilms are difficult to eradicate due to their resistant phenotype, but mechanisms by which bacteria in biofilms attain resistance are still unknown. Moreover, this resistance of biofilm-embedded bacteria to antimicrobial agents makes it necessary to search for agents to effectively kill them. Novel strategies are therefore required to deal with biofilm-mediated food borne toxicities and infections.

Several food types like beef, cheese, raw milk, pasteurized milk, curd, chilly powder, turmeric powder, coriander powder, soft drink, fresh fish, dried fish and dried prawn from the local stores and markets in Kochi, Kerala were screened for food borne bacterial pathogens; this yielded thirty six isolates, of which 20 (55%) were strong biofilm producers. The strength of the biofilm formation was assessed qualitatively by Congo red assay and quantitatively by microtiter plate assay.

Molecular identification of the twenty biofilm producers using 16S ribotyping identified the isolates as *Bacillus altitudinis* (BTMW1, BTMG1, BTCW2, BTMW3, BTPW1, BTTP1), *Bacillus pumilus* (BTMY2, BTMY4, BTMW2, BTCP1), *Pseudomonas aeruginosa* (BTRY1), *Brevibacterium casei* (BTDF1), *Staphylococcus warneri* (BTDF2), *Micrococcus luteus* (BTDF3, BTFF1), *Micrococcus sp* (BTDP2), *Bacillus niacini* (BTDP3), *Bacillus sp* (BTSD1), *Bacillus licheniformis* (BTSD2), *and Geobacillus staerothermophilus* (BTFF2). The nucleotide sequences were submitted to GenBank and accession numbers obtained.

Phylogenetic analysis of the biofilm strains using MEGA 6 software helped to understand their interrelatedness. The antibiogram was elucidated for all 20 strong biofilm producers. Nine of the strongest biofilm producers were selected based on their biofilm strength and MAR profile for further studies. Exoenzyme profiling showed the ability of most isolates to produce more than two enzymes,characteristic feature that indicates their capability in not only forming biofilms, but also in reducing the nutritional value of the food.

Two bioactive compounds, namely pyocyanin and rhamnolipids, were isolated and characterized from *Pseudomonas aeruginosa* strain *BTRY1*. Qualitative and quantitative analyses of both compounds confirmed their identity. The concentration of pyocyanin obtained was 1.245 ± 0.001414 µg/mL and that of rhamnolipids was 75 ± 0.007171 µg/mL. The colonies on CTAB methylene blue agar with a blue halo as well as oil spreading or the drop collapsing assay allowed facile identification of rhamnolipids. The FTIR and Proton NMR spectra of both compounds showed characteristic peaks which confirmed their identity. The compounds also showed high free radical scavenging activity with no cytotoxic effects.

The antibiofilm activity of four bioactive compounds, namely pyocyanin and rhamnolipids characterized in this study and melanin and bacteriocin BL8 which were previously characterized, were checked against the nine strong biofilm producers. Individually pyocyanin, rhamnolipids and melanin inhibited biofilm formation of six of the nine isolates, while bacteriocin BL8 inhibited all. Different combinations of the four compounds were tested against the pathogens and a synergistic effect was observed. The most effective combination was when all four compounds were present together, which inhibited biofilm formation in higher percentage.

Extracellular Polymeric Substances (EPS) production, an essential component for biofilm formation was quantified for each biofilm former before and after treatment by the four bio active compounds singly and in combination. The results conclusively proved the antibiofilm activity attributed to the compounds; with greater reduction in dry weight of EPS after treatment with the bioagents compared to the control. Biofilm Inhibitory Concentrations (BIC) of all four bioactive compounds against nine pathogens was in nanogram quantities, thereby proving their higher efficiency as antibiofilm agents.

Microscopic techniques are widely used for studying biofilms. In this study, Scanning Electron Microscopy (SEM) and Confocal Scanning Laser Scanning Microscopy (CLSM) imaging techniques were used to confirm the biocontrol of bacterial biofilms by the bioagents, pyocyanin, rhamnolipids, melanin and bacteriocin BL8. The SEM and CLSM micrographs clearly exhibit the differences in the treated and untreated groups of test pathogens; the reduced

microbial presence in the compound treated glass slide clearly confirmed their biofilm controlling ability.

The quantification of live cells in the CLSM images were by To-pro-3 stain. The reduced intensity due to decrease in live cells numbers on treatment with pyocyanin/rhamnolipids/melanin/bacteriocin was easily visible, which signified the shrinking of biofilm formation. The intensity data of the confocal images analyzed using Image J, produced Red Green Blue (RGB) graphs. The variations in the RGB plots in the treated compared to control to due to the change in the pixel intensity clearly indicated that biocontrol of bacterial biofilms can be attributed to the four bioactive molecules.

Pyocyanin and rhamnolipids at BIC concentrations inhibited biofilm formation of most strains from the culture collection of Microbial Genetics Laboratory, CUSAT, including some potent producers like *Vibrio diabolicus (TVMS3)* and *Salmonella Enteritidis (S49).*

Multispecies biofilms are more likely in nature. Biofilms consisting of multiple microbial species were developed in the polystyrene plates after checking antagonism between the twenty producer species. Biocontrol of multispecies biofilms by bioagents singly and in combination gave promising results, demonstrating their ability to control multispecies biofilms in nature. The four bioactive compounds were assayed for their capability to act as preservatives/additives that help to reduce the bioburden in the common foods in the market. The bioactive compounds thus proved to be capable of preventing food borne intoxications and infections in the food industry.

Phage therapy is gaining importance in countering the menace of antibiotic resistance. Although all the 20 test organisms were used for the isolation of lytic bacteriophages, phages against only two were obtained. ΦBAP-1 obtained from meat against *Bacillus altitudinis* strain BTMW1 and ΦPAP-1 obtained from milk against *Pseudomonas aeruginosa* strain BTRY1 produced large, clear plaques indicating their lytic nature, and were visualized by tetrazolium staining.

ΦBAP-1 and ΦPAP-1 were concentrated employing PEG-NaCl precipitation method before further characterization; and optimization of the factors for phage viability and propagation is summarised below (Table 8.1).

Table 8.1 Comparison of **ΦBAP-1 and ΦPAP-1**

Parameter	**ΦBAP-1**	**ΦPAP-1**
Host	*Bacillus altitudinis (BTMW1)*	*Pseudomonas aeruginosa (BTRY1)*
Structure	Non enveloped, icosahedral head with long contractile tail	Non enveloped, hexagonal head without any tail
Size	Head diameter = 80.40 ± 1.1 nm, Tail length = 168 ± 1.4 nm	Head diameter = 264.47 ± 0.91 nm
Shape	Tailed	Polyhedral
Family	*Siphoviridae*	*Tectiviridae*
Multiplicity of infection (MOI)	1 phage/ bacterium	5 phages/bacterium
Optimum temperature for phage viability	50°C	50°C
Optimum pH for phage viability	7	8
Optimum NaCl concentration for phage viability	0.1 M	0.1 M
Sugar favourable	Mannose	Mannitol
Host range	Narrow	Narrow
100 % phage adsorption	35 min	40 min
Latent period	30 min	30 min
Rise period	50 min	60 min
Burst size	56 phages/bacterium	60 phages/bacterium
Optimum temperature for phage adsorption	37°C	37°C
Optimum pH for phage adsorption	9	9
Optimum NaCl concentration for phage adsorption	0.5 M & 0.75 M	0.5 M
Optimum $CaCl_2$ concentration for phage adsorption	10 mM	10 mM
Latent period under optimized conditions	20 min	20 min

Rise period under optimized conditions	100 min	100 min
Burst size under optimized conditions	71 phages/bacterium	73 phages/bacterium
Propagation in nutrient deprived conditions	Growth under all types of starved conditions compared to stationary phase and thus be used as biocontrol agent.	Growth under all types of starved conditions compared to stationary phase and thus be used as biocontrol agent.
Nature of genome	Double stranded DNA	Double stranded DNA
Anti biofilm activity	Broad	Broad

Even though both phages exhibited narrow host range, their anti biofilm activity was very broad ranged. They were capable of controlling biofilm production of the other eight pathogens in greater percentage along with the complete lysis of their respective specific hosts. This finding was noteworthy in the counter or alternate strategies for biofilm biocontrol. Addition of acetone precipitated phage proteins caused a greater reduction in the biofilm formation by the pathogens when compared to the addition of intact whole phages. This finding necessitates the importance of taking a relook at the use of phage proteins in the food industry as preservatives/additives. The mixture of proteins were analysed by SDS-PAGE and their approximate molecular weights were determined. Further characterization of each protein is required, which will help in understanding not only their structure but also their mechanism of action, in controlling biofilms.

In food industry, biofilm are highly resistant to chemical and physical treatments. Residues of disinfectants are also undesirable, hence it warrants prospecting for safe biofilm inhibitors for use in the food industry. Thus, this work focused on various strategies for biocontrol of bacterial biofilms. The four bioactive compounds and two bacteriophages were proved promising. One of the highlight of the study was that the lytic phage ΦBAP-1 against *Bacillus altitudinis* (BTMW1) is the first reported phage against this host to the best of our knowledge. It was very potent in all aspects of biofilm control, along with another lytic phage ΦPAP-1 against *Pseudomonas aeruginosa* (BTRY1). The phage proteins added a completely new dimension to biofilm biocontrol and is worthy of more research. Since the bioagents used were all of microbial origin, the

production in large scale would be cost effective and eco friendly in nature and are microbiologically safe. These bio agents can be used as additives or as cleansers in the food processing environment. Hurdle technology which includes the combination of all the bioactive compounds in their BIC concentrations is a novel idea to resist food pathogens persisting in the food processing environments.

Future studies can be conducted in the characterization of the four bioactive compounds, their large scale production and elucidation of their other properties, like anti cancer, anti inflammatory, and anti tumor, to name a few. On the other hand, the analysis and complete characterization of these phage proteins in detail as well as of other phages will add to the existing knowledge on the role of phage proteins in biofilm mitigation, including their mechanism of action.

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APPENDIX – 1

NUTRIENT MEDIUM

Ingredients		**g/L**
Peptone	-	5
Sodium chloride	-	5
Beef extract	-	1
Yeast extract	-	2

Suspended 13g (Himedia, Mumbai, India) in 1000mL distilled water. Mixed well, autoclaved at 15 lbs pressure (121^{o}C) for 15 min and cooled to 50-55^{O}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 25 grams (Himedia) in 1000 mL distilled water. Heated to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121^{O}C) for 15 min. 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 (Himedia) in 1000 mL distilled water. Heated to boiling to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure (121^{O}C) for 10 min. Final pH was 7.3 ± 0.2. When used as solid agar medium, 2.0 % agar (w/v) was added to the medium.

MUELLER -HINTON BROTH

Ingredients		**g/L**
Beef infusion	-	300
Casein acid hydrolysate	-	17.5
Starch	-	1.5

Suspended 21 grams (Himedia) in 1000 mL distilled water. Heated to boil, to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure ($121^{O}C$) for 15 min. When used as solid agar medium, 2.0 % agar (w/v) was added to the medium for agar plate preparation. Final pH (at $25^{O}C$) was 7.4 ± 0.2.

MINIMAL MEDIA

5X minimal media – 20 mL
Distilled water – 80 mL
1 M MgSO4 - 0.2 mL
Autoclaved and added
20 % glucose - 2 mL
1 M CaCl2 - 0.01 mL

Minimal media (5X concentrate)

Ingredients	**g/L**
Na2HPO4.7H20	- 6.4
KH2PO4 (dibasic)	- 1.5
NaCl	- 0.25
NH4Cl	- 0.5

Ingredients were dissolved in 80 mL distilled water. Adjusted the volume to 100mL with distilled water and sterilized by autoclaving at 15lbs for 20 min before use.

PHYSIOLOGICAL SALINE

NaCl - 0.85g
Distilled water - 100 mL

Sterilized by autoclaving at 15 lbs pressure ($121^{O}C$) for 15 min, cooled and used

BRAIN HEART INFUSION MEDIUM

Bovine brain and heart tissue powder

Suspended 20 grams of media (HiMedia) in 1000 mL distilled water. Heated to boil to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure ($121^{o}C$) for 15 min.

CONGO RED AGAR

Suspended 37 grams of Brain heart infusion broth (BHI) (HiMedia) and 50 g of Sucrose (HiMedia) in 1000 mL distilled water. 2.0% agar (w/v) was added to the medium for agar plate preparation. Congo red stain (0.8 g/L) was made ready as a strong aqueous solution and sterilized ($121^{o}C$ for 15 min) separate from therest of the medium components and supplemented to the agar when the temperature reached $55^{o}C$.

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^{O}C for 15 min), separate from the rest of the medium components and supplemented to the agar when the temperature reached 55^{O}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 a aqueous solution and sterilized (121^{o}C for 15 min) separate from the rest of the medium components and supplemented to the agar when the temperature reached 55^{o}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^{o}C for 15 min) separate from the rest of the medium components and supplemented to the agar when the temperature reached 55^{o}C.

CARBOXY METHYL CELLULOSE AGAR

Ingredients	**g/L**
Yeast extract	- 0.02
KH_2PO_4	- 0.01
$MgSO_4$	- 0.05

Suspended 5 grams of carboxy methyl cellulose media (Himedia) in 1000 mL distilled water. Heated to boil, to dissolve the ingredients completely. 2.0 % agar (w/v) was added to the medium for agar plate preparation. Sterilized by autoclaving at 15 lbs pressure (121^{O}C) for 10 min.

PSEUDOMONAS ISOLATION HIGH VEG™ BROTH

Suspended 45.03 gram of medium (HiMedia) in 1000 mL distilled water. Heated to boiling to dissolve the ingredients completely. Sterilized by autoclaving at 15 lbs pressure (121^{O}C) for 15 min. When used as solid agar medium, 2.0 % agar (w/v) was added to the medium for agar plate preparation.

KAY'S MINIMAL MEDIUM
Ingredients g/L

$NH_4H_2PO_4$ - 0.03
K_2HPO_4 - 0.02
Glucose - 0.02
$FeSO_4$ - 0.05
$MgSO_4$ - 0.02

Suspended ingredients in 1 liter distilled water, mixed and Sterilized by autoclaving at 15 lbs pressure (121^OC) for 15 min.

PROTEOSE PEPTONE AMMONIUM SALT MEDIUM

Ingredients	**g/L**
NH_4Cl	- 0.02
Proteose Peptone	– 0.01
Glucose	- 0.05
KCl	- 0.02
Tris-HCl	- 0.12
$MgSO_4$	- 0.0016

Suspended ingredients in 1 liter distilled water, mixed and Sterilized by autoclaving at 15 lbs pressure (121^OC) for 15 min.

MINIMAL SALTS MEDIUM

Ingredients	**g/L**
KH_2PO_4	0.7
Na_2HPO_4	0.9
$NaNO_3$	2.0
$MgSO_4 \cdot 7H_2O$	0.4
$CaCl_2 \cdot 2H_2O$	0.1
$FeSO_4 \cdot 7H_2O$	2.0
$MnSO_4 \cdot H_2O$	1.5
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.6

Suspended ingredients in 1 liter distilled water, mixed and Sterilized by autoclaving at 15 lbs pressure (121^oC) for 15 min.

CETYL TRIMETHYL AMMONIUM BROMIDE (CTAB) AGAR

Ingredients	**g/L**
Peptone	1.38
$MgSO_4.7\ H_2O$	4.0
$Na_2HPO4.\ 2H_2O$	1.0
$NaH_2PO4.\ 2H_20$	1.0
$CaCl_2.\ 2H_20$	0.005
Glycerol	25 mL
Agar agar	20

Suspended ingredients in 1 liter distilled water, mixed and Sterilized by autoclaving at 15 lbs pressure (121^{o}C) for 15 min.

APPENDIX – 2

1M Glycine- HCl buffer (pH 2.0)
Solution A: 0.2 M glycine
Solution B: 1M HCl

Combined 25 mL of solution A and 22 mL of solution B and diluted to 100 mL with distilled water.

0.01M 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 was made up to 1L to get 0.01 M buffer.

SM buffer

Ingredients	**g/L**
NaCl	5.8 g
MgSO4.7H2O	2.0 g
1 M Tris HCl (pH 7.5)	50 mL
2% gelatin	5.0 mL

Ingredients were dissolved and was made up to 1 litre with milliQ water and autoclaved at 15lbs for 20 min and stored at 4^{o}C until use.

Phosphate Buffered Saline (PBS) (pH 7)

Ingredients	**g/L**
NaCl	8.0
KCl	0.2
Na2HPO4	1.44
KH2PO4	0.24

Ingredients were dissolved in 800 mL of distilled water, pH adjusted to 7.00 with 1N HCl. The volume was made up to 1 litre with distilled water, autoclaved at 15lbs for 20 min and stored at room temperature until use.

Citrate buffer (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 200 mL 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 mLand then filter sterilized.

Desired pH	Solution A (mL)	Solution B (mL)
3	46.5	3.5
4	33.0	17
5	20.5	29.5
6	9.5	41.5

Phosphate buffer (pH 7)

Solution A: 0.2 M NaH_2PO_4

Solution B: 0.2 M Na_2HPO_4

Mixed 39 mL of solution A with 61 mL of solution B and the volume was made up to 200 mL with distilled water, followed by filter sterilization.

Tris (hydroxymethylamino methane buffer system (pH 8 and 9)

Solution A: 0.2 M Tris buffer

Solution B: 0.2 M HCl

Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 mL and filter sterilized.

Desired pH	Solution A (mL)	Solution B (mL)
8	50	26.8
9	50	5

Carbonate – bicarbonate buffer (pH 10 and 11)
Solution A: 0.2 M Na2CO3
Solution B: 0.2M NaHCO3
Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 mL and filter sterilized.

Desired pH	Solution A (mL)	Solution B (mL)
10	27.5	22.5
10.7	45.0	5

Sodium hydroxide - Potassium chloride buffer (pH 12 and 13)

Solution A: 0.2 M KCl
Solution B: 0.2M NaOH
Referring to the table below for desired pH, mixed the indicated volumes of solutions A and B, then diluted with distilled water to 200 mL and then filter sterilized.

Desired pH	Solution A (mL)	Solution B (mL)
12	50	12
13	50	132

APPENDIX -3

TE buffer
1M Tris-Cl - 10mL
500mM EDTA (pH 8.0) - 2mL

1M Tris-HCl
Tris base - 60.57 g
Deionised water - 500mL
Adjusted to desired pH using concentrated HCl

0.5M EDTA
EDTA - 18.6 g
Deionised water - 100mL

50X TAE Buffer
Tris base - 121 g
Glacial acetic acid - 28.6 mL
0.5M EDTA pH 8.0 - 50 mL
Deionised water added to make volume to 500 mL.
1X TAE Buffer

50X TAE buffer – 10 mL
Deionised water – 490 mL

10X TBE
Tris base - 108g
Boric acid - 55g
0.5M EDTA (pH 8.0) - 20mL
Deionised water added to make volume to 1000mL.

0.5X TBE Buffer
10X TBE buffer – 50 mL
Deionised water - 1000mL

3M Sodium acetate (pH 5.2, 7.0)
Sodium acetate.3H2O - 408.3 g
Distilled water - 800 mL
pH adjusted to 5.2 with glacial acetic acid. Dilute acetic acid was used to adjust the pH to 7.0. Adjusted the volume to 1 liter with distilled water. Sterilized by autoclaving.

APPENDIX -4

Polyacrylamide Gel Electrophoresis

Stock acrylamide solution (30:0.8:1)
Acrylamide (30%) - 60.0 g
Bis-acrylamide (0.8%) - 1.6 g
Distilled water - 200.0 mL
Filtered through Whatman No. 1 filter paper and stored in amber colored bottle at 4^{o}C.

Stacking gel buffer stock (0.5M Tris-HCl, pH 6.8)
Tris buffer - 6 g in 40 mL distilled water
Titrated to pH 6.8 with 1M HCl (~ 48 mL) and made up to 100 mLwith distilled water. Filtered through Whatman filter paper No. 1 and stored at 4^{O}C.

Resolving gel buffer stock (3M Tris-HCl, pH 8.8)
Tris buffer - 36.3 g
Titrated to pH 8.8 with 1M HCl (~ 48 mL) and made up to 100 mL with distilled water. Filtered through Whatman No. 1 filter paper and stored at 4^{o}C.

Reservoir buffer for SDS-PAGE (pH 8.3)
Tris buffer - 3.0 g
Glycine - 14.4 g
SDS - 1.0 g
Dissolved and made up to 1L with distilled water. Prepared as 10X concentration and stored at 4°C.

Sample buffer for Non-reductive SDS-PAGE (2X)
Tris-HCl (pH 6.8) - 0.0625 M
Glycerol (optional) - 10% (v/v)

SDS - 2%
Bromophenol blue - 0.01%

SDS (10%) - 1 g in 10 mL distilled water

Sucrose (50%) - 5 g in 10 mL distilled water
Autoclaved at 121° C for 15 min and stored at 4°C until use.

Stacking Gel (5%) (2.5 mL)
Acryl : Bis – 0.425 mL
Stacking gel Buffer – 0.625 mL
10% SDS – 25 µL
TEMED – 2.5 µL
10 % APS - 25 µL

Distilled water – 1.425 mL

Resolving Gel (12%) (5.0 mL)
Acryl : Bis – 2 mL
Resolving gel Buffer – 1.25 mL
10% SDS – 50 µL
TEMED – 15 µL
10 % APS – 37.5 µL
Distilled water – 1.66 mL

Protein Marker for SDS-PAGE
Broad range molecular weight protein marker mix from New England BioLabs (UK) is a ready to load marker. The protein marker was mixed and 7µL taken in a tube. Heated for 5 min at 100°C. After a quick microcentrifuge spin, loaded directly on to the gel. The composition of the marker mix is as given below.

Components		**MW in Da**
Myosin	-	212,000
MBP-β- galactosidase	-	158,194
β- galactosidase	-	116, 351
Phosphorylase b	-	97,184
Serum albumin	-	66,409
Glutamic dehydrogenase	-	55,561
MBP2	-	42,710
Thioredoxin reductase	-	34,622
Triosephosphate isomerase	-	26,972
Trypsin inhibitor	-	20,000
Lysozyme	-	14,313
Aprotinin	-	6,517
Insulin A	-	3,400
B chain	-	2,340

pH was 6.8 at 25°C and stored at -20°C.

Silver staining
Fixing solution 1 – 50 mL methanol and 5 mL acetic acid in 45 mL water
Fixing solution 2 - 50 mL methanol in 50 mL water
Sensitizer - Sodium thiosulfate (20mg/100mL)
Staining solution* - Silver nitrate (200mg/100mL)

Developing solution*
Sodium carbonate (anhydrous) - 3g/100mL
Formaldehyde - 25µl/100mL
Sodium EDTA solution - 1.4 g/100 mL
* Mixed and prepared fresh before use.

APPENDIX -5

Statistical tests

1.Kruskal-Wallis test

This is a method for comparing several independent random samples and can be used as a nonparametric alternative to the one way ANOVA. If the test is significant, you can make multiple comparisons between the samples. You may choose the level of significance for these comparisons (default is a = 0.05). The term **significance level (alpha)** is used to refer to a pre-chosen probability and the term "P value" is used to indicate a probability that you calculate after a given study. The **alternative hypothesis (H_1)** is the opposite of the null hypothesis; in plain language terms this is usually the hypothesis you set out to investigate. The choice of significance level at which you reject H_0 is arbitrary. Conventionally the 5% (less than 1 in 20 chance of being wrong), 1% and 0.1% ($P < 0.05$, 0.01 and 0.001) levels have been used. Most authors refer to **statistically significant** as $P < 0.05$ and **statistically highly significant** as $P < 0.001$ (less than one in a thousand chance of being wrong) (StatsDirect statistical software (version 3.0, Cheshire, UK). Two types of methods can be used namely, Dwass-Steel-Chritchlow-Fligner method and Conover-Inman method.
In the present study, this was used for differentiating the weak, moderate and strong biofilm producers. Report for the data analysed in the present study:

Kruskal-Wallis test

Variables: Strong, Moderate, Weak

Groups = 3
df = 2
Total observations = 31

T = 20.957661
P < 0.0001

Adjusted for ties:

T = 20.970345

P < 0.0001

At least one of your sample populations tends to yield larger observations than at least one other sample population.

Kruskal-Wallis: all pairwise comparisons (Dwass-Steel-Chritchlow-Fligner)
Critical q (range) = 3.314493

Strong vs. Moderate	significant
(|-6.223787| > 3.314493)	P < 0.0001
Strong vs. Weak	significant
(|-2.337775| > 3.314493)	P <0.0001

Kruskal-Wallis: all pairwise comparisons (Conover-Inman)
Critical t (28 df) = 2.048407

Strong and Moderate	significant
(15 > 4.0991)	P < 0.0001
Strong and Weak	significant
(20.5 > 10.845199)	P = 0.0006

2. Sign Test

The sign test is a statistical method to test for consistent differences between pairs of observations, such as the weight of subjects before and after treatment. Given pairs of observations (such as weight pre- and post-treatment) for each subject, the sign test determines if one member of the pair (such as pre-treatment) tends to be greater than (or less than) the other member of the pair (post-treatment). If there are more positive as negative changes, then **P > 0.5 which means the test is significant.**
In the present study, the test is used in the context of biocontrol experiments using the bioactive compounds, EPS quantification experiments and in application studies of biocompounds.

1. For the biocontrol experiments with the bioactive compounds, the data can be commonly represented as:

Sign Test
For 18 observations with 9 on one side:

Cumulative probability
Two sided P > 0.9999 significant
One sided P = 0.5927

Normal approximate z = 0
Two sided P > 0.9999 significant

One sided P = 0.5

Exact (Clopper-Pearson) 95% confidence interval for the proportion:

Lower Limit = 0.260191
Proportion = 0.5
Upper Limit = 0.739809

2. For EPS quantification experiments, the data obtained from the software is as follows:

Sign test

For 8 observations with 4 on one side:

Cumulative probability
Two sided P > 0.9999 significant
One sided P = 0.6367

Normal approximate z = 0
Two sided P > 0.9999 significant
One sided P = 0.5

Exact (Clopper-Pearson) 95% confidence interval for the proportion:
Lower Limit =0.157013
Proportion = 0.5
Upper Limit = 0.842987

3. For application study of biocompounds, the data obtained is as follows:

Sign test

For 8 observations with 4 on one side:

Cumulative probability
Two sided P > 0.9999 significant
One sided P = 0.6367

Normal approximate z = 0
Two sided P > 0.9999 significant
One sided P = 0.5

Exact (Clopper-Pearson) 95% confidence interval for the proportion:
Lower Limit =0.157013
Proportion = 0.5
Upper Limit = 0.842987

4. For multispecies consortia studies, the data can be represented as follows:

Sign test

For 8 observations with 4 on one side:

Cumulative probability
Two sided P > 0.9999 significant
One sided P = 0.6367

Normal approximate z = 0
Two sided P > 0.9999 significant
One sided P = 0.5

Exact (Clopper-Pearson) 95% confidence interval for the proportion:
Lower Limit =0.157013
Proportion = 0.5
Upper Limit = 0.842987

3. Newman–Keuls Test and Tukey Test

Two of the most common methods of pairwise comparisons are the Tukey test and the Newman-Keuls test. Both tests are based on the "Studentized range" or "Student's q". They differ in that the Newman-Keuls test is a sequential test designed to have more power than the Tukey test. Choosing between the Tukey and Newman-Keuls tests is not straightforward and there is no consensus on this issue. The NewmanKeuls test is most frequently used in psychology, while the Tukey test is most commonly used in other disciplines. An advantage of the Tukey test is to keep the level of the Type I error (i.e., finding a difference when none exists) equal to the chosen alpha level (e.g., $\alpha = .05$ or $\alpha = .01$). An additional advantage of the Tukey test is to allow the computation of confidence intervals for the differences between the means. Although the Newman-Keuls test has more power than the Tukey test, the exact value of the probability of making a Type I error of the Newman-Keuls test cannot be computed due to the sequential nature of this test. In addition, because the criterion changes for each level of the Newman-Keuls test, confidence intervals cannot be computed around the differences between means. Therefore, selecting whether to use the Tukey or Newman-Keuls test depends upon whether or not additional power is required to detect significant differences between means.

In the present study, this was used for preparation of log- and stationary-phase, starved- and nutrient- depleted culture studies of phages. Report for the data analysed in the present study:

Newman-Keuls multiple comparisons for ΦBAP-1

Comparison	Mean difference L |L/SE(L)|	Separation
Expt1 vs. Expt2	-2.355	5
	47.724505	P < 0.0001
Expt1 vs. Expt5	-2.16	4
	43.772794	P < 0.0001

Expt1 vs. Expt4	-2.085	3
	42.252905	P < 0.0001
Expt1 vs. Expt3	-1.78	2
	36.072025	P < 0.0001
Expt2 vs. Expt3	0.575	4
	11.65248	P = 0.0016
Expt3 vs. Expt5	-0.38	3
	7.700769	P = 0.0066
Expt3 vs. Expt4	-0.305	2
	6.180881	P = 0.0072
Expt2 vs. Expt4	0.27	3
	5.471599	P = 0.0265
Expt2 vs. Expt5	0.195	2
	3.951711	P = 0.0383
Expt4 vs. Expt5	-0.075	2
	1.519889	P = 0.3316 stop

Variable	Mean Significant contrasts
Expt1	0.765 Expt2, Expt5, Expt4, Expt3
Expt2	3.12 Expt1, Expt3, Expt4, Expt5
Expt5	2.925 Expt1, Expt3, Expt2
Expt4	2.85 Expt1, Expt3, Expt2
Expt3	2.545 Expt1, Expt2, Expt5, Expt4

Tukey multiple comparisons for ΦPAP

Critical value (Studentized range) = 5.673125, |q*| = *
Pooled standard deviation = 0.069785

Comparison	Mean difference L (95% CI) |L/SE(L)|
Expt1 vs. Expt2	-2.355 (-2.634944 to -2.075056) 47.724505 P < 0.0001
Expt1 vs. Expt5	-2.16 (-2.439944 to -1.880056) 43.772794 P < 0.0001
Expt1 vs. Expt4	-2.085 (-2.364944 to -1.805056) 42.252905 P < 0.0001
Expt1 vs. Expt3	-1.78 (-2.059944 to -1.500056) 36.072025 P < 0.0001
Expt2 vs. Expt3	0.575 (0.295056 to 0.854944)

	11.65248
	P = 0.0023
Expt3 vs. Expt5	-0.38 (-0.659944 to -0.100056)
	7.700769
	P = 0.0147
Expt3 vs. Expt4	-0.305 (-0.584944 to -0.025056)
	6.180881
	P = 0.036
Expt2 vs. Expt4	0.27 (-0.009944 to 0.549944)
	5.471599
	P = 0.0572 stop
Expt2 vs. Expt5	0.195 (-0.084944 to 0.474944)
	3.951711
	P = 0.1676
Expt4 vs. Expt5	-0.075 (-0.354944 to 0.204944)
	1.519889
	P = 0.8129

Variable	Mean Significant contrasts
Expt1	0.765 Expt2, Expt5, Expt4, Expt3
Expt2	3.12 Expt1, Expt3
Expt5	2.925 Expt1, Expt3
Expt4	2.85 Expt1, Expt3
Expt3	2.545 Expt1, Expt2, Expt5, Expt4

Newman-Keuls multiple comparisons for BAP

Comparison	Mean difference L |L/SE(L)|	Separation
Expt1 vs. Expt2	-7.16	5
	241.363551	P < 0.0001
Expt2 vs. Expt4	5.82	4
	196.19216	P < 0.0001
Expt2 vs. Expt5	5.35	3
	180.348463	P < 0.0001
Expt2 vs. Expt3	5.16	2
	173.943565	P < 0.0001
Expt1 vs. Expt3	-2	4
	67.419986	P < 0.0001
Expt1 vs. Expt5	-1.81	3
	61.015088	P < 0.0001
Expt1 vs. Expt4	-1.34	2
	45.171391	P < 0.0001
Expt3 vs. Expt4	0.66	3

	22.248595	P < 0.0001
Expt4 vs. Expt5	-0.47	2
	15.843697	P < 0.0001
Expt3 vs. Expt5	0.19	2
	6.404899	P = 0.0062

Variable	Mean Significant contrasts
Expt1	1.14 Expt2, Expt3, Expt5, Expt4
Expt2	8.3 Expt1, Expt4, Expt5, Expt3
Expt4	2.48 Expt2, Expt1, Expt3, Expt5
Expt5	2.95 Expt2, Expt1, Expt4, Expt3
Expt3	3.14 Expt2, Expt1, Expt4, Expt5

Tukey multiple comparisons for BAP

Critical value (Studentized range) = 5.673125, |q*| = *
Pooled standard deviation = 0.041952

Comparison	Mean difference L (95% CI) |L/SE(L)|
Expt1 vs. Expt2	-7.16 (-7.328292 to -6.991708) 241.363551 P < 0.0001
Expt2 vs. Expt4	5.82 (5.651708 to 5.988292) 196.19216 P < 0.0001
Expt2 vs. Expt5	5.35 (5.181708 to 5.518292) 180.348463 P < 0.0001
Expt2 vs. Expt3	5.16 (4.991708 to 5.328292) 173.943565 P < 0.0001
Expt1 vs. Expt3	-2 (-2.168292 to -1.831708) 67.419986 P < 0.0001
Expt1 vs. Expt5	-1.81 (-1.978292 to -1.641708) 61.015088 P < 0.0001
Expt1 vs. Expt4	-1.34 (-1.508292 to -1.171708) 45.171391 P < 0.0001

Expt3 vs. Expt4	0.66 (0.491708 to 0.828292) 22.248595 P < 0.0001
Expt4 vs. Expt5	-0.47 (-0.638292 to -0.301708) 15.843697 P = 0.0005
Expt3 vs. Expt5	0.19 (0.021708 to 0.358292) 6.404899 P = 0.0313

Variable	**Mean** **Significant contrasts**
Expt1	1.14 Expt2, Expt3, Expt5, Expt4
Expt2	8.3 Expt1, Expt4, Expt5, Expt3
Expt4	2.48 Expt2, Expt1, Expt3, Expt5
Expt5	2.95 Expt2, Expt1, Expt4, Expt3
Expt3	3.14 Expt2, Expt1, Expt4, Expt5

ANNEXURE-1

ANTIBACTERIAL ACTIVITY OF PYOCYANIN, RHAMNOLIPIDS, MELANIN AND BACTERIOCIN BL8

The antibacterial activity of all the four bioactive compounds against the nine test food pathogens that are strong biofilm producers as proved in this study, was carried out using liquid broth assay (Jiang, 2011).

All the test pathogens were grown in nutrient broth at OD~1 at 600 nm which are in the log phase. Each organism was inoculated into six tubes containing 1 mL of nutrient broth of OD_{600}~1. To each tube, different weights (in μg) of the four different bioactive compounds was added and thus concentrations was be depicted as μg/mL for each test tube in all cases.

The concentration at which there is no visible growth for an organism was termed to be Minimum Inhibitory Concentration (MIC). The MIC for all four compounds was found by this method for each and every test organism. It was found that MIC of a particular compound was same for all nine test pathogens and the MIC of all the four bioactive compounds were different from each other. It is noticeable that these compounds showed activity at very minute microgram quantities. The data is represented in the tables 1(a) – 1(d) and proved their high antibacterial activity.

Table 1(a): Antibacterial activity of pyocyanin at different concentrations against the nine foodborne pathogens using broth assay

Test Organism	Positive control (in cfu/mL)	On addition of pyocyanin (0.1556 µg/mL)	On addition of pyocyanin (0.3113 µg/mL)	On addition of pyocyanin (0.6225 µg/mL)	On addition of pyocyanin (1.245 µg/mL)
Bacillus altitudinis(BTMW1)	3.1 ± 0.1414 x 10^8	1.1 ± 0.1414 x 10^4	85 ± 0.2121	0	0
Bacillus pumilus(BTMY2)	2.7 ± 0.2828 x 10^8	1.2 ± 0.2828 x 10^4	62 ± 0.1414	0	0
Pseudomonas aeruginosa(BTRY1)	3.6 ± 0.2828 x 10^8	3.5 ± 0.1414 x 10^4	3.5 ± 0.1414 x 10^4	3.3 ± 0.2121 x 10^4	3.4 ± 0.2121 x 10^4
Brevibacterium casei(BTDF1)	2.4 ± 0.2121 x 10^8	0.98 ± 0.0707 x 10^4	80 ± 0.2828	0	0
Staphlococcus warneri(BTDF2)	1.35 ± 0.2121 x 10^8	0.95 ± 0.1414 x 10^4	70 ± 0.2121	0	0
Micrococcus luteus (BTDF3)	2.05 ± 0.0707 x 10^8	1.13 ± 0.1414 x 10^4	91 ± 0.1414	20 ± 0.1414	22 ± 0.2828
Bacillus niacini (BTDP3)	2.15 ± 0.2121 x 10^8	1.32 ± 0.2828 x 10^4	73 ± 0.0707	0	0
Bacillus sp (BTSD1)	3.6 ± 0.1414 x 10^8	1.35 ± 0.0707 x 10^4	73 ± 0.2828	0	0
Geobacillus stearothermophilus(BTFF2)	2.2 ± 0.2828 x 10^8	1.2 ± 0.1414 x 10^4	72± 0.0141	30 ± 0.0707	34 ± 0.0021

*MIC of pyocyanin=0.6225 µg/mL (at which no visible growth is obtained)

Number indicate the cfu/mL at different concentrations of pyocyanin

Table 1(b): Antibacterial activity of rhamnolipids at different concentrations against nine foodborne pathogens using broth assay

Test Organism	Positive control (in cfu/mL)	On addition of rhmanolipids (9.375 µg/mL)	On addition of rhamnolipids (18.75 µg/mL)	On addition of rhamnolipids (37.5 µg/mL)	On addition of rhamnolipids (75 µg/mL)
Bacillus altitudinis(BTMW1)	3.1 ± 0.1414 x 10^8	1.5 ± 0.0707 x 10^4	85 ± 0.2121	25 ± 0.2121	28 ± 0.2828
Bacillus pumilus(BTMY2)	2.7 ± 0.2828 x 10^8	1.4 ± 0.1414 x 10^4	62 ± 0.1414	0	0
Pseudomonas aeruginosa(BTRY1)	3.6 ± 0.2828 x 10^8	3.7 ± 0.2121 x 10^4	3.7 ± 0.2121 x 10^4	3.6 ± 0.2828 x 10^4	3.8 ± 0.0014 x 10^4
Brevibacterium casei(BTDF1)	2.4 ± 0.2121 x 10^8	1.13 ± 0.0707 x 10^4	80 ± 0.2828	0	0
Staphlococcus warneri(BTDF2)	1.35 ± 0.2121 x 10^8	1.28 ± 0.1313 x 10^4	70 ± 0.2121	0	0
Micrococcus luteus (BTDF3)	2.05 ± 0.0707 x 10^8	1.29 ± 0.0707 x 10^4	91 ± 0.1414	0	0
Bacillus niacini (BTDP3)	2.15 ± 0.2121 x 10^8	1.8 ± 0.2828 x 10^4	73 ± 0.0707	16 ± 0.0021	18 ± 0.0002
Bacillus sp (BTSD1)	3.6 ± 0.1414 x 10^8	1.62 ± 0.2121 x 10^4	73 ± 0.2828	0	0
Geobacillus stearothermophilus(BTFF2)	2.2 ± 0.2828 x 10^8	0.97 ± 0.2121 x 10^4	72± 0.0141	0	0

*MIC of rhamnolipids=37.5 µg/mL (at which no visible growth is obtained)

Number indicate the cfu/mL at different concentrations of rhamnolipids

Table 1(c): Antibacterial activity of melanin at different concentrations against nine foodborne pathogens using broth assay

Test Organism	Positive control (in cfu/mL)	On addition of melanin (25 µg/mL)	On addition of melanin (50 µg/mL)	On addition of melanin (75 µg/mL)	On addition of melanin (100 µg/mL)
Bacillus altitudinis(BTMW1)	3.1 ± 0.1414 x 10^8	1.25 ± 0.2121 x 10^4	65 ± 0.1414	10 ± 0.0707	0
Bacillus pumilus(BTMY2)	2.7 ± 0.2828 x 10^8	2.1 ± 0.1414 x 10^4	198 ± 0.2828	65 ± 0.1414	50 ± 0.2121
Pseudomonas aeruginosa(BTRY1)	3.6 ± 0.2828 x 10^8	2.8 ± 0.1414 x 10^4	97 ± 0.0707	15 ± 0.0020	0
Brevibacterium casei(BTDF1)	2.4 ± 0.2121 x 10^8	1.9 ± 0.0707 x 10^4	78 ± 0.2121	18 ± 0.0014	0
Staphlococcus warneri(BTDF2)	1.35 ± 0.2121 x 10^8	0.97 ± 0.0070 x 10^4	39 ± 0.1414	11 ± 0.0707	0
Micrococcus luteus (BTDF3)	2.05 ± 0.0707 x 10^8	1.75 ± 0.0707 x 10^4	169 ± 0.1414	85 ± 0.0021	40 ± 0.2828
Bacillus niacini (BTDP3)	2.15 ± 0.2121 x 10^8	1.91 ± 0.0707 x 10^4	89 ± 0.2828	9 ± 0.2121	0
Bacillus sp (BTSD1)	3.6 ± 0.1414 x 10^8	2.85 ± 0.0707 x 10^4	94 ± 0.0707	13 ± 0.0050	0
Geobacillus stearothermophilus(BTFF2)	2.2 ± 0.2828 x 10^8	1.85 ± 0.0707 x 10^4	177 ± 0.2121	75 ± 0.0014	20 ± 0.0141

*MIC of melanin= 100 µg/mL (at which no visible growth is obtained)

Number indicate the cfu/mL at different concentrations of melanin

Table 1(d): Antibacterial activity of bacteriocin BL8 at different concentrations against nine foodborne pathogens by broth assay

Test Organism	Positive control (in cfu/mL)	On addition of bacteriocin (1500 µg/mL)	On addition of bacteriocin (2000 µg/mL)	On addition of bacteriocin (2380 µg/mL)	On addition of bacteriocin (2500 µg/mL)
Bacillus altitudinis(BTMW1)	3.1 ± 0.1414 x 10^8	0.945 ± 0.2121 x 10^4	120 ± 0.0141	0	0
Bacillus pumilus(BTMY2)	2.7 ± 0.2828 x 10^8	0.97 ± 0.0141 x 10^4	150 ± 0.0212	0	0
Pseudomonas aeruginosa(BTRY1)	3.6 ± 0.2828 x 10^8	0.87 ± 0.0141 x 10^4	70 ± 0.0707	0	0
Brevibacterium casei(BTDF1)	2.4 ± 0.2121 x 10^8	2.05 ± 0.0707 x 10^4	30 ± 0.0707	0	0
Staphlococcus warneri(BTDF2)	1.35 ± 0.2121 x 10^8	0.88 ± 0.0141 x 10^4	95 ± 0.0212	0	0
Micrococcus luteus (BTDF3)	2.05 ± 0.0707 x 10^8	1.815 ± 0.0212 x 10^4	85 ± 0.0141	0	0
Bacillus niacini (BTDP3)	2.15 ± 0.2121 x 10^8	0.93 ± 0.0707 x 10^4	110 ± 0.0212	0	0
Bacillus sp (BTSD1)	3.6 ± 0.1414 x 10^8	1.915 ± 0.0212 x 10^4	90 ± 0.0707	0	0
Geobacillus stearothermophilus(BTFF2)	2.2 ± 0.2828 x 10^8	1.31 ± 0.0141 x 10^4	80 ± 0.0141	0	0

*MIC of bacteriocin=2380 µg/mL (at which no visible growth is obtained)

Number indicate the cfu/mL at different concentrations of bacteriocin

ANNEXURE-2

The tables (Annexure-2) lists the comparison for percentage of reduction due to each of the eleven combinations to evaluate the most effective combination for each of the test pathogens used.

Table 2(a) Comparison in reduction of biofilm formation (in %) by food pathogens on treatment with different combinations of bioactive compounds (as per section 4.3.3)

Organism	**B+R***	**B+M***	**B+P***	**M+R***	**M+P***	**R+P***
Bacillus altitudinis (BTMW1)	36.5	65	42.3	28.5	34.3	5.9
Bacillus pumilus (BTMY2)	81	33.5	53.5	47.5	20	67.75
Pseudomonas aeruginosa (BTRY1)	38	70.5	38	32.5	32.5	0
Brevibacterium casei(BTDF1)	59.5	68	70.5	66.5	77.5	69.15
Staphylococcus warneri(BTDF2)	45	70.5	66	43.5	64.5	38.9
Micrococcus luteus (BTDF3)	44	32.5	32.5	11.5	0	11.3
Bacillus niacini (BTDP3)	39	57.5	53	18.5	32.5	14
Bacillus sp (BTSD1)	77.5	69	59.5	63.5	45.5	53.95
Geobacillus staerothermophilus (BTFF2)	77	38.5	38	39.5	0.5	38.8

*B- Bacteriocin, *P-Pyocyanin, *R- Rhamnolipids, *M- Melanin

Table 2(b) Comparison in reduction of biofilm formation (in %) of the food pathogens on treatment with different combinations of bioactive compounds (as per section 4.3.3)

Organism	**B+M +P***	**B+M+ R***	**B+P+ R***	**M+R+ P***	**M+P+R+B***
Bacillus altitudinis (BTMW1)	47.2	43.3	28.2	22.8	72
Bacillus pumilus (BTMY2)	35.6	54	67.3	45	97
Pseudomonas aeruginosa (BTRY1)	47	47	25.3	21.6	65
Brevibacteriumcasei(BTDF1)	72	64.6	66.3	71	75
Staphylococcus warneri (BTDF2)	67	53	50	49	81
Micrococcus luteus (BTDF3)	21.6	29.3	29.3	7.5	68
Bacillus niacini (BTDP3)	47.6	38.3	35.3	21.6	67
Bacillus sp (BTSD1)	58	70	63.6	54.3	83
Geobacillusstaerothermophilus (BTFF2)	25.6	51.6	51.3	26.25	87

*B- Bacteriocin, *P-Pyocyanin, *R- Rhamnolipids, *M- Melanin

ANNEXURE-3

Of the thirty six isolates, 11 isolates were obtained from beef, 4 from chicken, 6 from raw milk, 1 each from turmeric powder, chilly powder and curd, 3 each from dried fish and dried prawns, 2 each from soft drink, fresh fish and coriander powder. Table 3 lists all the thirty six isolates from different types of food samples and the food sources from which they were isolated.

Table 3: Thirty six isolates from different food samples and their respective sources.

Isolate	Source	Isolate	Source	Ioslate	Source	Isolate	Source
BTMW1	Beef	BTTP1	Turmeric powder	BTMP1	Beef	BTRC1	Raw Milk
BTMY2	Beef	BTDF1	Dried Fish	BTMY1	Beef	BTCP2	Coriander Powder
BTMG1	Beef	BTDF2	Dried Fish	BTCW1	Chicken	BTCP4	Coriander Powder
BTMW2	Beef	BTDF3	Dried Fish	BTMY3	Beef	BTDP1	Dried Prawn
BTCW2	Chicken	BTDP2	Dried Prawns	BTMP2	Beef	BTPC1	Curd
BTMW3	Beef	BTDP3	Dried Prawns	BTRB1	Raw Milk	BTCP2	Chicken
BTMY4	Beef	BTSD1	Soft Drink	BTPY1	Chilly powder		
BTRY1	Raw Milk	BTSD2	Soft Drink	BTRS1	Raw Milk		
BTPW1	Beef	BTFF1	Fresh Fish	BTRS2	Raw Milk		
BTCP1	Chicken	BTFF2	Fresh Fish	BTRW1	Raw Milk		

LIST OF PUBLICATIONS

FULL PAPER IN PEER-REVIEWED JOURNALS

1. **Laxmi M** & Sarita G Bhat (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

Accepted publications yet to be published

1. **Laxmi M**, Noble K Kurian, Smitha S & Sarita G Bhat, (2015). Melanin and bacteriocin from marine bacteria for biocontrol of biofilm forming food pathogens in *Indian Journal of Biotechnology.*
2. **Laxmi M** & Sarita G Bhat, (2015). Characterization of pyocyanin with radical scavenging activity & antibiofilm properties from *Pseudomonas aeruginosa* BTRY1 in *3 Biotech*.

BOOK CHAPTER

1. **Laxmi M** & Sarita G Bhat (2014) Biocontrol of Bacterial biofilms, Chapter 7, Microbial Bioproducts, ISBN-978-93-80095-51-6, published by Directorate of Public relations and Publications, CUSAT, for Department of Biotechnology.

POSTERS / ABSTRACTS IN NATIONAL / INTERNATIONAL SYMPOSIA

1. **Laxmi M** and Sarita G Bhat, (2013). Studies on biofilm producers from food samples available in the local markets. Oral session of 23rd Swadeshi Science Congress organized jointly by Swadeshi Science Movement, Kerala and Mahatma Gandhi University, Kottayam during 6-8 November 2013.
2. **Laxmi M** and Sarita G Bhat (2014) Biocontrol of Biofilm forming bacteria from different food samples using bioactive compounds. Oral session of 27th

Kerala Science Congress 2015 held at Alappuzha during 27-29th January, 2015.

AWARDS AND HONOURS

1. Secured the **Best Paper award** at 27th Kerala Science Congress 2015 held at Alappuzha during 27-29th January, 2015 for the research paper titled 'Biocontrol of Biofilm forming bacteria from different food samples using bioactive compounds'.
2. Received **Certificate of Appreciation** for securing the best paper award at 27th Kerala Science Congress from the **Honorable Governor of Kerala, Shri. Justice P. Sathasivam**, during an event conducted for the felicitation of top performers of the University under student enrichment programme held at CUSAT on 22nd June, 2015.

GENBANK SUBMISSIONS

1. **Laxmi M** & Sarita G B (2013) KF460551- Bacillus altitudinis strain BTMW1 16S ribosomal RNA gene, partial sequence.
2. **Laxmi M** & Sarita G B (2013) KF460552- Bacillus pumilus strain BTMY1 16S ribosomal RNA gene, partial sequence.
3. **Laxmi M** & Sarita G B (2013) KF460553 Bacillus altitudinis strain BTMG1 16S ribosomal RNA gene, partial sequence.
4. **Laxmi M** & Sarita G B (2013) KF460554- Bacillus pumilus strain BTMW2 16S ribosomal RNA gene, partial sequence.
5. **Laxmi M** & Sarita G B (2013) KF460555- Bacillus altitudinis strain BTCW2 16S ribosomal RNA gene, partial sequence.
6. **Laxmi M** & Sarita G B (2013) KF460556- Bacillus altitudinis strain BTMW3 16S ribosomal RNA gene, partial sequence.
7. **Laxmi M** & Sarita G B (2013) KF460557- Bacillus pumilus strain BTMY4 16S ribosomal RNA gene, partial sequence.
8. **Laxmi M** & Sarita G B (2013) KF460558- Pseudomonas aeruginosa strain BTRY1 16S ribosomal RNA gene, partial sequence.

9. **Laxmi M** & Sarita G B (2013) KF460559- Bacillus altitudinis strain BTPW1 16S ribosomal RNA gene, partial sequence.
10. **Laxmi M** & Sarita G B (2013) KF460560- Bacillus pumilus strain BTCP1 16S ribosomal RNA gene, partial sequence.
11. **Laxmi M** & Sarita G B (2013) KF460561- *Bacillus altitudinis* strain BTTP1 16S ribosomal RNA gene, partial sequence.
12. **Laxmi M** & Sarita G B (2013) KF573739 – *Brevibacterium casei* strain BTDF1 16S ribosomal RNA gene, partial sequence.
13. **Laxmi M** & Sarita G B (2013) KF573740 –*Staphylococcus warneri* strain BTDF2 16S ribosomal RNA gene, partial sequence.
14. **Laxmi M** & Sarita G B (2013) KF573741- *Micrococcus lutueus* strain BTDF3 16S ribosomal RNA gene, partial sequence.
15. **Laxmi M** & Sarita G B (2013) KF573742- *Micrococcus sp* strain BTDP2 16S ribosomal RNA gene, partial sequence.
16. **Laxmi M** & Sarita G B (2013) KF573743- *Bacillus niacini* strain BTDP3 16S ribosomal RNA gene, partial sequence.
17. **Laxmi M** & Sarita G B (2013) KF573744- *Bacillus sp* strain BTSD1 16S ribosomal RNA gene, partial sequence.
18. **Laxmi M** & Sarita G B (2013) KF573745- *Bacillus licheniformis* strain BTSD2 16S ribosomal RNA gene, partial sequence.
19. **Laxmi M** & Sarita G B (2013) KF573746- *Micrococcus luteus* strain BTFF1 16S ribosomal RNA gene, partial sequence.
20. **Laxmi M** & Sarita G B (2013) KF573747- *Geobacillus staerothermophilus* strain BTFF2 16S ribosomal RNA gene, partial sequence.

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RESEARCH ARTICLE

DIVERSITY CHARACTERIZATION OF BIOFILM FORMING MICROORGANISMS IN FOOD SAMPLED FROM LOCAL MARKETS IN KOCHI, KERALA,INDIA

[1]Laxmi M, *Sarita G Bhat

[1]Department of Biotechnology, Cochin University of Science & Technology, Kochi, Kerala

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ABSTRACT

Biofilms are of considerable interest in food hygiene given that it leads to serious health problems and economic loss due to food spoilage. Food samples including beef, cheese, raw milk, pasteurized milk, curd, chilly powder, turmeric powder, coriander powder, soft drinks, fresh fish, dried fish and dried prawn were tested for the presence of food borne pathogens using standard plate count assay. Quantification of biofilm formation was by microtitre plate assay. Thirty six isolates which are indicative of great threat in the food industry were characterized. Twenty isolates were strong biofilm producers, ten moderate biofilm formers, while only one was a weak producer. Five did not produce any biofilm. The antibiogram of the twenty strong producers showed multiple resistance to antibiotics. The enzyme profiling of the strong producers showed that most produced more than one enzyme, which is indicative of their competence in reduction the nutritional value of food and causing spoilage.

INTRODUCTION

Biofilms are microbially derived sessile communities characterized by many cells attached to an abiotic or living surface, and embedded in a matrix of extracellular polymeric substances produced by the cells. Biofilm formation has been noted in fossil records (~3.25 billion years ago) (Maric and Vranes, 2007). Antony Von Leeuwenhoek is credited with the discovery of biofilm on his own tooth surface. The first published report on biofilms by Zobell in 1943, used buried slide culture method, where the slides buried in the soil had an attachment of microorganisms (Kokar*eet al.*, 2009). Angst (1923) observed that the marine bacterial load on hulls of ships was higher than the free floating cells, and also proposed that bacterial biofilms led to serious corrosion of these hulls. By 1980s, bacteria were observed on the solid surfaces of many ecological environments including waste water treatment systems, equipments used in the manufacture of vinegar, industrial water systems, tooth decay, urinary tract and also on other implanted medical devices (Zottola and Sasahara, 1994). These observations led to the development of new electronic techniques including scanning electron microscopy, transmission electron microscopy and laser scanning confocal microscopy.

The formation of biofilm is a complex and dynamic process involving different steps (Costerton*et al.*, 1994(b) and Melo*et al.*, 1992), such as conditioning of the surface, adhesion of cells, formation of microcolony, biofilm formation, detachment and dispersal of biofilms.

In the food industry, biofouling causes grave problems, impeding heat flow across surfaces, increase in fluid frictional resistance and corrosion rate at the surface, all leading to energy and product losses. Biofilms due to spoilage and pathogenic microflora on surfaces of food like poultry, meat and in processing environments also pose considerable problems of cross contamination and post-processing contamination. Therefore in the context of food hygiene biofilms have been of considerable interest, as they may contain spoilage and pathogenic bacteria increasing contamination and risk to public health.

The microbes involved in biofilm formation and health risks include bacteria belonging to the genera *Vibrio, Salmonella, Pseudomonas, Listeria, Bacillus, Escherichia, Clostridium,* to name a few. With the emergence of resistance in pathogenic bacteria to traditional antibiotics, development of alternative control measures gained momentum. In addition, microorganisms produce saccharolytic, proteolytic, pectinolytic and lipolytic enzymes, whose metabolic end products are associated with food spoilage and poisoning. Thus the food industry faces multitude of challenges to keep products safe and free of pathogenic microorganisms for the consumers and also to augment product shelf life

The present study focuses on the bacterial biofilm formers in food available in the markets and their characterizations, since food poisonings and food pathogen related health risks and deaths are increasing by the day.

MATERIALS AND METHODS

Quantification of biofilm forming pathogens by microtitre plate assay

The food samples including beef, cheese, raw milk, pasteurized milk, curd, chilly powder, turmeric powder, coriander powder, soft drink, fresh fish, dried fish and dried prawn collected from the local stores and markets in Kochi, Kerala were analyzed using standard plate assay. 1 g of sample was serially diluted in10 mL of sterile distilled water. 0.1 mL of each dilution was plated on nutrient agar (HiMedia,

* Corresponding author: **Sarita G Bhat**
Department of Biotechnology, Cochin University of Science & Technology, Kochi, Kerala

Mumbai, India) plates using spread plate technique. The isolated bacterial colonies were picked and preserved in nutrient slants at 4^0C. These bacterial isolates were tested for their biofilm forming ability using microtitre plate assay (Rode *et al.*, 2007).

The wells of a sterile 96 well polystyrene microtitre plates were filled with 230 µL of tryptone soy broth (TSB) (HiMedia, Mumbai, India). 20 µL bacterial cultures (OD_{600} =1) were added into each well separately, with triplicates for each bacterial culture and incubated aerobically for 24 h at 37^0C. Negative control included only TSB. The contents of the plates were poured off, wells washed 3 times with phosphate buffer (0.01 M, pH 7.2) and the attached bacteria were fixed with methanol. After 15 minutes, the plates were decanted, air dried and stained with of 1% crystal violet for 5 minutes. The excess stain was rinsed under running tap water. After air drying, the dye bound to adherent cells was extracted with 33% (V/V) glacial acetic acid per well and the absorbance was measured at 570 nm using a UV-VIS spectrophotometer (Schimadzu, Japan). Based on the absorbance (A_{570}) they were graded A= A_c= No biofilm producers; A_c< A= Weak biofilm producers; $2A_c$<A= Moderate biofilm producers; $4A_c$<A= Strong biofilm producers; where cutoff absorbance A_c was the mean absorbance of the negative control. All tests were interpreted thrice independently and statistically analysed (Christensen *et al.*, 1988; Stepanovic*et al.*, 2000);

Molecular characterization of biofilm producers

Genomic DNA was isolated and purified (Ausubel*et al.*, 1987). A portion of the 16S rDNA was amplified using a primer pair for 16S rDNA (Reddy *et al.*, 2002). The identity of the sequences was determined by comparing the 16S rDNA sequence with the sequences available in the NCBI nucleotide databases using BLAST (Basic Local Alignment Search Tool) algorithm (Altschul*et al.*, 1990). A phylogenetic tree was also constructed for the biofilm producers by neighbor joining method (Saitou and Nei, 1987) using the MEGA 4 software (Tamura *et al.*, 2007)

Antibiotic sensitivity tests

All strong biofilm producers were tested for antibiotic sensitivity in accordance with the Kirby- Bauer method (Bauer *et al.*, 1966), with 12 antibiotics (HiMedia, Mumbai, India) belonging to different classes, namely ampicillin (5 µg/disc), azithromycin (15 µg/disc), cefixime (5 µg/disc), cefuroxime (30 µg/disc), ceftriazone (15 µg/disc), chloramphenicol (30µg/disc), ciprofloxacin (5 µg/disc), gentamicin (10 µg/disc), nalidixic acid (30µg/disc), norfloxacin (5 µg/disc), tetracycline (30 µg/disc), and trimethoprim (5 µg/disc). The results were interpreted as per the manufacturers' instructions.

Enzyme profiling of the biofilm producers

The qualitative assessment of enzyme activities including amylases, proteases, cellulases and lipases was using starch agar, skimmed milk agar, carboxymethyl cellulose agar and tributyrin agar respectively, as a part of characterization of the strong biofilm producers and consequently for the determination of their ability to degrade the nutritional substances in the food samples.

Amylases activity

For detecting amylase activity, organisms were patched onto 0.5% starch agar plate and incubated for 24 hours at room temperature. Gram's Iodine solution was flooded onto the inoculated plate. A clear zone around the colony indicates that amylase has hydrolysed the starch thereby giving no blue colour on reaction with iodine (Murray *et al.*, 2007).

Proteases activity

The test organisms were patched onto 10% skimmed milk agar plate and incubated overnight at room temperature. Clear zones produced around the colony indicate that the casein in the mediumhas been hydrolysed. . No clearance of the medium is seen as the negative test (Mahendran*et al.*, 2010).

Lipases activity

The test organisms were patched onto 1% tributyrin agar plate and incubated the plates for 48 - 72 hours at room temperature. Clear zones around the colony indicate the presence of lipases (Karnetova*et al.*, 1984).

Cellulases activity

For cellulolytic activity, the test organisms were patched onto 0.5% carboxy methyl cellulose agar and incubated for 48 hours at room temperature. The plates were flooded with 0.1% Congo red solution and kept for 20-30 minutes with intermittent shaking, drained flooded with 1N NaCl solution and kept for 15 minutes. A yellow colour around the colony leaving the other portion of the plate red indicates positive reaction (Eggins and Pughg, 1962).

RESULTS

Quantification of biofilm forming pathogens by microtitre plate assay

Screening of the various food sample types for bacterial food

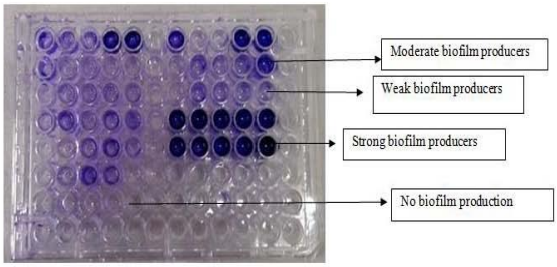

Figure 1 Microtitre plate for quantification of biofilm producers after the crystal violet staining

borne pathogens using standard plate count assay yielded thirty six isolates. Thirty one (86.11%)were biofilm producers. Figure 1 shows the quantification of biofilm production by microtitre plate assay. They were classified as strong, moderate and weak biofilm producers.

Figure 2 shows the strength of the biofilm production in all the 36 isolates obtained. Figure 3 shows biofilm production in the twenty strong producers in accordance to the A_C value.

The fig (1) shows varying intensities of crystal violet, which is indicative of the strength of the biofilm formed. The more intense the color, stronger the biofilm formed. From the figure, the levels of biofilm formed by different food pathogens is evident and can be clearly utilized to differentiate the strong, moderate and weak biofilm producers.

Molecular characterization of biofilm producers using 16S rDNA sequence analysis

Genomic DNA was isolated from the twenty isolates. Polymerase chain reaction based 16S rDNA amplification and sequence analysis thereafter was used for molecularcharacterization of the biofilm formers.. Following BLAST the identity of the biofilm formers was determined and the sequence data was submitted to the NCBI database and accession numbers obtained. Table 1 shows the identity of the twenty biofilm producers based on NCBI BLAST analysis.

The 16S rDNA analysis revealed that 14 of the biofim formers were *Bacillus* species, 4 were lactic acid bacteria and one each *Brevibacterium* and *Pseudomonas* species.

Table 1 The identity of the isolates with biofilm forming ability

Isolate	Organism	Genbank Accession number	Isolate	Organism	Genbank accession number
BTMW1	*Bacillus altitudinis*	KF460551	BTTP1	*Bacillus altitudinis*	KF460561
BTMY2	*Bacillus pumilus*	KF460552	BTDF1	*Brevibacteriumcasei*	KF573739
BTMG1	*Bacillus altitudinis*	KF460553	BTDF2	*Staphylococcus warneri*	KF573740
BTMW2	*Bacillus pumilus*	KF460554	BTDF3	*Micrococcus luteus*	KF573741
BTCW2	*Bacillus altitudinis*	KF460555	BTDP2	*Micrococcus sp*	KF573742
BTMW3	*Bacillus altitudinis*	KF460556	BTDP3	*Bacillus niacini*	KF573743
BTMY4	*Bacillus pumilus*	KF460557	BTSD1	*Bacillus sp*	KF573744
BTRY1	*Pseudomonas aeruginosa*	KF460558	BTSD2	*Bacillus licheniformis*	KF573745
BTPW1	*Bacillus altitudinis*	KF460559	BTFF1	*Micrococcus luteus*	KF573746
BTCP1	*Bacillus pumilus*	KF460560	BTFF2	*Geobacilluss tearothermophilus*	KF573747

Out of thirty six isolates obtained, 56% (n=20) were strong biofilm producers, 28% (n=10) were moderate producers while 3 % were weak producers. 14% (n=5) did not form biofilm. The biofilm producers were classified and this is depicted in figure (2).

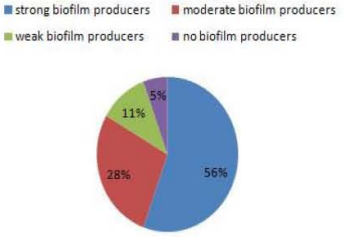

Figure 2 Classification of biofilm producers

Out of the twenty strong producers, the maximum biofilm production was shown by the strain BTSD2.. These biofilm formers were further identified using 16S rDNA sequence analysis.

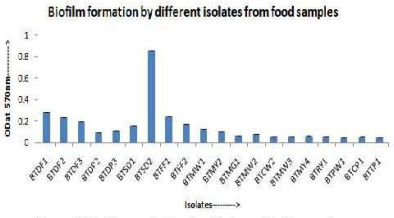

Figure 3 Biofilm production by 20 strong biofilm producers

This furthermore revealed that most of these strong biofilm producers are also food pathogens.

Phylogenetic analysis of the biofilm strains obtained in the study was done to understand their interrelatedness and is depicted as in figure (4).

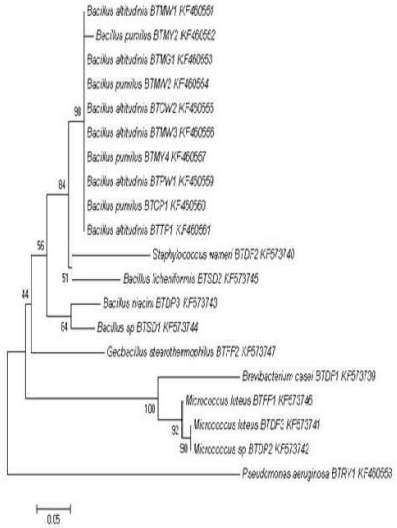

Figure 4 showing phylogenetic analysis of the obtained biofilm strains in the study

The number at the nodes of the phylogenetic tree are percentages indicating the levels of bootstrap support based on the Neighbour-Joining analysis of 1000 resampled data sets using MEGA 4 software.

It was observed that the *B.altitudinis* and *B. pumilus*strains grouped together in a single clade as did the three *Micrococcus* sp.

Antibiogram of the strong biofilm producers

Antibiotic Sensitivity Test was done and the antibiogram of the strong biofilm producers is as given in the figure (5). It was observed that percentage of biofilm producers resistant or intermediately resistant to most of the antibiotics was greater. From the figure (5) it was observed that eventhough the percentage of sensitivity was more, the sum total of intermediate resistance and total resistance was higher. This indicated that increased risks of food poisonings and food related deaths.

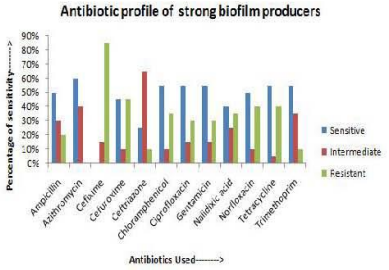

Figure 5 The antibiotic profile (%) of the twenty strong producers

Enzyme profile of biofilm producers

The enzyme profile showed that most of the strong biofilm producers were capable of producing more than one enzymes and thus were able to diminish the nutrient content of the food samples. The enzyme profile of is as listed in the table (4). The figures [6] – [9] depicts the different qualitative enzymatic assays using special media mentioned in the section 2.4.

The results of the qualitative enzymatic assays showed the ability of most of the isolates to produce more than one enzyme. This characteristic feature pointed out that these isolates, in addition to the biofilm formation, can reduce the nutritional value of the food.

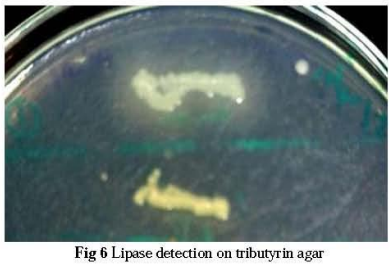

Fig 6 Lipase detection on tributyrin agar

Figure (6) – (9) shows the results of four qualitative enzymatic assays that include the lipase assay on tributyrin agar, cellulase assay on carboxy methyl cellulose agar, protease assay on skimmed milk agar and amylase assay on starch agar respectively

DISCUSSION

Several reports have been published on screening of food borne pathogens from different foods. The incidence of *Pseudomonas* sp in food items like beef, milk, anchovy and chicken was reported by Keskin and Ekmekci (2007). Agarwal*et al.*, 2011 evaluated the biofilm forming ability of different *Salmonella* serotypes using the microtitre plate assay

Table 2 showing the enzyme profile of strong biofilm producers

Strain	Amylase	Protease	Cellulase	Lipase
Bacillus altitudinis(BTMW1)	-	+	+	+
Bacillus pumilus (BTMY2)	-	+	+	+
Bacillus altitudinis (BTMG1)	-	+	+	+
Bacillus pumilus (BTMW2)	-	+	+	+
Bacillus altitudinis (BTCW2)	-	+	+	+
Bacillus altitudinis (BTMW3)	-	+	+	+
Bacillus pumilus (BTMY4)	-	+	+	+
Pseudomonas aeruginosa (BTRY1)	-	+	-	-
Bacillus altitudinis (BTPW1)	-	+	+	+
Bacillus pumilus (BTCP1)	-	+	+	+
Brevibacteriumcasei (BTDF1)	-	-	-	-
Staphylococcus warneri (BTDF2)	-	+	-	+
Micrococcus luteus (BTDF3)	-	+	-	-
Micrococcus sp (BTDP2)	-	-	-	-
Bacillus niacini (BTDP3)	-	-	-	+
Bacillus sp (BTSD1)	+	+	-	-
Bacillus licheniformis (BTSD2)	-	-	+	+
Micrococcus luteus (BTFF1)	-	+	-	+
Geobacillusstearothermophilus (BTFF2)	+	+	-	-

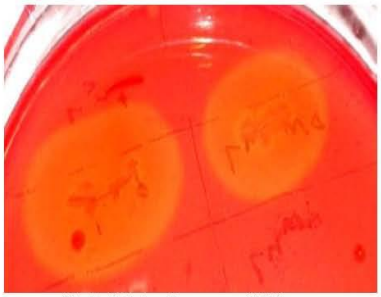

Fig 7 Cellulase detection on CMC agar

Fig 8 Protease detection on skimmed milk agar

Fig 9 Amylase detection on Starch agar

with the crystal violet staining and their results showed that most of the strains in the study formed biofilm on plastic surfaces; t this study also categorized the isolated pathogens as strong, moderate and weak biofilm producers.

Murmann*et al*., 2008 collected food samples from outbreaks of Salmonellosis and molecular characterization was done. They also checked the antimicrobial susceptibility using antibiotics like amikacin (30 µg), ampicillin (10 µg), cefaclor (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), tetracycline (30 µg), tobramycin (10 µg), streptomycin (10 µg), sulphamethoxazole-trimethoprim (23.75/1.25 µg) and sulfonamide (300 µg) and a low frequency of antimicrobial resistance was observed in their study. Bacteria in biofilms are normally reported to have intrinsic mechanisms that protect them from most aggressive environmental conditions, including the exposure to antimicrobials (Davies, 2003). Teh*et al*., 2012 reported that enzymes secreted from biofilms into raw milk during transportation can potentially reduce the quality of different dairy products and could lead to severe economic losses in the food industry.

Thus biofilms production by food pathogens pose a immense threat to the food industry. In the present study, 20 strong biofilm producers were characterized by 16S rDNA sequencing and their identity revealed. The strains belonged to the genera *Bacillus, Pseudomonas, Micrococcus, Staphylococcus, Brevibacterium and Geobacillus*. The strongest biofilm producer was *Bacillus sp* (BTSD1). The enzyme profiling showed that the strongest biofilm producers produced most of the important starch, cellulose, proteins and lipids hydrolyzing enzymes and were thereby capable of easily diminishing the quality of the food samples. Multiple antibiotic resistance was observed among the strong biofilm producers, which were also food pathogens.

According to the present study, most of the biofilm forming food pathogens were multiple antibiotic resistant and produced more than one enzyme responsible for the food perishability. Several bioactive compounds find application against the biofilm formation of most of the strains and their safety needs to be confirmed prior to application in the food industry.
Since biofilm formation a serious issue, their control must be considered since it directly influences public health.

Acknowledgement

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