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RESEARCH ARTICLE
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Bacillus spp. Isolated from White Shrimp *Fenneropenaeus merguiensis* (De Man, 1888) and Antagonistic Activity against *Vibrio* Pathogens

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ABSTRACT

Shrimp is an export commodity that has great potential in regional and international markets due to increasing demand every year. It is supported by the promotion of intensive and super-intensive cultivation; however, this method could be more efficient. Both can trigger stressful conditions and reduce water quality. The main problems in shrimp cultivation are low feed efficiency, decreased water quality, and vibriosis outbreaks. The proposed solution to this problem is to use anti-vibrio activity probiotics. This study aimed to determine the anti-vibrio activity of *Bacillus* spp. bacteria isolated from shrimp. Total of six *Bacillus* spp. strains isolated from the intestines of Jerbung shrimp *Fenneropenaeus merguiensis* (De Man, 1888) tested for anti-vibrio activity against *Vibrio parahaemolyticus, Vibrio harveyi and Vibrio alginolyticus* using the Kirby Bauer agar diffusion method. The research results showed that the *Bacillus* strains identified included *Bacillus subtilis* strain JR1 [RL.20], *Bacillus cereus* strain EGU510 [UM 16.1], *Bacillus pseudomycoides* strain LU2 [GS 14.1], *Bacillus cereus* strain 24 [GS 14.2], *Bacillus pseudomycoides* strain LU2 [GS 14.3], and *Bacillus* sp. strain Z96 [RS 19.1]. All strains of *Bacillus* spp. effectively inhibit pathogenic Vibrio bacteria, including six, four and two strains, producing *anti-Vibrio parahaemolyticus, anti-Vibrio harveyi and anti-Vibrio alginolyticus* activity, respectively. In conclusion, several strains of *Bacillus* spp. very effective in inhibiting pathogenic bacteria including *Vibrio parahaemolyticus, Vibrio harveyi,* and *Vibrio alginolyticus*.

Keywords: Anti-Vibrio Activity, *Bacillus* spp, Probiotic Bacteria, *Vibriosis*

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INTRODUCTION

Shrimp is one of the export commodities with great potential across both regional and international markets (Wijayanto et al., 2021) due to the progressively increasing demand every year. The production in Indonesia was estimated at 1.21 million tons in 2021 (Marine and Fisheries Ministry, 2020), increasing by 9.20% compared to the value in previous year at 1.11 million tons. The increase can be attributed to the promotion of intensive and super-intensive cultivation but this method is relatively risky. Intensive and super-intensive cultivation in shrimp production can trigger stressed conditions, decreased water quality, and low feed efficiency, potentially leading to disease outbreaks.

One of the disease outbreaks often encountered in shrimp cultivation is vibriosis caused by infection with *Vibrio* bacteria which may lead to mass deaths (Imtiyaz et al., 2023). *In shrimp farms, Vibrio spp. is extremely dangerous as they are Gram-negative, motile, facultatively anaerobic bacteria*. Studies related to pathogenicity of *Vibrio* sp. have been widely carried out, including Han et al. (2020) which reported the ability of *Vibrio parahaemolyticus* to cause acute hepatopancreatic

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necrosis [AHPND] in cultivated shrimp with a mortality rate of 100% (Orozco-Ochoa et al., 2023). Other *Vibrio* strains that cause vibriosis include *Vibrio harveyi*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio splendidus*, *Vibrio salmonicida*, and *Vibrio vulnificus* (Ramadhani et al., 2022). These *Vibrio* strains have a significant influence on the cultivation of shrimp, fish, crustaceans, and lobsters (Valente & Wan, 2021). Treatment for vibriosis is generally carried out using antibiotics but this method can increase resistance when used regularly. Moreover, the use of antibiotics can leave residue on shrimp meat which is dangerous when consumed by humans (Chau et al., 2021). To overcome problems related to vibriosis disease outbreaks, the use of antibiotics, water quality, and feed efficiency in shrimp cultivation, other safe and efficient alternatives are needed. The use of probiotic bacteria is highly recommended due to the ability to improve survival rates, immunity, and resistance of shrimp to vibriosis disease. Increasing the number of probiotic bacteria in ponds can suppress the growth and presence of pathogenic bacteria (Amin et al., 2023). El-Saadony et al. (2022) also stated that the application of probiotic bacteria in shrimp cultivation improved water quality and feed efficiency. Among these microorganisms are those that produce amylase, protease, lipase, and cellulase, which are enzymes secreted by their extracellular membranes (Dawood et al., 2019). Enzymes can also help reduce the amount of organic material contained in ponds such as dirt, waste, dead organisms, and uneaten pellets (Pratiwi et al., 2020). Probiotic bacteria commonly used in shrimp cultivation include the genus *Bacillus* sp. *Clostridium* sp. *Lactobacillus* sp. *Psychrobacter* sp. and *Arthrobacter* sp. (Martínez Cruz et al., 2012).

The probiotic *Bacillus* sp. is widely used in aquaculture due to its ability to increase shrimp growth, improve water quality, and inhibit pathogen growth (Truong et al., 2021). Several species have anti-vibrio activity against *Vibrio vulnificus* (Gao et al., 2017), *Vibrio parahaemolitycus* (Kewcharoen and Srisapoome, 2019) *Vibrio harveyi* (Rusmana et al., 2021), *Vibrio alginolyticus* (Xiu et al., 2017), and *Vibrio owensii* (Nguyen et al., 2021). Furthermore, *Bacillus* spp. has extracellular enzyme activity such as protease, amylase, lipase, and cellulase (Sulistiyani et al., 2021). These extracellular enzymes are used to break down organic material and increase feed digestibility. Studies related to anti-vibrio activity and extracellular enzymes of *Bacillus* spp. isolated from cultivated shrimp have generally been widely carried out. However, information on anti-vibrio activity and extracellular enzymes isolated is limited, presenting oppurtunities for further investigation. This study aimed to obtain *Bacillus* sp. isolates with the potential to function as probiotics within shrimp cultivation, specifically against pathogenic bacteria.

MATERIALS & METHODS

Ethical Approval

The conducted research is not related to either human or animal use.

Time, Place, and Collection of Samples

Research was conducted between August and December 2023 at the Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University. Jerbung shrimp *Fenneropenaeus merguiensis* (De Man, 1888) samples were obtained from three water locations representing the North and South Coasts of Central Java. Sampling was carried out in the Pemalang and Cilacap catchment areas with the length (22.2±1.6cm) and weight of shrimp ranged 92.95±10g. After collecting the samples, they were placed into a cool box and transported to the laboratory for further analysis.

Isolation of Bacteria *Bacillus* spp.

Isolation of bacteria from the intestine of shrimp samples followed the procedure of Nurhafid et al. (2021). Shrimp intestines were collected as a whole, followed by washing using phosphate buffer saline, and all waste contained in the intestines was removed. The shrimp intestine samples were dissolved in approximately 0.5mL of 0.9% NaCl, proportionately diluted to 10^{-1}, and then homogenized in a vortex using 4.5mL of the diluted solution. These steps were performed until the dilution tube reached 10^3. Incubation was conducted for 48 hours at 28^{o}C on de Man Rogosa Sharpe (MRS) agar media using the pour plate technique. Purification of bacteria growing in single colonies was determined by colony morphology after streaking.

Initial Identification and Molecular Identification based on the 16s rRNA Gene

The purified bacteria were identified as *Bacillus* by observing the simple biochemical characteristics through the 3% KOH, Gram staining, Catalase, motility, and hemolytic tests (Parvathi et al., 2009). The results were then assessed through molecular identification to determine the level of similarity to *Bacillus* spp. GenBank data were used to identify the sequence by its similarity to 16S rDNA sequences. The PrestoTM Mini gDNA Bacteria Kit [Geneaid] was used to extract bacteria DNA to obtain pure DNA solutions. The results of each DNA extraction were mixed with a mastermix containing a pair of primers, nuclease-free water, and Mytaq HS Redmix 2x [DNA polymerase, Buffer MgCl2, and dNTP]. Subsequently, PCR amplification was carried out with Primus 25 Thermocycler PCR [Peqlab]. The primers used followed the study of Marchesi et al. (1998) including 27f [5'- AGA GTT TGA TCC TGG CTC AG -3'] and 1492R [5'- GGT TAC CTT GTT ACG ACT T -3'] with results 1500bp amplification. The PCR results were subjected to electrophoresis to determine the base pair length of bacteria obtained (Fig. 1). The results of our sequence analysis were analyzed using NCBI [http://ncbi.nlm.nih.gov/] Basic Local Alignment Search Tool [BLAST].

Preparation of *Vibrio* sp.

The *Vibrio* sp. bacteria used include *Vibrio parahaemolyticus, Vibrio alginolyticus,* and *Vibrio harveyi*. All Vibrio strains were obtained from the Research Laboratory Collection of the Faculty of Fisheries and Marine Sciences, Jenderal Soedirman University.

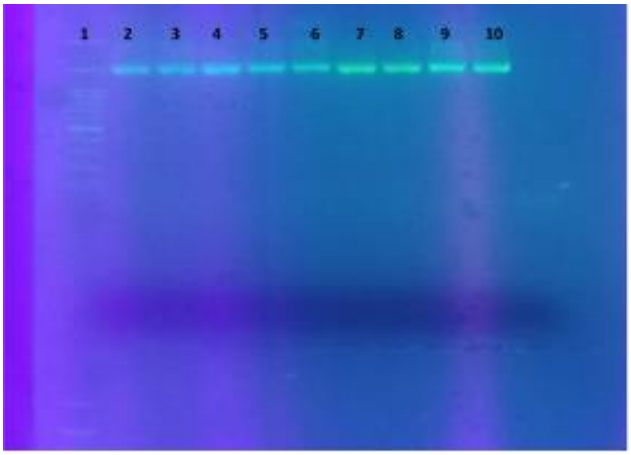

Fig. 1: Visualization of gel electrophoresis amplification results Well notes: 1. DNA markers have a size of 1500 bp, 2. *Bacillus cereus* strain EGU510, 3. *Bacillus pseudomycoides* strain LU2, 4. *Bacillus cereus* strain 24, 5. *Bacillus pseudomycoides* strain LU2, 6. *Bacillus* sp. strain Z96, 7. *Bacillus subtilis* strain _JR1, 8. Positive control [*Bacillus thuringiensis*], 9-10. Negative Control with Nucleous free water.

Bacteria strains were activated by taking 0.5µL, which was slanted on Tryptic Soy Agar (TSA) media, placed in Tryptic Soy Broth (TSB) media, and then incubated for 24 hours at 28ºC (Fitriadi et al*.,* 2023).

Testing for Anti-vibrio Bacteria *Bacillus* spp.

The media used was solid TSA in which vibrio bacteria were spread at 100µL with a density of 10^8 CFU/mL on the agar surface. *Bacillus* spp. bacteria suspension was dropped 10µL with a concentration of 10^9 CFU/mL on a blank disk placed on the agar surface followed by incubation at 28ºC for 48 hours (Nguyen et al*.,* 2021) Anti-vibrio activity was measured based on the inhibition zone that formed (Sulistiyani et al*.,* 2021).

Extracellular Enzyme Activity Screening

Protease Production

The protease enzyme activity was measured on skim milk agar medium made by mixing 2% skim milk powder dissolved in seawater with Nutrient Agar medium (Utomo et al., 2019). Both media were autoclaved separately, specifically, the Nutrient Agar medium was autoclaved at 121°C for 15min, while skim milk powder was autoclaved at 121°C for 5min. After the temperature reached ±45°C, the Nutrient Agar medium and skim milk powder were mixed in Erlenmayer, and poured into a glass cup. Each strain of *Bacillus* spp. was inoculated in a Petri dish and incubated at 28°C for 48 hours. Protease activity can be detected by a clear zone (Pailin et al., 2001).

Amylase Production

The amylase enzyme activity was determined by adding 1% starch dissolved in seawater to Nutrient Agar medium (Ayal et al., 2024). After the starch medium reached 45°C, it was autoclaved at 121°C for 15min and poured into a petri dish. Each strain of *Bacillus* spp. was inoculated in a petri dish and incubated at 28°C for 48 hours. The clear zone was visualized with iodine solution dripped on the petri dish (Ibrahim et al., 2012).

Cellulase Production

The cellulase enzyme activity was determined by making a Nutrient Agar medium with 1% carboxymethyl cellulose [CMC] dissolved in seawater (Artha et al., 2019). Cellulase medium was autoclaved at 121°C for 15min and poured into a petri dish when it reached 45°C. Each strain of *Bacillus* spp. was inoculated in a Petri dish and incubated at 28°C for 48 hours, then dripped with Congo red solution to visualize the clear zone (Gupta et al., 2012).

Lipase Production

Nutrient Agar medium was mixed with 1% Tween 80 dissolved in seawater for 15min then autoclaved at 121°C to measure lipase enzyme activity (Ni'matuzahroh et al., 2024). *Bacillus* spp. strains were inoculated into a Petri dish after medium reached 45°C, incubated for 48 hours at 28°C. Lipase activity could be identified by a clear zone (Nerurkar et al., 2013).

Data Analysis

The data obtained were preliminary identification results, molecular identification results, anti-vibrio activity and extracellular enzyme activity index analyzed statistically and presented in the form of figures, tables, and graphs. Therefore, the data were analyzed descriptively and compared with the literature.

RESULTS

Initial and Molecular Identification of *Bacillus* spp.

The results of 180 bacteria isolates obtained from three different locations led to the selection of six isolates that showed simple biochemical characteristics typical of *Bacillus* spp. namely Gram-positive, catalase-positive, motility positive or negative, and hemolytic positive or negative. The initial identification results of *Bacillus* spp. bacteria are presented in Table 1.

Based on the molecular identification results, six strains of *Bacillus* spp. were identified, namely, one *Bacillus subtilis*, two *Bacillus cereus*, two *Bacillus pseudomycoides*, and one *Bacillus* spp. (Table 2 and Fig. 1).

Anti-Vibrio Activity of *Bacillus* spp.

Anti-vibrio activity was evaluated to determine the potential of *Bacillus* spp. bacteria in inhibiting several Vibrio strains that cause vibriosis outbreaks, namely *Vibrio paraemolitycus, Vibrio alginolyticus,* and *Vibrio harveyi. Bacillus cereus* strain EGU510 [UM 16.1], *Bacillus cereus* strain 24 [GS 14.2], and *Bacillus subtilis* strain _JR1 [RL 20] had anti-*vibrio parahaemolyticus* activity and *Vibrio harveyi*. However, these strains did not inhibit *Vibrio alginolyticus*. *Bacillus pseudomycoides* strain LU2 [GS 14.1] had anti-vibrio *parahaemolyticus*, *Vibrio harveyi*, and *Vibrio alginolyticus. Bacillus* sp. strain Z96 [RS 19.1] showed anti-*Vibrio alginolyticus* and *Vibrio parahaemolyticus* but did not inhibit *Vibrio harveyi*. Meanwhile, *Bacillus pseudomycoides* strain LU2 [GS 14.3] and *Bacillus cereus* strain EGU510 [UM 16.1] had anti-vibrio *parahaemolyticus* activity. However, these strains did not inhibit *Vibrio alginolyticus* and *Vibrio harveyi*.

Table 1: Initial identification results of *Bacillus* spp.

Sample Code	Shape	Elevation	Edge	Color	Size	Texture	Gram	Catalase	Motility	Hemolytic
UM 16.1	Circular	Convex	entire	beige	Small	smooth	+	+	-	-
GS14 – 01	Irregular	Raised	undulate	beige	Big	Dry	+	+	+	-
GS14 – 02	Irregular	Raised	undulate	beige	medium	Dry	+	+	+	-
GS14 – 03	Irregular	Convex	undulate	beige	Big	Dry	+	+	+	-
RS 19.1	Irregular	Flat	undulate	beige	Big	Dry	+	+	-	-
RL. 20	irregular	crateriform	lobate	beige	medium	dry	+	+	-	-

Table 2: *Bacillus* spp. Sequencing Results of 16S RNA Gene Isolated from the Digestive Tract of Jerbung Shrimp from Northern and Southern Waters of Java

No	Code	Species	Identity [%]	Source
1	UM 16.1	*Bacillus cereus* strain EGU510	98.77	Jerbung Shrimp *Fenneropenaeus merguiensis* (De Man, 1888) Intestines, TPI Pemalang
2	GS 14.1	*Bacillus pseudomycoides* strain LU2	94.70	Jerbung Shrimp *Fenneropenaeus merguiensis* (De Man, 1888) Intestines, TPI Pemalang
3	GS14.2	*Bacillus cereus* strain 24	96.95	Jerbung Shrimp *Fenneropenaeus merguiensis* (De Man, 1888) Intestines, TPI Pemalang
4	GS 14.3	*Bacillus pseudomycoides* strain LU2	94.94	Jerbung Shrimp *Fenneropenaeus merguiensis* (De Man 1888) Intestines, TPI Pemalang
5	RS 19.1	*Bacillus* sp. strain Z96	92.47	Jerbung Shrimp *Fenneropenaeus merguiensis* (De Man 1888) Intestines, TPI Cilacap
6	RL 20	*Bacillus subtilis* strain _JR1	100	Jerbung Shrimp *Fenneropenaeus merguiensis* (De Man 1888) Intestines, TPI Cilacap

Table 3: *Bacillus* spp. tested for anti-vibrio activity with the agar diffusion method. Concentration of *Bacillus* spp. 10^9 CFU/mL and the Vibrio used was 10^8 CFU/mL

Strain *Bacillus*	Strain *Vibrio* sp. [mm]		
	Vibrio parahaemoliticus	*Vibrio alginolyticus*	*Vibrio harveyi*
Bacillus cereus strain EGU510	5,50+1,00	-	3,50+1,00
Bacillus pseudomycoides strain LU2	3,25+0,50	1,50+0,58	3,25+0,50
Bacillus cereus strain 24	4,00+0,00	-	4,75+1,71
Bacillus pseudomycoides strain LU2	5,00+0,00	-	-
Bacillus sp. strain Z96	4,75+1,71	6,00+1,15	-
Bacillus subtilis strain_JR1	4,00+1,63		4,00+1,63

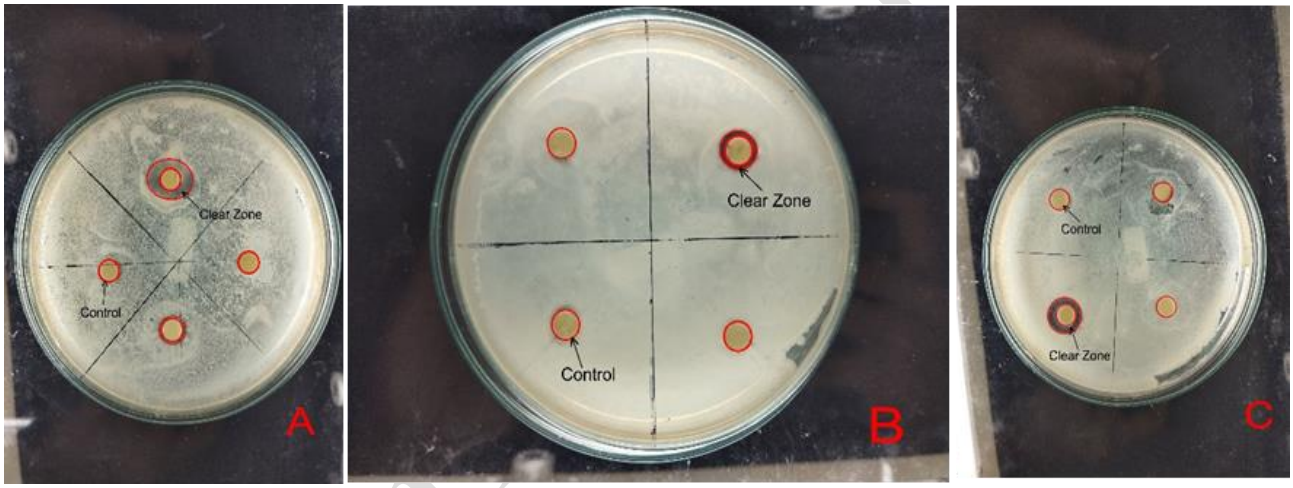

Fig. 2: Anti *Vibrio* Test Results; [A] *Vibrio parahaemolyticus*; [B] *Vibrio harveyi*; [C] *Vibrio alginolyticus.*

In general, the anti-vibrio activity of *Bacillus* spp. included in the weak and moderate categories, ranging from 1,50-6,00mm. Anti-vibrio activity results are shown in Table 3, while visualization is illustrated in Fig. 2.

Extracellular Enzyme Activity of *Bacillus* spp.

There was no difference in the diameter of the clear zone shown in (Fig. 3) between strains of *Bacillus* spp. Based on the results, all bacteria species obtained produced different extracellular enzymes with varying indices. *Bacillus pseudomycoides* strain LU2 [GS 14.1], *Bacillus cereus* strain 24 [GS14.2], and *Bacillus pseudomycoides* strain LU2 [GS 14.3] produced amylase, lipase, cellulase and protease enzymes. The strain EGU510 [UM 16.1] of *Bacillus cereus* produced amylase, lipase, and protease enzymes. Amylase and protease enzymes were produced by *Bacillus subtilis* strain _JR1 [RL 20], while only *Bacillus* spp. strain Z96 [RS 19.1] produced protease enzyme. Extracellular enzyme activity index results are presented in Fig. 4.

The results showed that five strains of *Bacillus* spp. effectively produced amylase enzyme, namely *Bacillus subtilis* strain _JR1 [RL 20], *Bacillus pseudomycoides* strain LU2 [GS 14.1], *Bacillus cereus* strain 24 [GS14.2], *Bacillus pseudomycoides* strain LU2 [GS 14.3], and *Bacillus cereus* strain EGU510 [UM 16.1]. *Bacillus pseudomycoides* strain LU2 [GS 14.3] had the highest amylase activity index of 2.2 after 48 hours, followed by *Bacillus subtilis* strain _JR1 [RL 20], *Bacillus cereus* strain EGU510 [UM 16], and *Bacillus cereus* strain 24 [GS14.2] with an index of 1.7. Furthermore, the amylase enzyme's peak production differed between bacteria, with *Bacillus cereus* strain 24 [GS14.2] producing a peak amylase index at 24 hours of incubation.

Four strains of *Bacillus* spp. effectively produced lipase enzyme, namely *Bacillus pseudomycoides* strain LU2 [GS 14.1], *Bacillus cereus* strain 24 [GS14.2], *Bacillus pseudomycoides* strain LU2 [GS 14.3], and *Bacillus cereus* strain EGU510 [UM 16.1]. The highest lipase activity index was obtained by *Bacillus cereus* strain EGU510 [UM 16.1], namely 1.8. This was followed by

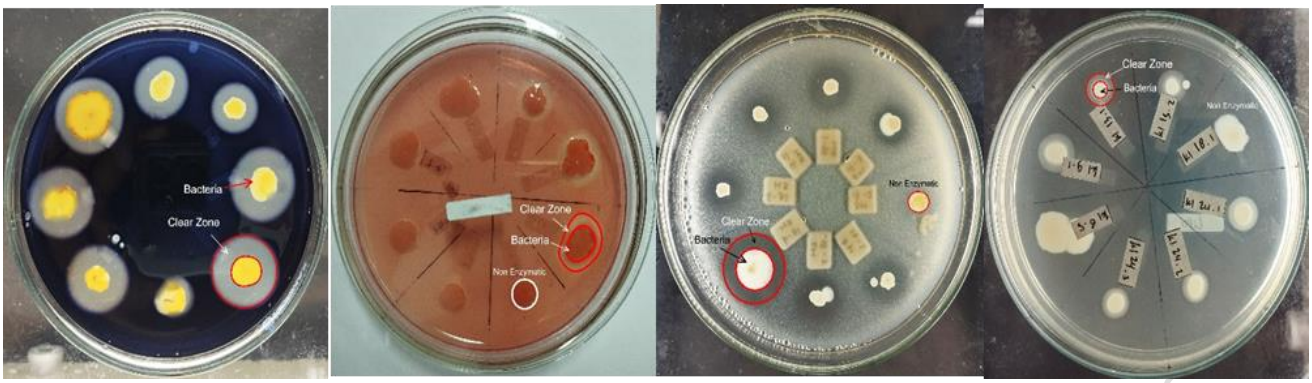

Fig: 3: Enzymatic activity of *Bacillus* spp. which was tested with incubation times [12, 24, 36, and 48 hours] (A) amylase enzyme activity; (B) Lipase enzyme activity; (C) Cellulose enzyme activity; (D) Protease enzyme activity.

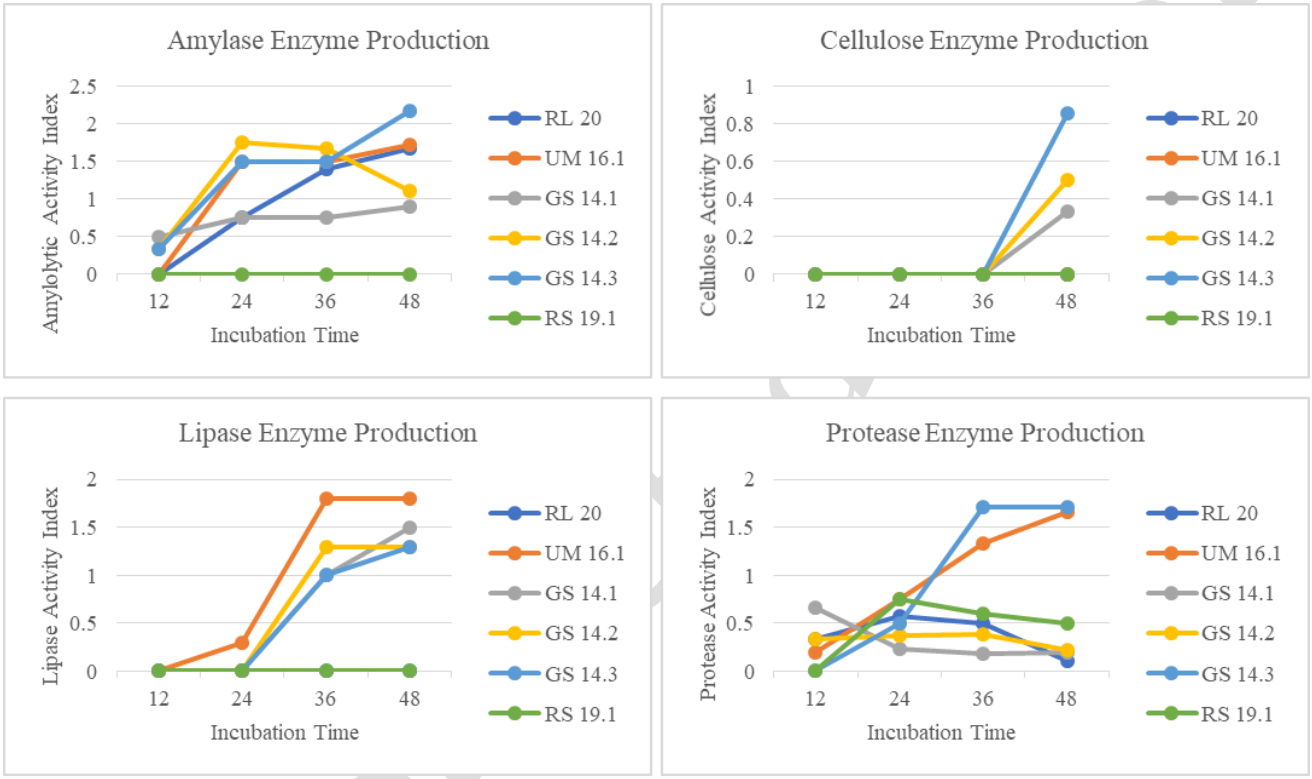

Fig. 4: Enzymatic activity of *Bacillus* spp. tested with incubation times [12, 24, 36, and 48 hours]. (A) amylase enzyme activity; (B) Lipase enzyme activity; (C) Cellulose enzyme activity; (D) Protease enzyme activity.

Bacillus pseudomycoides strain LU2 [GS 14.1] at 1.5, as well as *Bacillus cereus* strain 24 [GS14.2] and *Bacillus pseudomycoides* strain LU2 [GS 14.3] with a value of 1.3. The average lipase activity index of *Bacillus* bacteria appeared at an incubation time of 36 hours.

Based on the results, three *Bacillus* spp. strains produced cellulase enzyme, namely *Bacillus pseudomycoides* strain LU2 [GS 14.1], *Bacillus cereus* strain 24 [GS14.2], and *Bacillus pseudomycoides* strain LU2 [GS 14.3]. The highest cellulase activity index was obtained by *Bacillus pseudomycoides* strain LU2 [GS 14.3], namely 0.9 followed by *Bacillus cereus* strain 24 [GS14.2] and *Bacillus pseudomycoides* strain LU2 [GS 14.1] with index values of 0.5 and 0.3 respectively. The cellulase index value was obtained after 48 hours of incubation because the visualization of the clear zone obtained only became visible when the medium was covered by Congo Red solution.

All *Bacillus* spp. strains produced protease enzyme, with the highest protease activity index of 1.7 occurring in *Bacillus pseudomycoides* strain LU2 [GS 14.3] and *Bacillus cereus* strain EGU510 [UM 16.1] at an incubation time of 48 hours. This was followed by *Bacillus* spp. strain Z96 [RS 19.1] with a value of 0.8 at an incubation time of 24 hours, and *Bacillus pseudomycoides* strain LU2 [GS 14.1] with a value of 0.7 at an incubation time of 12 hours. Additionally, *Bacillus subtilis* strain _JR1 [RL 20] and *Bacillus cereus* strain 24 [GS14.2] had index values of 0.6 and 0.4 at an incubation time of 24 and 36 hours, respectively.

DISCUSSION

The results showed six, four, and two *Bacillus* spp. strains that had anti-vibrio activity against *Vibrio parahaemolyticus*, *Vibrio harveyi*, and *Vibrio alginolyticus*. Activity formed was in the weak adn medium category,

ranging from 1,50-6,00mm. According to Davis & Stout (1971), activity of the inhibition zone formed is separated to four categories, namely weak [≤5.0mm], medium [6-10mm], strong [11-20mm], and very strong [≥20mm]. *Bacillus* spp. bacteria are capable of producing more than 20 types of antimicrobial compounds, including antibiotics, bacteriocins, and lipopeptides (Knipe et al*.,* 2021; Devi et al., 2023). Anti-vibrio mechanism of *Bacillus* spp. bacteria is to inhibit growth, disrupt biofilm formation, and interrupt quorum sensing (Kasanah et al*.,* 2022). As stated by Gao et al. (2017), *Bacillus subtilis* H_2S bacteria produced anti-vibrio substances which inhibited the growth of 29 *Vibrio* strains by disrupting cell membranes and causing cell lysis. Tepaamorndech et al. (2018) also stated that *Bacillus aryabhattai TBRC8450* isolated from shrimp ponds showed antimicrobial activity against 35 pathogenic strains, including *Vibrio harveyi* and *Vibrio parahaemolyticus*. The presence of anti-Vibrio activity is caused by the production of inhibitory components including organic acids, hydrogen peroxide, and bacteriocins (Jlidi et al*.,* 2022). Additionally, Khan et al. (2021) stated that antibacterial substances not only inhibited the growth of pathogens but also increased host resistance.

In this study, Vibrio strains were used to refer to the occurrence of vibriosis disease outbreaks in fish and shrimp farming. *Vibrio parahaemolyticus* and *Vibrio harveyi* bacteria strains were selected due to frequent association with outbreaks of Acute Hepatopancreatic Necrosis Disease [AHPND] in shrimp cultivation (Muthukrishnan et al*.,* 2019). Meanwhile, *Vibrio alginolyticus* is one of the dominant causative agents of vibriosis outbreaks with high mortality rates in marine fish, shrimp, and shellfish (Bunpa et al*.,* 2020). The presence of antagonism or anti-vibrio activity from *Bacillus* spp. is helpful in treating diseases related to *Vibriosis* (Wang et al*.,* 2020). Based on the results, all *Bacillus* strains obtained in this study had anti-*Vibrio parahaemolyticus* activity. There are 4 strains of *Bacillus* that show anti-*Vibrio harveyi* activity, and 2 strains that show anti-*Vibrio alginolyticus* activity. These results were consistent with previous studies, for example, Basi-Chipalu et al. (2015) found that *Bacillus pseudomycoides* bacteria had antibacterial compound *pseudomycoicidin lanbiotic* capable of inhibiting the growth of *Esteria coli* bacteria. Antibacterial compounds from *Bacillus pseudomycoides* and *Bacillus cereus* are generally used to suppress pathogenic bacteria (Knežević et al*.,* 2021; Haque et al., 2021). According to Vidal et al. (2018), *Bacillus cereus* isolated from the intestines of Vannamei shrimp effectively reduced pathogenic bacteria *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. The level of anti-*vibrio* activity of bacteria is influenced by several factors including the type, number and growth phase, concentration of antimicrobial compounds, temperature and length of incubation time, as well as physico-chemical properties of the substrate namely pH, water content, and surface tension (Frazier & Westhoff, 1981).

The results showed that all *Bacillus* spp. strains isolated were capable of producing different extracellular enzymes. The presence of extracellular enzyme activity importantly contributes in accelerating the absorption of shrimp nutrients (Kurniaji et al., 2023). These enzymes break down complex compounds found in feed such as protein, starch, lipids, and cellulose, into simpler components. This activity maximizes the absorption of nutrients while also increasing shrimp growth performance and feed efficiency (Monier et al., 2023). *Bacillus* spp. bacteria can produce different extracellular enzymes such as protease, amylase, lipase, cellulase, xylanase, chitosanase, and esterase (de Veras et al., 2018), which assist in improving the digestive function of shrimp (Xie et al., 2019). The application of *Bacillus* spp. bacteria in optimizing shrimp growth has been widely carried out. In a study by Wang et al. (2020), lipase, amylase, cellulase and protease enzymes produced by *Bacillus subtilis* and *Bacillus licheniformis* significantly improved the digestive performance of *Panaeus monodon*. Jefri et al. (2020) also stated that activity of the amylase and protease enzymes produced by *Bacillus* spp. increased the survival and growth of Vannamei shrimp. Ziaei-Nejad et al. (2006) added that the use of *Bacillus* spp. bacteria as probiotics increased digestive enzyme activity, as well as survival and growth of *Fenneropenaeus indicus*.

Another potential application of extracellular enzymes produced by *Bacillus* spp. is water quality controller. Bacteria in the pond break down organic material into simpler forms (Hlordzi et al., 2020). Several previous studies stated that *Bacillus* spp. reduced organic matter found in shrimp ponds, leading to better water quality (Dalmin et al*.,* 2001). According to Sonune & Garode (2018) *Bacillus licheniformis* strain KR261405 produced protease and lipase enzymes, which reduced BOD, COD, nitrate, and phosphate in city wastewater. Truong et al. (2021) also stated that *Bacillus subtilis* CM3.1 producing amylase, protease, and cellulase enzymes reduced TAN and NO2-N concentrations in Vannamei shrimp ponds. As stated by Dat et al. (2019), bacteria from the genus *Bacillus* spp. have been widely used to reduce the amount of organic matter, pollutant loads, heavy metals, and pathogens in cultivation ponds. Additionally, enzyme-producing bacteria have been used to improve water quality and remove unwanted substrates from wastewaters, as well as to increase the growth rate and digestibility of cultivated animal feed (Ray et al., 2012).

Extracellular enzymes produced by *Bacillus* spp. in this study included protease, amylase, cellulase, and lipase, with activity index ranging from 0.2 to 2.2. The high activity of extracellular enzymes shows a significant ability to degrade substrates contained in the media (Su et al., 2020). The results showed an increase and decrease in extracellular enzyme activity index along with bacteria incubation period attributed to differences in the exponential phase. According to Rolfe et al. (2012), high extracellular enzyme activity occurs in the exponential phase, when bacteria cells are in optimum conditions for metabolism and reproduction. In this study, the average peak incubation period for producing extracellular enzymes was 24 and 48 hours. According to Islam et al. (2019), the production of extracellular enzymes in *Bacillus*

spp. increased linearly from 24 hours to 48 hours, but was optimal at an incubation time of 24 hours. According to Shrestha et al. (2022) the optimal incubation time for *Bacillus* bacteria to produce extracellular enzymes was 24 hours. Extracellular enzymes obtained from several strains of *Bacillus* bacteria increased with higher incubation time. This is consistent with Gofar et al. (2014) stating that extracellular enzyme activity tends to increase with higher incubation time. The decrease in extracellular enzyme activity index was due to an increase in bacteria colony diameter, but not in the clear zone diameter (Fitriadi et al., 2023). Factors that influence the rise and fall of extracellular enzymes are temperature, growth pH, incubation time, and substrates such as protein, starch, lipid, and cellulose (Saranraj et al., 2017).

In order for probiotics to be effective, pathogenic bacteria should be directly suppressed or indirectly increased through increased nutrient absorption, immunity, and improved water quality (Nayak, 2021). Consequently, probiotics in aquaculture are generally tested for safety, acidity, heat tolerance, and pathogen inhibition (Ruiz-Moyano et al., 2019). As part of the evaluation, probiotics are also evaluated for their ability to release anti-bacterial activity which can enhance water quality (Jiang et al., 2023). In this study, Bacillus spp. The strains obtained had anti-vibrio activity against *Vibrio parahaemolyticus, Vibrio alginolyticus,* and *Vibrio harveyi*. The presence of antivibrio activity indicates that bacillus bacteria are able to inhibit the growth of vibrio bacteria, so it is hoped that this potential can be further developed as probiotic bacteria that are able to inhibit the growth of vibrio bacteria found in shrimp cultivation.

Conclusion

In conclusion, the research results show that each strain of *Bacillus* spp. very effective in inhibiting pathogenic bacteria including *Vibrio parahaemolyticus, Vibrio harveyi,* and *Vibrio alginolyticus* while producing extracellular enzymes, namely amylase, lipase, cellulase, and protease. The highest anti-*Vibrio parahaemolyticus* activity was obtained by *Bacillus cereus* strain EGU510 (5.50±1.0), anti-*Vibrio harveyi* by *Bacillus cereus* strain 24 (4.75±1.71), and anti-*Vibrio alginolyticus* by *Bacillus* sp. strain Z96 (6.00±1.15). Meanwhile, the highest extracellular enzyme activity was obtained by *Bacillus pseudomycoides* strain LU2, which included the enzymes amylase (2.2), protease (1.7), and cellulase (0.9). While the highest lipase enzyme was obtained by *Bacillus cereus* strain EGU510 (1.8). The high anti-vibrio activity and extracellular enzyme activity of the *Bacillus* strains obtained in this study have the potential to be potential probiotic agents that can be developed further.

Authors' Contribution

RF: Writing - Original Draft, Conceptualization. AS: Writing - Original Draft, Methodology. PHTS: Writing - Review & Editing, Data Curation. SBP: Writing - Original Draft. S.S. and S: Writing - Review & Supervision. All authors critically revised the manuscript and approved the final version.

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Competing Interests

The authors declare that they have no competing interests.

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