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Original Research Article

Antibacterial Activity of Endophytic Bacterial Extracts Isolated from Pineapple Peel (*Ananas comosus* L.)

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ABSTRACT

Endophytic bacteria are known to contain various bioactive substances. Pineapple peel (*Ananas comosus* L.) is a waste product utilized in environmental preservation. The present study is focused on the isolation of endophytic bacteria from pineapple peel, and the assessment of their antibacterial activity. The ethyl acetate extracts of isolated bacteria were tested for their antibacterial activity using the disc diffusion method. The components of the bacterial extract were identified by UPLC-MS/MS analysis. Extract of the endophytic bacteria (*Bacillus velezensis* WSM-1) had the highest activity with an inhibition zone diameter of 17.71 mm and 15.87 mm against *Staphylococcus aureus* and *Escherichia coli*, respectively. UPLC MS/MS analysis identified several compounds, including; 6-Methoxyquinoline, 2-(6-amino-9*H*-purin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol, Aklavin, Maltotriose, Cholestanetriol, Piperic acid, Isoflavone, Paromomycin, and Mycaloide B. Paromomycin is known to have antibacterial activity. It is a type of antibiotic that belongs to the aminoglycoside class and is commonly used to treat infections caused by gram-negative bacteria. The results of the present study suggest that the endophytic bacterial isolate from pineapple peel have potential antibacterial activity and there is a need for further study to isolate and characterize the antibacterial active constituents.

***Keywords*:** Endophytic bacteria, Antibacterial activity, Pineapple peel, *Ananas comosus* L.

Introduction

Endophytes, which are microorganisms living within plants' tissues, are known for their ability to produce a diverse array of bioactive metabolites. These metabolites have been shown to exhibit a wide range of biological activities and are classified into various categories, including steroids, lactones, alkaloids, terpenoids, phenolic compounds, quinones, lignans, and more.[1] The relationship between endophytes and their host plants is influenced by genetic factors and environmental conditions.[2,3] The endophyte-plant interaction is a complex interplay between the two organisms. The endophyte sometimes provides beneficial services to the host plant, such as enhancing nutrient uptake, promoting growth, and protecting against biotic and abiotic stresses.[2,3] Moreover, the degree of dependence of endophytes on their host plant varies depending on the endophyte type. Obligate endophytes, for example, rely completely on their host plant for their growth and survival. In contrast, facultative endophytes can shift between living within plant tissues and in the soil.[3] This biphasic lifestyle allows them to take advantage of both the resources available within the plant and those found in the soil.

Compounds produced by endophytes have many advantages, such as having antibacterial activity. The active compound of plant endophytic microorganisms frequently has a more potent action than that produced by their host plants. As a result, the capacities of the active metabolites of plant endophytic microbes should be investigated.[4-5]

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As the prevalence of bacterial resistance continues to increase, there is a pressing need to identify novel sources of antimicrobial agents that possess greater efficacy and can combat this growing public health concern. To address this issue, research on the isolation of endophytic bacteria has become an increasingly popular area of investigation, as these microorganisms are known to produce a diverse range of bioactive metabolites that have the potential to serve as potent antimicrobial agents.

Despite the promising potential of endophytic bacteria, research on their isolation from various plant sources remains limited. Previous research has shown that various concentrations of ethanol extracts from pineapple peel can inhibit the growth of *E. coli* and *S. aureus*.[7] Specifically, to the best of our knowledge, no studies have been conducted on the isolation of endophytic bacteria from pineapple peel, a readily available agricultural waste product. Investigating the endophytic bacteria found in pineapple peel can potentially lead to the discovery of novel bioactive compounds that exhibit strong antimicrobial activity.[8] Therefore, the present study seeks to isolate the endophytic bacteria from pineapple peel, investigate their antibacterial activity, and identify the constituents from the extract of the bacterial isolate. Research on the isolation and characterization of endophytic bacteria from pineapple peel can potentially lead to the discovery of novel bioactive compounds that can serve as potent antimicrobial agents, contributing to the development of more effective treatments against bacterial infections and aiding in the fight against bacterial resistance.

Materials and Methods

Sample Preparation and Surface-Sterilization

The plant sample (*Ananas comosus L.*) was collected from Kuranji in West Sumatera's Padang city on November 15th, 2021. The sample was identified and authenticated at the Andalas University Herbarium with the voucher number 518/K-ID/ANDA/XI/2021. The peel was carefully removed, and was sterilized by immersion in 1% NaOCl for 2 min, then 70% alcohol for 2 min, and finally rinsed with sterile H_2O (5x). A 100

µL sample of the sterilized peel rinse was spread on a nutrient agar (NA) medium to confirm the sterilization procedure. The NA medium was then incubated at 37°C for two days, and the presence or absence of microbial growth was assessed.[6]

Isolation of the Endophytic Bacteria
The sterile pineapple peel was cut to 1×1 cm size, and then grown on Nutrient Agar (NA) medium. Bacteria that grow around the pineapple peel were purified by taking the growing colonies one by one and inoculate them on a new NA media until pure isolates were obtained. The colony colour and surface texture were observed.

Fermentation of the Endophytic Bacteria
Nutrient broth (NB) media was used to ferment the pineapple peel endophytic bacteria. Fermentation was carried out on a shaker incubator for 24, 48, 72, and 96 hours at a temperature of 37℃ and a speed of 120 rpm. The fermented bacteria were centrifuged at 5000 rpm for 15 min, then the sediment and substrate were separated by pipetting.

Extraction of the Endophytic Bacteria
The substrate at each incubation time was then extracted using ethyl acetate by maceration. Thereafter, the extracts were evaporated to dryness. The yields of the extracts were determined. The extracts were divided into two portions; one portion was used for antibacterial tests, and the other was subjected to Liquid Chromatography coupled mass spectrometry (UPLC-MS/MS) analysis to identify the compounds present in the extracts.

Antibacterial assay by Kirby-Bauer Disc Diffusion Method
The ethyl acetate extracts of the bacterial substrate at the different incubation time (24, 48, 72, and 96 hours) were examined for their antibacterial activity against *Staphylococcus aureus* ATCC 251577 and *Escherichia coli* ATCC 25922 using pathogenic microorganisms. Each ethyl acetate extract was placed on a 6-mm sterile paper disc (Advantec®) in 10 µL quantities. The discs were then placed on Nutrient Agar (Merck®) containing 0.5 McFarland of the bacterial suspension. A positive control was set up using Chloramphenicol (Oxoid®) at concentration of 30 µg/mL, while DMSO was used as the negative control by inoculating 100 µL on to the paper disc. Each treatment was replicated three times, and the Petri dishes were incubated at 37°C overnight. Antimicrobial activity was indicated by clear zones in the media, and the diameters of inhibition zones were measured.

LC-MS/MS analysis
The extract from *Klebsiella* sp. Was used for this analysis. The extract was dissolved in ethanol, then a 5 µL quantity was injected into the LC-MS/MS instrument using a microsyringe. The stationary phase was C18, with a column size of 1.8 µm, 2.1 × 100 mm. The mobile phase/eluent used was a combination of water: formic acid (99.9:0.1) (v/v) and acetonitrile:formic acid (99.9:0.1) (v/v) with a flow rate of 0,2 mL/min. The system running time was 23 minutes. Mass data analysis was done using Masslynx 4.1 software. Parent ion and daughter fragments were compared through several MS library websites such as MassBank, ChemSpider, and HMDB (Human Metabolome Database).

Statistical analysis
The antimicrobial analysis was done in triplicate and the results were expressed as mean ± Standard Deviation.

Results and Discussion

In this study, three isolates of endophytic bacteria; *Bacillus velezensis* strain WSM-1, Bacillus sp.1 and Bacillus sp.2 were successfully obtained from pineapple skin (*Ananas comosus* L) with different shapes, colours, and textures (Figure 1).
Bacillus velezensis strain WSM-1 has milky white colony colour, slightly protruding surface, and smooth texture. In contrast, *Bacillus sp*.1 has yellowish-white colony colour, wavy edges, and slightly dry texture, while *Bacillus sp*.2 has yellowish-white colony colour, root-like shape (rhizoid), wavy edges, slightly dry texture, and adheres firmly to the media.[7,8] The optimum growth time as revealed by fermentation results was at 72 hours, which was marked by the largest dry cell biomass of bacteria formed (Table 1).
The optimal time for bacterial growth is 96 hours. At that time, the bacteria generally have reached the stationary phase and have begun to produce secondary metabolites. The stationary phase of bacterial growth can be reached at a time < 96 hours or at a time > 96 hours. However, not all bacteria can grow optimally at that time.[9]
The results of the antibacterial activity test showed that the extract from *Bacillus velezensis* strain WSM-1 exhibited the highest antibacterial activity compared to other extracts. Specifically, it showed potent activity against both *S. aureus* and *E. coli* with inhibition zone diameter (IZD) of 17.71 mm and 15.87 mm, respectively after a 72-hour incubation period. In addition, the extract from *Bacillus sp.1* and *Bacillus sp.2* demonstrated strong activity against *S. aureus* with IZD of 12.08 mm and 12.25 mm, respectively, and *E. coli* with IZD of 12.18 mm and 12.27 mm, respectively) after 72 hours (Table 2).
It is interesting to note that Bhore and Sathisha's study classified bacterial growth inhibitory activity into four categories: very strong (≥ 20 mm), strong (10-20 mm), moderate (5-10 mm), and weak (≤ 5 mm). Based on this classification, the extract from *Bacillus velezensis* strain WSM-1 can be categorized as very strong, while the extract from *Bacillus sp*.1 and *Bacillus sp.2* can be classified as strong. Overall, these findings suggest that the endophytic bacteria present in host plants can vary in their genera and species, which can be influenced by plant growth conditions such as soil conditions.[12] Furthermore, the extract from *Bacillus velezensis* strain WSM-1 exhibited potent antibacterial activity, making it a potential candidate for further research into natural antibacterial agents.[10]

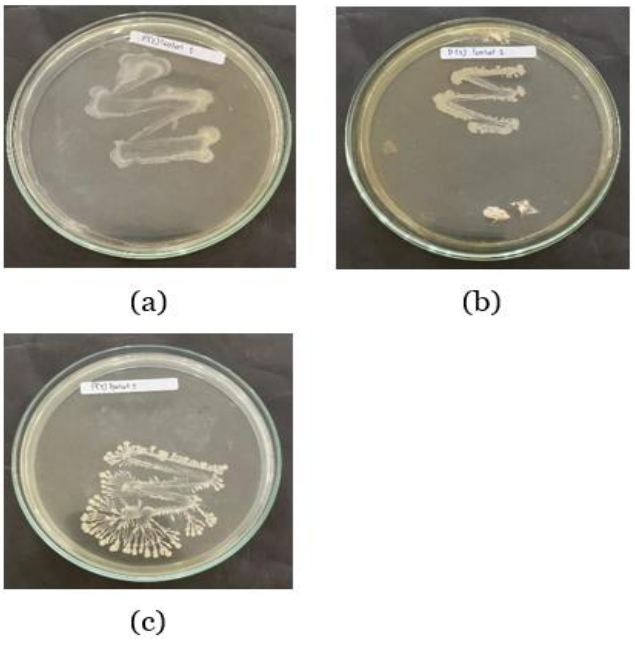

Figure 1: Morphology of endophytic bacteria isolated from pineapple peel (*Ananas comosus* L) (a) *Bacillus velezensis* WSM-1 (b) *Bacillus* sp.1 (c) *Bacillus* sp.2

Table 1: Yield of bacterial dry cell biomass after fermentation

Culture time (h)	Weight (g)		
	***Bacillus velezensis* WSM-1**	***Bacilllus* sp.1**	***Bacillus* sp.2**
24	0.05	0.07	0.08
48	0.12	0.15	0.26
72	0.15	0.22	0.28
96	0.13	0.19	0.24

The ethyl acetate extract from *Bacillus velezensis* strain WSM-1 bacterial isolates was further separated and analyzed using UPLC-MS/MS. From the chromatogram (Figure 2), 25 peaks were observed out of which only nine compounds were identified from MS database library by comparing parent and daughter ions (Table 3). Parent ion and daughter fragments were compared through several MS library websites such as MassBank, ChemSpider, and HMDB (Human Metabolome Database).

The identified compounds as shown in table 3 have fascinating history. For example, it is believed that aklavin compounds have the potential to be developed as an antibiotic.[11] These compounds were identified at a retention time of 3.96 minutes and m/z $[M+H]^+$ 570.2716. They have been shown to possess remarkable efficacy against various bacteriophages and gram-positive bacteria, fungi, and viruses.

Another noteworthy compound is paromomycin, an aminoglycoside that exhibits potent antibacterial activity against both Gram-negative and Gram-positive bacteria, along with certain protozoa and cestodes. Although it is no longer an antibiotic, it was approved in India in 2007 as a cost-effective and well-tolerated treatment for visceral leishmaniasis.[12] However, it is important to note that identifying these compounds is only based on a comparison with library data. Therefore, further investigation is needed to isolate and identify the specific compounds responsible for the activity. By so doing, the exact chemical composition of the bacterial isolate can be determined, and this will shed light on the potential use of these compounds for future research and development of new drugs.[13]

Conclusion

There are potential benefits of metabolites produced by endophytic microorganisms from pineapple peel. These endophytic microorganisms are a reservoir of promising antimicrobial agents that could be active against a number of human pathogenic organisms. The study has shown that the extract from *Bacillus velezensis* strain WSM-1 displayed the highest antibacterial activity, particularly against *S. aureus* and *E. coli*, with a "very strong" inhibitory activity. Extracts from *Bacillus sp*.1 and *Bacillus sp*.2 also showed strong inhibitory activity against these bacteria.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Table 2: Measurement of the inhibition zone diameter of the extracts based on the culture time

Extracts	Test Bacteria	Culture Time (h)	Inhibition Zone Diameter (mm)
Bacillus velezensis WSM-1	*Staphylococcus aureus* ATCC 25157	24	12.88
		48	15.30
		72	17.71
		96	13.50
	Escherichia coli ATCC 25922	24	-
		48	-
		72	15.87
		96	-
Bacillus sp.1	*Staphylococcus aureus* ATCC 25157	24	7.80
		48	8.65
		72	12.08
		96	12.02
	Escherichia coli ATCC 25922	24	8.94
		48	9.00
		72	12.18
		96	5.20
Bacillus sp.2	*Staphylococcus aureus* ATCC 25157	24	-
		48	-
		72	12.25
		96	9.63
	Escherichia coli ATCC 25922	24	-
		48	-
		72	12.27
		96	11.89

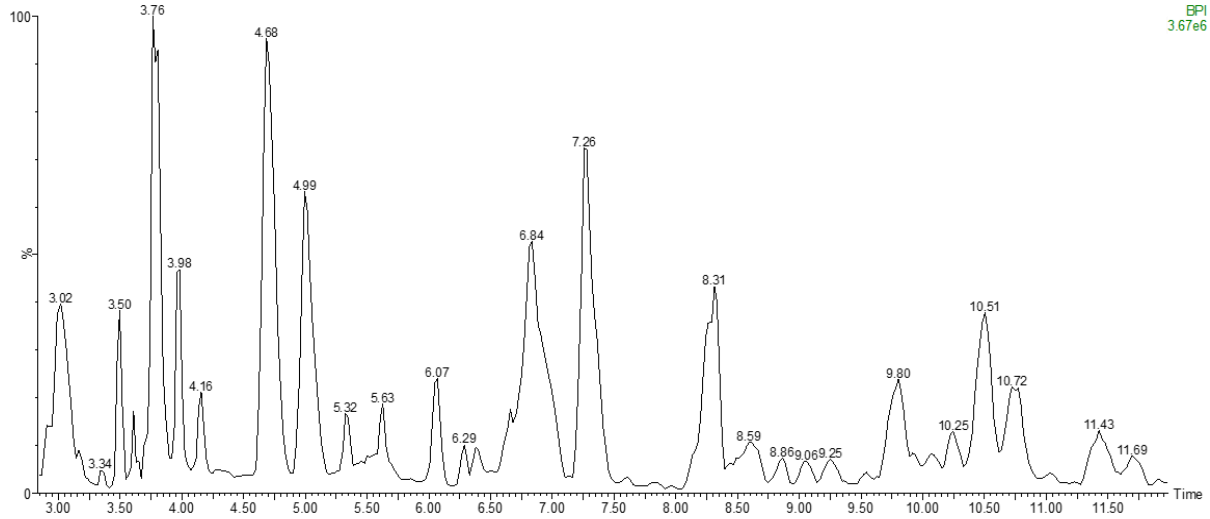

Figure 2: UPLC–MS/MS Chromatogram of Ethyl Acetate Extract from *Bacillus velezensis* WSM-1 Endophytic bacteria

Table 3: Identified secondary metabolites in the ethyl acetate extract of pineapple (*Ananas comosus* L) peel endophytic bacteria

Retention Time (Min)	Parent Ion $[M^+H]^+$	Daughter Ions $[M^+H]^+$	Molecular Formula	Compound name
3.018	160.0767	130.0658; 159.0673	$C_{10}H_{10}NO$	6-Methoxyquionoline(96)
3.760	268.1107	261.1245; 262.1267	$C_{10}H_{13}N_5O_4$	2-(6-amino-9H-purine-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol
3.960	570.2716	284.1396; 267.2708	$C_{30}H_{37}O_{10}$	Aklavin (95)
4.160	505.2338	243.0887; 284.1399	$C_{18}H_{34}O_{16}$	Maltotriose (96)
4.680	421.1878	169.0770; 395.1964	$C_{27}H_{49}O_3$	Cholestanetriol
5.320	219.0939	211.1449; 176.0713	$C_{11}H_{11}O_4$	Piperic acid
6.400	211.1233	146.0602; 130.0653	$C_{23}H_{46}N_5O_1$	Isoflavones
6.830	616.3622	260.1667; 611.1636	$C_{23}H_{46}N_5O_{14}$	Paromomycin
9.820	1023.182	362.1965; 1021.5183	$C_{52}H_{75}N_4O_{17}$	Mycaloide B

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