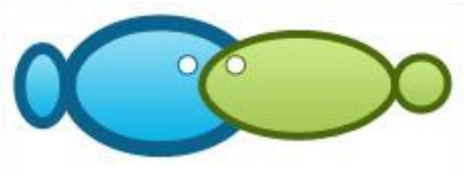

Study of antibiotic producing bacteria from anchovy fish (*Stolephorus* sp.) processing waste: isolation, characterization, and molecular identification

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Abstract. This research aims to: a) obtain bacterial isolates from waste of anchovy fish (*Stolephorus* sp.) processing that have the potential to inhibit *Staphylococcus aureus* and *Escherichia coli* bacteria, b) analyze the antibiosis potential of bacteria originating from anchovy fish processing wastewater in inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli*, c) analyze the morphological forms and potential antibiosis against *Staphylococcus aureus* and *Escherichia coli*, d) determine the types of potential antibiosis bacteria against *Staphylococcus aureus* and *Escherichia coli* through molecular identification. This study is expected to provide information on the presence of wastewater bacteria from anchovy fish processing that can produce antibiotics against *Staphylococcus aureus* and *Escherichia coli*. The results of this study indicate that: a) six bacterial isolates with potential antibiosis were obtained from anchovy fish washing wastewater and eight bacterial isolates from anchovy fish boiling wastewater; b) the Bg 1.1 bacterial isolate has strong antibiosis potential against *Staphylococcus aureus* and *Escherichia coli* bacteria in anchovy fish washing wastewater, and the Ag 1.2 bacterial isolate also has strong antibiosis potential against *Staphylococcus aureus* and *Escherichia coli* bacteria in anchovy fish boiling wastewater; c) the Bg 1.1 isolate has gram-positive bacterial cells, bacillus-shaped cells, aerobic nature, (β) hemolysis, optimum growth at pH 6-9, temperature range of 30-40°C, and salt concentration of 25-75 ppt; d) identification results using 16S rRNA sequences show that the Bg 1.1 isolate has 100% similarity with *Bacillus subtilis* strain soil GB2 bacteria. Based on its inhibitory activity and characterization, the Bg 1.1 isolate is a potential candidate for antibiotic production.
Key Words: 16S rRNA, antibiosis, anchovy processing liquid waste, potential antibiotic bacteria.

Introduction. The sustainable utilization of natural resources has become an urgent necessity in addressing global environmental and health challenges. Within the fisheries industry, waste generated from fish processing poses a significant environmental concern. Anchovy fish (*Stolephorus* sp.) are commonly processed on a mass scale in the fisheries industry, resulting in substantial waste production. The byproducts of anchovy processing contain various organic compounds and chemicals that may contaminate aquatic environments (Venugopal & Sasidharan 2021).

Concurrently, the escalating antibiotic resistance in pathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli* has emerged as a critical global health issue. The emergence of antibiotic resistance has diminished the effectiveness of treatments and heightened public health risks. Consequently, research aimed at discovering new sources of effective antibiotics is imperative (Chinemerem Nwobodo et al 2022).

The development of infectious diseases causes an increase in the need for antibiotics. Viruses, bacteria, fungi and protozoa are microorganisms that cause infectious diseases. One example is the bacteria *Escherichia coli* and *Staphylococcus*

aureus. Infectious diseases that are usually caused by *Escherichia coli* and *Staphylococcus aureus* bacteria are urinary tract infections, sepsis and meningitis (Karam et al 2019).

Infectious diseases can be treated by administering antibiotics. The activity of antibiotics is divided into two, namely inhibiting the growth of microorganisms (bacteriostatic activity) and killing microbes (bactericidal activity) (Pancu et al 2021). In the search for antibiotic substances, it is necessary to first explore the potential of microorganisms that have antibiosis power (da Cunha et al 2019). Antibiosis is the inhibition of pathogens by metabolite compounds produced by biological agents. The successful antibiosis test is indicated by the formation of a halo zone around the bacterial isolate (Rilda et al 2023).

There are several strains of microorganisms that are capable of producing antibiotics including *Brevibacillus brevis*, *Paenibacillus polymyxa*, *Bacillus subtilis*, *Streptomyces griseus*, *Streptomyces mediterranei*, which comes from the Eubacteriales (bacteria) group, *Streptomyces noursei*, *Streptomyces tenebrarius*, *Streptomyces verticillus* which comes from the Actinomycetes (fungi) group and *Penicillium chrysogenum*, *Cephalosporium* spp. which comes from the Ascomycetes (mold) group (Koilybayeva et al 2023; Nikolic & Mudgil 2023; Ribeiro et al 2023).

Microorganisms that have the potential to produce antibiotics are also obtained from dirty places. Many antibiotics come from feces, microorganisms isolated from soil have the ability to produce antibiotics such as the antibiotic Vancomycin (Chin et al 2023). Dried anchovy processing liquid waste is a production residue that comes from the washing process which is no longer used. Waste is a place to obtain microorganisms that are capable of producing antibiotics (Tiwari et al 2018). Periadnadi et al (2024) also conducted research searching for antibiotics in the sewage system of cattle slaughterhouses by finding seven bacterial isolates from the genus *Bacillus* (Periadnadi et al 2024).

In this context, investigating antibiotic-producing bacteria from fish processing waste is highly relevant. Waste from fish processing industries, particularly from anchovy fish, may harbor potential microorganisms capable of producing antibacterial compounds that can inhibit the growth of pathogenic bacteria. Through isolation, characterization, and molecular identification, this study aims to identify potential bacteria that could serve as a source of new antibiotics to combat the escalating antibiotic resistance crisis and mitigate the environmental impacts of fisheries industry waste. Isolation of antibiotic-producing bacteria from fish processing wastewater has never been reported. Therefore, exploring antibiotic-producing bacteria from anchovy fish processing waste holds the promise of dual benefits: contributing to public health by discovering novel antibacterial compounds and promoting sustainable management of fisheries industry waste. Based on this, it is important to carry out research on the isolation and molecular identification of the 16s rRNA gene of antibiotic bacteria from dried anchovy processing liquid waste.

Material and Method. The experiment in this study was conducted in March 2023 at the Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, Indonesia.

Materials. The materials for this research were liquid waste from washing anchovies (*Stolephorus* sp.) taken from the Pasie Nan Tigo Fisheries Processing Center Padang Indonesia, distilled water, *Staphylococcus aureus* and *Escherichia coli* test bacteria, McIlvaine buffer solution, Farland's 0.5, nutrient agar (NA) medium, nutrient broth (NB) medium, skim milk agar (SMA) media, glucose peptone (GP) broth media, 70% spirit and alcohol, crystal violet, safranin, malachite green, 3% H_2O_2 solution, trypticase soy agar (TSA) medium, tryptone broth medium, p-aminodimethylaniline, nitrate broth, Simmons citrate medium, methyl red, nutrient gelatin medium, NaOH, HCl, spectrophotometer, centrifuge, lysozyme solution, ammonium acetate, chloroform, PCR machine, ddH_2O reagent, dNTP, Taq buffer, DNA Taq polymerase enzyme, primer 27 F: 5'- -AGA GTT TGA TCC TGG CTC AG -3' and primer 1492 R: 5'- TAC GGY TAC CTT GTT ACG ACT T- 3',

Mupid mini cell (exu), tris acetate-EDTA (TAE) buffer, ethidium bromide solution, Gel Doc Printgraph, sodium acetate, dH_2O ultra-pure solution.

Isolation of bacteria. Isolation of bacteria begins with taking 1 ml of fishery processing liquid waste, then the sample is put into a test tube containing 9 ml of sterile distilled water and then homogenized (dilution 10^{-1}). Dilution 10^{-2} was done using 1 ml of sample suspension from dilution 10^{-1} that was then put it into a second test tube containing 9 ml of sterile distilled water and homogenized. Dilution was carried out to a dilution of 10^{-7}. Then 1 ml of sample suspension from dilutions 10^{-3}, 10^{-5}, and 10^{-7} and 1 ml of the test bacteria *S. aureus* and *E. coli* were taken. Each isolate was mixed with each other and poured into a sterile Petri dish, then planted in a pour plate on nutrient agar (NA) medium. Next, it was incubated at 37ºC for 24 hours (Wang et al 2020). If a halo zone forms around the colony, the colony is suspected to be a potential bacterium, and the isolate is continued to be purified to obtain a pure isolate.

Bacterial identification. Identification of bacterial isolates includes morphological and biochemical characteristics of bacteria, namely: Gram staining, spore staining, motility, catalase, oxidase, indole, nitrate reduction, H_2S test, glucose fermentation and gas formation, citrate test, MR-VP test, oxidative fermentative (O/F) test, and gelatin hydrolysis test (Fournière et al 2020). In molecular identification, this study used primer 27 F: 5`-- AGA GTT TGA TCC TGG CTC AG – 3` and primer 1492R: 5`-- TAC GGY TAC CTT GTT ACG ACT T -–3`.

Data analysis. The obtained data are analyzed descriptively and presented in a table of bacterial isolates. Character (morphology) of bacterial isolates consists of morphological characters and potential characters. Character (morphology) is used as the character identity of selected potential isolates that are antibiotics to pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli.* The potential of bacteria is analyzed by antibacterial zone parameters. The sequence data analysis was performed with the help of the DNA star software program. For sequence analysis, alignment analysis is performed by comparing the sequences obtained (question) with the existing sequences of the GenBank with the NCBI database search site (NCBI 2023). Existing sequences were identified from GenBank with the NCBI database search site (www.ncbi.nlm.nih.gov) using BLAST (Raliya & Tarafdar 2013).

Result and Discussion

The existence of bacterial isolates from fish processing liquid waste. The waste generated from the washing and boiling processes in fish processing constitutes liquid waste that can serve as a potential source of environmental contamination. One type of microorganism inhabiting such liquid waste is bacteria. To further understand the presence of bacteria in fish processing liquid waste, a bacterial isolation process is necessary.

Bacterial isolation is a method used to obtain pure cultures of a microorganism by separating or transferring it from the environment in which the microorganism resides. This isolation process is carried out with the aim of identifying and studying the characteristics of bacteria present in fish processing liquid waste. The results of bacterial isolation can provide valuable information regarding the types of bacteria present, as well as the potential risks or benefits they may pose.

The isolation of bacteria from fish processing liquid waste yields interesting results. The results of this bacterial isolation are presented in Table 1, which contains data on the bacteria successfully isolated using three different growth media.

Table 1

Results of bacterial colony counting in fish processing liquid waste

No.	*Total plate count agar (cfu/ml) (10^8)*		*Proteolytic bacteria (cfu/ml) (10^8)*		*Fermentative bacteria (cfu/ml) (10^8)*	
	Washing	*Boiling*	*Washing*	*Boiling*	*Washing*	*Boiling*
1	20.8	9	7	5.6	6.4	6.8
2	19.2	6.6	6.8	6	5.8	4.2
Average	20	7.8	6.9	5.8	6.1	5.5

The liquid waste generated from the processing of anchovy fish is divided into two types: liquid waste from washing (freshwater) and liquid waste from boiling (saltwater). The difference in types of liquid waste also affects the presence of bacteria. Factors such as the duration of fish processing and processing components will influence the amount of contamination sources, including water usage, salt usage, and other tools utilized in the process. The proliferation of bacteria on fish bodies is not only influenced by naturally occurring bacteria in the fish (concentrated in the gut contents, gills, and skin) but also by bacteria originating from other contamination sources, including pathogenic bacteria.

The results of bacterial colony count calculations isolated from three bacterial growth media show that the average bacteria living on plate count agar (PCA) media is $20x10^8$ cfu/ml in washing wastewater and $7.8x10^8$ cfu/ml in boiling wastewater. This occurs because the boiling process reduces the number of heat-sensitive and salt-tolerant bacteria. The average bacteria isolated on skim milk agar (SMA) media is $69x10^7$ cfu/ml from washing wastewater and $58x10^7$ cfu/ml from boiling wastewater. This is due to the ability of bacteria growing on SMA media to degrade proteins, which are the main components in fish waste. Furthermore, bacteria were also isolated on glucose peptone agar carbonate (GPA $CaCO_3$) media to assess fermentative ability. Calculation results show the average bacterial colony on washing wastewater is $61x10^7$ cfu/ml, while on boiling wastewater is $55x10^7$ cfu/ml. The presence of bacterial colonies in fish processing wastewater indicates that these bacteria have the ability to neutralize acid and have the potential as fermentative bacteria. Thus, the results of bacterial isolation from fish processing wastewater demonstrate the diversity of bacterial types thriving in that environment.

Isolation of bacteria from fish processing liquid waste. Isolating bacteria from wastewater generated during fish processing is a crucial step in environmental microbiology research. This process involves the characterization of bacterial colonies that grow on growth media through macroscopic and microscopic observations. Macroscopic observations, including colony shape, margin, color, and elevation, provide initial insights into the types of bacteria present in the waste sample. Microscopic observations are conducted using a microscope along with relevant biochemical tests. The characterization of bacterial isolates originating from fish processing wastewater can be observed in Table 2.

Based on the results of biochemical tests, it was determined that all bacterial isolates (6 bacterial isolates) belonged to the *Bacillus* group. *Bacillus* bacteria are classified as Gram-positive bacteria. Bacterial identification was conducted following the guidelines outlined in Bergey's Manual of Determinative Bacteriology (Guerrero 2001) and the Manual for the Identification of Medical Bacteria (Zhang et al 2023).

These bacteria exhibit Gram-positive characteristics and are motile due to peritrichous flagella. They produce oval-shaped endospores, although sometimes they may be round or even cylindrical, and under unfavorable conditions, they develop high resistance. These traits are consistent with those of *Bacillus* species. Sporulation occurs, with one spore per cell, and it is not resistant to exposure to open air. The bacteria are aerobic or facultative anaerobic, positive for catalase and oxidase, and may be pathogenic to vertebrates or invertebrates. Additionally, they possess diverse

physiological capabilities and are sensitive to factors such as heat, pH, salinity, and chemoorganotrophs in fermentation or respiratory metabolism (Rolim et al 2019).

These findings align with the research conducted by Periadnadi et al (2024), who identified 14 isolates of reactive powder concrete (RPS) waste channel bacteria with potential antagonistic effects against the test bacterium *Staphylococcus aureus*. Among these isolates, 7 were found to possess antibiosis potential from the genus *Bacillus* spp.

Bacteria possessing spores are categorized under Group I, which includes *Clostridium* and *Bacillus*. Upon conducting the motility test, it was determined that all bacteria were classified as motile (Haldar et al 2016). Bacterial movement is facilitated by the presence of flagella. Most bacteria with locomotion (flagella) belong to the *Bacillus* and *Spirillum* groups, while flagella or locomotion tools are rarely found in the coccus group of bacteria (Aizawa & Minamino 2024).

The microscopic morphology of bacteria from fish processing wastewater is classified as Gram-positive bacteria due to their purple coloration and bacillus shape. After Gram staining, if bacterial isolates appear purple, they are classified as Gram-positive bacteria, whereas if they appear red, they are classified as Gram-negative bacteria. The distinction between Gram-positive and Gram-negative bacteria resulting from this staining process is attributed to the treatment with alcohol, which hydrates the bacterial cell wall and the lower lipid content present (Kristensen et al 2023).

The purple coloration of Gram-positive bacteria occurs because the crystal violet dye becomes trapped within the cell due to the dehydrated cell wall, reducing its permeability and pore size. Conversely, the red coloration of Gram-negative bacteria results from the absorption of safranin dye and the loss of crystal violet dye during rinsing with alcohol. The observation of cell shape and Gram staining of bacteria is depicted in Figure 1.

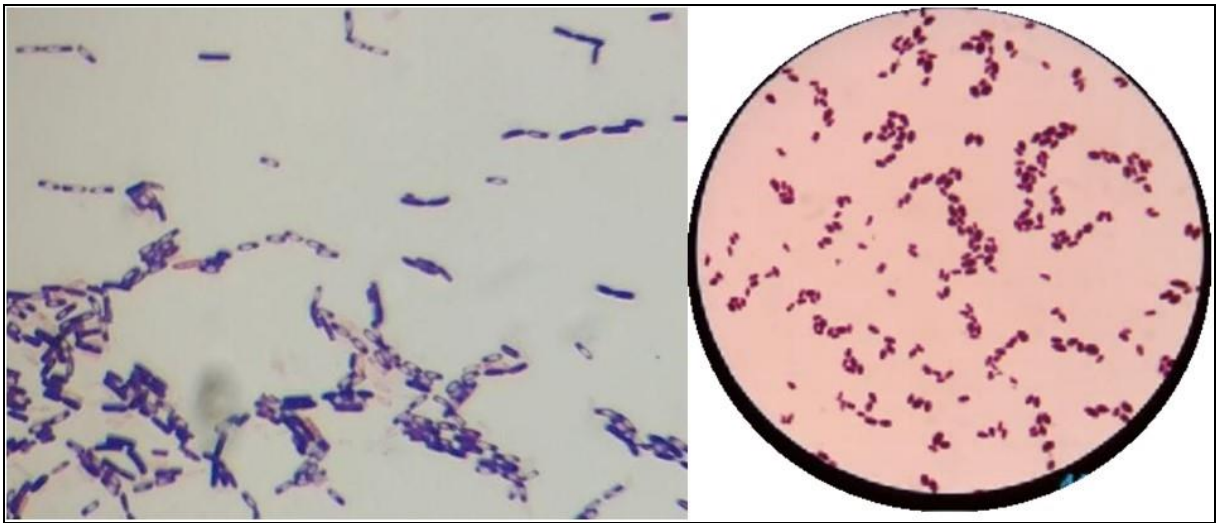

Figure 1. Gram staining: (a) Gram-positive bacteria, (b) bacteria in the form of bacilli.

Bacterial antibiosis test of anchovy processing liquid waste. The antibiosis activity test was conducted to observe and measure the halo zones of bacterial isolates from fish processing wastewater against test bacteria. *Staphylococcus aureus* and *Escherichia coli* bacteria were used as the test bacteria. The presence of halo zones around the paper discs indicates antibiosis activity. The presence of clear zones is attributed to the inability of the test bacteria to grow, indicating that metabolites and acidic compounds produced by bacteria from fish processing wastewater inhibit the growth of the test bacteria. The ability of microorganisms to degrade depends on their adaptability to the environment (Chunyan et al 2023). The results of the inhibition zone calculations for bacterial isolates from anchovy fish processing wastewater can be seen in Table 3.

Table 2

Morphological characterization of bacterial isolates from fish processing wastewater

Characterization	*Bacterial isolate*													
	Ag 1.1	*Ag 1.2*	*Ag 1.3*	*Bg 1.1*	*Bg 1.2*	*Bg 1.3*	*As 1.1*	*As 1.2*	*As 1.3*	*As 1.4*	*Bs 1.1*	*Bs 1.2*	*Bs 1.3*	*Bs 1.4*
Macroscopic														
Shape	Irregular	Irregular	Irregular	Filamentous	Circular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular
Margin	Undulate	Undulate	Undulate	Filamentous	Entire	Undulate	Undulate	Undulate	Undulate	Curled	Undulate	Undulate	Undulate	Curled
Colour	Cream	Cream	Cream	White	Milk-white	Cream	Cream	Cream	Cream	Cream	Cream	Cream	Cream	Cream
Elevation	Raised	Raised	Raised	Flat	Convex	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Raised
Microscopic														
Gram	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Endospora	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Biochemical test														
Catalase test	+	-	+	-	+	+	-	+	+	+	+	-	-	-
Oxidase test	+	-	-	-	+	+	+	+	+	+	+	-	-	-
Motility test	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indol test	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Reduction test nitrate	-	-	-	-	-	-	-	+	+	-	-	-	-	-
H_2S test	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate test	-	-	-	-	-	-	-	-	-	-	+	-	-	-
MR test	-	-	-	-	-	-	+	+	+	-	-	-	-	-
VP test	+	-	+	-	+	+	-	-	-	+	+	-	-	-
Oxidative test fermentative	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis test urea	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hydrolysis test gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 3

In vitro potential of bacterial isolates from anchovy processing liquid waste

Isolate code	*Bacterial isolate inhibition zone (mm)*	
	Staphylococcus aures	*Eschericia coli*
Washing		
Ag 1.1	7	6
Ag 1.2	8	9
Ag 1.3	11	10
Bg 1.1	12	13
Bg 1.2	9	7
Bg 1.3	7	8
Boiling		
As 1.1	10	9
As 1.2	11	12
As 1.3	9	8
As 1.4	10	11
Bs 1.1	9	7
Bs 1.2	8	8
Bs 1.3	9	6
Bs 1.4	7	10

Based on Table 3, it can be observed that in the wastewater from fish washing, the isolate with the highest halo zone is Bg 1.1, measuring 12 mm in the antibiosis test against *Staphylococcus aureus* and 13 mm in the widest halo zone against *Escherichia coli*. This aligns with Hwanhlem's et al (2011) findings, who isolated 14 bacterial isolates from traditional Thai fermented fish (plasom). In this study, the LPS17 isolate exhibited the widest inhibition zone against *S. aureus* at 38.3 mm, while the LPS04 isolate showed the widest inhibition zone against *E. coli* at 62 mm.

Furthermore, in the wastewater from fish boiling, the highest halo zone in the antibiosis test against *Staphylococcus aureus* was observed in the As 1.2 isolate at 11 mm, and against *Escherichia coli*, it was also in the As 1.2 isolate at 12 mm. Abdalaziz et al (2023) also found three bacterial isolates capable of producing inhibition zones against *Escherichia coli* and *Staphylococcus aureus*, measuring 11 mm.

Based on the size of the inhibition zones of bacteria from fish processing wastewater, it can be seen that the bacterial isolate Bg 1.1 from fish washing wastewater has potential. The control using the antibiotic Chloramphenicol exhibited an antibacterial zone diameter of 12 mm. The antibiotic (Chloramphenicol) composition used was 1 g in 9 mL of sterile aquadest. In El awady et al (2023) study, selected isolates were also tested with Chloramphenicol antibiotics, resulting in a clear zone diameter of 26.77 mm. Additionally, Graf et al (2023) found four isolates producing Chloramphenicol antibiotics, indicated by clear zones ranging from 19.54 mm to 22.31 mm.

There are three categories of inhibition zones: zones with a diameter less than 5 mm (≤5mm), which fall into the weak category, zones within the range of 6-10 mm, categorized as moderate, and those within the range of 11-20 mm, classified as strong (Rahman et al 2023). The inhibition zones of the bacteria obtained fall within the range of 11-20 mm. This proves that the bacterial isolates obtained from fish processing wastewater fall into the strong bacteria category.

Hemolysis test. Hemolysis testing, also known as pathogenicity testing, serves as a benchmark for categorizing bacteria as pathogens. Isolates exhibiting pathogenic characteristics are assessed based on the level of clarity formed on blood agar media. Blood hemolysis is categorized into three types: alpha (α) hemolysis, beta (β) hemolysis, and gamma (γ) hemolysis. Alpha (α) hemolysis involves the partial lysis of red blood cells and hemoglobin, beta (β) hemolysis refers to the complete lysis of red blood cells and hemoglobin, while gamma (γ) hemolysis indicates no hemolytic ability whatsoever. The principle of hemolysis testing is the change in color of bacterial colonies inoculated on

blood agar and the appearance of a clear zone surrounding the bacterial colonies (Aula et al 2014). The hemolysis testing results of the Bg 1.1 bacteria, selected due to its strong antibiosis potential against *Staphylococcus aureus* and *Escherichia coli* bacteria in Anchovy fish washing wastewater, can be observed in Figure 2.

Figure 2. Bg 1.1 Bacterial hemolysis test on blood agar.

Based on Figure 2, it can be observed that the bacterial isolate from anchovy fish processing wastewater (*Bacillus* spp.) is capable of lysing blood agar. This occurs because there is a clear zone surrounding the bacteria. This hemolysis falls into the category of beta (β) hemolysis, which involves the complete lysis of red blood cells and hemoglobin. *Bacillus* spp. bacteria exhibit hemolytic properties and can produce secondary metabolites capable of lysing blood cells. Blood is fully utilized by microbes. Streptolysin is an exotoxin produced by bacteria that causes complete lysis of red blood cells. *Bacillus cereus* sensitivity testing using the Vitek 2 identification system showed white bacterial colonies with beta hemolysis (β) on blood agar media (Zeeshan et al 2024).

Growth curves of selected bacterial isolates. This optimization is necessary to observe the growth of selected bacteria as potential antibiotic-producing candidates to ensure optimal functionality in the future. The optical density (OD) test of the Bg 1.1 bacterial isolate was conducted over a 60-hour period using a spectrophotometer at a wavelength of 600 nm. Microorganism growth typically exhibits four phases in its curve: lag phase, log phase, stationary phase, and death phase. Each phase of bacterial growth is influenced by several factors such as growth medium and environment, nutrients, temperature, and others (Thapa et al 2017). The growth curve of the Bg 1.1 bacteria can be seen in Figure 3.

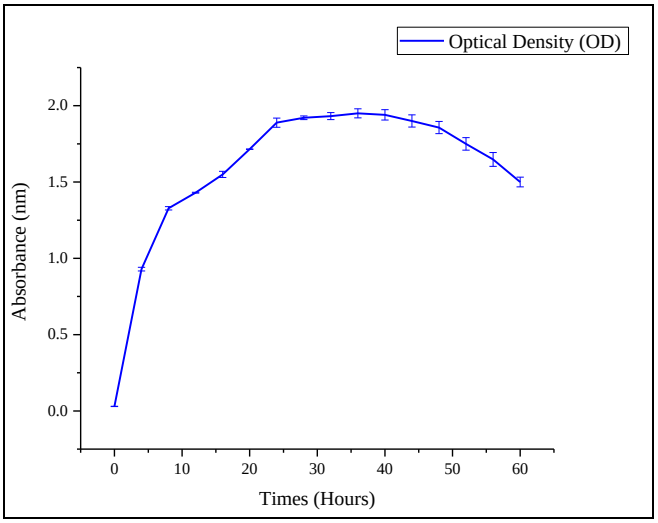

Figure 3. Growth curve of selected bacterial isolate Bg 1.1 during 60 hours of incubation measured from optical density (OD).

Microbes require generation time in their growth process. Generation time is the time needed for microbes to grow and double in number by dividing into two, then dividing again, and so on, resulting in each generation having twice the population of the previous one (Omoregie et al 2020). Microbes also go through four phases in their growth.

Based on Figure 3, the four growth phases of the Bg 1.1 bacteria can be observed. The first phase is called the lag phase, during which the bacteria adapt to the surrounding environmental conditions. The lag phase occurs from hour 0 to hour 4. During the lag phase, there is a rapid increase in the number of bacteria. The speed of the lag phase can be influenced by the medium and the inoculum size. The second phase is the log phase or exponential growth phase. Rapid and constant microbial cell division occurs, following a logarithmic curve during the log phase. Additionally, microbes require more energy during the log phase. The log phase occurs from hour 6 to hour 24. The next phase is the stationary phase, during which the population size remains constant because the number of growing cells equals the number of dying cells. The stationary phase occurs from hour 25 to hour 44. The final phase is the death phase. During this phase, the curve graph will start to decline. The death phase for the Bg 1.1 isolate is from hour 45 to hour 60. Microbial cell death occurs due to the depletion of nutrients in the medium and the exhaustion of energy reserves within the cells.

Secondary metabolites occur during bacterial growth, which are antibacterial substances synthesized directly in the ribosomes. Secondary metabolites are produced maximally during the exponential phase until the early stationary phase. The optimal production of secondary metabolites occurs at the beginning of the stationary phase or the end of the exponential phase (Dadi et al 2019). The production of secondary metabolites follows the pattern of primary metabolites.

The Bg 1.1 isolate grown in glucose peptone (GP) broth media can thrive and grow well because GP broth medium contains sufficient nutrients to be optimally utilized in its metabolic activities. The growth pattern of the Bg 1.1 bacterial isolate is similar to that of bacteria isolated from fermented tenggiri fish (*Scomberomorus guttatus*) products, where secondary metabolite production occurs from hour 6 to hour 22 (Yusra et al 2022). *Bacillus cereus* bacteria derived from milk and its derivatives found that bacteriocin production enters the stationary phase after 10-16 hours of incubation (Torii & Ohkubo 2023).

The isolation and identification of indigenous bacteria begin with bacterial isolation using NA medium. A total of 6 bacterial colonies were found based on the halo zone around the bacteria (Figure 4).

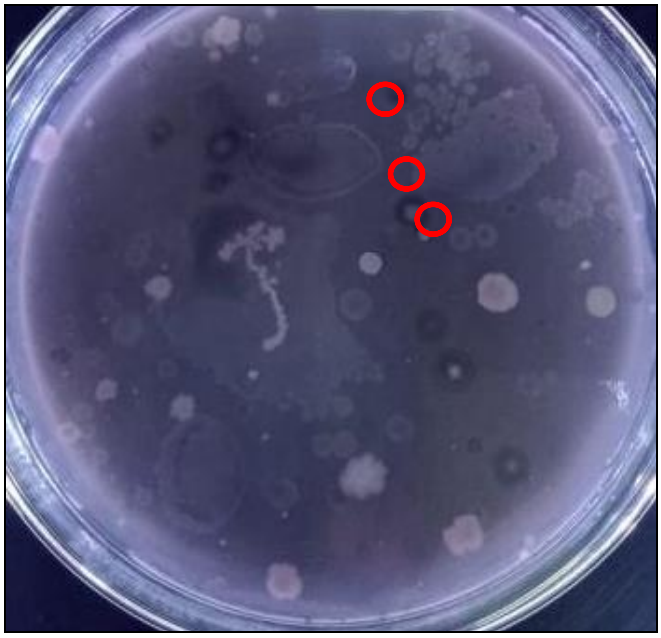

Figure 4. Antibiosis test of bacterial colonies against test bacteria.

Molecular identification was conducted on isolates exhibiting the highest inhibitory activity against the growth of the two test bacteria, namely *S. aureus* and *E. coli*. The bacterial isolate used for molecular identification was Bg 1.1. Molecular identification is performed using the polymerase chain reaction (PCR) technique. There are three main processes fundamental in bacterial identification: extraction, amplification, and electrophoresis. In the extraction stage, DNA strands are isolated from other cellular components. Subsequently, the extracted DNA is amplified via PCR. DNA amplification targets the 16S rRNA gene region. The extracted DNA serves as a template to amplify a segment approximately 500 or 1,500 base pairs in length of the 16S rRNA gene sequence (Regueira-Iglesias et al 2023). The results of gene amplification using PCR on the Bg 1.1 isolate can be observed in Figure 5.

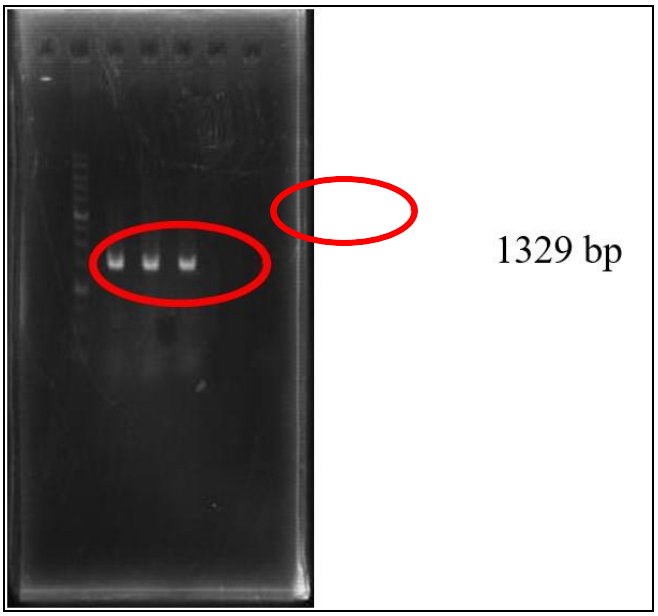

Figure 5. Results of 16S rRNA fragment amplification of isolate Bg 1.1

Isolate Bg 1.1 is known to possess a DNA length of approximately 1,500 base pairs (bp). Its substantial size of 1,500 bp is considered advantageous for informatics purposes. This aligns with Brown's et al (1992) assertion, which elucidates that the 16S rRNA gene

comprises a generally conservative region spanning between 500-540 bp with dispersed locations. Furthermore, the 16S rRNA gene is characterized by a high content of guanine and cytosine (G+C) nitrogen bases, with a length ranging from 1500 to 1550 bp.

Subsequently, sequencing results are utilized to search for sequence similarities within available databases. The commonly employed technique is the Basic Local Alignment Search Tool (BLAST), utilizing an online server (www.ncbi.nlm.nih.gov). A phylogenetic tree is constructed to ascertain the relationship or similarity between Bg 1.1 isolates and species presumed to exhibit similarity based on their nucleotide base sequences. The objective is to reconstruct the history of life and elucidate the diversity of living organisms (Claverie & Notredame 2007). The phylogenetic tree of superior isolates from anchovy processing wastewater (Bg 1.1) is depicted in Figure 6.

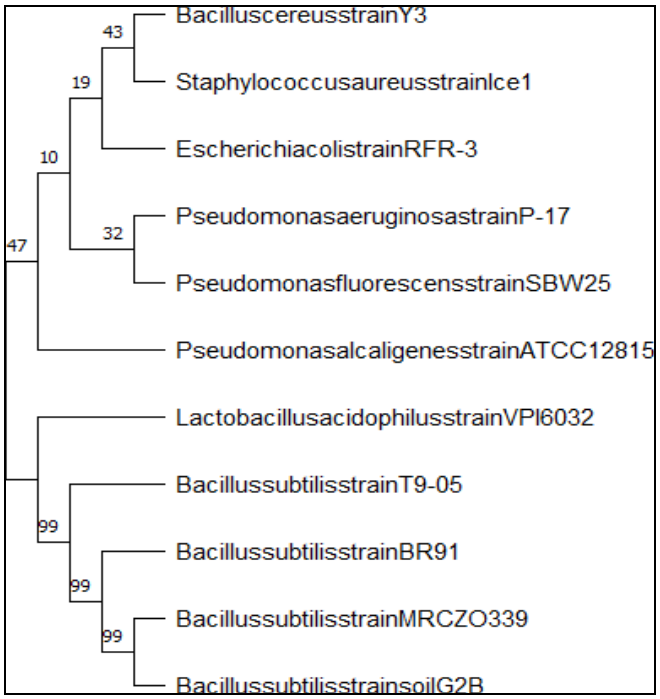

Figure 6. Phylogenetic tree of isolate Bg 1.1.

Based on the results of the BLAST analysis conducted on the DNA sequence of the superior isolate Bg 1.1, it was determined that it originates from the bacterium *Bacillus subtilis* soil strain G2B, exhibiting a homology or level of similarity of 100%, with an E-value of 0. An E-value of 0 indicates identical sequences between the two. The phylogenetic tree results, generated through comparison of 16S rRNA gene sequences, indicated that the type of strain of isolate Bg 1.1 belonged to *Bacillus subtilis* soil G2B. *Bacillus subtilis* soil G2B bacteria share a closer strain type with species such as *Bacillus subtilis* strain T9-05, *Bacillus subtilis* strain BR 91, and *Bacillus subtilis* strain MRCZO339. Isolate Bg 1.1 exhibited a bootstrap value of 99 alongside *Bacillus subtilis* strain T9-05, *Bacillus subtilis* strain BR 91, and *Bacillus subtilis* strain MRCZO339. To assess the reliability of the model data set, the bootstrap method was employed. If the bootstrap value is low, the sequence should be excluded from the analysis to ensure the reliability of the phylogenetic tree (Kin et al 2023).

Conclusions. Based on the research conducted on antibiotic-producing bacteria from anchovy (*Stolephorus* sp.) fish processing wastewater against *Staphylococcus aureus* and *Escherichia coli* bacteria, it can be concluded that six bacterial isolates with potential antibiosis were obtained from the wastewater of anchovy fish washing, and eight bacterial isolates from the wastewater of anchovy fish boiling. The Bg 1.1 bacterial isolate exhibited strong antibiosis potential against both *Staphylococcus aureus* and *Escherichia coli* bacteria in anchovy fish washing wastewater, while the As 1.2 bacterial isolate demonstrated similar potency against the same bacteria in anchovy fish boiling

wastewater. Additionally, Bg 1.1 isolate displayed characteristics of gram-positive bacterial cells, bacillus-shaped cells, aerobic nature, (β) hemolysis, optimal growth at pH 6-9, temperature range of 30°C-40°C, and salt concentration of 25-75 ppt. Molecular identification using 16S rRNA sequences revealed that the Bg 1.1 isolate exhibited 100% similarity with *Bacillus subtilis* strain soil GB2 bacteria.

These findings suggest the potential of wastewater bacteria from anchovy fish processing to produce antibiotics against harmful pathogens. The strong antibiosis activity exhibited by Bg 1.1 and Ag 1.2 isolates underscores their promise as candidates for antibiotic production. Moreover, the characterization of Bg 1.1 isolate provides valuable insights into its physiological traits, which could inform further studies on its antibiotic production capabilities. The molecular identification results further confirm the taxonomic affiliation of the isolates, enhancing our understanding of their genetic makeup and evolutionary relationships. Overall, this study contributes to the exploration of antibiotic-producing bacteria from anchovy fish processing waste and lays the groundwork for future research in antibiotic discovery and development.

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