

CHARACTERIZATION AND ANTIBACTERIAL ABILITY OF THE *Bacillus* sp. STRAIN VNUA16 ISOLATED FROM FISH POND SLUDGE IN HAI PHONG

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ABSTRACT

Aquaculture industry, which plays an important role in Vietnam economy, has been oriented for effective and sustainable development. However, aquaculture is facing with the appearance of more serious water pollution and aquatic animal diseases. *Bacillus* species are capable of synthesizing antibacterial substances and degrading organic compounds, contributing to the control of aquatic diseases and improve water quality. Therefore, the study was carried out to determine biological characteristics and antibacterial ability of the VNUA16 strain isolated from fish pond sludge in Hai Phong province. The study of morphological, biochemical characteristics and 16S rRNA gene sequence showed that the strain VNUA16 belongs to the genus *Bacillus*. The antagonistic activity of this strain was evaluated by the diffusion plate method. According to the results, the *Bacillus* sp. strain VNUA16 was able to antagonize *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa* P2.1 and *Staphylococcus aureus* SA12, recording inhibition zones of 6.43; 8.53; 9.57 mm, respectively. At pH6 and 30°C, this strain exhibited the strongest antibacterial activity against *Pseudomonas aeruginosa* P2.1. The strain *Bacillus* sp. VNUA16 has a promising potential to be used as a biological agent for control of aquatic animal diseases.

Keywords: Antibacterial activity, *Bacillus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio parahaemolyticus*.

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1. INTRODUCTION

Aquaculture has been practiced in Vietnam for a long time, which plays an important role in developing the economy of the country [1]. According to General Statistics Office, in the first five months of 2023, total fisheries and aquaculture production reached a record about 3.42 million tonnes, increased by 1.4% compared to the same period last year, comprising around 2.59 million tonnes of fish, 0.386 million tonnes of shrimp and 0.526 million tonnes of other aquatic animals [2]. Three species dominate the aquaculture sector, including the freshwater *Pangasius* (*Pangasianodon hypophthalmus*), tilapia and shrimp [3].

Aquaculture production has been significantly affected by bacterial diseases such as motile aeromonad septicemia, vibriosis, columnaris, edwardsielliosis and furunculosis, causing heavy mortalities and high economic losses [4]. Bacteria responsible for causing disease in aquatic animals belong to the genera *Vibrio*, *Aeromonas*, *Flavobacterium*, *Pseudomonas*, *Edwardsiella*, *Streptococcus* as well as *Staphylococcus* [4, 5, 6, 7, 8]. *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* are known as the most common opportunistic pathogens causing ulcerous disease [8]. While *Staphylococcus* species, namely *S. epidermidis*, *S. aureus*, *S. hominis*, *S. cohnii* and *S. warneri* generally induce hemorrhagic septicemia in fish [5]. *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. vulnificus* and *V. harveyi* are the major pathogenic species of *Vibrio* affecting fishes and shrimps in aquaculture [4].

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There are various methods to prevent diseases or reduce the losses caused by bacterial pathogens, including good farm management and the use of antibiotics. However, treatment by extensive feeding antibiotics may cause the rise of bacterial antibiotic resistance and antibiotic residues in cultured aquatic animals [9]. Therefore, the use of probiotics is an effectively alternative way to control bacterial diseases and improve farm production yields in aquaculture [9]. *Bacillus* species have been applied as potential probiotics in the aquaculture sector because of their rapid growth, ability to produce a wide range of extracellular enzymes as well as broad spectrum antimicrobial compounds [9, 10]. It was demonstrated that *Bacillus* spp., *Bacillus subtilis* BT23 had inhibitory effects against diseases caused by *Vibrio* spp. (*V. harveyi*, *V. alginolyticus*, *V. parahaemolyticus*) in tiger shrimp *Penaeus monodon* [11, 12]. Moreover, *B. subtilis* with a remarkable antimicrobial activity against *Edwardsiella ictaluri* was found in the channel catfish intestine [13]. *Bacillus licheniformis* isolated from the gastrointestinal tracts of Nile tilapia (*Oreochromis niloticus*) revealed pathogen inhibition and potential probiotics characteristics [14]. Other *Bacillus* species, such as *B. pumilus*, *B. megaterium*, *B. tequilensis*, *B. amyloliquefaciens* also were reported by producing extracellular enzymes and antagonistic ability against bacterial pathogens (*Aeromonas* spp., *Edwardsiella ictaluri*, *Streptococcus iniae*) in goldfish (*Carassius auratus*), Indian major carps (*Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala*), catfish, Nile tilapia and grass carp [13, 15, 16, 17, 18].

The aim of the present study is to determine biological characteristics as well as antimicrobial activity of the bacterial strain *Bacillus* sp. VNUA16 from fish pond sludge in Hai Phong province. The effect of temperature and pH on inhibitory activity of this strain against bacterial pathogen *Pseudomonas aeruginosa* was also conducted.

2. MATERIALS AND METHODS

2.1. Materials

Bacterial strain VNUA16 was isolated from fish pond sludge in Hai Phong province, Kien

Thuy district according to the method described by Duong Nguyen Anh Tam *et al.* (2022) [19]. The strain was purified and preserved on agar slant tubes in a refrigerator and in LB medium containing 20% glycerol at -20°C at the Department of Microbial Biotechnology, Faculty of Biotechnology, Vietnam National University of Agriculture for further examination.

Pathogenic strains, including *Pseudomonas aeruginosa* P2.1, *Vibrio parahaemolyticus* and *Staphylococcus aureus* SA12. These strains were provided by the Department of Microbial Biotechnology, Faculty of Biotechnology, Vietnam National University of Agriculture.

2.2. Methods

2.2.1. Biological characterization of the VNUA16 strain

The VNUA16 strain was streaked in Luria-Bertani (LB) agar medium, containing the following components (g/l): Peptone 10, yeast extract 5, NaCl 10, agar 20, pH7. After 24 hours of incubation at 30°C, the colony morphological characteristics of the strain such as size, form, elevation, margin, color, surface and shape were determined. This strain was also characterized by different biochemical tests, including the Gram staining, catalase, motility, citrate, indole, nitrate reduction, carbohydrate utilization, starch hydrolysis, cellulose hydrolysis, Methyl Red (MR) and Voges-Proskauer (VP) tests according to the method described by Barrow and Feltham (2004) [20].

The effect of temperature on the growth of the strain VNUA16 was also conducted. The strain was cultured at different temperature of 30, 35 and 40°C in a liquid LB medium at 160 rpm. After 48 hours of incubation, the growth of this strain was determined by measuring optical density (OD) at 620 nm wavelength. The experiment was tested three times and mean values were calculated [21].

2.2.2. Molecular identification based on 16S rRNA sequence analysis

Bacterial genomic DNA was extracted using a DNA isolation kit (iNtRON Biotechnology, Korea) described by the manufacturer's instruction and

stored at -20°C for further analysis. The 16S rRNA gene of the bacterial strain was amplified by PCR using the specific primers sequences, including 27F:5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R:5'-TACGGYTACCTTGTTACGACTT-3'. The program for PCR amplification was carried out in a Thermal Cycler (BIO-RAD) using following conditions: An initial denaturation at 95°C (5 min), 29 cycles of denaturation at 95°C (30 sec), annealing at 53°C (30 sec), then extension at 72°C (1 min), and a final extension at 72°C for 10 min. The PCR products were checked using gel electrophoresis on 1.5% agarose, then were sequenced by 1st BASE (Singapore). Subsequently, the obtained nucleotide sequences were aligned and compared with the sequences belonging to the other bacterial isolates published in GenBank database of NCBI using basic local alignment search tool (BLAST) program. A phylogenetic tree was created by the neighbour-joining method using MEGA 7.0 software with bootstrap replications (1000) [19, 22].

2.2.3. Determination of antimicrobial ability of the VNUA16 strain

An antimicrobial activity test was performed using the inhibition zone assay according to the procedures of Chen *et al.* (2016) with some modifications [23]. Bacterial pathogens, namely *Pseudomonas aeruginosa* P2.1, *Vibrio parahaemolyticus* and *Staphylococcus aureus* SA12 were incubated for 24 hours at 30°C and 160 rpm in proper media, including LB broth (for *Pseudomonas aeruginosa* P2.1 and *Staphylococcus aureus* SA12) and Tryptic Soy Broth (TSB), containing 17.0 g/l tryptone, 3.0 g/l soytone, 2.5 g/l dextrose, 2.5 g/l K₂HPO₄ with 2% NaCl (for *Vibrio parahaemolyticus*). After that, 50 µl of culture broth were thoroughly spread on the agar media and wells were created on plates.

Regarding the VNUA16 strain, it was cultured in LB broth at 30°C in a incubator shaker (160 rpm). After 48 hours of incubation, the obtained culture broth was centrifuged at 10,000 rpm at 10°C for 10 min to collect supernatants. 100 µl of cell - free supernatant was added into each well in the plates. The plates were placed at 4°C for 2 - 3 hours, then incubated at 30°C for 12 hours. The

presence of antimicrobial activity is indicated by the formation of a clear zone around the well. Diameter of inhibition zone was measured and recorded. Each experiment was tested in three replications and the diameter of the clear zone was measured.

2.2.4. The effect of temperature and pH on antibacterial activity of the VNUA16 strain

The strain was incubated in LB broth at 160 rpm at different temperature and pH conditions. The initial pH of culture media were adjusted at 2, 4, 6, 8, 10 and 12. In terms of temperature, the strain was cultured at temperature ranging from 30°C to 40°C at an interval of 5. Antimicrobial activity against *Pseudomonas aeruginosa* P1.2 was determined as described in 2.2.3. The experiment was performed in triplicate [24].

2.2.5. Data analysis

All experiments were performed in triplicate and results represented mean values. Based on GraphPad Prism software version 9.0, the data of these experiments were analyzed with one-way ANOVA ($p < 0.05$) and Tukey's test. A value of $p < 0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Characterization of isolated bacterial strain

The morphological and biochemical characteristics of VNUA16 strains were conducted according to the method described above, and the results were presented in figure 1 and table 1. As can be seen from the figure 1, the colony morphology of this strain cultured on LB medium was circular form with small diameters ranging from 1 to 2 mm, slightly raised elevation, entire margin, moist in texture, opaque in opacity and cream-yellow in color.

Observed under light microscope, cells of this strain were rod-shaped, arranging in singles or in pairs with rounded or square ends. It was also found that the VNUA16 strain was a Gram-positive and spore-forming bacterium, exhibiting positive for catalase, nitrate reduction, Methyl Red and Voges-Proskauer tests, but negative for indole and citrate tests. This strain produced acid from different carbohydrate sources such as D-glucose,

D-galactose and maltose. However, it was unable to utilize lactose, starch, sorbitol, sucrose and D-inositol. In terms of extracellular hydrolytic enzymes, the *Bacillus* sp. strain VNUA16 had the capacity to produce amylase and cellulase. Similarly, Ghosh *et al.* (2017) indicated that *Bacillus licheniformis* strain ONF1P isolated from

the gut of Nile Tilapia (*Oreochromis niloticus*) exhibited amylase and cellulase activities [14]. According to Pham Thi Tuyet Ngan *et al.* (2021), five strains of *Bacillus* spp., namely CM3.1, CM2.2, TV1.2, TV3.1 and BT1.2 isolated from shrimp pond sludge were also able to hydrolyze starch and cellulose [25].

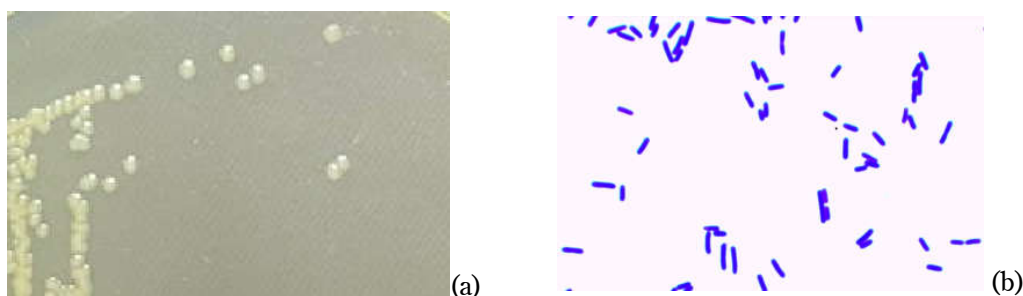


Figure 1. Morphological characteristics of the VNUA16 strain

a. Morphological colony of the VNUA16 strain; b. Cellular morphology of the VNUA16 strain

Based on the description in Bergey's Manual of Determinative Bacteriology, it is indicated that the VNUA16 strain has

morphological and biochemical characteristics similar to the genus *Bacillus* [26].

Table 1. Phenotypic and biochemical characteristics of the VNUA16 strain

Characteristics	VNUA16	Characteristics	VNUA16
Gram staining	+	Starch hydrolysis	-
Shape	Rod	Cellulose hydrolysis	-
Spore formation	+	Fermentation for carbon sources: Lactose	-
Motility	+	D-glucose	+
Catalase	+	D-galactose	+
Citrate utilization	-	Maltose	+
Indole production	-	D-inositol	-
Nitrate reduction	+	Starch	-
Voges-Proskauer (VP)	+	Sorbitol	-
Methyl Red (MR)	+	Sucrose	-

The bacterial growth was determined by measuring its absorbance at a wavelength of 620 nm after 48 hours of incubation in LB broth. The result was shown in figure 2. As indicated in this figure, there was a varied growth of the VNUA16 at different temperature. The VNUA16 strain had

the highest growth at 30°C, recording optical density (OD) of 2.48. Moreover, the growth of this strain decreased dramatically at 40°C (OD 620 nm = 0.73). As a result, the VNUA16 strain was considered as a mesophilic bacterium.

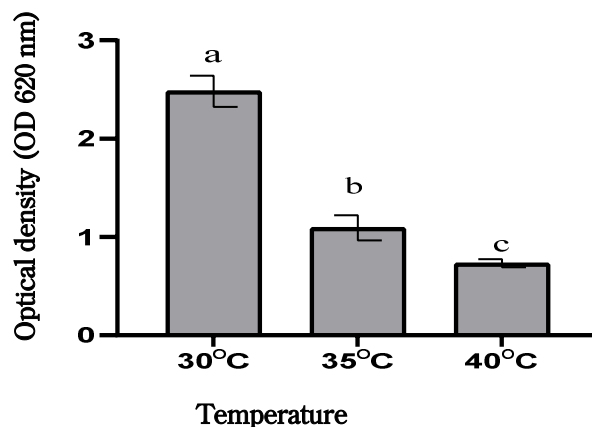


Figure 2. The effect of temperature on the growth of VNUA16 strain

Note: Different letters means significant differences according to Tukey's test at $p < 0.05$

Similarly, nine novel species of the *Bacillus cereus* group, including *Bacillus paranthracis* Mn5, *Bacillus pacificus* EB422, *Bacillus tropicus* N24, *Bacillus albus* N35-10-2, *Bacillus mobilis* 0711P9-1, *Bacillus luti* TD41, *Bacillus proteolyticus* TD42, *Bacillus nitratreducens* 4049 and *Bacillus paramycoides* NH24A2 isolated from sediments and seawater had an optimum temperature for the growth at 30°C [27]. Wekesa *et al.* (2022) recorded

that *Bacillus velezensis* isolated from Lake Bogoria grew optimally at temperature of 30-35°C [28]. Various *Bacillus* species, namely *Bacillus subtilis*, *Bacillus firmus*, *Bacillus flexus*, *Bacillus safensis* also had optimal growth at 30°C [29, 30]. However, *Bacillus* sp. Bsa 1 and *Bacillus flexus* APGI had maximal growth at 40°C [24, 31].

3.2. Identification of the VNUA16 strain by 16S rRNA sequence analysis

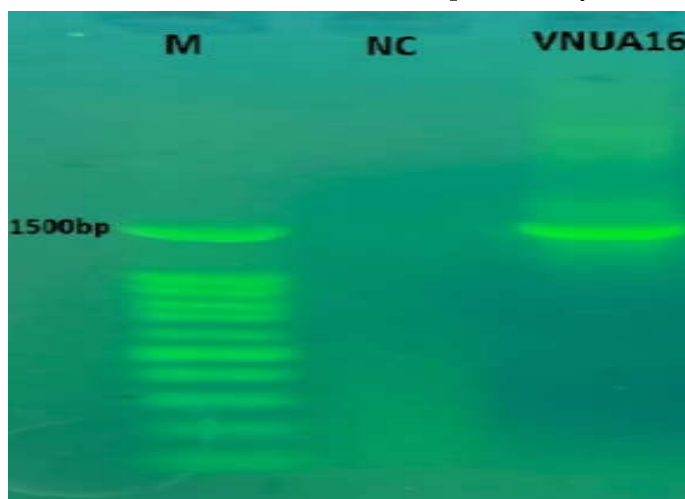


Figure 3. PCR product of 16S rRNA gene from the *Bacillus* sp. strain VNUA16

NC. Negative control (no template DNA added); M. DNA ladder marker 1,500 bp

PCR amplification of 16S rRNA revealed the possibility of amplifying PCR products with size of 1,500 bp. As can be seen from the figure 3, the PCR product of the VNUA16 strain was around 1,500 bp in length. After comparing for homology

of 16S rRNA sequence of the VNUA16 strain with other reference sequences in GenBank database, it is indicated that the highest genetic similarity 97.61% was with *Bacillus paramycoides* MCCC 1A04098.

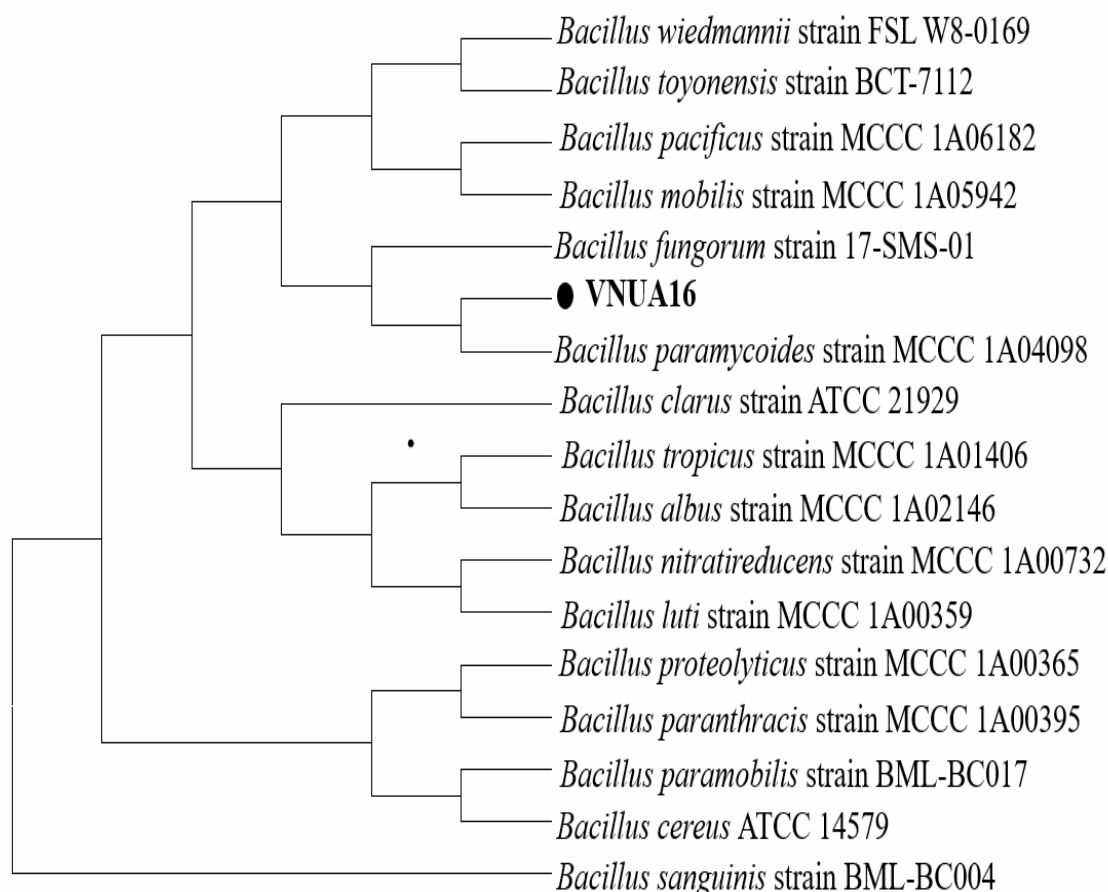


Figure 4. Phylogenetic tree of the VNUA16 and representatives of other related strains within *Bacillus* genus based on 16S rRNA sequence analysis

Based on the phylogenetic tree construction, it is apparent that the VNUA16 strain formed a clade with *Bacillus paramycoides* (Figure 4). The formation of clade in VNUA16 strain with *Bacillus paramycoides* showed that two bacterial strains in the taxonomic group has the same ancestor. The phylogenetic analysis of 16S rRNA gene sequences suggested that this strain was clustered within the taxonomic groups of *Bacillus* genus. Morphological and biochemical features also corresponded to those of this genus.

3.3. Determination of antimicrobial ability of the VNUA16 strain

The *Bacillus* sp. strain VNUA16 displayed moderate antibacterial activity against three bacterial pathogens, namely *Pseudomonas aeruginosa* P2.1, *Vibrio parahaemolyticus* and *Staphylococcus aureus* SA12 (Figure 5). The results showed that the VNUA16 strain had the highest antagonistic activity against *Staphylococcus aureus* SA12 with zone inhibition diameter of 9.57 ± 0.60 mm, followed by *Pseudomonas aeruginosa* P2.1 (8.53 ± 0.50 mm) and *Vibrio parahaemolyticus* (6.43 ± 0.49 mm). While among tested pathogenic bacteria, *Bacillus subtilis* 11A exhibited the lowest activity to

Staphylococcus aureus SA12 [32]. It is apparent that *Bacillus* sp. strain VNUA16 exhibited broad spectrum antibacterial activities because of inhibiting both Gram-positive and Gram-negative bacterial pathogens.

The genus *Bacillus* is known to be able to secrete different proteinase and antimicrobial secondary metabolites resulting in inhibiting a lot of pathogenic bacteria [22]. The results obtained by Sarjito *et al.* (2022) indicated that seven bacterial strains, belonging to *Bacillus* genus such

as *Bacillus cereus*, *B. aerius*, *B. paramycoides*, *B. thuringiensis*, *B. altitudinis*, *B. salarius* and *B. wiedmanni* had antibacterial activity against three species of *Vibrio* from vannamei shrimp (*Litopenaeus vannamei*) such as *Vibrio vulnificus*, *V. anguillarum*, *V. alginolyticus*. All these strains had no zone of inhibition against *V. parahaemolyticus*. Among them, *Bacillus paramycoides* was capable of inhibiting *V. anguillarum* with a clear zone of 9.73 mm [22].

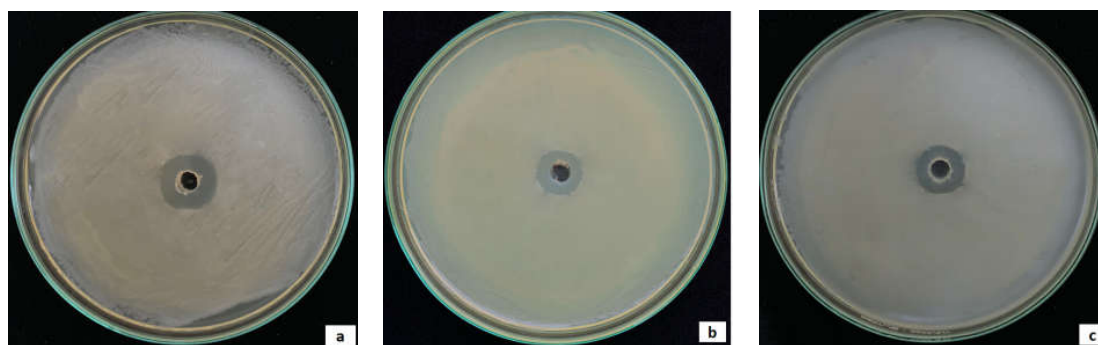


Figure 5. Antagonistic activity of *Bacillus* sp. VNUA 16 against bacterial pathogens

(a) *Pseudomonas aeruginosa* P2.1; (b) *Vibrio parahaemolyticus*, (c) *Staphylococcus aureus* SA12

According to Pham Thi Tuyet Ngan *et al.* (2021), 13 strains of *Bacillus* spp. isolated from sludge of extensive shrimp ponds were capable of antagonizing to *V. parahaemolyticus* with zone of inhibition ranging from 2.05 to 13.05 mm. Among them, there were less than half of isolates having lower inhibition activities compared to the VNUA16 strain [25]. Setiaji *et al.* (2020) explored the inhibitory effects of different *Bacillus* species

on the growth of *Vibrio alginolyticus*, *Aeromonas hydrophila* and *Pseudomonas aeruginosa* because they could produce terpenoid compounds. *Bacillus cereus* strain JS22 had antimicrobial activity against *Pseudomonas aeruginosa* similar to the *Bacillus* sp. strain VNUA16 [33].

3.4. The effect of temperature and pH on antagonistic activity of *Bacillus* sp. VNUA16

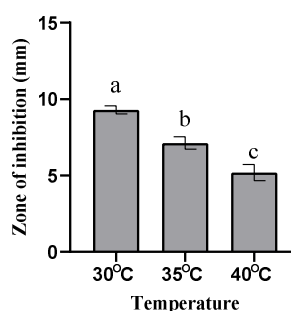


Figure 6. Effect of temperature on antagonistic activity of *Bacillus* sp. VNUA16 against *P. aeruginosa* P2.1 after 48 hours of incubation

Note: Different letters means significant differences according to Tukey's test at $p < 0.05$

The inhibitory effect of *Bacillus* sp. VNUA16 strain against *Pseudomonas aeruginosa* was conducted in different temperature and pH. The influence of different pH and temperature on the inhibitory activity against *P. aeruginosa* P2.1 of *Bacillus* sp. strain VNUA16 is presented in figure 6 and figure 7. As can be seen from figure 6, the optimum temperature for antagonistic effect of the *Bacillus* sp. VNUA16 against *P. aeruginosa* was at 30°C with a clear zone of inhibition of 9.3 mm, followed by 35°C with an inhibitory zone of 7.13 mm. Meanwhile, the antibacterial activity of this strain against *P. aeruginosa* considerably decreased at 40°C. The results were in agreement with the study of Iqbal *et al.* (2018). According to Iqbal *et al.* (2018), *Bacillus safensis* MK-12 isolated from waste dump soil revealed maximum antibacterial activity against different pathogens, including *Staphylococcus aureus*, *P. aeruginosa*, *Vibrio cholera*, *E. coli* at 30°C. However, the antibacterial activity of *B. safensis* MK-12 against *P. aeruginosa* was significantly higher than that of the VNUA 16 strain, recording zone of inhibition of around 17 mm [30]. Besides, unlike to the *Bacillus* sp. strain VNUA16, *Bacillus flexus* APGI and

Bacillus BSA1 strains isolated from the gut of common carp *Cyprinus carpio* had better level of zone of inhibition against the test bacterial pathogen at 40°C [24, 31].

With regard to pH, the VNUA16 strain recorded highest inhibitory effect against *P. aeruginosa* at pH6, indicating zone of inhibition of 9.53 mm. There was no significant differences in the antibacterial activity of the VNUA16 between pH 6 and pH 8, while zone inhibition diameters of this strain against test bacterial pathogen were lower at pH 2, 4 and 10, ranging from 5.43 mm to 8.17 mm. The bacterial pathogen *P. aeruginosa* was not inhibited by the strain VNUA16 at pH 12. According to Ramasubburayan *et al.* (2014) [24], *Bacillus flexus* APGI strain could not inhibit the growth of *P. aeruginosa* not only at pH 12 but also at pH 4. However, this strain had maximum antagonistic activity against *P. aeruginosa* at pH 8, which was still higher than that of the *Bacillus* sp. strain VNUA16. The study of Iqbal *et al.* (2018) [30] reported that *Bacillus safensis* strain MK-12 also exhibited strongest antagonistic effect against bacterial pathogen at pH 8.

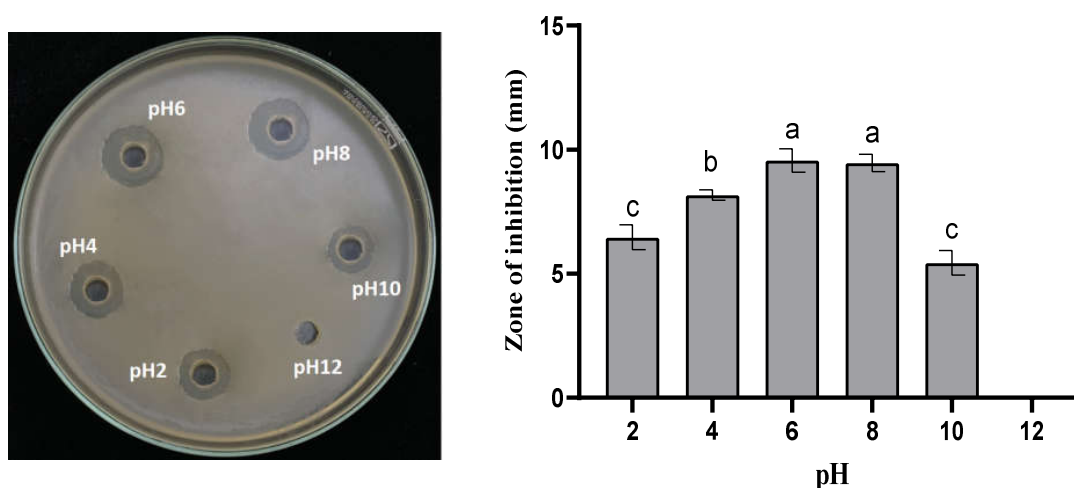


Figure 7. Effect of pH on antagonistic activity of *Bacillus* sp. VNUA16 against *P. aeruginosa* P2.1 after 48 hours of incubation

Note: Different letters means significant differences according to Tukey's test at $p < 0.05$

4. CONCLUSION

The present study demonstrated that the VNUA16 strain belongs to the *Bacillus* genus based on its morphological, biochemical characteristics and the analysis of 16S rRNA gene sequences. The optimal growth temperature of the strain was at 30°C. This strain possessed a broad spectrum antibacterial activity because of suppressing both Gram-negative bacteria (*P. aeruginosa*, *V. parahaemolyticus*) and Gram-positive bacterium (*S. aureus*). The antagonistic activity of the strain against *P. aeruginosa* was highest at pH6 and 30°C. The *Bacillus* sp. strain VNUA16 is considered as an important biological control agent against aquatic pathogenic bacteria, maintaining environmentally friendly and sustainable aquaculture.

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REFERENCES

1. Nguyen Nhu Tri, Nguyen Phuc Cam Tu, Dinh The Nhan, Nguyen Van Tu (2021). An overview of aquaculture development in Vietnam. *Proceedings of the International Conference on Fisheries and Aquaculture*, 7 (1): 53-71.
2. General Statistics Office (2023). Socio-economic situation in the first quarter of 2023. Retrieved at: <https://www.gso.gov.vn/tin-tuc-thong-ke/2023/accessed: 09/9/2023>.
3. Tran N., Chan C. Y., Aung Y. M., Bailey C., Akester M., Cao Q. L., Tring T. Q., Hoang C. V, Sulser T. B. & Wiebe K. (2022). Foresighting future climate change impacts on fisheries and aquaculture in Vietnam. *Frontiers in Sustainable Food Systems*, 1-15.
4. Öztürk R. C. & Altnok I. (2014). Bacterial and Viral Fish Diseases in Turkey. *Turkish Journal of Fisheries and Aquatic Sciences*, 14: 275-297.
5. Çanak O. & Timur G. (2020). An initial survey on the occurrence of staphylococcal infections in Turkish marine aquaculture (2013-2014). *Journal of Applied Ichthyology*, 36: 932-941.
6. Ayoub H. F., Tohamy E. Y., Salam H. M. & Mohamed S. S. (2021). Isolation, identification and antimicrobial profile of *Aeromonas* spp., *Pseudomonas* spp. and *Vibrio* spp. from the Nile Tilapia, *Oreochromis niloticus* in fish farms. *Egyptian Journal of Aquatic Biology & Fisheries*, 25(3): 171-185.
7. Metin S., Kubilay A., Onuk E. E., Didinen B. I. & Yildirim P. (2014). First isolation of *Staphylococcus warneri* from cultured rainbow trout (*Oncorhynchus mykiss*) broodstock in Turkey. *Bulletin of the European Association of Fish Pathologists*, 34 (5): 165-173.
8. Yaseen M. S, Abdelaziz M., Abdel-moneam D. A., Abd-Elhay E., Wassif I. M., Fawzy M. & Moustafa M. (2020). Phenotypic and genotypic characterization of the pathogenic *Pseudomonas aeruginosa* isolated from cultured *Pangasianodon hypophthalmus* in Egypt. *Egyptian Journal of Aquatic Biology & Fisheries*, 24 (6): 453-467.
9. Loh, J. Y. (2017). The role of probiotics and their mechanisms of action: An aquaculture perspective. *World Aquaculture*, 19-23.
10. Soltani M, Ghosh K., Hoseinifar S. H., Kumar V., Lymbery A. J., Roy S. & Ringo E. (2019). Genus *Bacillus*, promising probiotics in aquaculture: Aquatic animal origin, bioactive components, bioremediation and efficacy in fish and shellfish. *Reviews in Fisheries Science & Aquaculture*, 1-49.
11. Domrongpakkaphan V. & Wanchaitanawong P. (2006). In vitro antimicrobial activity of *Bacillus* spp. against pathogenic *Vibrio* spp. in black tiger shrimp (*Penaeus monodon*). *Kasetsart Journal Natural Science*, 40 : 949 – 957.
12. Vaseeharan B. & Ramasamy P. (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for

black tiger shrimp *Penaeus monodon*. *Letters in Applied Microbiology*, 36: 83-87.

13. Ran C., Carrias A., Williams M. A., Capps N., Dan B. C. T., Newton J. C., Kloepper J. W., Ooi E. L., Browdy C. L., Terhune J. S. & Liles M. R. (2012). Identification of *Bacillus* strains for biological control of catfish pathogens. *PLoS One*, 7(9): 1-9.

14. Ghosh K., Banerjee S., Moon U. M., Khan H. A. & Dutta D. (2017). Evaluation of gut associated extracellular enzyme-producing and pathogen inhibitory microbial community as potential probiotics in Nile tilapia, *Oreochromis niloticus*. *International Journal of Aquaculture*, 7: 143-158.

15. Ramirez-Torrez J. A., Monroy-Dosta M. C., Hernandez- Hernandez L. H., Castro-Mejia J., Bustos-Martinez J. A., & Hamdan-Partida A. (2018). Presumptive probiotic isolated from *Oncorhynchus mykiss* (Walbaum, 1792), cultivated in Mexico. *International Journal of Aquatic Science*, 9 (1): 3-12.

16. Banerjee S., Mukherjee A., Dutta D. & Ghosh K. (2016). Non-starch polysaccharide degrading gut bacteria in Indian major carps and exotic carps. *Jordan Journal of Biological Sciences*, 9 (1): 69-78.

17. Xu R., Ding F.-F., Zhou N.-N., Wang T., Wu H.-X., Qiao F., Chen L.-Q., Du Z.-Y. & Zhang M.-L. (2022). *Bacillus amyloliquefaciens* protects Nile tilapia against *Aeromonas hydrophila* infection and alleviates liver inflammation induced by high-carbohydrate diet. *Fish & Shellfish Immunology*, 127: 836-842.

18. Zhou P., Chen W., Zhu Z., Zhou K., Luo S., Hu S., Xia L. & Ding X. (2022). Comparative study of *Bacillus amyloliquefaciens* X030 on the intestinal flora and antibacterial activity against *Aeromonas* of grass carp. *Frontiers in Cellular and Infection Microbiology*, 12: 1-14.

19. Duong Nguyen Anh Tam, Pham Hoang Minh, Lam Hue Nghi, Pham Quoc Cuong, Le Duc

Trung, Tran Minh Bao, Tra Van Tung (2022). Amoxicillin degradation ability of *Bacillus cereus* C1 isolated from catfish pond sludge in Vietnam. *Heliyon*, 8: 1-8.

20. Barrow G. I. & Feltham R. K. A. (2004). Cowan and Steel's manual for the identification of medical bacteria. 3th ed., Cambridge University Press.

21. Kumari G., Kumar D., Singh D. & Kumar N. (2020). Characterization of microbial inoculants and its different formulation for mass production. *International Journal of Current Microbiology and Applied Sciences*, 9 (7): 721-627.

22. Sarjito S., Amalia R. & Sabdaningsih A. (2022). Screening of spore-associated bacteria to control vibriosis in vannamei shrimp (*Litopenaeus vannamei*). *Biodiversitas*, 23 (10): 5333-5341.

23. Chen Y., Li J., Xia P., Zhu W & Mo Z. (2016). The ability of marine *Bacillus* spp. isolated from fish gastrointestinal tract and culture pond sediment to inhibit growth of aquatic pathogenic bacteria. *Iranian Journal of Fisheries Sciences*, 15 (2): 704-714.

24. Ramasubburayan R., Titus S., Verma P. K., Immanuel G. & Palavesam A. (2014). Isolation, screening and optimization of culture conditions for enhanced antibacterial activity by a marine epibiotic bacterium *Bacillus flexus* APGI against fouling bacterial strains. *Journal of pure and applied microbiology*, 8 (4): 2909-2920.

25. Pham Thi Tuyet Ngan, Vu Hung Hai, Vu Ngoc Ut, Huynh Truong Giang (2021). Selection of *Bacillus* sp. bacteria having organic matter decomposition ability and resistance to *Vibrio parahaemolyticus* causing disease in Penaeid shrimp. *Can Tho University Journal of Science*, 57 (3B): 191-199.

26. Holt J. G., Krieg N.R., Sneath P. H. A., Staley J. T. & Williams S. T. (1994). Bergey's Manual of Determinative Bacteriology 9th ed.. Lippincott Williams & Wilkins.

27. Liu Y., Du J., Lai Q., Zeng R., Ye D., Xu J. & Shao Z. (2017). Proposal of nine novel species of the *Bacillus cereus* group. *International Journal of Systematic and Evolutionary Microbiology*, 67 (8): 2499-2508.
28. Wekesa T. B., Wekesa V. W., Onguso J. M., Wafula E. N. & Kavesu N. (2022). Isolation and Characterization of *Bacillus velezensis* from Lake Bogoria as a potential biocontrol of *Fusarium solani* in *Phaseolus vulgaris* L. *Bacteria*, 1: 279-293.
29. Setyati W. A., Martani E., Triyanto, Subagiyo & Zainuddin M. (2014). Selection, identification and optimization of the growth water probiotic consortium of Mangrove Ecosystems as Bioremediation and biocontrol in shrimp ponds. *JPHPI*, 14 (3): 243-253.
30. Iqbal S., Qasim M., Begum F., Rahman H., Sajid I. (2018). Screening, characterization and optimization of antibacterial peptides, produced by *Bacillus safensis* strain MK-12 isolated from waste dump soil KP, Pakistan. *BioRxiv*, 1-17.
31. Karthick M., Maheshkumar P., Krishnamoorthy D., Prakasham A., Balachandar M. & Raj R. A. (2018). Optimization of culture conditions for enhanced antibacterial activity by a fish gut bacterium *Bacillus* sp. Bsa1. *International Journal of Innovative Science and Research Technology*, 3 (8): 86-91.
32. Cahya V. A., Hanim C., Yusiati I. M., Bachruddin Z. & Erwanto Y. (2019). Growth optimization of *Bacillus subtilis* 11A isolated from Indonesian native chicken (*Gallus domesticus*) for bacteriocin production. *IOP Conference Series: Earth and Environmental Science*, 387: 1-4.
33. Setiaji J., Feliatra F., Teruna H.Y., Lukistyowati I., Suharman I., Muchlisin Z.A., Johan T.I. (2020). Antibacterial activity in secondary metabolite extracts of heterotrophic bacteria against *Vibrio alginolyticus*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*. *F1000 Research*, 9: 1-11.