STANDARDIZATION OF BACTERIOPHAGE THERAPY FOR LUMINOUS VIBRIOSIS PROBLEM IN SHRIMP HATCHERIES

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BY

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CERTIFICATE

This is to certify that the thesis entitled ***"Standardization of bacteriophage therapy for luminous vibriosis problem in shrimp hatcheries"*** submitted by **Mr.Tanmoy Gon Choudhury,** I.D. No. **MFK 810** in partial fulfillment of the requirements for the award of Master of Fisheries Science in Fishery Microbiology of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work done by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis of the award of any degree, diploma, association ship, fellowship or other similar titles.

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MANGALORE
July 2010

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(Shri. C. H. KRISHNA BHAT)

Dedicated to my loving

Parents and Brother

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Mangalore
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Tanmoy

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I. INTRODUCTION

Shrimp farming is one of the fastest growing food sectors in aquaculture during the last two decades. About 6 % of the world's farmed shrimp production comes from India. In commercial aquaculture, improper farm management practices and subsequent stress may lead to sporadic incidences of disease or disease epidemics. According to a recent FAO report, global *Penaeus monodon* production decreased worldwide by 131090 ton from year 2003 to 2007 due to various diseases (FIGIS; FAO, 2007). The sector was struck by several disease problems during mid 1990's resulting in economic loss to the farmer. Luminous bacteriosis that is caused by luminous *Vibrio harveyi* is one of the major diseases for penaeid larvae in hatcheries and juveniles in farms throughout Southeast Asia and South America (Sunaryanto and Mariam, 1986; Jiravanichpaisal *et al*., 1994; Pizzutto and Hirst, 1995; Liu *et al*., 1996; Alvarez *et al*., 1998; Vandenberghe *et al*., 1998; Karunasagar *et al*., 1994; Alabi *et al*., 1999; Chrisolite, *et al*., 2008). In 2004, aquaculture accounted for more than 41 % of total shrimp production (FAO, 2007).

V. harveyi, is a gram-negative bioluminescent marine bacterium, is not only ubiquitous in the marine environment and is also considered part of normal microflora of healthy shrimps and intestinal microbiota of marine animals (Makemson and Hermosa, 1999; Vandenberghe *et al*., 1999). This organism probably persists in hatchery environments by forming biofilm on various surfaces (Karunasagar *et al*., 1996).

Traditionally, to control the bacterial diseases, antibiotics have been used in shrimp farms and hatcheries as prophylactic and therapeutic agent (Baticados and Paclibare, 1992). The indiscriminate use of antibiotics in aquaculture has lead to the emergence and spread of

antibiotic resistant isolates of bacteria in the environment. Karunasagar *et al*. (1994) reported that the antibiotics used in the hatchery were ineffective in controlling luminous bacteria as antibiotic resistant *V. harveyi* emerged in larval tanks and caused serious mortality in *P. monodon* larvae. Another major concern associated with the use of antibiotics is the problem of residues which has already led to imposement of ban by the seafood importing countries on shrimp containing traces of antibiotics. Antibiotic therapy is therefore legally unavailable to treat vibriosis in shrimp culture practices and hence the search for an alternative and ecofriendly control methods for vibriosis.

An efficient ecofriendly and scientifically demonstrable solution to bacterial infections lies in the phage therapy. Phages are bacterial viruses that are extremely abundant in nature and are believed to be important in controlling bacterial populations in natural systems (Carlton, 1999; Imbeault *et al*., 2006). Phage therapy has potential to control disease in aquaculture systems depending on the isolation and identification of phage that will specifically kill relavant pathogenic bacteria (Nakai and Park, 2002; Park and Nakai, 2003; Skurnik *et al*., 2007). In recent years, the use of bacteriophages for biological control of pathogens of cultured fish and shrimp has aroused much interest since no drug residues and drug toxicity are associated with this type of therapy (Wu and Chao, 1982; Nakai *et al*., 1999). Bacteriophage therapy could be an attractive adjunct and viable proposition for controlling luminous vibriosis in shrimps (Vinod *et al*., 2006; Karunasagar *et al*., 2007).

Every organism required appropriate physicochemical parameters for its optimal activity. Salinity, temperature, pH and total dissolved solids are four important abiotic factors affecting the growth and survival of aquatic organisms (Prayitno *et al*., 1995; Boyd, C.E.,

1999; Kumlu *et al.*, 2000). During rainy season, changes in environmental factors such as temperature, salinity, pH and organic load might be involved in triggering the outbreak of luminous bacteria (Farghaly, 1950; Sunaryanto and Mariam, 1986; Ramesh, *et al.*, 1989; Prayitno *et al.*, 1995). It is also important to know the effect of various environmental factors on disease control measures to be used. Environmental factors could also influence the growth and activity of vibriophage and thus this improve the efficiency in disease control.

Recombinant lysozyme expressed from *P. monodon* is observed to have lytic activity against both Gram positive and Gram negative bacteria. Minimal inhibitory concentration (MIC) of purified shrimp lysozyme for *V. harveyi* was 0.47 µg ml^{-1} (Tyagi *et al.*, 2007). After attachment of phage to the cell wall of the bacterium by tail fibres, the penetration of phage DNA into the bacteria is promoted by lysozyme produced by the phage (Madigan *et al.*, 1997). Phage penetration may be enhanced in the presence of recombinant shrimp lysozyme.

Against the above background, the study was undertaken with the following objectives.

1. Studying the factors for optimal phage activity.
2. Studying the role of lysozyme on the vibriophage activity.

II. REVIEW OF LITERATURE

2.1. Bacteriophage

2.1.1. Definition

Bacteriophages (or phages) are obligate intracellular parasites that infect bacteria and reproduce by hijacking the host's biosynthetic pathways. Phages are classified as either lytic or lysogenic based on their replication strategy. A lytic phage infects its bacterial host, replicates its DNA, and produces progeny that are immediately released for further infection, destroying its host in the process. A lysogenic phage, on the other hand, integrates itself in the genome of its bacterial host, establishing a stable relationship with the bacteria that it has infected. This stable relationship is maintained until some stressor, such as DNA damage, disrupts it. Lysogenic phages are capable of transferring genes for toxin production or pathogenicity factors between bacterial populations (Waldor *et al*., 2005).

2.1.2. General characteristics

Phages like all viruses are composed of a nucleic acid core surrounded by a protein coat (Fig. 1). The majority of phages contain DNA but some contain RNA also. Phages are more resistant to physical and chemical factors than human viruses (Pelczar *et al*., 1996).

The research on the bacteriophages has some exceptional importance in the history of microbiology. The simplicity of their cultivation, short generation time and exact accountability help to clarify not only the structure but also the relationships between the bacterial and viral particles. Since bacteriophages are the smallest entities known which are capable of self-replication, they have been widely used in genetic research. Much has been learned about host-parasite relationships from the studies of bacterium-bacteriophage

interaction. This provides a better understanding of plant and animal infection with viral pathogens. Thus the bacterium-bacteriophage interaction has become the model system for the study of viral pathogenicity.

There is interdependency between the bacteriophage and the host cells. The interaction usually ends with the destruction of the microbial cells. Bacteriophages are host specific and have no adverse affects on humans, animals or on the environment.

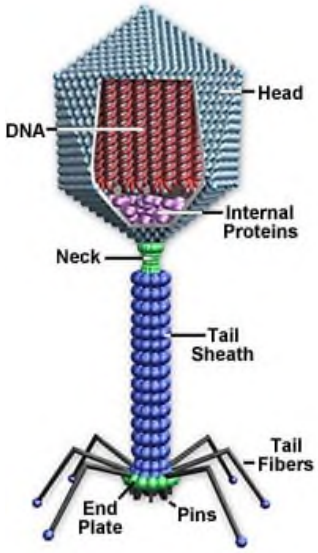

Fig. 1. Bacteriophage (Source: http://micro.magnet.fsu.edu/cells/viruses/images/bacteriophage.jpg)

Phages like all viruses are composed of nucleic acid surrounded by a protein coat which is called head, and a long, fairly complex tail, at the end of which is a series of tail fibers. During the attachment process, the virus particles first attach to cells by means of the tail fibers (Fig. 2). The ends of the fibers interact specifically with core polysaccharides that are part of the outer layer of the gram negative cell wall. These tail fibers then retract, and the core of the tail makes contact with the cell envelop of the bacterium. The action of a lysozyme-like enzyme results in the formation of a small hole. The tail sheath contracts, and the DNA of the virus passes into the cell through a hole in the tip of the tail, the majority of

the coat protein remaining outside (Madigan *et al*., 1997). Bacterial viruses occur in different shapes.

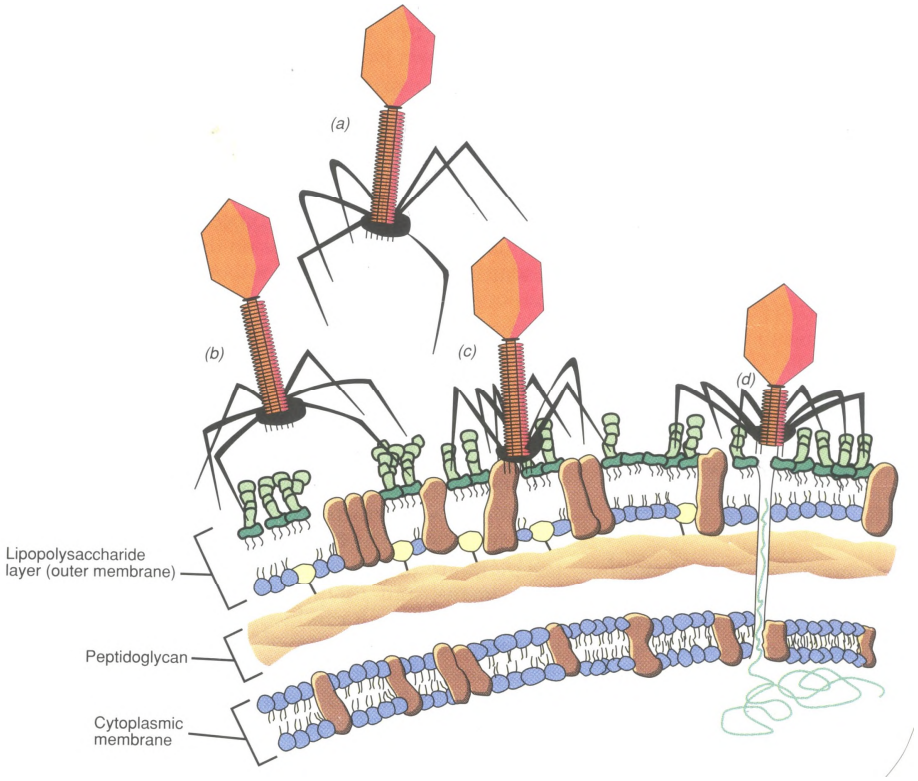

Fig. 2. Attachment of bacteriophage particle to cell wall of bacteria (Source: Madigan *et al*., 1997). (a) Unattached particles. (b) Attachment of the wall by the long tail fibers interacting with core polysaccharide. (c) Contact of cell wall by tail pins. (c) Contraction of the tail sheath and injection of the DNA.

Bacterial viruses are categorized into lytic and lysogenic types (Fig. 3). Lytic phages can infect the cells and the cells respond by producing large number of new virus. At the end of the incubation period, the host cell bursts and releases new phages to infect other host cells. In lysogenic ones, the viral nucleic acid is carried and replicated along with the host bacterial cells from one generation to another without any cell lysis. These phages may become virulent

at some subsequent generation and can lyse the host cells. There are also filamentous phages which simply leak out of the cells without causing any lysis (Pelczar *et al*., 1996).

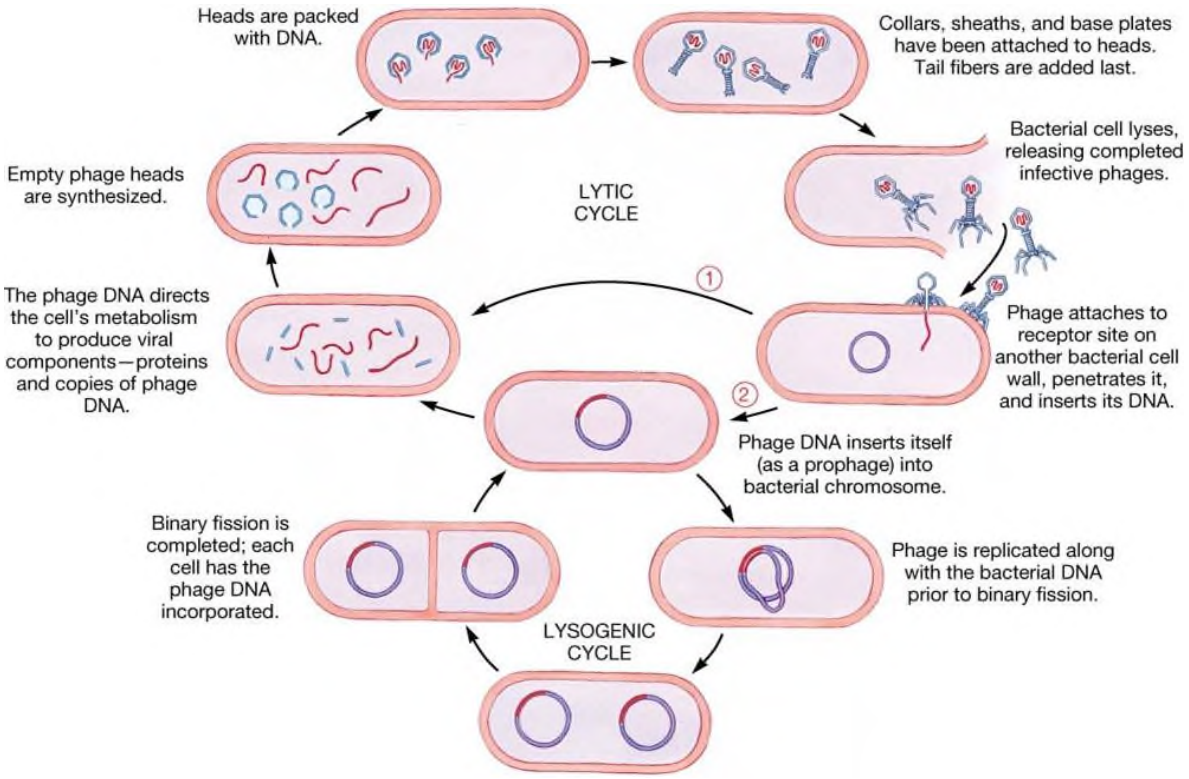

Fig. 3. Lytic and lysogenic cycles of a bacteriophage (Source: http://faculty.irsc.edu/FACULTY/TFischer/images/bacteriophage life cycle.jpg).

2.1.3. Discovery

The history of bacteriophages discovery has been the subject of a lengthy debate. It starts with the reports on the presence of marked antibacterial activity against *Vibrio cholerae* in the water of the Ganges and Jamuna River in India as reported by Ernest Hankin (1896). This antibacterial substance could pass through fine porcelain filter and has been thought to be responsible for limiting the spread of cholera epidemics. Two years later, the Russian bacteriologist, Gamaleya, observed the same phenomena while working with *Bacillus subtilis* (Samsygina *et al*., 1984). Almost twenty years later of Hankins's observation, a British

bacteriologist, Frederick W. Twort in 1915, actually isolated filterable entities capable of lysing bacterial cultures and producing small cleared areas on bacterial lawns (Twort, 1915). Two years after discovery by Twort, Felix d' Herelle, a French Canadian microbiologist at the Pasteur institute in Paris reported the same phenomenon and Felix d' Herelle officially named it as bacteriophage (d' Herelle, 1917).

2.1.4. Host range

Bacteriophages are host specific; they can be not only genus and species specific but even strain specific. Hence they lyse only the target bacteria unlike antibiotics that can have a wide spectrum. It is assumed that the host range of phages is narrow, *i.e*., they do not cross their generic boundaries (Ackermann *et al.,* 1987). This has been documented from studies of more than 4000 bacteriophage isolates described at that time and was also reported for isolated marine bacteriophages (Borsheim, 1993). A broad host range has also been demonstrated for cyanophages infecting different genera of freshwater and marine cyanobacteria (Suttle, 2000).

The role of bacterial outer membrane proteins serving as receptors for a broad-host-range vibriophage KVP40 has been described by Inoue and coworkers (Inoue *et al.,* 1995a, 1995b). Studies have shown that the presence of more than one specific tail fiber protein may allow phages to increase their host range, since these proteins are used by the phage to identify a host and attach to it (Nimmich *et al.,* 1991; Scholl *et al.,* 2001). The occurrence of conserved regions of tail fibre genes in different viral families suggests its wide host range (Ljungquist *et al.,* 1992).

2.1.5. Ecology

Campbell (2003) reported that bacteriophages are ubiquitous throughout the prokaryotic world. Ewert and Paynter (1980) reported the presence of bacteriophages in

ecosystems that support bacterial multiplication such as natural waters, soil, faeces, sediments, sewage, bovine and sheep rumens. The numerical abundance of phage like particles in aquatic system compared to that of prokaryotic cells might represent more than 10% of biomass of these cells (Wilhelm and Suttle, 1999; Wommack and Colwell, 2000; Campbell, 2003). Bacteriophages play a major role in microbial food chain and food web (Suttle, 1994) and also in ecological balance in nature (Fuhrman, 1999).

2.2. Distribution of bacteria and viruses in marine ecosystem

The importance of microbes in aquatic ecology is widely recognized (Sorokin, 1971; Pomeroy, 1974; Azam *et al.,* 1983). These microbes occur in a wide range of aquatic environments including estuaries, marine coastal waters, sediment and aquaculture environments (Barbieri *et al.,* 1999; Urakawa *et al.,* 2000; Suantika *et al.,* 2001; Vandenberghe *et al.,* 2003; Venter *et al.,* 2004). The world's ocean is estimated to contain $1.1x10^{29}$ prokaryotic cells (Whitman *et al.,* 1998). This vast abundance of bacteria represents a large proportion of the active biomass in marine environment. Heterotrophic bacteria have been estimated to represent up to 70% of the living carbon in the photic zone (Fuhrman *et al.,* 1989).

Several studies have shown that vibrios occur in high densities in association with marine organisms such as coral fish, shellfish, shrimp and zooplankton (Gomez-Gil *et al.,* 1998; Huys *et al.,* 2001; Heidelberg *et al.,* 2002; Rosenberg and Benhaim, 2002; Sawabe *et al.,* 2003).

d'Herelle (1926) was among the first to examine viruses in aquatic environments. Despite these early beginnings and occasional sojourns by other scientists into aquatic viral ecology (Safferman and Morris, 1967; Torrella and Morita, 1979) the potential significance of

viruses in marine system was largely ignored until 1980`s (Bergh *et al.,* 1989; Proctor and Fuhrman, 1990). Most marine viruses infecting bacteria are bacteriophages is seen from their strong correlation to bacterial abundance and less to chlorophyll a (Paul *et al.,* 1993). The first report of bacteriophage activity in marine ecosystem was by Zobell (1946). There are approximately 10^{10} phage/liter in surface seawater (Fuhrman, 1999) and 10^{7}-10^{9} cfu g^{-1} of sediment or top soil (Danovaro and Serreso, 2000; Danovaro *et al.,* 2001). Bacteriophages are widely distributed in the environment and in the aquatic environment, there are tenfold more phages compared to bacteria (Skurnik and Strauch, 2006).

2.3. Vibriophages

Phages that infect *Vibrio* species are named vibriophages. They not only lyse the vibrios but also confer virulence to the host and can be present in various aquatic environments. Vibriophages are most abundant in molluscs at density ranging from 10^{5} to10^{8} per gram (Weinbauer and Peduzzi, 1994; Maranger and Bird, 1995).

Many workers have demonstrated the ubiquity of vibriophages in aquatic environment. Phages infecting many *Vibrio* species such as *V. cholerae, V. parahaemolyticus*, (Nakanishi *et al.,* 1966; Baross *et al.,* 1978b; Raghu Patil, 2005, Surendranath, 2006), *V. harveyi,* (Ruangpan *et al.,* 1999; Shivu, 2004, Surendranath, 2006), *V. fischeri,* and *V. alginolyticus* (Levisohn and Nealson, 1987; Lin *et al.,* 1993; Nandana, 2002) have been isolated from the marine and other aquatic environment. Prophage was induced into the lytic cycle by addition of mitomycin C (Jing and Paul, 1998).

2.4. Phages of *V. harveyi*

Hidaka *et al.* (1990) reported bacteriophages of *V. harveyi* for the first time from Kagashima Bay in Japan. Depending on the lytic pattern and host bacteria, he classified 85

phage isolates into 3 groups and all these phages had hexagonal head and non-contractile tail with double stranded DNA as genetic material. Ruangpan *et al*. (1999) reported from Thailand phage induced virulence in *V. harveyi* that caused mortality in shrimp and the disease was called tea brown gill syndrome.

Oakey and Owens (2000) studied temperate bacteriophage VHML (*Vibrio harveyi* myovirus-like) having a narrow host range isolated from toxin producing strains of *V. harveyi* in Australia. Analysis of VHML by Oakey *et al*. (2002) revealed that the total genome consisted of 43,195 bp coding 57 putative open reading frames. Vinod (2002) isolated bacteriophages, which were virulent to *V. harveyi*. They had a contractile tail and a hexagonal head. He also reported on their potential application for the biocontrol of pathogenic *V. harveyi* in shrimp hatcheries due to its broad host range.

Rajeev (2003) isolated four phages of *V. harveyi* from shrimp farm water which had double stranded DNA as genetic material and he reported that among the 4 bacteriophages, 3 belonged to family *Siphoviridae* and one to family *Myoviridae*. Shivu (2004) isolated several phages that had double stranded DNA, were lytic to *V. harveyi* and belonged to family *Siphoviridae*. Their approximate genome sizes were 83, 63 and 44 kb. He also reported that out of seven phages, three (Viha1, Viha3 and Viha7) lysed over 65% of the global colaction of *V. harveyi* tested (183 Strains) while Viha2, Viha4 and Viha6 lysed over 40%. Viha5 had a narrow spectrum of lytic activity (14%).

Patil (2005) isolated 4 bacteriophages of *V. harveyi* from oyster and shrimp hatchery water which had a broad host range. Out of 4 phages, 3 belonged to family *Siphoviridae* and contained double stranded DNA as genetic material. Bacteriophages specific to *V. harveyi* 1114 was isolated by Pasharawipas *et al*. (2005) from shrimp ponds. It possessed an

icosahedral head, a rigid tail, without a terminal fiber and a genome of double stranded DNA. The phage belonged to the family *Siphoviridae* and tentatively named *V. harveyi* Siphovirus like (VHSL) phage.

Vidgen *et al.* (2006) observed that when *V. harveyi* was infected with VHML, it caused change in the phenotypic profile of the bacterium and resulted in a poorer level of assurance for identification of *V. harveyi*. It was also found that in different strains of *V. harveyi*, VHML integrates at different sites. Khemayan *et al.* (2006) produced low percentage of lysogenized clones of variable stability when *V. harveyi* (strain VH1114) was exposed to *V. harveyi* siphovirus like phage 1 (VHS1). They also found that VHS1 prophage was carried by VH1114 as an episome rather than integrated into the host chromosome.

Surendranath (2006) isolated three phage of *V. harveyi*, which had moderately broad host range. All 3 phages belonged to family *Siphoviridae*. Okana *et al.* (2007) isolated twelve bacteriophage strains of *V. harveyi* from sea water samples obtained from aquaculture tanks. Chrisolite *et al.* (2008) found luminescent *V. harveyi* and their bacteriophages distributed in a commercial shrimp hatchery in South India. Crothers-Stomps *et al.* (2009) isolated bacteriophage with lytic activity against *V. harveyi* from prawn farm samples. The purified phage had a clear lytic ability and no apparent transducing properties, indicating they are appropriate for phage therapy. They also reported that phage resistance is potentially a major constraint to the use of phage therapy in aquaculture as bacteria are not completely eliminated.

2.5. Incidence of luminescent vibriosis in aquaculture

V. harveyi is recognized as an important pathogen of cultured penaeid larvae throughout the Southeast Asian region (Lavilla-Pitogo *et al*., 1990; Karunasagar *et al*., 1994 Alabi *et al*., 1999; Chrisolite, *et al*., 2008). Virulent strains of *V. harveyi* cause devastating

mortality in the hatchery systems of *P. monodon* (Sunaryanto and Mariam, 1986; Lavilla-Pitogo *et al*., 1990; Karunasagar *et al*., 1994). Luminous vibriosis, was initially reported in *P. monodon* larvae till mysid stages (Ruangpan, 1997; Chrisolite, *et al*., 2008), further its incidence was observed upto the postlarval stages by Songserm *et al*. (1990). Since then, a rising trend in the larval stock has occurred in hatcheries all over the Asian countries.

Luminous bacteria can infect the tissues of a variety of marine crustaceans (Inman, 1927; Bowman and Phillips, 1984), fish (Kraxenberger-Beatty *et al*., 1990; Anderson and Norton, 1991) and bivalves (Pass *et al*., 1987). Some workers have examined the haemocoel of moribund spawns of *P. esculentus* and obtained a pure culture of *V. harveyi* (Owens *et al*., 1992). The incidence of *V. harveyi* from cultured lobster was reported by Diggles *et al*. (2000). Outbreaks of luminous vibriosis in phyllosoma larvae of the packhorse rock lobster, *Jasus verreauxi* (Milne Edwards, 1851) indicates that *V. harveyi* is a serious pathogen not only for the shrimps but also for other crustaceans.

Mortalities of *P. monodon* and *P. merguiensis* larvae associated with luminescence have been observed in hatcheries in Indonesia (Sunaryanto and Mariam, 1986), Thailand (Tansutapanit and Ruangpan, 1987), The Philippines (Lavilla-Pitogo *et al*., 1990; Baticados *et al*., 1991) and India (Karunasagar *et al*., 1994). Chen *et al*. (1992) recorded 43% of *V. harveyi* among all other bacterial isolates from the hatchery.

2.6. Influence of environmental factor on luminous vibriosis

Sunaryanto and Mariam, (1986) found that during the rainy season luminous bacterial disease was most common. Prayimo *et al*. (1995) reported change in environmental factors such as salinity and pH which enhance the virulence of the luminous bacteria and hence explain the seasonality of the disease outbreaks. The mechanism of increased virulence

is unknown but Farghaly (1950) demonstrated that environmental factors such as low salinity and altered pH reduced the growth rate of luminous bacteria. Salinity, temperature, pH and total dissolved solid are four of the most important abiotic factors affecting the growth and survival of aquatic organisms (Prayitno *et al*., 1995; Boyd. C.E., 1999; Kumlu *et al.,* 2000).

2.7. Application of bacteriophage in aquaculture

The first reported application of phages to treat infectious diseases in humans came from Bruynoghe and Maisin in France in 1921, who used bacteriophages to treat staphylococcal skin disease. There have been several reports on phage therapy, but many of them were not scientifically well-demonstrated and the simultaneous discovery of antibiotics as led to the phage therapy taking a backstage (Barrow and Soothil, 1997; Alisky *et al*., 1998). However, in recent years the increasing emergence of multidrug resistant bacteria has made researchers reconsider the use of phages for therapy.

Attempts have also been made on the use of phages for disease control in aquaculture. Wu and Chao (1982) reported the potential of phages to control disease caused by *Edwardsiella tarda* and this was probably one of the earliest investigations of the potential application of phages in aquaculture. Oral administration of bacteriophages against *Lactococcus garvieae* to young yellow tails (*Seriola quinqueradiata*) resulted in 100% survival following intraperitoneal challenge with the pathogen compared to 10% survival in control (Nakai *et al*., 1999). Park and Nakai (2003) documented by an experimental infection that oral administration of phages impregnated feed to ayu (*Plecoglossus altivelis*) brought down cumulative mortality from 65% to 22.5% following oral challenge with *Pseudomonas plecoglossicida* through feed. Bacteriophages have been used to prevent the occurrence of

furunculosis caused by *Aeromonas salmonicida* in farmed rainbow trout in Japan (Roberts *et al*., 2002).

Several studies have been carried out on bacteriphages of *V. harveyi*. Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *V. harveyi* infection in shrimp hatchery (Karunasagar *et al*., 1994) and their ability to form resistant biofilms on the surfaces of larval rearing tanks (Karunasagar *et al*., 1996) has been a cause for concern. *V. harveyi* bacteriophage isolated from shrimp hatchery and farm environment has the potential to control luminous vibriosis. Larvae of *P. monodon* (infected with *V. harveyi*) showed enhanced (80%) survival in presence of bacteriophage as compared to the control (20%) (Vinod *et al*., 2006). Surendranath (2006) found that a mixture of phages was more efficient to control biofilm of *V. harveyi* than the use of individual phage. One bacteriophage which lysed 70% of the 100 *V. harveyi* isolates tested, was used for biocontrol of *V. harveyi* in shrimp hatchery (Karunasagar *et al*. 2007).

Crothers-Stomps *et al.* (2009) isolated *V. harveyi* phage which has potential for use as a biocontrol agent to combat vibriosis in the rearing system of phyllosoma of the tropical rock lobster, *Panulirus ornatus*.

2.8. Bacteriophage lysin

Lysins are phage-encoded enzymes that break down bacterial peptidoglycan at the terminal stage of the phage reproduction cycle. Their action is tightly regulated by holins, by membrane arrest, and by conversion from their inactive to active state. Recent research has not only revealed the unexpected diversity of these highly specific hydrolases but has also yielded insights into their molecular organization and their three-dimensional structures. Lysin can be endo-beta-N-acetyl-glucosaminidases or N-acetylmuramidases (lysozymes). Their N-terminal

catalytic domains are able to target almost every possible bond in the peptidoglycan network, and their corresponding C-terminal cell wall binding domains target the enzymes to their substrate. Owing to their specificity and high activity, lysins have been employed for various *in vitro* and *in vivo* studies, in food science, in microbial diagnostics, and for treatment of experimental infections. Clearly, phage lysins represent great tools for use in molecular biology, biotechnology and in medicine, and their potential is being recognized for harnessing it even more. (Loessner, 2005).

Listeria bacteriophage lytic enzymes were found to be useful for *in vitro* applications such as rapid, gentle cell disruption, and they provide new approaches as selective antimicrobial agents for destruction of *L. monocytogenes* in contaminated foods (Loessner *et al*., 1996, Gaeng *et al*., 2000). Bacteriophage lytic enzyme was used for prevention and elimination of upper respiratory colonization of mice by group A streptococci (Nelson *et al*., 2001). Rydman and Bamford (2002) reported that Enterobacteria bacteriophage PRD1 encodes two proteins (P7 and P15) that associated with a muralytic activity. Protein P15 is a soluble β-1,4-*N*-acetylmuramidase that causes phage-induced host cell lysis. P15 was a structural component of the PRD1 virion and was connected to the phage membrane. The principal muralytic protein involved in PRD1 DNA entry seems to be the putative lytic transglycosylase protein P7. The incorporation of these two different lytic enzymes into virions reflects the broad host range of bacteriophage PRD1. Rapid killing of antibiotic resistant *Streptococcus pneumonia* occurred when treated with phage-coded lytic enzymes Cpl-1 lysozyme (Jado *et al*., 2003, Entenza *et al*., 2005, Grandgirard *et al*., 2008). PlyV12, a bacteriophage lytic enzyme was found to be have killing effect on antibiotic resistant *Enterococcus faecalis* and *E. faecium*, and therefore being considered as alternative

therapeutic tool (Yoong *et al*., 2004). *Bacillus anthracis* specific phage lytic enzymes kill the vegetative forms in the blood of infected individuals (Fischetti, 2006). Extensive studies established phages and phage-encoded lytic enzymes (virolysins) as two of the most promising families of alternative antibacterial for the treatment and prophylaxis of antibiotic resistance bacterial infections which shown great potential in veterinary and human medicine for the treatment and prophylaxis of infections (Courchesne *et al*., 2009).

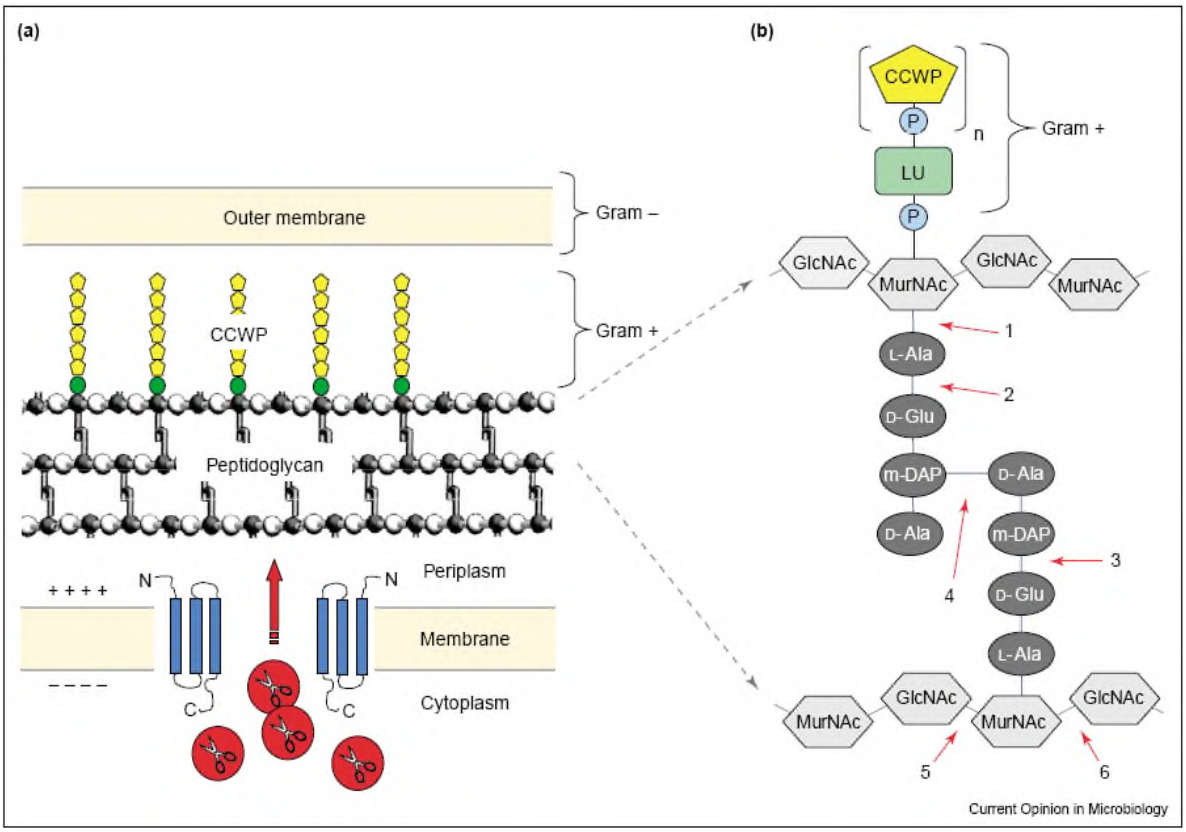

Fig. 4. Bacterial cell wall structure and endolysin targets (Source: Loessner *et al*., 2005).

(a) Schematic representation of the bacterial cell wall, and of how phage endolysins gain access to their substrate. Holin proteins (blue) insert themselves into the cytoplasmic membrane and can oligomerize, thereby forming membrane lesions. The endolysins (red) pass through these pores to access the peptidoglycan. Lysis from the outside is possible with Gram-positive cell walls, in which the thick and highly cross-linked peptidoglycan network is usually accessible from the outside. In Gram-negative cells, the outer membrane appears to represent an efficient barrier for prevention of lysis by free endolysin.

(b) This depicts the fine structure of a type A1g peptidoglycan, such as that of *E. coli*, *Listeria* and many other bacteria. The interpeptide bridge consists of a diamino acid (m-DAP) that is directly cross-linked to the terminal D-Ala of the opposite peptide chain. The bonds potentially attacked by endolysins of different enzymatic specificities are indicated by numbers: 1, N-acetylmuramoyl-L-alanine amidase; 2, L-alanoyl-D-glutamate endopeptidase; 3, D-glutamyl-m-DAP endopeptidase (note that this activity has not yet been identified in a phage endolysin); 4, interpeptide bridge-specific endopeptidases; 5, N-acetyl-b-D-glucosaminidase; and 6, N-acetyl-b-D-muramidase (also known as muramoylhydrolase and 'lysozyme') and lytic transglycosylase. Abbreviations: CCWP, carbohydrate cell wall polymer; GlcNAc, N-acetyl glucosamine; LU, linkage unit; m-DAP, meso-diaminopimelic acid; MurNAc, N-acetyl muramic acid; P, phosphate group.

2.9. Lysozyme

Lysozymes, also known as muramidase or N-acetylmuramide glycanhydrolase, are a family of enzyme which damage bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. It is abundant in a number of secretions, such as tears, saliva and mucus. Lysozyme is also present in cytoplasmic granules of the polymorphonucler neutrophils (PMN). Large amounts of lysozyme can be found in egg whites. C-type lysozymes are closely related to alpha lactalbumin in sequence and structure making them part of the same family. In humans, the lysozyme enzyme is encoded by the *LYZ* gene. Lysozyme is one of the earliest known antibacterial proteins, ubiquitous in many eukaryotes and prokaryotes and has been recognized as a molecule involved in non specific innate immunity. In invertebrates, it is well known that lysozyme is differentially expressed (Rojtinnakorn *et al.*, 2002) and expression responds to bacterial challenge (Somboonwiwat *et al.*, 2006, Burge *et al.*, 2007). Six types of lysozyme are recognized (Hikima *et al.*, 2003): chicken type (c-type), goose type (g-type), plant type, bacterial type lysozyme, T4 phage lysozyme (phage type) and invertebrate type (i-type). Expression of c-type lysozyme from *L. vannamei* and its activity against *V. alginolyticus*, *V.*

parahaemolyticus and *V. cholerae* have been documented (de-la-Re-Vega *et al.,* 2004; de-la-Re-Vega *et al.,* 2006). The kuruma shrimp lysozyme shows lytic activity to several infectious pathogens belonging to *Vibrio* spp. (Hikima *et al.*, 2003). Tyagi *et al.* (2007) found antivibrio activity of recombinant lysozyme expressed from black tiger shrimp and it was also able to reduce luminous *V. harveyi* numbers in sea water by 3 log units in 1 hour.

After attachment of phage to the cell wall of the bacterium by tail fibres, the penetration of phage DNA into the bacteria is promoted by lysozyme produced by the phage (Madigan *et al.*, 1997). It surmised that efficiency of the phage penetration may inprove in the presence of recombinant shrimp lysozyme.

III. MATERIAL AND METHODS

3.1. Bacteria and bacteriophages

V. harveyi stock cultures (*n* = 87) obtained from various sources (Table 1) and maintained in tryptone soy broth with 30% glycerol at −80ºC in deep freezer (Sanyo Corporation, Japan) was used in this study. Bacteria were grown overnight in tryptone soya broth with 1% sodium chloride **(**TSBS) at 28ºC in a shaker water bath (100 rpm). They were then subcultured onto luminescent agar (LA) plates (West and Colwell, 1984). An isolated luminescent colony of each culture was maintained in T_1N_1 butts for further work.

Previously isolated *V. harveyi* phage (seven in number) (Table 2) maintained in SM buffer at -20ºC freezer (Blue Star, India), with a known potential for biocontrol of luminous vibriosis in hatchery (Vinod *et al.*, 2006) were used in this study.

Tryptone soya broth with 1% NaCl (TSBS)

Tryptone	15 g
Yeast extract	5 g
Sodium chloride	10 g
pH	7.2 ± 0.2
Distilled water	1000ml

The above ingredients were dissolved to distilled water and distributed in 5 ml volumes to test tubes, sterilized at 121ºC for 15 minutes at 15 PSI.

Luminescent agar (LA)

Pancreatic digest of casein	17.5 g
Pancreatic digest of soya meal	3 g
Potassium dihydrogen phosphate	2.5 g
Sodium chloride	17.5 g
Magnesium chloride	4 g
Calcium chloride	1 g
Agar	15 g
pH	7.2±0.2
Distilled water	1000 ml

The medium was sterilized at 121^{o}C for 15 minutes at 15 PSI, and poured into sterile petriplates.

T_1N_1 Butts

Tryptone	1 g
Sodium chloride	1 g
Agar	0.5 g
pH	7.2 ± 0.2
Distilled water	100 ml

The above ingredients were boiled to dissolve agar and distributed in 3 ml volumes to test tubes, sterilized at 121^{o}C for 15 minutes at 15 PSI.

SM buffer

Sodium chloride	5.8 g
$MgSO_4$	2.0 g
1 M Tris- HCl (pH 7.5)	50 ml

The ingredients were mixed, made up to 990 ml using distilled water and sterilized at 121^{o}C for 15 minutes. The final volume was made up to one liter by adding 5 ml of

presterilized (110°C for 15 min at 15 PSI) 2 % gelatin and 5 ml of pre-sterilized distilled water.

3.2. Propagation of phage and determination of phage titers

The phages to be used in the study were propagated in their respective host grown as a lawn culture on TSAS in Roux bottles. The host bacteria was grown in 5 ml TSBS for 12 h to obtain a young activated culture. 4 ml host culture and 1 ml phage suspension as mixed and spread by gentle rocking on the surface of TSAS agar in Roux bottles. After 5–6 h incubation at ambient temperature (28 ±1°C), the phages were harvested in 25 ml SM buffer. A soft agar overlay technique (Adams, 1959) was used for determining the phage titer. Soft agar tubes were maintained at 48–50°C in a water bath. Phage suspensions were serially diluted in phage buffer. Hundred microliter of each phage dilution was then mixed with 900µl of 8 h old host culture in TSBS and added to 3 ml of molten soft agar in tubes. The mixture was rolled between the palms to allow proper mixing and overlaid on TSAS plates. After solidification the plates were incubated overnight at ambient (28±1°C) temperature. The plaques were counted and expressed as plaque forming units/milliliter (pfu ml^{-1}).

Tryptone soya agar with 1% NaCl (TSAS)

Tryptone	15 g
Yeast extract	5 g
Sodium chloride	10 g
Agar	15g
pH	7.2 ± 0.2
Distilled water	1000 ml

The medium was sterilized at 121°C for 15 minutes at 15 PSI, and poured into sterile petriplates.

Soft agar

Tryptone	1.5 g
Yeast extract	0.5 g
Sodium chloride	1.0 g
Magnesium chloride	0.1 g
1 M Calcium chloride	1ml/100ml
pH	7.2 ± 0.2
Agar	0.3 g
Distilled water	100 ml

The above ingredients were boiled to melt the agar and distributed in 3 ml volumes to test tubes, sterilized at 121^{o}C for 15 minutes and maintained in water bath at 45^{o}C.

3.3. Determination of host ranges of phages

The ability of seven phages (Table 2) to lyse eighty seven isolates of *V. harveyi* obtained from various sources (Table 1) was studied. A lawn of the bacterium was prepared on TSAS and 10 µl bacteriophage (10^{9} pfu ml^{-1}) was placed on each of the isolates to be tested. The plates were incubated at ambient temperature (28±1°C) and observed for the of clear zones after incubation at ambient temperature (28±1°C) for 12 h.

3.4. Phage activity under different physic-chemical parameters

Role of different parameters *viz.* salinity, temperature, pH, TDS of seawater on the activity of phage was studied under experimental setup. Three variations for each parameter was taken for the study. They were chosen from a range of values including optimum that are encountered during larval rearing in a hatchery. Conditions were as follows,

Salinity (parts per million (ppt))	20, 25 and 30
Temperature	20ºC, 30ºC and 37ºC
pH	6, 7 and 8
Total dissolved solid (mg ml^{-1})	11.25, 22.63, 38.43

3.4.1. Estimation of various parameters

Seawater was transported to the laboratory and salinity was estimated by salinometer (Alago, Germany). This way adjusted to various salinities by adding fresh water at required levels to 35 ppt sea water. Temperature was maintained by placing sterilized sea water in a water bath maintained at 20ºC, 30ºC and 37ºC. pH was determined in digital pH meter (Eutech, U.K.). Different seawater samples were brought and the appropriate samples were taken for the study. Total dissolved solids was estimated by the method outlined in 'Survey of methods to determine total dissolved solid concentrations' (U.S. Environmental protection agency, Washington, D.C.1988) with minor modification. Briefly, 100 ml filtered seawater sample was taken in a pre-weighed dry clean beaker; samples were evaporated in a steam bath, transferred to an oven maintained at 105ºC for 2 h, allowed to cool and final weight taken. By deducting final weight from the initial weight TDS of the sample was calculated and result as mg ml^{-1}.

3.4.2. Experimental setup

3.4.2.1. Salinity

Twelve glass troughs each containing 1L autoclaved (110ºC for 15 min. at 15 PSI) seawater with 30 post larvae (PL15) of *P. monodon* obtained from a commercial hatchery was setup in four groups with three troughs in each group (Fig. 5). First group of troughs

designated A_1, A_2, and A_3 had sea water with 20, 25 and 30 ppt salinity respectively and served as control to look for effect of salinity stress if any on the larval survival.

The second group of troughs was designated B_1, B_2 and B_3 with 20, 25 and 30 ppt salinity and contained larvae. *V. harveyi* was added at 10^6 cfu ml^{-1} (final concentration) and served as pathogen control.

The third set of experimental troughs was maintained in replicate and was designated C_1(a&b); C_2(a&b); C_3(a&b) with 20, 25 and 30 ppt salinity respectively. To these troughs, bacteria was added at 10^6 cfu ml^{-1} (final concentration). 0.1 ml phage suspension (10^9 pfu ml^{-1}) on day 0 followed by a second addition of 0.1 ml of phage at 24 h. The final concentration of the phage in all the troughs was maintained at 10^5 phage ml^{-1}.

PL were acclimated for 48 h before starting the experiment. Experiment was carried out for three days. Continuous aeration was done throughout the experiment to maintain oxygen level. At different time intervals (0, 24, 48 and 72 h) total plate count (TPC) and luminous bacterial count (LBC) were determined by spread plate method using TSAN and LA respectively. Survival of larvae was recorded in all the trough throughout the experiment.

Absence of viral pathogens, such as white spot syndrome virus (WSSV), monodon baculovirus (MBV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) and hepatopancreatic parvovirus (HPV) in PL used for this study were confirmed by polymerase chain reaction (PCR) as described earlier (Otta *et al*., 2003; Rai *et al.,* 2009; Safeena *et al*., 2010). The absence of luminous bacteria in PL was confirmed by plating 5 larvae on luminous bacteria agar (West and Colwell, 1984).

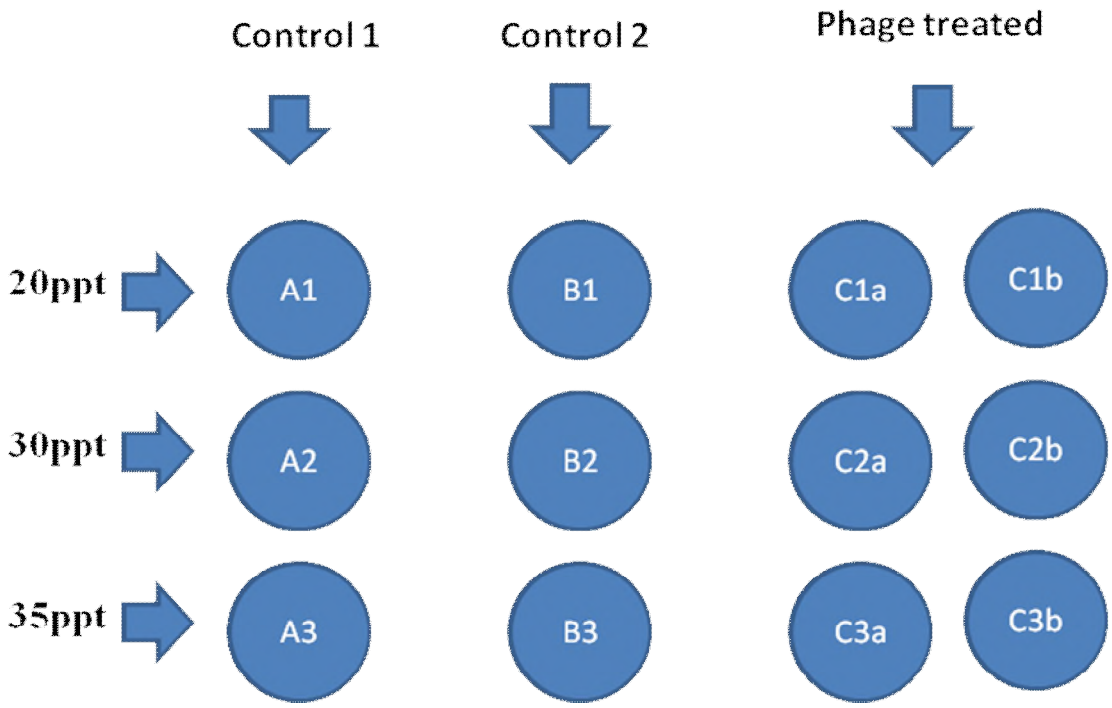

Fig. 5. Diagrammatic representation of experimental setup for standardizing salinity for phage therapy.

3.4.2.2. pH

In case of pH standardization, the experimental set up was similar as for salinity experiment. Twelve glass troughs each containing 1 L autoclaved (110°C for 15 min. at 15 PSI) seawater with 30 post larvae (PL15) of *P. monodon* obtained from a commercial hatchery was setup in four groups with three troughs in each group. First group of troughs designated A_1, A_2, and A_3 had sea water with pH 6, 7 and 8 respectively and served as control to look for effect of pH stress if any on the larval survival.

The second group of troughs was designated B_1, B_2 and B_3 having pH 6, 7 and 8 and contained larvae. *V. harveyi* was added at 10^6 cfu ml^{-1} (final concentration) and served as pathogen control.

The third set of experimental troughs was maintained in replicate and was designated C_1(a&b); C_2(a&b); C_3(a&b) with pH 6, 7 and 8 respectively. To these troughs, bacteria was added at 10^6 cfu ml^{-1} (final concentration). 0.1 ml phage suspension (10^9 pfu ml^{-1}) on day 0 followed by a second addition of 0.1 ml of phage at 24 h.

At different time intervals (0, 24, 48 and 72h) TPC, TVC and LBC were determined by spread plate method using TSAN, TCBS and LA respectively. Others experimental condition same as earlier.

Thiosulphate Citrate Bile salt Sucrose Agar (TCBS)

Peptone	10 g
Yeast extract	5 g
Sodium thiosulphate	10 g
Sodium citrate	10 g
Sodium cholate	3 g
Oxgall	5 g
Sucrose	20 g
Sodium chloride	10 g
Ferric citrate	1 g
Bromothymol blue	0.04 g
Thymol blue	0.04 g
Agar	15 g
pH (at 25^{o}C)	8.8 ± 0.2
Distilled water	1000 ml

The recommended quantity of dehydrated medium (HiMedia, Mumbai) was boiled in sterile distilled water, cooled to 50^{o}C and poured into sterile petriplates.

3.4.2.3. TDS

In case of TDS standardization, the experimental set up was similar as for salinity experiment. Twelve glass troughs each containing 1 L autoclaved (110°C for 15 min. at 15 PSI) seawater with 30 post larvae (PL15) of *P. monodon* obtained from a commercial hatchery was was setup in four groups with three troughs in each group. First group of troughs designated A_1, A_2, and A_3 had sea water with TDS of 11.25, 22.63 and 38.43 mg ml^{-1} respectively and served as control to look for effect of TDS stress if any on the larval survival.

The second group of troughs was designated B_1, B_2 and B_3 having TDS of 11.25, 22.63 and 38.43 mg ml^{-1} and contained larvae. *V. harveyi* was added at 10^6 cfu ml^{-1} (final concentration) and served as pathogen control.

The third set of experimental troughs was maintained in replicate and was designated C_1(a&b); C_2(a&b); C_3(a&b) with TDS of 11.25, 22.63 and 38.43 mg ml^{-1} respectively. To these troughs, bacteria was added at 10^6 cfu ml^{-1} (final concentration). 0.1 ml phage suspension (10^9 pfu ml^{-1}) on day 0 followed by a second addition of 0.1 ml of phage at 24 h.

At different time intervals (0, 24, 48 and 72h) TPC, TVC and LBC were determined by spread plate method using TSAN, TCBS and LA respectively. Others experimental condition same as earlier.

3.4.2.4. Temperature

Nine glass troughs each containing 1 L autoclaved (110°C for 15 min. at 15 PSI) seawater was setup in three groups with three troughs in each group. The first group of troughs designated A_1, A_2 and A_3 had sea water having temperature of 20, 30 and 37°C respectively. *V. harveyi* was added at 10^6 cfu ml^{-1} (final concentration) and served as pathogen control.

The second set of experimental troughs was maintained in replicate and was designated C_1(a&b); C_2(a&b); C_3(a&b) with temperature of 20, 30 and 37°C respectively. To these troughs, bacteria was added at 10^6 cfu ml^{-1} (final concentration). 0.1 ml phage suspension (10^9 pfu ml^{-1}) on day 0 followed by a second addition of 0.1 ml of phage at 24 h. The final concentration of the phage in all the troughs was maintained at 10^5 phage ml^{-1}.

At different time intervals (0h, 4h, 24h, 28h, 48h, and 72h) TPC, TVC and LBC were determined by spread plate method using TSAN, TCBS and LA respectively.

3.4.3. Statistical analysis

The bacterial count obtained at different time interval under various parameter during phage treatment were analyzed using analysis of variance (ANOVA). Duncan's multiple range test as perform to find out whether any significant difference existed among bacterial count (cfu ml^{-1}) in all the experiments. ANOVA was performed using statistical package http://www.physics.csbsju.edu/stats/anova.html and *p* (probability) value was estimated from online database http://www.danielsoper.com/statcalc/calc07.aspx. Duncan's multiple range test was performed as described by Bewick *et al*., 2004.

3.5. Standardization of bacteriophage dose application in different salinity and pH conditions

For experimental setup, seven conical flasks each containing 500 ml of sterile seawater was taken. To all seven flasks, 1 ml of a young culture of *V. harveyi* (OD 0.6) was added to have a final bacterial count of 10^6 cfu ml^{-1}. Flask 1 served as bacterial control to which no phage was added. To flask 2-7, phageV was added at 0, 24, 48 h at varying doses of 50, 100, 150, 200, 250, 300 µl to have a final phage count of $1.4x10^7$, $2.8 x10^7$, $4.2 x10^7$, 5.6

$x10^7$, 7 $x10^7$, 8.4 $x10^7$ pfu ml^{-1}.At 72 and 96 h, the flask 2-7 were replenished with phageJ and at 120 h, phageN was added at the volumes mentioned earlier (50, 100, 150, 200, 250 and 300 µl). At different time intervals (0 h, 24 h, 48 h, 72 h, 96 h, and 120 h) *V. harveyi* count was determined by spread plate method using TSAN.

The experiment was repeated in an identical manner with seawater of varying salinity (20, 25, 30, 35 ppt) and with varying pH (6, 7, 8). For standardization of bacteriophage doses, different doses of bacteriophage (50, 100, 150, 200, 250 and 300 µl) were added to sea water of different salinity (20 ppt, 25 ppt, 30 ppt, and 35 ppt) and pH conditions (6, 7, and 8).

3.6. Expression of recombinant shrimp lysozyme

3.6.1 Lysozyme clone:

The recombinant gene constructs containing gene for Lysozyme (LZ) was maintained in *E. coli* Top10 hosts in Luria-Bertanii (LB) broth with 30% glycerol at −80°C freezer (Sanyo Corporation, Japan) in the Department of Fishery Microbiology, College of Fisheries, Mangalore. This clone used for the production of lysozyme. For expression studies, plasmids were extracted from these cells and transformed to *E. coli* BL21 (DE3) pLysS. Expression vectors was used, pEXP5-NT/TOPO TA (Invitrogen, USA) (Fig. 6) in which shrimp LZ gene (GenBank accession no. EU095851) inserted.

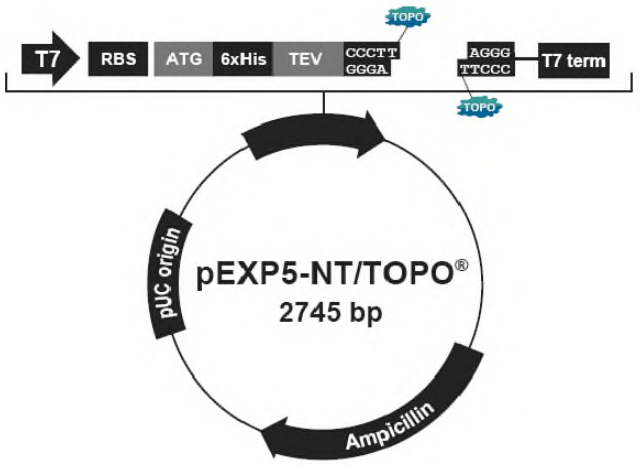

Fig. 6. Vectors used in this study. pEXP5-NT/TOPO TA linearized vector (Invitrogen, USA)

3.6.1. Plasmid DNA extraction

3.6.1.1 Reagents supplied with kit (Eppendorf Fast Plasmid Mini Kit)

Lysis solution, RNase solution, Lysozyme, Wash buffer concentrate, Elution buffer and Fast Plasmid Spin Column Assembly.

3.6.1.2 Plasmid extraction protocol

Young bacterial cultures at the logarithmic growth phase were used for plasmid DNA extraction. 1.5 ml of bacterial culture was centrifuged to sediment the cells to the bottom of the microfuge tube. The clear supernatant was discarded and to the pellet, 400 µl of ice cold complete lysis solution was added. The pellet was mixed thoroughly and incubated at room temperature for 3 min. Subsequently, the lysate was transferred to the spin column assembly and centrifuged at maximum speed for 60 sec. 400 µl of diluted wash buffer was added to the spin column and centrifuged for 1 min. For elution, 50 µl of elution buffer was added to the spin column placed in a collection tube and centrifuged. The purified plasmid was collected in the collection tube and stored (Fig. 7).

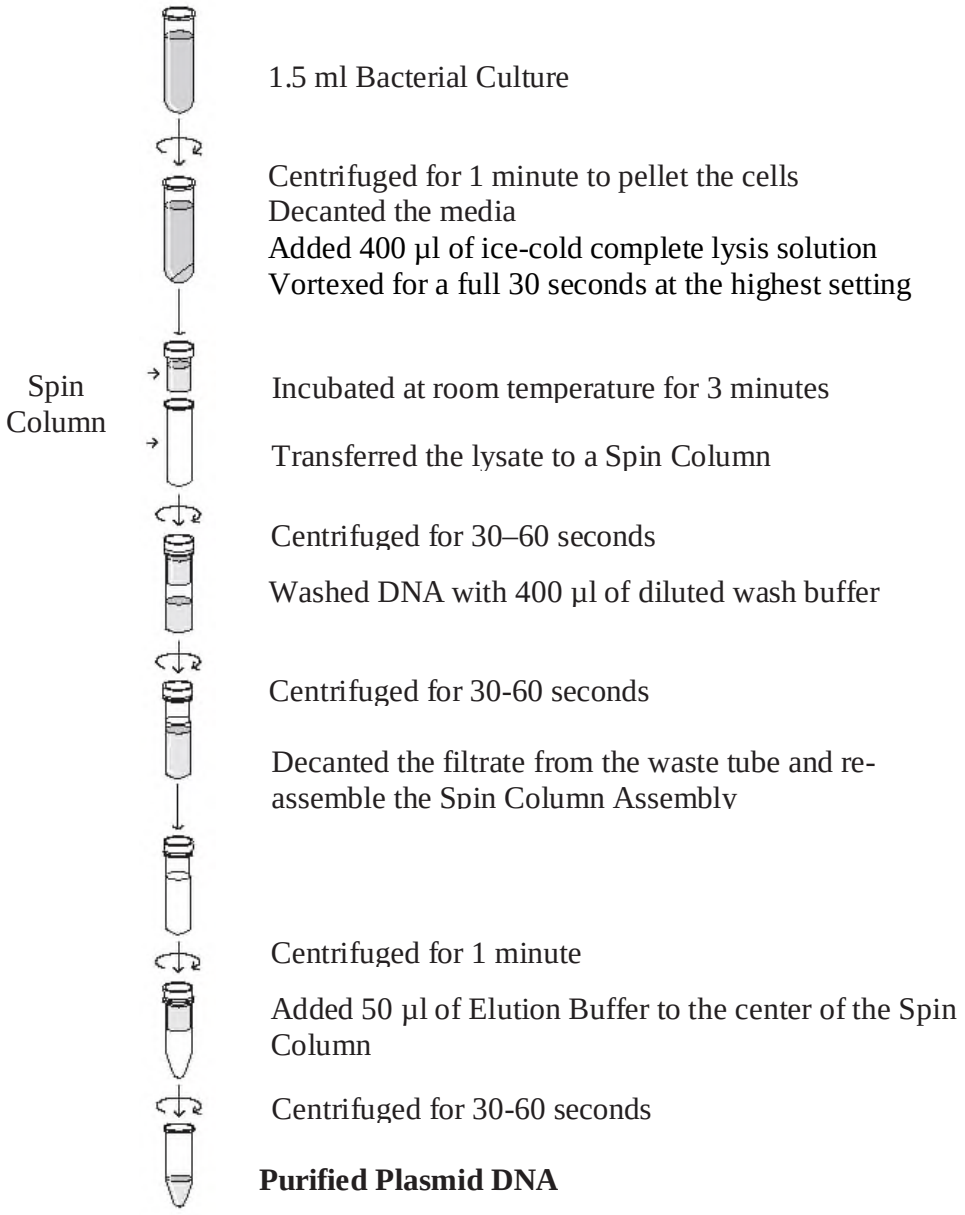

Fig. 7. Plasmid purification using Eppendorf fast plasmid mini kit

3.6.2. Preparation of competent cells

The procedure described by the suppliers (Qiagen, USA) was followed to prepare the competent cells. Aliquots of *E. coli* strains BL21 were removed from the supplied vial with a sterile tooth pick, streaked on LB agar containing antibiotics (34 µg/ml chloramphenicol) and

incubated overnight at 37ºC. Then single colony was inoculated into 10 ml of LB broth containing respective antibiotic and grown overnight at 37ºC with constant shaking at 150 rpm. The following day, 1 ml of overnight grown culture was transferred into 100 ml of LB broth containing respective antibiotics and incubated for 90-120 min with vigorous shaking at 200 rpm at 37ºC. Optical density at 600 nm (OD_{600}) was measured after every 30 min. When OD_{600} reached 0.4-0.5, culture was chilled on ice, transferred to pre-chilled 50 ml polypropylene tubes and centrifuged at 4000 x *g* for 10 min using cooling centrifuge (Hearus, Germany). The supernatant was discarded and the cells pellet was suspended gently without damaging the cells in 30 ml of ice cold TFB1 buffer and the suspension was kept on ice for an additional 90 min and centrifuged at 2500 rpm at 4ºC for 10 min. The supernatant was discarded carefully and the cell pellet was resuspended in 4 ml of ice cold TFB2 buffer. About 150 µl of suspension of competent cells was aliquoted in 1.5 ml microfuge tubes, frozen and stored at -80ºC.

Luria-Bertanii (LB) broth (Hi Media, Mumbai)

Tryptone	1%
Yeast extract	0.5%
Sodium chloride	1%

2.5 g of LB broth (readymade) was dissolved in 100 ml of distilled water and sterilized at 121 ºC for 15 min. To the cooled LB broth, antibiotics were added from the stock solutions to get the desired concentrations.

Luria-Bertanii (LB) agar (Hi Media, Mumbai)

Tryptone	1%
Yeast extract	0.5%
Sodium chloride	1%
Agar	1.5%

4 g of LB agar (readymade) was dissolved in 100 ml of distilled water and sterilized at 121 $^{\circ}$C for 15 min. Then it was cooled to 50ºC and antibiotics were added from the stock solutions to get the desired concentrations.

Chloramphenicol stock solution (34 mg/ml)

Stock solution was prepared by dissolving 0.0377 g of chloramphenicol powder (HiMedia, Mumbai) having the 90% purity in 1ml ice cold ethanol and stored in aliquots at -20°C.

TFB1 buffer

RbCl	100 mM
$MnCl_2$	50 mM
Potassium acetate	30 mM
Calcium chloride	10 mM
Glycerol	15%
pH	5.8

All the chemicals used were of molecular biology grade. pH was adjusted carefully to avoid formation of insoluble manganese precipitate. After adjusting the pH, the buffer was filter sterilized and stored at 4ºC.

TFB2 buffer

MOPS	10 mM
RbCl	10 mM
Calcium chloride	75 mM
Glycerol	15%
pH	6.8

After adjusting the pH, the buffer was filter sterilized and stored at 4°C.

3.6.3. Transformation

1 µl plasmids were added to tube containing 150 µl competent cells (*E. coli* BL21). The contents were mixed by gentle tapping and incubated on ice for 30 min. The cells were subjected to heat shock at 42ºC for 90 sec in a water bath. The tubes were then rapidly transferred to ice and allowed to chill for 1-2 min. 500 µl of LB broth was added to the tubes and incubated at 37ºC for 1hr with vigorous shaking. The cells were centrifuged at 4000 x *g* for 10 min. After discarding the 300 µl supernatant, transformed cells were resuspended in remaining 200 µl broth and plated on two LB plates (100 µl/plate) containing antibiotics (100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol). The plates were then incubated overnight at 37ºC.

3.6.3. Screening of transformants

Transformants were randomly selected and screened for the presence of insert by preparing crude lysate of DNA (Dileep *et al*., 2003). Briefly, each of the selected colonies was inoculated to 2 ml of LB broth and incubated overnight at 37ºC. To 50 µl of the culture, 450 µl of TE buffer was added and cells were lysed at 95ºC for 10 min, snap chilled, centrifuged

briefly for 5 min and 2 µl of supernatant was used as template for PCR using gene specific primers.

Table 1. Primers used, annealing temperature, expected PCR product size and vector used for cloning the genes.

Protein	GenBank Accession number	PCR product size(bp)	Anneling temperature (°C)	Vector used	Primer sequences
LZ	AF425673	477	48	pEXP5-NT TOPO TA expression vector	F 5' ATGAGGATCCTTCCTCTGGCG 3' R 5' CTAGAATTCGAAGACAGAGTT 3'

3.6.4. Expression of the recombinant proteins

To ascertain the expression, each of the positive clones was inoculated to to 5 ml of LB medium containing antibiotics (100 µg ml^{-1} ampicillin and 34 µg ml^{-1} chloramphenicol). Cultures were grown overnight at 37ºC with constant agitation at 150 rpm. One ml of overnight cultures were inoculated into 10 ml of LB broth with specific antibiotics and incubated at 37ºC with constant shaking until the OD_{600} was 0.5-0.7. The cultures were induced with 1 mM concentration of IPTG and further incubated for 4 h with the same conditions. Uninduced recombinant clone and *E. coli* strains (BL21 cells) were used as control. After incubation, 1 ml culture from each sample was centrifuged at 11,000 x *g* for 3 min and the supernatant was discarded. The pellet was resuspended in 30 µl distilled water and 30 µl of 2x sample buffer by continuous vortexing, lysed at 95ºC for 5-10 min in a dry bath. Then, samples were loaded to the polyacrylamide gel for electrophoresis.

For production of recombinant proteins in relatively large scale, 200 ml of LB broth was used. The culture of the recombinant *E. coli* was grown (as described above) and expression obtained under IPTG (1mM) induction and time course (4 h). Cells were harvested by centrifugation at 11,000 × g for 5 min after induction with IPTG for 4 h. Cell pellet was resuspended in lysis buffer (pH 8.0) and stirred using magnetic beads for 1 h at room temperature, sonicated on ice at 20-30 W with six 10 sec bursts. The lysate was centrifuged at 11,000 × *g* for 15 min at room temperature to pellet the cellular debris. The supernatant was added to dialysis sacks (Sigma Diagnostics, USA), sealed and kept in distilled water overnight. After dialysis protein was kept in -20º C freezers until further use.

Ampicillin stock solution (100 mg ml^{-1})

Stock solution was prepared by dissolving 0.1089 g of ampicillin powder (HiMedia, Mumbai) having 98% purity in 1 ml distilled water, filter sterilized, aliquotted and stored at -20ºC.

Isopropyl-thio- β-D-galactoside (IPTG, 1 M)

Stock solution was prepared by dissolving 2.3 g of IPTG in 10 ml of distilled water, filter sterilized and aliquots were stored at -20ºC.

Lysis buffer (pH 8.0)

NaH_2PO_4	100 mM
Tris-Cl	10 mM
Guanidine hydrochloride	6 M

pH was adjusted to 8.0 using 5N NaOH

3.6.5. SDS-polyacrylamide gel electrophoresis

Recombinant protein expressed in *E. coli* was analysed by the method of Laemmli (1970) using SDS-PAGE with some modifications. The various buffers and reagents used are as follows:

a) Acrylamide-bisacrylamide mixture

Twenty nine grams of acrylamide and 1.0 g of bisacrylamide (N, N'-methylene bisacrylamide) were dissolved in 80 ml of distilled water. The volume was made up to 100 ml and stored at 4°C.

b) Separating gel buffer (1.5 M Tris-Cl, pH-8.8)

181.7 g of Tris base was dissolved in 800 ml of distilled water and pH was adjusted using concentrated HCl. Then final volume was made up to 1 liter with distilled water and stored at 4°C.

c) Stacking gel buffer (1 M Tris-HCl, pH-6.8)

121.1 g of Tris base was dissolved in 800 ml of distilled water and pH was adjusted using concentrated HCl. Then final volume was made up to 1 liter with distilled water and stored at 4°C.

d) 10 % sodium dodecyl sulphate (SDS) solution

10 g of SDS was dissolved in 100 ml of distilled water and stored at room temperature.

e) 10% ammonium persulfate (APS) solution

1 g of APS was dissolved in 10 ml of the distilled water and stored at 4°C temperature. APS decomposes slowly and hence fresh solution was prepared after one week.

f) N,N,N',N'-tetramethylethylenediamine (TEMED)

Electrophoresis grade TEMED (Bangalore Genei, Bangalore) stored at 4°C was used.

g) Electrode buffer (5´ solution)

5´ stock solution was prepared by dissolving 15.1 g of Tris base, 94 g of glycine (electrophoresis grade) in 900 ml distilled water and then 50 ml of 10 % SDS solution of electrophoresis grade was added and the final volume was made up to 1 liter with distilled water and stored at room temperature. For running the gel, 1X buffer was prepared using 5´ electrode buffer.

h) SDS gel loading buffer (2´)

2´ gel loading buffer contained 100 mM Tris-Cl (pH 6.8); 4% (w/v) SDS; 20% (v/v) glycerol; 0.1% (w/v) bromophenol blue; 200 mM β-mercaptoethanol. Gel loading buffer lacking thiol reagent was stored at room temperature. 200 mM β-mercaptoethanol was added to buffer from 14 M stock mercaptoethanol just before use.

i) Staining solution

2.5 g of Coomassie Brilliant blue R250 (HiMedia, Mumbai), 450 ml of methanol and 100 ml of acetic acid were mixed, filtered and volume was made to 1 liter with distilled water. Solution was stored at room temperature.

j) Destaining solution

300 ml of methanol and 100 ml of acetic acid mixed with distilled water to make up 1 liter. Solution was stored at room temperature.

k) Standard protein molecular weight marker

A medium-range protein molecular weight marker ((PMW-M) Bangalore Genei, Bangalore) was used for determination of molecular weights of desired protein. The molecular weight protein standards included phosphorylase b (97,000 Da), bovine serum albumin (66,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (29,000 Da), soyabean trypsin inhibitor (20,100 Da) and lysozyme (14,300 Da).

3.6.6.1. Preparation of SDS-PAGE apparatus (Sambrook *et al.*, 1989)

In this study, all recombinant proteins were analyzed on 15% resolving and 5% stacking gel. Glass plates were rinsed by absolute alcohol and cleaned using tissue paper. Spacers (1 mm) were placed on both sides between two plates in such a way that any bubbles could not move through that and seated in stand and checked by water. 15% resolving gel was prepared and poured up to 3\4 portion, and kept for solidification. After solidification, 5% stacking gel was prepared, poured and 1 mm comb was inserted into the staking gel. Gel was allowed to solidify. Comb was removed and the solidified gel with plates was fixed in gel running apparatus. Gel running tank was filled with 1X electrode buffer and prepared samples were loaded into wells. Samples were resolved by applying constant current of 20 mA for 2 hr. After electrophoresis, the gel was transferred to a clean container and stained overnight at room temperature with shaking. After staining, the gel was destained until a clear background was obtained. Photographs of the gels were taken with Herolab gel documentation system (Germany).

Resolving gel mixture was prepared in a small beaker with a magnetic stirrer by mixing the components for a desired concentration of acrylamide according to the following chart (Harlow and Lane, 1998).

Recipe for resolving gels (pH 8.8)

Solution component	**15% gel**	
	Component Volumes (ml)	
Total volume	5.0	10.0
Deionised water	1.2	2.3
30% acrylamide mix	2.5	5.0
1.5 M Tris-HCl pH 8.8	1.3	2.5
10% (w/v) SDS	0.05	0.1
10 % (w/v) APS	0.05	0.1
TEMED	0.002	0.004

Immediately upon addition of APS and TEMED, the solution was poured into the assembled plates and overlaid with iso-butanol to prevent diffusion of oxygen into the gel and for obtaining a uniform margin of the gel. It was allowed to polymerize for about 15 min.

In a similar manner, stacking gel mixture with 5% acrylamide mix was prepared by mixing the components as below (Sambrook *et al.*, 1989).

Recipe for stacking gels (pH 6.8)

Component	1 ml	3 ml	5 ml	8 ml	10 ml
H_20	0.68	2.1	3.4	5.5	6.8
30% Acrylamide Mix	0.17	0.5	0.83	1.3	1.7
1.0 M Tris (pH-6.8)	0.13	0.38	0.63	1.0	1.25
10% SDS	0.01	0.03	0.05	0.08	0.1
10% APS	0.01	0.03	0.05	0.08	0.1
TEMED	0.001	0.003	0.005	0.008	0.01

3.6.6.2. Sample preparation for SDS-PAGE

One ml of test culture was pelleted by centrifugation at 11,000 × g for 3 min and supernatant was discarded. The pellet was resuspended in 30 µl distilled water and 30 µl of 2X sample buffer, vortexed and lysed at 95°C for 5-10 min in a dry bath. The samples were loaded to the SDS polyacrylamide gel.

3.6.7. Protein estimation

The protein content was measured by the method of Lowry (1951). The procedure is based on quantitating the color obtained from the reaction of Folin Ciocalteau phenol reagent with tyrosyl residues of unknown protein and comparing this with a standard protein, usually bovine serum albumin (BSA). Five standard BSA (1 mg/ml in distilled water) samples were prepared in 0.1 N sodium hydroxide to give concentrations of 20, 40, 60, 80 and 100 µg / ml, respectively, in glass tubes in duplicates. Test samples were taken in 2 different dilutions of 20 µl and 40 µl. Equal volume of alkaline copper reagent was added to all tubes and the mixture

was incubated for 15 min at room temperature. Folin's reagent (Sigma, USA) was added at a concentration of 50 µl ml^{-1}. The entire mixture was mixed well and incubated for another 30 min at room temperature. Optical density was measured at 690 nm. The protein concentration of the sample was obtained from the graph plotted for the standard BSA concentrations as optical density values.

Copper sulphate (1%) solution

Copper sulphate	0.1 g
Distilled water	50 ml

Sodium potassium tartarate (2%) solution

Sodium potassium tartarate	1 g
Distilled water	50 ml

Sodium carbonate (4%) solution

Sodium carbonate	2 g
Distilled water	50 ml

Sodium hydroxide (0.1 N) solution

Sodium hydroxide	0.2 g
Distilled water	50 ml

Alkaline copper reagent (ACR)

Copper sulphate (1%)	1 ml
Sodium potassium tartarate (2%)	1 ml
Sodium carbonate (4%)	48 ml

Bovine serum albumin (1%)

Bovine serum albumin	0.01 g
Distilled water	1 ml

3.7. Role of lysozyme on the phage activity

3.7.1. Solid phase assay

The role of recombinant tiger shrimp lysozyme (TSL) on vibrio phage activity was studied by solid phase assay. Bacterial cultures grown in TSB with 1% NaCl were used for the assay. For solid phase assay bacterial lawn was prepared on TSAS plates. After drying, 10 µl of recombinant lysozyme protein (crude protein) and bacteriophage separately and recombinant lysozyme protein & bacteriophage together were spotted separately on the plate in three sectors. The proportion of lysozyme and phage was 1:1, 2:1, 3:1, 4:1 respectively. Similarly phage and lysozyme proportion was made at 1:1, 1:2, 1:3 and 1: 4 respectively. Plates were incubated at 30^{o}C for overnight. All experiments were carried out in triplicate and diameter of the zone of inhibition was measured in all three cases.

3.7.2. Role of lysozyme on the vibriophage activity in seawater

To study the role of lysozyme on vibriophage activity in seawater, we added vibriophage alone lysozyme alone and vibriophage + lysozyme together to sea water to which of *V. harveyi* was added in three different set of experiments. Eight conical flasks (A, B_1, B_2, B_3, C_1, C_2, C_3 and D) containing 100 ml sterilized sea water (autoclaved at 110ºC for 15 min at 15PSI) were used for the experiment (Fig. 8). In all flasks *V. harveyi* was added to a final count of 1.19× 10^6 cfu ml^{-1}. 15 µl of bacteriophage (1.41×10^9 pfu ml^{-1}) was added to flask 'A'. 25 µl, 50 µl and 100 µl of TSL (1.6 mg ml^{-1} conc.) was added to B_1, B_2 and B_3 flasks respectively. 25 µl, 50 µl and 100 µl of TSL (1.6mg ml^{-1} conc.) together with 15 µl of bacteriophage to each flask was added to C_1, C_2 and C_3 respectively. Flask D kept as control, to which neither bacteriophage nor TSL was added. At different time intervals (0 h, 1 h, 2 h, 4 h and 24 h), *V. harveyi* counts was determined by surface spread method using TSAN.

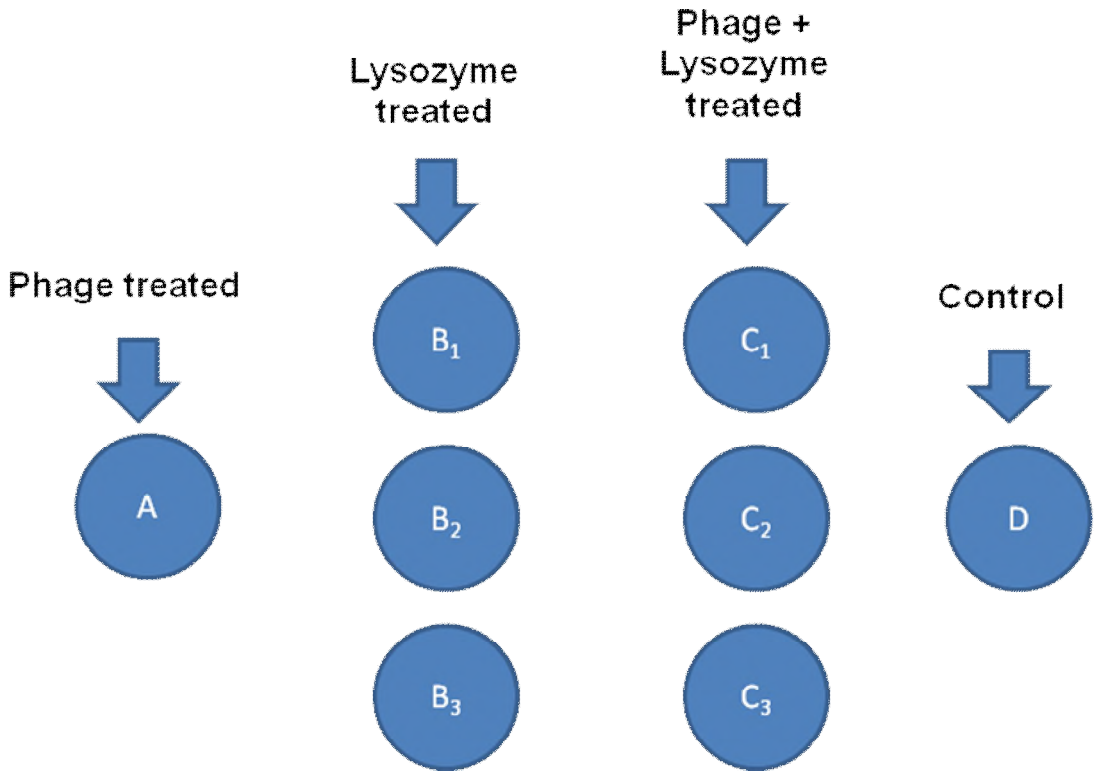

Fig. 8. Diagrammatic representation of experiment to see the role of lysozyme on the vibriophage activity in seawater

IV. RESULTS

4.1. Bacteria and bacteriophages

A global collection of eighty seven *V. harveyi*, isolated earlier from different sources was used in the study. Culture details are provided in Table 1.

Seven *V. harveyi* phage isolated earlier from various source in our laboratory were used in the study. Details of the phage are provided in Table 2.

4.2. Propagation of bacteriophages and estimation of phage titers

For phage propagation, host culture was grown on TSBS for 12 h. 4 ml of this young culture and 1 ml phage suspension was mixed and spread on the surface of TSAS agar in Roux bottles. After 5–6 h incubation at room temperature (28°C), the phages were harvested in SM buffer. A soft agar overlay technique (Adams, 1959) was used for determining the phage titres (Fig. 8). The titers were found was found to be in the range of 10^9 - 10^{11} pfu ml^{-1} for all seven phage (Table 2).

4.3. Determination of host range of phages

Of seven phages tested against 87 *V. harveyi* isolates, PhageV isolated from shrimp hatchery water had broad range activity lysing 64% of isolates while VHphageJ isolated from oyster lysed 61%. Other phages *viz* A, N, M isolated from shrimp hatchery water lysed 43%, 53% and 49% of isolates respectively. PhageK and PhageR had very narrow activity and lysed only their specific hosts. On the basis of broad host range activity (Fig. 9) phageV was used for further phage application studies.

4.4. Standardization of phage application under various parameters

Every organism has an optimum physicochemical parameter for its activity. Keeping this point in mind, we tried to examine various parameters required for optimum phage activity against luminous vibriosis of shrimp. In all the experiments, we also recorded the percentage survivability of PL in whole experiment.

4.4.1 Salinity

Of the three different salinities (20, 25 and 30 ppt) used in the study, 25 ppt was the optimum for *V. harveyi* phage activity against *V. harveyi*. In 25 ppt salinity, there was 2.11 $\log_{10}$ reduction in TPC and 3.87 $\log_{10}$ reduction in LBC after 72 h. On the other hand in 20ppt and 30 ppt, approximately one $\log_{10}$ reduction in TPC and 3.5 $\log_{10}$ reduction in LBC was observed in each case after 72 h (Fig. 10 and Fig. 11). In the control 1 group, less than 10 luminous bacteria was present in all three salinity troughs. In control 2, less than one $\log_{10}$ increase in LBC was obtained (Table 3).

Larval survivability was significantly higher in phage treated troughs compared to untreated troughs. Among phage treated tubs, 25 ppt salinity presented higher larval survivability than that at 20 ppt and 30 ppt (Fig. 21). In case of control 1 group no larval mortality was found.

One way ANOVA revealed the counts of LBC to be significantly different in three different salinities. *F* value was found to be 24.93. Duncan multiple range test showed significant difference in the decreasing pattern of LBC at different time intervals among the three different salinity range in phage application experiment.

4.4.2. pH

pH standardization was done by carrying out the experiment in acidic pH (6), neutral pH (7) and alkaline pH (8). Neutral pH was the best for *V. harveyi* phage activity. In neural pH there was 4 log_{10} reduction in LBC (Fig. 13), 3.5 log_{10} reduction in TVC (Fig. 14) and 2.5 log_{10} reduction in TPC (Fig. 15), whereas in acidic and alkaline pH log_{10} reduction was comparatively less than in neutral pH (Table 4). In control 2, almost constant count was obtained in LBC, and half log increases were found in both TVC and TPC (Table 4).

Larval survivability was also higher in neutral pH troughs compared to other condition, whereas in control 2, larval survivability was less (Fig. 21). In case of control 1, no larval mortality was seen.

To see the difference in decreasing pattern in LBC at different time interval in three different pH used, one way ANOVA was performed. ANOVA revealed that the decreasing patter of LBC was significantly different in three different pH. *F* value was found to be 22.84. Duncan multiple range test showed significant difference among LBC at three different pH.

4.4.3. Total dissolved solid

Lower the TDS, higher was the phage activity in the experiment. Three different TDS (11.26, 22.63 and 38.43 mg ml^{-1}) were used for the experiment. TDS of 11.25 mg ml^{-1} showed highest log_{10} reduction in all bacterial counts, followed by TDS of 22.63 mg ml^{-1} and 38.43 mg ml^{-1}. In case of 11.26 mg ml^{-1} TDS, there was 3.3 log_{10} reduction in LBC (Fig. 16), 3.18 log_{10} reduction in TVC (Fig. 17) and 2.16 log_{10} reduction in TPC (Fig. 18).

Larval survivability was highest in troughs with lowest TDS. In Control 2 troughs, survivability of larvae was low (Fig. 21). No larval mortality was found in control 1 group troughs.

One way ANOVA gave *F* value of 23.67 which indicated that the decreasing pattern of LBC was significantly different in three different TDS. After obtaining significant difference in ANOVA, to look for differences in each possible group at time intervals, Duncan multiple range test was performed which also showed that significant difference exists in decreasing pattern of LBC at different time intervals among three different TDS used in the experiment.

4.4.4. Temperature

Of the three temperature range (20, 30 and 37ºC) used in study, 30ºC was found to be best for phage activity. There was four $\log_{10}$ reduction in LBC (Fig. 19), three and half $\log_{10}$ reduction in TVC (Fig. 20) and two and half $\log_{10}$ reduction in TPC at 30ºC (Fig. 21). At 20ºC and 37ºC there was less reduction in the counts as compared to 30ºC (Table 6). In control 2, close to one $\log_{10}$ increase in LBC was found both at 30ºC & 37ºC and about half $\log_{10}$ increase in LBC at 20ºC (Table 6).

Counts were performed at 0, 4, 24, 28, 48 and 72 h. After four hours of addition of phage, the decrease seen in all the counts was significant as compared to after 24 h. Phage addition at 0 h and 24 h, resulted in significant reduction in all the counts at 4 hours and 28 hours.

One way ANOVA revealed that the decreasing patter of LBC was significantly different in three different temperatures. *F* value was found to be 11.96. Duncan multiple range test showed significant difference in the decreasing pattern of LBC at different time intervals among the three different temperatures.

4.5. Standardization of bacteriophage dose application in different salinity and pH conditions

In all the experiments relating to the use of three phages added in different volumes (varying pfu), maximum reduction of bacterial numbers in all cases was observed within 48 h of first phage application. Upon addition of the second phage, the decrease was again observed within 24 h of addition. However in all the cases, no significant reduction of bacterial numbers was found after third phage addition.

4.5.1. Salinity

At 20 ppt salinity, out of six variable volumes of phage added, reduction of bacterial number to less than 1000 was observed on addition of 150 µl phage ($4.2x10^7$ pfu ml^{-1} of seawater) doses (Table 8, Fig.23). At 25 ppt salinity 100 µl phage ($2.8x10^7$ pfu ml^{-1} of seawater), brought about reduction in counts from 3400000 to 1020 in 120 h (Table 9, Fig.24). At 30 ppt salinity 250 µl phage ($7x10^7$ pfu ml^{-1} of seawater), brought about reduction in counts from 4700000 to 820 in 120 h (Table 10, Fig.25). At 35 ppt salinity 300µl phage ($8.4x10^7$ pfu ml^{-1} of seawater), brought about reduction in counts from 4700000 to 690 in 120 h (Table 11, Fig.26).

4.5.2. pH

At 6 pH, out of six variable volumes of phage added, reduction of bacterial number to less than 1000 was observed on addition of 150 µl phage ($4.2x10^7$ pfu ml^{-1} of seawater) doses (Table 12, Fig.27). At 7 pH 100µl phage ($2.8x10^7$ pfu ml^{-1} of seawater), brought about reduction in counts from 2800000 to 760 in 120 h (Table 13, Fig.28). At 8 pH 150 µl phage ($2.8x10^7$ pfu ml^{-1} of seawater), brought about reduction in counts from 7200000 to 910 in 96 h (Table 13, Fig.29).

4.6. Expression of recombinant shrimp lysozyme:

4.6.1. Screening of transformants

After antibiotic (ampicillin and chloramphenicol) selection, positive clones (transformed to *E. coli* BL21 (DE3) pLysS competent cells) were confirmed for the presence of gene by PCR using the lysozyme-specific primers. Positive colonies showed a 477 bp amplicon (Figure 21). Along with vector ATG initiation codon, this 477 bp sequence coded for a recombinant lysozyme protein of molecular weight of 21.1 kDa.

4.6.2. Expression of recombinant lysozyme

PCR positive clone was used for expression study. Induction and subsequent analysis of recombinant clones by SDS PAGE generated a distinct protein band of approximately 21 kDa. However, no such band was observed for induced non recombinant cells and uninduced recombinant clones thus, confirming the expression of recombinant lysozyme (Figure 22).

4.7. Role of lysozyme on the vibriophage activity

4.7.1. Solid phase assay

Solid phase assay was performed to determine the role of recombinant lysozyme on the vibriophage activity against *V. harveyi* culture resulting in clear zone. The lytic activity on *V. harveyi* was observed by adding phage alone, lysozyme alone and phage+ lysozyme together. It was found that in most of the cases, phage+lysozyme together gave higher zone of inhibition on *V. harveyi* lawn, compare to phage alone and lysozyme alone (Fig. 26) (Table 8). Mean±SD of diameter of zone of inhibition on *V. harveyi* lawn was calculated in all three cases. Graphical representation of data also shows higher zone of inhibition in Phage+lysozyme compared to phage and lysozyme individually (Fig. 25).

In case of phage+ lysozyme, it was also tested at different ratio (1:1, 1:2, 1:3, 1:4 and vice versa) and with two different volumes (10 µl and 20 µl) (Table 10). It was found that 1:1 to 2:1 ratio of phage:lysozyme had higher activity compare to combinations of varying proportions..

4.7.2. Role of lysozyme on the vibriophage activity in seawater

To see the role of recombinant lysozyme on vibriophage activity three possible applications as in section 4.7.1.was used: Phage alone, lysozyme alone and phage+lysozyme together was applied to sea water to which *V. harveyi* was added. Three different volumes (25, 50 and 100 µl) of lysozyme (1.6 mg ml^{-1}) was added to constant amount phage (15 µl having 1.41×10^{11} pfu ml^{-1} phage). There was greater reduction in *V. harveyi* counts when both phage and lysozyme were added together compared to phage alone and lysozyme alone when 50 and

100 µl lysozyme was tested with phage (Fig. 28, 29). However not much effect was was seen when 25 µl lysozyme was added with phage (Fig. 27). It was seen that recombinant lysozyme alone (50 µl and 100 µl)) was more active against *V. harveyi* in seawater (Table 11).

In case of phage alone there were 2.81 $\log_{10}$ reductions in *V. harveyi* counts. 100 µl of lysozyme alone brought about a 2.4 $\log_{10}$ reductions in *V. harveyi* counts after 24 hours of experiment. But when lysozyme (100 µl) and phage were added together, there was 3.27 $\log_{10}$ reduction in *V. harveyi* counts after 24 hours. In case of 50µl lysozyme and phage combination there was moderatel decrease in *V. harveyi* counts but in case of 25 µl of lysozyme and phage combination there was hardly any activity found and also a decrease in *V. harveyi* counts when compared to phage alone.

Table 1. Details of *V. harveyi* isolates used in the study.

Serial no.	Isolates nos	Isolated from
1 – 18	Vh1, Vh2, Vh3, Vh4, Vh5, Vh6, Vh7, Vh8, Vh9, Vh10, Vh11, Vh12, Vh34, Vh35, Vh36, Vh37, Vh44, Vh45	Shrimp farm water (India)
19 - 24	Vh13, Vh14, Vh16, Vh21, Vh22, Vh23	Shrimp larvae (India)
25 – 27	Vh17, Vh42, Vh43	Dead shrimp larvae (India)
28 – 32	Vh24, Vh25, Vh26, Vh27, Vh28	Shrimp pond sediment (India)
33 – 37	Vh29, Vh30, Vh31, Vh32, Vh33	Water (India)
38 – 41	Vh38, Vh39, Vh40, Vh41	Healthy shrimp larvae (India)
42 – 48	STD3-0942, STD3-0947, STD3-0949, STD3-0953, STD3-0983, STD3-0986, STD3-1007	Ecuador
49	STD3-0957	Shrimp heamolymph (Costa Azul Sinaloa-Mexico)
50	LMG04043	NCMB 24
51 – 55	LMG04044, LMG07890, LMG11660, LMG11659, LMG10948	USA
56 – 58	LMG10946, BB886, BB120	India
59	LMG10947	Seawater (Red sea)
60	LMG11225	Seawater (Italy)
61	LMG11226	Sea water (Hawaii, USA)
62	LMG11755	Shark mouth (Bahamas)
63-64	LMG13949, LMG16874	Shrimp (Thailand)
65 – 69	LMG16828, LMG16830, LMG16831, LMG16832, LMG16853,	Black Tiger prawn (Thailand)
70 – 71	LMG16862, LMG16863	Oyster (Spain)
72	LMG19643	*Trachurus japonicas* (Japanese horse mackerel) (Japan)
73 – 80	STD3 0999, STD3 1000, STD3 1009, STD3 1011, STD31012, STD3-1020, STD3-1022, STD3-1024	Diseased *P. chinensis* (China)
81	ACMM20	Sea water (ACMM)
82	VLB571	Sea bass (*Dicentrarchus labrax)* (Spain)
83	VLB645	Sea bass (*D. labrax*) (Tunisia)
84-86	BB152, BB, BB170	Laboratory of AARC
87	ACMM642	*P. monodon* larvae (ACMM)

Table 2. Details of *V. harveyi* bacteriophage used in the study.

V. harveyi Phage	Isolated from	Specific host for isolation	Titer (pfu/ml)
PhageV	shrimp hatchery water	VH 17	1.41×10^{11}
PhageJ	oyster	VH 36	2.61×10^{10}
PhageM	shrimp hatchery water	VH 36	1.17×10^{10}
PhageN	shrimp hatchery water	VH 10	2.91×10^{11}
PhageA	shrimp hatchery water	VH 25	9.6×10^{9}
PhageK	shrimp larvae	VH 39	2.71×10^{11}
PhageR	Shrimp larvae	VH 39	1.12×10^{11}

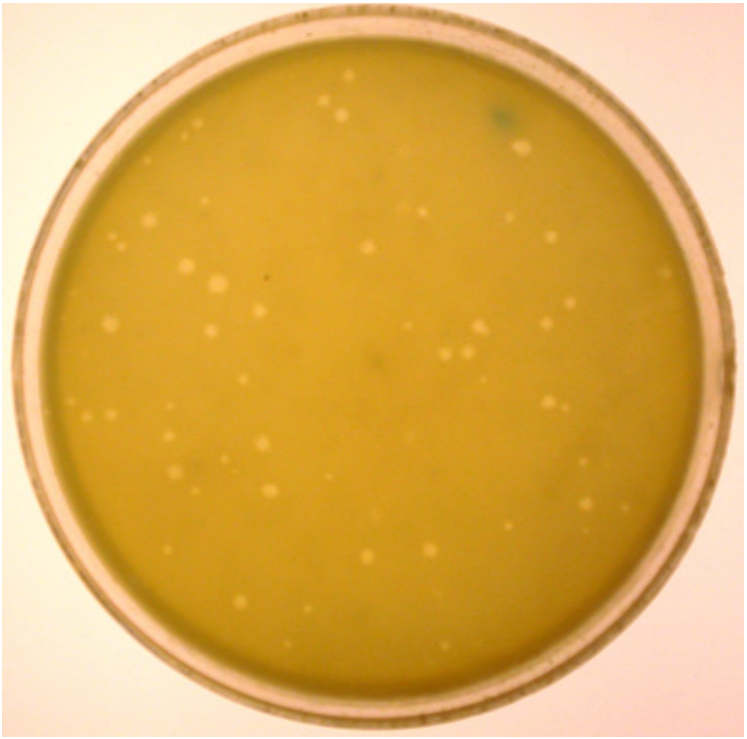

Fig. 9. Plate showing plaques formed by phage (Soft agar overlay method)

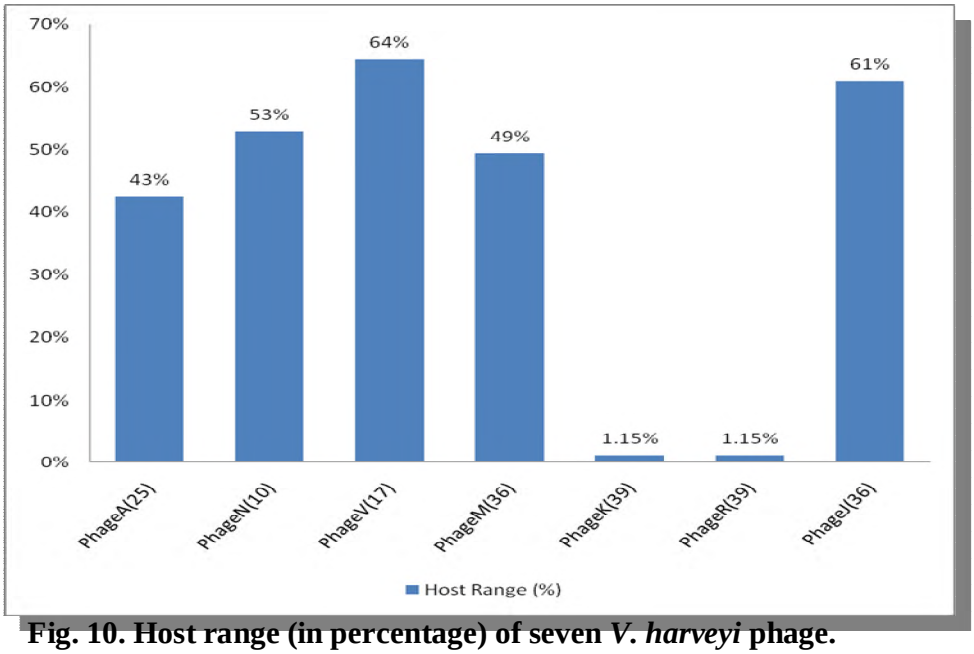

Fig. 10. Host range (in percentage) of seven *V. harveyi* phage.

Table 3. TPC and LBC at different time interval in various salinities during phage therapy.

Time	**0** hour						24 hours						48 hours						72 hours					
Salinity (ppt)	Bacterial no (cfu/ml)						Bacterial no (cfu/ml)						Bacterial no (cfu/ml)						Bacterial no (cfu/ml)					
	Control 1		Control 2		Phage treated		Control 1		Control 2		Phage treated		Control 1		Control 2		Phage treated		Control 1		Control 2		Phage treated	
	LBC	TPC	LBC	TPC	LBC	TPC	LBC	TPC	LBC	TPC	LBC	TPC	LBC	TPC	LBC	TPC	LBC	TPC	LBC	TPC	LBC	TPC	LBC	TPC
20	<10	1.14×10^{3}	0.97×10^{6}	1.1×10^{6}	1.24×10^{6}	1.34×10^{6}	<10	8.89×10^{3}	1.13×10^{6}	1.28×10^{6}	1.10×10^{5}	2.68×10^{5}	<10	8.89×10^{3}	1.32×10^{6}	1.41×10^{6}	6.7×10^{3}	1.92×10^{5}	<10	8.89×10^{3}	1.39×10^{6}	1.54×10^{6}	1.92×10^{3}	1.3×10^{5}
25	<10	6.11×10^{3}	0.93×10^{6}	1.24×10^{6}	0.97×10^{6}	1.19×10^{6}	<10	7.16×10^{3}	0.98×10^{6}	1.41×10^{6}	2.84×10^{4}	9.92×10^{4}	<10	7.16×10^{3}	1.19×10^{6}	1.5×10^{6}	8.63×10^{2}	1.51×10^{4}	<10	7.16×10^{3}	1.41×10^{6}	1.68×10^{6}	2.86×10^{2}	9.14×10^{3}
30	<10	4.9×10^{3}	1.07×10^{6}	1.34×10^{6}	1.18×10^{6}	1.41×10^{6}	<10	7.1×10^{3}	1.19×10^{6}	1.47×10^{6}	1.70×10^{5}	3.11×10^{5}	<10	7.1×10^{3}	1.28×10^{6}	1.59×10^{6}	6.61×10^{3}	1.47×10^{5}	<10	7.1×10^{3}	1.35×10^{6}	1.81×10^{6}	2.81×10^{3}	1.16×10^{5}

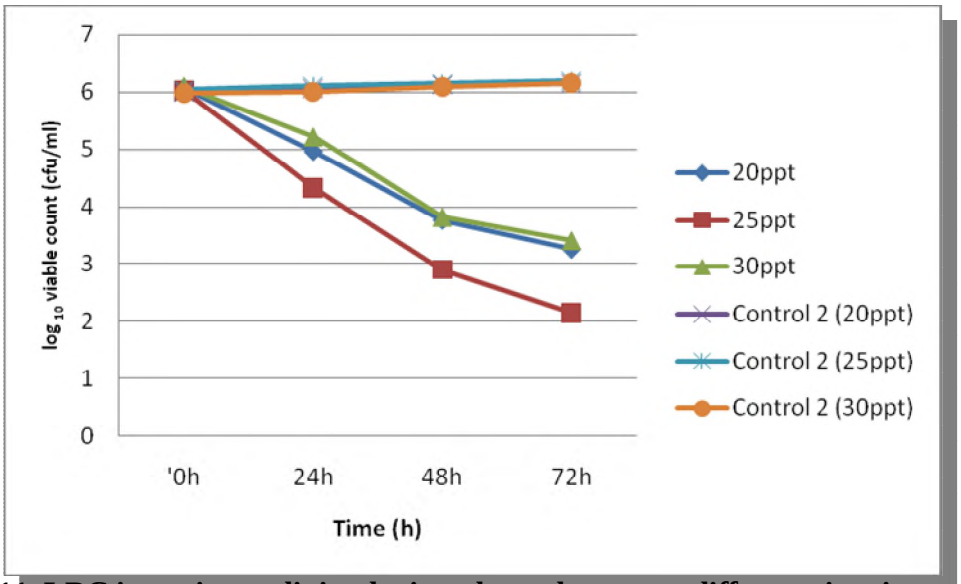

Fig. 11. LBC in various salinity during phage therapy at different time intervals.

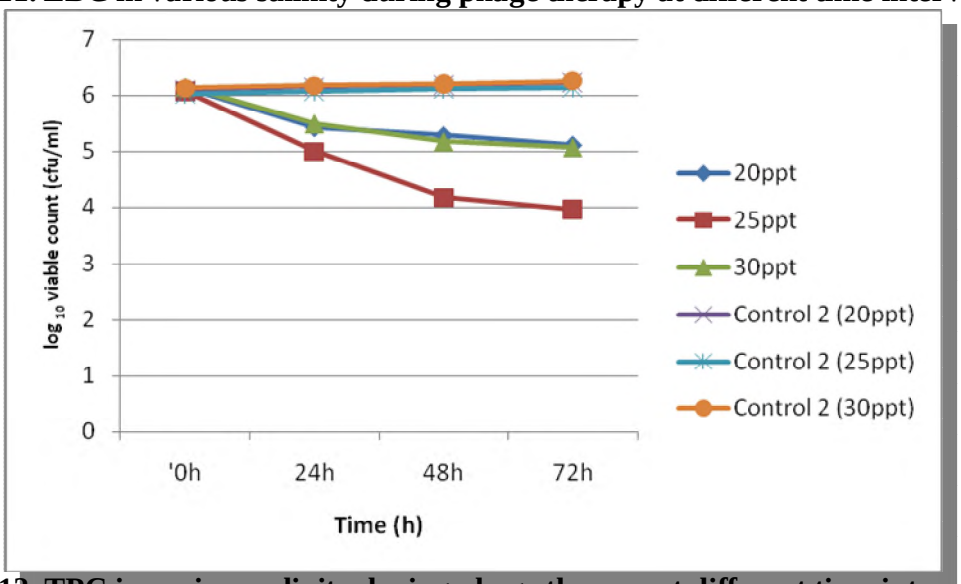

Fig. 12. TPC in various salinity during phage therapy at different time intervals.

Table 4. TPC, TVC and LBC at different time interval in various pH during phage therapy.

Time	**0** hour									24 hours									48 hours									72 hours								
pH	Bacterial no (cfu/ml)									Bacterial no (cfu/ml)									Bacterial no (cfu/ml)									Bacterial no (cfu/ml)								
	Control 1			Control 2			Phage treated			Control 1			Control 2			Phage treated			Control 1			Control 2			Phage treated			Control 1			Control 2			Phage treated		
	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC
6	<10	<10	4.51×10^{3}	1.13×10^{6}	1.23×10^{6}	1.31×10^{6}	1.07×10^{6}	1.21×10^{6}	1.34×10^{6}	<10	<10	3.35×10^{3}	1.19×10^{6}	1.27×10^{6}	1.38×10^{6}	9.71×10^{4}	1.04×10^{5}	4.28×10^{5}	<10	<10	3.39×10^{3}	1.08×10^{6}	1.24×10^{6}	1.41×10^{6}	8.69×10^{3}	9.04×10^{3}	3.18×10^{5}	<10	<10	2.73×10^{3}	1.12×10^{6}	1.31×10^{6}	1.55×10^{6}	4.91×10^{3}	5.12×10^{3}	2.24×10^{5}
7	<10	<10	2.51×10^{3}	1.31×10^{6}	1.38×10^{6}	1.41×10^{6}	1.12×10^{6}	1.28×10^{6}	1.49×10^{6}	<10	<10	3.48×10^{3}	1.68×10^{6}	1.84×10^{6}	2.11×10^{6}	6.27×10^{4}	8.21×10^{4}	3.44×10^{5}	<10	<10	5.42×10^{3}	1.93×10^{6}	2.14×10^{6}	4.21×10^{6}	8.13×10^{2}	1.01×10^{3}	9.49×10^{4}	<10	<10	5.65×10^{3}	2.12×10^{6}	3.20×10^{6}	4.61×10^{6}	1.23×10^{2}	2.71×10^{2}	5.18×10^{4}
8	<10	<10	4.67×10^{3}	1.22×10^{6}	1.32×10^{6}	1.34×10^{6}	1.09×10^{6}	1.19×10^{6}	1.36×10^{6}	<10	<10	5.18×10^{3}	1.52×10^{6}	1.61×10^{6}	1.91×10^{6}	8.91×10^{4}	9.19×10^{4}	6.64×10^{5}	<10	<10	5.52×10^{3}	2.02×10^{6}	2.32×10^{6}	3.04×10^{6}	4.29×10^{3}	6.31×10^{3}	2.14×10^{5}	<10	<10	7.21×10^{3}	2.18×10^{6}	2.48×10^{6}	4.43×10^{6}	3.02×10^{3}	3.31×10^{3}	1.45×10^{5}

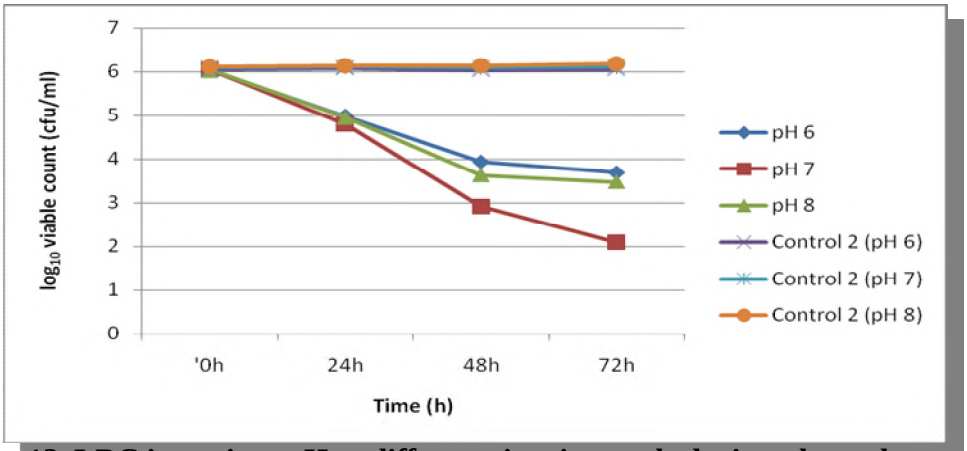

Fig. 13. LBC in various pH at different time intervals during phage therapy.

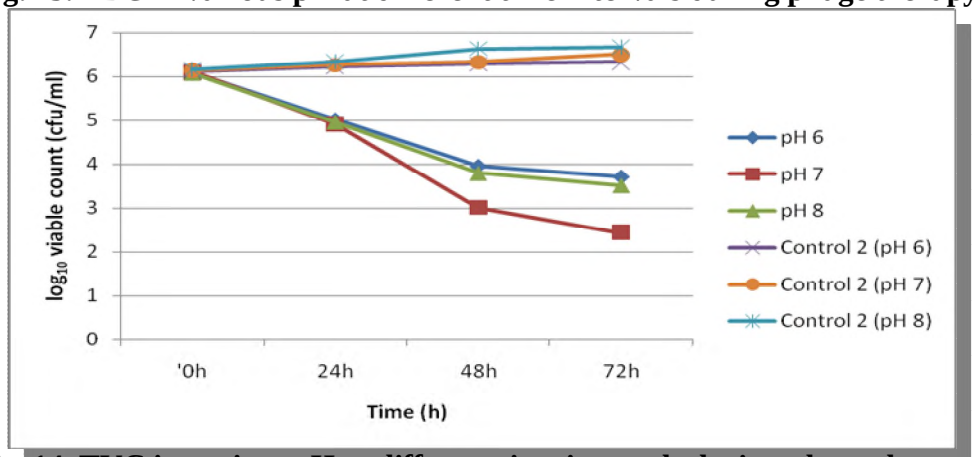

Fig. 14. TVC in various pH at different time intervals during phage therapy.

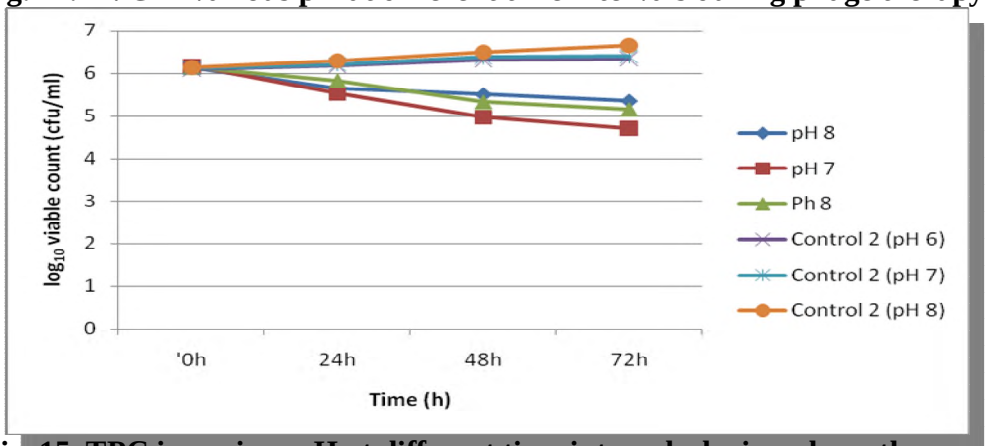

Fig. 15. TPC in various pH at different time intervals during phage therapy.

Table 5. TPC, TVC and LBC at different time interval in various TDS during phage therapy.

Time	**0** hour									24 hours									48 hours									72 hours								
TDS (mg/ml)	Bacterial no (cfu/ml)									Bacterial no (cfu/ml)									Bacterial no (cfu/ml)									Bacterial no (cfu/ml)								
	Control 1			Control 2			Phage treated			Control 1			Control 2			Phage treated			Control 1			Control 2			Phage treated			Control 1			Control 2			Phage treated		
	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC
11.26	<10	<10	6.45×10	1.1×10^{6}	1.23×10^{6}	1.41×10^{6}	1.27×10^{6}	1.22×10^{6}	1.54×10^{6}	<10	<10	7.31×10	1.29×10^{6}	1.44×10^{6}	1.67×10^{6}	7.41×10^{4}	9.21×10^{4}	3.28×10^{5}	<10	<10	7.91×10	2.31×10^{6}	3.23×10^{6}	4.91×10^{6}	3.13×10^{3}	4.15×10^{3}	9.63×10^{4}	<10	<10	8.73×10	2.29×10^{6}	2.9×10^{6}	5.54×10^{6}	6.04×10^{2}	7.81×10^{2}	1.06×10^{4}
22.63	<10	<10	2.72×10	1.03×10^{6}	1.28×10^{6}	1.44×10^{6}	1.15×10^{6}	1.42×10^{6}	1.79×10^{6}	<10	<10	3.91×10	1.39×10^{6}	1.41×10^{6}	1.81×10^{6}	1.12×10^{5}	1.93×10^{5}	3.95×10^{5}	<10	<10	6.92×10	6.1×10^{6}	5.41×10^{6}	7.3×10^{6}	5.96×10^{3}	8.2×10^{3}	2.21×10^{5}	<10	<10	9.93×10	8.21×10^{6}	7.41×10^{6}	7.68×10^{6}	4.63×10^{3}	4.94×10^{3}	1.44×10^{5}
38.43	<10	<10	3.71×10	1.17×10^{6}	1.14×10^{6}	1.24×10^{6}	1.04×10^{6}	1.18×10^{6}	1.37×10^{6}	<10	<10	4.8×10^{4}	1.25×10^{6}	1.31×10^{6}	1.97×10^{6}	1.73×10^{5}	1.81×10^{4}	4.01×10^{5}	<10	<10	4.94×10	3.18×10^{6}	5.13×10^{6}	5.29×10^{6}	3.11×10^{4}	3.51×10^{4}	1.87×10^{5}	<10	<10	8.73×10	4.3×10^{6}	5.45×10^{6}	6.81×10^{6}	9.52×10^{3}	1.02×10^{4}	1.66×10^{5}

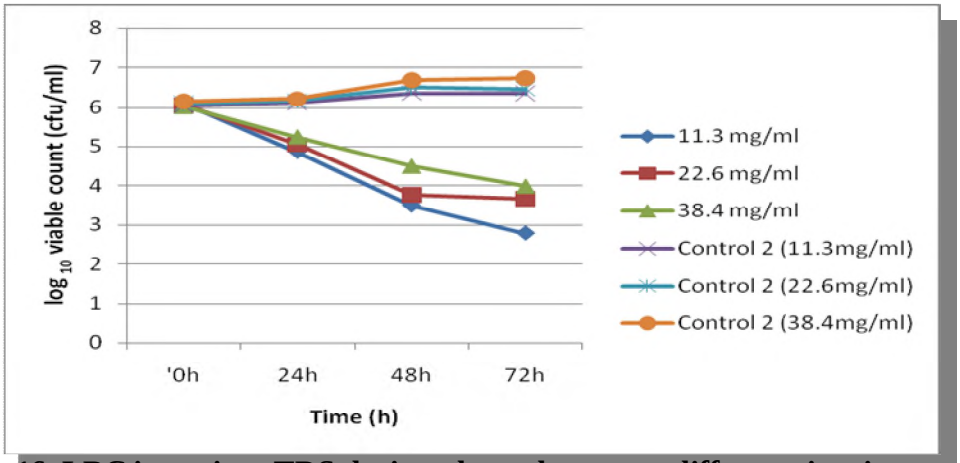

Fig. 16. LBC in various TDS during phage therapy at different time intervals.

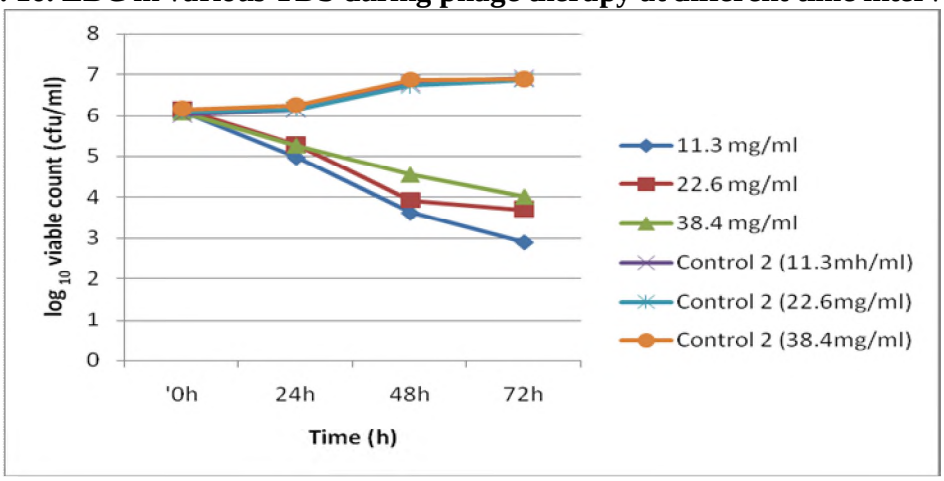

Fig. 17. TVC in various TDS during phage therapy at different time intervals.

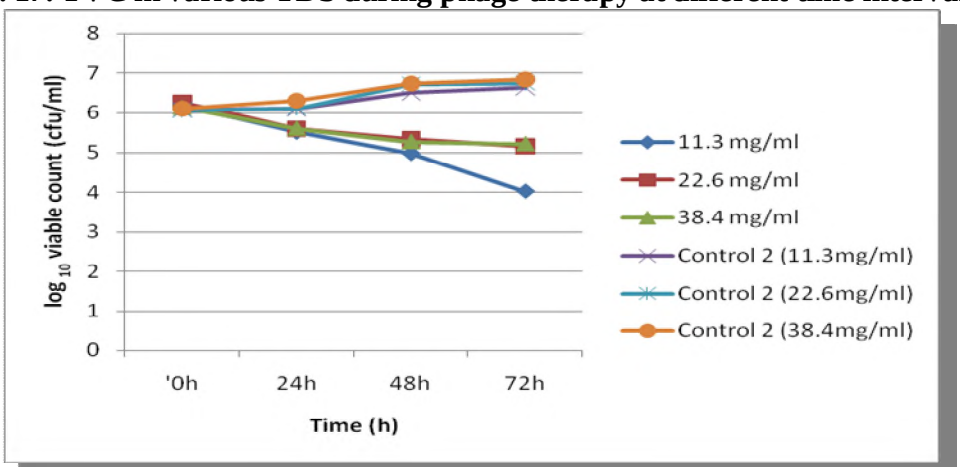

Fig. 18. TPC in various salinity during phage therapy at different time intervals.

Table 6. TPC, TVC and LBC at different time interval for various temperatures during phage therapy.

Time	0 hour						4 hours						24hours						28hours						48hours						72hours					
	Bacterial no (cfu/ml)						Bacterial no (cfu/ml)						Bacterial no (cfu/ml)						Bacterial no (cfu/ml)						Bacterial no (cfu/ml)						Bacterial no (cfu/ml)					
	Control 2			Phage treated			Control 2			Phage treated			Control 2			Phage treated			Control 2			Phage treated			Control 2			Phage treated			Control 2			Phage treated		
	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC	LBC	TVC	TPC
20	1.13×10^{6}	1.1×10^{6}	1.21×10^{6}	1.2×10^{6}	1.12×10^{6}	1.09×10^{6}	1.18×10^{6}	1.14×10^{6}	1.21×10^{6}	3.71×10^{5}	4.21×10^{5}	7.28×10^{5}	1.31×10^{6}	1.73×10^{6}	2.01×10^{6}	4.13×10^{5}	4.15×10^{5}	6.96×10^{5}	1.29×10^{6}	2.41×10^{6}	2.54×10^{6}	1.04×10^{4}	1.81×10^{4}	9.63×10^{4}	1.81×10^{6}	4.46×10^{6}	5.54×10^{6}	6.04×10^{3}	9.07×10^{3}	6.63×10^{4}	2.21×10^{6}	5.17×10^{6}	7.18×10^{6}	6.73×10^{3}	7.31×10^{3}	6.92×10^{4}
30	1.03×10^{6}	1.08×10^{6}	1.24×10^{6}	1.05×10^{6}	1.02×10^{6}	1.11×10^{6}	1.59×10^{6}	1.53×10^{6}	1.91×10^{6}	3.42×10^{4}	6.93×10^{4}	8.15×10^{4}	4.17×10^{6}	5.41×10^{6}	7.3×10^{6}	3.16×10^{4}	5.2×10^{4}	6.21×10^{4}	6.21×10^{6}	7.41×10^{6}	7.98×10^{6}	4.63×10^{2}	7.94×10^{2}	1.44×10^{3}	7.21×10^{6}	9.41×10^{6}	1.98×10^{7}	1.51×10^{2}	4.13×10^{2}	1.72×10^{3}	9.81×10^{6}	9.71×10^{6}	2.16×10^{7}	1.05×10^{2}	3.97×10^{2}	2.02×10^{3}
37	1.04×10^{6}	1.14×10^{6}	1.18×10^{6}	1.01×10^{6}	1.11×10^{6}	1.17×10^{6}	1.45×10^{6}	1.21×10^{6}	1.87×10^{6}	7.63×10^{4}	9.51×10^{4}	1.10×10^{5}	5.18×10^{6}	5.83×10^{6}	6.29×10^{6}	5.11×10^{4}	5.51×10^{4}	9.07×10^{4}	6.93×10^{6}	7.45×10^{6}	7.81×10^{6}	1.52×10^{3}	4.02×10^{3}	1.66×10^{4}	6.98×10^{6}	8.45×10^{6}	9.81×10^{6}	9.47×10^{2}	3.14×10^{3}	1.95×10^{4}	8.18×10^{6}	9.26×10^{6}	9.73×10^{6}	8.82×10^{2}	3.08×10^{3}	2.07×10^{4}

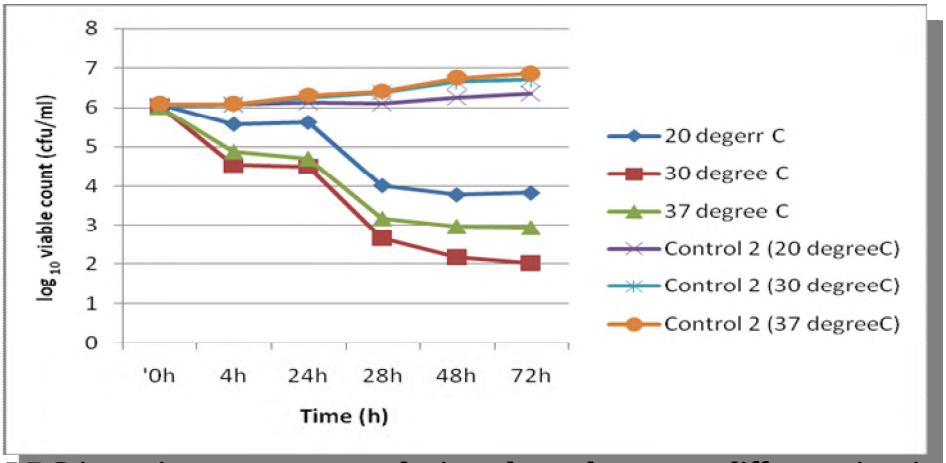

Fig. 19. LBC in various temperature during phage therapy at different time intervals

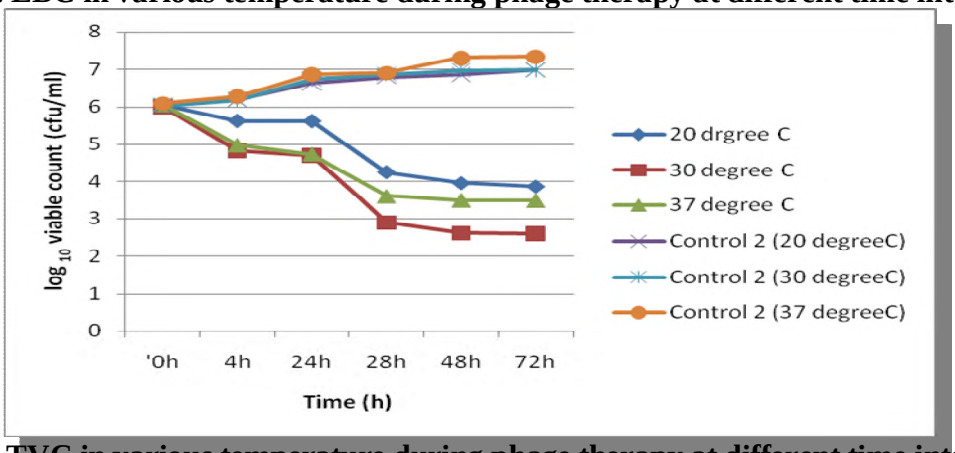

Fig. 20. TVC in various temperature during phage therapy at different time intervals.

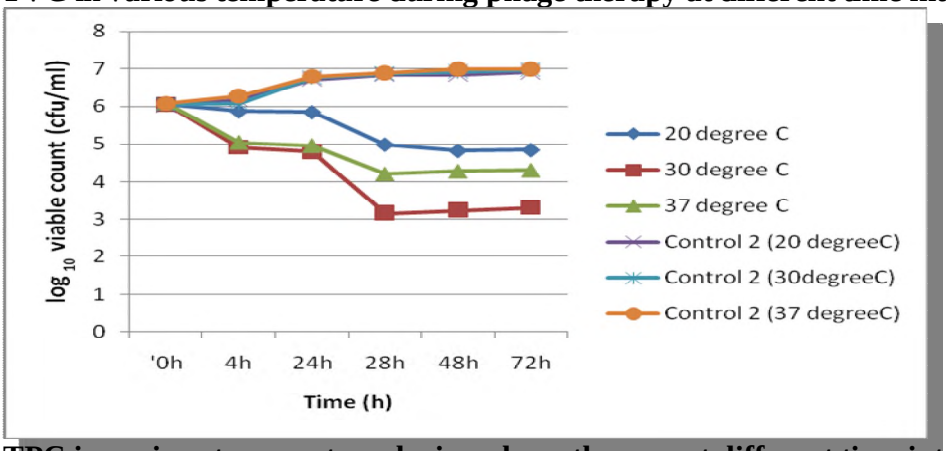

Fig. 21. TPC in various temperature during phage therapy at different time intervals.

Table 7. Percentage survivability of *P.monodon* larvae in control and phage treated troughs with different salinity, pH, TDS levels.

	Salinity			pH			TDS		
	20	25	30	6	7	8	11.25	22.63	38.43
Control 1	100	100	100	100	100	100	100	100	100
Control 2	36.7	36.7	33.3	30.0	40.0	46.6	36.7	33.3	33.3
Phage treated (Mean±SD)	68.35± 2.333	81.65± 2.333	75± 2.404	70± 4.667	81.65± 2.333	78.3± 2.404	85± 2.404	76.65± 4.738	75± 2.404

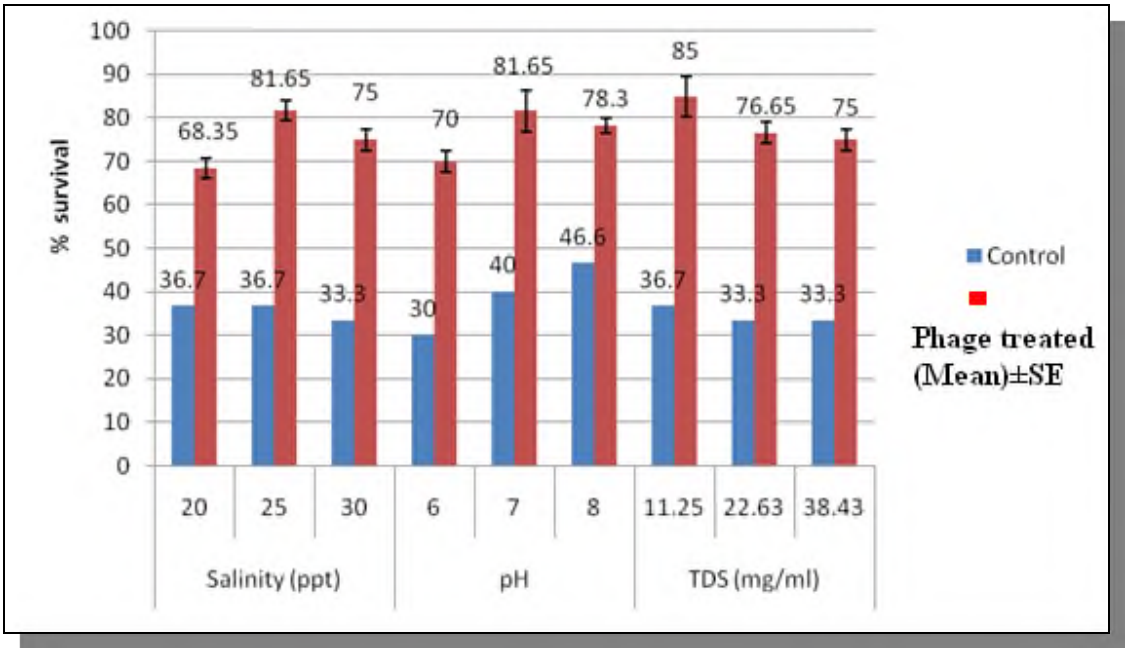

Figure 22. Percentage survivability of *P.monodon* larvae in control and phage treated troughs with different salinity, pH, TDS levels.

Table 8. *V. harveyi* counts at different time interval for various quantity of phages treatment at 20 ppt salinity.

Phage	PhageV			PhageJ		PhageN	
	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour	144 hour
50 µl	3400000	720000	220000	95000	12000	5700	4250
100 µl	3400000	440000	62000	24500	4200	1710	1560
150 µl	3400000	590000	31000	17100	4400	890	760
200 µl	3400000	86000	17900	9200	2800	670	450
250 µl	3400000	140000	36000	6100	2100	590	620
300 µl	3400000	74000	11000	3700	910	350	310
No Phage	3400000	1800000	470000	580000	310000	690000	810000

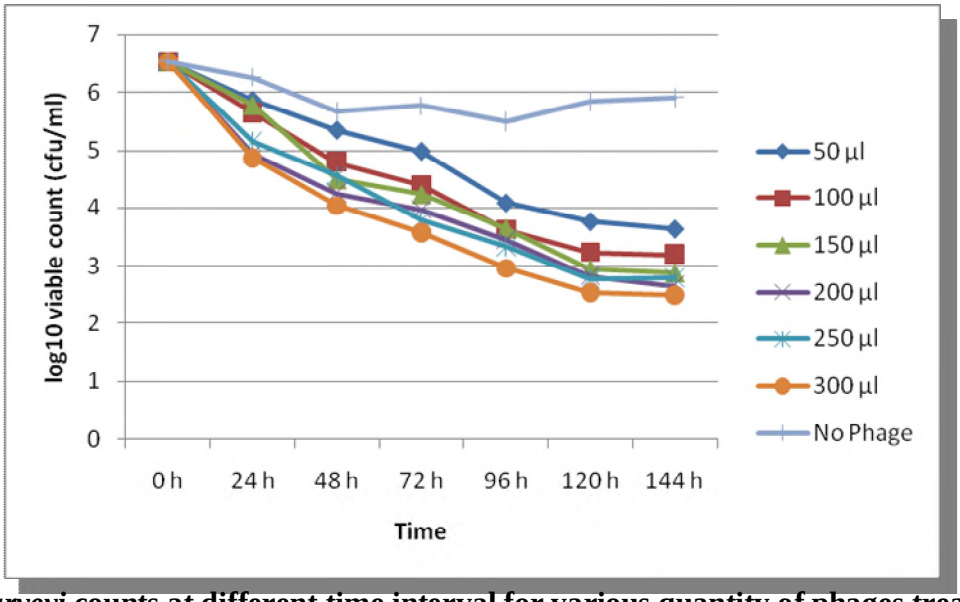

Fig. 23. *V. harveyi* counts at different time interval for various quantity of phages treatment at 20 ppt salinity

Table 9. *V. harveyi* counts at different time interval for various quantity of phages treatment at 25 ppt salinity

Phage	PhageV			PhageJ		PhageN	
	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour	144 hour
50 µl	3400000	470000	74000	50000	4500	1940	1070
100 µl	3400000	280000	44000	17500	3400	1020	620
150 µl	3400000	72000	15000	7400	1240	750	520
200 µl	3400000	82000	32000	5100	1040	560	990
250 µl	3400000	19000	7700	2100	670	390	360
300 µl	3400000	8200	2600	1800	590	143	220
No Phage	3400000	7200000	980000	510000	750000	590000	670000

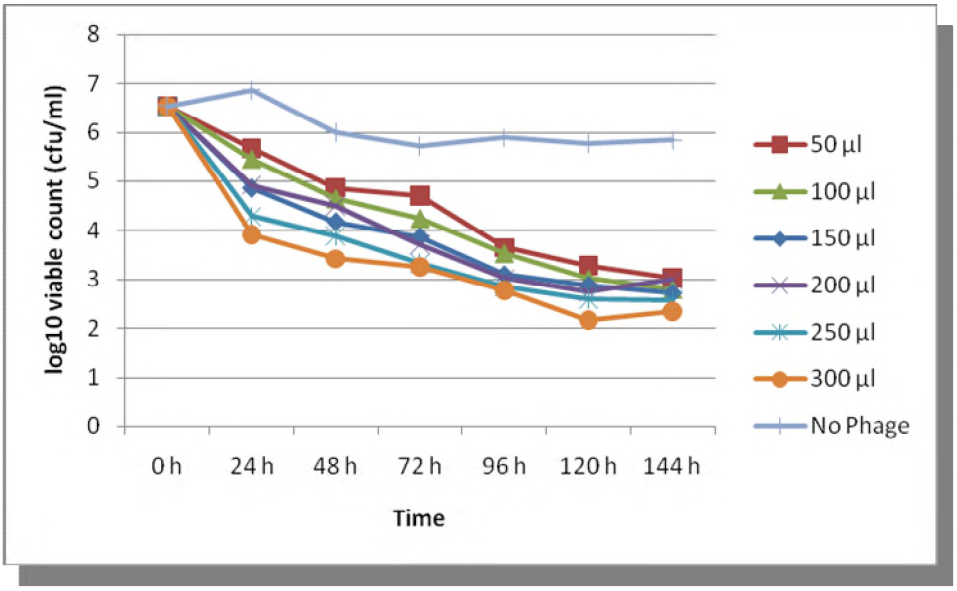

Fig. 24. *V. harveyi* counts at different time interval for various quantity of phages treatment at 25 ppt salinity

Table 10. *V. harveyi* counts at different time interval for various quantity of phages treatment at 30 ppt salinity

Phage	PhageV			PhageJ		PhageN	
	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour	144 hour
50 µl	47000000	520000	74000	50000	7100	4700	3900
100 µl	47000000	460000	26000	13100	3600	3100	2400
150 µl	47000000	57000	24000	4300	2100	2010	2300
200 µl	47000000	28000	7300	3200	1600	1200	1050
250 µl	47000000	17800	5400	2900	1000	820	670
300 µl	47000000	9500	2300	2000	690	580	440
No Phage	47000000	5700000	4300000	2800000	990000	1240000	3200000

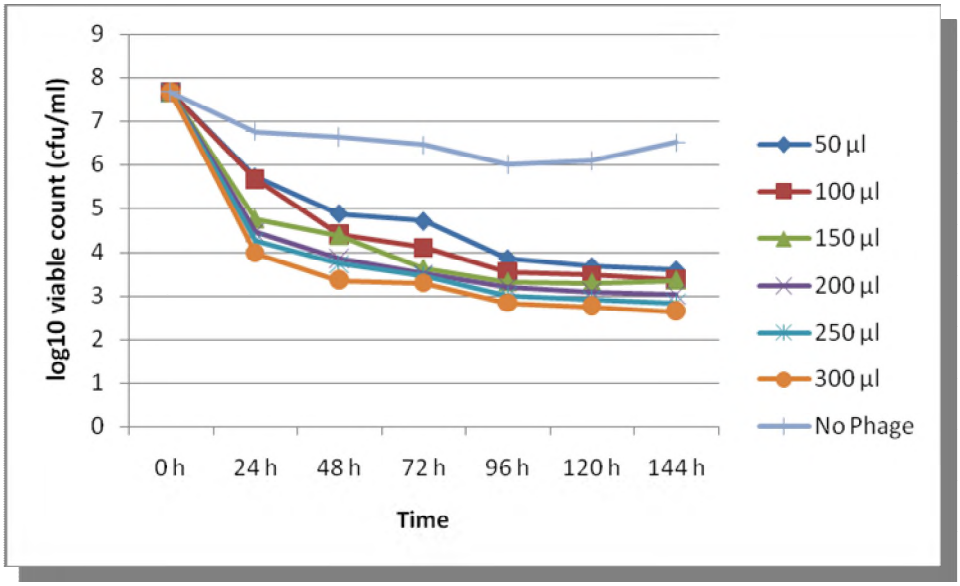

Fig. 25. *V. harveyi* counts at different time interval for various quantity of phages treatment at 30 ppt salinity

Table 11. *V. harveyi* counts at different time interval for various quantity of phages treatment at 35 ppt salinity

Phage Phage/l	PhageV			PhageJ		PhageN	
	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour	144 hour
50 µl	4700000	790000	137000	61000	11000	9700	8500
100 µl	4700000	350000	25000	8200	5600	5200	4400
150 µl	4700000	57000	32000	2900	3600	2810	2100
200 µl	4700000	51000	21000	6900	3200	2520	1180
250 µl	4700000	23000	3400	2700	2000	1220	920
300 µl	4700000	7500	1900	1300	710	690	720
No Phage	4700000	9400000	4300000	2100000	1900000	1690000	1820000

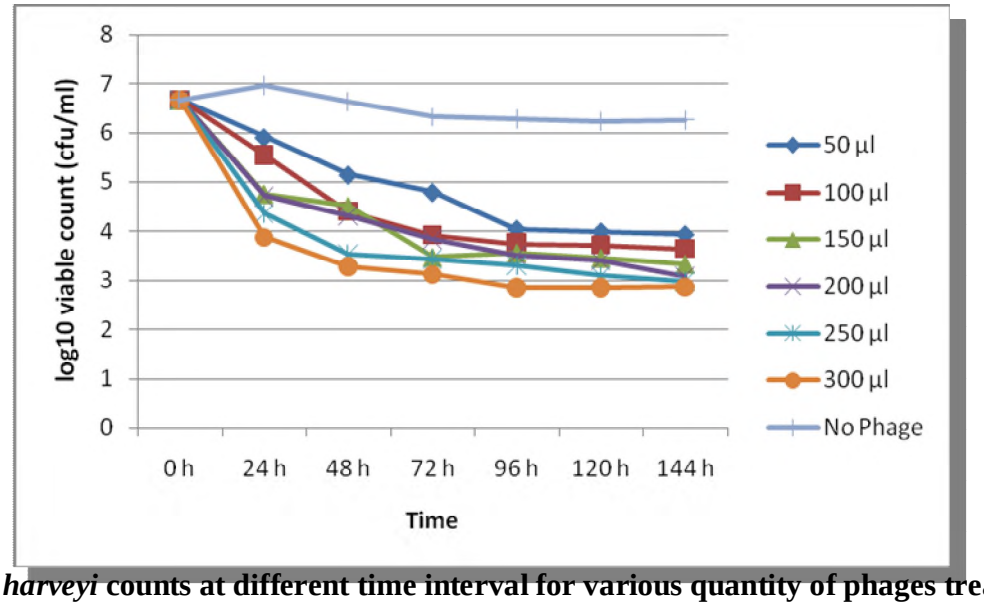

Fig. 26. *V. harveyi* counts at different time interval for various quantity of phages treatment at 35 ppt salinity

Table 12. *V. harveyi* counts at different time interval for various quantity of phages treatment at pH 6

Phage	PhageV			PhageJ		PhageN	
	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour	144 hour
50 µl	2800000	88000	29100	16400	8900	5900	2700
100 µl	2800000	71000	15000	4900	2710	1020	930
150 µl	2800000	41000	5500	3620	1100	820	510
200 µl	2800000	44000	6900	2730	1020	790	500
250 µl	2800000	61000	4400	2330	1070	610	480
300 µl	2800000	15000	3100	1410	790	670	430
No phage	2800000	61000000	7100000	3800000	4100000	2910000	1810000

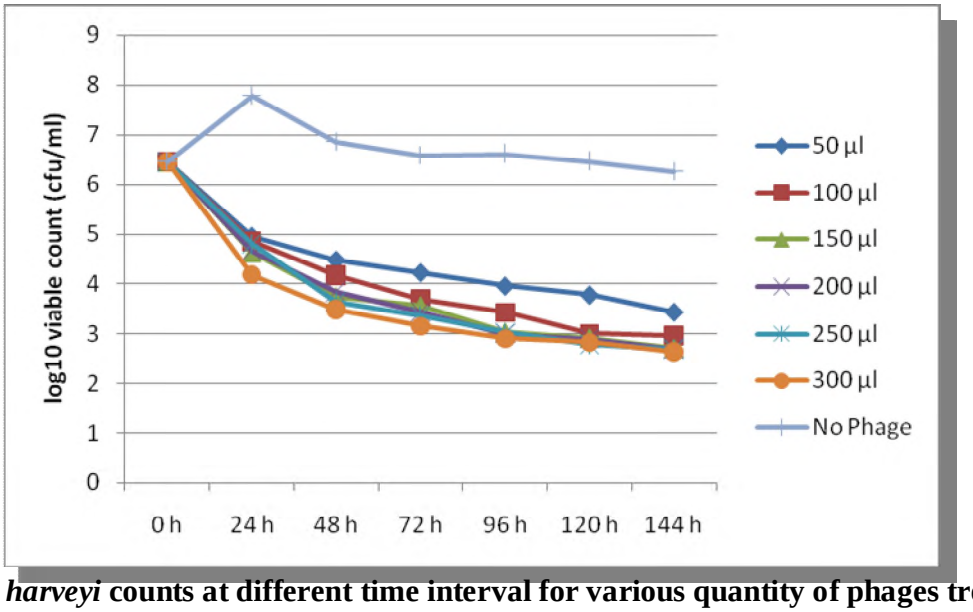

Fig. 27. *V. harveyi* counts at different time interval for various quantity of phages treatment at pH 6

Table 13. *V. harveyi* counts at different time interval for various quantity of phages treatment at pH 7

Phage Phage/l	PhageV			PhageJ		PhageN	
	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour	144 hour
50 µl	2800000	184000	31000	22100	3900	2010	1920
100 µl	2800000	46000	6200	3700	830	760	670
150 µl	2800000	55000	2100	3100	890	340	440
200 µl	2800000	24200	3090	2400	510	420	340
250 µl	2800000	31000	4700	1690	660	380	350
300 µl	2800000	9100	820	320	120	160	130
No Phage	2800000	4100000	7100000	2600000	3200000	6700000	7100000

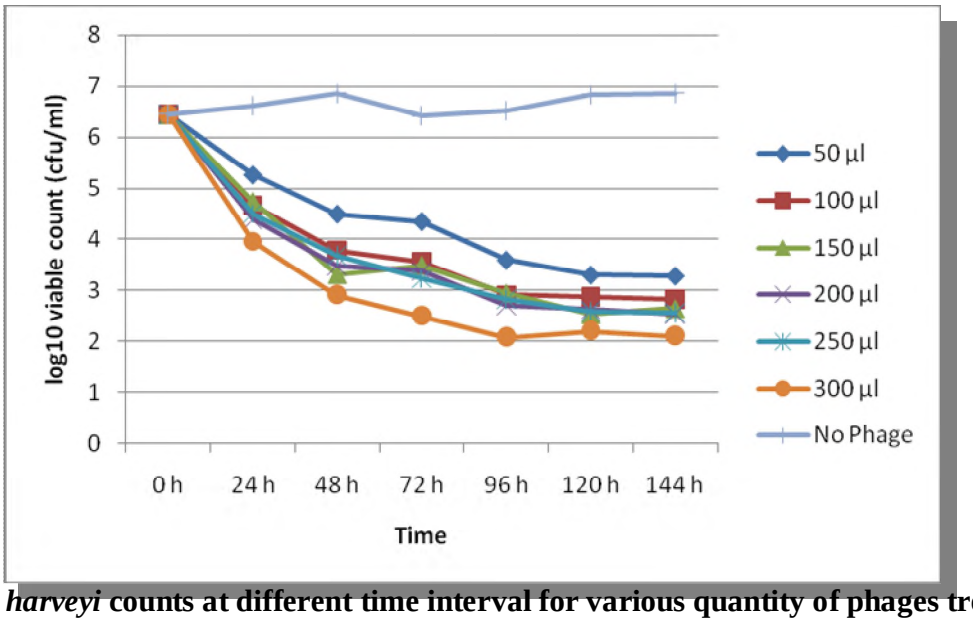

Fig. 28. *V. harveyi* counts at different time interval for various quantity of phages treatment at pH 7

Table 14. *V. harveyi* counts at different time interval for various quantity of phages treatment at pH 8

Phage Phage/l	PhageV			PhageJ		PhageN	
	0 hour	24 hour	48 hour	72 hour	96 hour	120 hour	144 hour
50 µl	7200000	340000	79000	11100	6300	3110	2190
100 µl	7200000	280000	9300	6400	1900	930	960
150 µl	7200000	97000	7500	5200	910	620	720
200 µl	7200000	19200	4500	4100	710	530	450
250 µl	7200000	13100	3900	1750	560	470	330
300 µl	7200000	7000	3200	1090	252	150	110
No Phage	7200000	6100000	2600000	1900000	1200000	3400000	5800000

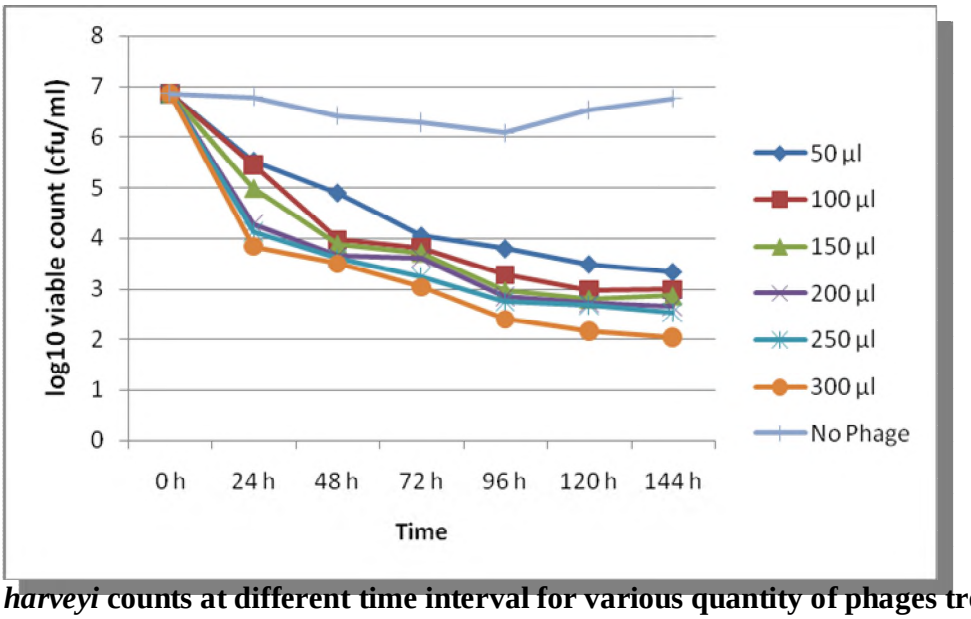

Fig. 29. *V. harveyi* counts at different time interval for various quantity of phages treatment at pH 8

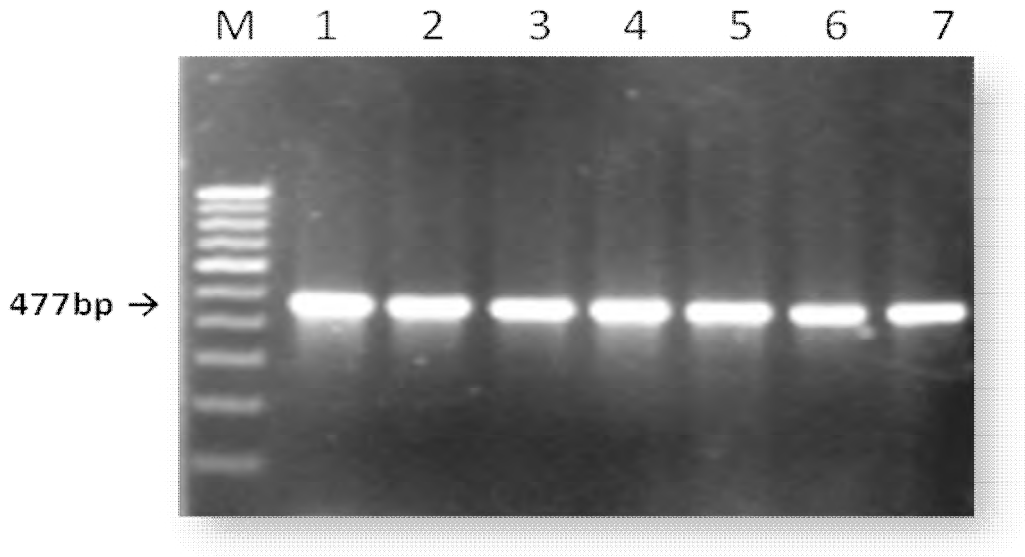

Fig. 30. Agarose gel electrophoresis of PCR products from recombinant lysozyme clones by gene specific primers. Lane M: 100 bp DNA marker; Lane 1-7: Recombinant lysozyme clones.

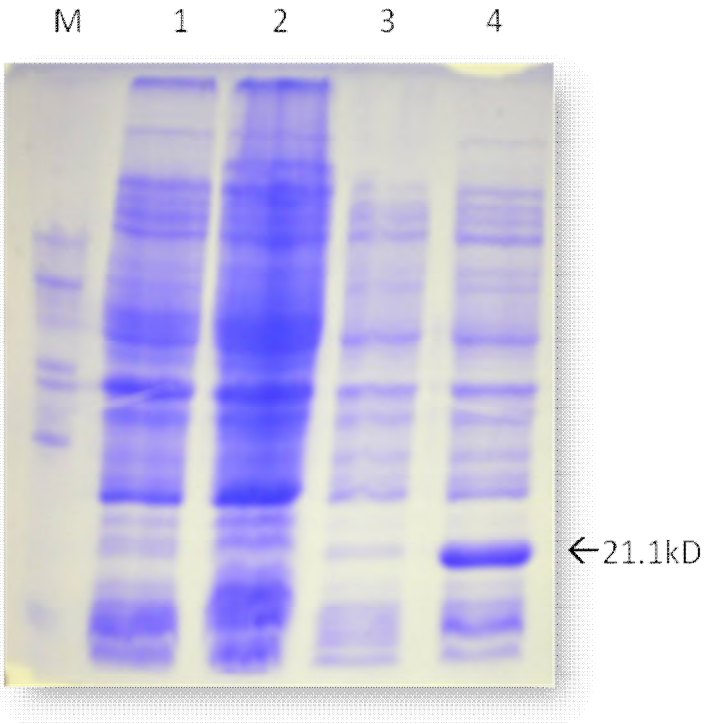

Fig. 31. SDS PAGE showing expression of recombinant lysozyme after induction with IPTG. Lane M: Protein molecular weight marker (Perfect Protein™ Markers, 10-225 kDa, Novagen, Merck)
Lane 1: Non recombinant BL21 *E. coli* cells without IPTG;
Lane 2: Non recombinant BL21 *E. coli* cells with 1 mM IPTG;
Lane 3: Recombinant lysozyme clone without IPTG;
Lane 4: Recombinant lysozyme clones with 1 mM IPTG.

Table 15. Zone of inhibition (in mm) of *V.harveyi* (n=87) by phage alone (A), lysozyme alone (B) and phage+lysozyme (C).

	VHPhageA(25)			VHPhageN(10)			VHPhageV(17)			VHPhageM(36)			VHPhageK(39)			VHPhageR(39)			VHPhageJ(36)		
Culture	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
1	0	8	10	0	8	9.5	8.5	11	12.5	9	9.5	11	0	7	8.5	0	8	9.5	8	9.5	11.5
2	0	9	12	0	8.5	11	0	9	11	0	9	11.5	0	8.5	10	0	9.5	11	0	8	11
3	0	8.5	11	0	9	11.5	9	12	13.5	8	9	10.5	0	8	10	0	9	10.5	8.5	9.5	11.5
4	0	8	11	0	8.5	10.5	0	8	10	0	8	10	0	9	10.5	0	10	11	0	8.5	11
5	0	8	13	7	9	11	7.5	10	12	7	9.5	10.5	0	8	10	0	9	10.5	9	10	11.5
6	0	7	11	0	9	11.5	0	9	11.5	0	8.5	9.5	0	9.5	11	0	9	10.5	0	8	9.5
7	8	8.5	11	7.5	8.5	11.5	8	11	12.5	7.5	9	10	0	7.5	9.5	0	8.5	10	8	9.5	11
8	8	8	12	8	9	12	9	11.5	13	8	9	10.5	0	10	12	0	8	11	9.5	11	12.5
9	9	9	13	8.5	9.5	12	10	12	13.5	8.5	9.5	11	0	9.5	11	0	8	10	9	10	13
10	9	10	12	9.5	10.5	12.5	0	9.5	12	9	10	11.5	0	8	10	0	9	11.5	8	9.5	11
11	0	8	12	9	10	11.5	9	12	13	8	9.5	11	0	10	11.5	0	8	11	9	10.5	11.5
12	7	9.5	13	8	10	12	9.5	12.5	13.5	8.5	10	11.5	0	9.5	11	0	9.5	12	8.5	10	12
13	0	9	12	0	9	12.5	0	9	11	0	8.5	10	0	11	12.5	0	7.5	9.5	0	9	11.5
16	9	11	14	9	9.5	12	7	9.5	11	9	9.5	11	0	10.5	11.5	0	8.5	10	10	11.5	12.5
17	10	11	16.5	10	11	13	9.5	12	13.5	9	10	11.5	0	10	11	0	8	10	9	11	12.5
21	8	9.5	12	9	10.5	13.5	8	11	12.5	8	10	11.5	0	9	10	0	8	9	8	10.5	12
22	9	12	14	8	9.5	12	9	11.5	13	9.5	10.5	12	0	8.5	11	0	9	10.5	7.5	10	12.5
23	8	11	13	7.5	9	11.5	9.5	12	14	7.5	8.5	10.5	0	9	11	0	9	11	9	10.5	11
24	8	10	13	8	9	11	9	12.5	14	9	10	11.5	0	9.5	11.5	0	8	10.5	8	10	11.5
25	10	12	14.5	9	10	12.5	9	11	13	9	10.5	11.5	0	9.5	10.5	0	9	11	8.5	9.5	11
26	9	10	12.5	8.5	9.5	12	8.5	11.5	13.5	9.5	11	12	0	10	11	0	9.5	12.5	10	11.5	13
27	9	9.5	13	9	10	12	9	12	14	9	10	11.5	0	10.5	11	0	9.5	12	10.5	11.5	14
28	0	9	13.5	0	9	11.5	0	9	11	0	9	10.5	0	11	12	0	8	9	0	9.5	11
29	0	8.5	11	9	11	13	8.5	11	13	8	9.5	11	0	10	12	0	7	9.5	9	10	12

30	0	11	14	0	8.5	10	0	9.5	11	9	10.5	11.5	0	9.5	11	0	10	12	9.5	11	11.5
31	0	10	13	8.5	9.5	11	9	10.5	12	9	11	13	0	9	11.5	0	10	13	8.5	10.5	13
32	0	10	12.5	0	10	12	0	8.5	10	0	9	10	0	8	10	0	9.5	12.5	0	8	10.5
33	8	9	11	8	9.5	11.5	9	11	13	8.5	9.5	11	0	7.5	10	0	9	10.5	8	9	12.5
34	8	9	12	9	10	12	7.5	11	12.5	7.5	9	11.5	0	8	10.5	0	8.5	10	8	9.5	13
35	0	10	13	9.5	11	12.5	8	10.5	12	0	8.5	11	0	8	10	0	10	11.5	7.5	9	11.5
36	9	8.5	11.5	8	9.5	12	9	11	12.5	10	10.5	12	0	9	10.5	0	10.5	12	9.5	11.5	14
37	0	9	11	0	9	11	0	10	11.5	0	8	10	0	8	10	0	11	12.5	0	8.5	11
38	0	9	12	0	8	10.5	0	9	11	0	7.5	9.5	0	9	10.5	0	9	11	0	9	12.5
39	10	10	13.5	8.5	10	12.5	7	9.5	11.5	9.5	10.5	12	9	8.5	10	10.5	9.5	11.5	9	11	13
40	0	11	13.5	0	8	10	0	9.5	11	0	8	10	0	7.5	10	0	9	10.5	0	10	13
41	8	11	14	7.5	9	11	8	10	12	8	9	10.5	0	9	11	0	8.5	11	9.5	10.5	11.5
42	0	10	13	8.5	9.5	12	9.5	11	13	0	9	11	0	10	11.5	0	10	12.5	0	8.5	11
43	0	10	12	0	8.5	11	0	10	13	0	8.5	10	0	9	11	0	11	13	0	9.5	12.5
44	8	10	12.5	9.5	11	13.5	8	10.5	12.5	8.5	9.5	11	0	9.5	11.5	0	10.5	13	9	11	13
45	0	9	11	0	10	12.5	0	8	10	0	9	11.5	0	9	10.5	0	9	11	0	10	12
46	9	9.5	12	8	9	11	8.5	10	11.5	9	9.5	11	0	8.5	9.5	0	9.5	11.5	10	11.5	14
ACMM642	0	6.5	7.5	0	7	8.5	9	9	11	0	7.5	8.5	0	7	9	0	8	9	9	9	10
STD3-0942	0	8	8.5	0	9	9	0	8.5	9.5	8	8	9	0	7.5	8.5	0	8	9.5	0	8	9
STD3-0947	0	8.5	9.5	0	8.5	10	0	8	9.5	0	9	9.5	0	8	9	0	8.5	9	0	8.5	9.5
STD3-0949	0	7.5	8.5	0	9	9.5	9	8.5	10	0	8	9	0	7	8	0	8	9.5	0	9	10
STD3-0953	0	8.5	9	0	8.5	9	0	9	11	0	7.5	8.5	0	8.5	8	0	9	9.5	0	9.5	10
STD3-0957	0	9	9.5	0	9	9.5	0	9.5	11	0	9	9.5	0	9	9.5	0	8.5	8	0	8	9.5
STD3-0983	0	8	8.5	0	8.5	10	8	7.5	9.5	9	9.5	10	0	9	10	0	7.5	8.5	0	9	9.5
STD3-0986	0	8	9	0	9	10	0	8	9	0	8	9	0	8.5	9	0	8	8.5	0	8.5	10
STD3-1007	0	8.5	9.5	0	9.5	11	8.5	8.5	9.5	9.5	8	9	0	8	9	0	8	9.5	0	8	8.5
STD3-1020	0	8	8.5	0	10	11	0	8	9.5	0	9	9.5	0	8	9.5	0	9	10	9	9.5	9
STD3-1022	0	8	9.5	0	7.5	9	0	8	9.5	0	9.5	10	0	8.5	9	0	7.5	9	10	9.5	10.5

STD3-1024	0	8.5	9	0	7	9	0	7.5	9	0	9	10	0	7	8.5	0	7	8.5	10	10	11
LMG04043	8.5	8	8	0	8	8	0	9	9	0	9	9.5	0	9	9	0	7.5	9	7.5	8	9.5
LMG04044	0	7	8	8.5	7.5	9.5	9	9.5	11	9	9.5	9	0	9	9.5	0	9	9.5	0	8	9
LMG07890	10	8.5	8	0	8	9	9.5	9	11	9	9.5	9	0	8.5	9	0	8	8.5	8	8.5	9
LMG10946	9	8.5	9	9	8.5	10	8	8.5	10	8	9	10	0	8	9	0	8	9	0	9	9.5
LMG10947	8	9	9.5	10	8	10	9	9	10	7.5	8.5	9.5	0	9	9.5	0	8.5	9.5	0	8.5	9
LMG10948	0	8.5	10	9	8.5	9.5	9	9	11	0	8	8.5	0	8.5	9	0	8.5	9.5	9.5	9.5	10
LMG11225	0	8	9.5	0	9	10	0	8.5	9.5	0	8.5	9	0	9	9	0	8	8.5	9.5	10	10.5
LMG11226	7	8	9.5	0	8.5	9.5	0	8	9	0	8	9.5	0	8.5	9	0	8	9	0	8	9
LMG11659	0	9	9.5	8	9	10	9.5	9.5	9	8	9	9.5	0	8.5	9	0	9	9.5	0	8	9
LMG11660	8	8	8.5	0	9.5	9	9	8.5	9.5	9	9.5	10	0	9	10	0	9	9.5	8	8.5	9.5
LMG11755	9	9.5	10	9	8.5	10	8.5	9	10	8	9	10	0	8.5	9.5	0	9.5	10	8.5	9	9.5
LMG13949	0	8	9.5	9	8	9	8	9	9.5	0	8.5	10	0	9	9.5	0	8.5	10	0	9.5	10
LMG16828	9	9	9	8	8	9.5	9	9.5	11	0	8	9.5	0	8.5	9	0	9	10	9	9.5	11
LMG16830	7.5	9	9	7	8.5	10	0	8.5	9.5	0	8	9	0	9	9.5	0	8.5	9.5	0	9	10
LMG16831	6.5	7.5	9	8	9	10	8	8.5	9.5	8	8.5	9.5	0	8.5	9.5	0	8	10	9.5	10	9
LMG16832	7	7.5	8.5	9	8.5	9.5	9	9	11	9	9	9.5	0	9	10	0	8.5	8	8.5	10	11
LMG16853	0	7	9	0	8	9	9	9.5	11	8	9	10	0	8.5	8	0	7.5	9.5	8	8.5	9.5
LMG16862	8.5	8	9.5	8	8.5	9.5	8	9	10	8	8.5	9.5	0	9	10	0	9	9.5	8.5	9	9
LMG16863	0	7	8.5	8	9.5	9	8	8.5	10	8	9	10	0	7	8.5	0	8	9.5	0	9.5	10
LMG16874	9	8.5	8.5	9	8	10	8.5	9	11	0	9.5	9	0	8	9	0	8.5	9.5	9	9.5	10
LMG19643	9	9	9.5	8	8	9.5	9	9.5	9	8	9	10.5	0	8	9	0	8.5	10	0	10	11
STD3 0999	0	9	10	0	7.5	8.5	9	9.5	11.5	0	8.5	9.5	0	8.5	8.5	0	9.5	10	10	10	11
STD3 1000	0	7.5	10	0	7	8	0	8	9	0	8	8.5	0	7.5	9	0	8.5	9.5	0	9	10
STD3 1009	0	8	9.5	0	8	8.5	0	8.5	9.5	9	9.5	10	0	9	9	0	9	9.5	8.5	9.5	9
STD3 1011	0	8.5	10	0	8	8	0	8.5	9.5	0	8	9	0	8.5	9	0	9	10	9	9.5	10.5
STD3 1012	0	9	9.5	0	8.5	9.5	9	8.5	10	9.5	9	10	0	7.5	8	0	8.5	8	8.5	9	10
ACMM20	0	9	9	0	7.5	8	0	8	10	0	8	8.5	0	9	9.5	0	8	9.5	0	9	9.5

VLB571	0	8	9.5	0	7.5	8.5	0	8	9.5	0	8.5	9.5	0	7	8.5	0	8	9	0	8.5	10
VLB645	0	8.5	9	8	8	9	9	8.5	9.5	0	7.5	8.5	0	8	9.5	0	7.5	8.5	9.5	9.5	11
BB886	7	7.5	8	8	7.5	8.5	8.5	9	9.5	0	8	9	0	9	9.5	0	8	8.5	8	9	9.5
BB152	7.5	8	8	8	9	9.5	7.5	8	9.5	7	8	9	0	9	10	0	8	8.5	0	9	10
BB	0	8	8.5	0	9	10	0	8.5	9.5	0	8	9	0	8.5	9.5	0	7.5	9	9	9.5	11
BB120	0	8	8	0	8	8.5	9	9	10	8.5	8.5	9.5	0	8	9.5	0	8	8.5	9.5	9.5	10
BB170	0	8	8.5	7	7.5	9	9	9.5	11	10	9	11	0	9	10	0	9	9	0	9	10

Table 16. Mean ±SD of diameter of zone of inhibition of *V. harveyi* (in mm) by phage alone, lysozyme alone and phage+lysozyme.

	Phage (Mean±SD)	**Lysozyme (Mean±SD)**	**Phage+Lysozyme (Mean ±SD)**
VHPhageA(25)	9.53±0.81	9.5±1.14	12.48±1.25
VHPhageN(10)	8.42±0.65	9.43±0.83	11.68±0.9
VHPhageV(17)	8.61±0.77	10.46±1.23	12.23±1.12
VHPhageM(36)	8.52±0.73	9.37±0.84	10.96±0.77
VHPhageK(39)	9±0	9±0.99	10.71±0.81
VHPhageR(39)	10.5±0	9.01±0.93	11.02±1.09
VHPhageJ(36)	8.81±0.8	9.91±1.02	12±1.01

Table 17. Zone of inhibition on Solid phase assay by phage alone, lysozyme alone and phage + lysozyme together at different ratio and different quantity.

Quantity	Phage (mm)	Lysozyme (mm)	Phage : Lysozyme			
			1:1	1:2	1:3	1:4
10 µl	9.5	13	19.5	18	17.5	17
20 µl	18	19	23	21	21	20
Quantity	Phage (mm)	Lysozyme (mm)	Phage : Lysozyme			
			1:1	2:1	3:1	4:1
10 µl	10	14	19	21	18	20
20 µl	18.5	18.5	22.5	24.5	22	21

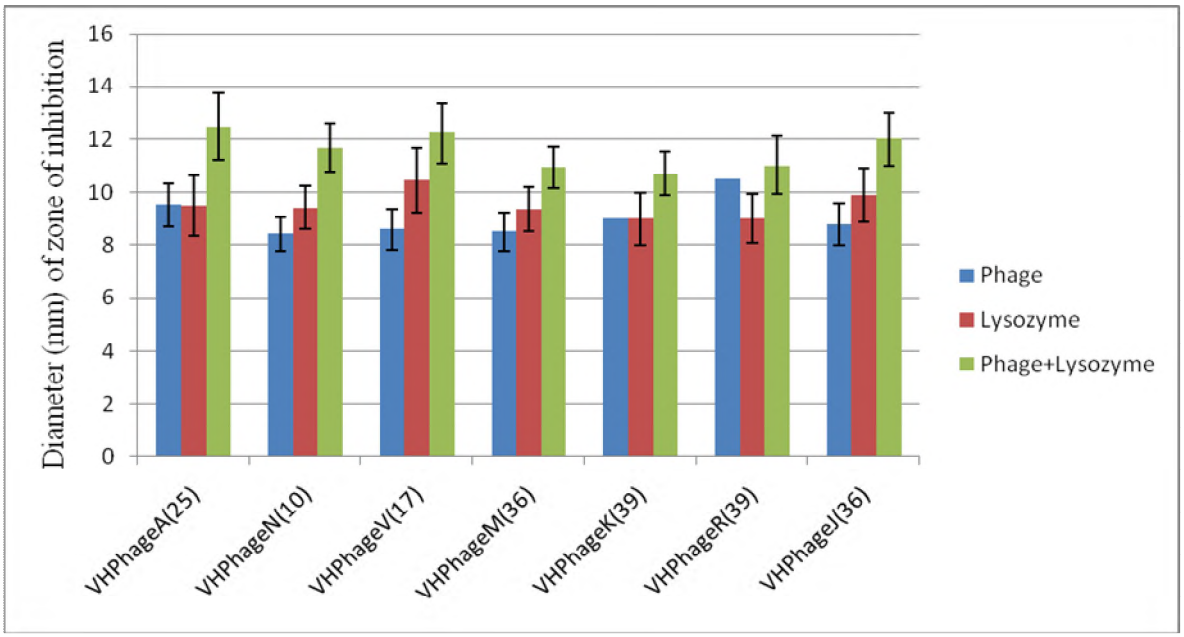

Fig. 32. Mean ±SD of diameter of zone of inhibition of *V. harveyi* (in mm) by phage alone, lysozyme alone and phage+lysozyme.

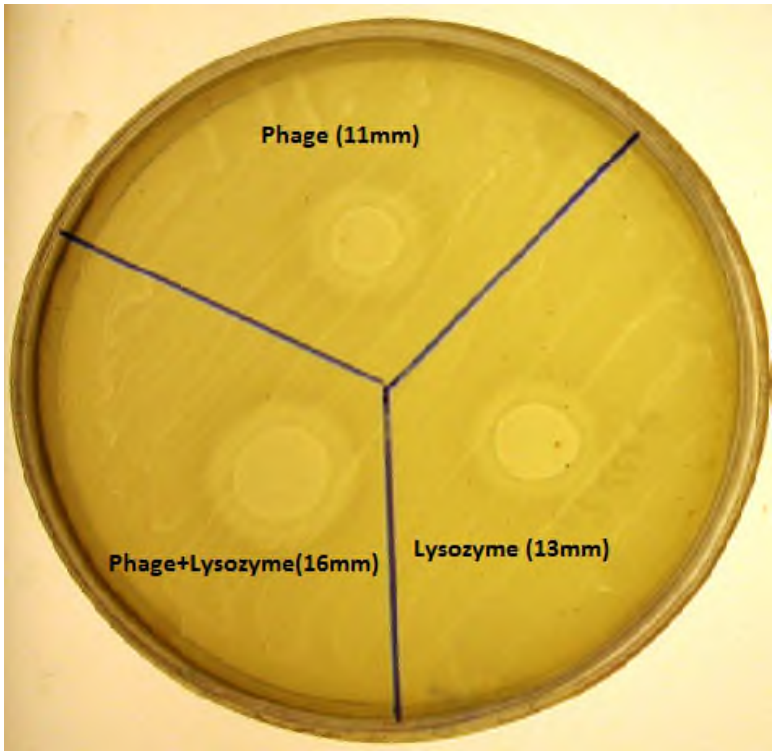

Fig. 33. Zone of inhibition on Solid phase assay by phage alone, lysozyme alone and phage + lysozyme together.

Table 18. *V. harveyi* counts at different time intervals in role of lysozyme in vibriophage activity experiment.

Phage 15 µl, Lysozyme (µl)	0 hour		1 hours		2hours		4hours		24hours	
	Bacterial no (cfu/ml)		Bacterial no (cfu/ml)		Bacterial no (cfu/ml)		Bacterial no (cfu/ml)		Bacterial no (cfu/ml)	
	Lysozyme alone	Phage + Lysozyme	Lysozyme alone	Phage + Lysozyme	Lysozyme alone	Phage + Lysozyme	Lysozyme alone	Phage + Lysozyme	Lysozyme alone	Phage + Lysozyme
25	1.21×10^{6}	1.09×10^{6}	6.31×10^{5}	4.9×10^{5}	2.42×10^{5}	1.49×10^{5}	2.83×10^{5}	8.48×10^{3}	2.79×10^{5}	4.93×10^{3}
50	1.15×10^{6}	1.12×10^{6}	7.45×10^{4}	2.41×10^{4}	3.93×10^{4}	8.47×10^{3}	2.31×10^{4}	3.51×10^{3}	3.03×10^{4}	1.22×10^{3}
100	1.08×10^{6}	1.26×10^{6}	1.37×10^{4}	1.29×10^{4}	8.89×10^{3}	6.84×10^{3}	$6.92.\times10^{3}$	1.92×10^{3}	4.24×10^{3}	6.72×10^{2}
Phage alone (15µl)	1.13×10^{6}		1.06×10^{6}		4.61×10^{5}		2.48×10^{3}		1.74×10^{3}	
Positive Control	1.11×10^{6}		1.24×10^{6}		1.71×10^{6}		2.78×10^{6}		3.11×10^{6}	

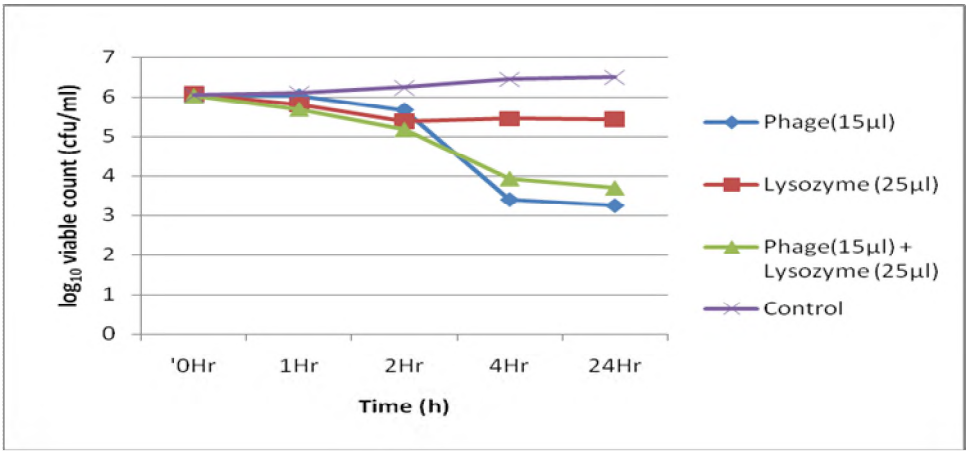

Fig. 34. Role of lysozyme on vibriophage activity experiment (*V. harveyi* count, Lysozyme 25 µl).

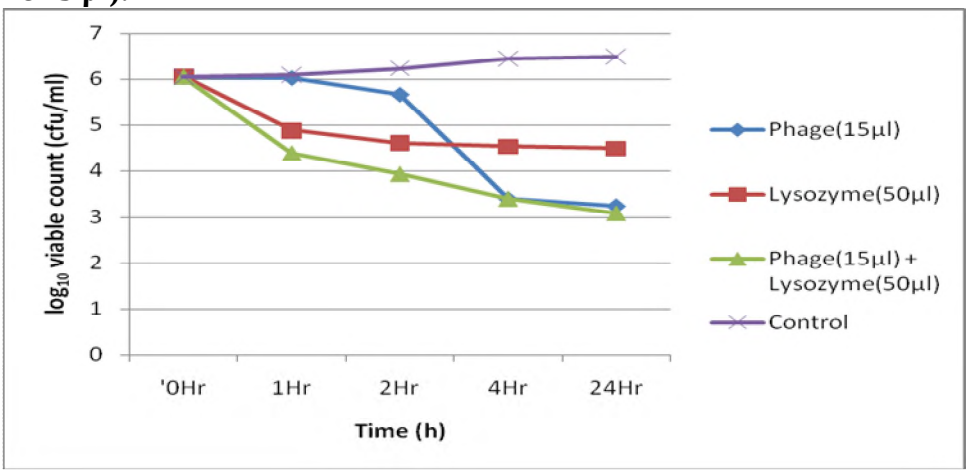

Fig. 35. Role of lysozyme on vibriophage activity experiment (*V. harveyi* count, Lysozyme 50 µl).

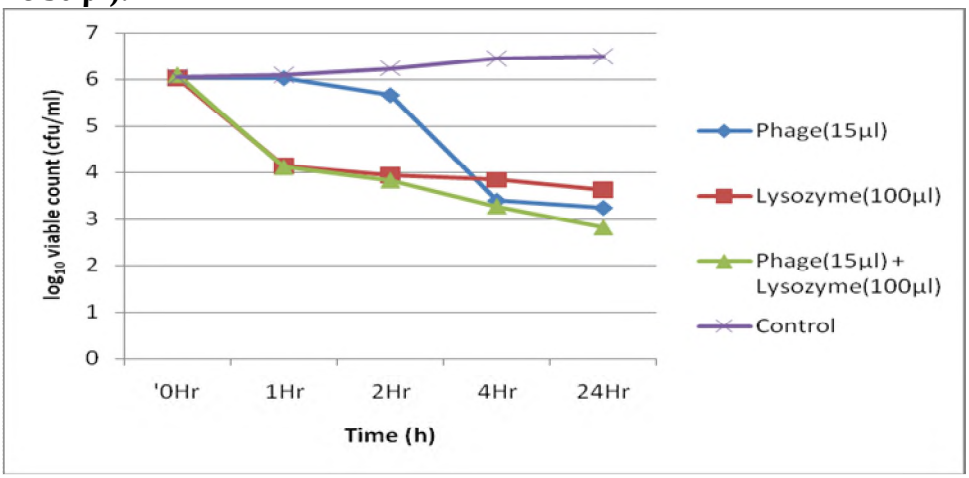

Fig. 36. Role of lysozyme on vibriophage activity experiment (*V. harveyi* count, Lysozyme 100 µl).

V. DISCUSSION

Outbreak of diseases in shrimp aquaculture results in huge economic losses. The causative agents of infectious diseases in shrimp are mainly viruses and bacteria. Bacteria such as *V. harveyi* can be primary or secondary pathogens. *V. harveyi*, belonging to family Vibrionaceae. *V. harveyi* causes luminous vibriosis resulting in rapid mortalities in shrimp hatcheries in different parts of the world.

Bacteriophages are viruses that specifically infect and lyse bacteria. Phage therapy, a method using phages for the treatment of bacterial infectious disease, was first suggested by d'Herelle who discovered phages about the year 1917**.** Report of treating bacterial diseases by applying phages came from France in 1921 for the first time. Following this many people reported using phages for controlling bacterial disease, but as they were not well demonstrated scientifically, combined with the discovery of antibiotics about the same time for treatment of infectious bacteria led to the replacement of phage therapy (Barrow and Soothil, 1997; Alisky *et al*., 1998). Using phages for therapy gained prominence in recent years once again among the scientists with increasing problems of multidrug resistance in bacteria.

Though bacteriophage therapy of human bacterial disease has been subject of several studies, little attention has been given to phages as agents of therapy against bacterial disease in aquaculture. There have been several instances where phages were used in aquaculture in an attempt to control disease. One of the earliest report of the potential of the phages to control disease in aquaculture came from Wu and Chao (1982) who reported use of phage to control disease caused by *Edwardsiella tarda*. *Lactococcus garvieae* infections of yellow tail have been successfully controlled by phages, which indicates potential for phage therapy in aquaculture (Nakai *et al.,* 1999). Park and Nakai

(2003) documented that oral administration of phages of *Pseudomonas plecoglossicida* through feed to ayu increased resistance to experimental infection with *P. plecoglossicida*. Roberts *et al*. (2002) have also demonstrated preventing the occurrence of furunculosis caused by *Aeromonas salmonicida* in farmed rainbow trout using bacteriophages.

Karunasagar *et al*. (1994) reported mass mortality of black tiger shrimp (*P. monodon*) larvae due to multiple antibiotic resistant *V. harveyi* infection in shrimp hatchery. Their ability to form resistant biofilm on the surfaces of larval rearing tanks (Karunasagar *et al*., 1996) has been a cause for concern. Luminous vibriosis can be controlled by using *V. harveyi* bacteriophage isolated from shrimp hatchery. This is authenticated by the report that larvae of *P. monodon* (infected with *V. harveyi*) treated with bacteriophage showed 80% survival compared to the untreated control (20%) (Vinod *et al*., 2006**)**. Bacteriophages have been used for biocontrol of *V. harveyi* in shrimp hatchery (Karunasagar *et al*., 2007).

5.1. Host range

For successful phage therapy, the phage must have a broad host range and should overcome problems of phage resistance. In the present study, out of seven phages, VHPhageV and PhageJ showed more than 61% lytic activity, whereas PhageA, N and M showed lytic activity between 43 to 53%. PhageK and R had very narrow range of llytic activity as they lysed only their specific host. In this study PhageV was used for standardization of phage application due to lytic activity of large number of isolates (64%). In a similar study, Karunasagar *et al*. (2007) found, bacteriophage of *V. harveyi* isolated from diverse environment having a broad host range and lysing around 55-70% of *V. harveyi* strains. In their study to the broad host range of phage Viha10 and Viha8 (70% and 68% respectively) were found suitable for biocontrol of pathogens in shrimp hatcheries.

Rajeev (2003) isolated *V. harveyi* phages that could lyse only *V. fischeri* and were not lytic against any of the other *Vibrio* species indicated above. This observation further supports the findings of Shivu (2004) and Patil (2005) reported that the *V. harveyi* phage isolated by their independent studies could lyse 8 different *Vibrio* species. Further, Payne *et al.* (2004) reported that a temperate phage *Vibrio harveyi* myovirus-like (VHML) could infect *V. cholerae* and *V. mimicus.* All these observations put together reveal that *V. harveyi* phages are abundant in the aquatic environment and such phages possess broad host range with a potential for phage therapy.

5.2. Standardization of phage application under various parameters

The impact of the seasonal variations on the virulence of the luminous bacteria and consequently the disease outbreaks has been well documented by Sunaryanto and Mariam, (1986) who recorded more disease outbreaks during the rainy season Later Prayimo *et al*. (1995) concluded that change in environmental factors such as salinity and pH can enhance the virulence of the luminous bacteria though the mechanism of increased virulence was unknown. Farghaly (1950) reported that environmental factors such as low salinity and pH could reduced the growth rate of luminous bacteria and might in turn lead to the overexpression of their virulence genes thus making them more virulent.

In this study we tried to see, whether any relation exists between *V. harveyi* phage activity and various environmental factors. The experiment was performed under four varying environmental conditions such as salinity, pH, TDS and temperature. This was done to study the optimum for phage activity. Further during change, what quantity of phage would be required for best results was also required to be known if phage therefore was to be applied in hatcheries and farms to control luminous vibriosis.

For standardization of salinity, out of three salinity (20 ppt, 25 ppt and 30 ppt) used, it was found that 25ppt salinity is best for *V. harveyi* phage activity over 20ppt and 30ppt. In 25ppt, the reduction in LBC and larval survivability was comparatively high than the other two salinity. In the case of temperature, 30ºC was best for *V. harveyi* phage activity. This is also the optimum temperature of growth for *V. harveyi*. Therefore it is to be expected that the optimum of *V. harveyi* phage activity, will be the optimum for host. Bacteriophages may predominte in the environment where the temperature is near to the optimum temperature of host. With regard to pH, neutral pH was found to be suitable for *V. harveyi* phage activity. In pH 7, the reduction in LBC and larval survivability was higher than that of acidic and alkaline pH. As *V. harveyi* host cell grow well in neutral pH, it is not surprising that the same pH optimum exists for *V. harveyi* phage. Higher the TDS lower was the *V. harveyi* phage activity. This could be due to the inactivation of phage practical by soluble materials and thus there are not enough phages attaching to target cell. Larval survivability also gradually decreased as TDS value of sea water increased as higher numbers of *V. harveyi* are known to be pathogenic to shrimp larvae.

Statistical analysis using one way ANOVA and Duncan's multiple range test showed significant difference in LBC in three conditions for all four parameters. It also showed that phage activity was highest within first four hours of addition.

From our experiments the knowledge and information we generated will help in standardizing application of phage for biocontrol of luminous vibriosis in shrimp hatchery and farm. PhageV is suitable to use for phage therapy against luminous vibriosis as it lysed 64% *V. harveyi* isolates (n=87). Its optimum activity was found to be 25 ppt salinity at 30°C and neutral pH (7) with low TDS (less than 11.25mg ml^{-1}).

5.3. Standardization of bacteriophage dose application in different salinity and pH conditions

5.3.1. Salinity

In case of 20 ppt salinity, it was found that in 150 µl, 200 µl, 250 µl, 300 µl phage doses, the bacterial number decreases to less than 1000 after 120 h of phage treatment. 150 µl phage ($4.2x10^7$ pfu ml^{-1} of seawater) is sufficient dose for application at 20 ppt salinity. If rapid treatment is necessary, then 300µl phage dose would be suitable as it decreases the bacterial number to less than 1000 within 96 h of phage treatment.

In case of 25 ppt salinity, 100 µl of phage ($2.8x10^7$ pfu ml^{-1} of seawater) dose was suitable for application as it decreases the *V. harveyi* numbers to less than 1000 within 120 h of phage treatment. For rapid reduction in counts, 250 µl to 300 µl phage dose is recommended, as they decrease the *V. harveyi* numbers to less than 1000 within 96 h of treatment.

In case of 30 ppt salinity, 250 µl phage ($7x10^7$ pfu ml^{-1} of seawater) dose was found suitable for reduction in 120 h. Hence for rapid reduction of the pathogen, 300µl phage dose may be necessary for 96 h period. In case of 35 ppt salinity, 300 µl phage ($8.4x10^7$ pfu ml^{-1} of seawater) dose was found ideal for reduction in counts in 96 h.

It was also determined experimentally that the addition of third phage was not necessary for treatment. Two phage treatments were found to be sufficient to reduce bacterial number in 120 h treatment period.

5.3.2. pH

In case of pH 6, the 150 µl phage ($4.2x10^7$ pfu ml^{-1} of seawater) dose was found to be suitable for treatment, as it reduces the bacterial number to less than 1000 after 120 h treatment. But, for rapid treatment, 300 µl phage dose was found to be suitable as it showed similar decrease within 96 h of treatment. In case of pH 7, 100 µl phage ($2.8x10^7$

pfu ml^{-1} of seawater) dose was found suitable for 120 h treatment. Whereas in 48 h to 96 h treatment by 300 µl phage dose was found suitable. In case of pH 8, 150 µl phage ($4.2x10^7$ pfu ml^{-1} of seawater) dose was found suitable for 96 h treatment.

Not much effect of third phage was found in all three cases, so, there is no need of third phage addition for treatment.

Generally in a hatchery setting, the *V. harveyi* counts during a disease outbreak is in the range of $10^4 – 10^6$. The levels of phage to be added to reduce the counts to negligible level would have to depend on the initial counts. If the counts are greater, large volume of phage would be more effective. However even the lowest volume used had a killing effect on *V. harveyi*. When low counts are encountered low volume of phage can be used.

Reduction in counts by phage activity is dependent on other physicochemical parameters of the water body. In this study, at varying salinity, pH the quantity of phage application was standardized. It was seen that in the range that was not the optimum for *V. harveyi* or the phage, larger volume of phage were required to bring about reduction in counts.

5.4. Expression of lysozyme

Lysozyme is one of the potent antimicrobial peptides secreted by many organisms as part of their innate defense mechanism. In invertebrates, expression of lysozyme is regulated and responds to bacterial challenge (Somboonwiwat *et al.*, 2006, Burge *et al.,* 2007). Attempts have been made to clone and express lysozyme gene of shrimp. de-la-Re-Vega *et. al.* (2004) and Tyagi *et al.* (2007) cloned and expressed lysozyme gene of *Litopenaeus vannamei* and *P. monodon* and showed that lysozyme is expressed in inclusion bodies in *E. coli.* In our study, the method was successfully used to get functionally active recombinant lysozyme from *P. monodon*.

5.5. Role of lysozyme on the vibriophage activity

The recombinant lysozyme from *P. monodon* has been observed to be active against both Gram positive and Gram negative bacteria while hen's egg white lysozyme is active against only Gram positive bacteria (Tyagi *et al*., 2007). The i-type lysozyme, chlamysin from Icelandic scallops (Nilsen *et al.,* 1999) has been found to be active against both Gram positive and Gram negative bacteria. de-la-Re-Vega *et al.* (2006) reported that *L. vannamei* c-type lysozyme was active against both Gram positive and Gram negative bacteria. The activity of *P. monodon* lysozyme against *Vibrio* spp. seen in our study has been earlier reported (Tyagi *et al.,* 2007). Similar antivibrio activity of lysozyme from kuruma shrimp, *P. japonicus* (Hikima *et al*., 2003) and Pacific white shrimp, *L. vannamei* (de-la-Re-Vega *et al.*, 2006) suggests potential for application of this protein in shrimp health management since *Vibrio* spp. are important pathogens of these animals (Karunasagar *et al.,* 1994). In this study *P. monodon* lysozyme showed activity against *Vibrio* spp. in solid phase assay. Activity of recombinant lysozyme against various *Vibrio* spp. and other pathogenic bacteria by solid phase assay has been described earlier (Hikima *et al.,* 2003; de-la-Re-Vega *et al.,* 2006). In this study, the *P. monodon* lysozyme was able to reduce counts of luminous *V. harveyi* in seawater by 3.27 log units in 24 h experiment. Tyagi *et al*. (2007) also found antivibrio activity of recombinant lysozyme expressed from black tiger shrimp which was able to reduce luminous *V. harveyi* counts in sea water by 3 log units in 1 hour. The possibility of lysozyme improving the phage entry and further lysis has been studied for the first time.

After adsorption of phage particles on the surface of host bacteria, the actual penetration of phage into the host cell is facilitated by localized digestion of certain cell surface structure by phage enzyme (e.g., lysozyme) carried on the tail of the phage (Pelczar

et al., 1996). In our study, use of recombinant shrimp lysozyme along with *Vibrio* phage increased the activity of *Vibrio* phage. This demonstrates that the lysozyme acts in a manner similar to phage lysozyme and thus increases the phage activity by making holes on cell surface. The results showed increased activity in both solid phase assay as well as in sea water. Application of *Vibrio* phage along with lysozyme for controlling luminous vibriosis in hatchery setting can be a useful strategy for enhanced activity of phages and to address situations of phage resistance due to the inability of phage to enter the bacteria.

VI. SUMMARY

In this study out of seven *V. harveyi* phage used, PhageV was found to be suitable for biocontrol of luminous vibriosis due to its highest host range (68%). Phage activity was evaluated under four important parameters of the aquatic environment *viz* salinity, pH, TDS and temperature. The salinity range used was 20, 25 and 30 ppt; pH range selected was 6, 7 and 8; TDS level chosen for the experiment were 11.25, 22.63 and 38.43 mg ml^{-1} and temperatures selected were 20, 30 and 37ºC. It was found that of the three salinity tested, 25 ppt was best for *V. harveyi* phage activity. In case of temperature, 30ºC was found to be better than 20 and 37ºC. Neutral pH was found to be optimum for phage activity. At lower TDS values, the activity was much better.

In studies on standardization of bacteriophage dose in varying salinity condition of seawater, 150 µl ($4.2x10^7$ pfu ml^{-1} of seawater), 100 µl ($2.8x10^7$ pfu ml^{-1} of seawater), 250 µl ($7x10^7$ pfu ml^{-1} of seawater) and 300 µl ($8.4x10^7$ pfu ml^{-1} of seawater) was required for optimum activity in 20, 25, 30 and 35 ppt salinity respectively over a 96 h period.

For standardization of bacteriophage dose in varying pH condition of seawater, a dose of 150 µl ($4.2x10^7$ pfu ml^{-1} of seawater) at pH 6 and 100 µl ($2.8x10^7$ pfu ml^{-1} of seawater) at pH 7 and 8 over a 96 h period had the desired activity of reducing *V.harveyi* counts.

Application of Two phage types was found ideal is total reduction of luminous bacterial count. The first phage V was applied at 0, 24 and 48 h followed by phage J at 72 and 96 h. The counts were reduced from 10^6 to 10^3.

It was also found that in the presence of recombinant shrimp lysozyme, the lytic activity of vibrio phage increased dramatically in solid phase assay. However in seawater, the activity of the combination of phage and lysozyme was not marked.

In conclusion, PhageV can be used for biocontrol of luminous vibriosis in hatchery setting with best activity at 25ppt salinity, 30ºC temperature, neutral pH and low TDS (<11.25mg/ml). At varying salinity, pH, TDS and temperature, different volumes (indicative of different pfu) have to be used for best result. Application of more than one phage is ideal to overcome resistance of isolates to any one phage. The activity is enhanced in the presence of lysozyme which probably facilitates the entry of more phage particles.

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VIII. ABSTRACT

Bacteriophage therapy is a viable proposition for controlling luminous vibriosis in shrimp aquaculture. Environmental factors influence the growth and activity of phages thereby increasing efficiency in controlling bacterial diseases.

After attachment of phage to the cell wall of the bacterium by tail fibres, the penetration of phage DNA into the bacteria is facilited by lysozyme produced by the phage. We surmise the performance of the phage may improve in the presence of externally added recombinant shrimp lysozyme. Hence recombinant shrimp lysozyme which exhibits bacteriolytic activity against both Gram positive and Gram negative bacteria.

Of seven *V. harveyi* phage used, PhageV was found to be best for biocontrol of luminous vibriosis due to its highest host range. Out of four parameters used, the activity of phage was best at a salinity of 25 ppt, temperature of 30ºC, neutral pH and low total dissolved solids (<11.25 mg ml^{-1}). At varying salinity, pH, TDS and temperature, different volumes (indicative of different pfu) have to be used for best result. Application of more than one phage is ideal to overcome resistance of isolates to any one phage.

In the presence of recombinant shrimp lysozyme, the activity of vibrio phage improved markedly. Combination of lysozyme and phage experiments in sea water showed better activity in comparison to lysozyme and phage applied individually. Thus confirmed lysozyme helped bacteriophage to inject into the bacteria by making pore in cell surface, which resulting in increased phage activity.