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Research Article

Exploration and Molecular Identification of Proteolytic Bacteria as Probiotic Candidates from Shrimp Ponds in West Sumatra, Indonesia

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Abstract

Background and Objective: The existence of intensive shrimp aquaculture faces serious challenges in the form of a decrease in pond water quality due to overfeeding. Efforts are needed to improve pond water quality by utilizing proteolytic bacterial isolates to break down suspended or accumulated feed on the pond bottom. The research aims to find proteolytic bacterial isolates from pond sediments and the digestive tract of shrimp (*Litopenaeus vannamei*). **Materials and Methods:** The materials needed are pond sediment samples, shrimp digestive tract, seawater complete agar (SWCA) medium and skim milk agar medium (SMA). The study used survey methods to determine sampling locations and continued with experimental methods in the laboratory. Nine isolates were obtained from pond sediments and two bacterial isolates were from the shrimp digestive tract. **Results:** The proteolytic potency test showed that two isolates from pond sediments and one isolate from the digestive tract of shrimp were positive for proteolytic. The largest proteolytic index value reached 6.357. Molecular identification by analyzing the *16S rRNA* gene sequence shows that PC23 isolate is closely related to the bacterium *Exiguobacterium indicum* strain KR6 with percent identity 99.44-99.58% and PU32 isolate with *Bacillus cereus* strain 125 with percent identity 100%. **Conclusion:** The bacteria obtained can be used as probiotic candidates for the future are *Exiguobacterium indicum* strain KR6 and *Bacillus cereus* strain 125.

Key words: Bacteria, ponds, proteolytic activity, shrimp, water quality

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Vannamei shrimp (*Litopenaeus vannamei*), is a type of aquaculture commodity that has the highest ratio of aquaculture production and various biological advantages compared to several other types of crustaceans¹. Of the total production costs incurred, around 60-70% are expenses for procuring feed. The protein composition in feed is the highest portion, reaching 25-35%. Feed efficiency in shrimp farming activities is minimal. It is estimated that only about 22% of the feed is converted to growth. The rest is suspended or accumulated in the waters as organic waste. The accumulation of organic waste in pond waters has an impact on decreasing DO (dissolved oxygen) values and increasing BOD (Biological Oxygen Demand) values². This condition will disrupt the shrimp's physiological activities, metabolism and growth. In addition, an increase in organic waste in pond waters also spurred the growth of pathogenic microorganisms and the rate of anaerobic decomposition of organic matter³. The decomposition of organic matter in the form of protein by heterotrophic bacteria will produce ammonia, nitrate and nitrite, which are toxic to organisms in the waters. Ammonia is the primary end-product of protein metabolism produced by most aquatic animals and is also generated after the breakdown of organic waste by microbes. An increase in nitrogen levels in shrimp ponds leads to reduced food intake, increased oxygen consumption, elevated nitrogen excretion and changes in protein concentration, resulting in high mortality⁴, thus threatening the sustainability of shrimp farming activities.

For this reason, there is a need to bioremediate pond waters and increase feed efficiency through the use of proteolytic bacteria as bioremediation agents and at the same time as probiotic bacteria to increase feed efficiency. Proteolytic bacteria can be a group of bacteria that produce extracellular protease enzymes to hydrolyze proteins into amino acids. Shrimp production in intensive ponds uses probiotics, this aims to maintain air quality, the bacteria in probiotics can degrade food waste and organic material that settles at the bottom of the pond. These bacteria are expected to live and develop in pond waters by producing protease enzymes so that they can degrade the remaining organic material and makes the pond rich in nutrients⁵. As a biocatalyst, proteases hydrolyze the peptide bonds of proteins, with the resulting compounds depending on the enzymes produced by the bacteria⁶.

Several studies have been conducted on protease bacteria, such as *Rhodovulum sulfidophilum* PS342, which

have potential in shrimp cultivation because they produce good proteolytic enzymes and anti-vibration compounds⁷. *Lactococcus lactis* was also found to increase the proteolytic content from 40.6-94.3%⁸. *Bacillus* had a high enzymatic activity between 24.6-15.9 U/mL⁹. Several previous studies have reported various types of proteolytic producing bacteria from shrimp ponds, for example *Lactobacillus* sp. and *Pseudomonas* sp.¹⁰. The *Bacillus* sp., *Lactobacillus* sp. and *Pseudomonas* sp., has amylolytic, cellulolytic and lipolytic activities in the digestive tract of *Vannamei* shrimp¹¹.

The study aimed to obtain bacterial isolates from pond sediments and shrimp digestive tracts. Furthermore, testing the proteolytic ability of isolates and identifying them molecularly to determine the type.

MATERIALS AND METHODS

Description of the study sites: Ulakan is located at 100°12'36"E; 0°43'12"S, one of the areas on the coast of Padang Pariaman, West Sumatra, Indonesia, which has the potential to become the Center of the Shrimp Aquaculture Industry.

Study area: The research was carried out at the Microbiology Research Laboratory, Department of Biology, The Faculty of Mathematics and Natural Sciences, Andalas University. The study was carried out from April to June, 2022.

Materials and methods: Seawater complete agar (SWCA) and skim milk agar (SMA) medium were taken from Brand of Himedia from India.

Bacterial isolation and testing the proteolytic potential:

Bacterial isolation from pond sediments and shrimp digestive tract using dilution technique and inoculation on seawater complete agar (SWCA) medium by pour plate. Furthermore, the purification of bacterial isolates was carried out². The proteolytic potential of bacterial isolates was tested by growing bacterial isolates on skim milk agar (SMA) medium. The formation of a clear zone around the colony indicates the ability of the bacterial isolates to produce protease enzymes. Furthermore, the colony's and clear zone's diameters were measured. Proteolytic potential isolates are those with a proteolytic index (IP) value of ≥ 2 . The calculation of the IP value uses the formula¹²:

$$IP = \frac{\text{Clear zone diameter} - \text{Colony diameter}}{\text{Colony diameter}}$$

Identification molecular: Identification of bacterial isolates was carried out molecularly on selected bacterial isolates. The identification process began with isolating bacterial DNA isolates using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA). Measurement of the quality and quantity of DNA was analyzed through electrophoresis techniques. The marker was λ DNA (50 ng/ μ L) to determine DNA concentration. Biodrop UV-Vis spectrophotometer (Biodrop Inc., UK) quantifying bacterial genomic DNA. The DNA products whose quality and quantity are known are ready to be amplified. The DNA amplification used KOD Blue Mastermix (Toyobo, Japan) and the primers used were 16S *rRNA*_F (5'AGA GTT TGA TCM TGG CTC AG 3') and 16SrRNA_R (5'AAG GAG GTG WTC CAR CC 3') with an estimated product size of 1.498 bp. Running PCR was carried out for 35 cycles. Some of the PCR products were electrophoresed and visualized using a UV transilluminator (Uritace cambridge, type, UV Table BXT-15.M V1From French).

Most of the other PCR products were stored at -20°C for sequence analysis. The electropherogram obtained from the sequencing was then edited and continued using the SeqMan TM application. Subsequently, BLAST is on the NCBI website¹³. Several bacterial DNA sequence data were selected in the Genbank for alignment, phylogenetic tree construction and determination of genetic distances using the MEGA X program¹⁴. Alignment was performed using the Clustal W algorithm. The phylogenetic tree was constructed using the

Neighbor-joining method¹⁵ and the evolutionary distance was analyzed using the Kimura 2-parameter method¹⁶. The bootstrap value used is 1.000¹⁷. Genetic distances were analyzed using the pairwise distances method.

RESULTS AND DISCUSSION

The results of bacterial isolation from pond sediments and shrimp digestive tract and the environmental conditions of pond waters were described in Table 1.

Table 1 shows the total number of bacterial isolates found from shrimp ponds as many as 11 isolates consisting of; nine isolates from pond sediments and two isolates from the digestive tract of *L. vannamei* shrimp. Environmental factor conditions in shrimp pond waters are pH 7.73-7.89, temperature 27-28°C and salinity 25-30 ppt. The growth of bacterial isolates is strongly influenced by environmental factors such as temperature, pH and salinity. Bacteria that are unable to adapt to environmental conditions will die because their metabolic processes will be hampered¹⁸. Meanwhile, salinity affects the growth rate, the number of generations and the generation time of bacteria¹⁹. The mechanism of bacteria that live at high salinity will adapt to osmotic pressure²⁰.

The proteolytic potential of bacterial isolates was qualitatively carried out on eleven bacterial isolates. The test results showed that three bacterial isolates were indicated as producers of protease enzymes, characterized by forming clear zones around the colonies (Table 2 and Fig. 1).

Table 1: Bacterial isolates found as a result of isolation from pond sediments and the digestive tract of shrimp and environmental factors in shrimp pond waters

Sampling point	Isolate code	Σ Isolate	Abiotic factor		
			pH	Temperature (°C)	Salinity (ppt)
Pool A5	PA5-1	2	7.83	27	25
	PA5-2				
Pool A2	PA2-2	3	7.89	28	25
	PA2-3				
	PA2-4				
Pool C1	PC1-1	2	7.78	28	25
	PC1-2				
Pool C2	PC2-1	2	7.73	27	30
	PC2-2				
Intestines	PU-1	2	-	-	-
	PU32				
Number of isolates		11			

Table 2: Average colony diameter and clear zone, as well as the proteolytic index (IP) of protease enzyme-producing bacteria from *Litopenaeus vannamei* shrimp ponds

Point	Isolate code	Σ diameters (mm)		
		Colony	Clear zone	Proteolytic index (IP)
Pool A2	PA22	1.35	2.20	0.692
Pool C2	PC23	1.40	10.30	6.357
Intestines	PU32	5.77	18.77	2.251

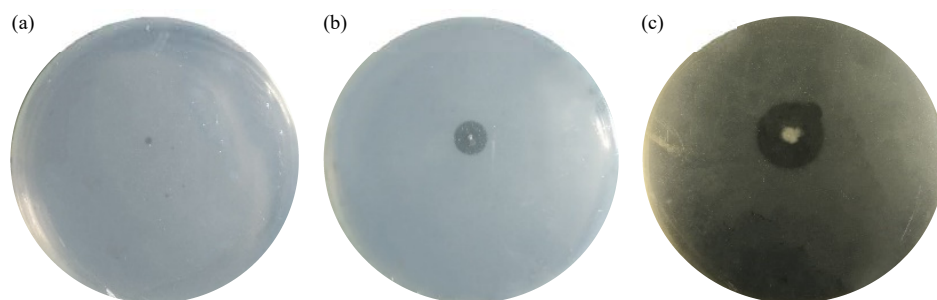


Fig. 1 (a-c): Bacterial isolates are indicated by the proteolytic potential clear zone formation around the bacterial colonies grown on SMA medium, (a) Isolate PA22 (b) PC23 and (c) PU32

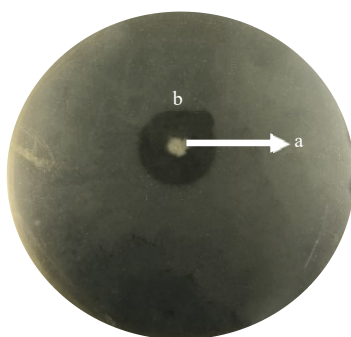


Fig. 2: Bacterial isolates that have proteolytic potential
a: Bacterial colonies and b: Clear zone

Proteolytic potential bacterial isolates were characterized by the formation of clear zones around the bacterial colonies Fig. 2.

The results of the proteolytic activity test obtained two potential bacterial isolates that had a clear proteolytic index value of ≥ 2 . The clear zone formed was the result of the hydrolysis of protein (casein), which is an opaque white colloidal suspension, into derivative compounds that are more soluble and transparent²¹. Proteolytic activity is the ability of bacterial isolates to produce protease enzymes to decompose proteins into amino acids²². The size of the index value is determined by the growth and division of bacteria in the exponential phase. Enzyme production is specific depending on the type of bacteria and environmental factors such as substrate, temperature, pH and others²³. Proteolytic bacterial isolates hydrolyze proteins into peptides and amino acids which dissolve in the medium to form clear zones around bacterial colonies²⁴. Lee *et al.*²⁵ stated that gastrointestinal microbes have enzyme activity to break down complex molecules into simpler ones.

According to Ajitha *et al.*²⁶, bacteria that have proteolytic activity have the ability to produce protease enzymes which

are secreted into their environment. These extracellular proteolytic enzymes then work to hydrolyze protein compounds into oligopeptides, short chain peptides and amino acids. The diameter of the inhibition zone formed can qualitatively indicate the high proteolytic ability of the protease enzymes produced or also the high amount of enzymes produced and released. The existence of this extracellular protease enzyme is very important for the life of bacteria because it provides the need for nitrogenous compounds that can be transported into the cell. The types of bacteria that have the ability to secrete protease enzymes have great potential to be used as cleaning agents for protein contaminants. Shrimp feed is rich in protein so the remaining feed and excreta products also contain high protein. So the presence of this extracellular protease enzyme will break down these protein compounds into simple compounds that can be directly used by bacteria as nutritional components for their growth.

Potential bacterial isolates were identified on isolates with IP values ≥ 2 , namely isolate PC23 and isolate PU32. The DNA isolation in the form of electrophoresis and quantification of the DNA of the two isolates can be seen in Fig. 3 and Table 3.

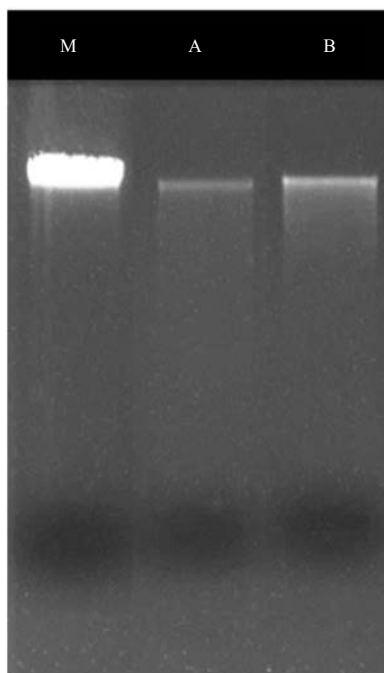


Fig. 3: Visualization of bacterial genome DNA

M: Marker λ DNA (concentration 100 ng/ μ L), A: PCR product of *16S rRNA* gene isolate PC23 and B: PCR product of gene *16S rRNA* isolate PU32

Table 3: Quantification data of bacterial genomic DNA samples

Bacterial isolates code	Concentration (ng/ μ L)	Absorbance (λ A260/A230)	Absorbance (λ A260/A280)
PC23	22	2.204	1.923
PU32	39	2.294	1.857

Figure 3 shows the visualization of bacterial genomic DNA with various band intensities, which also shows the difference in DNA concentration. The higher the band intensity, the thicker the band formed, indicating the higher DNA concentration. The DNA bands obtained from each sample also showed no contaminants in the form of RNA or protein. The DNA purity was measured based on the absorbance ratio of λ 260/230 nm and λ 260/280 nm Table 3.

Purity was measured at absorbance ratios of 260/230 nm and 260/280 nm with ratio values of 2.0-2.2. If the measurement ratio is lower than 2.0 it indicates sample contamination by EDTA, carbohydrates and phenolic compounds. Table 3 also shows that the absorbance values of λ 260/230 are 2.204 and 2.294, this indicates the purity of the bacterial genomic DNA. The DNA with a good level of purity, because it has an absorbance ratio value of λ 260/280 optimal range of 1.7-2.0. If the ratio value is lower, it indicates that the sample contains protein, phenolic compounds and other contaminants. The absorbance value is 260/280 (Table 3), namely 1.857-1.923, belonging to the category of good purity. This was also supported by the

visualization of DNA on the agarose gel in the form of DNA bands that did not appear to show any other bands of contaminants.

Figure 3 shows that the thick and bright DNA bands correspond to an estimated target size of 1,498 bp. But apart from the target band, smears also appear. It is suspected that the *16S rRNA* gene is multicopied in the genome of the bacterial sample resulting in primary attachment in other regions of the genome.

Visualization of the *16S rRNA* gene amplification results of bacterial isolates was shown in Fig. 4.

Isolate PC23: The results of sequencing of PC23 isolates were not contiguous, resulting in sequence editing done separately. Electropherogram of forward preliminary reading results size 500 bp as follows; the BLAST results of sequences with reverse primers showed that the PC23 isolates belonged to the genus *Exiguobacterium*. From 100 sequence data from BLAST results, the query cover value is 100%. The percent identity value ranges from 99.44 to 99.58%. The phylogenetic tree obtained was as follows Fig. 5.

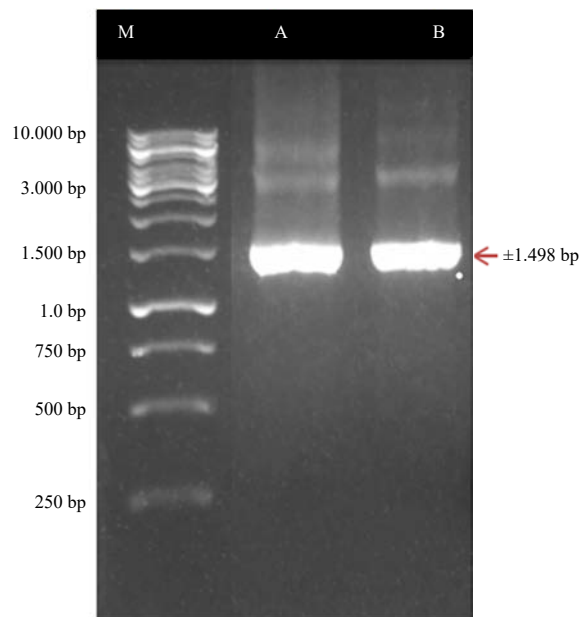


Fig. 4: Visualization of *16S rRNA* gene amplification results of bacterial isolates

M: Marker 1 kb gene ruler (Thermo Scientific, USA), A: PCR product of *16S rRNA* gene isolate PC23 and B: PCR product of gene *16S rRNA* isolate PU32

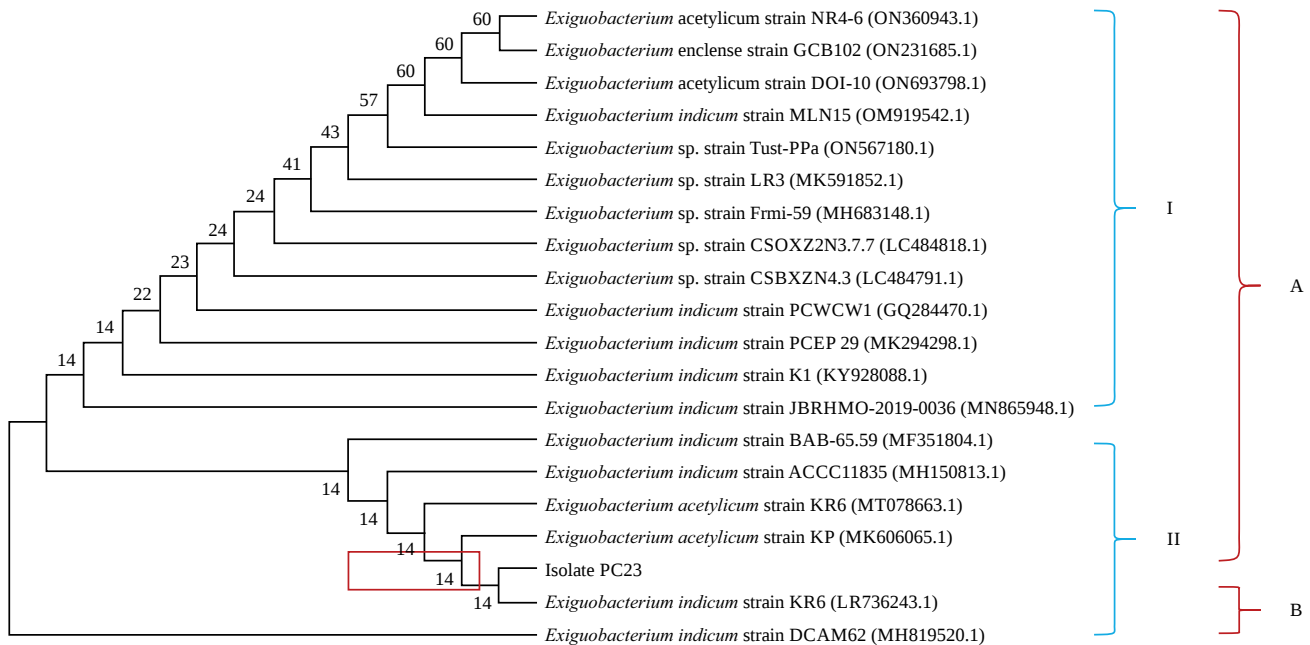


Fig. 5: BLAST results and phylogenetic construction of PC23 prayers

Phylogenetic tree, showing two groups (group A and group B). Group A consists of two sub-groups (A-I and A-II). Bacterial isolate PC23 is in the sub-group A-II. Based on the position on the phylogenetic tree, PC23 isolate is on the same branch as the KR6 strain *Exiguobacterium indicum*

The results of sequencing of PC23 isolates were not contiguous, resulting in sequence editing done separately. Electropherogram of forward primary reading results size 500 bp as follows:

- Forward primer reads is:** CTTAAGCTCTCCCGGAAGGGA
ACGCAGCGACAATGTGAGTAACGACTGGGTAACCTCACC
TGTAAGACTGTGCATAACTCCATTGAAACCGGGTGCTAA
TACATCGATGGTTGTTTGGAGATACATGCGTTCAGACCAGTC

ATGGTGCCGACTGTATGGACTAGCGCAGCTCACGAGCA
TGGCACCTCGCAGATGCGCCTTGCTAGTGCATTGAGTAAT
TGGTGGGGTAAGGGGCCATCCATGCGACAACGCATAAC
GGTACTTGACAGGGTGATGACTGACACTGGGACTGAAAC
ACTGCCCCAAGTCCTACTGGAGGCTCCTCCACGGAATCTT
ACACTCTGGACGAACGTCTGATGGAGCGTGACCACGGGA
TTGATAAAGTTTTCTTCTCTTAAAGGATGATCTAATGCA
AGAACTTAACGAGAGGCATTGCTCGTACCTTGACGGAAC
CCTACGACAACTCCCGGCTAACTACCTCCCACTACCTA
GGTA

Readings were obtained with a sequence of 718 bp. The electropherogram of the *16S rRNA* gene sequencing results is as follows:

- **718 bp base sequence with reverse primer is:**
TGAGGCGCGAAAGGGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGT
TGGGGGGTTTCCGCCCTCAGTGGTGAAGCTAACGCATT
AAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAA
CTCAAAGGAATTGACGGGGACCCGCAAGCGGTGGAG
CATGTGGTTTAATTGAAGCAACGCGAAGAACCTTACCA
ACTCTTGACATCCCTTGACCGCTGAGAGATCAAGTTTTT
CCTTCGGGGACAATGGTGACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGCTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCCTATCCTTAGTTGCCAGCATTGAGTTGGGC
ACTCTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGT
GGGGATGACGTCAAATCATCATGCCCCCTATGAGTTGGG
CTACACACGTGCTACAATGGACGGTACAAAGGGCAGCGA
GACCGCGAGGTGGAGCCAATCCCATAAAGCCGTTCCAG
TTCGATTGCAAGGCTGCAACTCGCCTGCATGAAGTCGGA
ATCGCTAGTAATCGCAGGTGAGCATACTGCGGTGAATAC
GTTCCCGGGTCTGTACACACCGCCGTCACACCACGAG
AGTTTGCAACACCCGAAGCCGGTGAGGTAACCGCAAGG
AGCCAGCCGTCGAAGGT

Isolate PU32: Electropherogram sequencing results with forward primer isolate PU32 obtained 905 bp, while the reverse primer was 1.229 bp. The edited electropherogram of the *16S rRNA* gene sequencing of the bacterial Isolate PU32 had a size of 1.404 bp. The electropherogram of isolate PU32 is as follows: BLAST results of sequences with reverse primers showed that PC32 isolates belonged to the genus *Bacillus*. From 100 sequence data from BLAST results, the query cover value is 100%. The percent identity value ranges from 100%. The phylogenetic tree obtained was as follows Fig. 6.

Electropherogram sequencing results with forward primer isolate PU32 obtained 905 bp while the reverse primer was 1,229 bp. The edited electropherogram of the *16S rRNA*

gene sequencing of the bacterial Isolate PU32 had a size of 1,404 bp. The electrophoregram of isolate PU32 is as follows.

- **Sequence of the 1,404 bp isolate PU32 is:**
GAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTA
ACACGTGGGTAACTGCCCATAAGACTGGGATAACTCCG
GGAAACCGGGCTAATACCGGATAACATTTGAACCGCA
TGGTTCGAAATTGAAAGCGGCTTCGGCTGTCACTTATGG
ATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGG
CTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGT
GATCGGCCACACTGGGACTGAGACACGGCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTCCGCAATGGACGA
AAGTCTGACGGAGCAACGCCGCTGAGTGATGAAGGCTT
TCGGGTCTGTAATACTCTGTTGTTAGGGAAGAACAAGTGC
TAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGTAATAC
GTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAG
CGCGCGCAGGTGGTTTCTTAAGTCTGATGTAAAGCCCA
CGGCTCAACCGTGAGGGTCATTGGAAGTGGGAGACTT
GAGTGCAGAAGAGGAAAGTGAATTCCATGTGTAGCGGT
GAAATGCGTAGAGATATGGAGGAACACCAAGTGGCGAAG
GCGACTTTCTGGTCTGTAAGTACACTGAGGCGCGAAAG
CGTGGGGAGCAACAGGATTAGATACCTGGTAGTCCAC
GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTCCGC
CCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCTGG
GGAGTACGGCCGCAAGGCTGAACTCAAAGGAATTGAC
GGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTCTG
AAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCT
GAAAACCTAGAGATAGGGCTTCTCTTCGGGAGCAGAG
TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCTGA
GATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCCTGAT
CTAGTTGCCATCATTAAAGTTGGGCACTTAAGGTGACTG
CCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAA
TCATCATGCCCCCTATGACCTGGGCTACACACGTGCTACA
ATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAG
CTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTG
CAACTCGCTACATGAAGCTGGAATCGCTAGTAATCGCG
GATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCGTCACACCACGAGAGTTTGAACACCCGA
AGTCGGTGGGGTAACCTTTTGGAGCCAGCCGCTAAGGT

Based on the phylogenetic tree, all bacteria are on the same branch. This is due to the sequence data that make up the *16S rRNA* gene compared to the sequences of the bacterial isolate PU32 and the 20 comparison bacteria from the genbank did not have any differences. This is also supported by data on genetic distance calculations, between bacteria compared to a genetic distance value of 0.00. Therefore, it is certain that the bacteria Isolate PU32 is a bacterium of the *Bacillus cereus* species.

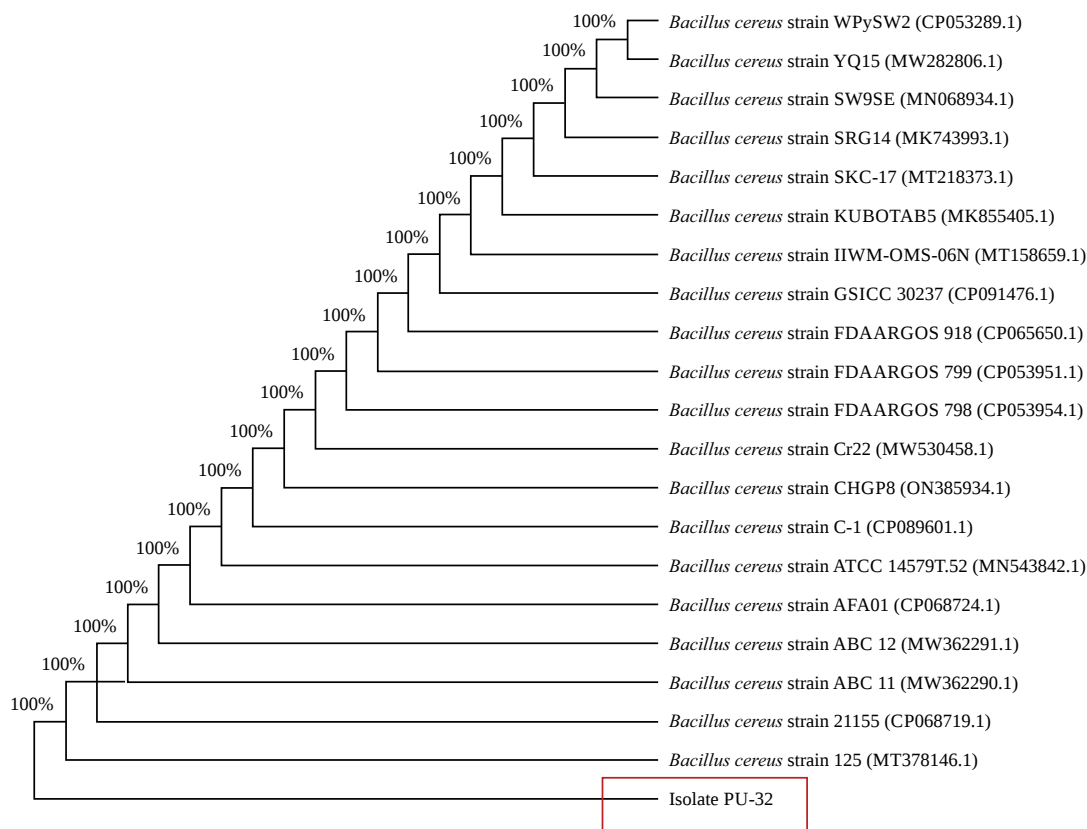


Fig. 6: BLAST results and phylogenetic construction of PU32 isolate

The implication of this research is to provide information about bacteria that can degrade waste containing protein and be used as a component of making probiotics that can be used by sustainable shrimp cultivation businesses to overcome existing problems in ponds. The application of the results of this research is to try directly in enlargement of vannamei shrimp to improve pond water quality. The recommendations from this research are good for the first month during the disease-susceptible period in vannamei shrimp cultivation and the limitations of this research are research funds and the time required for molecular analysis.

CONCLUSION

In this study, two potential proteolytic bacterial isolates were obtained in shrimp ponds, namely; *Exiguobacterium indicum* strain KR6 with a proteolytic index of 6.357 and *Bacillus cereus* strain 125 with a proteolytic index of 2.251. The bacteria obtained are very important for the world of vannamei shrimp cultivation because they can be used as probiotic candidate bacteria so that problems in shrimp cultivation in the future can be resolved and provide benefits to cultivation farmers.

SIGNIFICANCE STATEMENT

This research was carried out because there are many problems faced in the world of vannamei shrimp cultivation, one of which is the problem of diseases in shrimp which are increasingly increasing and resistant, so it is necessary to search for proteolytic bacteria candidates for probiotics that will be used in the shrimp cultivation process so that the production process runs smoothly and do not experience losses due to disease, therefore the selection of proteolytic bacteria in this study can be used to make probiotics for shrimp ponds isolated from the shrimp ponds themselves.

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