Journal of Applied Biology & Biotechnology Vol. 11(6), pp. 59-65, Nov-Dec, 2023
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2023.63868

Isolation of toxic gas-producing bacteria (*Desulfovibrio* spp.) from shrimp ponds and potential of bacteriophages as biocontrol

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ARTICLE INFO

Article history:
Received on: March 20, 2023
Accepted on: August 06, 2023
Available online: October 25, 2023

Key words:
16S rRNA gene sequence,
Bacteriophages,
Black colonies,
Desulfovibrio spp.,
H_2S gas-producing bacteria,
Organic pollution.

ABSTRACT

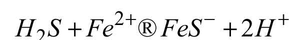
Desulfovibrio spp. is the main causes of hydrogen sulfide H_2S gas and organic pollution in shrimp ponds. The amount of food deposited at the pond bottom provides favorable conditions for the growth of H_2S gas-producing bacteria. Many studies have shown that the use of antibiotics to treat bacteria led to the emergence of multidrug resistant strains, which has raised awareness of the critical need for alternative antimicrobials. Bacteriophages – viruses that infect and inhibit bacterial cells – are currently of great interest as highly viable alternative to antibiotics. The purpose of this study is to evaluate the effectiveness of bacteriophage in controlling H_2S producing bacteria in shrimp ponds. The bacterial strains were identified based on morphological, physiological, and biochemical criteria. The bacterial species was molecularly identified using the 16S rRNA gene sequencing, and phage strains capable of inhibiting H_2S gas-producing bacteria were investigated by agar spot test and plating method. The result investigated six strains of bacteria, *Desulfovibrio* spp., that were isolated from the shrimp pond. This study showed that phages L12 and T0 could infect bacteria *Desulfovibrio* spp. and prevent the growth of the bacteria 11D as well as prevent the generation of H_2S gas.

1. INTRODUCTION

In shrimp culture pond sediments, hydrogen sulfide (H_2S) – producing bacteria are a group of strictly anaerobic bacteria living in anaerobic environments that are capable of reducing sulfates to sulfides [1]. They are a taxonomically diverse group of bacteria with representatives in several phyla within the domain Bacteria. The majority of known species to date belong to Deltaproteobacteria, of which *Desulfovibrio* is a widely studied genus [2,3]. *Desulfovibrio* spp. is fermenting, anaerobic, and Gram-negative curved rods characterized by the presence of a pigment, desulfoviridin [4,5].

H_2S producing bacteria carry out a special anaerobic respiration process, in which sulfate is used as the final hydrogen acceptor. Commonly used hydrogen donors are alcohols, organic acids, or molecular hydrogen. During the reduction process from sulfates to sulfides, H_2S gas, a toxic colorless gas with rotten egg smell, is produced and its buildup affects shrimp health, thereby decreasing production. This gas combines with metal ions such as Fe and Cu (e.g., in shrimp feed ingredients) to form insoluble black precipitates [2]. The mechanism of this process can be presented as follows:

$$8(H) + SO_4^{-2} ® H_2S + 2H_2O + 2OH^-$$



$$H_2S + Fe^{2+} ® FeS^- + 2H^+$$

H_2S causes severe oxygen deficiency and has a very bad impact on the growth rate and survival rate of cultured shrimp. An H_2S concentration of 0.01–0.02 ppm in pond water may poison shrimp and lead to eventual death. According to Fast and Boyd (1992), the appropriate H_2S content for shrimp growth and development must be zero [6]. According to Boyd (1990), H_2S is a toxic gas that will bind with Fe of hemoglobin or Cu of hemocyanin, making blood cells lose the ability to transport oxygen, leading to slow growth and a low survival rate due to lack of oxygen [7].

Given the unfavorable roles of H_2S producing bacteria, such as *Desulfovibrio* spp., in shrimp ponds, their control is necessary. In general, antibiotics have been widely used to treat bacterial infections in aquaculture systems, especially in hatcheries and intensive farming ponds. However, accumulation of antibiotic residues in water and mud, as well as the development of resistance to antibiotics, is a great concern. Therefore, there is need for better treatment options for bacterial infections in aquaculture systems. The use of bacteriophages is one of such options, and a promising approach at that.

Phages are the most abundant biological entities on the planet [8-10]. Scientists all over the world have shown the effectiveness of bacteriophages to inhibit pathogenic bacteria [11-15]. Phages are specific viruses, because they infect and multiply in numbers on one or several respective strains of bacteria. Bacteriophages eliminate only

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target bacteria without harming other groups of bacteria [16]. Recently, there have been many studies on the application of bacteriophages to control *Desulfovibrio* species, with remarkable results. Eydal *et al.* isolated bacteriophages effective against *Desulfovibrio aespoeensis* [17]. Furthermore, Handley *et al*., (1973) reported phages produced from the culture of *Desulfovibrio vulgaris* NCIMB 8303 by UV irradiation or treatment with mitomycin [18], Rapp and Wall found phages that could mediate transmission load in *Desulfovibrio desulfuricans* [19]. Kamimura and Araki isolated a lytic phage infecting *Desulfovibrio salexigens* [20]. However, studies on the application of bacteriophages for controlling H_2S gas-producing bacteria in shrimp ponds are still very limited. This study, therefore, evaluates the effectiveness of bacteriophage in the control of H_2S producing bacteria in shrimp ponds.

2. MATERIALS AND METHODS

2.1. Samples Collection

Water and shrimp samples were collected at shrimp ponds around the Mekong Delta (e.g., Soc Trang, Kien Giang province). The bottom sludge samples in the pond were collected at a depth of 2–5 cm. Lytic phages were provided by the Molecular Biology laboratory, Biotechnology Research and Development Institute, Can Tho University, Viet Nam [Table 1].

2.2. Isolation, Characterization and Identification of H_2S Gas-Producing Bacteria

To increase bacterial proliferation, about 7 mL of pond water mixed with black mud was added to 13 mL of liquid Postgate B medium (a specialized medium for isolating H_2S gas-producing bacteria) and left overnight. Furthermore, shrimp samples were added to 20 mL of liquid Postgate B medium after removing the gills and viscera. They were spread on Thiosulfate Citrate Bile Sucrose (TCBS) medium and incubated at room temperature for 48 h. The number of colonies formed were counted and their morphology was observed. A typical black colony was picked and grow in liquid Postgate B medium [21]. After 12–24 h, bacterial suspensions were streaked cultured to select stock colonies. Bacteria were then stored in 20% glycerol at −20°C.

The bacterial strains were identified using morphological, physiological, and biochemical indicators (such as motility test, Gram staining, and oxidase test) as described in Bergey's Manual of Systematic Bacteriology [22].

Further, bacterial DNA extraction was performed using crude method, and H_2S producing bacteria were identified by amplifying the 16S rRNA sequence using the primer pairs – 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [23]. Polymerase chain reaction (PCR) was performed with these conditions – initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 2 min; and final extension at 72°C for 10 min. Electrophoresis of the PCR products was carried out on a 2% agarose gel with TAE buffer 0.1X at 50 V for 45 min, and H_2S producing bacteria were identified through the presence of an expected band size of 1500 bp.

PCR products were sequenced by an automatic sequencing system at 1st BASE company, Malaysia. The results of 16S rRNA gene sequencing were compared with the Basic Local Alignment Search Tool program sequence on the bank database of National Center for Biotechnology Information (NCBI) to determine the species name of the bacterial strain.

2.3. Isolation and *In Vitro* Evaluation of Bacterial Hosts Susceptible to Bacteriophage Infection

To select a bacteriophage strain capable of inhibiting H_2S gas-producing bacteria, we performed the agar spot test, using TCBS agar plate supplemented with 0.5% agar. H_2S gas-producing bacteria were cultured in liquid Posgate B medium for 48 h before this experiment. TCBS medium layer with 0.5% agar was added to the Petri dish. Next, liquid culture bacteria were added to Eppendorf tube, centrifuged at 10,000 rpm for 10 min, removed the supernatant, leaving 500–700 µL of the filtrate with the residue, and mixed well. Then, using a sterilized cotton swab, three layers of the bacterial solution were spread on the surface of the medium. The plates were incubated for 30–45 min at room temperature. Thereafter, a micropipette was used to add 2 µL of bacteriophages for each drop. On drying of the drop zones, plates were incubated for 24 h at room temperature and plaques were observed. The experiment was arranged in a completely randomized design with three replications and using the double-layer agar method [24].

2.4. Evaluation of Bacteriophages' Ability to Inhibit H_2S Gas-Producing Bacteria by Plating Method

The purpose of this experiment was to evaluate the inhibitory effects of bacteriophages on the growth of H_2S producing bacteria based on colony forming units. In the control treatment (1A), only H_2S gas-producing bacteria with no bacteriophages were added. In the bacteriophage treatment (1B), H_2S producing bacteria and bacteriophages were added.

The H_2S gas-producing bacteria strains were cultured in Postgate B medium for 12–24 h before this experiment. The medium used to spread the bacteria was TCBS medium supplemented with 0.5% agar. First, the bacteria were diluted to the desired concentration (10^{-6} CFU/mL) and used in each of the treatment. Treatment 1A consisted of 100 µL of bacterial suspension and 10 µL of TSB medium, while treatment 1B consisted of 100 µL of bacterial suspension and 10 µL of phage.

Incubation time with bacteriophages was 1 h, which was informed by the bacteriophage life cycle of 1.5 h previously described [25]. After sufficient incubation for each treatment, the mixture was spread on

Table 1: Code of phage strains isolated from pond water and shrimp samples (August 22, 2021).

Number	1	2	3	4	5	6	7	8	9	10	11	12
Phage code	1F	CHD	T3	S7	S10	V12	L12	T0	T1	BD	V22	V23
Source	BL	TV	TV	TV	TV	BT	TV	TV	T1	BT	BT	BT
Number	13	14	15	16	17	18	19	20	21	22	23	24
Phage code	B2	V8	D6	D7	N3	2B	3B	Vy	R	4B	T2	S10
Source	TV	TV	TV	TV	TV	BL	BL	BT	CM	BL	TV	TV

BL: Bac Lieu province, TV: Tra Vinh province, BT: Ben Tre province, CM: Ca Mau province

TCBS plate. Each experiment was repeated 3 times. The bacteria were allowed to proliferate for 48 h before counting the colonies, calculating the CFU, and observing the change in colony shape in the bacteriophage treatment and the control treatment. Collected data were calculated as mean ± standard deviation using Microsoft Excel (2013) software. Experimental results were analyzed by one-factor ANOVA ($P < 0.05$) using Minitab 16 software.

3. RESULTS AND DISCUSSION

3.1. Isolation of H_2S Gas-Producing Bacteria

The H_2S gas-producing bacteria strain was identified by the morphological characteristics of the cultures pattern (black color) on TCBS medium [Figure 1]. A total of 26 strains were obtained that are likely to be H_2S producing bacteria [Table 2]. In this medium, an iron compound and a sulfur compound were included in the TCBS medium for the growth of bacteria. In addition, H_2S gas is produced if sulfur compounds are reduced by the bacterial strain. This medium is, therefore, intended to identify bacteria that reduce sulfur-containing compounds to sulfides during metabolism. H_2S gas is produced by some bacteria through the reduction of sulfur containing amino acids such as cysteine and methionine, or through the reduction of inorganic sulfur compounds such as thiosulfate, sulfate, or sulfite during protein decomposition or during anaerobic respiration that converts electrons to sulfur instead of oxygen. In both cases, the generated H_2S gas reacts with the ferric citrate compound to form a black precipitate of ferric sulfide. The black color acts as an indicator of the presence of H_2S.

After conducting net culture on TCBS medium, six strains of bacteria capable of producing H_2S were selected, namely, 8D, 10D, 11D, 11X, 12D, and 12V with the selection criteria being black color, colony morphology, and smell of H_2S gas. These six isolates were used for further experiments [Figure 2]. Besides, these bacteria were cultured in liquid in the Postgate B specialized medium to confirm their H_2S gas-producing abilities. Because the composition of this medium includes yeast extract, which provides amino acids and long-chain peptides for the growth of microorganisms, NaCl maintains the balance osmotic, $MgSO_4$, and $FeSO_4$ provide essential ions, they have simple nutritional requirements and grow well in the medium with stable carbon/energy sources [21]. H_2S producing bacteria oxidize sodium lactate and reduce sulfate to sulfide, producing H_2S. At that time, H_2S will react with metal ions in water such as Fe, Mg, or solid to create metal sulfate compounds, and this compound is insoluble in water. Metallic compounds with a sulfide group, such as iron (II) sulfide, are usually black or brown in color, similar to the color of mud. Iron (II) sulfide occurs commonly in nature as iron-sulfur protein. Below is a comparison image of H_2S producing bacteria grown in liquid in the iron-free TSB medium [Figure 2a] and the iron-containing Postgate B medium [Figure 2b].

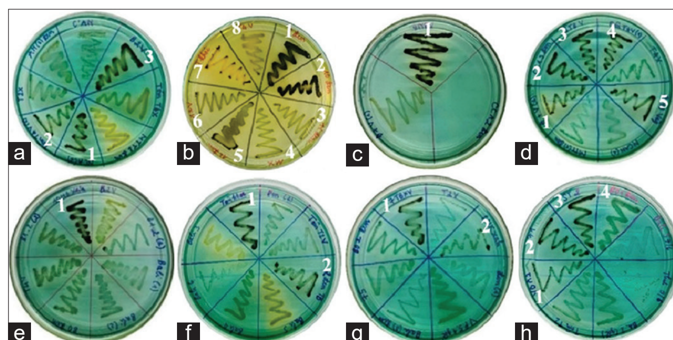

Figure 1: The H_2S gas-producing bacteria pattern is black when cultured on TCBS medium. (a) 1: NCA(2), 2: TT4V(2),3: B2V; (b) 1: 8D, 2: 10D, 3: 11D, 4: 11X, 5: 12D, 6: 12V, 7: TK(1), 8: T4V; (c) 1: 4NBV; (d) 1: DV(2), 2: CS13D, 3: 3TBV, 4: TT4V(1), 5: 9NBV; (e) 1: 10NBX; (f) 1: TK(2), 2: 2DTB; (g) 1: 7TBXV, 2: 8NKX; (h) 1: VBA2, 2: 71, 3: ST2, 4: B01D).

3.2. Morphological, Physiological, and Biochemical Characteristics of Suspected *Desulfovibrio* spp.

Based on visual observations, including color, shape, and size of colonies when grow on TCBS media, the bacterial isolates were inferred to be *Desulfovibrio* spp. Of note, all the selected six bacterial isolates had similar macroscopic characteristics, with colony sizes ranging from 1–3 mm in diameter [Table 3]. Morphological observations of bacterial colony show that all bacterial colony is circular, black color, and the size of the diameter of bacterial colonies range from 1–3 mm. These characteristics are typical *Desulfovibrio* colony.

Further, biochemical characteristics of the isolates showed that they were motile, oxidase and catalase negative, and Gram-negative and could produce H_2S gas [Table 3]. The test on SIM medium [Figure 3] showed that the isolated bacterial strains produced H_2S gas. There is evidence of a black precipitate along the strain in the medium, because the SIM medium contains colored ammonium sulfate and sodium thiosulfate. Therefore, the bacteria are positive when black precipitate emerges in the medium, FeS. In contrast, bacteria that do not produce H_2S will give a negative result on SIM medium, and no black color is present.

3.3. PCR Amplification of the 16S rRNA Gene and Sequencing

The gel electrophoresis analysis showed that the six suspected H_2S gas-producing bacteria had band sizes of 1500 bp [Figure 4]. According to (De Lillo *et al.* 2006), if 16S rRNA gene of bacteria has been correctly amplified, then the length of the PCR product would be around ± 1500 bp [23]. The sequence analysis from the database

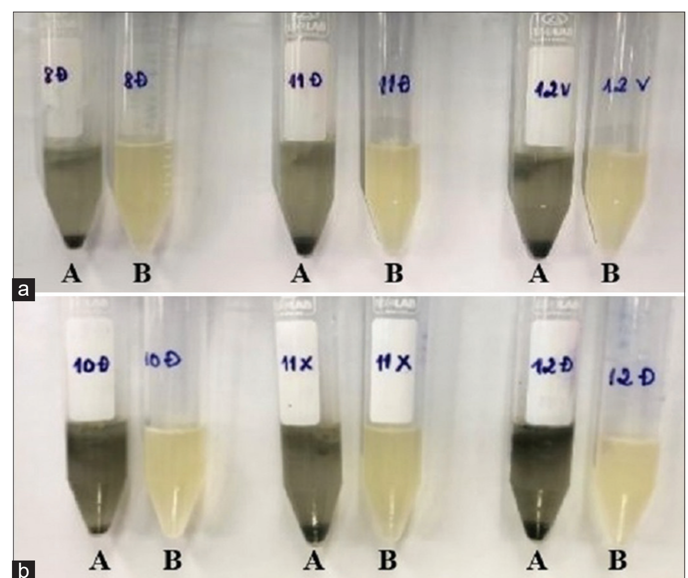

Figure 2: The 8D, 11D, 12V, 10D, 11X, 12D strains are liquid cultured. (a) Samples black precipitate insoluble in Postgate B medium, (b) Samples no precipitation occurred in TSB medium.

in GenBank shows that the six strains isolates were identified to have similarities 91–99% to the species *Desulfovibrio* spp. Hagstrom *et al.* (2000) stated that isolates with a 16S rRNA sequence similarity >97% could represent the same species [26]. According to a study by author Clark *et al.*, (2007) showed that *Desulfovibrio* spp. is a Gram-negative bacterium, capable of motility using flagella, producing H_2S gas, negative for catalase, and oxidase [27]. Therefore, it can be inferred that the isolated H_2S gas-producing bacteria are strains of *Desulfovibrio* spp.

3.4. Isolation and *In Vitro* Evaluation of Bacterial Hosts Susceptible to Bacteriophage Infection

3.4.1. Diffusion on agar surface (Agar spot test)

To evaluate the potential of bacteriophages to infect the bacterial isolates, we selected 11X bacterial strain and performed agar spot tests using all the phages in TCBS medium. Results showed that ten phages were capable of infecting 11X [Figure 5a]. At the respective drop sites, faint spots appeared after 24 h [Figure 5b], which could be explained by the initial interaction of the phage and bacteria. However, after 48 h, the plaques were more transparent at the drop site and the signature black residues of *Desulfovibrio* spp. changed from black yellow to black green. This can be explained, because the bacteria live in anaerobic conditions, so when exposed to oxygen,

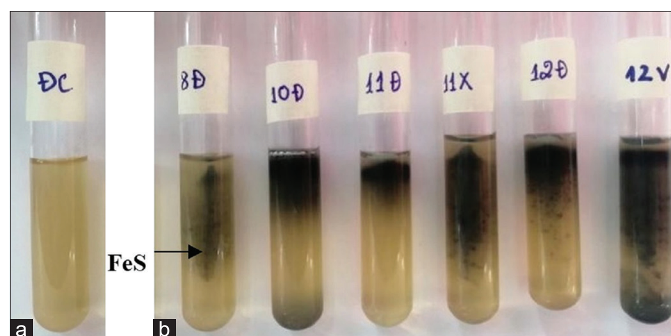

Figure 3: The bacteria produce H_2S gas in SIM medium. (a) *E. coli* (control) does not produce H_2S gas. (b) The results showed that this bacterium was positive for H_2S.

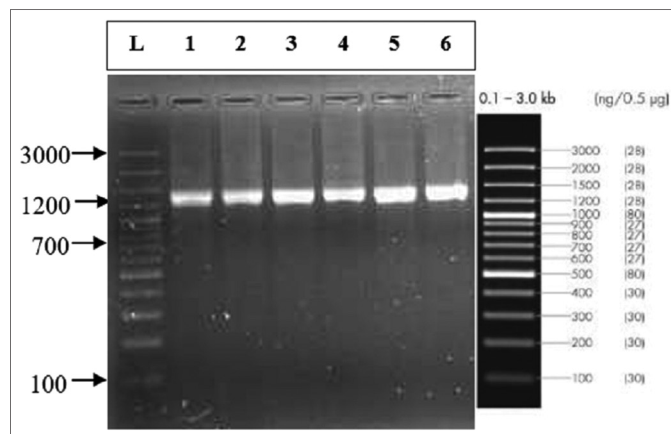

Figure 4: 16S rRNA gene amplification product (*L: Ladder; 1: 8D; 2: 10D; 3: 11D; 4: 11X; 5: 12D; 6: 12V*).

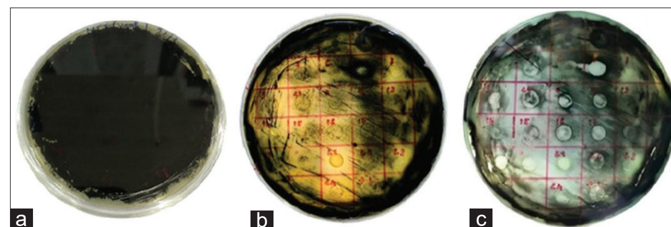

Figure 5: Plaque appearance at the drop site. (a) 11X strain spread on TCBS medium. (b) Phage drop site after 24 h. (c) Phage drop site after 48 h and is exposed to oxygen for 5 min.

Table 2: Presumptive H_2S gas-producing bacteria.

Number	Bacteria Strain	Number	Bacteria Strain
1	NCA (2)	14	CS13D
2	TT4V (2)	15	3TBV
3	B2V	16	TT4V (1)
4	8D	17	9NBV
5	10D	18	10NBX
6	TK (1)	19	TK (2)
7	12V	20	2DTB
8	12D	21	7TBXV
9	11D	22	8NKX
10	11X	23	VBA2
11	T4V	24	71
12	4NBV	25	ST2
13	ĐV (2)	26	B01D

Table 3: Morphological, physiological, and biochemical characteristics of the suspected *Desulfovibrio* spp. isolates in samples of black mud, shrimp pond found in the Mekong Delta.

Bacteria Biochemical properties	8D	10D	11D	11X	12D	12V
Cell shape	Comma	Comma	Comma	Comma	Comma	Comma
Size	1.5→2	1→2	2→3	1.5→2	2→3	1→1.5
Colony	Black	Black	Black	Black	Black	Black
Gram	Negative	Negative	Negative	Negative	Negative	Negative
Motility	+	+	+	+	+	+
Catalase	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
H_2S gas generation	+	+	+	+	+	+

Positive (+), negative (-)

Table 4: Host strain of ten phages on six of *Desulfovibrio* spp.

Bacteria code	Phage code										Total number of phages infecting bacteria
	ϕ1B	ϕCHD	ϕT3	ϕS7	ϕS10	ϕV12	ϕL12	ϕT0	ϕT1	ϕBD	
8D	-	-	-	-	-	-	+	+	-	-	2
10D	-	-	+	+	-	-	+	+	+	+	6
11D	-	-	-	-	-	-	+	+	-	-	2
11X	+	+	-	+	+	+	+	+	-	+	8
12D	-	-	-	-	-	-	+	+	-	-	2
12V	-	-	-	-	-	-	+	+	-	-	2
Bacteria	1	1	1	2	1	1	6	6	1	2	

The sign (+) indicates a bacteriophage strain capable of infecting bacteria, the sign (–) indicates a bacteriophage strain that cannot infect bacteria.

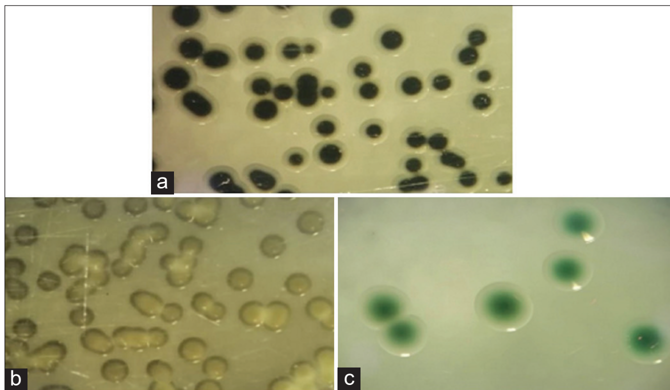

Figure 6: Phages changed the phenotype of bacteria colonies after 1 h of incubation. (a) 11D, (b) 11D + ϕL12, (c) 11D + ϕT0 (The image was taken by the KRUSS MSZ5000 stereo microscope with ×7 focal length).

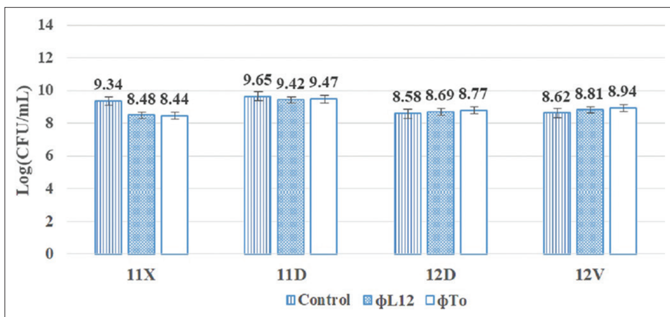

Figure 7: Column chart comparing the number of colonies of 11X, 11D, 12D, and 12V with phage L12 and T0.

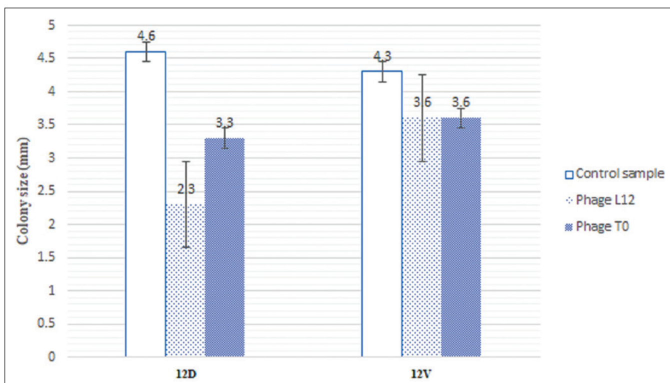

Figure 8: The diagram shows the colony size of 12D and 12V bacteria when the phage infects.

the environment changed to the characteristic color of the TCBS medium, which means that H_2S gas had escaped at this time. We conclude that the phages had significant inhibitory effects on the bacteria [Figure 5c].

The plaque by the drop method were collected, processed, and stored in SM buffer for further experiments.

3.4.2. Host strain of bacteriophages

Bacteriophages infect their hosts by attaching to specific receptors on bacteria. As a result, most phages have narrow host ranges. Using the two-layer agar method, we attempted to determine the host range of each of the ten phages that showed an initial killing effect on 11X. These phages were evaluated for infectivity against the six strains of *Desulfovibrio* spp. The results were recorded based on the visibility of clear spots (plaques) corresponding to the site of the phage drop after 24 h of incubation [Table 4]. The bacterial strains most infected were 10D and 11X, by 6 and 8 phages, respectively. Only two phages each could infect strains 8D, 11D, 12D, and 12V. This could mean that these bacterial strains do not possess the specific receptors needed by non-infecting phages. Furthermore, bacteria possess various defense mechanisms against bacteriophages, including mutation of phage receptors [28]. These results provide an opportunity to show that these bacterial strains might actually not be the same. Furthermore, most of the phage strains were shown to have no overlap with each other due to the different host numbers of each strain. Two phages, ϕL12 and ϕT0, infected all the six bacterial strains; therefore, they were used for further experiments.

3.5. Effects of Phages T0 and L12 on *Desulfovibrio* spp.

3.5.1. Effect of phages on colony morphology Desulfovibrio spp.

Strain 11D was used to evaluate the effects of phages ϕL12 and ϕT0 on bacterial colony appearance and morphology. Results showed that ϕL12 and ϕT0 almost completely inhibited the production of H_2S gas by bacteria, causing the colony to change color from black [Figure 6a] to yellow [Figure 6b] and green [Figure 6c]. However, these results showed that the phages could only partially inhibit the bacteria, as some could still survive in the presence of phages.

3.5.2. Bacteriophages effect on the number of bacteria Desulfovibrio spp.

Here, we show that bacteriophage treatments reduced colony forming units of bacteria. Phages ϕL12 and ϕT0 significantly ($P<0.05$) reduced the number of bacterial strains 11X and 11D, especially 11X, decreased 0.86- and 0.9-fold in the presence of L12 and T0 phages, respectively [Figure 7], as opposed to controls without phage treatment. This indicates that the phages were able to infect a large proportion of the bacteria. However, not all the tested phages had the ability to inhibit

the growth of bacteria, such as phages 12D and 12V. When infecting bacterial cells, phages do not kill bacterial cells but have the ability to stimulate growth and development increasing the vitality of bacteria and causing them to grow and develop more strongly, so the number of colonies formed was higher than that of the treatment without phage.

In addition, the increase in bacterial population in the treatment with bacteriophages was explained by the fact that the phage was in the latent stage, so it did not reduce the number of bacteria [29]. According to Eriksen *et al.* (2018), bacteria tend to have herd immunity by creating overlapping layers of bacteria when there is a bacteriophage attack on the outer surface [30]. That way, the inner layer of bacteria remains protected and thrive. This may explain the increase in bacterial population in the treatment with bacteriophages. In the treatment with the presence of bacteriophage strains L12 and T0 with the host *Desulfovibrio* spp., the number of colonies increased, namely, 12D and 12V.

3.5.3. Effect of bacteriophages on colony size of bacteria Desulfovibrio spp.

The colony size of bacterial strain 12D after infected by phage T0 was reduced by 28.3% compared to the control, especially when treated with phage L12, in which there was a 50% decrease in colony size compared to the control [Figure 8]. Both phages L12 and T0 reduced colony size of 12V by 16.3% [Figure 8]. All of which could explain that the phage had a strong effect on gene of the bacteria, causing the colony size to decrease, especially the L12 phage, which reduced the size of 12D by half compared to the following control 48h spread counting.

4. CONCLUSION

The H_2S gas-producing bacteria isolated from shrimp pond water, black mud, and diseased shrimp samples were Gram-negative, comma-shaped bacteria. The results of 16S rRNA gene sequence analysis showed that this strain belongs to the species *Desulfovibrio* spp. (91–99% similarity). Bacteriophages L12 and T0 have the ability to infect and inhibit bacteria, *Desulfovibrio* spp., besides also changing the color of bacteria 11D. In addition, the phage inhibits the ability to produce H_2S gas by this bacterium. The results of this study show that phage therapy is promising for its application as an antimicrobial agent in the treatment of diseases caused by the bacteria *Desulfovibrio* spp. in aquatics.

5. ACKNOWLEDGMENT

The authors would like to thank the Department of Research Affairs, Institute of Food and Biotechnology, Can Tho University for their valuable support. The study was funded by the Ministry of Education and Training, Vietnam (Project code: B2020-TCT-03).

6. AUTHORS' CONTRIBUTIONS

Truong Thi Bich Van conceived and designed the study. Tran Vo Minh Thu conducted the experiments for bacteria. All authors read and approved the manuscript.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All generated and analyzed data are included in this research paper.

10. PUBLISHER'S NOTE

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How to cite this article:
Van TTB, Thu TVM. Isolation of toxic gas-producing bacteria (*Desulfovibrio* spp.) from shrimp ponds and potential of bacteriophages as biocontrol. J App Biol Biotech. 2023;11(6):59-65.
DOI: 10.7324/JABB.2023.63868