Isolation of Bacteriophage against Salmonella typhi and its Potential to Control Biofilm

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DEDICATED TO

MY PARENTS, SIBLINGS AND NIECES WHO'S LOVE, SUPPORT AND PRAYERS HELP ME PASS THROUGH LIFE

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Aimen Saleem

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LIST OF ABBREVIATIONS

ARB's	Antibiotic Resistant Bacteria
$CaCl_2$	Calcium Chloride
CFU	Colony Forming Unit
C-LSM	Confocal Laser Scanning Microscopy
CV	Crystal Violet
CVC	Central Venous Catheters
CRA	Congo red Agar
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
EPS	Extracellular Polymeric Substances
ICTV	International Committee on Taxonomy of Viruses
Kb	kilo Bases
KDa	kilo Dalton
LB	Luria-Bertani
$MgCl_2$	Magnesium Chloride
MDR	Multi Drug Resistant
mM	Milli mole
MOI	Multiplicity of Infection
NaCl	Sodium Chloride
NIH	National Institutes of Health
nm	Nano-meter
NS	Normal Saline
OD	Optical Density
PBS	Phosphate Buffer Saline

PEG	Poly Ethylene Glycol
PFU	Plaque Forming Unit
Rpm	Revolution per minute
rRNA	Ribosomal RNA
SD	Standard Deviation.
SEM	Scanning Electron Microscopy
SS	Stainless Steel
TAE	Tris Acetate EDTA
Taq	Thermus aquaticus
TBE	Tris Borate EDTA
TCP	Tissue Culture Plate
TEM	Transmission Electron Microscopy
TSB	Tryptic Soy Broth
WHO	World Health Organization

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ABSTRACT

Bacteriophages are known to be the most abundant entity in the universe. They infect bacteria and replicate inside bacterial cells either by lytic or lysogenic cycle. Bacteriophages have been employed for many applications i.e. for transfer of gene/protein vaccines, an alternative to antibiotics, screening of peptides/proteins, identification of pathogens etc. They also have an important role in controlling biofilm, an aggregation of microbial cells that are adhered to inert/biological surface or with each other. Biofilms are resistant to antibiotics and other disinfectants so they a play major role in the spread of disease, so an alternative should be find out to control biofilm formation. Amongst biofilm forming bacteria, Salmonella typhi is gram negative, rod shaped bacterium that is involved in spread of typhoid fever. Due to poor administrative measures of antibiotics, this bacterium has developed resistant towards 3rd generation antibiotics. This study aimed to isolate bacteriophage against Salmonella typhi and to control biofilm formation. Bacteriophages were isolated from sewage water against clinical bacterial strains and characterization of isolated phage showed it to be stable at long temperature range (37-65 ºC) and pH range (5-11) with adsorption rate increased by presence of divalent metal ions. The burst size of bacteriophage is 300 virions per cell with 24 minutes latent time period. The isolated phage reduced bacterial population to 2 folds in planktonic culture and 2.2 folds in the biofilm. In conclusion our findings suggest that waste water is a good source for finding bacteriophages against newly emerging antibiotic resistant bacteria Salmonella typhi. Phages can be used to control bacteria both in planktonic forms, as well as in biofilms. In conclusion our findings suggest that waste

water is a good source for finding bacteriophages against newly emerging antibiotic resistant bacteria Salmonella typhi. Phages can be used to control bacteria both in planktonic forms, as well as in biofilms.

Chapter 1

INTRODUCTION

Bacteriophages are viruses that are composed of protein coats that enclose genome (DNA/RNA). They are the most common entity in the biosphere (Grath and Sinderen, 2007). They infect bacteria and replicate within bacteria either by lysogenic cycle (incorporates viral genome in host genome and replicates using host replicating machinery) or by lytic cycle (multiply within host cells and get released by disrupting bacterial membrane). After their discovery, initially they got much attention due to their antibacterial activity but later on, the discovery of antibiotics set back their importance in the field of medicine (Clark and March, 2006).

Prokaryotic phage includes fourteen officially accepted families while five families are waiting for classification. Among these families, only two have RNA genome while remaining has DNA genome. Among bacteriophages having DNA genome, eight have circular DNA while nine have linear genome. The majority of viruses approximately 96.2% is tailed and belongs to three families while remaining 3.7% comprises of polyhedral, filamentous and Pleomorphic phages and belongs to 17 families (Ackermann, 2007).

Phages are either pleomorphic or polyhedral, filamentous or tailed. Nine families of phages infect only archaea, nine infect only bacteria and only one infects both archaea and bacteria (Grath and Sinderen, 2007).

Table1.1 Morphology, nucleic acid type and example of bacteriophages classified into 13 families; ds: double stranded, ss: single stranded, L: linear, C: circular, T: super helical, S: segmented. (Ackermann, 2003)

Shape	Nucleic Acid	Families	Characteristics	Example
Tailed	DNA, ds, L	*Myoviridae*	Tail contractile	T4
		Siphoviridae	Tail long, non-contractile	Λ
		Podoviridae	Tail short	T7
Polyhedral	DNA, ss, C	*Microviridae*		Φ X174
	DNA, ds, C, T	*Corticoviridae*	Complex capsid, lipids	PM2
	DNA, ds, L	*Tectiviridae*	Internal lipoprotein vesicle	PRD1
	RNA, ss, L	*Leviviridae*		MS2
	RNA, ds, L, S	*Cystoviridae*	Envelope, lipids	Φ6
Filamentous	DNA, ss, C	*Inoviridae*	Filaments or rods	Fd
	DNA, ds, L	*Lipothrixviridae*	Envelope, lipids	TTV1
	DNA, ds, L	*Rudiviridae*	Resemble TMV	SIRV1
Pleomorphic	DNA, ds, C, T	*Plasmaviridae*	Envelope, lipids, no capsid	L2
	DNA, ds, C, T	*Fuselloviridae*	Spindle shaped, no capsid	SSV1

Phages like other viruses are inert outside their host cells so they need to infect bacteria to get replicated. The life cycle of phages involve five steps i.e. adsorption, penetration, replication, maturation, release. During adsorption, phages bind to cell receptor of bacterial cells. This step can reversible or irreversible (Adams, 1959). In bacteria cells, phages get replicated by either lysogenic cycle or lytic cycle. If phages multiply within bacteria and kill their host during their propagation, this is a lytic cycle. During the lysogenic cycle, viral genome incorporated within host genome and gets replicated without causing any side effect to bacterial cells. These phages are termed as

temperate phages harsh conditions can cause a switch from temperate phage to lytic life cycle (Inal, 2003).

Bacteriophages have certain applications in modern biotechnology industries: they have been used as an alternative to antibiotics, as carrier for transferring gene or protein vaccines, for detection of pathogenic bacteria, for targeted gene delivery and for screening peptide, antibodies and protein libraries (Clark and March, 2006). They also have role in prevention of biofilm formation on infectious wounds and surgical instruments (Weiling *et al.*, 2009).

Phages are used to synthesize protein with desired properties. The DNA encoding desired polypeptide is fused with phage coat protein, so desired protein is expressed on surface of phages (Sidhu, 2000). Mostly filamentous phage M13 of E.coli is used for protein expression (Benhar.2001).

As phages are specific to host bacteria so this property can be utilized in the identification of pathogenic bacteria. The sensitivity of detection can be increased by binding antibodies to phages specific to bacteria to be identified (Watson and Eveland, 1965).

Bacteriophages also have in important role in food industry. They can be used as food additives and bio preservatives in food. This aspect of bacteriophages needs research so they can be used without any harmful effects (Garcia *et al.*, 2008).

Therapeutic use of bacteriophages as the antibacterial agent had been started shortly after their discovery but later on their use was stopped due to the discovery of antibiotics. Although they continued to be used in the Soviet Union as they had less

access to antibiotics in those days. Nowadays scientists are again keen towards the use of phages as an alternative to antibiotics, due to increasing resistance of bacteria towards antibiotics. So by controlling little problems encountered due to phage therapy, they can be used as potential antibacterial agents. Phage therapy has been tested in animals, plants and human and it gives positive results in all (Sulakvelidze, 2001).

Biofilm is a group of microorganisms in which cells stick to each other on the surface and these adherent cells are embedded in self-produced extracellular polymeric substance (EPS). This EPS is composed of proteins, DNA and polysaccharides (Hall *et al*., 2004). Biofilms are prevalent in the hospital, industrial settings as well as in natural environment and may form on living or nonliving objects.

Biofilms play a role in chronic infections in humans and sometimes it may prove fatal and cannot be cured. These infections are resistant towards antibiotics and many other antimicrobial agents and have wide capacity to overcome host defense mechanism (Bjarnsholt T, 2013). Bacteriophages have a role in the degradation of biofilm, thus prevents the organism from chronic infection. They possess polysaccharide depolymerase enzyme that is specific to break EPS. The degradation of biofilm is due to combined action of depolymerase enzyme and lytic life cycle of phage that causes disruption of bacterial cells (Kevin *et al.,* 1998).

One of the major infections caused by biofilm is of medical devices such as catheter related infections that cause an increase in mortality and morbidity rate among patients. Phages have potential to reduce these infections. Thus pretreatment of the catheter with

specific phage or phage cocktail greatly reduces the risk of infections caused by biofilm (Weiling *et al*., 2009).

Typhoid fever is intestinal or blood infection caused by bacterium Salmonella typhi. This infection occurs due to drinking and eating of contaminated water and food, poor sanitation conditions and developing countries are at high risk to exposure of this disease (Crump and Mintz, 2010). Vaccines are available to prevent this disease and it is effective up to 60-70% of cases. Treatment of this disease is possible by 3rd generation antibiotics but increasing resistance to these antibiotics is making treatment of this disease difficult (Wain *et al*., 2015).

Salmonella typhi is seravor of *Salmonella enteric* subspecie enterica. It is rod-shaped, Gram-negative, flagellated and facultative anaerobic bacterium (Murray *et al.,* 2009). It belongs to family Enterobacteriaceae. This bacterium poses major problem for countries like Pakistan as there are increasing cases of typhoid fever reported in this region and this bacterium is getting more and more resistant towards antibiotics.30% strains are resistant towards second generation antibiotics (Rathore et al., 1996). 17.6% Salmonella typhi are getting resistant towards ceftriaxone. This is an alarming situation raising a question what would be next to ceftriaxone (Mushtaq.M.A. 2006).

Due to increasing resistance towards antibiotics, there is requirement to look for an alternative to control infections caused by Salmonella typhi. Throughout the world, researchers are focusing on phage therapy against multi drug resistant bacterium and much work is being done on isolation and characterization of phages against different microbes.

For bio control of microbes, it is advisable to not disturb natural microflora of specific region. To isolate phages against specific microbes it is recommended to isolate it from region of isolation of microbes. So this research aims to isolate bacteriophages against Salmonella typhi from waste water of different regions of Pakistan that can be used as therapeutics against antibiotics resistant Salmonella typhi and check their role to prevent biofilm formation.

HYPOTHESIS

Since bacteriophages are responsible for bacterial lysis, the bacteriophage against multi drug resistant Salmonella typhi found in blood sample of Pakistani population will help to control infections and biofilm formed by this bacterium.

AIMS AND OBJECTIVES

The main aims of this study were:

1. Isolation of bacteriophage against multi drug resistant Salmonella typhi
2. Characterization of bacteriophage isolated against Salmonella typhi
3. Determine the potential of bacteriophage to control biofilm

Chapter 2

REVIEW OF LITERATURE

2.1 BACTERIOPHAGES

A bacteriophage is a virus that infects bacterium and replicates inside it. The word bacteriophage is derived from "bacteria" and "phaegin", a Greek word that means "to devour". Bacteriophages are among the most diverse entities in the universe. They inject their genome in the cytoplasm of bacteria where it incorporates with the bacterial genome and get replicated (Grath and Sinderen, 2005).

2.2 HISTORY OF BACTERIOPHAGES

In 1896, a British bacteriologist, Ernest Hankin, reported the presence of creature in the water of rivers of India that causes lysis of bacteria against *Vibrio cholerae* that were able to pass through very fine porcelain fibers. (Sulakvelidze, Alavidze, & Morris, 2001). After two years of this discovery, the same phenomenon was observed against *Bacillus subtilis* by Russian biologist. After 20 years of Hankins's discovery, a British bacteriologist, Frederick Twort observed similar phenomenon and called that creature might be a virus (Twort, 1936). Two years after Twort's hypothesis, the official discovery of bacteriophages was done by a French microbiologist, Felix d'Herelle (Sulakvelidze, et al., 2001).

In 1969, Max Delbrück, Alfred Hershey and Salvador Luria were awarded the Nobel Prize in Physiology and Medicine for their discoveries of the replication of viruses and their genetic structure (Keen, 2012).

Phage therapy was also introduced in the United States to treat different diseases and wounds, but the discovery of antibiotics caused a major setback to phage therapy (Abedon, Kuhl, Blasdel, & Kutter, 2011).

2.3 STRUCTURE AND HABITAT OF BACTERIOPHAGES

Bacteriophages consist of double or single stranded DNA or RNA as genetic material encapsulated by protein coating known as the envelope. Most bacteriophages consist of proteinaceous icosahedral head that contains genome. In some cases lipid envelope parts of head and in other cases head has filamentous geometry. The tailed bacteriophage shows three types of architectural design each one adapting for different strategies for infection of the host (Ceurvo et al., 2012). Phages are filamentous, polyhedral, tailed or pleomorphic. 96% of viruses belong to tailed family while 3-4 % does not. Tail has fibers that facilitate phages for attachment to bacteria (Ackermann, 2007). They lack universally conserved genome but possess specific genes present in the specific viral community (Short & Suttle, 2002). Bacteriophages show similarities in their fnctions and structure although their genome do not show similarity (Ceurvo et al., 2012).

Bacterial phages are ubiquitous present in the different ecological environment. They are the most abundant entities in the universe. Bacteriophages are extremely abundant in the aquatic system however they can be found anywhere where their host (bacteria) is present. According to an observation there are approximately 10^7 viruses per mL of water (Paul, Rose, Jiang, Kellogg, & Dickson, 1993).

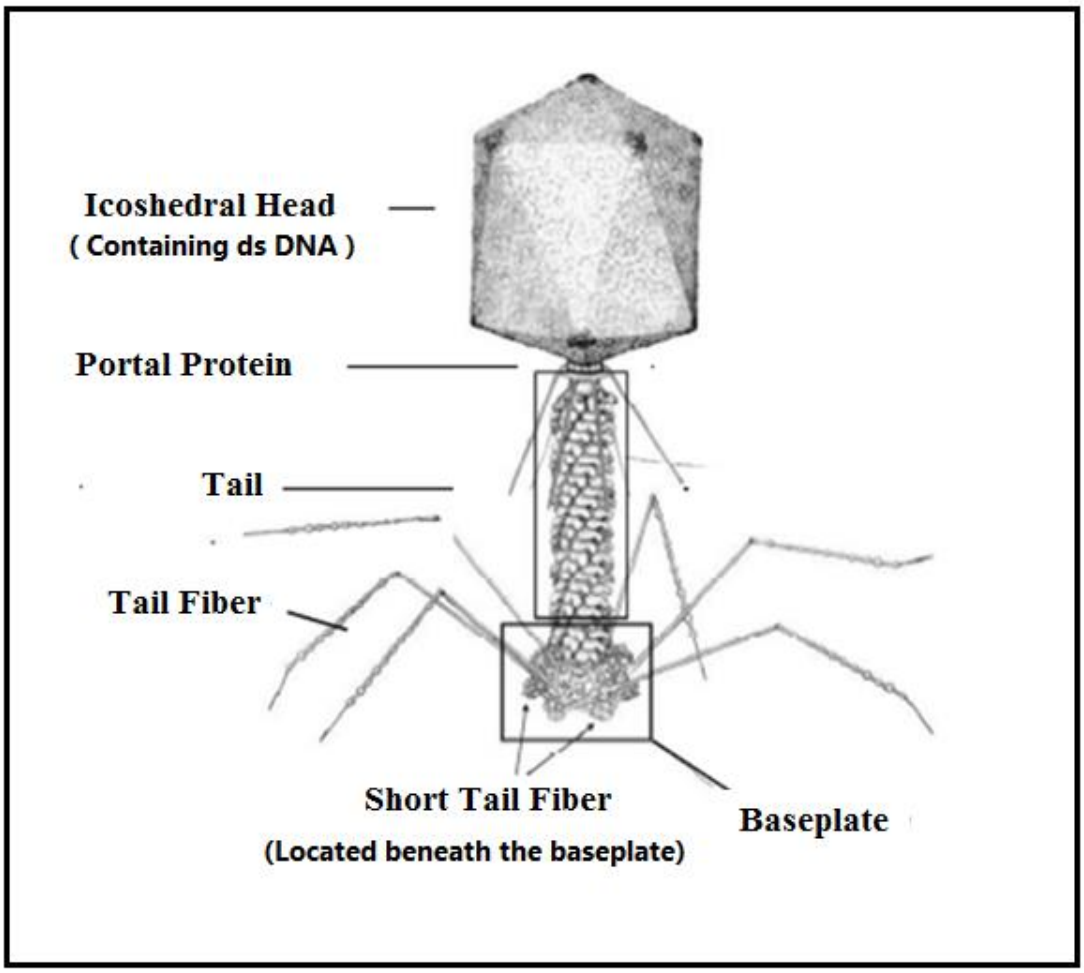

Figure 2.1: The typical morphological structure of a bacteriophage. Phage has an icosahedron head, long tail and tail fiber (Serwer *et al.*, 2008).

2.4 CLASSIFICATION OF BACTERIOPHAGES

Prokaryote phages are classified into 14 officially accepted families and five families need to be classified. The structure and taxonomical position of viruses hold great importance in describing viruses. Among these families, only two have RNA genome while remaining has DNA genome. Among bacteriophages having DNA genome, eight have circular DNA while nine have a linear genome. Phages are either pleomorphic or polyhedral, filamentous or tailed. The majority of viruses approximately 96.2% is tailed and belongs to three families while remaining 3.7%

comprises of polyhedral, filamentous and Pleomorphic phages and belongs to 17 families. The bacterial genera with most abundant phages are entering bacteria (Ackermann, 2007). Nine families of phages infect only archaea, nine infect only bacteria and only one infects both archaea and bacteria (Grath and Sinderen, 2007).

2.5 LIFE CYCLE OF BACTERIOPHAGES

Once viruses genome get enter into bacteria it replicates via one of two pathways i.e. Lytic life cycle or Lysogenic life cycle. The phages that follow lytic cycle are called virulent phages while those enter lysogenic life cycle are temperate phages.

2.5.1 Lytic Life Cycle

The first step in the lytic cycle of bacteriophage is its attachment to receptors on bacterial cells followed by injection of bacterial genome either by its tail or by mechanical force (Inamdar *et al*., 2006). The binding or attachment to cells are enhanced by the presence of divalent metal ions or sugar molecules (Guttman *et al.*, 2005). After adsorption, penetration of viral genome takes place depending upon type of phage. The phage capsid and tail remain outside the cell while bacterial genome get enters into a host cell (Letellier *et al*., 2004). This exploits host cell machinery like DNA polymerase, proteins RNA polymerase and nucleotides for the synthesis of phage components (Engelkirk *et al.*, 2011). After synthesis assembly of viral components take place. Once specific number of virions gets assembled, depending upon the type of phage and bacterium, the cell membrane of bacterial cell gets burst that leads to the release of new viruses (figure 2.2). However, bacterial lysis is not fate

in every case. In some cases phage release occurs by extrusion of the bacterial cell membrane (Young *et al.,* 2002).

2.5.2 Lysogenic Life Cycle

In this type of life cycle, phage genome gets incorporated with the bacterial genome. This phage is known as a prophage. Prophage remains in latent state and it's not infectious for bacteria. Bacteria having incorporated phage genome are known as lysogens (Stansfield *et al*., 1996). This incorporated genome replicates with the bacterial genome. Temperate phage also possesses lytic genes but these are not expressed due to the presence of repressors but can be triggered on by inducers or environmental factors. The switching on of lytic gene depends upon different factors for different phages. Lambda phage is best studied phage in this aspect. It requires activator CII to switch on lytic genes (Little, 2005).

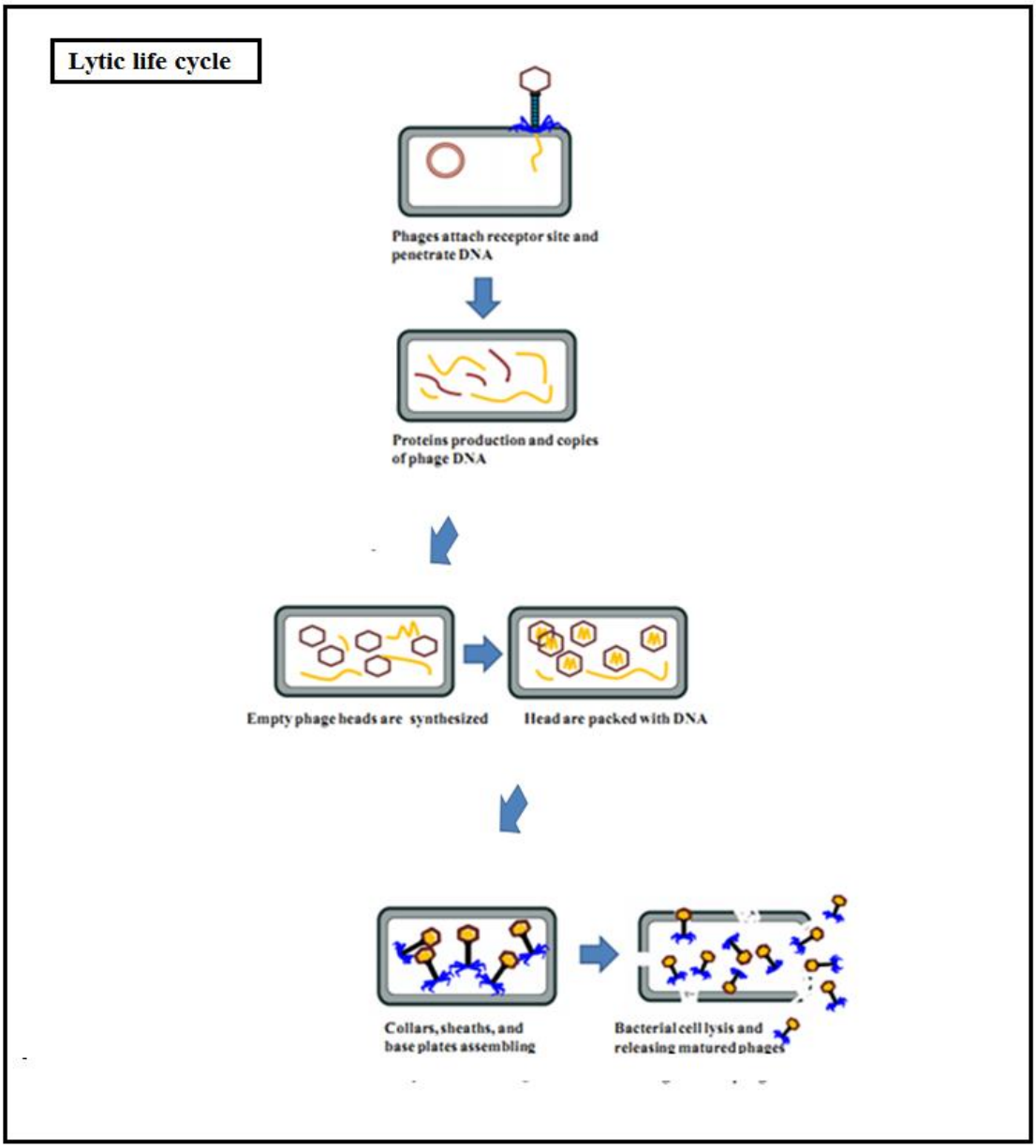

Figure 2.2: **Lytic life cycle of bacteriophage infection**. Different events that occur during lytic life cycle of phage, such as attachment, adsorption, transfer of nucleic acid, penetration, biosynthesis, assembly and release of progeny virions (Ahiwale *et al.* (2012)

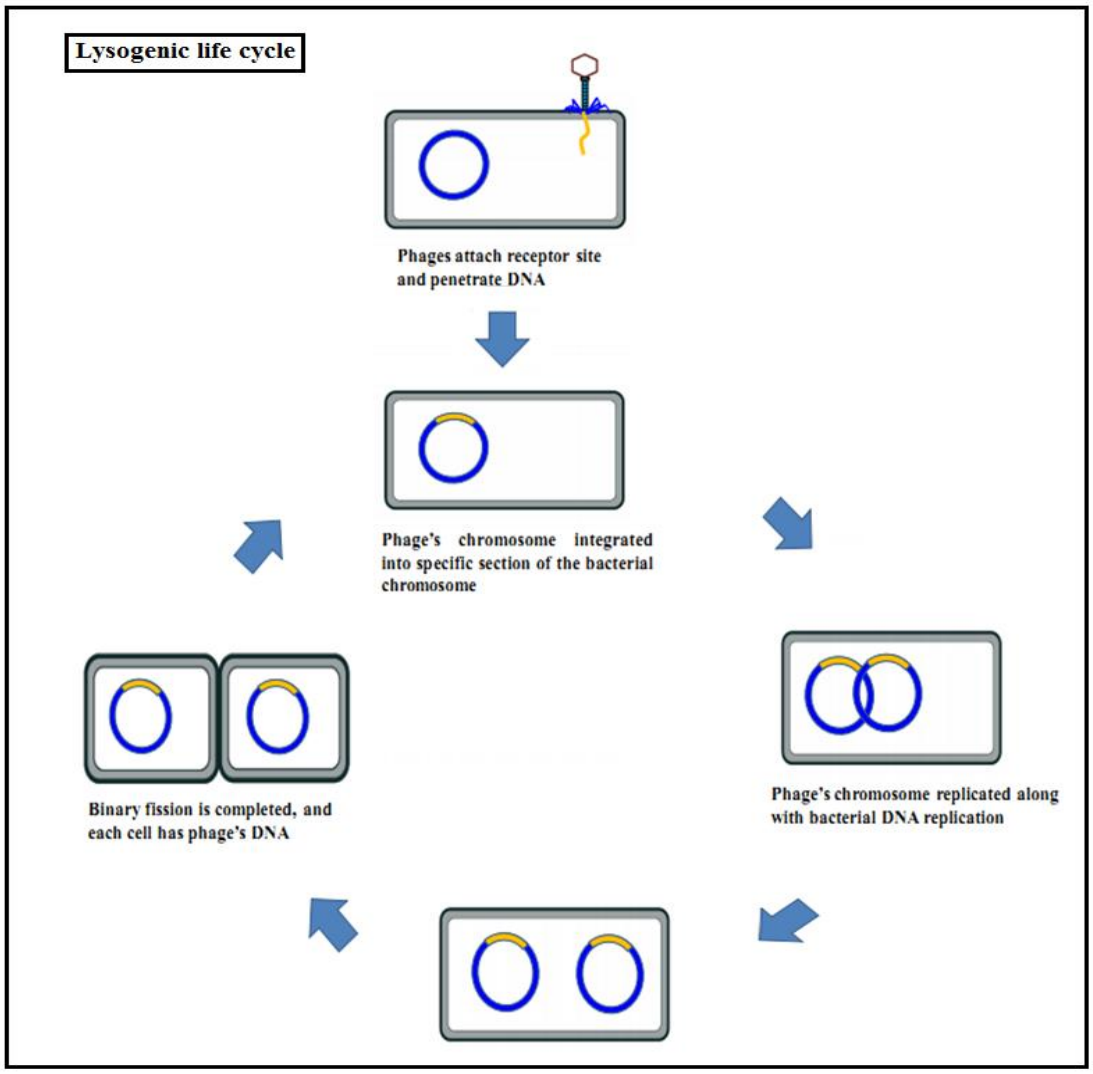

Figure 2.3: **Lysogenic life cycle of bacteriophage infection**. Figure describing different events that occur during lysogenic life cycle of phage, such as attachment, adsorption, transfer of nucleic acid, incorporation of phage DNA into bacterial chromosome (prophage), replications of virions (Ahiwale *et al.*2012).

2.6 APPLICATIONS OF BACTERIOPHAGES

Bacteriophages have a wide range of applications in biotechnological processes including gene therapy, drug delivery, vaccine development, Nano biotechnology, antimicrobial activity, controlling food spoilage, sewage water

treatment etc. (Hagens & Loessner, 2007; Withey, Cartmell, Avery, & Stephenson, 2005).

To prevent food from spoilage, bacteriophages are gaining attention nowadays as they are the natural enemy of bacteria, so they control bacteria without affecting natural microflora. Moreover, phages or phage-derived proteins can also be used to detect the presence of unwanted pathogens in food or the production environments, which allows quick and specific identification of viable cells (Hagens and Loessner, 2007).

Bacteriophage found its application in the field of medicine, shortly after their discovery but due to lack of knowledge of bacterial phage interaction and phage purification it did not find much practical importance. Moreover the discovery of antibiotics caused a major setback to therapeutic application of bacteriophage (Merril et al. 2003, 2006). But now due to increasing resistance towards antibiotics, researchers are again focusing on using phage for therapeutic purpose.

The components of bacteriophages are also gaining attention as prophylaxis. Lysozymes have been used as prophylactic agent for destroying cell wall of gram positive bacteria. Phage encoded lysozymes are playing a role to destroy Gram positive bacteria. These are of two types ; (i) endolysin formed during phage replication inside bacterial cell and destroy bacterial cell wall from inside (ii) phage tail associated murein lytic enzyme (TAME) which hydrolyze cell wall from outside after phage adsorption (Adhya et.al, 2015).

Although vaccine development had been started centuries ago when Edward Jenner developed vaccine to control small pox still there are certain limitations in the development of vaccines against different infectious agents. So this causes to look for

an alternative approach to developing vaccines (Burdin et al. 2004; O'Hagan and Rappuoli 2004). Recombinant protein molecules displaying subunits of pathogens are being used as vaccines, which although is major advancement in vaccine development yet they are not as effective due to lack of efficient delivery system. The same problem encounters with DNA based vaccines (Petrovskyand Aguilar 2004). Vaccinologists are now looking for utilizing viruses for vaccine delivery as they handle both humoral or cell mediated immunity (Adhya et.al, 2015).

Water shortage is becoming problem day by day so it is advisable to utilize biologically treated water for agriculture use. Biologically treated waste water contains bacteria, viruses and metazoans. This water can be used for certain purpose if it is pathogen free, so phages can be used to treat water. But it has certain limitations and scientists need to work on them for efficient use (Withey et al., 2005).

Biofilm formation, an important strategy for bacterial survival in harsh conditions, can take place on both living and inert surface. A major problem presented by biofilm is its ability to resist antibiotics. Therefore there is an urgent need to develop an alternative to control biofilm formation. In humans, biofilm formation poses severe health issues, most of them associated with the use of medical devices. In vitro experiments have shown that phages can control biofilm as they induce production of depolymerase causing degradation of inner layers of biofilm. In practice, treatment of medical devices with phage solution can control biofilm formation on these surfaces. Another approach has been use of phage encoding phage polysaccharide lyase to control Pseudomonas aeruginosa in cystic fibrosis patients. All these approaches require complete understanding of phage and polysaccharide

depolymerase enzyme that degrade polysaccharide within biofilm (Azeredo and Sutherland, 2008).

2.7 BIOFILM

Biofilm consists of bacteria that adhere to some object in moist condition by secretion of glue like slimy substance and started to reproduce. Biofilm formation takes place at any surface that is moist and has nutrient supply. Biofilm can be formed by single type of organism but in nature mostly it is aggregation of different bacteria, protozoa, algae, fungi etc. Biofilms are natural part of ecology. In some cases biofilms are harmful and must be treated while in some cases they are beneficial.

Biofilm forming microbes give out polymers called as extracellular polymeric substances (EPS). Biofilm forming microorganisms have the role in spread of diseases. Endotoxins are released by these microbes that help to survive harsh environment. Biofilm forming microbes develops the property of resistance against antibiotics and other agents (Donlan, 2009).

The architecture of biofilm is composed of two components i.e. water channel system and closely packed cells with no pores (Watnick and Kolter, 2000; Wimpenny *et al.*, 2000). Biofilm is not just adherence of cells to surface rather its water channel system when compared with circulatory systemt, it shows that biofilm is primitive multicellular organism Gilbert *et al.*, 2001). Biofilm has complex chemical composition consisting of DNA, proteins, enzymes, EPS, lipids and most important microbial cells. Moreover it contains high water content which plays role in circulation of nutrients and waste into and out of biofilm.

Table 2.1: Biofilm chemical composition (adapted from Vasudevan, 2014).

Sr. No	Components	Percent of matrix
1	Water	Up to 97 %
2	Microbial cells	2-5 %
3	Polysaccharides	1-2 %
4	Proteins	<1-2 % (including enzymes)
5	DNA and RNA	<1-2 %
6	Ions	Bound and free

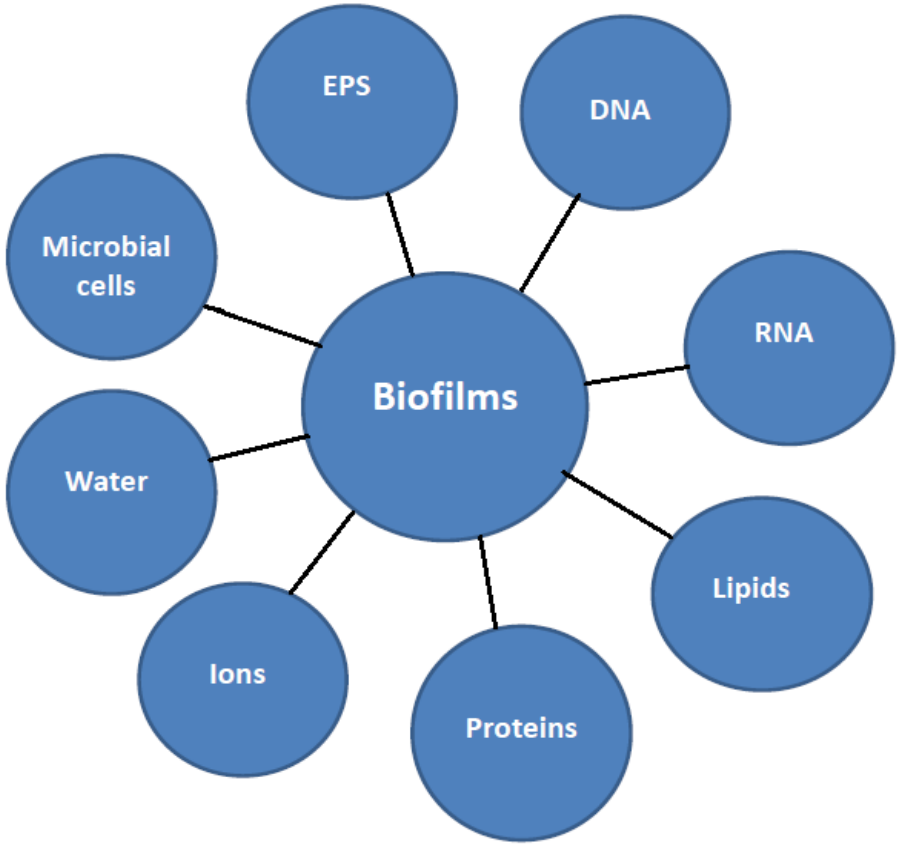

Figure 2.4: Diagram showing various components of bacterial biofilm (Jamal, 2015)

2.8 HISTORY OF BIOFILM

The study of biofilm started with invention of microscope when a Dutch scientist, Antone ven Leawnhocke" named a creature "animalcule" observed under light microscope. This discovery led to foundation of study of biofilms (Costerton *et al.,* 1999). In 1940, it was stated by Heukelekian and Heller that bacteria depend upon surface to grow on substrate. This growth takes place either as colonial attachment to surface or as bacterial slime. In 1973, Chracklis conducted a research to observe behavior of biofilm in industrial water and it was observed that it was resistant to any disinfectants. In 1978, the term biofilm was coined by Costerton and had diverged the attention of the world towards importance of biofilm. He put forward theory describing about mechanisms of attachment of bacteria to living and nonliving objects. In last 20 years, study of biofilm has been revolutionized from basic plating techniques to microarray and scanning electron microscope. The use of confocal laser microscope to study ultra-structure of biofilm and identification of genes that play role in formation of biofilm had gave better understanding of nature of biofilms (Characklis, 1973). Biofilm helps bacteria inside it to survive under harsh conditions. Biofilm can grow at any moist condition such as plaque teeth, a flower vase having gel-like film inside, infected tissues and slippery slimy river stones (Costerton *et al.,* 1999).

Biofilm forming bacteria has great tendency of growth. Individual cell separated from biofilm has ability to grow separately and form new biofilm. Biofilms are known for contamination of drinking water and corrosion of pipes (Donlan, 2001). Biofilms also has role in destroying medical devices, spread of diseases, environment and industrial damage due to its resistance towards antibiotics, enhancement in

secondary metabolite production, avoiding degradability by UV and chemical agents, altered biodegradability property and change in genetic frequencies (Costerton *et al.,* 1987; O'Toole *et al.,* 2000).

2.9 FORMATION OF BIOFILM

Biofilm formation is multistep process in which microbes undergo certain changes after adhering to surface. (i) Attachment, (ii) formation of micro colony (iii) formation of 3 dimensional structure, (iv) biofilm formation, maturation and detachment (Costerton *et al.,* 1999).

Biofilm formation starts when bacteria get close to surface and adhered to surface. The presence of moist surface provides an excellent environment for biofilm formation. Locomotary structures like flagella, pili, fimbriae, polysaccharide and proteins also contribute towards formation of biofilm (Donlan, 2002). After microbes get adhere to surface or biological tissue, they get stabilize and result in micro colony formation. Bacterial cells started giving chemical signals and resulted in multiplication of bacteria. When chemical signals reach threshold level genetic mechanism for production of exopolysaccharide started (Costerton *et al.,* 1999). So bacterial cell division, triggered by chemical signals, takes place in exopolysaccharide matrix leading to formation of micro colony (McKenney *et al.,* 1998). After formation of micro colony, certain genes in biofilm get switched on that trigger production of different components of biofilm. After formation of extra cellular matrix, water filled channel system formation take place that is involved in supply of nutrients within biofilm. Researchers proposed these water channels to be circulatory system that distribute nutrients to different components of biofilm and remove waste material from it (Prakash *et al.,* 2003). After biofilm formation, researcher has noticed

detachment of microbial cells from biofilm as natural phenomenon. This process takes place on regular basis and bacteria undergo multiplication and get dispersed (Costerton *et al.,* 1999). In some cases mechanical stress leads to dispersal of cells from biofilm into the surrounding. In most cases, bacteria stop producing EPS that leads to dispersion of cells (Baselga *et al.,* 1994). Dispersion of cells from biofilm can cause change in phenotypic characters of dispersed bacteria. However planktonic cells retain some properties of biofilm like insensitivity towards antibiotics (Baselga *et al.,* 1994).

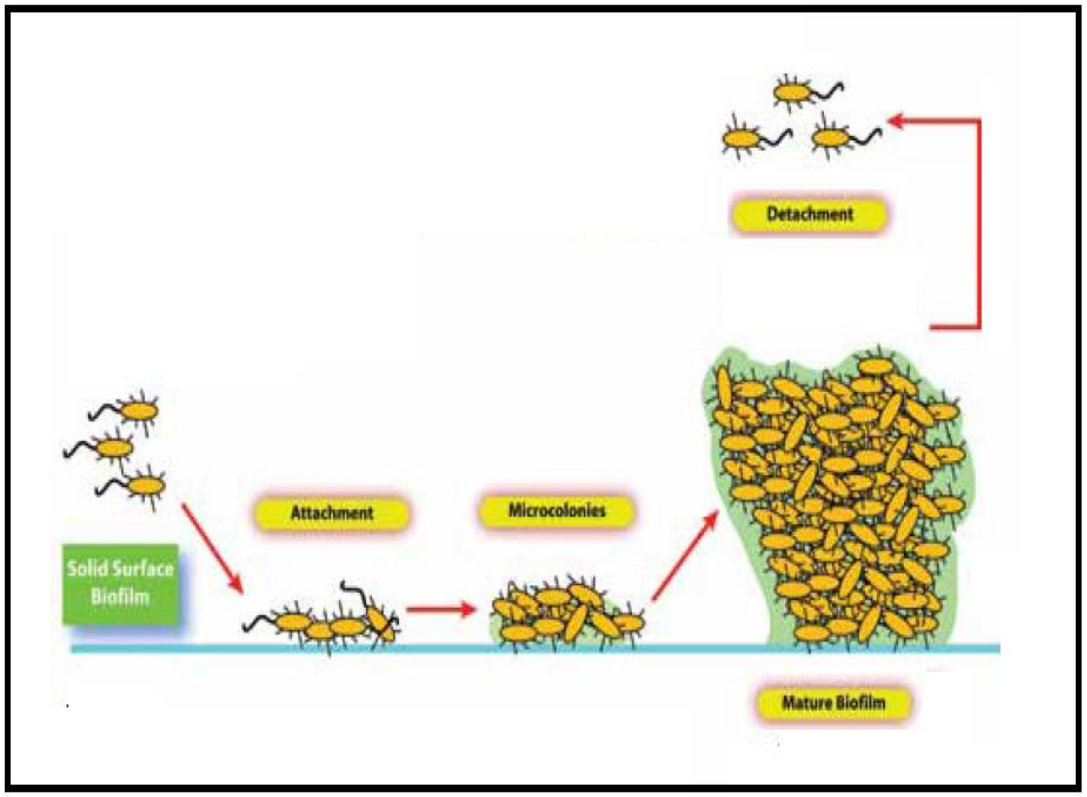

Figure 2.5: Figure describing process of biofilm formation (Hung and Henderson, 2008). Different events occurring during biofilm formation involves, (a) Initial surface attachment (b) micro-colony formation (c) formation of three dimensional structure (d) biofilm establishment and maturity and detachment (dispersal).

2.10 BIOFILM FORMING BACTERIA

Almost 99% microorganisms have capability of adhesion and biofilm formation(Sekhar *et al.*, 2009). When microorganisms get adhere to a surface, EPS is produced that leads to formation of biofilms. Due to its role in contaminating medical devices and resistance against antibiotics, biofilm poses great threat to public health (Costerton *et al.*, 1978). Some bacterial biofilm has been identified that are unaffected by human immune system as of *H. influenza* (Sekhar *et al.*, 2009). Biofilm forming capability has been reported in large number of bacterial species such as *P. aeruginosa, Streptococcus epidermidis, E. coli, S. aureus, Pasteurella multocida,*

Salmonella and Streptococcus mutans (Miller and Bassler, 2001; Parsek and Singh, 2003; Fux *et al.*, 2005; Ma *et al.*, 2009).

Escherichia coli are rod shaped gram negative bacteria and is considered related to nosocomial and community infections such as urinary tract infections (UTIs), prostatitis. It has the ability to form biofilm by secreting polysaccharide adhesion and also release toxins (Naves *et al.*, 2010). Among community-acquired illnesses, about 80 % to 90 % of all UTIs are caused by *E. coli* and 90 % of first episodes of UTI in children (Shapiro, 1992). Biofilm produced by E.coli is quite thicker which creates hurdle in eradicating infection caused by it (Surette and Bassler, 1998).

Pseudomonas aeruginosa is a gram negative pathogenic microbe that is present along with nonpathogenic natural micro flora o human skin. (Larson *et al.*, 2003; Mooij *et al.*, 2007). *P. aeruginosa* is human pathogenic organism able to withstand harsh environmental conditions and persist in environmental and hospital settings (Lutz and Lee, 2011).

P. aeruginosa has ability to form effective biofilm (O'Toole *et al*., 1999) and this ability is responsible for infections caused by *P. aeruginosa* (Costerton, 2001; Hall-Stoodley *et al*., 2004). *P. aeruginosa* is resistant towards wide variety of antibiotics including ciprofloxacin that are used in treatment of lung infections (Lyczak *et al*., 2002).

Salmonella enterica is one of the most important causes of foodborne illness in humans. It includes different seravors like Salmonella Typhimirium, Salmonella enteridis, Salmonella typhi and Salmonella Agona etc. All these seravors have ability to form biofilm on different surfaces that are in contact with food, causing food borne illness (Simm *et al.*, 2014). Salmonella Typhimirium has ability to form biofilm that

can withstand harsh acidic condition thus causing spoilage of food even in acidic environment (Xu *et al.,* 2010). Research was conducted to eradicate biofilm by *Salmonella enterica* using different chemicals like Sodium hydroxide, Sodium hypochlorite and benzalkonium. This shows that only sodium hydroxide successful to eradicate early biofilm but it is not effective against mature biofilm. So it is suggested to look for methods to control contamination by *Salmonella* (Corcoran *et al.,* 2014).

2.11 BACTERIOPHAGES AND BIOFILM CONTROL

Research was conducted to study effect of phages on biofilm of *L. monocytogens*. Biofilm was prepared on stainless steel and is treated with phage for 1-2 hrs and it shows 4-fold reduction in biofilm (Roy *et al*., 1993).

Researchers studied effect of T4 phage on E.coli biofilm. Biofilm was formed on polyvinylchloride and phage treatment with 0.6 MOI was given and its effect was analysed that showed 6 log reduction in five hours (Doolittle *et al.,* 1995).

Doolittle et al., (1996b) studied reduction of biofilm of *P.aureginosa* using fluorescently labelled bacteriophages. These phages did not penetrate biofilm and affected only upper layer. The reason behind this behaviour is unknown. One possibility is that EPS might block penetration of phages into biofilm.

In vitro study of biofilm removal from catheter was studied. Phages were applied on biofilm formed by *S. epidermidis* and reported success in biofilm eradication. Biofilm was completely eradicated in single trial (Wood *et al*., 2001).

Goodee et al., (2003) studied effect of phages on *Salmonella enterica*. When phage with 0.6 MOI titer was applied on chicken skin contaminated with Salmonella, 1 log reduction in bacterial density was observed. Phages applied in 100 or 1000 MOI reduced bacterial density by 2 $\log_{10}$ reduction in 48 hrs.

Sillankorva *et al*. (2004) studied effect of phage treatment on planktonic form of bacteria. Broth suspended biofilm of *P. fluorescens* were treated with phage sand observed one log reduction on planktonic population.

Sharma et al., (2005) studied comparison of phage and chemical treatment to kill E.coli O157:H7 biofilm formed on SS plates. More than one log reduction was observed with phage treatment as compared to 4-5 log reduction for alkaline cleanser. The potential of phage to eradicate biofilm can be enhanced by increasing phage adsorption to bacterial surface.

Lu and Collins (2007) used engineered phage to eradicate biofilm. Using genetic engineering, Phage T7 was constructed t express EPS depolymerase enzymes which is normally not present in phages. The engineered T7 phage reduced biofilms 4.5 logs as compare to both wild-type T7 phage as well as a control (phage untreated) of biofilm. So they have shown that the engineered phage have shown greater potential to eradicate biofilm as compare to wild type phage.

Jamal *et al.* (2015) have described application of bacteriophage Z to control MDR *K.pneumonia* in planktonic form as well as biofilm. Bacteria were grown both as biofilm and as suspension and then effect of phages was analyzed in both conditions. Biofilm reduction of 2log and 4 logs was observed after 24 and 48hrs respectively.

2.12 SALMONELLA TYPHI

Salmonella is genus belonging to family Enterobacteriaceae, has two species i.e. *enterica, bongori*. *Salmonella enterica* has further 6 subspecies: arizonae, diarizonae, enterica, salamae, indica, houtenae. Salmonella enterica sub specie

enterica has 2500 serotypes including Typhi, Paratyphi, Typhimirium, Entriiditis, and Choleraesuis (Gianella RA, 1996).

It is a rod-shaped, flagellated, facultative anaerobic, Gram negative bacterium. Unlike other salmonellas, Salmonella typhi can infect human and is causative agent for spread of typhoid disease that is most prevalent disease in underdeveloped areas (Hensel M, 2009). S. typhi usually invades the surface of the intestine in humans, but have developed and adapted to grow into the deeper tissues of the spleen, liver, and the bone marrow. Symptoms most characterized by this disease often include a sudden onset of a high fever, a headache, and nausea. Other common symptoms include loss of appetite, diarrhea, and enlargement of the spleen (depending on where it is located). These bacteria possess multi drug resistance genes that make it difficult to eradicate infections (Falkow *et al.*, 2004).

2.13 VIRULENCE OF SALMONELLA

Salmonella produces endotoxins, property of gram negative bacterium and it also possess Vi gene that contribute towards increase in its virulence. It is not affected by innate human immune system as it has ability to inhibit oxidative burst of leukocytes. It also produces invasion protein that facilitates bacterium to enter into non phagocytic cells (Feasey et al., 2012).

2.14 ANTIBIOTIC RESISTANCE OF SALMONELLA:

Since 1989, Salmonella had been source of many outbreaks in Africa and other under developed countries due to its resistance against many antibiotics like ampicillin, trimethoprim and chloramphenicol. Chromosomally inserted resistance

against ciprofloxacin has also been now observed in some strains found in United Kingdom (Rowe et al., 1997).

Increase in resistance of bacteria towards antibiotics has led to find out an alternative way for eradication of this bacterium. One such alternative is the one proposed by Goodridge & Bisha, 2011.

Chapter 3

MATERIALS AND METHODS

3.1 SAMPLING

3.1.1Water Sampling

Water samples were collected in sterilized 500ml bottle from canals, waste water treatment plant, waste water channels and industrial waste from different areas of Pakistan. More than one sample had been collected from one particular location (Table 3.1). Samples had been stored at 4ºCfor processing.

Table 3.1; Cities, localities and no. of samples collected

Sr.no	City	Area	No. of samples
1.	Lahore	Green town, Township, Allama Iqbal town	25
2.	Islamabad	Waste water channel near Shifa International Hospital, Waste water channel at G-10	10
3.	Rawalpindi	Nala lai, Girja road Union council, Tench bazar	15

3.1.2 Bacterial Sampling

20 samples of Salmonella typhi were collected from Armed Forces of Institute of Pathology (AFIP), Military Hospital Rawalpindi, during 10 October to 20 November in form of glycerol stocks. In AFIP, bacterial samples were isolated from blood samples of patients suffering from typhoid (data given in Table 2.2) and series of tests were performed to confirm the type of bacterium present. Bacterial strains were cultured and

sub cultured on plates. Fresh glycerol stocks were prepared and stored at -80ºCto use it in future.

Table 3.2 Patients data suffering from typhoid fever

Sample no.	Age (years)	Gender	Source
01	04	Male	Blood
02	06	Female	Blood
03	10	Female	Blood
04	05	Male	Blood
05	13	Male	Blood
06	02	Male	Blood
07	18	Female	Blood
08	11	Male	Blood
09	07	Female	Blood
10	13	Female	Blood
11	11	Female	Blood
12	13	Female	Blood
13	08	Male	Blood
14	06	Female	Blood
15	12	Male	Blood
16	20	Male	Blood
17	13	Female	Blood
18	11	Male	Blood
19	06	Female	Blood
20	15	Female	Blood

3.2 BACTERIAL SUB CULTURING

For sub culturing of bacterial strains taken from AFIP, a single colony of strain from nutrient agar Petri plate was picked up and inoculated in a test tube containing Luria Broth (LB). The inoculated tube was incubated at 37ºCfor 24 hours in shaking incubator (memmert). LB media consisted of 5.0 g Yeast extract (Uni chem.), 10 g NaCl (Scharlu), 10 g Tryptone (Oxoid) in 1000 mL distilled water (dH2O).

3.2.1 Growth on Differential Media

Bacterial strains were allowed to grow on MacConkey agar plates. 20 MacConkey agar plates were prepared and each bacterial strain was streaked on plate and plates were allowed to grow overnight at 37 ºC. On next day bacterial colonies were analyzed. MacConkey agar was consisted of 17 g Peptone, Protease peptone - 3 g, Lactose - 10 g, Bile salts - 1.5 g, Sodium chloride - 5 g, neutral red - 0.03 g, Crystal Violet - 0.001 g, Agar - 13.5 g, Water - add to make 1 liter. Adjust pH to 7.1 +/-0.2. Boiled it to dissolve agar then sterilized at 121° C for 15 minutes.

3.2.2 Gram Staining

A single colony from nutrient agar plate was selected and picked up using sterilized inoculating loop and smeared on a glass slide. The smear was then heat fixed. After heat fixation, crystal violet was applied on a plate and it was allowed to stain for 2 minutes. Crystal violet was then washed using water and then drops of Gram iodine were added for one minute. Then slide was flooded with tap water. 95% ethanol was poured on the slide as decolorizing agent to remove primary strain. After washing with water, safranin was added as counter-stain and allowed to stain for one minute. The slides were then washed with water and air dried. A light microscope was used to observe slide with 100X lens using emersion oil (Merchant and Packer, 1967).

3.3 SELECTION OF BACTERIAL STRAINS FORMING EFFECTIVE BIOFILM

Bacterial strains were selected which exhibits good capability of biofilm formation. To check this, bacterial strains were subjected to biofilm formation using 96 wells plate method. This method is derived from the method described by Christensen (1985). For this method bacterial culture were allowed to grow up to stationary phase in 3-5 ml culture tube using LB media in static incubator (memmert) 37 ºC. On next day, bacterial culture was diluted in LB media in ratio of 1:100. About 100ml of diluted bacterial culture was poured into wells of 96 well culture plate. The plate was covered with a lid and incubated at 37ºCfor 48 hours in static incubator. After 48 hours, the planktonic cells and culture media was removed from wells and pipetted into collection chamber containing 10% bleach solution. This step was repeated three times. After this, added 125 µl of 1% crystal violet solution. The adhered cells were allowed to stain for 10-20 minutes at room temperature. After this, crystal violet solution was removed by pipetting out into a collection chamber. Remaining crystal violet solution was removed using distilled water. This step was repeated 3-4 times. After washing, 200 µl of dimethyl sulfoxide (DMSO) was added and contents of well were mixed thoroughly by pipetting in and out. By using ELISA plate reader, optical density (OD) of each sample was measured at 500-600 nm.

3.4 ANTIBIOTIC SENSITIVITY OF THE CLINICAL BACTERIAL STRAINS

According to Clinical and Laboratory Standards Institute (CLSI) guidelines, sensitivity of bacterial strain to different antibiotics was determined (Wayne, 2007). The susceptibility of Salmonella was determined by conventional disc diffusion method using *Staphylococcus aureus* as a control. Five different antibiotics were used for disc diffusion method. These are Ampicillin (AMP-25), Erythromycin (E-15), Streptomycin (S-10), Ciprofloxacin (CP-10) and Chloramphenicol. The strain was uniformly swabbed on LB agar Petri plates and then discs of different antibiotics were placed at a suitable distance from each other on these plates. Plates were allowed to incubate overnight at 37 ºC.

3.5 PHAGE ISOLATION, AMPLIFICATION AND TITRATION

Waste water samples collected from different areas of Pakistan were used to isolate phages against Salmonella typhi. For phage isolation, an established methodology with some modifications was used (Jamalludin *et al.*, 2007). Bacterial culture was grown in Laurie Both (LB) media at 37ºCfor 24 hrs. Before using waste water samples were centrifuged for removal of algae and other suspended particles. After centrifugation, a 20ml water sample was poured into specific sterilized 250 ml flask in aseptic condition. Then 40 ml of LB media supplemented with $MgSO_4$ and 20 ml of bacterial strain (OD=1) grown in LB media were added into flask aseptically and allowed to incubate at 37ºCin shaking incubator at 130rpm for 18 hrs. After incubation, the mixture was centrifuged at 6000rpm for 30 minutes and the supernatant was filtered using membrane syringe filter (0.45mm) and was collected in separate sterile falcon tubes. This process was done for

every water sample separately. The filtrate was then subjected to spot assay to check its phage activity. For this purpose, different strains of Salmonella typhi were swabbed on different plates to make bacterial lawn and then 10-15 µl of each filtrate was spotted on each plate and allowed to incubate for 37 ºC. After analyzing results, filtrates showing good results were selected for further processing. The serial dilutions of filtrates were made up to 10^{-10} in phage buffer {Tries Hall (1 M, pH 7.5), NaCl (5.8 g), $MgSO_4.7H_2O$ (2.0 g) and gelatin (5.0 mL/L in distilled water}.100µl of each phage dilution was poured in separate test tube and 100µl of bacterial cultures was added in it. Then mixture was allowed to incubate for 2-3 minutes at room temperature. 2-3 ml of top agar (7% agar) was added in mixture, mixed it and immediately spread it on separate LB agar Petri plates and allowed it to cool down. Then these plates were placed in an incubator for overnight at 37 $^{\circ}C$. For purification of phage, isolated plaques were cut using sterile blades and poured in a flask containing 50ml bacterial culture in LB media. Flask was allowed to incubate at 37ºCovernight at 120 rpm. After incubation, phage filtrate was centrifuged at 6000rpm for 20 mints to settle bacterial cells and supernatant was syringe filtered (0.45mm) and stored at 4 ºC.

3.6 CHARACTERIZATION OF BACTERIOPHAGES

3.6.1 Host Range Determination

The host range of phage was determined by checking susceptibility of different clinical bacterial strains against the isolated phage. The bacterial strains included are *Staphylococcus aureus, E.coli and Pseudomonas aeruginosa.* Spot test was used to analyse phage susceptibility against these strains ((Zimmer *et al.*, 2002). For this purpose,

bacterial lawn of each strain was made and 10-20 µl of phage was spotted on each plate and then allowed to incubate at 37 ºC. For cross checking soft agar overlay method was used described by Sillankorva *et al.* (2008). After incubation, plates were checked for plaque formation against uninfected negative controls.

3.6.2 Thermal Tolerance of Phages

Heat tolerance for phage was analysed by the method described by Cepra et al., (2007) with some modifications. Phage suspensions were taken in different Eppendorf tubes and incubated at different temperatures i.e. 37ºC(control), 45 ºC, 55 ºC, 65 ºC, 70ºC and 80ºCfor 1 hour. After incubation, soft agar overlay method was used to check the survival rate of each phage.

3.6.3 pH Stability

To check pH tolerance of phages, a procedure given by Cepra et al., (2006) was adopted with modifications. A pH gradient was established at 3,5,7,9 and 11. One ml of phage suspension was added in 9ml of tryptic soya broth containing bacteria at specific pH and was incubated at 37^{o}C for 16 hrs. After incubation, soft agar overlay method was used to check the viability of phages.

3.6.4 Calcium Ions Effect on Adsorption Rate of Phages

To check the effect of divalent metal ions on phage adsorption, phages were treated with $CaCl_2$ and $MgCl_2$ separately. A 75ml bacterial culture was taken and was divided into three flasks. One flask is inoculated with phages, second with phages as well as $CaCl_2$ (10Mm) and in third flask added phages as well as $MgCl_2$. Samples were taken out of flasks after the regular interval from control and $CaCl_2$ and $MgCl_2$ treated flask.

Soft agar overlay method was used to determine the number of free phages in each flask (Capra *et al.,* 2006). The difference in a number of phages in all flasks gave the effect of divalent metal ions on adsorption of phages.

3.6.5 One Step Growth

The burst size and latent period of phage were calculated by one step growth method that was described by Sambrook *et al.* (1989). In this method, 50ml of bacterial culture with OD of 0.4-0.6 was centrifuged for cell harvesting. The cells were pelleted down and then the pellet was resuspended in 0.5ml LB media. Then 0.5 ml phages ($3x10^8$phages/cell) were added in it and allowed the phage for adsorption to bacterial cells. After this, mixture was centrifuged at 14000rpm for 30 sec to remove unabsorbed phages. The pellet was added in 100ml LB media and allowed to incubate at 37 ^oC with constant shaking (120rpm). After each 3 minutes, samples were collected from the flask, serially diluted and soft agar overlay method was used to calculate a number of phages.

3.7 SUSCEPTIBILITY OF PLANKTONIC CULTURES TO PHAGES

Susceptibility of bacterial strains towards phages was analyzed by the method described by Cerca et al., (2007). Cell suspension of bacterial strain was adjusted to < 2 x 10^8 cells/ml using normal saline and was added in flasks containing Tryptone soya broth (TSB) media followed by incubation at 37 $^\circ C$ with continuous shaking at 120 rpm until bacterial density reached up to > 2 x 10^8 cells/ml. Then phage suspension was added in flasks with different multiplicities of infection (MOI) i.e. 0.1, 0.5 and 5. One flask was taken as control that did not contain phages. All these flasks were incubated for 5hrs at shaking incubator (120 rpm, 37 $^\circ C$). Samples were collected from each flask after every

hour and its OD was taken at 600 nm. The serial dilutions of each sample were made, and spread on LB agar plates in triplicates followed by incubation at 37 °C. For calculation of phage titer serial dilutions were made and soft agar overlay method was used. This method was repeated three times.

3.8 SUSCEPTIBILITY OF BIOFILMS TO PHAGES

Biofilm of susceptible bacterial strain was developed by the method described earlier by Cesar *et al.*, (2005) with some modifications. The bacterial culture was grown overnight up to 2 x 10^8 cells/ml was diluted in TSB media in 1:100 ratio. Bacterial culture was then poured into wells of 96 wells plate (Corning incorporated), its lid is covered and placed in shaking incubator at 120 rpm at 37 °C for 24 hrs. After that bacterial culture along with planktonic cells were pipetted out of wells and poured into collection chamber containing 10% bleach solution. After washing step, phage diluted in normal saline was added in half of wells and other half wells were taken as control and placed it in an incubator under same conditions. On next day, phage titer was removed, and distilled water was used to wash wells. Then added crystal violet solution (1 %) in all wells and allowed to stain for 10-20 minutes. After that, crystal violet solution was pipetted out of wells and wells were washed using distilled water. Plates were kept inverted to get dry. The OD of the plate was taken using ELISA reader at 570 nm. The experiment was repeated three times.

3.9 BIOFILM STUDY USING STAINLESS STEEL PLATES

3.9.1 Development of Biofilm on Stainless Steel Plates

In this method stainless steel plates (1mm x 1mm) were used to study biofilm formation in six wells plate. For this purpose, stainless steel (SS) plates were first washed by 100 % acetone followed by immersion in NaOH for about one hour. Then plates were rinsed with distilled water and 70 % ethanol was sprayed on them. These plates were then placed in incubator at 60 °C for one hour followed by autoclaving at 121 °C for 15 minutes. Biofilm formation was taken place at two conditions i.e. static and dynamic without removal of media after 12 hrs. Biofilm formation on SS plates was studied at different time intervals. The conditions for the study of biofilm formation on SS plates were given in Table 3.3.

Table 3.3: Conditions for Biofilm Development

Sr.no	Conditions	Time of biofilm development incubation at 37 °C	
1.	Static without removal of media	24 hrs	48 hrs
2.	Dynamic without removal of media	24 hrs	48 hrs

3.9.2 Development of Biofilm and its CFU Determination

The method used for the study of biofilm formation on SS plates was the one described by Cerca *et al.*, (2005) with slight modifications. The SS plates after sterilization were placed in 6 well plates (Corning incorporated), and 50µl bacterial culture was poured onto the plate. Then 6ml TSB was added in wells in the way as to not disturb the bacterial culture on SS plates. The six well plates were then allowed to incubate at different conditions i.e. one plate at 37 °C at the static condition and another plate at shaking incubator (120 rpm, 37 °C). After 24 hrs one SS plates were taken out from each well of plate and immersed in freshly prepared 1X PBS so as to not disturb biofilm and then placed in a falcon tube. Then normal saline was added and falcon tube was vortexed vigorously. Finally, serial dilutions up to10^{-8} were made from this suspension and spread on nutrient agar plates for CFU count followed by incubation at 37 °C. The same steps were repeated after 48 hrs incubation of SS plates in 6 well culture plates. This process was repeated three times.

3.9.3 Application of Bacteriophage

Bacterial biofilms formed under different conditions were subjected to phage treatment. The biofilm formed on SS plates after 24 and 48 hrs were treated with phages for 4 hrs and then the activity of phages was analysed by taking its CFU and PFU count. After phage treatment, SS plates were immersed in freshly prepared 1X PBS and then put into falcon tubes, and 6ml normal saline was added in it. Then vortexed it vigorously and made dilutions from these suspensions. These dilutions were spread on nutrient agar

plates and allowed for incubation at 37 °C for calculating CFU. For calculating phage titre, dilutions were made soft agar overlay method was used.

3.10 Scanning Electron Microscopy

Biofilm formed on SS slides were fixed by the method as described previously of by Sillankorva *et al.* (2008) with some modification. After biofilm formation on SS plates these were removed form media with the help of sterilized forceps and carefully dipped in PBS (1X) solution for washing. Repeat washing step thrice. After PBS washing the SS plates were placed in glutaraldehyde (2.5 %) at 4 °C and fixed for one hour. After fixation the SS plates were subjected to drying procedure by using ethanol series. Biofilm samples were dehydrated with an ethanol series ranging from 30 % to 100 %. Each step of ethanol treatment was ranging from 5–10 min. After drying the biofilm SS plates were further processed for SEM analysis. Biofilm formed on SS plates were mounted on carbon adhesive tabs on aluminium specimen mounts. SS plates were rendered for conduction with Au/Pd, (Denton Desk II sputter coater). For biofilm detection, both treated and control SS plates were examined under scanning electron microscope at Institute of Space Technology (IST), Islamabad, Pakistan.

3.11 DOCKING STUDIES

To study phage bacterial interaction, docking studies were done. It helped to study how phage protein interacted with bacterial protein to get adsorbed on bacterial cells. Based on extensive literature study, phage and bacterial proteins were identified which are involved in bacterial-phage interaction. So a hypothetical model based on this literature was proposed to explain interaction of ST1 phage with Salmonella typhi.

The literature showed that Salmonella typhi had outer membrane proteins that are involved in the interaction of bacteria with antibiotics and phages. These proteins were being searched in RCSB protein databank and their data was collected.

TABLE 3.4 Proteins of Salmonella typhi involved in phage-bacterial interaction

Sr.no	Protein Name	PDB ID
1.	OMPF	3NSG
2.	Oligo Peptide Binding Protein	1QKA

Study of phages of different seravors of Salmonella enterica showed that they involved tail spike proteins for adsorption to the bacterial receptors. Different tail spike proteins were being searched in RCSB protein databank and their data was collected.

TABLE 3.5 Proteins of phages involved in phage-bacterial interaction

Sr.no	Protein Name	PDB ID
1.	Tail spike protein	1TYU
2.	Tail spike protein	1TYV

Using Z dock database, docking was done between phage and bacterial proteins in the order given in Table 3.6 and results were obtained within few minutes that were visualized in Discovery software.

TABLE 3.6 Order of docking between bacterial and phage protein

SR.NO	BACTERIAL PROTEIN	PDB ID	PHAGE PROTEIN	PDB ID
1.	OmpF	3NSG	TAIL SPIKE PROTEIN	1TYW
2.	Oligo peptide Binding Protein	1QKA	TAIL SPIKE PROTEIN	ITYU

CHAPTER 4

RESULTS

4.1 BACTERIAL Sub Culturing

Bacterial strains Salmonella typhi obtained from AFIP were further sub cultured on agar plates. On MacConkey agar, white colonies appear which showed these bacterium to be non-lactose fermenting bacterium (Figure 4.1) Furthermore, biochemical test (Gram staining) were performed and Gram staining results revealed that these were Gram-negative bacteria (Figure 4.2).

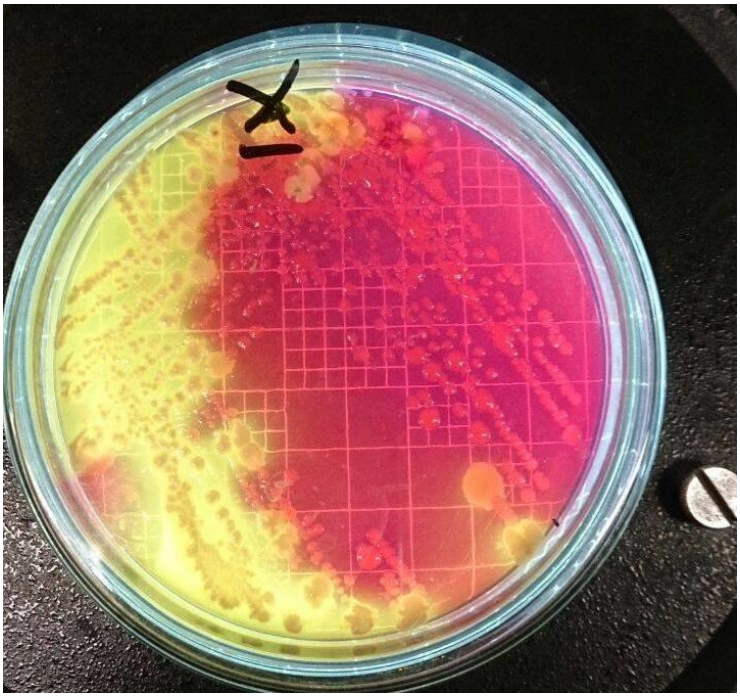

Figure 4.1: White colonies on MacConkey agar

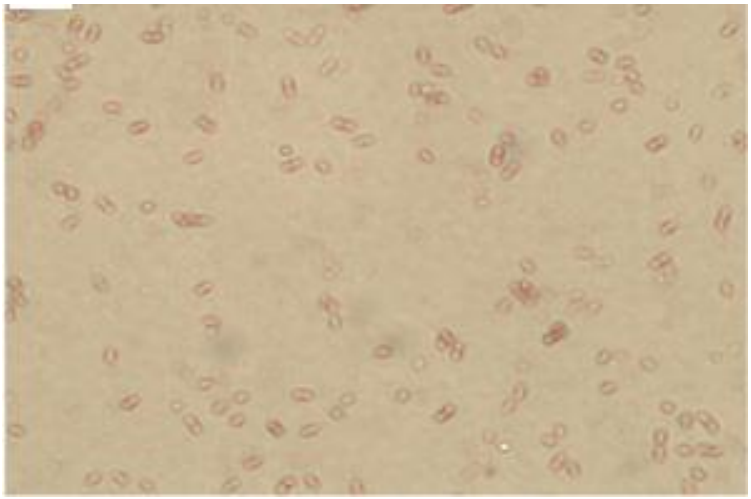

Figure 4.2: Gram staining of bacterial strains.

4.2 SELECTION OF BACTERIAL STRAINS FORMING EFFECTIVE BIOFILM

Twenty bacterial strains were tested for biofilm formation on 96 well cell culture plates and OD was measured at 600nm and among these eight strains form significant biofilm. Of these eight strains, only one (strain 6) was further processed for studying the effect of phages on biofilm formation.

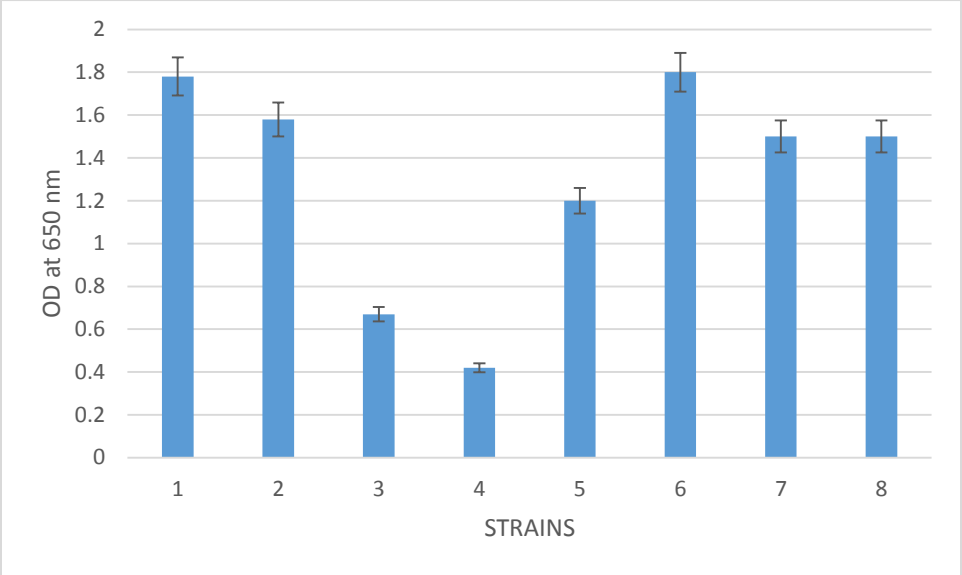

Figure 4.3: Selection of bacteria on biofilm formation: Bacterial strains were cultured in 96 well plates for biofilm assay and then OD was measured at 650 nm.Values show mean of 3 readings ± 2(SD).

4.3 ANTIBIOTIC SENSITIVITY OF CLINICAL BACTERIAL STRAINS

Antibiotic sensitivity of was Salmonella typhi was checked according to CLS1 guidelines. Results are shown in Table 4.2. The result shows that bacterial strains are resistant towards Ampicillin, Erythromycin, Streptomycin and Tetracycline. Ciprofloxacin and Chloramphenicol are still active towards Salmonella.

TABLE 4.1: Antibiotic Sensitivity of Salmonella typhi

Sr.no	Antibiotics	Antibiotic activity in mm
1.	Ampicillin	0
2.	Erythromycin	0
3.	Tetracyclin	0
4.	Ciprofloxacin	10
5.	Streptomycin	0
6.	Chloramphenicol	16

4.4 ISOLATION OF PHAGES

Using Salmonella typhi as host organism, phages were isolated by soft gar method. All the phages gave a clear zone on spot test (Figure 4.4). One phage is selected that give significant plaque towards strain 12. This phage was designated ST1. The presence of clear plaques on bacterial lawn shows these phages to be virulent phages. Phage ST1 has plaque ranging in size from 1 to 3mm. Each phage showed plaques with well-defined boundaries (Figure 4.4). Soft agar overlay method gives plaques with count of $5x10^7$ PFU/ml (Figure 4.5).

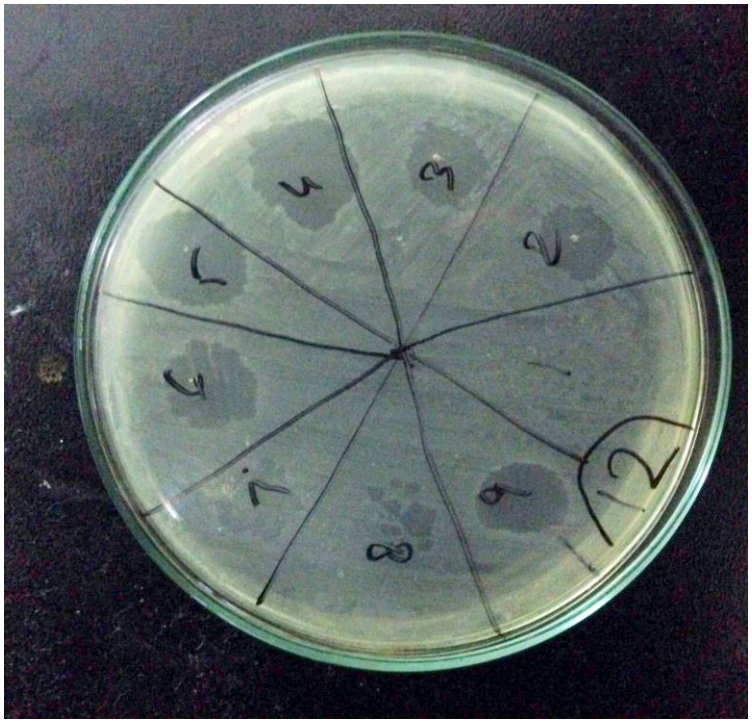

Figure4.4 Spot Assay: Soft agar plates are showing spot test. Phages form a clear spot on the bacterial lawn.

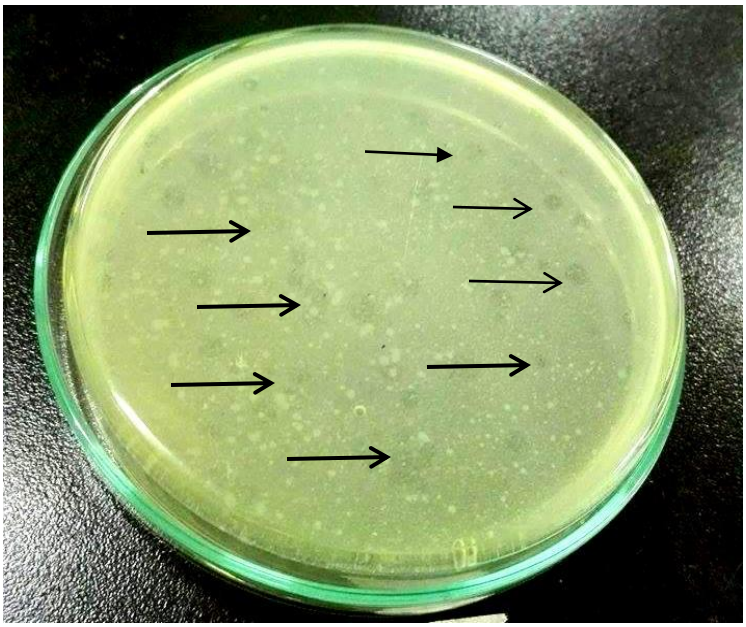

Figure 4.5: Soft Agar Overlay Assay: Soft agar overlay plate showing plaques of phage ST1. Phage shows plaque with clear boundaries.

4.5 CHARACTERIZATION OF PHAGES

4.5.1 Host Range Determination

About six bacterial strains were used to check host range of phages by spot assay. Phage ST1 shows activity against *E.coli* 1968, *Escherichia coli* 3051, *Staphylococcus aureus 2962, Staphylococcus aureus 2938* while phage showed no activity against *Pseudomonas aeruginosa 2949* and *Pseudomonas aeruginosa 3178.*

Table 4.2: Spot test of phage ST1 on different bacterial species.

Sr.no	Bacterial Strains	Activity
1.	*Pseudomonas aeruginosa 2949*	-
2.	*Pseudomonas aeruginosa 3178*	-
3.	*Staphylococcus aureus 2962*	+
4.	*Staphylococcus aureus 2938*	+
5.	E.coli LF 1968	+
6.	E.coli LF 3051	+

- **+= LYSIS, ─ = NO LYSIS**

4.5.2 Thermal Stability

Thermal stability test was carried out to check temperature range for phage ST1. One hour incubation at 37 $^{\circ}$C shows that phage retained its activity but gradual increase in temperature reduces capability of phage to survive with ultimately no phages at 70 $^{\circ}$C. The phage is stable at temperature ranging between 37 and 60 $^{\circ}$C (Figure 4.4).

The P value obtained is 2.2×10^{-8} that is less than 0.05. Thus data is statistically significant.

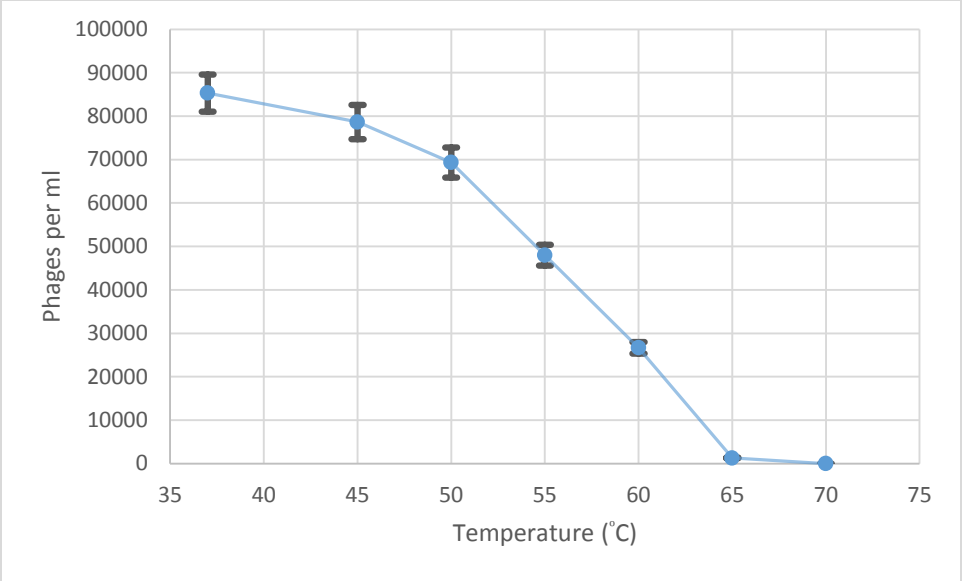

Figure 4.6: Thermal Stability: Stability of Phage ST1 treated with different temperature for 60 minutes. Values are mean of 3 readings ± 2(SD).

4.5.3 pH Stability

Optimum pH range for phage ST1 was determined by incubating it at different pH range (1, 3, 5, 7, 9, and 11) for 16 hours. The phage show stability at pH 7 and increase or decrease in pH causes decrease in phage titer. It shows good stability at pH 5, 7 and 9 but have no survival at pH 3 and 11.

The data is statistically significant as P value obtained is $1.1x10^{-8}$ which is less than 0.05.

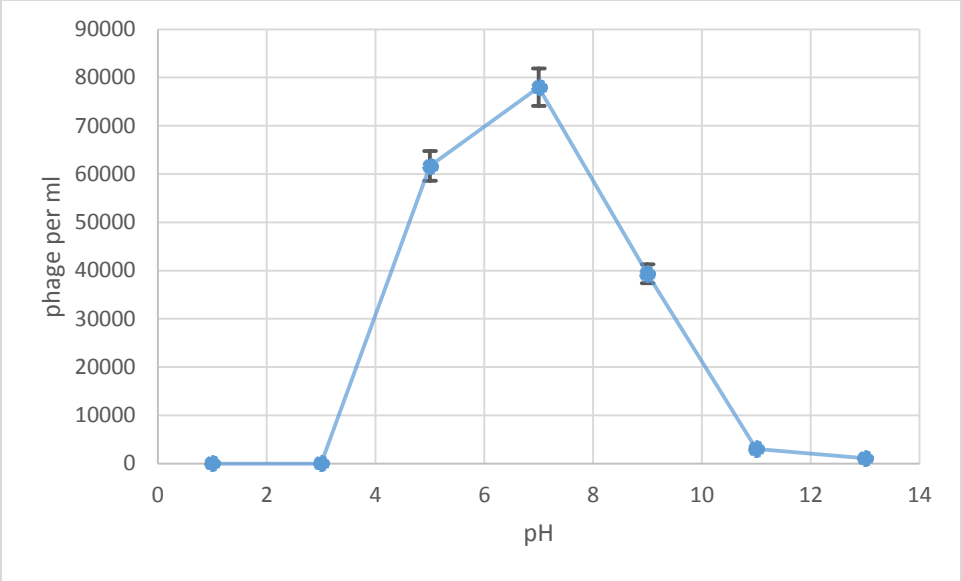

Figure 4.7: pH stability test phage ST1 grow on different pH range and shows stability at pH 5, 7 and 9. Values are mean of 3 readings ± 2(SD).

4.5.4 Calcium and Magnesium Ions Effect on the Adsorption Rate of Phages

The effect of Calcium (Ca^{+2}) or Magnesium (Mg^{+2}) ions on the adsorption of each phage was analyzed separately by adding (10 mM) Calcium Chloride or Magnesium Chloride to the phage and the host bacterial strain mixture. The number of free phages left in the solution (which was not bound to the bacteria) was detected for each phage separately at different time intervals of 0, 10, 20, and 30 min using the plaque assay. Data analysis showed significant differences between the control and the Calcium or Magnesium ion-treated phage ST1 (Figure 4.7). The statistical analysis shows result to be significant as P-value is less than 0.5 i.e. 0.0002, 0.0004 and 0.0002 for 10, 20 and 30 minutes respectively.

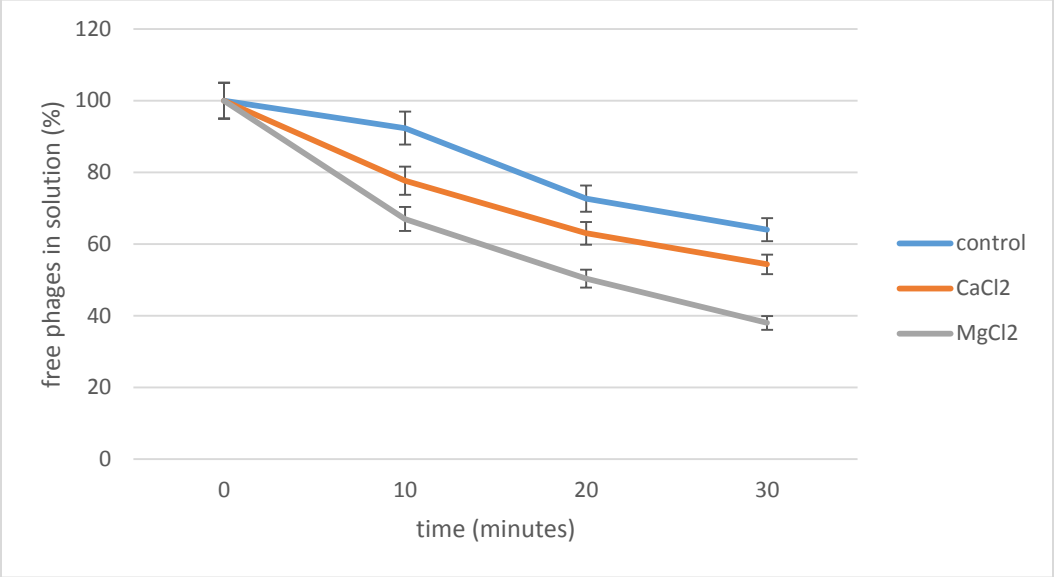

Figure 4.8: Adsorption rate test the effect of divalent metal ions on phage adsorption was observed at different time intervals and it turns out that $MgCl_2$ and $CaCl_2$ facilitate phage adsorption to bacteria. Values are mean of 3 readings.

4.5.5 ONE STEP GROWTH:

The single-step growth experiment was performed for determining the latent time periods and burst sizes of phage ST1. A triphasic curve was obtained for each phage that has the latent phase, log phase, and stationary phase. Determination of burst size was based on the ratio of the mean yield of phage. Phage ST1 had latent time period of 24 min, while showed burst sizes of 300 phages per cell.

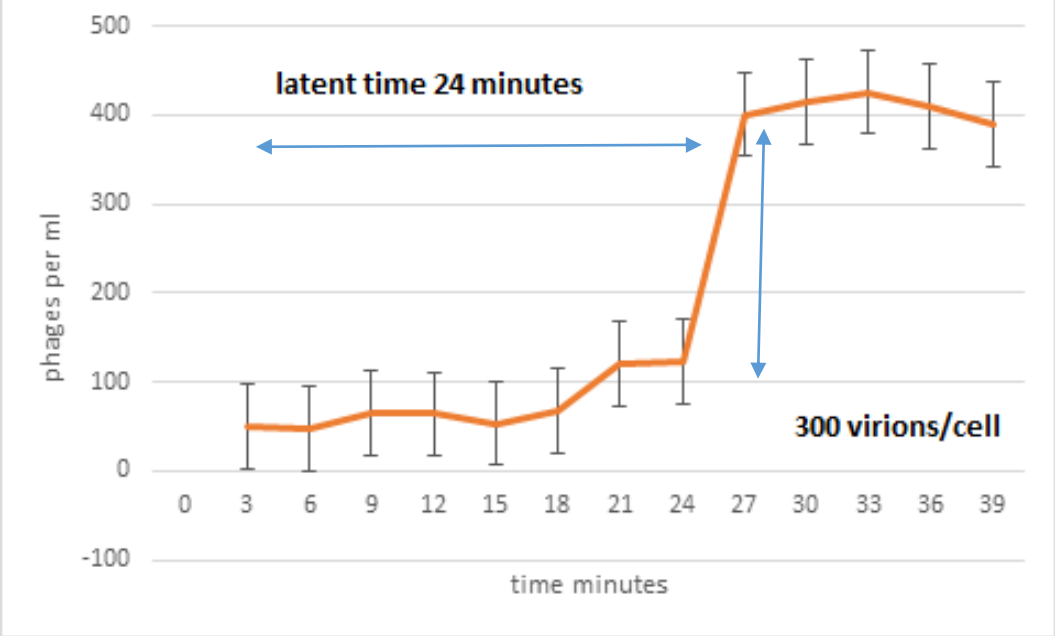

Figure 4.9 One step growth performed to calculate burst size and latent time period of ST1 and it found to be 300 virions per cell and 24 minutes respectively.

4.6 SUSCEPTIBILITY of BACTERIAL PLANKTONIC CULTURES to PHAGE

The highly susceptible bacterial strains were treated with different MOI of its respective host phages. The lytic activity of phage ST1 against planktonic cultures of Salmonella typhi in the exponential phases of growth are illustrated in Figure 4.9(A) and Figure 4.9(B).Salmonella typhi shows high susceptibility at all MOIs particularly at 5MOI. Cell growth was observed by taking ODs at different intervals {figure 4.9(A)} and also performed CFU count experiment at those intervals {figure4.9 (B)}.

The P value obtained for OD measurement at 1 hr., 2 hr., 3hr, 4hr and 5 hr. is .00002, ,00000004, 3.1 $x10^{-8}$, 4.5 $x10^{-8}$ and 1.2 $x10^{-7}$ respectively. As these values are less than 0.5 so the results are statistically significant. The P value for CFU count at

different time intervals is $3.7x10^{-8}$ $6.2\ x10^{-12}$, $4.6x10^{-8}$, 0.02 and $1.1\ x10^{-10}$ respectively. Thus this result is statistically significant.

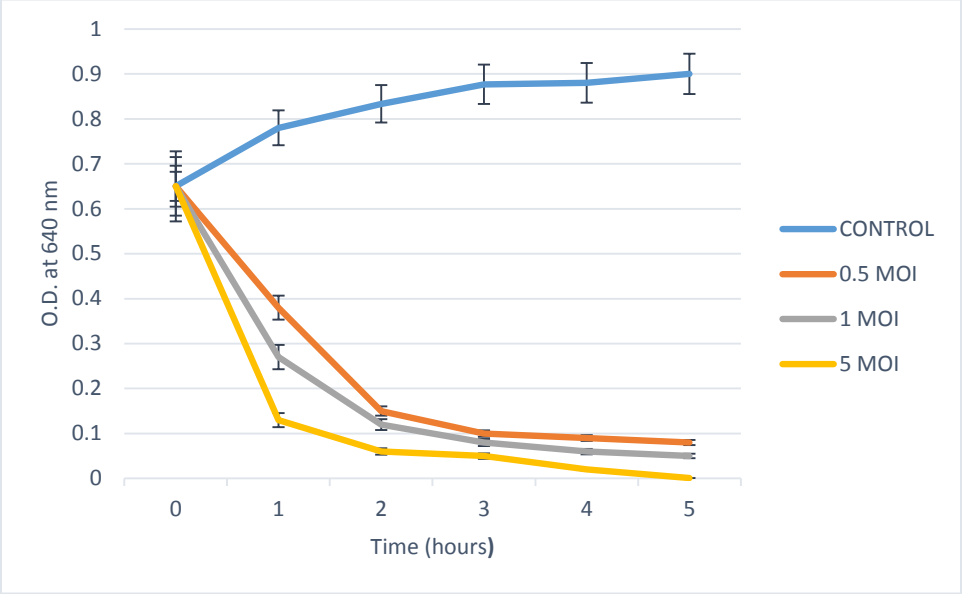

FIGURE 4.10(A): OD measurement Cell growth was observed in different samples (control, 0.5MOI, 1MOI and 5MOI) by taking OD at different time intervals. Values are mean of 3 readings ± 2(SD).

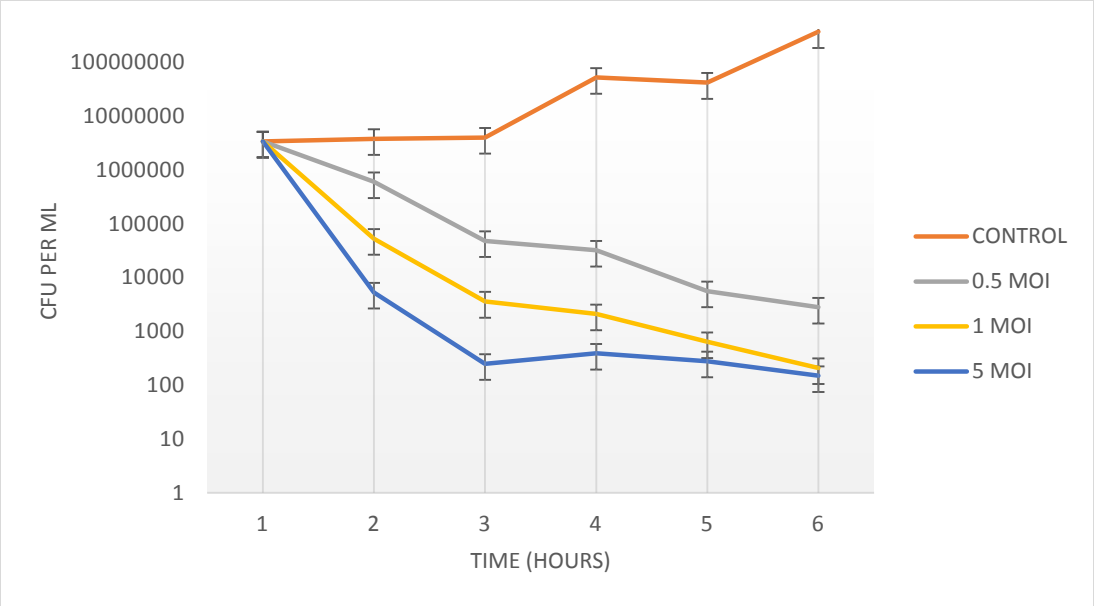

Figure 4.10(B): Bacterial growth reduction curve. Bacterial growth reduction curves of exponential growth phase planktonic Salmonella typhi by phage ST1, at different multiplicity of infection (MOI): 0.5, 1 and 5. Values show mean of 3 readings ± 2(SD).

4.7 SUSCEPTIBILITY OF BIOFILMS TO PHAGE

To determine action of phage on biofilm, 96 well cell culture plates were used to form biofilm for 24 hours and 48 hrs. Biofilm is then treated with phage ST1 and then reduction in biofilm was observed by taking difference between ODs of treated and untreated biofilm. It shows a clear reduction of biofilm (figure 4.11).

The P value obtained for biofilm formation and its treatment with phages at 24 hours and 48 hours is less than 0.05 i.e. 0.002 and 0.2 respectively.

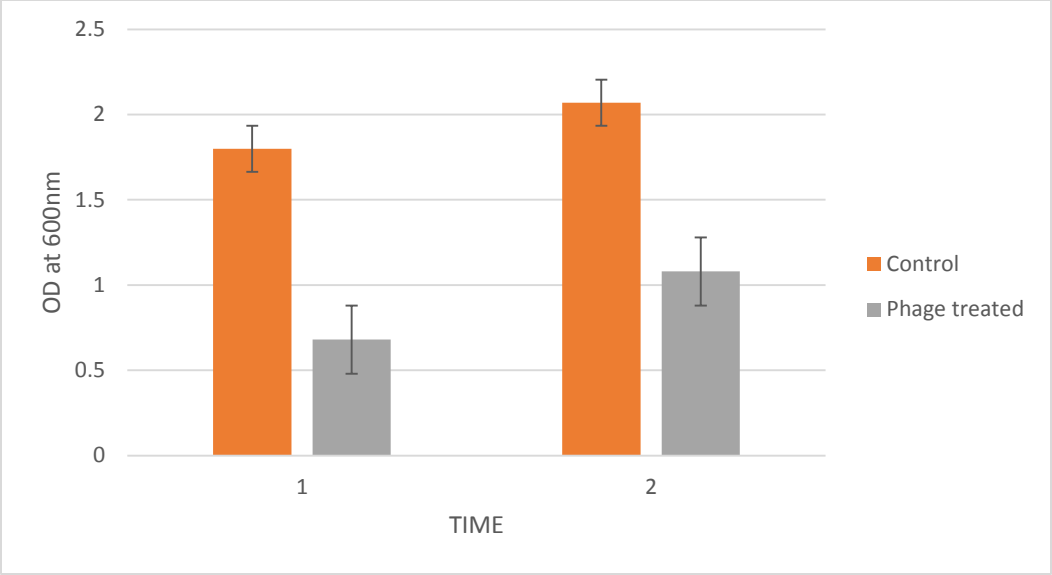

Figure4.11: Biofilm susceptibility to phage Reduction of biofilm biomass Salmonella typhi after treatment with ST1 phage at 24 and 48 hours respectively. Blue bars represent control biofilms without phage and orange bands represent biofilm infected with phage.

4.8 BACTERIAL BIOFILMS FORMED ON SS PLATES

Salmonella typhi biofilm formed, under static conditions with nonrenewal of media for 24 h and 48 h, were phage treated for 4 hour showing biomass reduction of 2.5 log and 2.8 log respectively {Figure 4.12(A)}

Biofilm formed, under dynamic condition with nonrenewal of media under dynamic conditions for 24hrs and 48 hours, formed were phage treated for 4 hour showing biomass reduction of 2 log and 2.2 log respectively {Figure 4.12(B)}.

The statistical analysis of data shows result to be significant as P value obtained for biofilm formation under static condition at 24 hours and 48 hours to be .001 and 0.2 respectively.

The P value obtained for biofilm formation under dynamic condition at 24 hours and 48 hours to be 0.02 and $7x10^{-5}$ respectively.

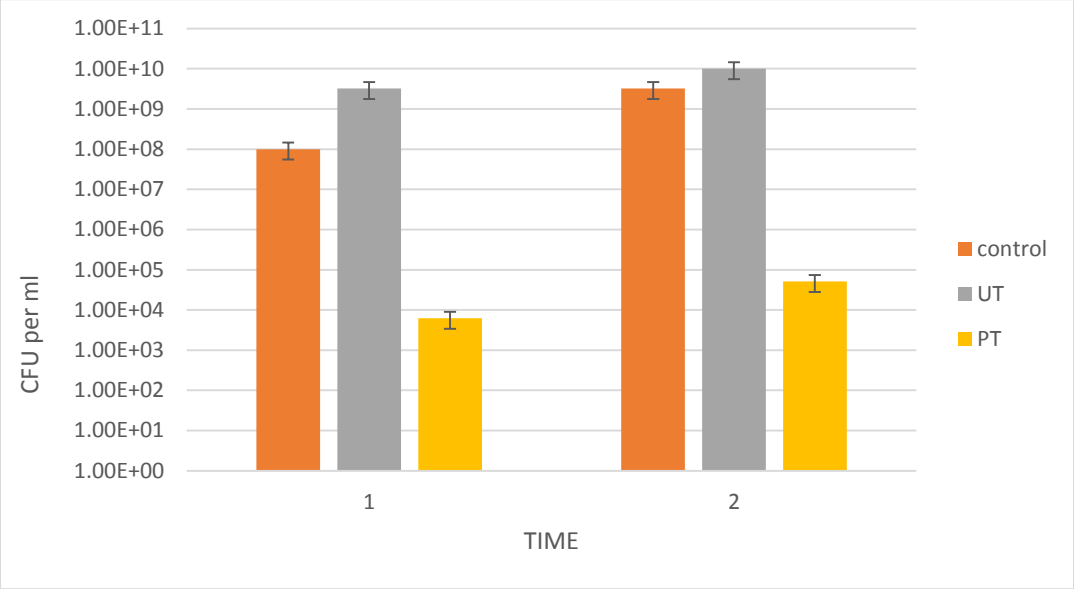

Figure 4.12(A) Biofilm formed on SS plates at static condition: Biofilm formed on SS plates was treated with phage for four. Brown bar represents control with 0hr, grey bar represents control 4hr and yellow bar represents 4hr phage treated biofilm. Value shows mean of 3 readings ± 2(SD).

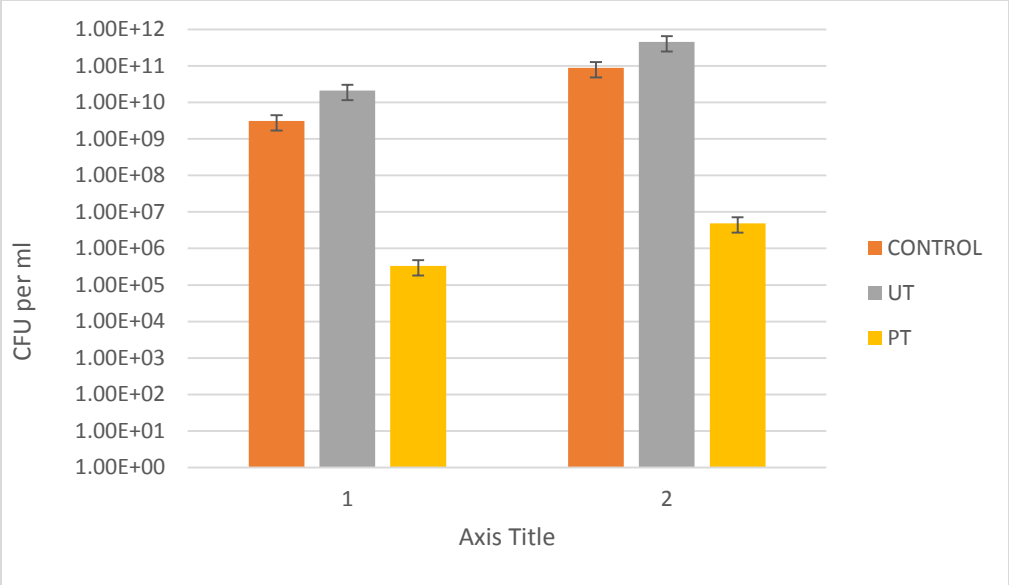

Figure 4.12(B) Biofilm formed on SS plates at dynamic condition: Biofilm formed on SS plates was treated with phage for four hours. Brown bar represents control with 0hr, grey bar represents control 4hr and yellow bar represents 4hr phage treated biofilm. Value shows mean of 3 readings ± 2(SD).

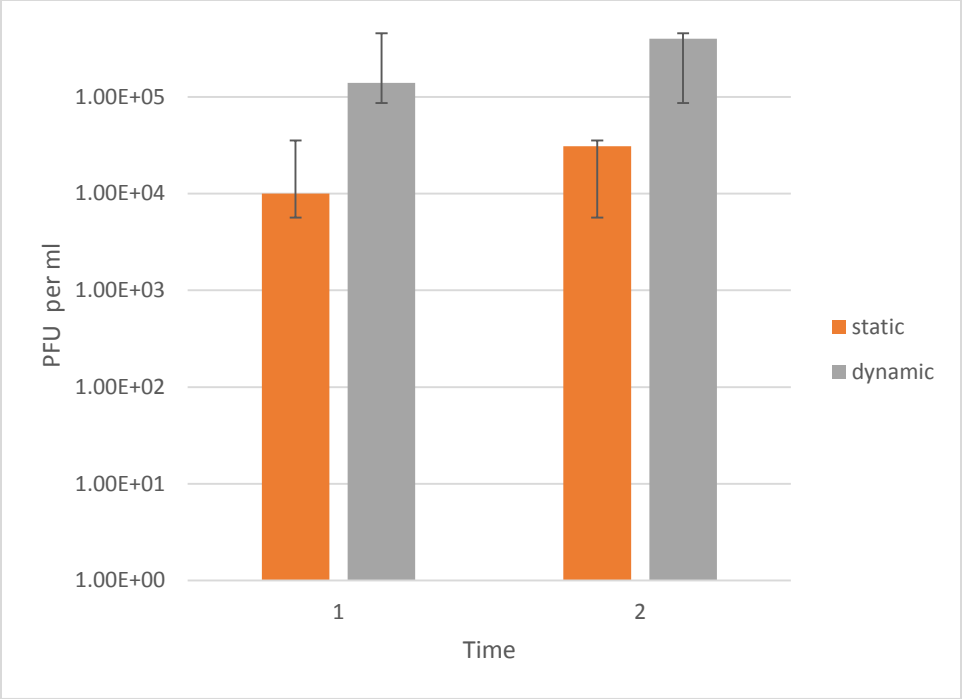

FIGURE 4.12(C): PFU counts on SS plates: Phages were counted on treated biofilm (24 and 48 hr.) under static and dynamic conditions. Brown bar represents phage count under static condition and grey bar represents phage count under dynamic conditions. Value shows mean of 3 readings ± 2(SD).

4.9 SCANNING ELECTRON MICROSCOPY

SEM was done to observe the pattern of biofilm formation .Figure 4.13(A) shows biofilm formation on SS plates after 48 hours while figure 4.13 (B) shows degradation of biofilm on SS plates due to phage treatment.

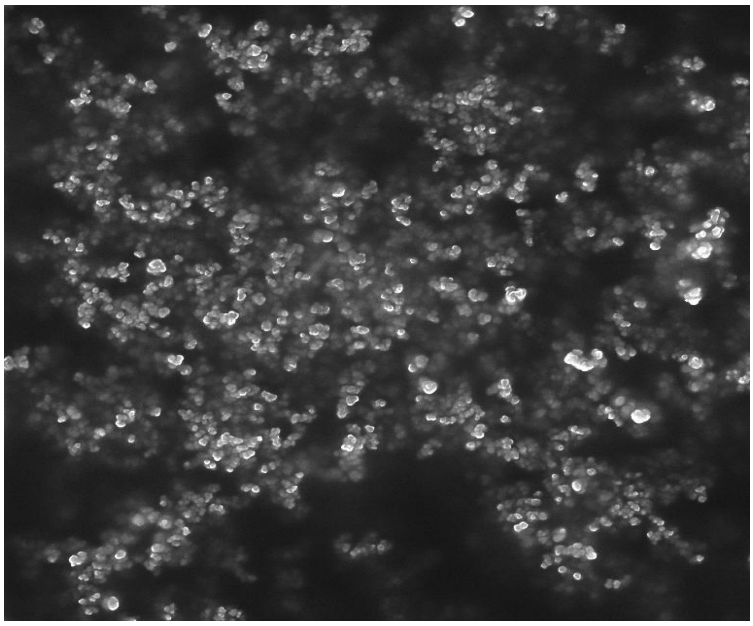

Figure 4.13(A) Micrograph showing Biofilm formation on SS plates after 48 hours

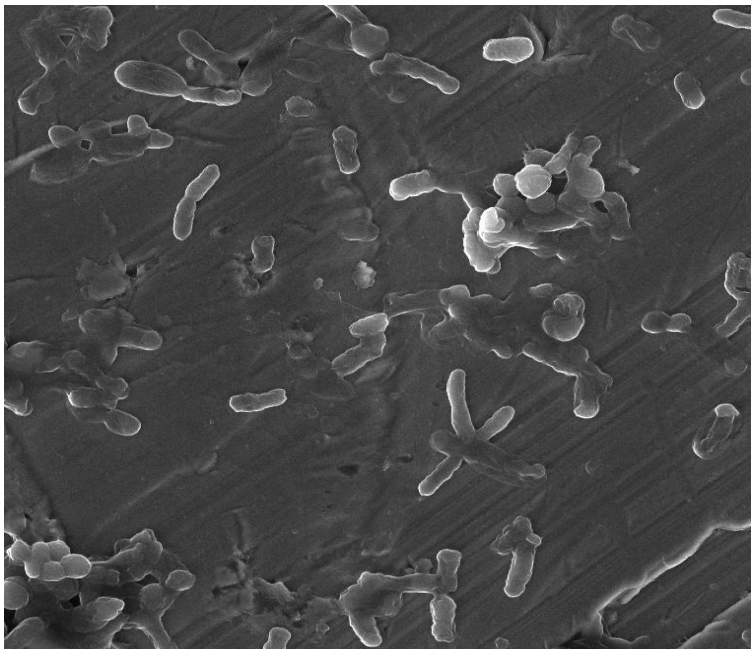

Figure 4.13(B) Micrograph showing effect of phage treatment on 48 hours biofilm

4.10 DOCKING STUDIES

As result of docking studies, two models were obtained which showed the interaction between phage proteins and bacterial proteins.

4.10.1 Model1

Model1 showed that phage protein bound with bacterial protein at 2 points. At one point, amino acids involved in binding include PRO93, GLY 342, TRP 494 of phages and LYS 492, GLU 344 and PHE343 of bacteria. Supporting amino acids of bacteria include THR499, GLY496 AND TYR 498 while that of phages include TRY417 and GLN416.

At other point, bacterial amino acids playing role in binding include VAL6, GLY7 and phage amino acid include LEU427, ILE 428.

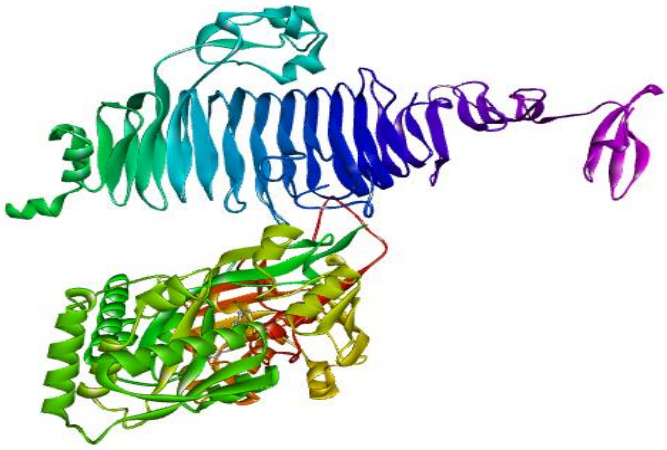

Figure 4.14 (A) Docking of bacterial protein (3NSG) and phage protein (1TYW)

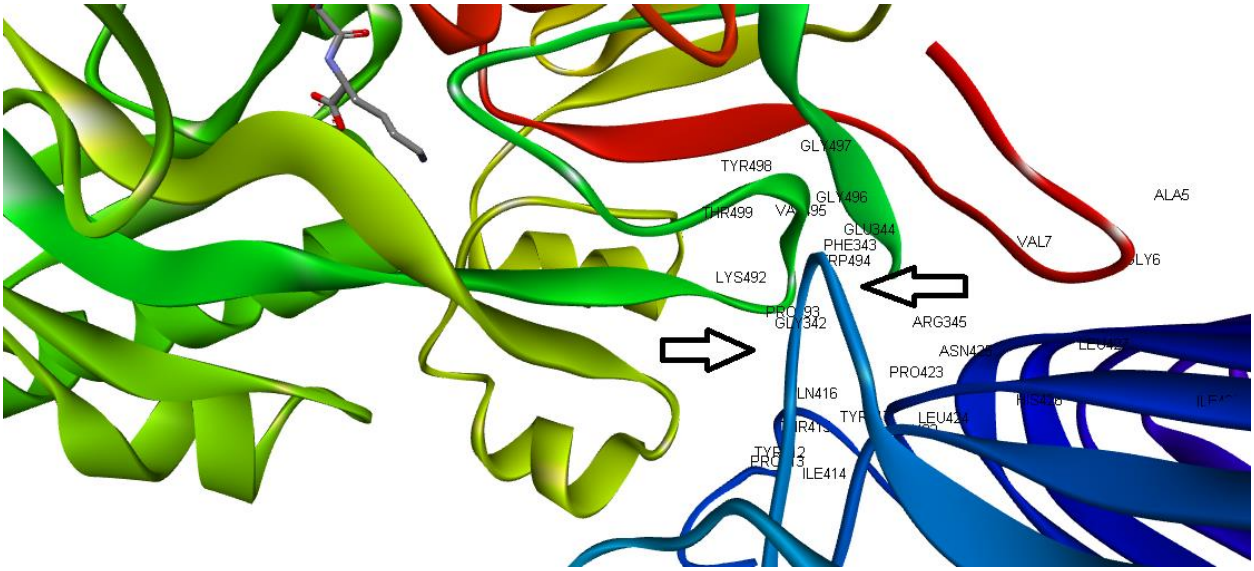

Figure 4.14 (B) Amino acids involved in binding

4.10.2 Model2

Model2 showed that phage protein bound with bacterial protein at one point and phage amino acids involved in this binding are GLY 342, ARG 345, ASP 346 and PHE 343 while bacterial amino acid include TRY 85, ALA 86. LEU 83, LYS 84 of bacteria plays a supportive role in binding of the protein.

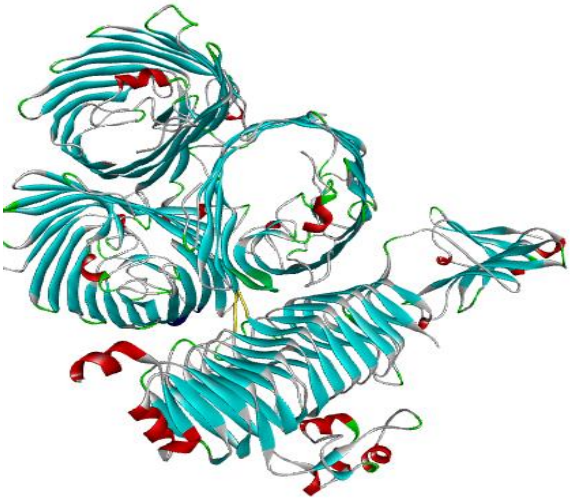

Figure 4.15(A) Docking of bacterial protein (1QKA) and phage protein (1TYU)

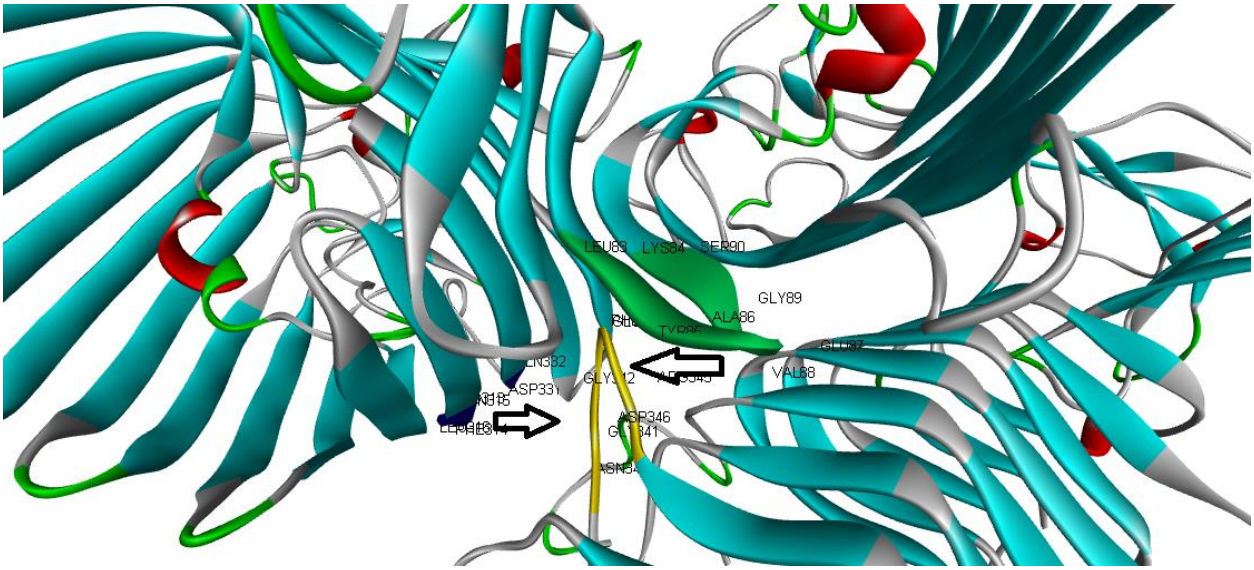

Figure 4.15(B) Amino acids involved in binding

Based on these two models, it is suggested that ST1 phage interacts with Salmonella typhi in the way presented in either model.

CHAPTER 5

DISCUSSION

Bacteriophages, the most common entity in the biosphere, composed of protein coat enclosing genome in the head (Grath and Sinderen, 2007).They infect bacteria and replicate within bacteria either by lysogenic cycle or by lytic cycle (Clark and March, 2006). Prokaryotic phages include 19 families. Among these 97% are tailed while remaining are either filamentous, polyhedral and pleomorphic (Ackermann, 2007).

The increased use of antimicrobials in healthcare and other settings has selected for resistant organisms (Archibald *et al.,* 1997; Sieradzki *et al.,* 2003). Bacteriophages are often considered alternative agents for controlling bacterial infection and contamination (Nakai and Park, 2002). Many phages have the ability to produce depolymerase that destroy bacterial biofilm.

Bacteriophages were discovered in 1896 and at first got much attention due to its antibacterial activity but with discovery of antibiotics, phage therapy faced major setback ((Abedon, Kuhl, Blasdel, & Kutter, 2011). Bacteriophages have many applications in industries as an alternative to antibiotics, carrier for protein/DNA vaccines, detection of pathogenic bacteria, targeted gene delivery and for screening protein libraries (Clark and March, 2006). They also have role in prevention of biofilm formation on infectious wounds and surgical instruments (Weiling *et al.,* 2009).

Biofilms are aggregates of microorganisms adhering to biological (living) or inert (non-living) surfaces, and are surrounded by an adhesive matrix of extracellular

polymeric substance (EPS) produced by the microorganisms within the biofilm (Rao *et al.,* 2005; Harper *et al.,* 2014). Donlan and Costerton (2002) reported that Leeuwenhoek was the first person to observe the bacterial biofilm of his own teeth (plaque) by using a simple microscope. Heukelekian and Heller (1940) observed increased growth of microorganisms in a container by providing surfaces to which these microorganisms could adhere. Zobell (1943) noticed that in sea water higher numbers of bacterial cells were adhered to surfaces when compared to bacteria floating in the surrounding medium.

According to Vasudevan (2014) the chemical composition of biofilms is very complex and comprised of various components such as microbial cells, extracellular polymeric matrices (EPS), proteins, lipids, DNA, RNA and enzymes. In addition to these components, water is the major part of biofilm that is responsible for the inflow of nutrients inside to the biofilm matrix.

Bacterial biofilm is infectious in nature and can result in nosocomial infections (Donlan, 2009). It has been estimated that biofilm is involved in more than 60 % of nosocomial infections (Donlan and Costerton, 2002; Vinh and Embil, 2005). According to National Institutes of Health (NIH) about 80 % of all chronic infections are associated with infectious biofilms (Monroe, 2007), and among all microbial infections about 65 % are related to biofilms (Potera, 1999).

It has been postulated that about 10^{31} bacteriophages are present in the environment (Ashelford *et al.*, 2000). It is very important to isolate and characterize new phages especially in light of the observation that most of the disease-causing organisms

live in the matrix-enclosed environments of the biofilms (Watnick and Kolter, 2000) that inherently show increased resistance toward all antibiotics (Gilbert *et al.*, 1997).

Due to poor sanitation conditions in South Asian countries, including Pakistan food borne diseases is very common like infection by Salmonella typhi known as typhoid fever. In some case sit may prove fatal. Mostly children are prone to this infection. This bacterium has developed resistance towards many antibiotics due to negligence in the administration of antibiotics. Even third generation antibiotics are proving to be ineffective in some areas. So the aim of our study was to isolate bacteriophages against Salmonella typhi and to check its effectiveness against biofilm formed by Salmonella typhi.

Samples of Salmonella typhi were isolated from a blood sample of patients suffering from fever. The patients were mostly young children of age about 8-11 including both males and females. Some adults were also diagnosed with this disease in the age of 18-20 but their ratio is too low. These bacterial strains were taken from AFIP where many tests had already performed to confirm its strains. The samples were being taken in the form of glycerol stocks and after taking these samples to our lab, they were grown on MacConkey agar plates, white colonies were observed on these plates. Gram staining of this bacterium showed it to be Gram negative bacterium.

Since our study was focused on the isolation of phages to control biofilm formation so our focus was selection of those strains of bacteria that have more potential to form a biofilm. Out of 20 strains of bacteria, eight bacterial strains have potential to form effective biofilm. Of these strains, only one (strain 12) was selected, which gave maximum biofilm formation for further research.

Due to increasing use of antibiotics in an improper way, pathogenic bacteria started to develop resistant towards them and narrowing down the potential of antibiotics to remove pathogens. A similar case was observed in the case of strains of Salmonella typhi. These strains were resistant towards 2nd generation antibiotics and resistance is increasing towards 3rd generation antibiotics at an alarming rate.

Phages are generally isolated from environments that are habitats for the respective host bacteria (Nakai and Park, 2002). It is known that sewage generally contains a large diversity of microorganisms due to the contamination from fecal material and hospital drainage water (Piracha *et al.*, 2014). As bacteriophages are present on the site of presence of the bacteria so phages against Salmonella typhi were isolated from sewage water. Among 20 strains taken from AFIP, phages were isolated against 18 strains. The phage that shows lysis against most bacterial strains was selected and was designated as ST1. This phage was stored in SM buffer at 4 ^{o}C.

To use bacteriophage in an effective way it is necessary to characterize these phages. Different properties of ST1 were studied like its host range, thermostability, pH stability, effect of different divalent metal ions on its adsorption and its burst size.

The study of host range of phage showed that ST1 was effective against a range of bacterium like different strains of E.coli and *Staphylococcus aureus*. But it was proved ineffective against *Pseudomonas aeruginosa.* The reason behind effectiveness of phages towards some bacterial strains and ineffectiveness towards other is that phages bind with bacterial surface utilizing specific receptors or alternative receptors on bacterial surface. Bacterial strains exhibiting those receptors were recognized by phages and their lysis take place while other remains ineffective. Many phages have been reported that are greatly

specific for their receptors present on the host cell surface. They only show interaction with their specific receptors, but do not interact with receptors having different structures (Piracha *et al.,* 2014).

ST1 phage can withstand temperature around 60 ^{o}C with an optimum temperature range between 37 ^{o}C to 55 ^{o}C and pH stability around 5 to 11 with 7 as optimum pH. Ackermann (2007) states that most of the phages can withstand a wide range of environmental conditions and most of the phages with tails exhibit a pH stability ranging from 5 to 9 and are normally inactivated by heating at 60 ^{o}C for 30 min. Our results for pH stability are consistent with the previous observations by Carey-Smith *et al.* (2006). They stated that most phages were able to survive well within a wide range of pH (5 to 9) at physiological conditions that maintain the native virion structure and stability.

The ability of phages to get adsorbed on bacterial surface was increased by presence of 10mM $CaCl_2$ and $MgCl_2$. $MgCl_2$ was proved to be more effective as compared to that of $CaCl_2$. According to Gill and Abedon (2003), adsorption of phages to bacterial surface depends upon physiochemical properties of bacteria and phages. According to Guttmann *et al.* (2005) cofactors such as Ca^{2+}, Mg^{2+}, divalent cations or sugars may be required for successful binding to occur. It is stated that ions cause electrostatic bonding of bacterium with phages (Briandet *et al.,* 2008). It is also suggested that increase of phage adsorption on bacterial surface due to Ca^{2+} ions may be due to increase in the concentration of phage particles at the host surface or they may alter the structure of a cell surface receptor thereby increasing accessibility to the receptor molecules or the transfer of phage nucleic acids (Watanabe and Takesue, 1972; Russel *et al.*, 1988).

One step growth was performed to determine the burst size and latent time period of phages. The longer the latent period is, the bigger the burst size of the phage. A phage's burst size is defined as the average number of phage particles produced per bacterial cell upon infection (Piracha *et al.*, 2014). The determination of the burst size was based on the ratio of the mean yield of the phage and it came out to be 300 virions per cell. The latent time period for ST1 was 24 minutes. Phage latent time period and burst size depend upon bacterial strain as well as phages. Moreover environmental factors also play crucial in this regard (Delbriick, 1946). . Heden (1951) stated that the phage burst size is much larger in nutrient-rich broth, compared to synthetic media. . Hence it can be concluded that burst size and latent time period are affected by various parameters.

In our next experiment, we analyze the effect of phages on planktonic cells and it turned out that ST1 phage is quite effective against bacterial culture. Planktonic cells were treated using different MOIs i.e., 0.5,1 and 5 and it turned out those phages at 0.5 MOI proved to be effective during 2-3 hours of phage treatment while in case of MOI 1 and 5 this treatment was effective during 1-3 hours and 1-2 hours respectively. So using fewer phages for more time is more effective strategy than using a large number of phages for less time. If more phages are used for treatment they may induce phage resistance in bacterial strains.

Salmonella typhi also has a tendency to form biofilm and is reason for causing infections so effect of phage should be checked at biofilm level. To check formation of biofilm, various methods have been employed like tissue culture plate, Congo red method, scanning electron microscopy etc. Among these tissue culture pate is the most

sensitive and reliable method (Mathur *et al.*, 2006). This method was used during this research to check capability of bacterial strains to form biofilm and was involved in other experiments involving biofilm formation.

Biofilms are resistant to antibiotics and are the main cause of infections in hospitals. Phages play great in controlling biofilm. So the experiment was designed to check the effect of ST1 against biofilm formed by Salmonella typhi. The biofilm was allowed to grow in 96 wells culture plate and after 24 and 48 hours of the formation of biofilm, they were treated with phages with a titer of $2x10^7$PFU/ml. The wells that were treated with phages showed reduction in biofilm up to two folds in both 24 hours plate and 48 hours plate. So ST1 proved to be effective to control biofilm even when it got mature. Phages specific for a certain bacterium can infect biofilm cells by first degrading the EPS and then ultimately lysing the bacterial cell (Hughes *et al.*, 1998). Carson *et al.* (2010) have reported the potential use of bacteriophages to reduce bacterial biofilms on medical-device surfaces and for the prevention of biofilm formation via direct incorporation of phages.

The experiment was also conducted to check the formation of biofilm and phage treatment against biofilm on SS plates at different conditions i.e. static and dynamic. Biofilm was allowed to for 24 and 48 hours and 4 hour phage treatment was given to each plate in each condition. This study revealed that biofilm formation is slightly more effective in dynamic state and phage treatment was also more effective in this state as compared to that of static state.

Peters *et al.* (1982) stated that scanning electron microscopy (SEM) is a well-established technique for the observation of morphology of bacteria adhering to the

surface, bacterial biofilm and also the relationship between them. So we performed SEM of our samples one with a biofilm formed by Salmonella typhi without treatment and other with treated biofilm. Peters *et al.* (1982) stated that scanning electron microscopy (SEM) is a well-established technique for the observation of morphology of bacteria adhering to the surface, bacterial biofilm and also the relationship between them.

To study phage-bacterial interaction that causes adsorption of ST1 on Salmonella typhi, hypothetical models were presented using docking. Docking studies suggest a relationship between two proteins and in our case it showed phage protein and bacterial protein interaction. Phage protein sequence/structure and bacterial protein sequence/structure were taken from protein databank and using Z-dock their interactions was studied and amino acids involved in these interactions were also found out. One model (3NSG-1TYW) present interaction of proteins at two points while second model (1QKI-1TYU) present interaction of phages with bacteria at one point. Both models are effective and hence we concluded that st1 interacts with bacterial protein via either of these models.

CONCLUSION

The bacteriophage (ST1) isolated against Salmonella typhi is an important step to control infections caused by this bacterium. The burst size of the isolated phage is 300 virions per cell with 24 minutes of latent time period making it suitable for use in phage therapy. The isolated phage shows stability towards high range of temperature, 37ºC - 65 ºC and pH 5-11 despite having narrow host range. The uptake of phages by bacterial cells increase by supplementing medium with divalent metal ions. The phage proves to be effective in reducing bacterial planktonic culture and biofilm up to 2 folds and 2.2 folds

respectively, thus ST1 can be employed to control contaminations of food products and medical devices.

FUTURE PERSPECTIVES

Future perspectives of the study include molecular characterization of the isolated phage, checking potential of this phage for phage therapy without developing resistance, checking therapeutic ability of this phage in animal model and cell lines, using phage to prevent contamination of medical devices by biofilm and checking potential of this phage for treatment of waste water and contaminated food.

CHAPTER8

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