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Isolation and Molecular Identification of Termite Nest Associated Bacteria with Potential as Vibriosis Biocontrol Agents in Shrimp Farming

Aldy Mulyadin [a], Tiara Amalia [b++], Siska Haerunnisa [b++], Sunarti [b++], Seniati [b#], Nurhayati [b#], Ifhan Dwinhoven [b†*], Khusnul Khatimah [b†], Ardiansyah [b†] and Amrullah [b†]

[a] Department of Aquaculture, Aquaculture Technology Study Program, Pangkep State Polytechnic of Agriculture, Makassar-Parepare Km. 83, Pangkep 90655, Indonesia.
[b] Department of Aquaculture, Fish Hatchery Technology Study Program, Pangkep State Polytechnic of Agriculture, Makassar-Parepare Km. 83, Pangkep 90655, Indonesia.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The potential for aquaculture development in Indonesia, especially the shrimp farming industry, is projected to continue to increase. However, the current growth in aquaculture production is in line with the increasing number of disease outbreaks and can affect the production, profitability, and sustainability of the industry worldwide. Vibriosis is a major challenge in aquaculture that causes fish mortality in large numbers and rapid times. Vibriosis treatment is still limited to the same

[++] *Student;*
[#] *Laboran;*
[†] *Lecturer;*
**Corresponding author: Email: ifhan.dwinhoven@polipangkep.ac.id;*

antibiotics in humans and animals. For this reason, alternative microorganisms that are more specific and selective in inhibiting vibrio are needed, one of which is the exploration of termite nest association bacteria. This study aims to obtain isolates of termite nest association bacteria that are antivibrio. Isolation of bacteria using ISP2 media. Furthermore, antagonistic screening was carried out against *V. alginolyticus* and *V. harveyi* bacteria. Isolates performed morphological and biochemical characterization followed by molecular identification with 16s rRNA sequence analysis using universal primer 27F 1492R. Isolation results obtained 15 isolates with varying colors and shapes. Ten isolates are Gram-positive bacteria and 12 isolates have a positive catalase test. Seven isolates have antivibrio activity, namely isolates SR9, SE1, SE4, SE5, SE6, SE7 and SE8. Isolate SE1 showed the best antivibrio activity capable of inhibiting the growth of *V. alginolyticus and V. harveyi* bacteria with an inhibition zone value of 3.5 cm and 4 cm, respectively. The results of the molecular analysis showed that isolate SE1 has 99.65% similarity with *Bacillus amyloliquefaciens* strain HY2-1. These bacteria have characteristics that have the potential to be used as probiotics in the aquaculture industry, especially vibrio biocontrol agents.

Keywords: Aquaculture; bacillus; termite; shrimp; vibrio.

1. INTRODUCTION

The aquaculture industry is the fastest growing sector in providing high protein demand [1]. The potential for aquaculture development in Indonesia, especially the shrimp farming industry, is projected to continue to increase. Where Indonesian shrimp export activities 2015-2019 have a positive trend with an increase in volume of 4.21% and an increase in value of 0.55%. This makes Indonesia one of the world's main *P. vannamei* shrimp supplier countries so it is targeted to increase national shrimp production by 250% until 2024 [2].

However, the current growth in aquaculture production goes hand in hand with an increasing number of disease outbreaks and can affect the production, profitability, and sustainability of the industry worldwide. Among the group of pathogenic microorganisms, shrimp diseases such as vibriosis are one of the challenges in increasing production that can cause mass mortality of cultured shrimp, marine and freshwater fish, and shellfish, resulting in economic losses for farmers [3].

Vibriosis caused by Vibrio bacteria can infect freshwater, brackish water, and seawater fish and cause death in large numbers in a short time [4]. Some of them are zoonotic, and even the prevalence of *Vibrio cholerae* infecting humans continues to increase [5]. In the field of aquaculture, the handling of Vibriosis disease is currently still limited and relies on the use of antibiotics, but the antibiotics used are the same types of antibiotics as animals and humans [6]. Inappropriate and uncontrolled use of antibiotics has accelerated the emergence of resistant bacterial strains [7]. *Vibrio* spp. have been resistant to the antibiotics erythromycin, streptomycin and ciprofloxacin [8].

The existence of bacterial strains that are resistant to antibiotics encourages various studies in the exploration of bacteria in producing new antibiotic compounds. Exploration of microorganisms continues to be carried out to find novel sources of new antibiotics that are more specific and environmentally friendly such as fish mucus extracts that are able to fight Vibrio [3] or the exploration of microbes from termite nests that play a role in the synthesis of antibiotic compounds [9].

Termite nests are a very rich source of bacterial strains that produce bioactive compounds. Bacterial isolates from termite nests collected from Pananjung Pangandaran Conservation Area, West Java, Indonesia are able to fight various pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus, Bacillus subtilis*, *Serratia marcescens* and pathogenic fungi such as *Fomitopsis palustris*, *Fusarium oxysporum,* and *Trichoderma* viride [10] The bioactivity test of termite nest-associated bacteria isolate SE1 is known to have bioactive peptide compounds that can inhibit the growth of Vibrio [9]. Therefore, the exploration of termite nest bacteria is expected to be a new alternative source in obtaining new, sensitive, specific and safe vibrio biocontrol bacteria for aquaculture.

2. MATERIALS AND METHODS

2.1 Materials

The equipment used in this study were plastic bags, refrigerators, analytical scales, water

baths, bunsen, micropipettes, petri dishes, test tubes, centrifuge, eppendorf tubes, erlenmeyer flasks, cryotubes, glass objects, shakers, vectors and microscopes.

The materials used were termite nest samples obtained from three different points, test bacteria *V. alginolyticus* and *V. harveyi*. The media used in this study were *International Streptomyces Project-2 (ISP2)*, TCBS agar (*Thiosulfate-Citrate-Bile Sucrose Agar*), *Crystal violet*, *iodine*, *ethanol* 96%, *safranin, chloroform*, disc paper, concentrated sulfuric acid, *methanol,* 70% alcohol, H_2O_2*, distilled water,* nystatin, and ciprofloxacin.

2.2 Procedure

2.2.1 Sampling

Sampling of termite nests was carried out at the Pangkep State Polytechnic of Agriculture with three different source points, namely termite nests attached to walls (isolate code SR), termite nests on board walls (isolate code RE) and termite nests on leaves and twigs of plants (isolate code SE).

2.2.2 Isolation of bacteria from termite nests

Samples that have been dried, cleaned, and mashed are then taken as much as 10 g and given pretreatment, namely the sample is heated in the oven at 50°C for one hour to kill pathogenic bacteria with rapid growth. The samples were then subjected to stratified dilutions of 10^{-1} to 10^{-5}. The microbial inoculation technique used was the spread technique. Each isolate was grown and purified using ISP2 media with a media composition of 4 g/L *yeast extract*, 10 g/L *malt extract*, 4 /L *dextrose* and 20 g/L *bacto agar* dissolved in 1 L of sterile distilled water. Then incubated for 3 days.

2.2.3 Screening antivibrio antagonist test on bacterial isolates

Antivibrio screening against *V. alginolyticus* and *V. harveyi*. The antibiotics rifampicin and tetracycline at a dose of 1 mg/ml were used as positive controls. The test was conducted using paper disks with three replicates. The test bacterial culture of termite nest association isolate was incubated at 37°C for 24 hours and then the inhibition zone formed was observed.

2.2.4 Characterization of termite nest associated bacteria

Bacterial characterization includes direct observation of bacterial morphology (cell shape, cell color) bacterial Gram staining is carried out using a modified method [11]. Characterization of biochemical tests by observing motility and the presence of catalase enzyme was carried out by dripping 3% hydrogen peroxide (H_2O_2) on a clean object glass. Isolates of termite nest-associated bacteria were applied to the object glass that had been dripped with hydrogen peroxide with an ose. The suspension is gently mixed using an ose, positive results are characterized by the formation of air bubbles.

2.2.5 Molecular identification of termite nest associated bacterial isolates

Molecular identification of bacteria was carried out only on selected isolates that had the best antivibrio ability. Molecular analysis was carried out at PT Genetics Science using a modified method [12]. Genomic DNA isolation and amplification with 16S rRNA gene primers (27F and 1492R), DNA extraction using Quick-DNA Magbead Plus Kit (Zymo Research, D4082). PCR amplification using MyTaq HS Red Mix (Bioline, BIO-25048). For sequence alignment analysis, it was carried out by comparing the sequences obtained (query) with those already in the Gene Bank with database searches NCBI internet site (http//www.ncbi.nlm.nih.gov) using BLAST (*Basic Local Alignment Search Tool*).

3. RESULTS AND DISCUSSION

3.1 Characterization of Termite Nest Associated Bacteria

The results of isolation of termite nest-associated bacteria from three different location sample points obtained 15 isolates that were successfully purified. Isolates that grow have different colors, shapes, cell edges, and colony elevations can be seen in Table 1.

Observations on isolates made after 14 days of age show varying colors such as brown, yellow, orange, and white as well as the shape and colonies of isolates. In the gram staining physiology test, ten isolates were Gram positive bacteria and five isolates were Gram negative bacteria. Gram positive are bacteria that have thick peptidoglycan cell walls that will retain the color of crystal violet when washing with 95%

alcohol in the gram staining process. This group of bacteria is widely recommended as an aquaculture probiotic that supports growth or fights pathogens [13].

The results of the catalase test showed that the dominant termite nest association bacteria were catalase positive. There are 13 probiotic candidate bacteria that are catalase positive (+) because of the formation of gas bubbles and have the enzyme catalase while the other 2 isolates are catalase negative (-). The presence of catalase enzyme in bacterial isolates serves to neutralize the bactericidal effect of hydrogen peroxide which will catalyze the decomposition of one of the free radicals, namely hydrogen peroxide into water and oxygen so as to protect cells from oxidative damage [14].

3.2 Antagonistic Test of Termite Nest Association Bacteria against Vibrio Bacteria

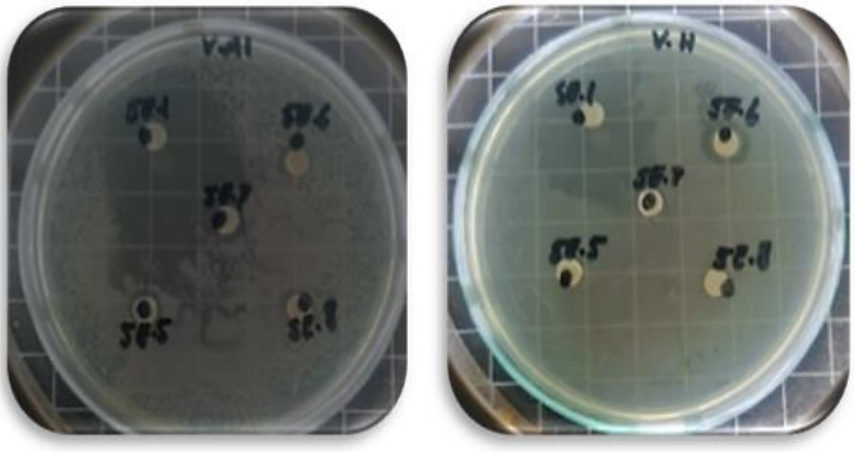
Antagonistic tests against Vibrio bacteria were carried out on *V. harveyi* and *V. alginolyticus* bacteria. There are seven isolates that have antivibrio activity, namely isolates SR9, SE1, SE4, SE5, SE6, SE7 and SE8. Isolates that showed antivibrio activity were dominantly derived from termite nest associations attached to plant twigs and leaves. Isolate SE1, which is very high, is isolate SE-1. Isolates SE1, SE4 and SE5 were known to inhibit both test bacteria.

SE1 isolate is the selected isolate for molecular analysis test, where this bacterium is able to inhibit the growth of *V. alginolyticus* bacteria with an average value of inhibition zone of 3.5 cm and *V. harveyi* with an average value of inhibition zone of 4 cm. Isolate SE1 showed higher antivibrio activity than the positive control of commercial antibiotics, rifampicin. This shows the potential of the isolate to be developed as an antibacterial candidate. The inhibition zone produced is categorized as very strong with an inhibition zone value of >2cm [15].

3.3 Molecular Identification of Selected Isolates Isolate SE1 Termite Nest Associated Bacteria

Molecular identification of SE1 isolate as a potential isolate to inhibit the growth of pathogenic bacteria *V. harveyi* and *V. alginolyticus* was carried out with PCR *(Polymerase Chain Reaction)* technique. *The* molecular testing phase begins with isolating bacterial DNA using the Quick-DNA Magbead Plus Kit. The results of DNA isolation were tested quantitatively and qualitatively to determine the level of purity. DNA extraction results showed a purity value of 2.01. DNA isolation results that have good purity have an A260 / A280 ratio value of 1.8-2.0.

Amplification products of 16S rRNA DNA fragments in PCR are visualized and their quality is visualized using gel electrophoresis. The results of gel electrophoresis of amplification products of 16S rRNA gene fragments of bacterial isolate SE1 can be seen in Fig. 2.



A **B**

Fig. 1. Zone of inhibition of SE1 isolate against *V. harveyi* (A) and *V. alginolyticus* (B)

Table 1. Isolation and characterization results of bacteria associated with termite nests

Code Isolate	Morphology			Gram stain	Enzyme Catalysis	Cell Shape	Zone of antibacterial inhibition	
	Color	Colony type	Elevation				*V. harveyi*	*V. alginolitycus*
SR 1	Chocolate	Circular	Flat	Gram (+)	Catalase (+)	Spherical (*coccus*)	-	-
SR 2	Chocolate	Rhizoid	Embossed	Gram (-)	Catalase (+)	Spherical (*coccus*)	-	-
SR 8	Yellow	Circular	Flat	Gram (+)	Catalase (+)	Stem (*capsule*)	-	-
SR 9	Yellow	Circular	Flat	Gram (+)	Catalase (+)	Stem (*capsule*)	2.5 cm	-
RE 1	White	Irregular	Flat	Gram (-)	Catalase (+)	Stem (*capsule*)	-	-
RE 2	White	Filamentous	Convex	Gram (+)	Catalase (+)	Spherical (*coccus*)	-	-
RE 3	Nila	Irregular	Flat	Gram (+)	Catalase (-)	Stem (*capsule*)	-	-
RE 5	Orange	Spindle	Convex	Gram (+)	Catalase (-)	Stem (*capsule*)	-	-
SE 1	Chocolate	Irregular	Flat	Gram (+)	Catalase (+)	Stem (*capsule*)	4.0 cm	3.5 cm
SE 2	White	Straight	Convex	Gram (-)	Catalase (+)	Spherical (*coccus*)	-	-
SE 4	Yellow	Circular	Convex	Gram (+)	Catalase (+)	Stem (*capsule*)	2.5 cm	1.8 cm
SE 5	Yellow	Circular	Flat	Gram (-)	Catalase (+)	Stem (*capsule*)	0.7 cm	0.8 cm
SE 6	Yellow	Irregular	Flat	Gram (+)	Catalase (+)	Stem (*capsule*)	1.4 cm	-
SE 7	Chocolate	Circular	Flat	Gram (-)	Catalase (+)	Spherical (*coccus*)	-	1.0 cm
SE 8	Chocolate	Circular	Convex	Gram (-)	Catalase (+)	Stem (*capsule*)	0.6 cm	-
K+ Ab							3 cm	3 cm

Table 2. Nanodrop quantification results absorbance value of isolate SE1

Sample	Cons. Nuclet Acid (ng/µl)	A260/280	A260/230	Volume (ng/µl)
SE1	50.7	2.01	1.96	50

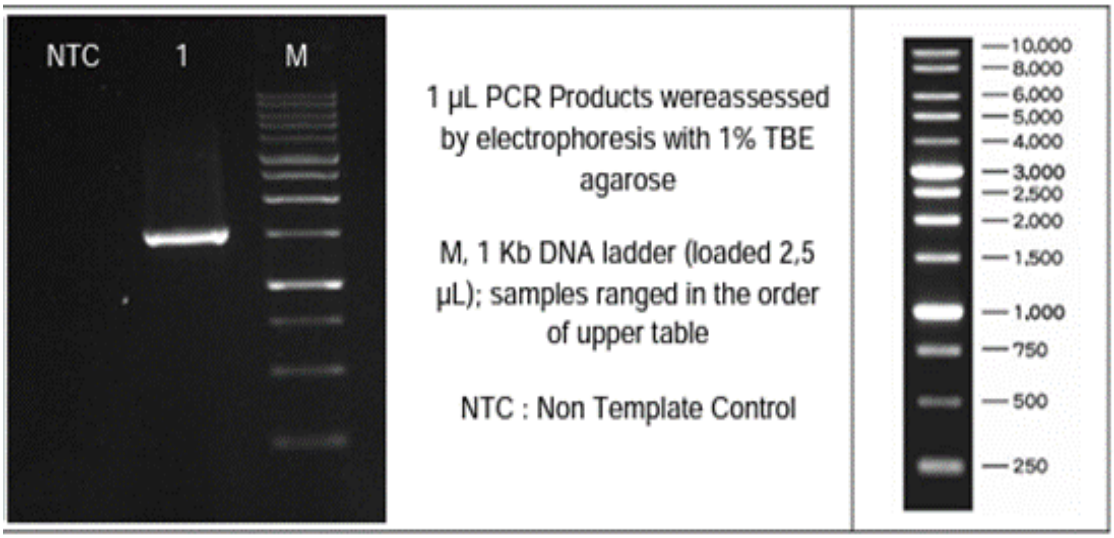


Fig. 2. DNA band electrophoresis results of isolate SE1

Sample isolate SE1 produces a single band with a size of about 1420 bp (*base pair*) in accordance with the value indicated by the DNA marker. The size of this size is in accordance with the expected size of the bacterial 16S rRNA genes of 1500 bp. Determination of the DNA sequence (sequencing) was carried out through the commercial services of 1st Base DNA Sequencing, Jakarta. The sequences obtained from 1st Base DNA Sequencing are:

```
1    TACTGCAGTC GAGCGGACAG ATGGGAGCTT GCTCCCTGAT GTTAGCGGCG GACGGGTGAG
61   TAACACGTGG GTAACCTGCC TGTAAGACTG GGATAACTCC GGGAAACCGG GGCTAATACC
121  GGATGGTTGT TTGAACCGCA TGGTTCAGAC ATAAAAGGTG GCTTCGGCTA CCACTTACAG
181  ATGGACCCGC GGCGCATTAG CTAGTTGGTG AGGTAACGGC TCACCAAGGC GACGATGCGT
241  AGCCGACCTG AGAGGGTGAT CGGCCACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG
301  GAGGCAGCAG TAGGGAATCT TCCGCAATGG ACGAAAGTCT GACGGAGCAA CGCCGCGTGA
361  GTGATGAAGG TTTTCGGATC GTAAAGCTCT GTTGTTAGGG AAGAACAAGT GCCGTTCAAA
421  TAGGGCGGCA CCTTGACGGT ACCTAACCAG AAAGCCACGG CTAACTACGT GCCAGCAGCC
481  GCGGTAATAC GTAGGTGGCA AGCGTTGTCC GGAATTATTG GGCGTAAAGG GCTCGCAGGC
541  GGTTTTCTTA AGTCTGATGT GAAAGCCCCC GGCTCAACCG GGGAGGGTCA TTGGAAACTG
601  GGGAACTTGA GTGCAGAGG  AGAGTGGAAT TCCACGTGTA GCGGTGAAAT GCGTAGAGAG
661  ATGTGGAGGA ACACCAGTGG CGAAGGCGAC TCTCTGGTCT GTAACTGACG CTGAGGACCG
721  AAAGCGTGGG GAGCGAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGAGTG
781  CTAAGTGTTA GGGGGTTTCC GCCCCTTAGT GCTGCAGCTA ACGCATTAAG CACTCCGCCT
841  GGGGAATACG GTCGCAAGAC TGAAACTCAA AGGAATTGAC GGGGGCCCGC ACAACCGGTG
901  GAGCATGTGG TTTAATTCGA AGCAACGCCA AGAACCTTAC CAGGTCTTGA CATCCTCTGA
961  CAATCCTAGA GATAGGACGT CCCCTTCGGG GGCAGAGTGA CAGGTGGTGC ATGGTTGTCG
1021 TCAGCTCGTG TCGTGAGATG TTGGGTTAAG TCCCGCAACG AGCGCAACCC TTGATCTTAG
1081 TTGCCAGCAT TCAGTTGGGC ACTCTAAGGT GACTGCCGGT GACAAACCGG AGGAAGGTGG
1141 GGATGACGTC AAATCATCAT GCCCCTTATG ACCTGGGCTA CACACGTGCT ACAATGGACA
1201 GAACAAAGGG CAGCGAAACC GCGAGGTTAA GCCAATCCCA CAAATCTGTT CTCAGTTCGG
1261 ATCGCAGTCT GCAACTCGAC TGCGTGAAGC TGGAATCGCT AGTAATCGCG GATCAGCATG
1321 CCGCGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCACACCACG AGAGTTTGTA
1381 ACACCCGAAG TCGGTGAGGT AACCTTTATG AGCCAGCCG
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The sequence of nitrogenous bases obtained by sequencing with the BLAST program shows that isolate SE1 has a significant level of homology with several bacterial species contained in the *GenBank* database on the NCBI website where isolate SE1 has a percentage of *similarity* level of 99.65% (Fig. 3) with *Bacillus amyloliquefaciens* strain HY2-1. The percentage of sequence homology with a range of 91% or smaller than 91% is said to be insignificant, the range of 92% to 96% indicates quite significant and the range of 97% to 100% indicates significant.

Phylogenetic tree construction is needed to determine the relationship of SE1 isolates with other species. The topology of the SE1 isolate phylogenetic tree can be seen in Fig. 4.

Bacillus amyloliquefaciens is a gram-positive, non-pathogenic, aerobic, motile bacterium found mainly near the root region of plants. This bacterium is one of the many Bacillus species used as probiotics, providing health benefits to its host where it is capable of producing enzymes viz. cellulase, amylase, and protease, which aid in digestion. *B. amyloliquefaciens is* capable of producing a broad-spectrum antibacterial/ antifungal protein, Baciamin, along with a lipopeptide compound known as Bacillomycin D, which also exhibits antifungal properties [16].

It is similar in appearance to *Bacillus subtilis* and has many homologous genes similar to it. Its cells regularly appear as long chains in contrast to a variety of different Bacillus species that are structured as single cells. The ideal temperature for cell development is between 30 and 40°C. This species is able to survive high temperatures, pressure, and extreme pH conditions [17]. Therefore, this isolate has the potential to be used as a probiotic in the shrimp farming industry, especially as a biocontrol for Vibriosis.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Bacillus amyloliquefaciens strain HY2-1 16S ribosomal RNA gene, partial sequence	2591	2591	100%	0.0	99.65%	MZ709015.1
Bacillus sp. (in: Bacteria) strain LRB-5 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.58%	MN726441.1
Bacillus sp. (in: Bacteria) strain QSB-6 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.58%	MN726440.1
Bacillus amyloliquefaciens strain BH5 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.58%	MN174660.1
Bacillus amyloliquefaciens strain BH4 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.58%	MN174659.1
Bacillus velezensis strain LHSB1 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.58%	MN044879.1
Bacillus siamensis strain HBUM07072 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.58%	MF662498.1
Bacillus sp. (in: Bacteria) strain SXAUPHD-B12 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.58%	MG645387.1
Bacillus sp. (in: Bacteria) strain SXAUPHD-B3 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.58%	MG645386.1
Bacillus siamensis strain G3-6 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.58%	KY401429.1

Fig. 3. DNA sequencing analysis results of SE1 isolate

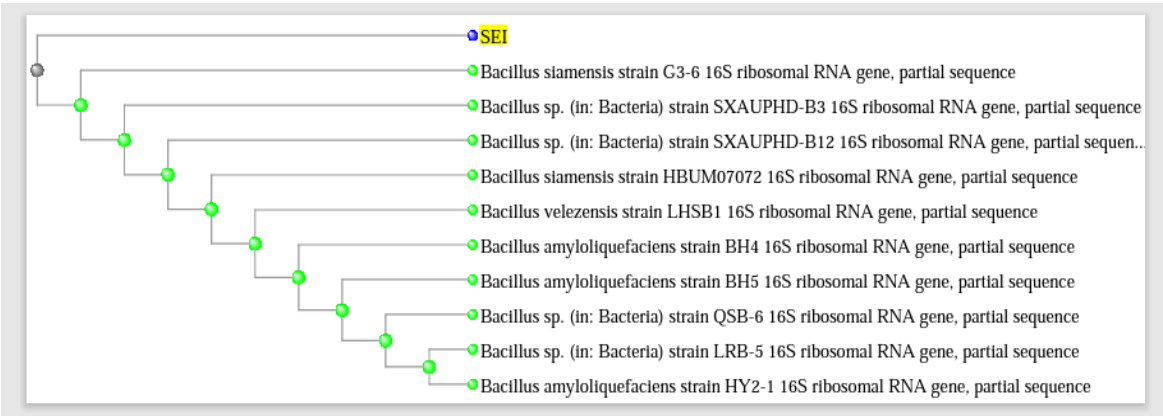

Fig. 4. Phylogenetic tree of isolate SE1 based on 16S rRNA sequence comparison

4. CONCLUSION

Based on the research conducted, it can be concluded that isolate SE1 is a termite nest association isolate that can significantly inhibit the growth of *V. alginolyticus* and *V. harveyi bacteria*. The results of molecular analysis showed that SE1 isolate has 99.65% similarity with *B. amyloliquefaciens* strain HY2-1. These bacteria have characteristics that have the potential to be used as probiotics in the aquaculture industry, especially vibrio biocontrol agents.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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