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Research Article
Antagonistic Activity Against Pathogenic *Vibrio* Isolates of Bioflocculant-Producing Bacteria Isolated from Shrimp Ponds

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Abstract

Background and Objectives: Biofloc culture system has been used in aquaculture as an effective technology for water treatment due to many advantages of being biodegradable and environmentally friendly. This study aims to isolate bioflocculant-producing bacteria antagonistic to pathogenic *Vibrio* species from Pacific white shrimp ponds in Thua Thien Hue, Vietnam. **Materials and Methods:** *Vibrio* isolates were isolated by screening on medium with and without antibiotics. The resistance of *Vibrio* to antimicrobial agents was assessed by Minimum Inhibitory Concentration (MIC). Bioflocs formed in shrimp cultures were used to screen bioflocculant-producing bacteria. The identification of bacteria was performed by 16S rRNA sequencing. The flocculating activity was measured by a test with kaolin clay suspension. To evaluate the antagonistic activity against *Vibrio* isolates, an agar well diffusion assay was used. **Results:** The screening results have found that *Vibrio* isolates such as *V. parahaemolyticus* KS02 and *V. alginolyticus* KS08 from shrimp ponds can be resistant to many antibiotics with the highest resistance rate up to 66.49%. Four bioflocculant-producing isolates were obtained and identified as *Bacillus* species. Among them, *Bacillus velezensis* B9 when grown in YPG medium supplemented with 3% sucrose and 0.7% peptone had the highest bioflocculation with an activity of 49.2%. Two isolates of *B. subtilis* B2 and *Bacillus* sp. B6 had quite strong antagonistic activities against vibriosis shown in the zones of inhibition on the assay plates with diameters of about 20 mm. **Conclusion:** The present study has found some *Bacillus* isolates had bioflocculant-producing efficiency and inhibited pathogenic *Vibrio* bacteria. These *Bacillus* isolates will potentially be used as inoculum for bioflocculation to improve shrimp production.

Key words: Antagonism, antibiotic resistance, *Bacillus*, bioflocculant-producing bacteria, *Vibrio*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The Pacific white shrimp (*Litopenaeus vannamei*) is the most important commercial aquatic species in the world, especially in many Asian countries. However, shrimp production has been mainly affected by infectious diseases caused by *Vibrio* bacteria[1]. During recent decades, antibiotics have been widely used in aquaculture to prevent and control vibriosis. Nevertheless, the massive and long-term use of antibiotics has led to the natural emergence of antibiotic-resistant bacteria and the accumulation of chemicals in aquaculture products that impact human health[2,3].

Several studies have reported a rise of antibiotic-resistant *Vibrio* bacteria in aquaculture, most of which show multiple-antibiotic resistance[4,5]. Consequently, antibiotics are ineffective in treating vibriosis[6]. The alternatives to antibiotics are urgently needed for vibriosis control and sustainable development of the aquaculture industry. Numerous bacterial species, including *Lactobacillus*, *Bacillus*, *Pseudomonas* and *Enterococcus*, have been reported as promising biocontrol agents for pathogenic *Vibrio* bacteria in shrimp aquaculture[7].

Some other factors also affect the productivity of shrimp, such as dissolved oxygen, pH, temperature, nitrogen and microbial community. The microbial community in aquaculture plays an important role that provides a healthy environment for shrimp by competing for food with pathogens, maintaining stable levels of nutrients in the water and transformation of harmful organic waste[8,9]. Recently, a new technology known as Biofloc Technology (BFT) has been developed based on these diverse heterotrophic bacterial communities. Bioflocculants have attracted considerable interest due to their advantages of being biodegradable, non-toxic and lack secondary pollution[10,11]. Biofloc forms naturally as aggregates of microorganisms with kinds of extracellular biopolymers including proteins, polysaccharides, nucleic acid produced during their growth and other types of organic matter such as feces and unused feed[10-12].

The BFT was conducted with the main principle of recycling waste nutrients into microbial biomass which can be used *in situ* by cultured animals; thus, providing a nutritious food source and improving the feed utilization efficiency[13,14]. In addition, biofloc systems are composed of some nitrifying bacteria which can convert nitrogen released from unutilized feed and feces. Thus, the BFT is able to maintain or improve water quality and eliminate water exchange during shrimp cultivation[15,16]. The BFT has been gaining global acceptance recently as an effective alternative for aquaculture water treatment without affecting shrimp production[17]. Several bioflocculant-producing microorganisms from aquaculture have been studied, including *Halomonas* sp., *Nitratireductor* sp. and *Pseudoalteromonas* sp.[18] and *Bacillus megaterium* SP1[19]. However, reports on antagonism activity of microorganisms producing bioflocculant against pathogens are meager.

Hence, the present study aimed to isolate and characterize bacteria with bioflocculant-produced ability from shrimp culture systems and evaluate their activity against pathogenic *Vibrio* bacteria.

MATERIALS AND METHODS

Sample collection: Water samples and diseased juvenile Pacific white shrimps (*L. vannamei*) were collected from shrimp ponds located in two districts Quang Dien and Phu Vang, Thua Thien Hue Province, Vietnam within a period of time from June-October, 2020. Six shrimp culture ponds (3 ponds/district) were chosen for sampling. Five water samples (500 mL/sample) were taken from the bottom of each pond and mixed together by shaking for 5 min, an aliquot of the mixture (50 mL) was then collected for screening pathogenic *Vibrio* bacteria. Ten shrimps from each sampling pond were obtained to perform a microbial assessment. All samples were kept in sterile plastic bottles or polyethylene bags and transferred to the laboratory in an icebox, then stored at 4°C. The infected shrimp samples were washed several times with sterile saline and followed by distilled water. These samples were processed immediately to isolate pathogenic *Vibrio* bacteria.

Screening and identification of pathogenic *Vibrio* bacteria: The infected shrimps were aseptically dissected to collect the intestine and hepatopancreas separately, which were then homogenized in sterile saline with a pestle. The ten-fold serial dilutions in saline solution of shrimp homogenates and the mixtures of water samples from ponds were used for screening pathogenic *Vibrio* bacteria. One hundred microliters of each dilution were spread on Thiosulfate Citrate Bile salt Sucrose (TCBS) agar (HiMedia, India) supplemented with one of ten types of antibiotics belonging to 4 different classes (per mL: 30 µg ampicillin, 30 µg cephalexin, 5 µg ciprofloxacin, 10 µg gentamycin, 30 µg kanamycin, 30 µg neomycin, 30 µg tetracycline, 30 µg oxytetracycline hydrochloride, 30 µg doxycycline and 10 µg streptomycin). All cultures were incubated at 37°C overnight until the morphology of the colonies could be distinguished. The single-irregular well-defined colonies were further transferred to the same fresh medium for pure

culture. The isolates were then identified using Gram stain and based on their morphology as described by Cowan and Steel[20].

Determination of MIC of antibiotics: The MIC of antibiotics against *Vibrio* isolates were determined by the method of Ataee *et al.*[21] with slight modifications. Ten mentioned antibiotics were dissolved in appropriate solvents to the concentration of 10 mg mL^{-1}, then diluted with distilled water to different concentrations before use. *In vitro* susceptibility test of *Vibrio* isolates was carried out by the broth microdilution MIC method with 10 different concentrations employed for each antibiotic (5-50 μg mL^{-1} for ciprofloxacin with the increment of 5 μg mL^{-1}, 30-300 μg mL^{-1} with the increment of 30 μg mL^{-1} for others). The 96-well microplates with antibiotic solutions were stored at 4°C before use. Subsequently, 152.5 μL Mueller Hinton broth (Himedia, India) at pH 7.4 was added to each well. Each well row on the microplate corresponding to one type of antibiotic with 10 different dilutions. Negative control containing antibiotic at concentration of 25 μg mL^{-1} for ciprofloxacin or 150 μg mL^{-1} for others, positive control as bacteria without antibiotic.

Vibrio isolates were grown overnight in 5 mL of Mueller Hinton broth at 37°C on a rotating shaker with a speed of 180 rpm. Bacterial cell density was then adjusted to an OD600 of 1 (approx. 1.5×10^8 CFU mL^{-1}) and 12.5 μL of bacterial suspension (approx. 1.88×10^6 CFU) was inoculated into each well. Cultures were continued for another 24 hrs under the same conditions. MIC was calculated for each antibiotic based on MIC_{50} and MIC_{90} values, MIC_{50} and MIC_{90} defined as the minimum concentration of an antibiotic type at which 50% and 90% of the *Vibrio* isolates were inhibited, respectively.

Isolation of bioflocculant-forming bacteria: Bioflocculant forming cultures were performed in six composite circular tanks (500 L $tank^{-1}$) filled with diluted seawater and were stocked with Pacific white shrimp post-larvae as previously described[22]. The Floc Volume (FV) (mL L^{-1}) in the culture tanks was measured every 7 days by using Imhoff cones to observe the forming of biofloc. When FV in culture tanks reached 5-15 mL L^{-1}, 5 mL of biofloc were collected and serially diluted in 10 folds. Isolation of bacteria from biofloc was conducted by spreading 50 μL of each dilution on YPG agar plates and incubated at 30°C for 2 days. Single colonies of different morphology appeared on YPG agar plates purified by repeated culture on the same fresh medium. Bacterial isolates were identified through colonical morphology and 16S rRNA gene sequencing.

Molecular identification of isolates: Five milliliters of overnight culture of isolates in Luria-Bertani (LB) broth was used for total genomic DNA extraction with cetyltrimethylammonium bromide (CTAB) method following protocol of Wilson *et al.*[23] with slight modifications. 16S rRNA sequences were amplified with universal primers: 16S rRNA_27F (5'_AGAGTTTGATCCTGGCTCAG_3') and 16S rRNA_1492R (5'_GGTTACCTTGTTACGACTT_3'). PCR amplification was done in a thermocycler Veriti (ABI) with a program as follows: a genomic denaturation at 95°C for 5 min; 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1.5 min and a final extension at 72°C for 10 min. The PCR reaction mixture consisted of 6 μL of Master Mix (Thermo Fisher Scientific), 10 pmol each primer, 50 ng of total DNA and double distilled water added to a final volume of 12 μL. The amplicons were sequenced by dideoxy chain termination method on the Applied Biosystem 3130 (ABI). Comparison of 16S rRNA sequences was performed using BLAST with database of GenBank (NCBI). The multiple representative sequences were aligned in the ClustalW implementation and a neighbor-joining phylogenetic tree of 16S rRNA genes was constructed by MEGA X software with the bootstrap test of 1,000 data re-samplings.

Determination of flocculating activity: The bioflocculant-producing bacteria were inoculated into 20 mL of YPG broth using 1% of sucrose instead of glucose (YPS) and incubated at 30°C on rotating shaker at 220 rpm for 8 hrs. Then, YPS broth was used to adjust the turbidity of cultures to an OD600 of 0.1 and the cultures were further incubated under the same conditions for 3 days. Finally, the broth was centrifuged at 13,000 rpm for 10 minutes at 4°C to obtain the cell-free supernatants for flocculating activity determination.

The flocculation activity was measured according to Agunbiade *et al.*[24] with some modifications. Kaolin clay was used as test material in assessing the efficiency of bioflocculation. Kaolin suspension was prepared by dissolving 5 g of kaolin clay in 1 L distilled water, then adjusted pH to 7. A volume of 240 mL kaolin suspension was added to 10 mL cell-free supernatant in 500 mL conical flask. The solution was shaken at 230 rpm for 2 min, followed by 80 rpm for 1 min and then 20 rpm for 30 min. Finally, the mixture in the flask was allowed to settle for 30 min. The OD of the clarifying solution was spectrophotometrically measured at 550 nm (Evolution 60S, Thermo Scientific). Control was distilled water instead of kaolin. The flocculation activity (FA, %) was calculated by the following Eq.:

$$FA = \frac{A - B}{A} \times 100$$

where, A and B are the respective absorbance of control and sample at 550 nm.

Effect of carbon and nitrogen source on bioflocculation: Sucrose and peptone used as carbon and nitrogen sources to investigate flocculating efficiency. Bacterial flocculation was produced by adding different concentrations of sucrose (1-7%) to culture and incubated under same conditions as described above. The optimal concentration of sucrose will be then selected for the next investigations with peptone from 0.2-1.2%. The bioflocculant activity of all treatments were evaluated according to modified method of Kurane and Matsuyama[25].

Antagonism assay against *Vibrio*: The antagonism activity against *Vibrio* isolates of bioflocculant-producing bacteria was investigated using agar well diffusion method of Bauer *et al*.[26] with some modifications. The pathogenic bacteria used in this study including 3 *Vibrio* KS02, KS05 and KS08 isolates from infected shrimps and 2 *Vibrio* KS03 and KS06 isolates provided by Laboratory of Enzyme and Protein Technology (Institute of Biotechnology, Hue University).

The *Vibrio* isolates were inoculated in peptone alkaline broth (peptone 20 g, NaCl 20 g, distilled water added to the final volume of 1 L, pH 8.6) and incubated at 37°C for 24 hrs on a rotating shaker at 180 rpm. Hundred microliter of suspension of each isolate was spread onto TCBS agar plates. Then, wells of 10 mm in diameter were punched into the agar using a sterile test tube. Bioflocculant-producing isolates were cultured in YPS broth at 30°C with a shaking speed at 220 rpm overnight. One hundred microliters of cultures were carefully applied to each well and the plates were then incubated at 37°C overnight. The diameter of inhibition zone around each well was measured in millimeters.

Statistics: All treatments were performed with three replications, each treatment was 10 samples. The results are presented as the mean of the repeats±standard error. The data were analyzed using one-way ANOVA, mean comparisons were performed by Duncan's test at a significance level of 0.05.

RESULTS

Antibiotic resistance of bacterial isolates from shrimp culture ponds: Thirty water samples and sixty homogenates of diseased Pacific white shrimp samples from Quang Dien and Phu Vang were spread onto TCBS agar plates supplemented with 10 types of different antibiotics, the plate without antibiotics used as control. The ratio of colony number (CFU mL^{-1}) that appeared on plates with antibiotic to that on plates without antibiotic was determined as the resistance rate of bacterial isolates. The results shown in Table 1 revealed that the strongest resistance of isolates was observed against streptomycin (10 μg mL^{-1}) up to 57.84% for samples from Quang Dien and 66.49% for Phu Vang samples. Whereas, all isolates from Quang Dien and Phu Vang exhibited high sensitivity towards ciprofloxacin (5 μg mL^{-1}) with a low resistance rate of 2.03% and 0.84%, respectively. In addition, the isolates showed moderate sensitivity against other tested antibiotics with the rate ranged from 33.78 to 55.84% (Quang Dien) and from 42.77 to 54.83% (Phu Vang).

Isolation and identification of antibiotic-resistant isolates: Three isolates named KS02, KS05 and KS08 were selected from the screening cultures based on colonial morphology according to Bergey's Manual of Systematic Bacteriology[27]. The colony of isolates KS02 and KS05 showed green and yellow color, respectively. While the KS08 isolate changed color during cultivation from yellow to light green and then turned black (Fig. 1). All of these three isolates exhibited resistance against nine antibiotics tested above except for ciprofloxacin. Gram staining of these isolates indicated that they were Gram-negative, pink-stained and rod-shaped bacteria.

Phylogenetic analysis of 16S rRNA gene sequences of three isolates revealed that they were the closest homology with members of the genus *Vibrio* (Fig. 2a). The 16S rRNA gene of KS02 shared high similarity (100%) to *Vibrio parahaemolyticus* 2012AW-0224. Whereas, 16S rRNA sequence of KS05 showed 99.7% identity with *V. owensii* 20160513VC2W. The 16S rRNA gene from KS08 was 100% identity with those of *V. alginolyticus* FDAARGOS 114. Three isolates could be hereafter referred to as *V. parahaemolyticus* KS02, *V. owensii* KS05 and *V. alginolyticus* KS08. The 16S rRNA nucleotide sequences of isolates KS02, KS05 and KS08 were deposited in the GenBank (NCBI) with temporary accession numbers MZ148455, MZ148456 and MZ148457, respectively.

Antimicrobial susceptibility of *Vibrio* isolates: The minimum inhibitory concentrations of the 10 common antibiotics against the three *Vibrio* isolates in this study were shown in Table 2. Overall, these isolates showed differences in sensitivity to the tested antibiotics. Two antibiotics of the β-lactam class showed the lowest inhibition of bacterial growth. Both KS02 and KS08 isolates were strongly

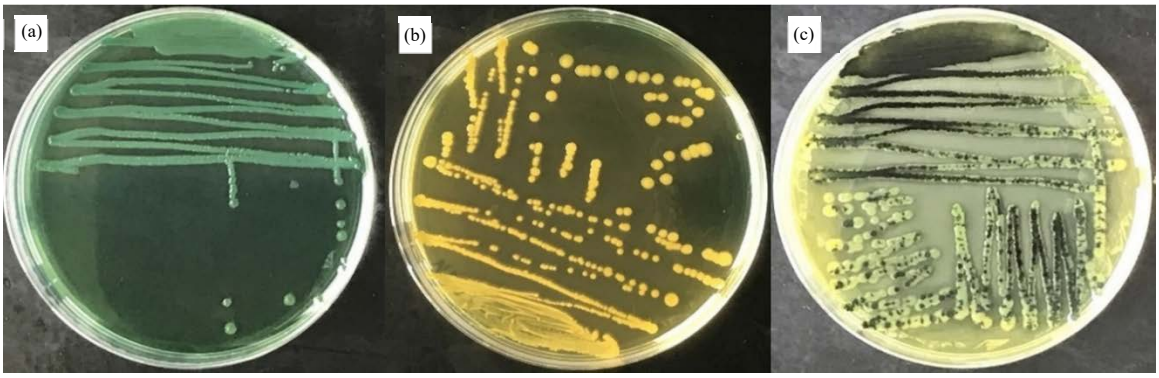

Fig. 1(a-c): Colonial morphology of antibiotic-resistant isolates, (a) KS02, (b) KS05 and (c) KS08 on TCBS agar plate

Table 1: Antibiotic resistance rate of *Vibrio* isolates (%)

Class of antibiotic	Antibiotics	Quang Dien	Phu Vang
β-lactam	Ampicillin (30 μg mL^{-1})	41.53±2.51	50.45±2.66
	Cephalexin (30 μg mL^{-1})	54.87±3.44	45.29±3.01
Aminoglycoside	Gentamycin (10 μg mL^{-1})	55.84±3.02	46.88±2.18
	Kanamycin (30 μg mL^{-1})	33.78±2.01	42.77±2.24
	Neomycin (30 μg mL^{-1})	35.22±2.33	54.08±3.67
	Streptomycin (10 μg mL^{-1})	57.84±3.05	66.49±3.19
Tetracycline	Tetracycline (30 μg mL^{-1})	53.25±3.16	44.23±3.22
	Oxytetracycline hydrochloride (30 μg mL^{-1})	39.27±1.69	49.10±4.04
	Doxycycline (30 μg mL^{-1})	36.68±1.22	54.83±3.98
Quinolone	Ciprofloxacin (5 μg mL^{-1})	2.03±0.18	0.84±0.09

Each treatment was repeated three times and data were expressed as Mean±standard error

Table 2: Minimum inhibitory concentration of antibiotics for *Vibrio* isolates

	MIC (μg mL^{-1})/inhibition (%)					
	Vibrio parahaemolyticus KS02		*Vibrio owensii* KS05		*Vibrio alginolyticus* KS08	
Antibiotics	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
β-lactam						
Ampicillin	-	-	150/55.69±2.87	-	-	-
Cephalexin	-	-	-	-	-	-
Aminoglycoside						
Gentamycin	180/53.00±2.98	-	60/68.84±4.41	-	120/77.33±3.33	-
Kanamycin	-	30/92.51±4.45	60/88.53±4.27	210/93.76±5.05	30/61.87±3.56	-
Neomycin	-	-	30/89.18±5.11	-	90/53.74±2.14	-
Streptomycin	90/60.08±3.17	-	-	-	-	-
Tetracycline						
Tetracycline	60/50.59±4.23	-	30/78.45±4.03	-	90/51.66±3.23	180/91.70±4.28
Oxytetracycline hydrochloride	-	-	-	-	-	-
Doxycycline	30/70.76±3.35	-	60/88.26±5.12	-	30/84.25±4.65	-
Quinolone						
Ciprofloxacin	-	5/97.88±3.96	-	5/93.54±4.27	-	5/97.82±3.01

Each treatment was repeated three times and data were expressed as Mean±standard error

resistant to cephalexin and ampicillin. Meanwhile, over 55% of KS05 isolate was inhibited by 150 μg mL^{-1} of ampicillin at MIC_{50}. Significant differences were found in the susceptibility of isolates to aminoglycoside antibiotics. In particular, the MIC_{90} value of kanamycin was very different between KS02 (30 g mL^{-1}) and KS05 (210 g mL^{-1}). KS08 was also sensitive to kanamycin concentrations of 30 g mL^{-1} in MIC_{50}. Of the tested kanamycin concentrations, no MIC_{50} and MIC_{90} values were found for KS02 and KS08, respectively.

Two KS02 and KS08 isolates exposed to gentamycin showed higher MIC_{50} values (180 and 120 μg mL^{-1}, respectively) compared to those of KS05 isolate (60 μg mL^{-1}). For tetracycline class, all three *Vibrio* isolates were resistant even to highest concentration (300 μg mL^{-1}) of

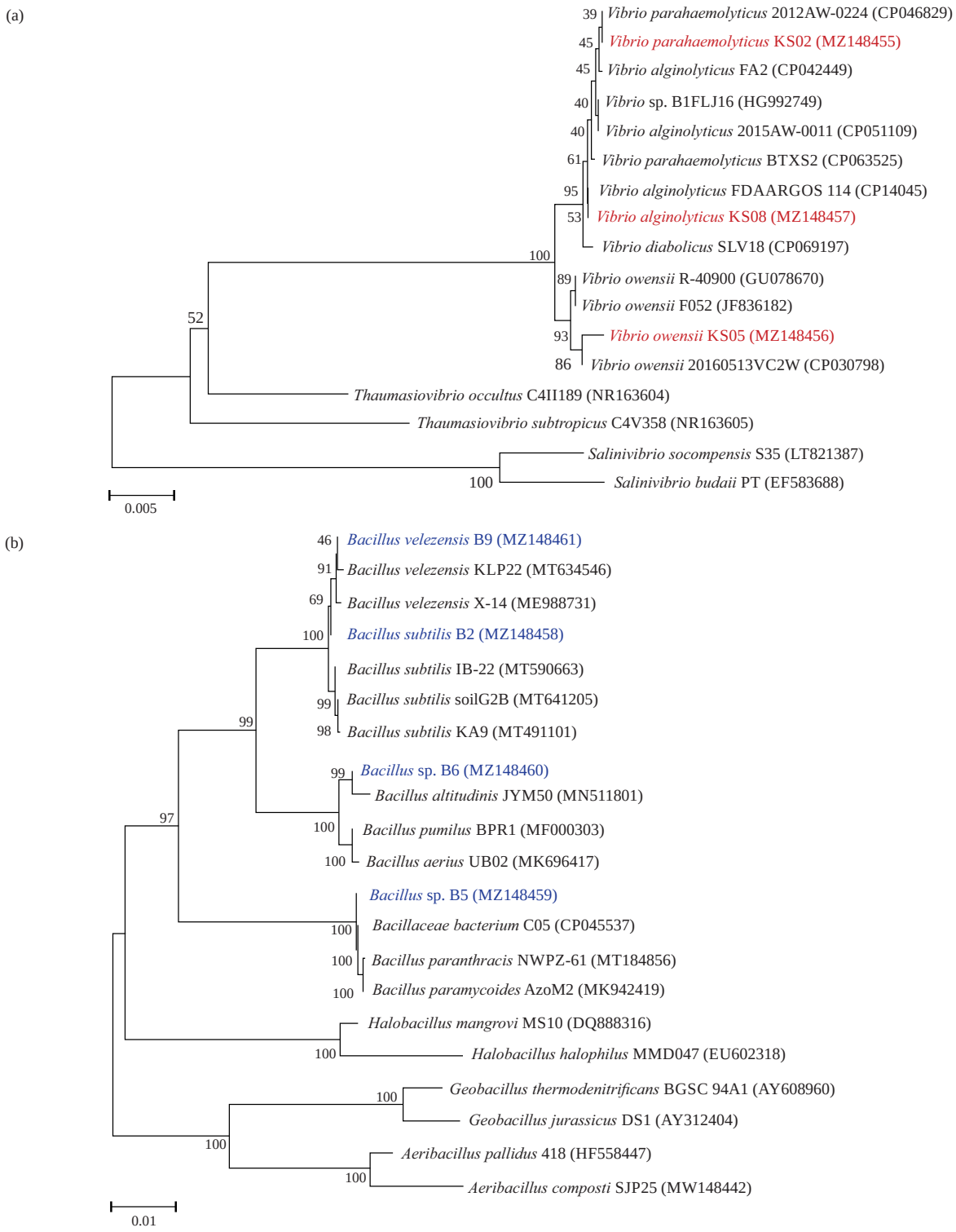

Fig. 2(a-b): Phylogenetic tree of 16S rRNA gene sequences of the *Vibrio* and *Bacillus* isolates with species in taxa constructed by MEGA X with the neighbor-joining algorithm, (a) Phylogenetic tree between 3 pathogenic *Vibrio* isolates (KS02, KS05, and KS08) and other *Vibrio* species and (b) Phylogenetic tree between 4 bioflocculant-producing *Bacillus* isolates (B2, B5, B6 and B9) and other *Bacillus* species

a: Numbers at nodes indicated bootstrap values based on 1,000 resampled data sets, Bar: 0.005. b: Numbers at nodes indicated bootstrap values based on 1,000 resampled data sets, Bar: 0.01

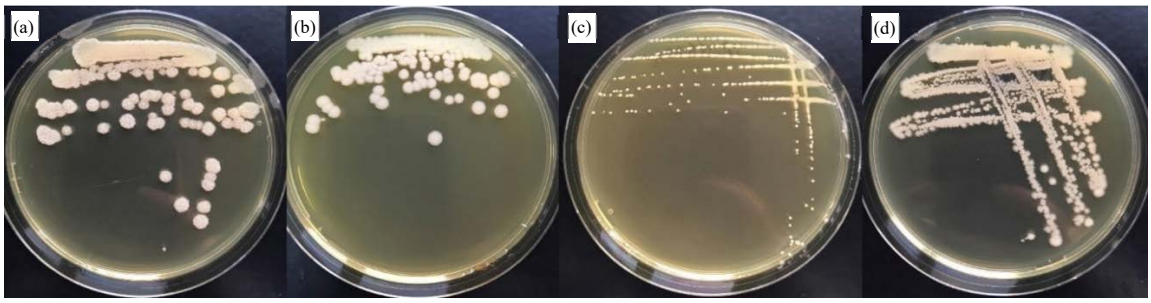

Fig. 3(a-d): Colonial morphology of bioflocculant-producing isolates (a) B2, (b) B5, (c) B6 and (d) B9 on YPG agar plate

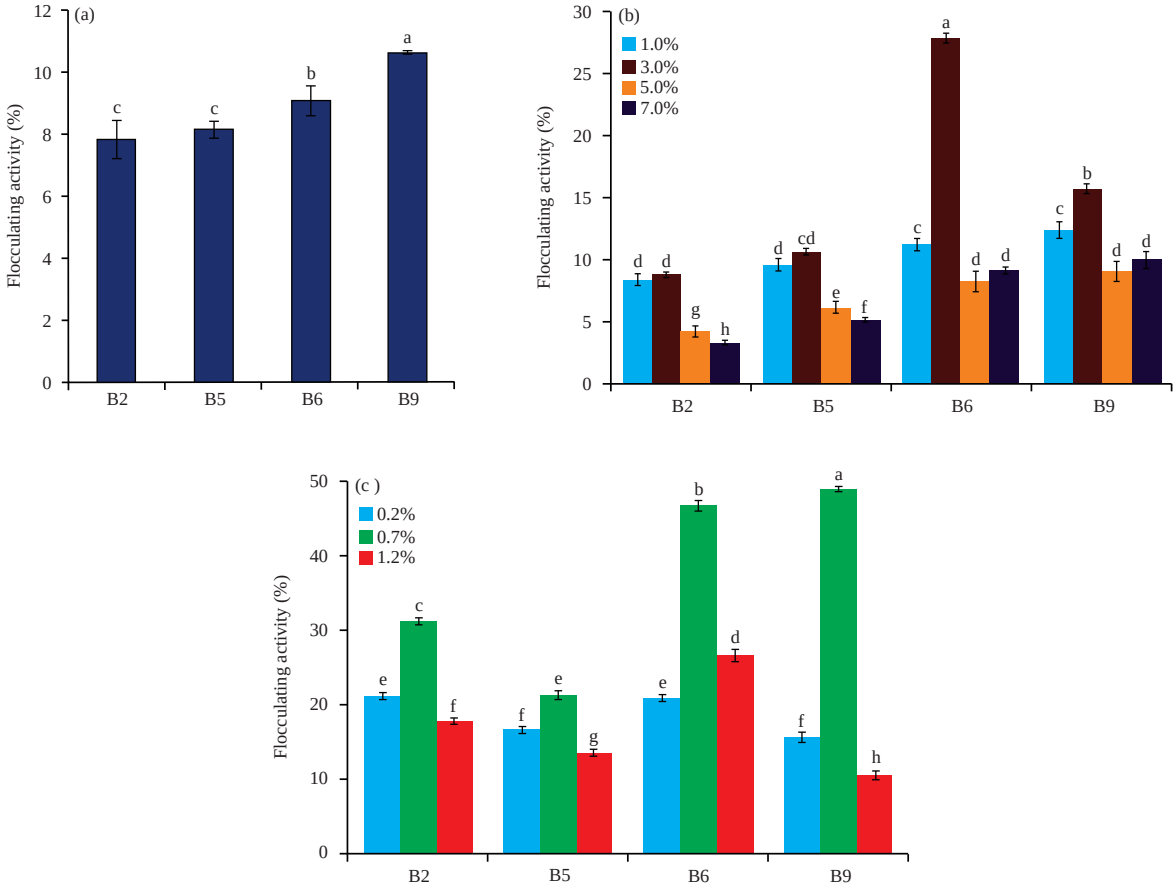

Fig. 4(a-c): Bioflocculation of *Bacillus* isolates, (a) Bioflocculating activity on YPG broth, (b) Bioflocculating activity on YPG broth supplemented with various concentrations of sucrose and (c) Bioflocculating activity on YPG medium supplemented with 2% sucrose and various concentrations of peptone
Different letters on a chart express statistically significant differences with $p<0.05$ (Duncan's test)

oxytetracycline hydrochloride. KS08 isolate was more susceptible to tetracycline than other two isolates with MIC_{50} of 90 μg mL^{-1} and MIC_{90} of 180 μg mL^{-1}. In addition, three *Vibrio* isolates were found to be sensitive to ciprofloxacin (MIC_{90} value 5 μg mL^{-1}) with the highest rate from 93.54-97.88%. In general, KS02 and KS08 isolates exhibited resistance to many antibiotics such as ampicillin, cephalexin, oxytetracycline hydrochloride, neomycin or streptomycin.

Screening for flocculant-producing bacteria: Ten bacterial isolates named B1-B10 were obtained on YPG agar medium from floc samples in Pacific white shrimp culture tanks. Morphologically, all colonies appeared as white to creamy in color. The colonies formed mostly circularly shape, smooth, raised with entire margin, others observed to be irregular, flat, rough and undulate. Four isolates (Fig. 3a-d) that showed the ability to produce flocculant were selected for further identification through molecular identification and

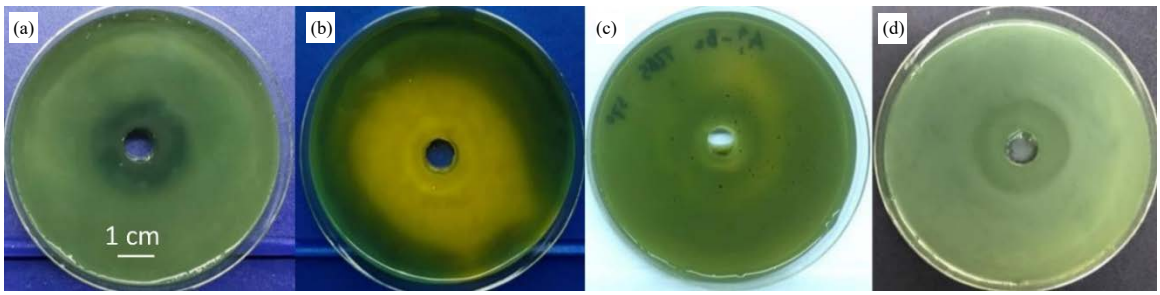

Fig. 5(a-d): Inhibitory effects of *Bacillus* isolates on the growth of pathogenic *Vibrio* isolates, (a) *B. subtilis* B2 vs. *V. parahaemolyticus* KS02, (b) *B. subtilis* B2 vs. *V. owenii* KS05, (c) *B. subtilis* B2 vs. *Vibrio* sp. KS03 and (d) *Bacillus* sp. B6 vs. *Vibrio* sp. KS06

phylogenetic analysis. The homology searches by BLAST of 16S rRNA nucleotide sequences revealed that all 4 isolates belonged to the genus *Bacillus*. The phylogenetic tree of these isolates was constructed as shown in Fig. 2b. A similarity comparison of 16S rRNA gene sequences showed that the gene of B2 isolate was 100% homologous with those of *B. subtilis* IB-22. The genes of two isolates B5 and B6 also were 99.9-100% similar to those of various *Bacillus* species. Meanwhile, the gene of the B9 isolate was identical with those of *B. velezensis* X-14 and *B. velezensis* KLP22. The 16S rRNA nucleotide sequences of isolates B2, B5, B6 and B9 were deposited in the GenBank (NCBI) with temporary accession numbers MZ148458, MZ148459, MZ148460 and MZ148461.

Flocculation activity: The flocculation of 4 isolates of *Bacillus* (B2, B5, B6 and B9) occurred after 3 days of incubation on YPG broth at a shaking speed of 220 rpm. The highest flocculation activity shown by *B. velezensis* B9 was 10.54%, following by *Bacillus* sp. B6 with an FA of 9.05%. There was no significant difference in flocculation activity between isolates B2 and B5 (Fig. 4a). The constituent of the culture medium plays an important role in enhancing bioflocculation. Thus, the assessment of flocculating activity when using various concentrations of sucrose and peptone was investigated. The results shown in Fig. 4b revealed that 3% sucrose had a significant effect on the bioflocculation of *Bacillus* isolates. Among them, *Bacillus* sp. B6 exhibited the highest activity with an FA of 28%. A decrease in bioflocculation of all isolates was observed in the range of sucrose 5-7%. As shown in Fig. 4c, it was found that adding 0.7% peptone to the medium yielded the highest bioflocculant activity with FA of 46.9% in B6 and 49.2% in B9. Flocculation activities of isolates B5 and B6 were decreased when peptone concentration raised from 1.2-1.7%, while isolates B2 and B9 showed opposite efficiency. Overall, the bioflocculation activity of all *Bacillus* isolates was significantly improved by adding 3% sucrose and 0.7% peptone to YPG broth in particular, the FA of B9 increased nearly 5 times.

Antagonistic activity against *Vibrio* of bioflocculant-producing bacteria: Four *Bacillus* isolates were screened for their antagonism to 5 pathogenic *Vibrio* isolates obtained from shrimp culture ponds by agar well diffusion assays. Among them, two isolates *B. subtilis* B2 and *Bacillus* sp. B6 showed high inhibitory ability against *V. parahaemolyticus* KS02, *V. owensii* KS05, *Vibrio* sp. KS03 and *Vibrio* sp. KS06. As shown in Fig. 5(a-d), the clear zones (*D-d*) of approximately 23, 22 and 18 mm in diameter were produced by *B. subtilis* B2 in assays of isolates *Vibrio* KS02, KS05 and KS03, respectively. Where *D* is the diameter of the inhibitory zone and *d* is the diameter of the pre-punched hole. Meanwhile, *Bacillus* sp. B6 exhibited an antagonistic effect with only *Vibrio* sp. KS06 (24 mm).

DISCUSSION

The present study obtained four bioflocculant-producing *Bacillus* isolates. Among them, *B. velezensis* B9 had the highest bioflocculation with an activity of 49.2%. Two isolates of *B. subtilis* B2 and *Bacillus* sp. B6 had quite strong antagonistic activities against vibriosis shown in the zones of inhibition on the assay plates with diameters of about 20 mm. *Bacillus* bacteria have been widely known as probiotics used in shrimp aquaculture because of their effects on shrimp growth, immune responses and resistance to pathogenic *Vibrio* bacteria[28-30]. On the other hand, several studies indicated that *Bacillus* species were able to produce bioflocculant that used for water quality maintenance and wastewater treatment in aquaculture[18,31-33]. However, little is known about the potential probiotic activities of bioflocculant-producing bacteria in shrimp cultures[34].

The *Vibrio* bacteria obtained from shrimp ponds in Thua Thien Hue showed resistance to all tested antibiotics with the highest rate for streptomycin (Table 1). From colonies that appeared on TCBS plates added with antibiotics, three *Vibrio* isolates were identified as *V. parahaemolyticus*, *V. owenii* and *V. alginolyticus*. They were strongly resistant to 2-4 antibiotics at a high concentration of 300 μg mL^{-1} (Table 2). High rate of multiple antibiotic resistance of *Vibrio* bacteria derived from shrimp ponds was also recorded in previous studies. Chikwendu *et al.*[35] revealed 81.3-97.8% of *Vibrio* strains resistant to 6 tested antibiotics. Among 119 *Vibrio* strains isolated from brackish water and seawater in Kerala (India), 55.5% strains showed resistance to 4-10 antibiotics and 14.14% strains are resistant to more than 10 antibiotics[36]. Adeyemi *et al.*[37] indicated that 18% of 44 *Vibrio* strains obtained from aquaculture water in Lagos (Nigeria) showed resistant abilities to 10 antibiotics. Theoretically, *Vibrio* species are considered highly sensitive to antibiotics and the most common way to resolve the vibriosis problem in aquaculture is using feed plus antibiotics[38]. However, the recent emergence of antibiotic-resistant bacteria was reported to be relevant with the excessive use of antimicrobials in either clinical use, animal therapy, or aquaculture systems[3,6].

In this work, screening of biofloc-producing bacteria was performed from floc samples in shrimp cultures, followed by conducting the antagonism assay in order to obtain bacteria species used as promising probiotics and also enhance water quality. From seawater, 4 isolates produced bioflocculant after 3 days of culture were identified as species belonging to genus *Bacillus*. In previous reports, *Bacillus* species considered one of the most common bioflocculant-producing bacteria were found in diverse environments from aquaculture farms, waste water, to crude petroleum oil[39,40]. For instance, *B. cereus* and *B. pumilus* strains isolated from seawater[31], *B. licheniformis* and *B. thuringiensis* strains obtained from intensive shrimp culture system[34] were identified as bioflocculant-producing bacteria.

However, the flocculating activity of these 4 *Bacillus* isolates was quite low (10.54%). As was known, many factors can affect flocculation activity such as carbon source, nitrogen source, initial pH, temperature and cation[39]. This study preliminarily optimized the flocculation activity by using different concentrations of sucrose and peptone. As shown in Fig. 4b and c, the flocculating activity of all *Bacillus* isolates was improved significantly with 5-fold higher FA. Carbon and nitrogen sources have been considered the crucial factor affect bioflocculant production, but they might vary in different microorganisms. It was found that *Virgibacillus* sp. XQ-1 used glucose and peptone as favorite carbon and nitrogen source which yielded the highest flocculating activity of 70.4%[11]. In the case of *Aspergillus parasiticus*, corn starch and peptone were found to be the best carbon and nitrogen source[41].

Antagonism among bacteria is considered an important factor for the evaluation of aquaculture probiotics[42]. Interestingly, two of our bioflocculant-producing bacteria, *B. subtilis* B2 and *Bacillus* sp. B6, showed inhibitory ability against four pathogenic *Vibrio* isolates from shrimp ponds with inhibition zones approximately 20 mm in diameter. Similar results have shown that *Bacillus* species had activity against pathogen bacteria in aquaculture. The culture of *Bacillus* sp. B16 and *Bacillus* sp. J7 displayed antimicrobial activity and formed inhibition zones of 15 and 22 mm in diameter, respectively[43]. A study by Peng *et al.*[44] revealed that *B. lichenformis* G showed antagonistic activity to 3 *Vibrio* strains with the inhibition zones ranged from 16-20 mm. The inhibitory effects of *Bacillus* are explained by the ability to produce antimicrobial substances such as bacteriocins[45].

CONCLUSION

The present study gave evidence that *Bacillus* isolates were potential sources of bioflocculant and able to inhibit the pathogen *Vibrio* bacteria which are resistant to multiple antibiotics. This result might provide promising candidates for bioflocculant-producer and probiotics applied in aquaculture. These results may also facilitate studies on applications of bioflocculants to improve the shrimp culture in ThuaThien Hue.

SIGNIFICANCE STATEMENT

The present study successfully isolated four bioflocculant-producing *Bacillus* isolates from Pacific white shrimp (*Litopenaeus vannamei*) culture ponds in Thua Thien Hue, Vietnam and demonstrated their antagonistic activity against pathogenic *Vibrio* bacteria. These results suggest that the *Bacillus* isolates would be potentially used as bioflocculant-producers and probiotics for the sustainable development of aquaculture.

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