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SCREENING OF POTENTIAL BACTERIA WITH ANTAGONISM PROPERTIES ISOLATED FROM SOIL AND STREAM IN HUTAN UiTM CAWANGAN NEGERI SEMBILAN

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Abstract

Infectious diseases caused by bacteria are increasing nowadays, and this cause the production of synthetic drugs also in demand. However, synthetic drugs give side effects to some people, so to reduce or as an alternative, biotherapeutic treatment can be used using bacteria taken from natural sources that have not been explored by humans which are very sustainable. Therefore, bacteria which are common microorganisms will help to serve critical roles in the health and operation of land and aquatic ecosystems. The study was conducted at Hutan UiTM Cawangan Negeri Sembilan, Kuala Pilah Campus to isolate and characterize the bacterial colonies from soil and stream. The main focus of this study is to identify Gram positive bacteria with negative haemolytic activity and to determine the antagonism activity against pathogenic bacteria. A total of 20 pure bacterial colonies were isolated from soil samples, and 30 from stream samples collected at various locations. Gram positive bacterial identification indicated that 12 out of the 20 soil colonies and 10 out of the 30 stream colonies exhibited characteristics consistent with Gram positive bacteria. Haemolysis test and catalase test were conducted on both Gram positive bacteria isolated from soil and stream samples where soil samples have seven bacterial isolates and stream samples have eight isolates showed no hemolytic activity, while for catalase test five and four bacterial isolates showed positive activity correspondingly. However, no inhibition zones were observed in the antagonism assays of all isolates from both samples against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Although the antagonism results were negative, this study contributes to our understanding of the microbial diversity in soil and stream water ecosystems. Further research is recommended to refine the experimental procedures, optimize conditions, and employ selective media for more targeted analysis. Such investigations will help unravel the potential applications of bacteria present in soil and stream samples and their ecological roles.

Keywords: Soil bacteria, stream water bacteria, antagonism activity,

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Introduction

Water is a valuable resource with numerous applications such as food production, cleaning, transportation, power generation, recreation, and also as medicine. Furthermore, the use of water to treat diseases is one of the oldest medical therapies (Mooventhan & Nivethitha, 2014). One of the bacteria found in streams is cyanobacteria that have a variety of health benefits and specific applications which include production of pharmaceuticals, fuels, cosmetics, food additives and plastics (Tiwari & Tiwari, 2020). Whereas, soils offer a lot of ecosystem services to humans, many of which could relate to human health (Steffan et al., 2020) and also crucial in the mineralization of organic matter, the biogeochemical cycling of carbon and nitrogen, and more soil activities (Liu et al., 2019). For example, Bacillus subtilis also known as spore-forming bacteria produces spores that can resist extreme circumstances such as

stomach acid, irradiation, and high temperature. Soil-based probiotics, like other commercially clinically utilized probiotic microorganisms, aid with digestive health and immune system regulation (Dalal et al., 2022).

Gram positive bacteria are chosen because they lack an outer membrane, resulting in a peptidoglycan layer that is significantly thicker than the outer membrane in Gram negative bacteria (Silhavy et al., 2010), therefore, antibiotic resistance is higher in Gram negative than in Gram positive bacteria (Buthaina, et. al., 2020). Besides that, haemolysis test is important for safety purposes to avoid a bacterium which can lyse the red blood cells of the host. Bacteria with hemolysins can discolor or clear the blood agar in the vicinity of their colonies because hemolysins, the substances formed by a variety of bacterial species that are thought to be important virulence agents. To supply nutrients, particularly iron, to the toxin-producing bacteria, these compounds cause membrane disruption, cell lysis, and tissue destruction (Mogrovejo et al., 2020).

Globally, infectious diseases are the leading cause of death. Not only are new infectious diseases emerging, but the re-emergence of lethal infectious diseases, as well as the increasing prevalence of antibiotic resistant strains, constitute a substantial threat to public health and welfare. The production of synthetic drugs is in demand due to the expanding worldwide antimicrobial resistance disease, there is a sense of urgency that need for alternatives to sustainable antibiotics, particularly in disadvantaged countries. However, synthetic drugs give side effects to some people because the broad spectrum antibiotic can kill the normal microbiota in gut microbiota in our body. The gut microbiota is known to be essential for the normal development and functioning of the human body, particularly for the priming and maturation of the adaptive immune system (Ramirez et al., 2020). Therefore, to reduce or as an alternative, biotherapeutic treatment can be used using bacteria taken from natural sources that have not been explored by humans which are very sustainable (Tamma et al., 2017). So, in this research, new therapeutic agents will be found by using natural sources from streams and soil.

Over the last few decades, the utilization of natural resources has diminished in light of the advancements in molecular biology and combinatorial chemistry, as artificial chemical compounds have been engineered to replicate their functions (Atanasov et al., 2015). Thus, this project will be helpful to find potential bacteria with the most important characteristic for this research which is the antagonism properties, the bacteria ability to grow against pathogenic bacteria, Gislin et al. (2018) managed to isolates total of eight Gram-positive bacterial from soil sample of Kochi, India with this antagonism properties to combat against antimicrobial resistance disease. Researchers are working on exciting new ways to fight infection which aim to find alternatives to antibiotics that are both effective and better for the long run. This study aims to investigate the microbial diversity in Hutan UiTM Cawangan Negeri Sembilan, considering the influence of abiotic factors such as climate, temperature, pH, and salinity on bacterial properties. The pristine condition of this unexplored environment is anticipated to provide valuable insights into the quality of soil and stream samples, offering a unique perspective on less-polluted ecosystems.

Methods

Materials

Water and soil samples were collected from the streams and soil of Hutan UiTM Cawangan Negeri Sembilan at different locations. For the soil samples, collection involved excavation and placement into sealed plastic containers. In the case of stream samples, water was procured directly from the flowing stream and transferred into designated sample containers. The pathogenic bacteria, specifically *Staphylococcus aureus* exhibiting large yellow colonies, *Pseudomonas aeruginosa* characterized by medium-sized and smooth-textured colonies, and *Escherichia coli* with a smooth texture and large size that has been requested prior seven days and kept inside incubator under controlled laboratory conditions were obtained from Unit Bank Culture, Faculty Applied Sciences, School of Biology, Kuala Pilah Campus, UiTM Cawangan Negeri Sembilan.

Chemicals
Blood agar, crystal violet stain, iodine solution, glycerol stock, De Man, Rogosa and Sharpe agar (MRS agar), MRS broth, 70% alcohol, safranin, nutrient agar and nutrient broth and distilled water.

Isolation of bacteria
Streak-plate methods were used to isolate pure culture of bacteria colonies. Five-fold serial dilution was carried out used for inoculation. Approximately, 1 of 10^{-5} dilution were inoculated on sterile Petri dishes, after the sterilized media (De Man, Regosa and Sharpe agar) were poured aseptically on the inoculated plates. Then, the inoculation loop was used to lightly drag back and forth across the agar's surface. The inoculation loops were sterilized and were placed against the far end of the first streak. After that, the procedure was repeated with a sterile loop on the second and third streaks of Zigzag a notion until the final section at the center of the plate. Then, the plates were closed. The completed agar plates were inverted, on the front desk's incubation rack in the incubate section. The plates were incubated at 37ºC for 24 hours.

Purification of bacteria
The different morphology of bacteria was observed under a light microscope and a single colony of predominant bacteria were picked and inoculated into a new nutrient agar. The total strains of bacteria were counted and identified. Then, the streak-plate method was repeated on a single colony that was chosen so pure culture was obtained.

Preservation of bacteria
Glycerol stock in -20ºC was used in the preservation of bacteria. Approximately, the first 50% of the glycerol solution were made by diluting 100% of glycerol in distilled water. Once the bacteria had grown, 500 µL of the culture were mixed with 500 µL of 50% glycerol in a tube. Then, the glycerol stock tube froze in -20º C.

Identification of Gram positive bacteria
First, the slide smear was prepared by using an inoculation loop with streak plate technique to transfer the culture to a microscope slide, the slide was dried over gentle heat. Then, a few drops of crystal violet were dropped off to the fixed culture on the microscope slide and waited for 1 minute before rinse with distilled water. Iodine solution was used next with the same method of crystal violet. After that, the slide was washed using 70% alcohol as decolouriser and again waited for 3 to 5 seconds. The slide was stained using safranin and waited for 45 seconds before the slide was washed off using distilled water. The slide was carefully dried using filter paper before the light microscope was used to observe the bacteria and identify the Gram positive bacteria.

Haemolysis test
Firstly, the blood agar powder was added to 1L of distilled water. The Bunsen burners were used to boil the solution until fully dissolved. Next, the dissolve solution, which is the blood agar were poured into sterilized petri plate and were let the agar to cool down and become solid. After that, the isolated bacteria were transferred to the blood agar media by using streak plate method with the inoculation loop. Then, the blood agar media were placed into the 35-37ºC incubator for 24 hours. Next, the blood agar was observed for their hemolysis activity.

Catalase test
A sterile inoculating loop was used to take a small number of bacteria from a well isolated single colony and were placed onto a microscope slide. Then, a dropper was used to place a drop of 3% hydrogen peroxide on the microscope slide that had the bacteria. The bacteria were not mixed and immediately observed for immediate bubble formation.

Antagonism activity by agar well diffusion assay
The bacteria with Gram Positive and negative haemolysis activity were selected for the antagonism

activity. Agar well diffusion assay method was used to test the antagonism activity of the bacteria. The *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* is already cultured on a petri plate and were obtained from Unit Bank Culture, Faculty Applied Sciences, School of Biology, Kuala Pilah Campus, UiTM Cawangan Negeri Sembilan. After that, a sterile cork borer was used to bore a hole with 6mm or 7mm wells of each petri plate. then, the bacteria isolates ($10^5 CFU/ml$) were pipetted into the hole using micropipette. After that, the media was incubated for 24 hours at 37°C. Lastly, the inhibition zone (mm) of different pathogenic bacteria were observed and evaluated.

Result and Discussion

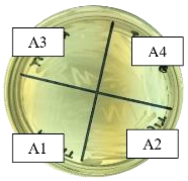
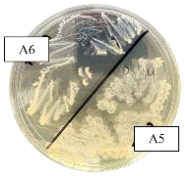
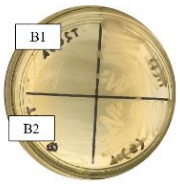
Bacterial colonies isolated from soil samples

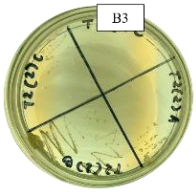
Soil samples were taken from four distinct locations on the Hutan UiTM Kuala Pilah Campus in Cawangan Negeri Sembilan. After incubating the samples on the agar plates for 24 hours at 37° C, a total of 20 pure bacterial colonies were obtained. This finding is supported by Ali and Naseem (2012), found around 28 bacterial colonies from different locations in Lahore and Changa manga, Pakistan. However, another study from Prashanthi et al. (2021), managed to find 263 bacterial isolates from soil samples obtained in Bangalore, India. The different number of bacterial colonies obtained because soil is different in their physical and chemical properties which can affect their composition of soil microbiota (Gagelidze et al., 2018)

Morphology of pure bacterial colonies obtained

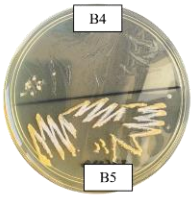
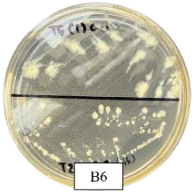
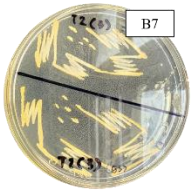
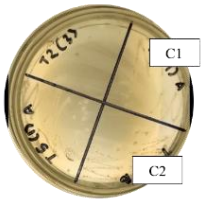
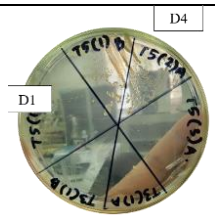
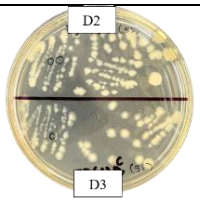
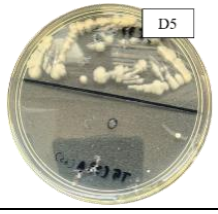
From the result observed, from the 20 pure colonies obtained, 12 pure colonies identified as Gram positives. Past study made by Xue et al. (2018) mentioned that the biogeographic distribution of soil microbes is depending on their environmental filter, for example their soil properties, environment situation and land cover. *Bacillus* species are Gram positive bacteria that are abundant in soils (Liu et al., 2019). According to Yahya et al. (2021) *Bacillus subtilis* has been used to produce a lot of precious antibiotics against pathogenic bacteria that are drug resistant. Table 1 showed the characteristics and morphology of bacterial colonies with total of 20 bacterial isolates.

Table 1. Characteristics and morphology of bacterial colonies with total of 20 bacterial isolates.

Isolates	Characteristics and morphology
	A1: Smooth, white color and has a few single colony A2: Smooth, white color and has some single colony A3: Smooth, white color and has some single colony A4: Smooth, white color and has a single colony
	A5: Rough, white color, sticky and has a few single colony A6: Rough, white color, mucoid and has some single colony
	B1: Smooth, white color and has a few single colony B2: Smooth, transparent and has a few single colony

	B3: Smooth, transparent and has some single colony

(Continue Table 1.)

	B4: Dry, creamy yellow and has some single colony B5: Dry, creamy yellow color and has some single colony
	B6: Smooth, mucoid, white color and has some single colony
	B7: Dry, creamy yellow and has a few single colony
	C1: Smooth, transparent, mucoid and has some single colony C2: Dry, white color and has a few single colony
	D1: Smooth, transparent, sticky and has some single colony D4: Smooth, white color, mucoid and has some single colony
	D2: Smooth, white color and has some single colony D3: Smooth, white color, mucoid and has some single colony
	D5: Smooth, white color, sticky and has some single colony

Haemolysis test

Bacteria that have hemolysin substance have the ability to break down the red blood cell which is dangerous for humans and mammals especially since red blood cells are important for supplying oxygen and nutrients throughout the whole body (Mogrovejo et al., 2020). The result show that among 12 bacterial Gram positive, only seven isolates showed negative haemolysis that is Gamma haemolysis shown in Figure 1 which are isolates A4, A5, A6, B4, B5, B6 and B7 that does not lyse the blood agar media. However, three other isolates which are isolates A3, D2 and D7 that show positive haemolysis where they lyse the blood agar causing clear zone surrounding the bacterial colonies that growth on the blood agar which can be seen in Figure 2.

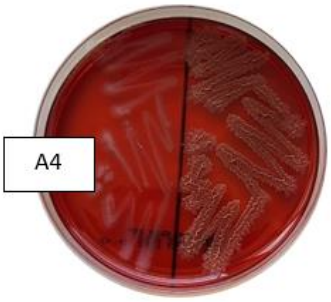


Figure 1. The observation of the Gamma-hemolysis of the bacteria growth on the blood agar

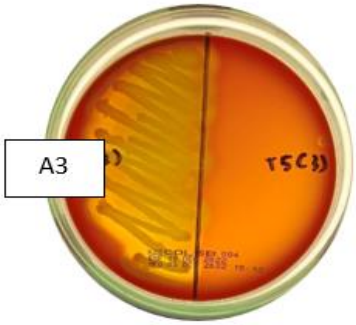


Figure 2. The observation of the Beta-hemolysis of the bacteria growth on the blood agar

Catalase test

The Catalase test helps to relieve the reactive oxygen species (ROS) by acting as an antioxidant which helps in protecting human or mammalian cells from oxidative stress (Jyoti, et. al., 2018). The oxidative stress situation could be prevented which will cause damage to the cells and tissues in human's body by separating hydrogen peroxide into water and oxygen (Pizzino, e.t al., 2017). Out of 12 Gram positive five bacterial colonies shown positive catalase test which are bacterial colonies for A3, A4, A5, A5 and D3. Previous study by Abdulkadir and Umaru, (2012) discover five bacterial colonies from 10 soil samples that was collected to show positive reaction to hydrogen peroxide proving that there are the bacterial colonies in soil sample contain catalase enzyme.

Antagonism activity of pure isolates against pathogenic bacteria

Antagonism activity of the isolates against the pathogenic bacteria using agar well diffusion assay to test the ability of bacterial isolates to inhibit the growth of other pathogenic bacteria. As shown in Table 2, all total 12 Gram positive bacteria with concentration 10^5 CFU/ml tested for antagonism test against pathogenic bacteria which are *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* with concentration of 1.5×10^5. However, after 24 hours incubation under 37ºC, no inhibition zone was managed to be observed on the Mueller-Hinton agar (MHA) except for positive control.

Similar study by Abdulkadir and Umaru (2012) stated that bacterial colonies that was obtained tested against pathogenic bacteria and found that *Escherichia coli* is resistant against all bacterial isolates as they were no inhibition zone observed. No inhibition zones formed might be because of not enough concentration or volume of antimicrobial compound produced to fight against pathogenic bacteria. The production of antimicrobial compounds could also be affected by factors such as nitrogen, carbon and temperature and need to be grown under suitable conditions (Abdulkadir & Umaru, 2012). Another study made by Tharmaraj & Shah, (2009) specified that factors such as bacterial load, growth conditions and resistance of the pathogenic bacteria to the bacterial isolates could led to the reason of the inhibition zone did not produce.

Table 2. Antagonism test of bacterial isolates against pathogenic bacteria using agar well diffusion assay

Isolates	**Zone of Inhibition (mm)**		
	Escherichia coli	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
A3	-	-	-
A4	-	-	-
A5	-	-	-
A6	-	-	-
B4	-	-	-
B5	-	-	-
B6	-	-	-
B7	-	-	-
D2	-	-	-
D3	-	-	-
D4	-	-	-
D5	-	-	-
Ampicillin	18.5	24	12.8
Sterile distilled water	-	-	-

(-) = no inhibition zones

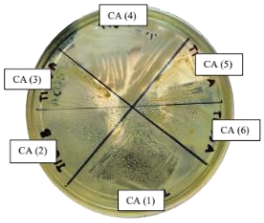
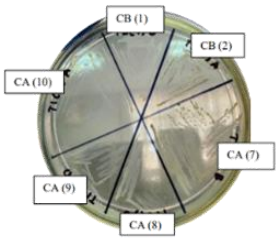
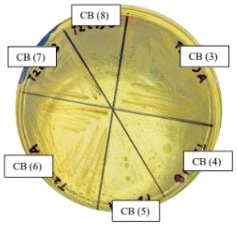
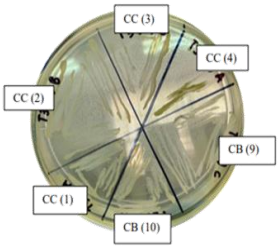
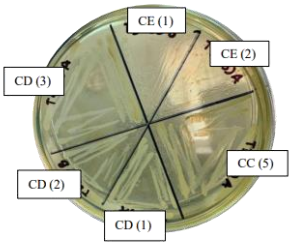
Bacterial colonies isolated from water samples collected from the stream

Furthermore, in the study conducted at Hutan UiTM Cawangan Negeri Sembilan, Kuala Pilah Campus, UiTM Negeri Sembilan, bacterial colonies were isolated from five different checkpoints in the stream. After incubation at 37°C for 24 hours, a total of 30 pure bacterial colonies were successfully obtained. the checkpoint that labeled CA, CB, CC, CD, and CE, displayed variations in the number of isolates, with CA having ten isolates, CB with ten isolates, CC with five isolates, CD with three isolates, and CE with two isolates. Notably, there was a decrease in isolate count as we moved from CA, which was closest to the campus and had orange-yellowish water, to CE, which was the farthest and had clearer water quality (Wen et al., 2020).

Morphology of pure bacterial colonies obtained

The morphology of the isolated stream bacteria included characteristics such as smooth, white, white-yellowish, yellowish, mucoid, dry, and dry-smooth, which was similar to findings in previous research on water samples from different lakes (Bumbla et al., 2020). Gram staining allowed differentiation between Gram positive and Gram negative bacteria, with 10 isolates being Gram positive and 20 being Gram negative. Gram negative bacteria were more prevalent in aquatic environments due to their adaptable cell wall structure and motility characteristics, which suited them for water-based niches (Kristensen et al., 2023). Table 3 showed the characteristics and morphology of bacterial colonies with total of 30 bacterial isolates.

Table 3. Characteristics and morphology of bacterial colonies with total of 30 bacterial isolates.

Isolate	Characteristics and morphology
	CA (1) – smooth, white color and has a few of single colony CA (2) – smooth, white colour and has a few of single colony CA (3) – smooth, yellowish colour and has a few of single colony CA (4) – dry, white colour and has some of single colony CA (5) – dry-smooth, yellowish colour and has a some of single colony CA (6) – smooth, yellowish colour and has some of single colony
	CA (7) – smooth, white colour and has single colony CA (8) – dry-smooth, white colour and have single colony CA (9) – dry-smooth, white colour and has a few of single colony CA (10) – smooth, mucoid, white colou and has single colony CB (1) – smooth, white colour and has some of single colony CB (2) – smooth, mucoid, white-yellowish colour and has some of single colony
	CB (3) – smooth, white-yellowish colour, has some of single colony CB (4) – smooth, white colour, single colony CB (5) – smooth, white colour and has single colony CB (6) – smooth, mucoid, yellowish colour and has single colony CB (7) – dry-smooth, white colour and has some of single colony CB (8) – dry-smooth, white colour and has some of single colony
	CB (9) – smooth, white colour and has single colony CB (10) – smooth, white-yellowish colour and has a few of single colony CC (1) – smooth, white-yellowish colour and has some of single colony CC (2) – smooth, white-yellowish colour and has a few of single colony CC (3) – smooth, yellowish colour and has some of single colony CC (4) – smooth, yellowish colour and has some of single colony
	CC (5) – smooth, mucoid, yellowish colour and has a few of single colony CD (1) – smooth, white colour and has some of single colony CD (2) - smooth, white colour and has some of single colony CD (3) – smooth, mucoid, white-yellowish colour and has a few of single colony CE (1) – smooth, white colour and has single colony CE (2) - smooth, white-yellowish colour and has single colony

Haemolysis test

A haemolysis test was conducted, revealing that only two out of the ten Gram positive bacterial colonies which was colonies CC (1) and CC (4) displayed positive beta hemolysis, indicating they were not suitable for human consumption. Figure 1 and figure 2 show the observation of bacteria growth on blood agar that resulting hemolysis beta and hemolysis gamma respectively. Haemolytic bacteria can release toxins or enzymes harmful to host tissues, making them less desirable for biotherapeutic production (Savardi et al., 2018). Given the importance of safety in biotherapeutic production, non-hemolytic bacteria are often preferred, as they can produce therapeutic products effectively without posing risks to patients (Saha et al., 2021).

Catalase test

A catalase test was performed to determine if the bacteria were catalase positive or negative. Four out

of the ten bacterial colonies were catalase positive that can be seen from colonies CA (6), CB (9), CC (3) and CC (5), suggesting their ability to neutralize reactive oxygen species, enhancing survival in aerobic environments. Water bacteria were more likely to be catalase negative due to their adaptation to low-oxygen aquatic habitats (Silaban et al., 2020).

Antagonism activity of pure isolates against pathogenic bacteria

Finally, by referring the data in table 4, the antagonism activity of the isolated bacteria against pathogenic strains (*Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) was evaluated using agar well diffusion. Unfortunately, none of the tested bacteria displayed an inhibition zone, which could be attributed to various factors, including the ineffectiveness of the prospective bacteria against the specific pathogenic strains, intrinsic resistance mechanisms of the pathogens, and the concentration of bacteria used (Kowalska-Krochmal & Dudek-Wicher, 2021).

Table 4. Antagonism activity against pathogenic bacteria using well diffusion assay

Zone of inhibition (mm)			
Isolates	**Inhibition zone against pathogenic bacteria**		
	Escherichia coli	***Pseudomonas aeruginosa***	***Staphylococcus aureus***
CA (30	-	-	-
CA (6)	-	-	-
CB (7)	-	-	-
CB (8)	-	-	-
CB (9)	-	-	-
CB (10)	-	-	-
CC (1)	-	-	-
CC (3)	-	-	-
CC (4)	-	-	-
CC (5)	-	-	-
Ampicilin	19	25	30
Sterile distilled water	-	-	-

Ampicillin, used as a positive control, exhibited a positive inhibition zone, confirming its inhibitory effects on the target microorganisms. This aligns with previous studies that found certain bacterial strains may not produce active inhibitory compounds against specific microbes (Da Silva et al., 2018; Khusro et al., 2014).

Thus, the study provides insights into the characteristics of bacterial isolates from a stream in Hutan UiTM Cawangan Negeri Sembilan, Kuala Pilah Campus. The findings emphasize the importance of selecting non-hemolytic bacteria with specific characteristics for biotherapeutic production. Additionally, the results highlight the complexity of antagonistic interactions between bacteria and their potential limitations in inhibiting pathogenic strains. Further research is needed to explore these interactions and their implications fully.

Conclusion

In conclusion, the bacterial isolates from both soil and stream sources exhibit potential characteristics of beneficial bacteria. In the soil samples, 12 bacterial colonies were identified as Gram positive bacteria, with seven displaying a negative haemolysis test and five showing positive catalase activity, indicative of catalase enzyme presence. While an inhibition zone was not observed, the limited antagonism testing conducted using two methods leaves room for further investigation with more comprehensive methodologies, equipment, and time allocation.

Similarly, in the stream samples, out of 30 pure colony bacterial isolates, ten were identified as Gram

positive bacteria, with eight exhibiting negative hemolysis and four demonstrating positive catalase activity. Unfortunately, antagonism activity testing using well diffusion and spot lawn assays did not yield inhibition zones on the Muller-Hinton agar. Despite this, the positive characteristics observed in some stream water bacterial isolates suggest their potential utility.

To advance this research, future studies should employ more refined procedures, appropriate apparatus, and a precise time frame to enhance the chances of obtaining conclusive results. Additionally, selective media for gram staining, focusing specifically on Gram positive bacteria, could be employed as a recommendation to streamline the identification process. Furthermore, exploring the enzyme activity within these bacterial isolates, particularly those sourced from soil, may unveil their potential health benefits when consumed.

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Author Contribution

Anis Shazlyn - carried out all the experimental work on stream and contributed in discussion and paper writing; NurFasihatul Husna - carried out all the experimental work on soil and contributed in discussion and paper writing; Ida Muryany - supervised on this work and contributed significantly in discussion and paper writing.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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