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Characterization of antibiotic-producing bacteria from cassava *tape* fermented by traditional *ragi tape*

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Abstract. *Muhiddin NH, Herawati N, Ramlawati, Hidayah N. 2024. Characterization of antibiotic-producing bacteria from cassava tape fermented by traditional* ragi tape. *Biodiversitas 25: 3359-3368.* This study was carried out to determine the phenotypic and molecular characteristics and identification of antibiotic producing bacterial isolates from cassava tape fermented by traditional *ragi tape*. The type of research is exploratory, including isolation and characterization of antibiotic-producing bacterial isolates from cassava tape fermented by traditional *ragi tape* from Bone District and Gowa District, South Sulawesi Province, and Polewali Mandar District, West Sulawesi Province. Bacterial isolation was done using the pour cup method, and an antibiotic test was performed using the Kirby-Bauer method with *Escherichia coli* and *Staphylococcus aureus* as indicator bacteria. The isolation process yielded two bacterial isolates that exhibited antibiotic properties, namely isolates Bb1 and Bb2. The phenotypic characterization of the isolate Bb1 revealed an irregular shape, bacillus-cell shape, Gram-positive, the presence of endospores, positive catalase, motile, aerobic, positive citrate, the capacity to ferment glucose, producing acid and acetyl methyl carbinol. Meanwhile, the Bb2 isolate exhibits a round colony shape, bacillus-cell shape, Gram-positive, produces endospores, positive catalase activity, motile, facultatively anaerobic, negative citrate, can ferment glucose and lactose, produces acid and acetyl methyl carbinol. The results of molecular identification demonstrated that bacterial isolate Bb1 exhibited a 99.72% homology level with *Bacillus velezensis* strain CR-502. In comparison, bacterial isolate Bb2 showed a 99.58% homology level with *Bacillus paramycoides* strains MCCC 1A04098. The findings indicate that bacterial isolates derived from cassava tape fermented by traditional *ragi tape* have the potential to be developed into functional foods and novel antibiotic compounds for application in the healthcare sector. This study highlights the importance of phenotypic and molecular characterization in explaining the diversity of microorganisms present in cassava tape as a traditional food fermentation.

Keywords: Antibacterial, antibiotics, bacteria, cassava tape, fermented food

INTRODUCTION

Indonesia is famous for its wide variety of traditional foods that rely on fermentation as their main processing method, and these culinary treasures can be found easily in both traditional and modern markets. Most of these traditional foods undergo fermentation when produced on a small scale or in households, resulting in unique and delicious products. One type of traditional food that is very common in Indonesia, especially in the provinces of South Sulawesi and West Sulawesi, is cassava *tape* or *tapai singkong*, known as *poteng*. *Tape* is a snack made through the fermentation process of carbohydrate substrates or foodstuffs using *ragi tape*. The *ragi tape* used generally contains microorganisms from the group of bacteria, molds and yeasts that play a role in producing a distinctive flavor and texture that many people prefer. The important role of bacteria in the fermentation process significantly influences the development of distinctive flavors, preservation ability, and nutritional value of food (Cempaka 2021; Muhiddin et al. 2023).

Traditionally, fermented tapes in South Sulawesi show tremendous diversity in terms of raw materials, fermentation processes, and end-product characteristics that reflect local cultural heritage. This diversity also affects the composition of microorganisms in the *tape*, including antibiotic-producing bacteria. This is per research carried out by Savadogo et al. (2016), that the fermentation process involving yeast and lactic acid bacteria has probiotic properties or bacteria that can produce antibiotics.

Several studies have shown that tape from different regions in South Sulawesi has different microorganism compositions, including antibiotic-producing bacteria such as species of *Bacillus* and *Lactobacillus. Bacillus cereus* was detected in several samples of *ragi tape*, a traditional Indonesian food starter. The different species of *Bacillus* spp. are capable of producing various chemical compounds, including antibiotics (Yu et al. 2020).

Bacterial phenotypic characters refer to directly observable traits, such as colony and cell morphology and biochemical and physiological properties (Duarte et al. 2019; Simpson et al. 2023). The phenotypic characteristics of bacteria can vary depending on the type of bacteria being studied. Some methods that can help characterize the phenotypic characteristics of bacteria include morphological analysis, the shape and structure of bacterial

cells observed under a microscope, and biochemical tests that help reveal the metabolic capabilities of bacteria (Kumar et al. 2016). Bacterial species diversity based on phenotypic characters can be identified through several methods, such as the ability to reduce sugar (Khushboo et al. 2023), survival in the pH range, citrate test (Pradhan and Tamang 2019), survival in the temperature range (Bautista et al. 2021), oxidation ability (Hidayata et al. 2017), starch hydrolysis, ability to reduce gelatin (Dsouza et al. 2022; Agustiani et al. 2023), sulfuric acid production capacity and lysine decarboxylation capacity (Capozzi et al. 2021).

Bacteria can be round, rod, and spiral and can be classified as Gram-positive or Gram-negative based on their reaction to Gram stain. They can also be classified as catalase positive or negative, depending on their ability to make the enzyme catalase. Bacteria can be motile or immobile depending on their ability to move (Amelia et al. 2020). Bacteria have an appropriate pH range for growth; for example, lactic acid bacteria produce maximum enzyme activity at acidic pH. Bacteria require a certain time to reach maximum enzyme activity, depending on the type of bacteria and growth conditions. Lactic acid bacteria generally produce maximum enzyme activity at an incubation time between 22-40 hours.

The microorganisms engaged in the *tape* fermentation process possess an exceptional capacity to generate natural antibiotic compounds. In light of this, this study sought to elucidate the phenotypic and molecular characteristics of these bacteria, with the ultimate goal of identifying those capable of producing antibiotics from cassava *tape*.

MATERIALS AND METHODS

Study area

The research was conducted at the Science Laboratory and Biology Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Negeri Makassar. Molecular characterization stages were carried out at the Microbiology Laboratory and the Laboratory of Molecular and Environmental Biology, Faculty of Mathematics and Natural Sciences, Universitas Halu Oleo.

Equipment and materials

The tools used in this study were a set of microbiological and molecular equipment. The material used as a source of antibiotic-producing bacterial isolates in this study was cassava tape which was collected from three regions, namely Bone, Takalar and Polewali Mandar districts. Pure cultures of *E. coli* and *S. aureus* bacteria were used for antibiotic activity testing. Some media, reagents and other chemicals used for isolation and characterization of antibiotic-producing bacterial isolates.

Procedures

Isolation of antibiotic-producing bacteria

This study began by collecting *tape* samples from traditional *ragi tape* fermentation from Bone District, Takalar District, South Sulawesi Province, and Polewali Mandar District, West Sulawesi Province. Bacteria from these samples were isolated using the pour plate method containing Plate Count Agar (PCA) and Nutrient Agar (NA) media and de Man, Rogosa, and Sharpe Agar (MRSA). Separate colonies with different characteristics were purified using the streak plate method to obtain pure cultures of bacterial isolates (Cappuccino and Sherman 1987; Hogg 2005). The isolates obtained were then further characterized and tested for antibiotic capacity.

Antibiotic activity test

The purified bacterial isolates were then tested for potential antibiotic activity through antibiotic sensitivity testing using the Kirby-Bauer method (Cappuccino and Sherman 1987). The Chloramphenicol and Ampicillin antibiotics were used as positive controls in sensitivity testing. This step allows us to evaluate the production potential of antibiotic compounds and provides further understanding of the characteristics of the isolates obtained in the previous stages. These isolates were tested for inhibition against *E. coli* and *S. aureus* bacteria. The clear zone formed when the microorganism isolate is placed adjacent to these bacteria will provide information about the potential antimicrobial ability of the isolate against *E. coli* and *S. aureus* bacteria. The presence of clear zones on the isolates is a potential indicator that these microorganisms show the ability to produce antibiotic compounds. The clear zone formed indicates that the microorganism produces compounds that inhibit surrounding bacteria growth, which is a characteristic commonly associated with antibiotic compounds.

Phenotypic characterization of antibiotic-producing bacteria

Isolates that show the ability to produce antibiotics were further characterized for phenotypic properties, including cell morphology under a microscope and various biochemical properties (Cappuccino and Sherman 1987; Hogg 2005). Phenotypic characterization provides a more comprehensive picture of the development and antimicrobial ability of the isolates during the incubation period.

Characterization and Molecular Identification of Antibiotic-Producing Bacteria

The antibiotic-producing bacterial isolates were then isolated for molecular identification. The genomic DNA of bacterial isolates was isolated using the versatile, quick prep method. Bacterial isolates were grown as much as 5 mL on NB media and incubated at 37°C for 24 hours. Bacterial culture of 2 mL was centrifuged at 13,000 rpm for 5 minutes. The pellet was resuspended with 750 µL of lysis buffer (100 mM Tris HCL pH 8.0, 100 mM NaCl, 50 mM EDTA, 1% SDS), then added 10 µL proteinase-K (20 mg/mL) and vortexed for 10 minutes. 40 µL lysozyme (100 mg/mL) was added to the pellet mixture and incubated at 55°C for 30 minutes, then centrifuged at 13,000 rpm for 5 minutes. The supernatant was transferred into a new test tube, 750 µL of phenol was added and vortexed for 15 minutes, then centrifuged at 13,000 rpm for 10 minutes. The resulting supernatant was transferred to a new tube and

cold chloroform was added in a ratio of 1:1 (v/v), then vortexed and centrifuged at 13,000 rpm at 4°C for 10 minutes. The supernatant was transferred to a new tube, and the DNA was precipitated with cold absolute ethanol 1:1 (v/v) and then incubated at -20°C overnight. The DNA solution was centrifuged at 13,000 rpm at 4°C for 10 min, and the pellet was washed with 500 µL of cold 70% ethanol, then centrifuged again for 10 min, then the ethanol was discarded. The pellet was dried and then resuspended using 30-50 µL of TE buffer. Amplification of the 16S rRNA gene was performed using the Mytaq Mix kit (Bioline). The primers used for 16S rRNA gene amplification were primers 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'GGTTACCTTGTTACGACTT-3'), which are universal primers for various bacterial strains (Chen et al. 2015). These primers are complementary to the 16S rDNA ends of all strains and produce a single band of approximately 1,500 bp in length.

16S rRNA gene fragments of bacterial isolates were amplified using PCR (Polymerase Chain Reaction) technique. The total PCR volume was 10 µL consisting of several components, namely: master mix 5 µL, dH_2O 3 µL, 27F 0.5 µL, 1429R 0.5, and DNA template 1 µL. PCR program used for amplification of 16S rRNA gene each with a specific temperature, time and cycle. Pre-PCR/Initial denaturation was carried out at 94°C for 5 minutes then the PCR process lasted 30 cycles, which included denaturation at 94°C for 1 minute, primer attachment (annealing) at 55°C for 1 minute, and chain extension at 72°C for 1 minute, then post-PCR/Final extension at 72°C for 10 minutes. PCR products were electrophoresed using 1% agarose gel and 1x TAE buffer. Electrophoresis was carried out at 80 volts for 30 minutes, then immersed in ethidium bromide and visualized using a UV transluminator, and 1 Kb DNA ladder was used as a comparison marker. Furthermore, molecular identification was carried out using BLAST analysis on NCBI to obtain a description of the similarity of each isolate with several key species (Ibrahim 2016).

RESULTS AND DISCUSSION

Isolation of antibiotic-producing bacteria

The results of isolation and characterization of colony morphology obtained 2 bacterial isolates from cassava *tape* from Bone District and 2 bacterial isolates from cassava *tape* from Polewali Mandar District (Table 1). Based on the observations, it was revealed that the four isolates have different colony morphological characters. Bacterial isolate Bb1 has a white colony color, round with a raised margin shape, flat elevation, and undulated edges. Bb2 bacterial isolate has a white colony color, round shape, convex elevation, and entire edge. Bacterial isolate Wb1 has a white colony color, round shape, raised elevation, and whole edge. Bacterial isolate Wb2 has a white colony color, round shape, flat elevation, and whole edge. Bacterial isolates found in cassava fermentation are mostly round with raised elevations and have whole edges (Renner et al. 2024). The four bacterial isolates were then tested for antibiotic activity using the Kirby-Bauer method. The presence of clear zones on the isolates is a potential indicator that these microorganisms show the ability to produce antibiotic compounds.

Based on the results of antibiotic activity testing of the four bacterial isolates, two bacterial isolates showed antimicrobial power (Figure 1). The results of antibiotic activity testing revealed interesting variations in clear zone diameter with the test bacteria *E. coli* and *Staphylococcus aureus*. The bacterial isolate Bb1 from cassava *tape* from Bone District showed the greatest inhibition activity against *E. coli* with a clear zone diameter of 38.29 mm and inhibition against *S. aureus* with a clear zone diameter of 13.62 mm. The Bb2 bacterial isolate from cassava *tape* from Bone District showed inhibition activity against *E. coli* with a clear zone diameter of 15.97 mm and inhibition against *S. aureus* with a clear zone diameter of 6.39 mm (Table 2). The isolates Wb1 and Wb2 from cassava *tape* from Polewali Mandar District also have the ability of antibiotic activity but in producing clear zones with diameters only reaching 5.44 mm against *E. coli* and 5.83 mm against *Staphylococcus aureus* and Wb2 with diameters only reaching 5.50 against *E. coli* and 5.20 mm against *Staphylococcus aureus.* The presence of a clear zone around the bacteria indicates antibiotic properties. The larger the clear zone, the more effective the antibiotic compound is in inhibiting microbial growth (Lázár et al. 2022).

Based on the results of the antibiotic activity test , it can be seen that 2 bacterial isolates, namely Bb1 and Bb 2, have the greatest inhibitory activity (Figure 1). This underscores the need for immediate and comprehensive characterization of these two isolates to understand their potential as antibiotic agents fully.

Table 1. Colony morphological characteristics of bacterial isolates from cassava *tape*

Isolate origin	Isolate code	Character			
		Color	Shape	Elevation	Edge
Bone	Bb1	White	Round with raised margin	Flat	Undulated
Bone	Bb2	White	Round	Convex	Entire
Polewali Mandar	Wb1	White	Round	Raised	Whole
Polewali Mandar	Wb2	White	Round	Flat	Whole

Phenotypic characterization of antibiotic-producing bacteria

Observation of the cell morphology was followed by Gram and endospore staining. Microscopic observations showed that bacterial isolate Bb1 is purple, which means that Bb1 bacteria are Gram-positive bacteria, and the results of endospore staining show that these bacteria have endospores (Figure 2). The purple color of Gram-positive bacteria is due to the binding of crystal violet. The cell wall of Gram-positive bacteria consists of layers of peptidoglycan, where the outermost part is the peptidoglycan layer so that when staining is done, it will produce a purple color from the color of crystal violet in the peptidoglycan section (Pukhrambam 2019).

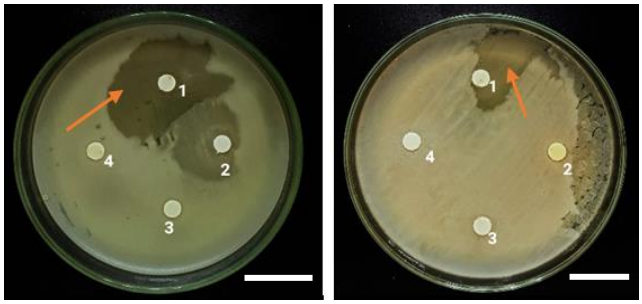
The composition of the cell wall of Gram-positive bacteria consists of several layers of peptidoglycan that join together and form a thick and rigid structure. In Gram-positive bacteria, there are about 40 layers of peptidoglycan, also called the Murein/mucopeptide layer, which constitutes 50% of the cell wall material. In addition to the Gram type, observation of Bb1 bacteria shows the shape of the bacteria, which is rod-shaped (*Bacillus*), specifically monobacillus, which is a single rod (Figure 2). Balogun et al. (2021) also stated that bacterial isolates collected from traditional magnetic starters, fermented cassava, and fermented food samples showed that all bacterial cells from cassava fermentation products were Gram-positive and round or rod-shaped (*Bacillus*). Hence, the bacteria found were also included in Gram-positive bacteria.

Observation of bacterial endospores Bb1 shows the presence of endospores, which a specific to *Bacillus sp*., which have a thick wall structure and a fresh subcaste on bacterial cells formed on the inside of the cell membrane. Endospores can repel chemicals and repel extreme heat and radiation. Srivastava et al. (2022) reported that phylogenetic analysis of the sequences showed that *Pratylenchus penetrans* is nearly related to *Bacillus* and possesses colorful types of collagen-suchlike filaments allowed to be involved in endospore attachment and resistance. Collagen-specific bodies were used to characterize the face of *Pasteuria* endospores.

The results from Simon's citrate test on Bb1 bacteria showed positive results, which was seen when the color of the medium changed to blue. The isolates tested preferred citrate as the sole carbon source of energy. The green-to-blue color indicates the efficient use of citrate by the isolates, as this medium contains citrate and is acidic. Hence, the pH of the medium increases and becomes alkaline (Brindavathy et al. 2023). The TSIA test aims to determine the ability of a bacterium to ferment sugar to produce a base or acid. If a yellow color forms on the agar medium, while if it can maintain the red color of the media, then it indicates a basic reaction.

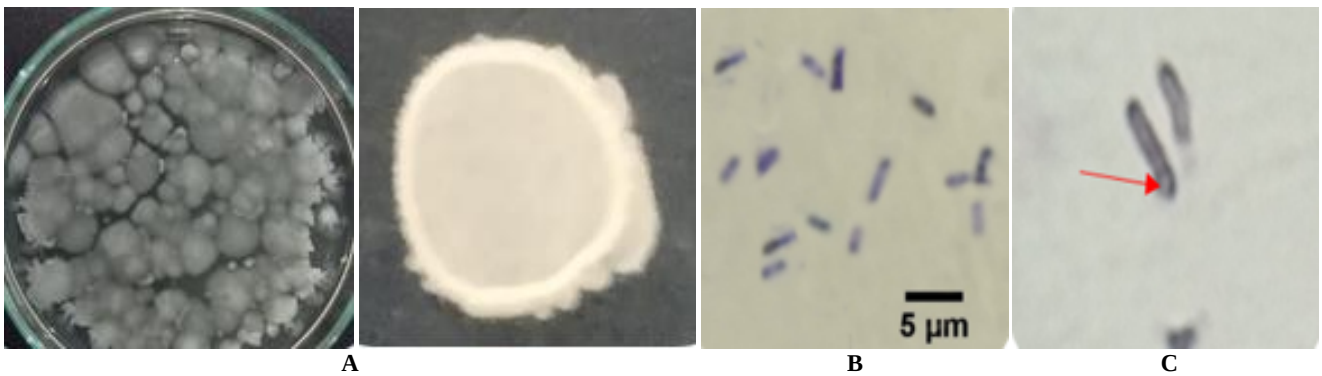
Table 2. The diameter of the clear zone formed (mm) from the activity of bacterial isolates

Control and isolate	Inhibition zone diameter (mm)	
	Escherichia coli	*Staphylococcus aureus*
Chloramphenicol	41.59	32.16
Ampicillin	18.54	6.05
Aquades	6.46	6.10
Medium NB	6.35	6.00
Bb1	38.29	13.62
Bb2	15.97	6.39
Wb1	5.44	5.83
Wb2	5.50	5.20



A B

Figure 1. Clear zone in the inhibition test of isolates 1. Bb1; 2. Bb2; 3. Wb1; and 4. Wb2 against A. *Escherichia coli*; B. *Staphylococcus aureus*. Scale bar = 2 cm



A B C

Figure 2. Morphological characteristics of A. Colonies; B. Gram-positive bacillus-shaped cells; C. Endospores of bacterial isolate Bb1

The media in the TSIA test also contains ferrous sulfate and sodium thiosulfate to detect H_2S gas production. Based on bacterial isolate Bb1, the top is red, and the bottom is yellow, indicating the fermentation of glucose and the absence of black sediment and does not produce gas. In addition, an oxidase test was also conducted to determine the presence of oxidase enzymes in bacteria. This enzyme can be produced by several microorganisms to catalyze the process of oxidation and reduction of electrons (Panjaitan et al. 2020). Positive test results (+) on bacterial isolate Bb1 are marked by the onset of blue color on oxidase indicator paper (Figure 3).

The formation of a cherry red ring on the surface of the Bb1 isolate media indicates a positive result in the indole test. A positive indole test indicates that bacteria can form indole from tryptophan as a carbon source (Latifah and Sofyanita 2023). Tryptophan is an essential amino acid that is oxidized by some bacteria to form indolic acid, pyruvic acid, and ammonia. The Methyl Red (MR) test is performed to show the organism's ability to produce and retain acidic end products from glucose fermentation. MR is a pH indicator that turns red at pH 4.4 or less (Mahe et al. 2021). Positive results on bacterial isolate Bb1 are characterized by the formation of red color in the media. At the same time, negative results are characterized by no color change in the media (Latifah and Sofyanita 2023). Based on the biochemical characterization of bacterial isolates from freshly harvested and fermented cassava water samples, it was found that in the methyl red test, 8 out of 9 samples showed positive results, including HCBA, HCBB, HCBC, HCBD, HCBE, FCBF, FCBG, and FCBI. There was one bacterial sample, FCBH, which showed negative results in the methyl red test, FCBH, which was identified as *Bacillus* sp. (Renner et al. 2024). The Voges Proskauer (VP) test is performed using a glucose phosphate medium to determine the ability of bacteria to produce acetylmethylcarbinol (acetoin) from glucose fermentation. Acetylmethylcarbinol (acetoin) is an intermediate in the formation of butylene glycol (Mahe et al. 2021). Negative results are characterized by no change in media to a red color. Meanwhile, the positive results of sample Bb1 is characterized by a change in the media to a red color (Latifah and Sofyanita 2023).

Furthermore, microscopic observations were made by Gram staining and endospore staining on bacterial isolate Bb2. The results showed a purple rod (*Bacillus*) shaped bacterial form. Bb2 bacterial isolates are Gram-positive, and the results of endospore painting show that these bacteria have endospores (Figure 4). Gram-positive bacteria exhibit a layer of peptidoglycan fibers that can be between 30 and 100 nm in size or thickness. This layer can maintain the crystal blue color (Rohde 2019). The results of research by Renner et al. (2024) reported that some bacterial isolates from fermented cassava samples were Gram-positive and had a bacillus-shape.

The catalase test on sample Bb2 shows positive with the appearance of gas bubbles on the object glass, and it is found that the process of breaking H_2O_2 or hydrogen peroxide by the catalase enzyme can produce microbes themselves. Positive catalase functions were to break H_2O_2 into H_2O and O_2, which provides parameter evidence that oxygen bubbles mark catalase activity. The catalase test is used to determine the presence of catalase, an enzyme that catalyzes the toxic substance hydrogen peroxide into water and oxygen. A positive result can be determined by the presence of bubbles (Khatoon et al. 2022).

The results of the citrate test on sample Bb2 showed negative results indicated by the absence of a change in the color of the media to blue. The Simon citrate test is negative for *E. coli* bacteria because *E. coli* cannot use citrate as a carbon source (Latifah and Sofyanita 2023). The TSIA test showed that the upper side of the Bb2 sample is red and the lower side is yellow, indicating that the bacteria can oxidize sugar without black sediment and gas production.

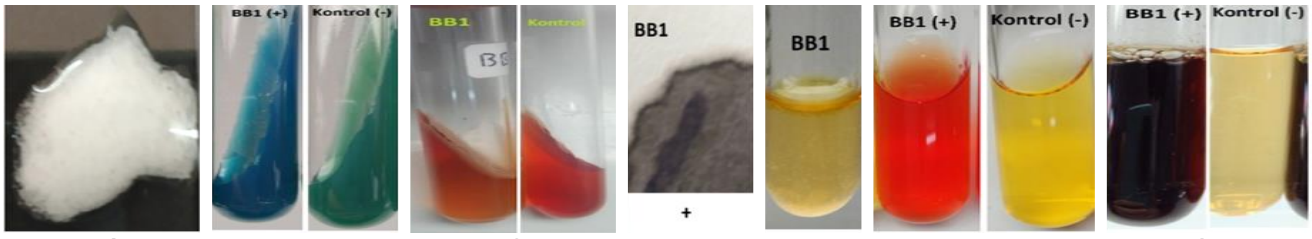


Figure 3. Biochemical characteristics test of bacterial isolate Bb1: A. Catalase test; B. Citrate test; C. Triple Sugar Iron Agar (TSIA) test; D. Oxidase test; and E. Indole test; F. Methyl Red (MR) test; and G. Voges Proskauer (VP) test

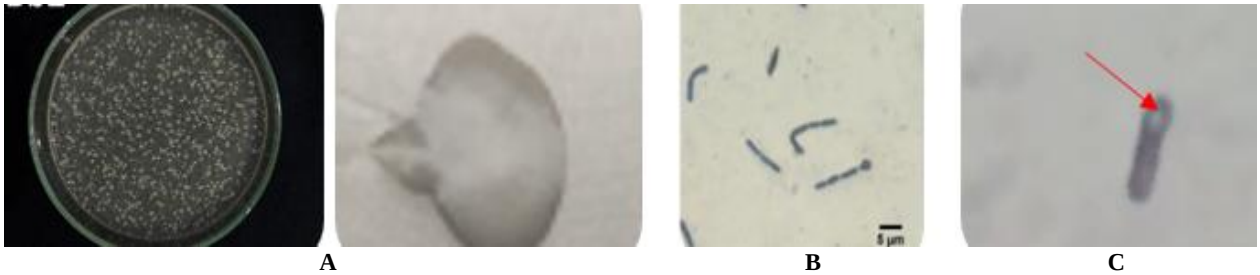


Figure 4. Characteristics of A. Colony morphology; B. Cell morphology; and C. Endospores of bacterial isolate BB2

The TSIA K/A H_2S (-) test means that only bacteria that metabolize glucose do not produce H_2S (Ningrum et al. 2019). Negative results in the oxidase test of sample Bb2 were indicated by the absence of color change in the oxidase reagent (tetramethyl-p-phenylenediamine) after being applied to the bacterial colony. The reagent remains unchanged in color (usually remains colorless or pale yellow), which indicated that the bacteria do not produce the oxidase enzyme and are unable to oxidize the reagent (Figure 5).

The presence of indole is detected by adding the *Ehrlich/Kovac* reagent containing paradimethylamine benzaldehyde. The formation of a red ring on the surface of the bacterial culture indicates a positive result (+) in the Bb2 sample. This test involves a tryptase enzyme that can hydrolyze tryptophan-type amino acids that have an indole side group so that the indole reacts with the test reagent to produce a red color. The results of the MR test on the Bb2 sample showed positive results, indicated by a change in color to red after the reduction of the MR signal. A pH indicator that remains red at pH 4.4 or less and *E. coli* can produce and store the end product of glucose fermentation, which is acidic. Bacteria can break down sugar and produce carbonic acid (Ningrum et al. 2019). The VP testing showed positive results in sample Bb2, marked by a change in color to red or pink. This change occurs due to the presence of acetoin compounds during carbohydrate fermentation. The fermentation process is carried out in Voges Proskauer Broth media with the addition of 40% KOH and alpha naphthol reagent, then reacted for 1-3 hours, resulting in positive VP forming a copper red color (Sultana et al. 2022).

The purpose of the MR-VP test is to determine the ability of bacteria to oxidize glucose to produce high concentrations of acid as the final product. The MR test serves to determine the presence or absence of mixed acid fermentation (methylene glycol) in bacterial colonies. MR test results can be known to be negative (-), characterized by after the addition of MR, the media does not change color to red. The Indole test is said to be positive, characterized by the formation of a red color on the growth medium, which indicates that the bacteria have a triptonase enzyme that can hydrolyze tryptophan-type amino acids that have indole side groups so that indole will react with the test reagent and form red indole (Figure 5).

Phenotypic characteristics that include cell morphology and biochemical properties of antibiotic-producing bacterial isolates showed that the cell shape of isolates Bb1 and Bb2 have the same characteristics, namely bacillus-shaped, Gram-positive nature, have endospores, motile, positive catalase test can ferment glucose and produce acid and acetyl methyl carbinol. The different characteristics of Bb1 and Bb2 are in the form of irregular and round colonies. Bb1 is aerobic, has positive oxidase and a positive citrate test, and cannot ferment lactose. While Bb2 is a facultative anaerobic, negative oxidase, negative citrate test, and can ferment lactose (Table 3).

Characterization and molecular identification of antibiotic-producing bacteria

The DNA sequence of PCR results of bacterial isolates showed that the length of the 16S rRNA gene sequence was successfully amplified from bacterial isolate Bb1 with a length of 1,430 bp (Figure 6.A). Similarly, the result of PCR that produces 1 single DNA band on isolate Bb1 (Figure 6.B). The band formed is aligned with the DNA ladder (M) 1 Kb. This indicates that the successfully amplified DNA has a size of about 1,500 bp. Figure 6.C is the identification results of bacterial isolate Bb1 showing a homology level of 99.72% to *Bacillus velezensis* strain CR-502 with a Max Score and total score of 2586, Query cover 98%, E-value is 0 and Percent Identity of 99.72%. Based on the phylogenetic tree in Figure 6.D, it can be seen that isolate Bb1 is closely related to *B. velezensis* strain CR-502, and *Bacillus amyloliquefaciens* strain NBRC15535 is most distantly related or not related to Bb1 (Figure 6).

Table 3. Cell morphological and biochemical characteristics of antibiotic-producing bacterial isolates

Character	Isolate Code	
	BB 1	BB 2
Cell shape	*Bacillus*	*Bacillus*
Gram staining	+	+
Endospore staining	+	+
Catalase test	+	+
Motility test	+	+
Oxygen demand test	Aerob	Anaerobic facultative
Citrate test	+	-
Glucose fermentation	+	+
Lactose fermentation	-	+
Oxidase test	+	-
Indole test	-	-
H_2s production	-	-
Methyl Red test	+	+
Voges Proskauer test	+	+
Reference genus	*Bacillus* sp.	*Bacillus* sp.

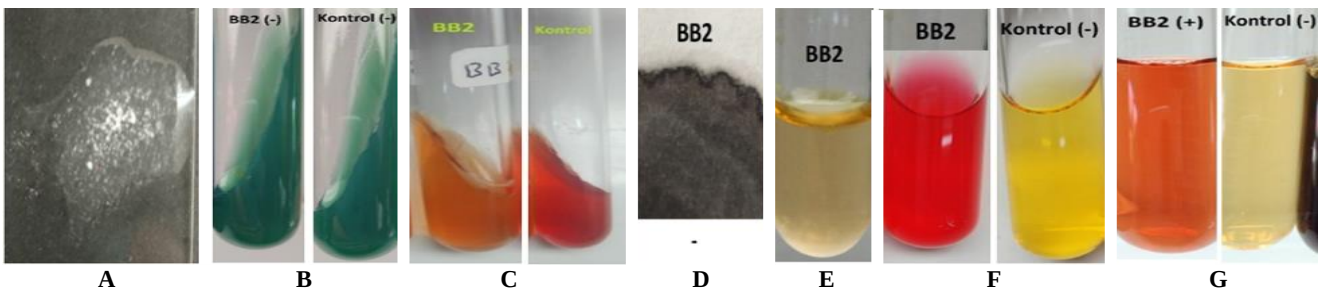


Figure 5. Biochemical characteristics test of bacterial isolate Bb2: A. Catalase test; B. Citrate test; C. Triple Sugar Iron Agar (TSIA) test; D. Oxidase test; and E. Indole test; F. Methyl Red (MR) test and G. Voges Proskauer (VP) test

GCTATACATG CAGTCGAGCG GACAGATGGG AGCTTGCTCC CTGATGTTAG CGGCGGACGG GTGAGTAACA
CGTGGGTAAC CTGCCTGTAA GACTGGGATA ACTCCGGGAA ACCGGGGCTA ATACCGGATG GTTGTTTGAA
CCGCATGGTT CAGACATAAA AGGTGGCTTC GGCTACCACT TACAGATGGA CCCGCGGCGC ATTAGCTAGT
TGGTGAGGTA ACGGCTCACC AAGGCAACGA TGCGTAGCCG ACCTGAGAGG GTGATCGGCC ACACTGGGAC
TGAGACACGG CCCAGACTCC TACGGGAGGC AGCAGTAGGG AATCTTCCGC AATGGACGAA AGTCTGACGG
AGCAACGCCG CGTGAGTGAT GAAGGTTTTC GGATCGTAAA GCTCTGTTGT TAGGGAAGAA CAAGTGCCGT
TCAAATAGGG CGGCACCTTG ACGGTACCTA ACCAGAAAGC CACGGCTAAC TACGTGCCAG CAGCCGCGG
TAATACGTAG GTGGCAAGCG TTGTCCGGAA TTATTGGGCG TAAAGGGCTC GCAGGCGGTT TCTTAAGTCT
GATGTGAAAG CCCCCGGCTC AACCGGGGAG GGTCATTGGA AACTGGGGAA CTTGAGTGCA GAAGAGGAGA
GTGGAATTCC ACGTGTAGCG GTGAAATGCG TAGAGATGTG GAGGAACACC AGTGGCGAAG GCGACTCTCT
GGTCTGTAAC TGACACTGAG GCGCGAAAGC GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCCACGC
CGTAAACGAT GAGTGCTAAG TGTTAGAGGG TTTCCGCCCT TTAGTGCTGA AGTTAACGCA TTAAGCACTC
CGCCTGGGGA GTACGGCCGC AAGGCTGAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA
CGCCTGGGGA GTACGGCCGC AAGGCTGAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA
GCTTCTCCTT CGGGAGCAGA GTGACAGGTG GTGCATGGTT GTCGTCAGCT CGTGTCGTGA GATGTTGGGT
TAAGTCCCGC AACGAGCGCA ACCCTTGATC TTAGTTGCCA TCATTCAGTT GGGCACTCTA AGGTGACTGC
CGGTGACAAA CCGGAGGAAG GTGGGGATGA CGTCAAATCA TCATGCCCCT TATGACCTGG GCTACACACG
TGCTACAATG GACGGTACAA AGAGCTGCAA GACCGCGAGG TGGAGCTAAT CTCATAAAAC CGTTCTCAGT
TCGGATTGTA GGCTGCAACT CGCCTACATG AAGCTGGAAT CGCTAGTAAT CGCGGATCAG CATGCCGCGG
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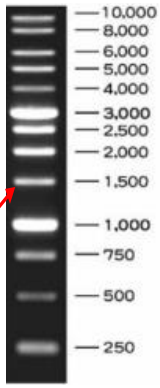
AGGTAACCTT TAGGAGCCAG CCGCCGAAGG

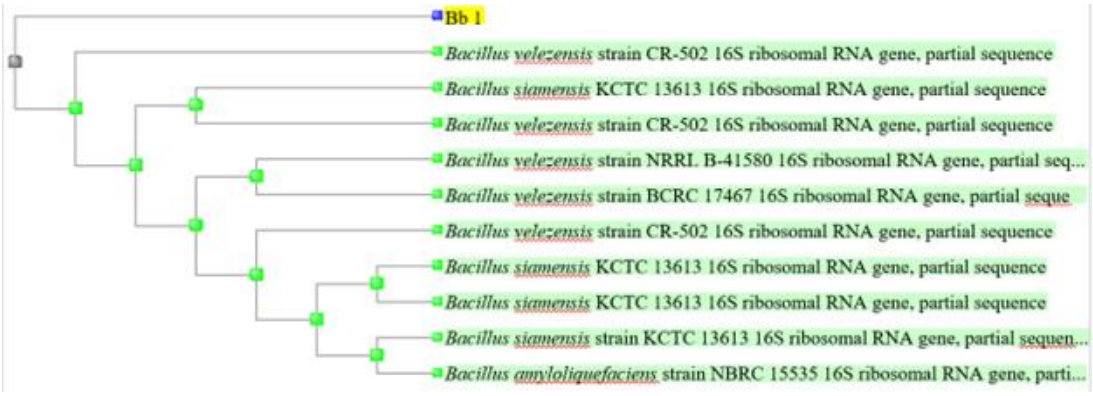
A



B

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Bacillus siamensis KCTC 13613 16S ribosomal RNA gene, partial sequence	2597	2597	100%	0.0	99.51%	MW578390.1
☑	Bacillus velezensis strain NRRL B-41580 16S ribosomal RNA gene, partial sequence	2593	2593	99%	0.0	99.51%	KY694464.1
☑	Bacillus velezensis strain BCRC 17467 16S ribosomal RNA gene, partial sequence	2593	2593	99%	0.0	99.51%	EF433407.1
☑	Bacillus siamensis KCTC 13613 16S ribosomal RNA gene, partial sequence	2588	2588	99%	0.0	99.44%	MN176482.1
☑	Bacillus siamensis strain KCTC 13613 16S ribosomal RNA gene, partial sequence	2588	2588	99%	0.0	99.44%	KY643639.1
☑	Bacillus siamensis KCTC 13613 16S ribosomal RNA gene, partial sequence	2588	2588	99%	0.0	99.58%	KT781674.1
☑	Bacillus velezensis strain CR-502 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.37%	ON597433.1
☑	Bacillus velezensis strain CR-502 16S ribosomal RNA gene, partial sequence	2588	2588	99%	0.0	99.58%	MW578391.1
☑	Bacillus velezensis strain CR-502 16S ribosomal RNA gene, partial sequence	2586	2586	98%	0.0	99.72%	MZ276294.1
☑	Bacillus amyloliquefaciens strain NBRC 15535 16S ribosomal RNA gene, partial sequence	2582	2582	99%	0.0	99.37%	MK182997.1

C



D

Figure 6. Molecular identification of bacterial isolate Bb1 A. DNA sequence; B. Visualisation of PCR results; C. BLAST results;D. Phylogenetic tree

The results of the molecular test showed that the bacterial insulate Bb1 was linked to the *B. velezensis* strain. In agreement with the results of the characterization that has been mentioned, the bacterial insulate Bb1 is in agreement with the results of exploration by Zhou et al. (2022) who conducted biochemical characterization using sucrose, glucose, maltose, and citrate which were linked as *B. velezensis* proved to have the ability to produce antimicrobial composites and benefit the intestinal microbiota. This is also in line with the research of Su et al. (2024) which stated that *B. velezensis* can regulate intestinal microbiota by increasing the number of beneficial microorganisms, reducing the number of pathogens, and increasing the nutritional value and taste of fermented foods.

DNA sequence showed the length of the 16S rRNA gene sequence that was successfully amplified from bacterial isolate Bb2 with a sequence length of 1,428 bp (Figure 7.A). The results of PCR, which produces 1 single DNA band on isolate 2 (Bb2) (Figure 7.B). The band formed is aligned with the DNA ladder (M) 1 Kb. This indicates that the successfully amplified DNA has a size of about 1,500 bp. Figure 7.C shows the identification results of bacterial isolate Bb2 shows a homology level of 99.58% to *B. paramycoides* strain MCCC 1A04098 with Max Score and total score of 2,603, Query cover 99%, E-value worth 0, and Percent Identity of 99.58%. Based on the phylogenetic tree in Figure 7.D, it can be seen that isolate Bb2 is closely related to *B. paramycoides* strain MCCC 1A04098, and *Bacillus cereus* strain CCM 2010 is most distantly related or not related to Bb2 (Figure 7).

CTATACATGC AGTCGAGCGA TGGATTAAGA GCTTGCTCTT ATGAAGTTAG CGGCGGACGG GTGAGTAACA
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TAATACGTAG GTGGCAAGCG TTATCCGGAA TTATTGGGCG TAAAGCGCGC GCAGGTGGTT TCTTAAGTCT
GATGTGAAAG CCCACGGCTC AACCGTGGAG GGTCATTGGA AACTGGGAGA CTTGAGTGCA GAAGAGGAAA
GTGGAATTCC ATGTGTAGCG GTGAAATGCG TAGAGATATG GAGGAACACC AGTGGCGAAG GCGACTTTCT
GGTCTGTAAC TGACACTGAG GCGCGAAAGC GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCCACGC
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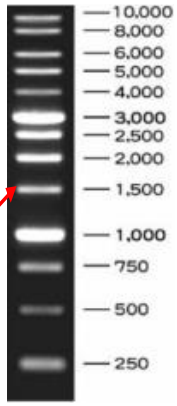
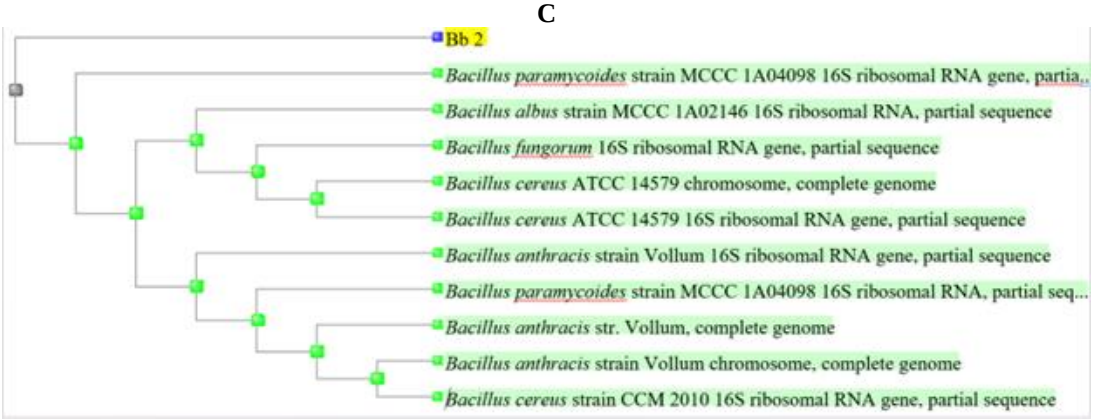


A **B**

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA gene, partial sequence	2603	2603	99%	0.0	99.58%	MW065486.1
☑	Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, partial sequence	2593	2593	99%	0.0	99.44%	KY628813.1
☑	Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	2593	2593	99%	0.0	99.44%	NR_157734.1
☑	Bacillus albus strain MCCC 1A02146 16S ribosomal RNA, partial sequence	2593	2593	99%	0.0	99.44%	NR_157729.1
☑	Bacillus anthracis strain Vollum chromosome, complete genome	2593	28493	99%	0.0	99.44%	CP076225.1
☑	Bacillus anthracis str. Vollum, complete genome	2593	28441	99%	0.0	99.44%	CP007666.1
☑	Bacillus anthracis strain Vollum 16S ribosomal RNA gene, partial sequence	2593	2593	99%	0.0	99.44%	AF290553.1
☑	Bacillus cereus ATCC 14579 chromosome, complete genome	2588	33642	99%	0.0	99.37%	CP034551.1
☑	Bacillus fungorum 16S ribosomal RNA gene, partial sequence	2588	2588	99%	0.0	99.37%	MG601116.1
☑	Bacillus cereus ATCC 14579 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99.37%	OK058270.1

C



D

Figure 7. Molecular identification Bacterial isolate Bb2: A. DNA sequence; B. Visualisation of PCR results; C. BLAST results; D. Phylogenetic tree Phylogenetic tree

Based on the results of molecular tests, bacterial isolate Bb2 was identified as a strain of *B. paramycoides*. In accordance with the characterization results that have been mentioned, bacterial isolate Bb2 is in accordance with the results of research by Yadav et al. (2022) that isolate *B. paramycoides* strain Bb2 was found to be Gram-positive, motile, non-acid resistant, and endospore-forming bacteria and biochemical characterization confirmed that the isolate was positive for catalase and Methyl Red (MR) tests. Besides that, *B. paramycoides* shows antimicrobial activity, can be added to food supplements, and is effectively used in treating hospital wastewater (Sukmawati et al. 2023).

The results of this study indicates that the isolation process obtained two bacterial isolates namely isolates Bb1 and Bb2 have antibiotic activity. The results of this study showed that from the isolation process, two bacterial isolates were obtained, namely isolates Bb1 and Bb2 which have antibiotic activity. Isolates Bb1 and Bb2 show the results of phenotypic characteristics that have a bacillary cell shape, Gram positive, endospores, positive catalase, motile, ferment glucose, and produce acids and acetyl methyl carbinol. The results of molecular identification of Bb1 have a homology level of 99.72% to *B. velezensis* strain CR-502 and Bb2 of 99.58% to *B. paramycoides*

strain MCCC 1A04098. The results of these findings indicate that bacterial isolates derived from cassava *tape* fermented with traditional *ragi tape* have the potential to be developed into functional foods and new antibiotic compounds that can be utilized in the health sector. This research underscores the importance of phenotypic and molecular characterization in further understanding the diversity of microorganisms in traditional fermented products.

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