

## Isolation and characterization of a moderately halophilic *Marinobacter* phage-host system from the Arabian Sea

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### Abstract

*Marinobacter* is an ecologically important genus of Gammaproteobacteria found in diverse marine habitats, many species of which are capable of degrading hydrocarbons. In this study, we isolated a *Marinobacter* phage-host system from the surface waters of the Arabian Sea using enrichment culture methods, studied their growth characteristics and investigated the effect of salinity and nitrate concentrations on phage-host interactions. The bacterial isolate had maximum identity to *Marinobacter salsuginis* based on 16S rRNA similarities and was termed as *Marinobacter* sp., strain D1S9. It could tolerate up to 14% of NaCl with maximum growth at 11% NaCl. The host grew optimally between 35-40°C and at pH 8. It had a generation time of 3.7 h with a mean growth rate of 0.27 h<sup>-1</sup>. The phage infected the host forming clear, round plaques of 1-2 mm diameter. It had a narrow host range restricted to the strain *Marinobacter* D1S9. The latent period and burst size of the phage were estimated to be 30 min and 106 phages per infected cell, respectively. The phage had an adsorption rate of 3.4 × 10<sup>-8</sup> ml min<sup>-1</sup> and retained 40.4% of its adsorption efficiency at 16% NaCl with a maximum at 4% NaCl (76.1%). Inorganic nitrate was found to have a direct role in controlling host growth and phage burst size.

### Keywords

Marine virus, phage-host interactions, latent period, burst size, nitrate

## Introduction

Viruses are present in the marine environments in copious numbers and their distribution vary spatially and temporally (Bergh et al.1989; Wommack et al.1992; Yang et al. 2010; Parsons et al. 2012). Their activities have far-reaching influences in various marine ecosystems. Viral lytic infection results in host mortality and consequent recycling of organic nutrients in the oceans (Evans et al. 2003; Suttle 2007; Mojica et al. 2016). Majority of marine viruses are bacteriophages (Torrella and Morita 1979). Recent studies across the tropical and subtropical oceans revealed viral lysis as one of the major causes of prokaryotic mortality in the deep ocean when compared to the surface waters resulting in the release of dissolved organic carbon. It is estimated that viruses release 145 Gt of carbon per year in these oceans (Lara et al. 2017). Host specific infection and lysis aids in the suppression of prevalence of a single group and encourages the coexistence of diverse groups of prokaryotic organisms (Suttle 2007).

Phage-host interactions in the natural environment are influenced by physico-chemical factors such as temperature, salinity, nutrients, UV radiation etc. (Finke et al. 2017). Availability of nutrients control the physiological state of host which in turn determines the virus life strategies and lytic characteristics such as latent period and burst size (Mojica and Brussard 2014). Several phage-host model systems were used to study the influence of nutrients on the interactions between the phage and its host (Moebus 1996a, 1996b, 1997a, 1997b; Wolf et al. 2004). However, salinity, temperature and radiation have direct impact on viral stability and infectivity (Kukkaro and Bamford 2009).

Phage-host interactions in the natural environment are so complex that it is essential to monitor them under controlled laboratory conditions to understand how phages modify the functioning of their hosts and overall ecosystem. *Marinobacter* is a genus comprising numerous highly versatile marine bacteria. They have been isolated from different marine habitats such as oil producing wells and hydrothermal systems and from natural-gas wells. Some of them are known to degrade hydrocarbons and others can influence the biogeochemical cycling of many inorganic elements (Huu et al. 1999; Handley and Lloyd 2013; Evans et al. 2018). In addition, there are reports that members of the genus *Marinobacter* could be used in bioreactors for drainage treatment from oil terminals. Phages infecting these bacteria have also been identified from these bioreactors which were supposed to improve the efficiency of the petroleum bioremediation process through phage driven microbial loop (Rosenberg et al. 2010). The phages specific to hydrocarbon degrading *Marinobacter* could also be used for the screening of potential hosts in other waste water treatment plants as well. Information on the isolation of phages infecting *Marinobacter* are scarce with only one detailed study available to date (Zhu et al. 2018). In this study, we isolated and characterized a *Marinobacter*

strain and the phage infecting it from surface water of the Arabian Sea. We hypothesize that nitrate concentration has a positive influence on host growth and subsequently on phage production.

## **Materials and methods**

### Isolation of bacterial host and phage

Both the bacterium and the phage were isolated from surface water of the Arabian Sea off the coast of Kochi (9.9586° N and 76.0825° E), southwest coast of India, during December 2014. Culturable bacteria were isolated by serial dilution and spread plating of the collected water sample on Zobell marine agar (ZMA). Pure cultures of morphologically different colonies were then established. Bacteriophages in the sample were screened against the bacterial isolates by soft agar overlay method after enriching 25 mL seawater with 10× Zobell marine broth (Middelboe et al.2010). Plates were examined for the appearance of plaques after 48 h of incubation at ambient temperature. Clear plaques formed were scraped into phage buffer (450 mM NaCl, 50 mM MgSO<sub>4</sub>, 50 mM Tris, 0.01% gelatin, pH 8) and incubated overnight at 4°C. This was followed by centrifugation at 10,000× *g* for 10 min and subsequently, the supernatant was passed through a 0.22 µm pore size Acrodisc syringe filter (Sigma-Aldrich, India) to remove any bacterial cells. The filtrate was then plated to isolate a single plaque and this was repeated five times to get the purified phage stock. In order to check the chloroform sensitivity of the phage, phage stock having 10<sup>10</sup> PFU mL<sup>-1</sup> was treated with 20% final concentration of chloroform and incubated at 37°C for 30 min. Plaque counts were determined by soft agar overlay method. The phage was characterized by whole genome sequencing (Aparna et al. 2019). Briefly, the viral particles were concentrated by PEG precipitation and ultrafiltration using Amicon Ultra-15 centrifugal filters. Viral DNA was extracted from the concentrated viral particles and sequenced using Illumina MiSeq platform. The detailed protocols and genome analyses are described by Aparna et. al.(2019). The genome sequence of the isolated phage termed Marinobacter phage AS1 is available at GenBank under accession number MK088078. Transmission electron microscopy was used for the morphological characterization of the isolated phage (Aparna et. al. 2019).

Phylogenetic, morphological and biochemical characterization of the host .The bacterial host of the isolated phage was identified using 16 rDNA analysis. 16S rDNA region in the genome was amplified by colony PCR using primers: UF (GCACAAGCGGTGGAGCATGTGG) and UR (GCCCGGGAACGTATTCACCG) (Amann et al. 1995). A single isolated colony of the bacterium was mixed with 100µl TE buffer and incubated at 100°C for 10 min. The mixture was centrifuged at 1000× *g* for 10 min, and the supernatant was used as template in PCR with 16S rDNA universal

primers. Amplified DNA was sequenced by Sanger dideoxy method and the sequence was compared against NCBI non-redundant database to analyze the phylogenetic lineage of the bacterium. The 16S rRNA partial sequence was submitted in GenBank with accession number MG948170.

Morphology of the bacterium was determined by Grams staining. Various biochemical tests for catalase, oxidase, citrate utilization, nitrate reduction, DNase, amylase, xylanase, lipase, laccase, protease, cellulase, asparaginase, urease, gelatinase, pectinase, phosphatase, indole production, methyl red and Voges-Proscauer were carried out to identify metabolic potential of the isolated bacterium (Krieg and Holt 1984; Sanchez-Perro et al. 2003; Kasana et al. 2008).

Generation time and mean growth rate of the host

Generation time ( $G$ ) and mean growth rate ( $\mu$ ) of the bacterium were estimated from its growth curve. For that, an overnight bacterial culture was added to fresh Zobell marine broth to obtain a final OD<sub>600</sub> of 0.05 and incubated for 48 h at 37°C. OD values were measured at regular intervals and plotted against time to generate the growth curve. Generation time and mean growth rate were calculated using the formulae:

Generation time,  $G = t/n$

Mean Growth Rate,  $\mu = n/t$

Optimum temperature, pH and salinity for the growth of host bacterium

The host bacterium was grown in Zobell marine broth at different temperatures (10, 20, 30, 35, 40 and 45°C) and pH (6.5, 7.0, 7.5, 8.0 and 8.5) and in tryptone broth having different NaCl concentrations (0.5, 3, 5, 7, 9, 11, 14 and 16) for 24 h. Optical densities (OD<sub>600</sub>) were measured at the start and at the end of incubations.

Host specificity and life cycle characterization of the phage

In order to investigate the host range of the phage, soft agar overlays were performed as described previously in the section with 10 *Marinobacter* strains and 4 *Alteromonas* strains obtained from microbial culture collection of the Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute (CMFRI), Cochin, India.

Latent period and burst size, the two important parameters in phage characterization, were estimated by one step growth experiment as described previously (Middelboe et al. 2010). An overnight culture of host was added to 50 mL Zobell marine broth and incubated until the culture reached a cell density of  $\sim 10^8$  CFU mL<sup>-1</sup>. One millilitre of this culture was added to phage stock of  $\sim 10^6$  PFU mL<sup>-1</sup> in triplicates, incubated for 10 min and centrifuged at 6000×  $g$  for 10 min and the

pellet was resuspended in 1 mL of growth medium to remove any unadsorbed viruses. The centrifugation and washing processes were repeated thrice. Finally, 50  $\mu\text{L}$  from the resuspended pellet was added to 50 mL of growth medium and incubated at 37°C. Subsamples were taken at every 15 min interval to estimate the number of viruses as plaque forming units over a period of 2 h.

#### Determination of adsorption rate constant of the phage

A flask containing 9 mL of temperature equilibrated fresh Zobell marine broth at 37°C was inoculated with 1 mL of overnight host bacterial culture. 1 mL of phage stock having  $10^5$  PFU  $\text{mL}^{-1}$  was added to the flask and mixed well. A control flask with 9 mL of the fresh medium and the same amount of phage particles was also maintained along with the experimental flask. 50  $\mu\text{L}$  aliquots were removed from the flask and transferred to chilled tubes containing 950  $\mu\text{L}$  growth medium at every 2 min interval until  $t = 16$  min. A 50  $\mu\text{L}$  subsample was also transferred to 950  $\mu\text{L}$  medium to calculate the phage count at  $t = 0$ . All the tubes were chilled on ice and after completion of 16 min, the tubes were centrifuged at  $10,000\times g$  for 10 min at 4°C. 100  $\mu\text{L}$  of the supernatant mixed with molten soft agar and the host culture was plated and the plaque forming units were calculated for each tube (Kropinski 2009). Bacterial counts were estimated by serial dilution and plating on Zobell marine agar plates. The adsorption rate constant was calculated as described by Kropinski (2009) using the formula:

$$k = \frac{2.3}{Bt} \log \frac{P_0}{P}$$

Where,  $k$  is the adsorption rate constant;  $B$  is the bacterial cell concentration;  $t$  is the time taken for achieving 50% adsorption;  $P_0$  is the initial phage titre and  $P$  is the phage titre at time  $t$ .

#### Influence of NaCl concentration on phage adsorption efficiency

A logarithmic phase culture of the host bacterium ( $\sim 10^8$  CFU  $\text{mL}^{-1}$ ) was centrifuged at  $8000\times g$  and the pellet was resuspended in tubes with nutrient broth having different added NaCl concentrations (0.5-18%). Equal volume of phage stock having a final concentration of  $\sim 10^7$  PFU  $\text{mL}^{-1}$  was added to each tube and the tubes were incubated for 20 min. Titre of unbound viruses in the supernatant was determined for each NaCl concentration by soft agar overlay method after centrifugation at  $10,000\times g$  for 10 min (Kukkaro and Bamford 2009).

#### Phage infection under varying nitrate concentrations

Seawater samples with varying nitrate concentrations (5  $\mu\text{M}$  and 0.3  $\mu\text{M}$ ) were used in the infection experiment after removing any bacteria by filtration. For each sample three preparations were made: one flask with only virus and one with only host (V and B) and a third flask with both host and virus (B+V). To the water samples, equal number of host cells were added and incubated overnight to acclimatize with the growth conditions. Phages were added to a final multiplicity of infection (MOI) 0.1 and incubated at 37°C. Plaque titre assay and plating on Zobell marine agar were performed at 0, 6, 24 and 48 h to estimate the number of phages and host respectively. Latent period and burst size of the phage were also estimated under the two different conditions (Middelboe et al. 2010).

## Results and discussion

### Identification and biochemical characterization of the host

Based on 16S rDNA sequence similarities with the sequences in NCBI database, the host bacterium was closely related to *Marinobacter salsuginis* (100% similarity) and was designated as *Marinobacter* sp. strain D1S9. It is Gram negative, rod shaped bacterium forming raised, translucent, circular colonies of 2-3 mm diameter on Zobell marine agar after 32-36 h of incubation. It was oxidase, catalase and protease positive and negative for all other tests.

### Generation time and mean growth rate

A growth curve of D1S9 was plotted as OD<sub>600</sub> vs time after 48 h incubation in Zobell marine broth at 37°C (Fig. 1). The bacterium had a generation time of 3.7 h with a mean growth rate of 0.27 h<sup>-1</sup>.

### Optimum temperature, pH and salinity for *Marinobacter* D1S9

Optimum growth of the host occurred between 35-40°C, at pH 8.0 and at 11% NaCl concentration (Fig.2). The host was moderately halophilic with good growth in the range 3-14% NaCl. Optimum pH of the bacterium is 8, closer to the pH of seawater. Factors such as salinity, temperature and pH influence the growth of bacteria in marine systems. Temperature tolerance of bacteria is governed by the temperature sensitivity of key enzymes, membrane permeability etc. (MacLeod 1965). Salt dependence, on the other hand, is a fundamental difference between marine and freshwater bacteria in terms of their physiology and ecological distribution. Na<sup>+</sup> ion pumps are required for membrane transport of metabolites from the environment. Moreover, the osmotic effect of the ions helps in the retention of solutes within the cell (MacLeod 1986). Many strains of the genus *Marinobacter* have been reported to be moderately halophilic with specific enzyme activities and may play crucial roles in different marine environments (Yoon et al. 2003; Martin et al. 2003; Montes et al. 2008; Kumar and Khare 2012). They efficiently degrade aliphatic and polycyclic aromatic hydrocarbons and acyclic compounds and hence play important roles in mitigation of oil pollution (Duran 2010). They

have frequently been isolated from hydrocarbon polluted sites (Lal et al. 2013). *Marinobacter sp.* has been used for saline industrial wastewater treatment due to its denitrifying efficiency, salt tolerance, pH tolerance and broad carbon use range (Li et al. 2013).

#### Plaque characteristics and Host specificity of the phage

The phage formed clear, round plaques of 1-2 mm diameter on bacterial lawn indicating complete lysis of the cells. Transmission electron microscopic analysis revealed the virus to be a Podovirus having a dsDNA genome with an icosahedral head and a short non-contractile tail. The detailed genome analysis of this phage is already described by Aparna et al. (2019). Phage AS1 lost 99.6 % of its activity after treatment with 20 % chloroform. In spite of the absence of lipids, one third of the tailed viruses are reported to be chloroform sensitive (Ackermann 2006). For example, Filamentous, dsDNA viruses of family *Inoviridae* are chloroform sensitive, despite the fact that they contain no lipid component (Cann 2016).

The isolated phage did not infect any of the *Marinobacter* or *Alteromonas* strains tested in this study, indicating a narrow host range restricted to the strain *Marinobacter* D1S9. Phage infection mechanisms and host resistance mechanisms are two possible factors determining host range of a particular phage. Adaptation to specialize for a single host bestows the phage with advantages over selecting for a broad host range (Koskella and Meaden 2013).

#### One-step growth curve of the phage

The latent period as well as burst size of infection of phage AS1 were estimated from one step growth curve (Fig. 3). The latent period lasted for about 30 min and followed by a steady increase in phage counts. This rise in counts of about 2 hours resulted in a burst size of  $\sim 10^6$  plaque forming units per infected cell. Compared to *Marinobacter* phage B23 (Zhu et al. 2018) which has a longer latent period and smaller burst size, AS1 has shorter latent period and a larger burst size. The two parameters vary largely among phages. A short latent period at high host densities helps the phage to establish multiple infections (Abedon 1989).

#### Adsorption rate constant and adsorption efficiency of the phage at varying NaCl concentrations

Phage AS1 had a fairly fast adsorption rate of  $3.4 \times 10^{-8}$  ml min<sup>-1</sup> when used to infect the host in Zobell marine broth at 37°C. The phage showed maximum adsorption efficiency at 4% NaCl and had considerable stability up to 16% NaCl (Fig. 4). 40% of adsorption efficiency was retained up to 16% NaCl and dropped to 29.2% at 18% NaCl. Study by Kukkaro and Bamford (2009) on virus-host interactions in a wide range of saline environments reported drastic variations in phage adsorption rates ranging from  $2.9 \times 10^{-13}$  to  $1.2 \times 10^{-9}$ . Membrane bound viruses were more sensitive to variations

in salt concentration than non-lipid containing ones. The efficiency of phage AS1 to infect the host under a broad salinity range indicates its ability to thrive under varying natural salinity conditions.

#### Influence of nitrate on phage life cycle characteristics

The latent period of infection was estimated to be three hours for both 5  $\mu\text{M}$  and 0.3  $\mu\text{M}$  nitrate incubations (Fig. 5). The viral burst size varied greatly between the two nitrate levels. In the presence of 5  $\mu\text{M}$  nitrate, the burst size was 325, while in the presence of 0.3  $\mu\text{M}$  nitrate only 28 viral progenies released per bacterial cell. At higher nitrate concentration, a gradual increase in viral counts was observed reaching a maximum ( $2.9 \times 10^8$  PFU  $\text{mL}^{-1}$ ) in 6 h, followed by a steady decline. Bacterial counts did not vary during first six hours, but decreased gradually thereafter. Also at lower nitrate concentration of 0.3  $\mu\text{M}$ , the viral counts showed a rapid increase for the first 6 hours ( $3.2 \times 10^7$  PFU  $\text{mL}^{-1}$ ) which subsequently decreased till 24 h, stabilized and increased to reach  $3.2 \times 10^7$  PFU  $\text{mL}^{-1}$  at 48h. Initial bacterial count at lower nitrate level was half of that at 5  $\mu\text{M}$  at 0 h. No considerable variation in growth was observed until 6 hours of incubation ( $6.7 \times 10^6$  CFU  $\text{mL}^{-1}$ ), decreased substantially ( $4.8 \times 10^5$  CFU  $\text{mL}^{-1}$ ) by 24 h and increased thereafter till 48 h ( $9.6 \times 10^5$  CFU  $\text{mL}^{-1}$ ). This increase in bacterial counts after 24 h (Fig. 5b) may have contributed by the increased availability of recycled nutrients as a result of the high viral lysis rates after 6 h. In the bacterial control flask without viruses (BC), a continuous decrease was observed up to 48 h ( $3.4 \times 10^5$  CFU  $\text{mL}^{-1}$ ). This might be due to the depletion of nutrients in the flask. In both low and high nitrate concentrations, bacterial counts at the end of the experiment were higher than the counts in control incubations indicating the replenishment of nutrients due to viral lysis in the experimental flasks (B+V). Viral lysis of host cells in (B+V) flasks release dissolved nutrients from dead cells which can be sources of nutrients for surviving bacteria. In viral control flasks (VC), counts remained stable till the end of the experiment.

Several studies have reported the influence of nutrient addition on virus-host interactions (Wolf et al. 2004; Motegi et al. 2009; Motegi and Nagata 2007; Williamson and Paul 2004). Experimental evidences show that burst size is linked to the supply of nutrients to the host cells, which increases with the increase in bacterial growth rate (Middelboe 2000; Nabergoj et al. 2017). In a study, investigating the influence of physiological status of the marine haptophyte *Phaeocystis pouchetii* on its interactions with virus PpV01, host growth conditions had no significant effect on phage latent period, but burst size varied substantially (Bratbak et al. 1998). Studies have shown that viral production was positively influenced by nitrate addition to seawater (Motegi and Nagata 2007). Viral lytic production is strongly influenced by the trophic status of the system (Payet and Suttle 2013). Nitrogen is an essential element for protein synthesis, the depletion of which indirectly affects phage



production by reducing the host growth. In our experiment, the decline in host cell number was faster in high nitrate than in low nitrate concentration, implying the importance of this nutrient in host growth and phage production.

### **Conclusions**

Studies on phage-host interactions are key to understanding the specific interplay between the two in the marine ecosystem. Cultivation experiments combined with genomics provide insights into the role of phages in the functioning of hosts in the environment. In this paper, we describe the isolation and characterization of a *Marinobacter* phage-host system and discuss the importance of nitrate and salinity status on phage life cycle and adsorption efficiency, respectively. Both the host and the phage exhibited tolerance to a wide range of salinities with the host growing at salinities up to 11% NaCl and the virus retaining 40% of its adsorption efficiency at 16 % NaCl. Further, nitrate concentrations have significant positive influence on host growth and phage burst size. Further mesocosm studies are necessary to understand the effects of natural nutrient conditions on phage-host interactions.

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## Figure legends

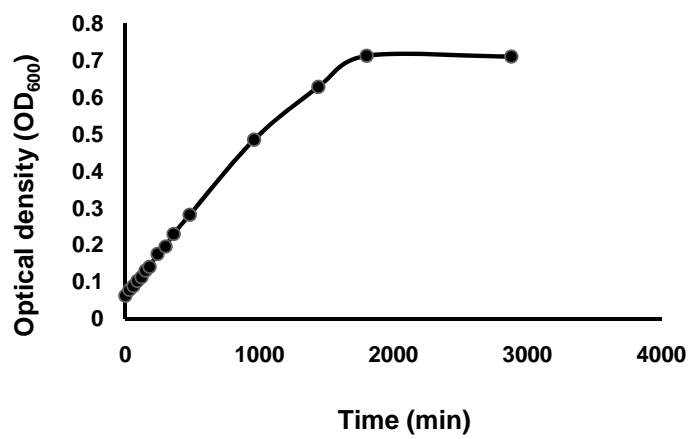
**Fig.1** Growth curve of *Marinobacter* sp. D1S9 displaying optical densities (OD) of the culture at different time points

**Fig.2** Growth of *Marinobacter* sp. D1S9 at varying (a) temperature, (b) salinity and (c) pH

**Fig. 3** One-step growth curve of phage AS1 displaying viral particles produced (represented as plaque forming unit (PFU)) at different points of time

**Fig. 4** Adsorption efficiency of the phage at varying NaCl concentrations

**Fig. 5** Influence of varying nitrate concentrations on the *Marinobacter* phage-host system (a) counts of phage and host (represented as plaque forming units and colony forming units, respectively) at different time intervals under high nitrate concentration (5  $\mu$ M) and (b) counts under low nitrate concentration (0.3  $\mu$ M). BC, bacterial count in the control flask; VC, viral count in the control flask; B, bacterial count in the experimental flask with both bacteria and virus; V, viral count in the experimental flask with both bacteria and virus



**FIGURE 1**

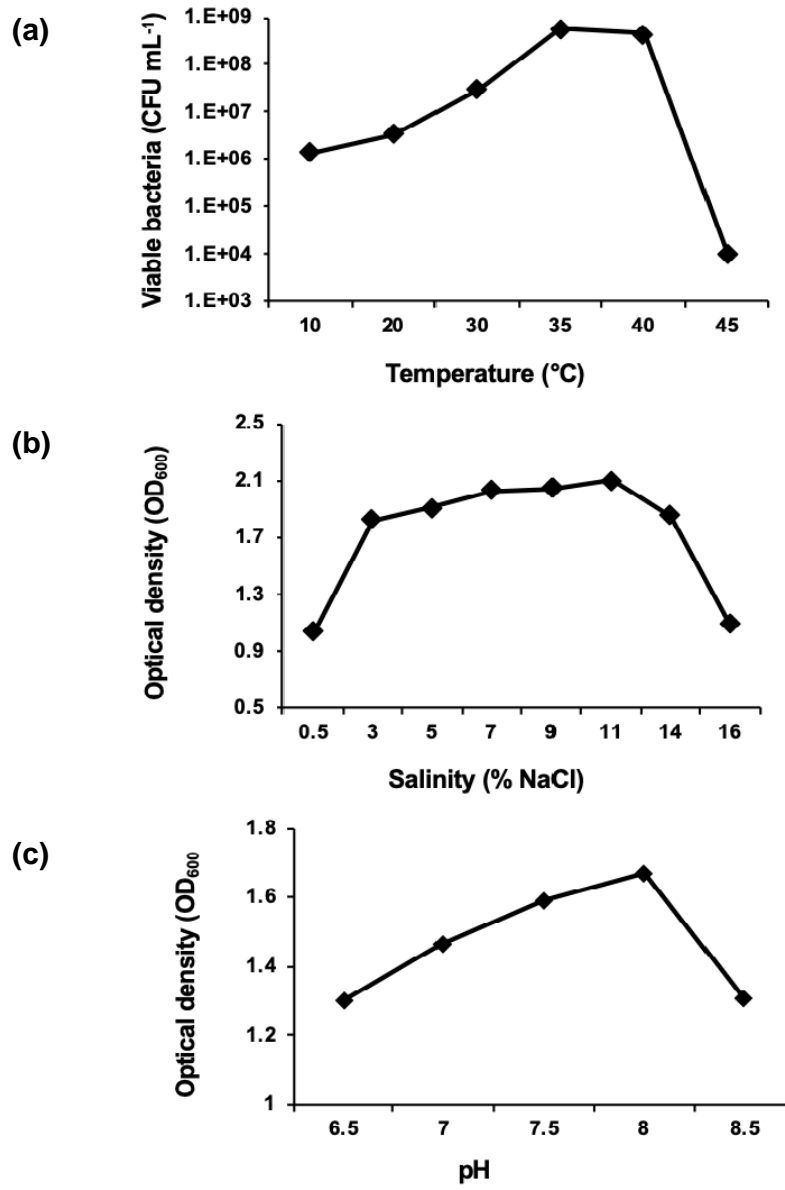


FIGURE 2



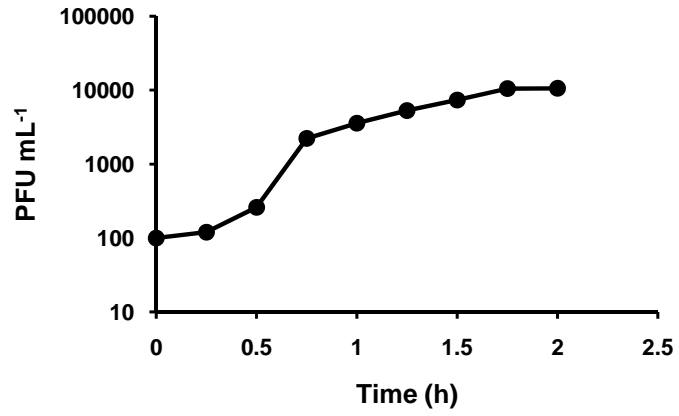


FIGURE 3

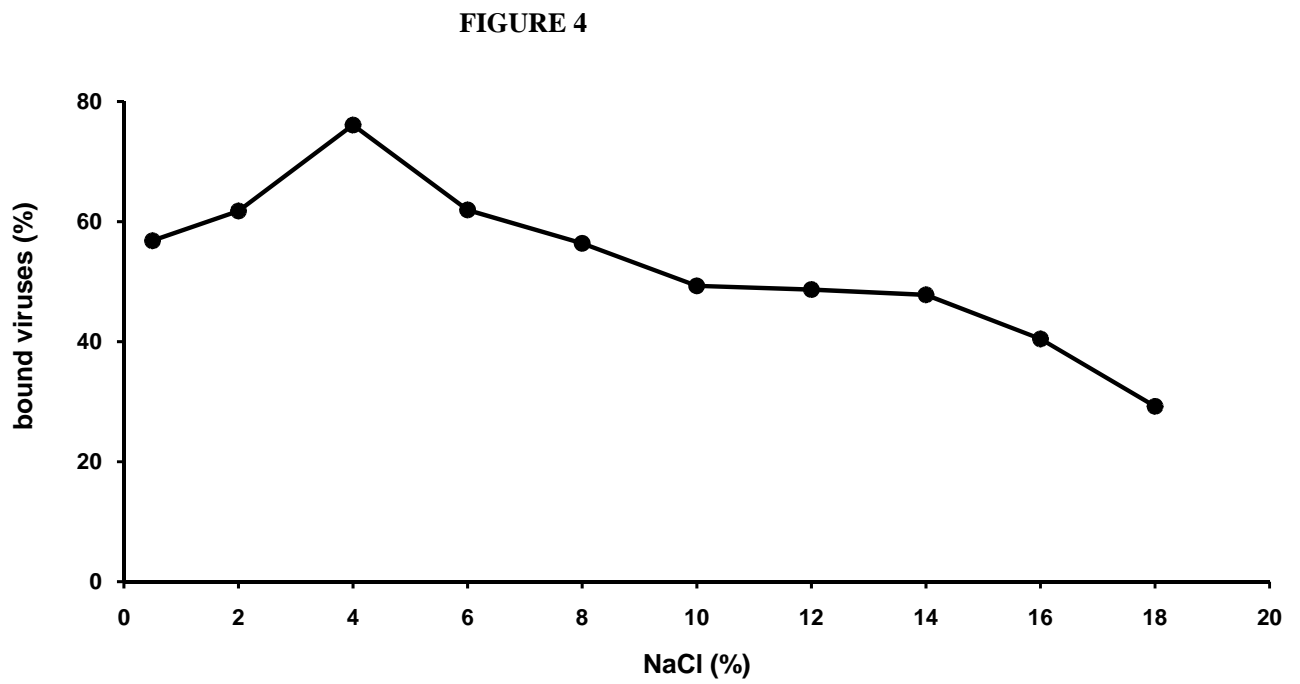


FIGURE 4

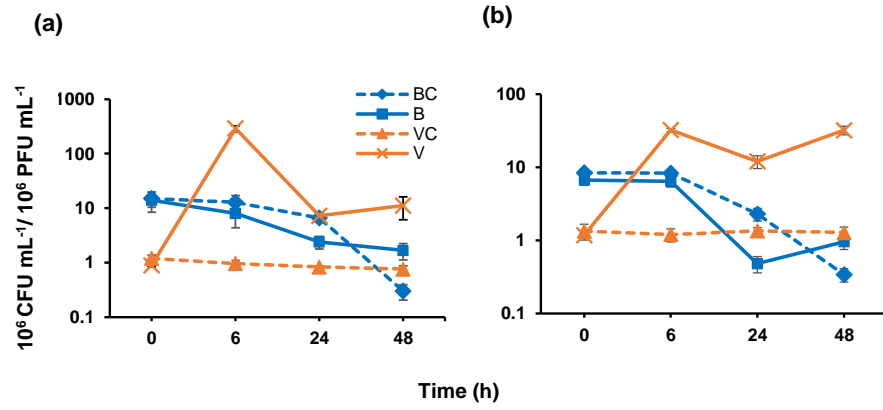


FIGURE 5