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Bioactive Compounds in Nest-Associated Bacteria Termites and Their Bioactivity as Antivibrio in Aquaculture Industry

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Abstract

Vibriosis is a major problem in aquaculture that causes large numbers and rapid mortality of fish. Vibriosis treatment is still limited to the same antibiotics in humans and animals. This study aims to obtain bacterial isolates from termite nests that are antivibrio and determine the profile of the antivibrio bioactive compounds. Bacterial isolates were obtained from three types of termite nests. All isolates were cultured on ISP media and then characterized and conducted *bioassay* tests against Vibrio *alginolyticus* and Vibrio *harveyi*. Secondary metabolite fermentation products from selected bacterial isolates were extracted using ethyl acetate and detection of bioactive compounds by Thin Layer Chromatography (TLC) method. The isolation results obtained 15 isolates, 10 Gram-positive bacteria namely isolates SR1, SR2, SR8, SR9, RE2, RE3, RE5, SE1, SE4, SE6 while the other 5 isolates are Gram-negative bacteria. Antivibrio screening obtained 7 isolates that have antivibrio activity. The strongest antivibrio activity is SE1, which was able to inhibit the growth of V. *alginolyticus* bacteria with an inhibition zone of 3.5 cm and V. *harveyi* with an inhibition zone value of 4 cm and was also positive for catalase. TLC showed that SE1 contained peptide compounds with purple color changes after dyeing using ninhydrin.

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Introduction

Indonesia is a major contributor to aquaculture products in the world with a production volume of 14.8 million tons with two million fish farmers and is a source of protein food for the people of Indonesia and even the world (FAO, 2022). One of the main and excellent aquaculture products is the shrimp commodity. In this case, the government consistently encourages the acceleration of shrimp production to achieve a target of two million tons by the end of 2024 with an increase in export value of up to 250% (KKP, 2021).

However, shrimp farming activities can have a major impact on ecosystem changes in the environment. Semi-intensive and intensive technologies with high stocking and feeding densities (Zaenab et al, 2022) lead to high levels of waste suspended or deposited on the bottom of the pond, which causes degradation of water quality (Khatimah et al., 2023;

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Ritonga, 2021). This can accelerate various problems such as disease problems, viruses, and pathogenic bacteria that can cause mass mortality (Jamal et al., 2019).

Vibriosis caused by Vibrio bacteria is a major concern in aquaculture because these bacteria can infect freshwater, brackish water and marine fish and even humans. Vibriosis is developing rapidly as global warming increases the temperature in the waters (Trinanes & Martinez-Urtaza, 2021). In the field of aquaculture, the handling of vibriosis is currently still focused on the use of antibiotics, but the antibiotics used are the same types of antibiotics as animals and humans (Guo et al., 2022). Inappropriate and uncontrolled use of antibiotics has accelerated the emergence of resistant bacterial strains. *Vibrio spp*. have been resistant to the antibiotics erithromicin, kanamicin, pencillin G and streptomicin and are sensitive to oxytetracycline, norfloxacin and ciprofloxacin (Castello et al., 2022).

Exploration of microorganisms continues to be carried out to find novel sources of new antibiotics that are more specific and environmentally friendly, one of which is the exploration of microbes from termite nests with the detection of bioactive peptide compounds that play a role in the synthesis of antibiotic compounds. 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 *Eschericia coli*, *Staphylococcus aureus, Bacillus subtilis*, *Serratia marcescens* and pathogenic fungi such as *Fomitopsis palustris*, *Fusarium oxysporum,* and *Trichoderma viridae* (Putu et al., 2018). Therefore, the exploration of termite nest bacteria is expected to be a new alternative source in obtaining new, specific and safe antibiotics for aquaculture.

Materials and Methods

Isolation of Bacteria from Termite Nests

Samples that have been mashed are then taken as much as 10 grams and then given pretreatment, namely the sample is heated in the oven at 50 ° C for one hour to kill *fast-growing* bacteria. The samples were then subjected to stratified dilutions of 10^{-1} to 10^{-5}. The microbial inoculation technique used was the spread technique on ISP2 media.

Antivibrio Bioactivity Screening on Actinobacteria

Screening of actinobacteria for antivibrio bioactivity against *V. alginolyticus* and *V. harveyi*. The antibiotics rifampicin and tetracyclin at a dose of 1 mg/ml were used as positive controls. The test was performed using paper disks made in triplicate. The test bacterial culture was incubated at 37°C for 24 hours and then the inhibition zone (clear zone) formed.

Identification of Actinobacteria Isolates

Bacterial identification is only carried out on selected isolates that have the best antivibrio ability. The stages of bacterial identification include morphological observations (cell shape, cell color, Gram staining) biochemical tests (motility and catalase).

Metabolite Production and Extraction

Scale-up fermentation of secondary metabolites from selected bacterial isolates was carried out for three days and placed on an orbital shaker. The fermentation results were then centrifuged to separate the cells and supernatant at 4500 rpm for 15 minutes. The fermentation results were extracted using ethyl acetate solvent. The solvent was then removed using a rotary evaporator at 90 rpm, 253 nmbr vacuum, and 35°C.

Detection of Bioactive Compounds from Selected Isolates

Development of Thin Layer Chromatography (TLC) using stationary phase is aluminum silica gel plate and mobile phase using a mixture of solvents n-hexane: methanol (9:1) and (8:2). The TLC plate was then dried and observed under UV light with wavelengths of 254 nm and 366 nm and then developed in eluent, dipped in ninhydrin reagent and H_2SO_4 and then heated on a hot plate at 110°C for 15 minutes until a color change occurred. Ninhydrin reagent is used to detect the content of peptide compounds and universal reagent H_2SO_4 other bioactive compounds.

Results and Discussion

Sample Collection

Termite nest sampling was carried out at the Pangkep State Agricultural Polytechnic campus. Sampling was carried out at 3 different locations. Based on the location of the nests at the 3 sampling locations, it shows that there are 2 types of termite nests, namely *arboreal mounds* attached to tree branches and tree trunks and *subterranean nests,* which nest in the ground/mounds of soil forming hills. Location of termite nests are divided into several types including *aboreal mounds / arboreal nests* associated with trees, *wood nesting in* dead wood/dead trees, and *subterranean nests* that nest in the ground/mounds of soil forming hills. While the termite *Longipeditermes longipes* (1 colony) has a *subterranean nest* type. *Subterranean* nests have a stronger construction than *arboreal mounds* and *wood nests* because they are made of soil material attached with salivary fluid. In addition, it also allows termites to build large nests because the main materials for making nests are easily available (Ervany et al. 2019).

Figure 1. Three types of termite nest sample

Bacteria Isolation from Termite Nests

Isolates that grow from the dilution of samples at several different locations and have different appearances are purified to obtain pure isolates. This was done by transferring each isolate that grew during isolation into 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. A total of 15 bacterial isolates were successfully purified on ISP2 media. The isolates that grew had different colors, shapes, edges, and elevations of the colonies. This can be used as an initial parameter for morphological characterization because the results show the differences produced by each isolate (Table 1). Observations on isolates after 14 days of age showed varying colors, mainly brown, yellow, orange, and white. In the gram staining physiology test, 10 isolates were gram-positive bacteria and 5 isolates were gram-negative bacteria.

Table 1. Morphology and Biochemical characterization of bacteria isolated from termite nest

Code Isolate	Morphology			Gram staining	Catalese	Cell type	
	Color	Edge	Elevation			Shape	Coupling type
SR 1	Brown	Wavy	Flat	Gram (+)	Catalase (+)	Spherical (*coccus*)	Group
SR 2	Ash brown	Straight and fibrous	Embossed	Gram (-)	Catalase (+)	Spherical (*coccus*)	Single
SR 8	Yellow	Transparent white	Flat	Gram (+)	Catalase (+)	Stem (*capsule*)	Single
SR 9	Yellow	Fiber	Flat	Gram (+)	Catalase (+)	Stem (*capsule*)	Teaming up
RE 1	White	Wavy	Flat	Gram (-)	Catalase (+)	Stem (*capsule*)	Single
RE 2	White	Filamentous	Convex	Gram (+)	Catalase (+)	Spherical (*coccus*)	Single
RE 3	Nila	Serrated	Flat	Gram (+)	Catalase (-)	Stem (*capsule*)	Single
RE 5	Orange	Slippery round	Convex	Gram (+)	Catalase (-)	Stem (*capsule*)	Chain
SE 1	Old Cream	Fiber	Flat	Gram (+)	Catalase (+)	Stem (*capsule*)	Teaming up
SE 2	Ivory white	Straight	Convex	Gram (-)	Catalase (+)	Spherical (*coccus*)	Single
SE 4	Pale yellow	Straight	Convex	Gram (+)	Catalase (+)	Stem (*capsule*)	Teaming up
SE 5	Pale yellow	Wavy	Flat	Gram (-)	Catalase (+)	Stem (*capsule*)	Single
SE 6	Yellow	Wavy	Flat	Gram (+)	Catalase (+)	Stem (*capsule*)	Teaming up
SE 7	Thick cream	Thin wave	Flat	Gram (-)	Catalase (+)	Spherical (*coccus*)	Single
SE 8	Cream	Wavy	Convex	Gram (-)	Catalase (+)	Stem (*capsule*)	Single

Antivibrio Bioactivity Screening on Isolate Bacteria

Based on the research that has been done, there are a total of 15 pure isolates that have been successfully inoculated from termite nest samples, but only 7 isolates have antivibrio activity. Where 4 isolates can inhibit the growth of *V. alginolyticus* bacteria and there are 6 isolates that can inhibit the growth of *V. harveyi.*

Table 2. Antivibrio activity of selected isolates

Isolate Code	Zone of Bacterial Inhibition					
	V. alginolyticus			*V. harveyi*		
	Vertical	Horizontal	Average	Vertical	Horizontal	Average
SR 9	-	-	-	2 cm	3 cm	2.5 cm
SR 9	-	-	-	0.6 cm	0.6 cm	0.6 cm
SE 1	3 cm	4 cm	3.5 cm	4 cm	4 cm	4 cm
SE 1	2 cm	1.5 cm	1.75 cm	3.5 cm	2.4 cm	2.95 cm
SE 4	1.7 cm	1.9 cm	1.8 cm	2.5 cm	2.5 cm	2.5 cm
SE 4	0.7 cm	0.7 cm	0.7 cm	1.9 cm	1.4 cm	1.65 cm
SE 5	0.8 cm	0.8 cm	0.8 cm	0.7 cm	0.7 cm	0.7 cm
SE 5	0.6 cm	0.6 cm	0.6 cm	0.6 cm	0.6 cm	0.6 cm
SE 6	-	-	-	1.5 cm	1.4 cm	1.45 cm
SE 6	-	-	-	1 cm	1.2 cm	1.1 cm
SE 7	1.1 cm	1 cm	1.05 cm	-	-	-
SE 7	0.7 cm	0.7 cm	0.7 cm	-	-	-
SE 8	-	-	-	0.6 cm	0.6 cm	0.6 cm
SE 8	-	-	-	0.5 cm	0.5 cm	0.5 cm
Control (+)	3 cm	3 cm	3 cm	1 cm	1 cm	1 cm
Control (+)	3 cm	3 cm	3 cm	1 cm	1 cm	1 cm
Control (-)	-	-	-	-	-	-
Control (-)	-	-	-	-	-	-

In 7 isolates that have antivibrio activity. SE-1 isolate was 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. SE 1 isolate showed higher antivibrio activity than commercial antibiotics, namely rifampicin. This is a very potential result to be developed as an antibacterial candidate (Schinke et al. 2017).

Bioactive Compounds Detection from Selected Isolates

Isolate SE1 extract was tested by TLC using specific reagents to determine the components contained in the isolated extract. Ninhydrin reagent is used to detect the content of peptide compounds p-anisaldehyde reagent terpenoid compounds universal reagent H_2SO_4. Based on the research that has been done, the detection results of bioactive compounds from isolate SE1 contain peptide compounds marked by purple color changes and fluorescent at UV 254nm. The staining of the TLC plate using ninhydrin reagent on the extract of the bacterial isolate shows the consistency that the bacterial isolate is positive for peptide compounds where the color change has shown changes to purple color (Friedman, 2004). Amino acid staining using ninhydrin reagent will show a wide variety of colors with a dominant mauve color shown by 15 amino acids such as alanine, arginine, glutamine, glutamic acid, histidine, isoleucine, leucine, methionine, norleucine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine. The amino acid 2,3 -diamino propinic acid (DAP) shows a blue appearance. The amino acids asparagine, glycine, proline, hydroxyproline, gly-gly-gly tripeptide, ser-gly dipeptide, and ala-ala dipeptide showed yellow discoloration while lysine shows a brown appearance Scheiner et al. 2002).

Conclusion

The results of the research that has been done can be concluded that isolate SE1 is the selected isolate, significantly able to inhibit the growth of bacteria V. alginolyticus and V. harveyi. TLC results showed that the SE1 bacterial isolate isolated from termite nests contained peptide compounds with a mauve color change after dyeing using ninhydrin. Therefore, this isolate has the potential to be used as a biocontrol for vibriosis in the aquaculture industry.

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