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PRODUCTION OF ANTIMICROBIAL COMPOUNDS USING THERMOPHILIC BACTERIA SPECIES *BACILLUS SUBTILIS* AND *BACILLUS TEQUILENSIS*

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ABSTRACT

Infections caused by resistant bacteria are a growing public health problem, consequently, a new source of microorganisms that can be used for antimicrobial production is needed. One of the microorganisms capable of producing antimicrobials is the thermophilic bacteria, namely *B. subtilis* and *B. tequilensis.* Due to having hot temperature-resistant enzymes, they are not easily damaged. Therefore, this study aims to produce new antimicrobials from *B.subtilis* and *B.tequilensis*. The antimicrobial activity was observed in 5 thermophilic bacterial isolates using the disk diffusion method. The results showed the strongest zone of inhibition (disk diameter = 6mm) or antimicrobial activity against *S.aureus* which was classified as gram-positive was discovered in *B.subtilis* UTMP15 (10.27 mm) at the incubation time of 24 hours, and against *E.coli* classified as gram-negative,it was in *B. Subtilis* UTMP12 (8.59 mm) at 48 hours. Hence, the isolate is a potential antimicrobial agent.

I. INTRODUCTION

Thermophilic bacteria have an important role in energy metabolism and matter cycling [1]. Besides, an important parameter of microbial culture for survival is temperature with different ranges, causing microorganisms to be divided into psychrophiles, mesophiles, and thermophiles [2].The thermophiles are categorized as moderate (50ºC-60ºC), extreme (60ºC-80ºC) andhyperthermophiles (80ºC-110ºC) [3]. Thermophilic bacteria at temperatures of 90ºC-95ºC include *Termotoga maritime* and *Aquifex pyrophilus* [4]. Members of the genera *Pyrobaculum, Pyrodictium, Pyrococcus,* and *Melanopirus* which belong to *Archaea* are found at 103ºC-110ºC, while in Fungi, classes *Ascomycetes* and *Zygomycetes* grow under high temperatures. Because the described bacteria play an important role in various fields, studies have been conducted to obtain new genera and species around the world [5]. According to Madigan and Martinko (2006), the reason behind the places where thermophilic microorganisms live is a heat source (water or geothermal resources) from the ocean, making the protein structure of thermophilic microorganisms stable and resistant to chemical reagentes [2].

Microorganisms' enzymes are cultured in large quantities within a short time due to being more stable than those from plants or animals and they can be stored under less than ideal conditions for weeks without losing biological activity. Therefore, many microbial enzymes on the market are mechanical or cellular [6]. Niehaus et al (1999) said enzymes are proteins produced by animals, plants, and microorganisms to catalyze biochemical reactions through metabolism in cells [2]. Thermostable enzymes including amylase, cellulase, chitinase, pectinase, xylanase, protease, lipase, and DNA polymerase in thermophilic microorganisms growing at ≥ 50ºC, are very suitable for biotechnological processes at high temperatures [7]. According to Khalil (2011), currently, only two thermophilic bacterial enzymes such as cellulase, etc. from the *Thermus aquaticus* and *alkaliphiles* groups are widely used, because of their high thermostability and resistance to physical or chemical factors [8].

The thermophilic bacterial enzymes that have the potential to be used for antimicrobials and new antibiotics production include protease. Antibiotics produced with fungi, bacteria, and streptomyces are one of the important secondary metabolites exploited commercially. Drugs used in infectious disease chemotherapy are divided into two, namely synthetic drugs (synthesized by chemical procedures in the laboratory) and antibiotics [9]. The lack of antibiotic innovation and pharmaceutical industry funding for new drugs development is not in line with the elevation in irrational drug use that exacerbates this problem. Also, the increasing emergence of resistant pathogenic bacterial infections that create significant problems in human global health is due to the lack of production and introduction of new and effective antibiotic/antibacterial drugs in clinical practice [10]. The most recent threat to human populations worldwide is the continual rise in many drug-resistant microbes in the water and atmosphere [11]. Common antibiotics such as ampicillin, Streptomycin, ciprofloxacin, cefotaxime, and azithromycin are no longer useful for curing bacterial infections, leading to a frightening situation in society [12]. Studies showed COVID-19 is a viral infection that initiates direct antibiotic stress on microorganisms. Based on the data, 72% of COVID-19 patients had received antimicrobial agents, while 8% were co-infected by bacteria or fungi [13][14]. Up to 68.9% of such patients were also reported to have been using antibiotics (mainly azithromycin and ceftriaxone) before hospital admission with a self-medication rate of 33%. This relates to low & middle-income countries, where the lack of knowledge about antibiotics usage causes the associated control measures to be weak [15]. Antibiotic resistance is a major challenge in several fields including biomedical and pharmaceutical studies. For instance, *Staphylococcus aureus* is resistant to methicillin, while *Salmonella typhi* to ciprofloxacin, which extends to other pathogenic bacteria [16]. Antibiotics are needed by the wider community, therefore investigation on antibiotics production from various microorganisms needs to be performed. Since thermophilic bacteria survive at high temperatures, hot springs are one of their habitats which can be found in several parts of Indonesia [17] due to having a lot of geothermal activity and high biodiversity. This country also has the largest geothermal resources in the world, with 252 locations spread across 26 provinces [18][19], hence one of the sources of hot springs is in North Sumatra. Moreover, thermophilic microorganisms have the potential to synthesize antimicrobial compounds as a candidate for antibiotic production. The isolated and identified bacteria, namely *Bacillus subtilis* UTMP11, *Bacillus subtilis*UTMP12, *Bacillus tequilensis*UTMP14, *Bacillus tequilensis*UTMSA14, and *Bacillus subtilis* UTMP15 were previously reported to produce enzymes such as inulinase, protease, cellulase, and carbohydrase [20][21][22]. Moreover, antimicrobial activity tests have never been conducted, therefore this study aims to examine antimicrobial activity using the thermophilic bacteria isolated from hot springs in North Sumatra for antibiotic production.

II. MATERIALS AND METHODS

II.1 THERMOPHILIC ISOLATE SAMPLING

The samples used were obtained from a collection of thermophilic bacteria isolates from the molecular biology laboratory of Prima Indonesia University with the code *Bacillus subtilis* UTMP11, *Bacillus subtilis*UTMP12, *Bacillus tequilensis*UTMP14, *Bacillustequilensis*UTMSA14, and *Bacillus subtilis* UTMP15.

II.2 BIOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION

The thermophilic bacteria were characterized biochemically and morphologically in terms of color, size, colony shape, edges, elevation, gram staining, motility, catalase, citrate, and starch hydrolysis.

II.3 ISOLATION OF THERMOPHILIC BACTERIAL CULTURE

A total of 10ml of NutrientAgar medium was poured and solidified in a petri dish. Thermophilic bacteria were cultured on the solid media using the quadrant streak plate method by taking 1 tube of pure bacterial culture and scratching it on the media surface, then incubated at 45°C for 24 hours.

II.4 ANTIBIOTIC PRODUCTION MEDIA

Synthetic media consisting of 5.0g L-glutamic acid, 0.01g $CuSO_4.7H_2O$, 0.015g $CaCl_2.2H_2O$, 0.01g $FeSO_4.7H_2O$, 0.5g KH_2PO_4, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.01g $MnSO_4.H_2O$, 0.01g NaCl, &1% glucose of the total production medium, weighed and dissolved in 1 liter sterile distilled water in an Erlenmeyer flask, was used as the antibiotic production media. Furthermore, up to 30ml of the antibiotic production media was filtered with a sterile Millipore filter and inserted into each of the flasks (50ml). Thermophilic bacteria inoculum was prepared in NB (Nutrient Broth) liquid media, then putintoeach test tube, and incubated at 45°C for 24 hours (10% inoculum in liquid media). Inoculum NB was added into each of the previous flasks, then placed in a shaker incubator that was run at a speed of 150rpm at 45°C. Every 24 hours, samples were collected for up to 72 hours, by pipetting 10 times (10ml) using a micropipette into each Eppendorf tube (sterile), followed by centrifuging for 10 minutes at 10,000 rpm to obtain a cell-free supernatant. Afterward,the cell-free supernatant were removed from the Eppendorf tube using a 10ml syringe & filtered again using a Millipore filter (disposable), then put inside a sterile test tube, and stored in the refrigerator. In the process of making antibiotic production media, the tools and materials used need to be sterile [23].

II.5 ANTIMICROBIAL ACTIVITY

About 10 ml of sterile NA (Nutrient Agar) media was poured into a petri dish until it solidified. The sterile cotton swab dipped in the test bacteria suspension was swabbed evenly on the surface of the media in a petri dish using the disk diffusion method. Next, the blank disks were dipped into each of the supernatants which was later placed on the surface of the swabbed media. This was followed by incubation for 24 hours in an incubator at 37°C. The results were observed and the visible clear zone was calculated using a caliper. Antimicrobial activity test was performed in triplicate in each sample, with observations within 24, 48, and 72 hours or for 3 consecutive days [24].

II.6 EFFECT OF INCUBATION TIME

Samples were incubated at 45°C in an incubator by setting a speed of 150rpm and they were taken for 24 hours from 0 to 72 hours. Cell-free supernatants from the samples were obtained at different times and used against test bacteria such as *Escherichia coli*&*Staphylococcus aureus*, then the clear zone formed around the bacterial colonies was measured [23].

III. RESULTS AND DISCUSSIONS

Results from the morphological and biochemical characterization *of Bacillus subtilis* UTMP11, *Bacillus subtilis* UTMP12, *Bacillus tequilensis* UTMP14, *Bacillus tequilensis* UTMSA14, and *Bacillus subtilis* UTMP15 can be seen in Tables 1 and 2, as well as Figures 1, 2, & 3.

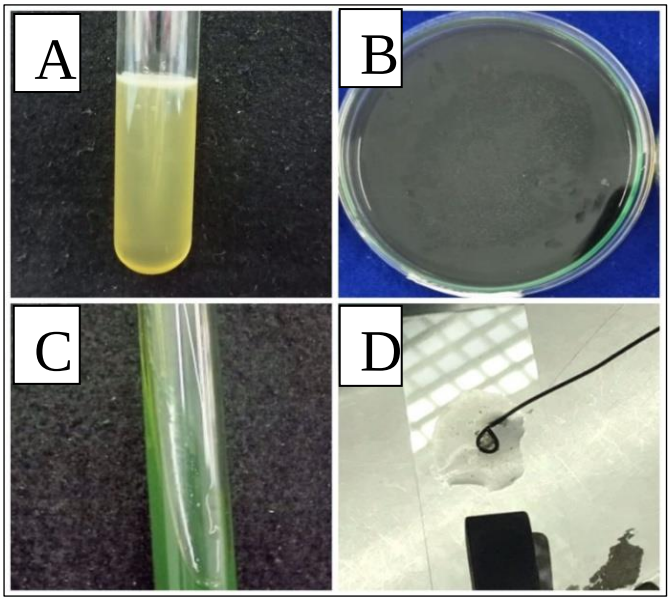

Figure 1: Results of biochemical characterization tests for *Bacillus subtilis* UTMP11, *Bacillus subtilis* UTMP12, *Bacillus tequilensis* UTMP14, *Bacillus subtilis* UTMP15 and *Bacillus tequilensis* UTMSA14 consisting of (a) motility, (b) starch hydrolysis, (c) citrate, and (d) catalase tests.
Source: Authors, (2021).

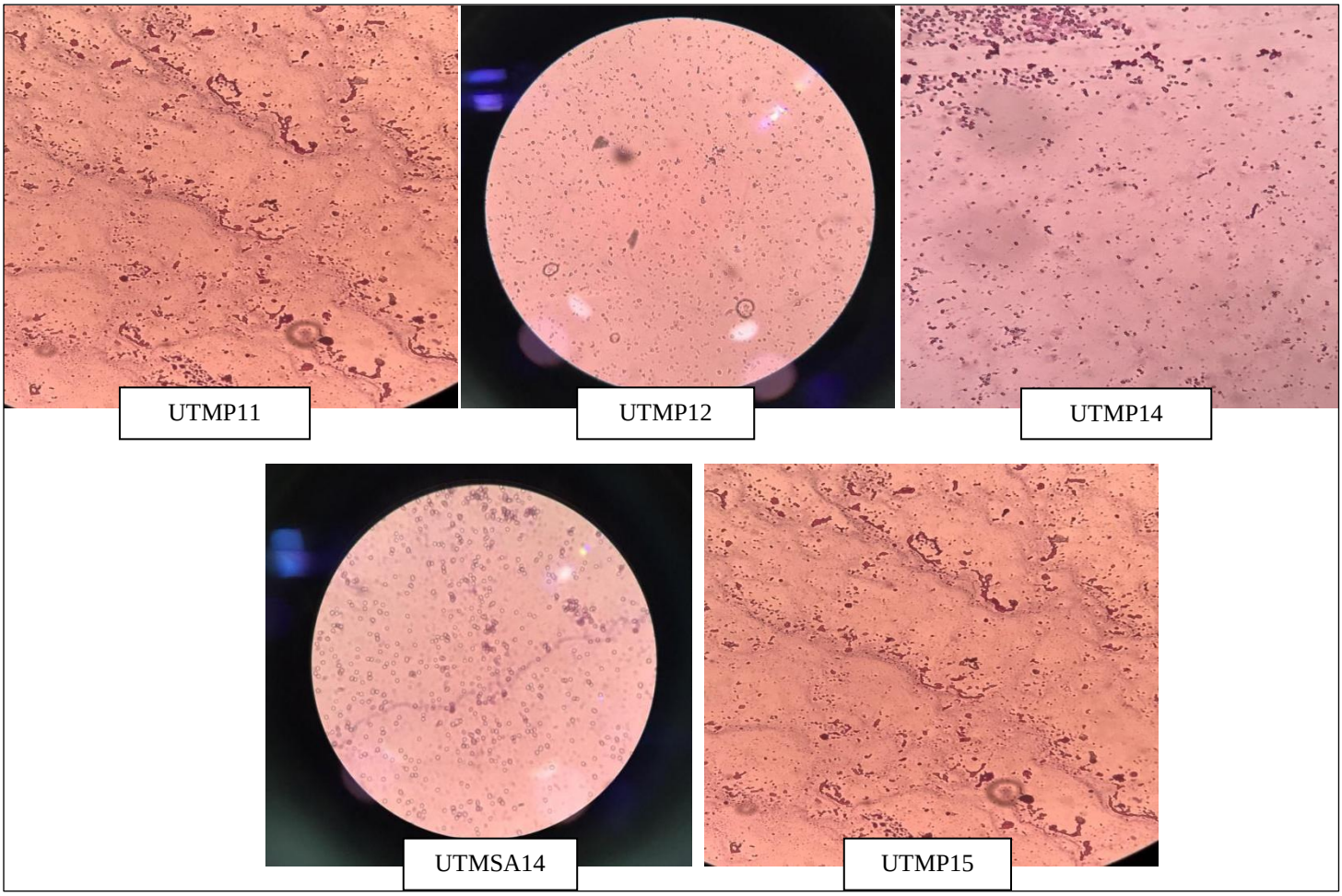

Figure 2: Morphological test results for microscopic observations of *Bacillus subtilis* UTMP11, *Bacillus subtilis* UTMP12, *Bacillus tequilensis* UTMP14, *Bacillus tequilensis* UTMSA14 and *Bacillus subtilis* UTMP15.
Source: Authors, (2021).

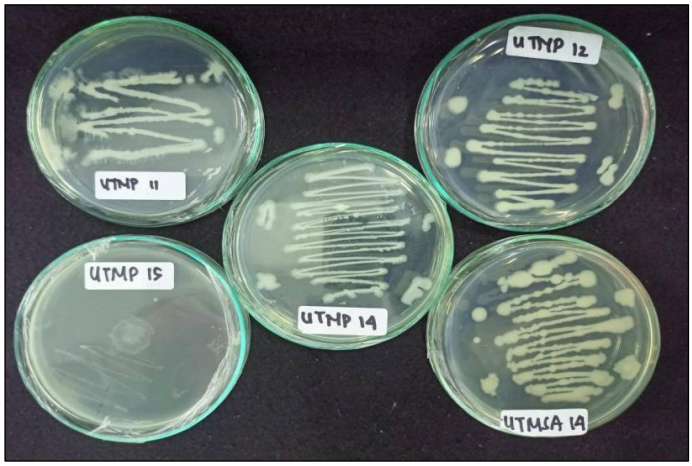

Figure 3: Culture results for macroscopic observations of *Bacillus subtilis* UTMP11, *Bacillus subtilis* UTMP12, *Bacillus tequilensis* UTMP14, *Bacillus tequilensis*UTMSA14, and *Bacillus subtilis* UTMP15 culture in NA (Nutrient Agar) media.
Source: Authors, (2021).

Table 1: Biochemical characterization of *Bacillus subtilis* UTMP11, *Bacillus subtilis* UTMP12, *Bacillus tequilensis* UTMP14, *Bacillus tequilensis* UTMSA14, and *Bacillus subtilis* UTMP15.

Isolates code	Motility test	Starch hydrolysis test	Citrate test	Catalase test
*Bacillus subtilis*UTMP11	+	-	-	-
*Bacillus subtilis*UTMP12	+	-	-	-
Bacillus tequilensis UTMP14	+	-	-	-
*Bacillus tequilensis*UTMSA14	+	-	-	-
*Bacillus subtilis*UTMP15	+	-	-	-

Source: Authors, (2021).

Table 2: Microscopic and macroscopic morphological tests for *Bacillus subtilis* UTMP11, *Bacillus subtilis* UTMP12, *Bacillus tequilensis* UTMP14, *Bacillus tequilensis* UTMSA14, and *Bacillus subtilis* UTMP15.

Isolates Code	Colony Morphology in Nutrient AgarMedia						
	Colony Shape	Colony Edges	Colony Elevation	Colony Size	Cell Color	Cell Shape	Gram Staining
Bacillus subtilis UTMP11	Filamentous	Filamentous	Convex	Moderate	Purple	Coccus	Gram-Positive
Bacillus subtilis UTMP12	Irregular	Undulate	Convex	Moderate	Purple	Coccus	Gram-Positive
Bacillus tequilensis UTMP14	Filamentous	Filamentous	Convex	Moderate	Purple	Coccus	Gram-Positive
Bacillus tequilensis UTMSA14	Irregular	Undulate	Convex	Moderate	Purple	Coccus	Gram-Positive
Bacillus subtilis UTMP15	Irregular	Undulate	Convex	Small	Purple	Coccus	Gram-Positive

Source: Authors, (2021).

Isolates *Bacillus subtilis* UTMP11, *Bacillus subtilis* UTMP12, *Bacillus tequilensis* UTMP14, *Bacillus tequilensis* UTMSA14, and *Bacillus subtilis* UTMP15 showed positive results on motility tests and negative on starch hydrolysis, citrate & catalase tests. The positive results characterized by the presence of culture spreading out of the inoculation line in the motility media indol sulfide proved bacterial motility [22]. Meanwhile, the negative results on starch hydrolysis tests characterized by the non-formation of clear zones around bacterial colonies during iodine solution administration indicated the absence of the hydrolysis process. Negative results on the citrate test were characterized by the absence of color changes,causing the media to remain green.Citrate tests are used ininvestigating the ability of microorganisms to use citrate. Meanwhile, the negative catalase tests were characterized by the absence of a reaction to gas bubbles' emergence. Catalase tests are performed to determine whether the test organism produces this enzyme.

The results of microscopic morphological tests seen in Table 2 and Figure 2 for the five isolates were obtained on gram-positive bacteria, with coccus cell shape and purple color. Having a thick peptidoglycan layer allows gram-positive organisms to maintain the crystal violet-iodine complex and their cell is stained as a purple color. The macroscopic test results, as seen in Figure 3 and Table 2, showed that irregular colony forms are more dominant than filamentous shapes. The edges of the colony undulate are more dominant in the aforementioned isolates than filamentous and they had convex elevation. In terms of size, 4 isolates codes show moderate colony and 1 indicates a small colony.

Bacteria of the genus *Bacillus* are a group characterized by the ability to produce strong spores and can be found in soil, water, or air [25]. *Bacillus* strains have biotherapeutic potential to interact with the host'sinternal environment by producing a variety of antimicrobial peptides and small extracellular effector molecules. They are also gram-positive, rod-shaped, spore-forming, and aerobic or facultative anaerobic bacteria that are found in soil, air, water, intestines of humans and animals, as well as from vegetables &and foods [26]. *B. subtilis* is the most prolific species which devotes 4-5% of its genome to synthesis, producing 66 antibiotics.Hence, 795 antibiotics have been identified from *Bacillus* species mainly frompeptides [25][27].

Commercially,*B. subtilis* is very important because it produces secondary metabolites in high and diverse quantities such as antibiotics, chemicals, and enzymes, as well as heterologous proteins, antigens, & vaccines to avoid being harmful to mammals including humans [25][28]. This bacterium grows in many environments and exhibits considerable genomic diversity. Therefore, the marine products *sp.*Hasa variety of secondary metabolites (lipopeptides,polypeptides,macrolactones, fatty acids, polyketides, lipoamides, and isocoumarins) [29]. There is a 99% similarity between *B. tequilensis* and *B. subtilis* based on the 16S rRNA gene sequence. *B. tequilensis* grows anaerobically, degrades tryptophan & starch but not urea, and uses citrate as a carbon source, while the motile, oxidase &catalase tests are positive.In addition, it withstands salt concentrations up to 8% physiologically and is gram-positive spore-forming [30].

III.1 ANTIMICROBIAL ACTIVITY

In this case, all thermophilic bacterial isolates with codes *Bacillus subtilis* UTMP11, *Bacillus subtilis* UTMP12, *Bacillus tequilensis* UTMP14, *Bacillus tequilensis* UTMSA14, and *Bacillus subtilis* UTMP15 were tested for inhibition zone to determine whether they had antimicrobial activity in the sample to inhibit the growth of pathogenic bacteria that were used as test bacteria namely *Escherichia coli* and *Staphylococcus aureus.*

Table 3: Zone of inhibition results for the thermophilic bacteria isolates against pathogenic bacteria (disk diameter = 6mm).

No.	Isolates code	Zone of inhibition (mm) (disk diameter = 6mm)					
		Escherichia coli			*Staphylococcus aureus*		
		24 Hours	48 Hours	72 Hours	24Hours	48 Hours	72 Hours
1.	*Bacillus subtilis*UTMP11	7.87	7.71	6.73	9.17	8.54	7.91
2.	*Bacillus subtilis*UTMP12	7.96	8.59	7.47	9.56	9.27	8.28
3.	*Bacillus tequilensis*UTMP14	7.55	7.74	6.0	8.52	7.86	7.84
4.	*Bacillus tequilensis*UTMSA14	7.53	7.82	7.46	8.80	8.64	8.2
5.	*Bacillus subtilis*UTMP15	7.14	7.85	7.72	10.27	8.32	8.90

Source: Authors, (2021).

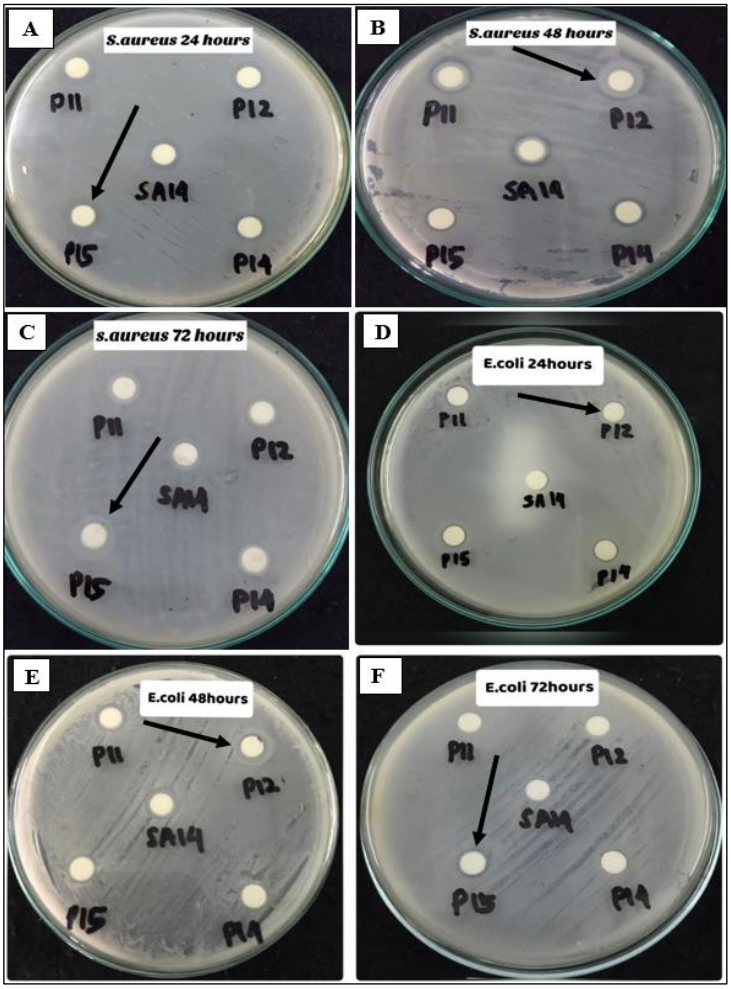

Figure 4: The results of the inhibition zone test for *Bacillus subtilis* UTMP11, *Bacillus subtilis* UTMP12, *Bacillus tequilensis* UTMP14, *Bacillus tequilensis* UTMSA14, and *Bacillus subtilis* UTMP15, where black arrows indicate a stronger inhibition zone at (a) 24 hours of *S.aureus* inhibition, (b) 48 hours of *S. aureus* inhibition, (c) 72 hours of *S. aureus* inhibition, (d) 24 hours of *E. coli* inhibition, and (e) 72 hours of *E. coli* inhibition.
Source: Authors, (2021).

Table 4 and Figure 4 show the results of the five isolates' zone of inhibition against pathogenic bacteria. The diameter of zone of inhibition against *Staphylococcus aureus* at incubation times of24, 48, and 72 hours was between 7.84 mm – 10.27 mm (disk diameter = 6mm), where the strongest was in *the Bacillus subtilis* UTMP15 at 24 hours and the weakest was in *the Bacillus tequilensis* UTMP14 at 72 hours. The diameter of the zone of inhibition against*Escherichia coli*at the same incubation time as the previous was between 6.0 mm – 8.59 mm (disk diameter = 6mm), where the strongest zone was in the *Bacillus subtilis* UTMP12 at 48 hours and the weakest was in the *Bacillus tequilensis* UTMP14 at 72 hours. *Bacillus subtilis* UTMP15 had better antimicrobial activity on *Staphylococcus aureus* than *Bacillus subtilis* UTMP12 tested with *Escherichia coli.*Furthermore, its zone of inhibition against *Staphylococcus aureus* was 10.27 mm (disk diameter = 6mm) while *Bacillus subtilis* UTMP12 was had 8.59 mm (disk diameter = 6mm). The results showed a stronger antimicrobial activity was exerted against the *Staphylococcus aureus* compared to *Escherichia coli.* This is also supported by Syukur Sumaryanti et al (2016) that stated *Staphylococcus aureus* is included in gram-positive bacteria which have a larger zone of inhibition usually when compared to gram-negative bacteria. This is due to several resistance mechanisms such as, the nature of the permeability barrier which in the outer layer inhibits an antimicrobial compound's entry. Another mechanism also disables the specific resistance of the compound to prevent cytoplasmic membrane penetration or intracellular increases [31].

The cell envelope of gram-positive bacteria consists of an inner plasma membrane surrounded by a permeable cell wall (not restrictive to antibiotics diffusion into the cell), and a thick layer of peptidoglycan, composed of an outer layer of cells, in contrast to gram-negative bacteria which have a distant peptidoglycan layer. Furthermore, it is surrounded by a second membrane consisting of a phospholipid and lipopolysaccharide (LPS) double layer called the outer membrane which serves to provide extra protection for the cell and plays a major role in preventing the diffusion of hydrophobic molecules, including many antibiotics, into the cell to ensure these compounds only enter through selective porins, providing intrinsic resistance [32].

The results in table 3 show the strongest zone of inhibition in pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli* at 24 and 48 hours compared to the incubation time of 72 hours. These observations are supported by a report from Syed Aun Muhammad (2009) which conducted an inhibition zone test and examined antimicrobial activity in *Bacillus* SAT4, where the strongest activity (24mm) was obtained at the incubation time of 24 hours and 48 hours [23].

Some of the factors constituting the inhibition zone are described by Muaaz *et al* (2007) which said, at an incubation time of 48 hours in sample bacteria, a relatively large zone of inhibition is produced. Therefore, it can be interpreted that the antimicrobial compounds synthesized are derived from secondary metabolites. In other studies, Ren *et al* (2010) reported that strains of bacteria isolates from extreme habitats such as thermophiles derived from hot water temperatures exhibit antimicrobial activity against some common pathogenic bacteria such as. *S.aureus* which are classified as gram-positive. The antimicrobial activity at 45°C is indicative of the isolates' thermotolerant properties as well as their antimicrobial compounds. Moreover, the antimicrobial activity from bacteria with extreme temperatures shows the possibility of bioprospecting these organisms for use [33]. Glucose is one of the factors in inhibition zone formation where the effect of its concentration on antibiotic production is studied and increased concentration is found to have a positive effect. Also, as an excellent source of carbon for bacterial growth, it interferes with the synthesis of many secondary metabolites. In some microorganisms, the inhibition effect of this molecule has been associated with a decrease in pH and is due to the acidification caused by organic acids accumulation [23].

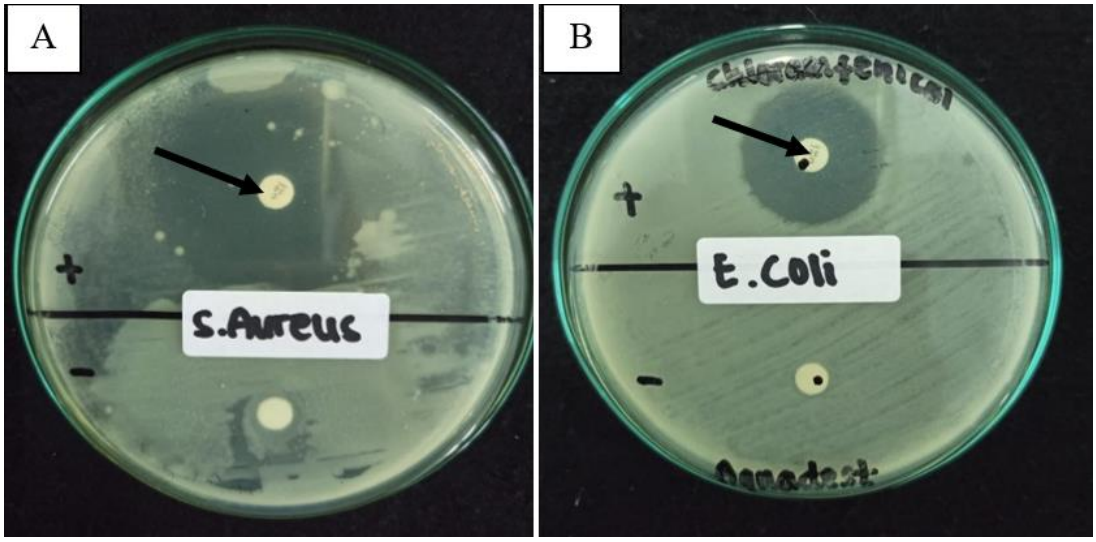

Figure 5: Test results for (a) *Staphylococcus aureus* and (b) *Escherichia coli* bacteria, where positive control used chloramphenicol antibiotic discs with a concentration of 30 and negative controls employed aquadest. The black arrow indicates the inhibition zone in the positive control test with the chloramphenicol used against both bacteria.
Source: Authors, (2021).

According to Figure 5, in the positive control test performed using a disc of antibiotic chloramphenicol with a concentration of 30, clear zones were formed in the *Staphylococcus aureus* and *Escherichia coli*. In the negative control where blank disks that have previously been dipped in the aquadest were employed, no clear zone was formed.

IV. CONCLUSIONS

Based on the results, thermophilic bacteria namely *Bacillus subtilis* and *tequilensis* species are potential producers of antimicrobials. They can be used for this purpose due to being stable at higher temperatures and having the strongest antimicrobial activity against gram-positive bacteria. Moreover, the strongest zone of inhibition against *Staphylococcus aureus* is in *the Bacillus subtilis* UTMP15 at an incubation time of 24 hours at 45ºC, while that of *E. coli* is in *Bacillus subtilis* UTMP12 at 48 hours within the same temperature. Compared to *E.coli* which is classified as gram-negative, the strongest antimicrobial activity against *S.aureus* classified as gram-positive bacteria is in *B.subtilis* UTMP15 (10.27 mm) at 24 hours, hence the isolate is a potential antimicrobial agent.

V. AUTHOR'S CONTRIBUTION

Conceptualization: Helmi Andriyani,Natacia and Edy Fachrial.
Methodology: Edy Fachrial.
Investigation: Helmi Andriyani and Natacia.
Discussion of results: Helmi Andriyani, Natacia and Edy Fachrial.
Writing – Original Draft: Helmi Andriyani and Natacia.
Writing – Review and Editing: Helmi Andriyani,Natacia and Edy Fachrial.
Resources: Helmi Andriyani, Natacia, and Edy Fachrial.
Supervision: Edy Fachrial.
Approval of the final text: Edy Fachrial.

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