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Exploration *Streptomyces* sp. on the Soil of Several Vegetations in the Forest Zone Area of Alas Purwo Banyuwangi National Park and Tests of its Inhibitory on *Escherichia coli* O157:H7

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ABSTRACT

Problems related to antibiotic resistance are getting higher every time. Therefore, studies related to the exploration of new sources of antibiotics are very important to do. One source of antibiotics that has the potential to be utilized is derived from *Streptomyces* sp. This bacteria belongs to the Gram-positive bacteria of the Streptomycetaceae family which has the ability to produce enzyme and antibiotic is often found in natural soils. This study aims to explore the bacteria *Streptomyces* sp. in grassland vegetation and tropical rain forests in the jungle zone in the Alas Purwo National Park Area East Java Indonesia and tested its ability to inhibit the growth of *Escherichia coli* O157:H7. Sampling in this study was carried out in two different vegetation areas of the Alas Purwo National Park. The first vegetation is Sadengan grassland and the second is forest in the jungle zone. Soil samples were taken for each type of vegetation at five points. To isolate *Streptomyces* sp. used pour plate method with selective media YEMA (*Yeast Extract Malt Agar*). Bacterial isolates suspected as *Streptomyces* sp. and different from one another are separated to be re-isolated and identified macroscopically and microscopically Seung and Good fellow (2002). Twenty *Streptomyces* sp. isolates were successfully isolated from 2 vegetations. The highest inhibition was *Streptomyces* sp. O (2.725 cm) against *Escherchia coli* O157:H7, while the one with the lowest inhibition was *Streptomyces* sp .G (0.542 cm). In the future, after carrying out molecular tests, *Streptomyces* sp.O can be used as an alternative new antibiotics to combat pathogenic bacteria.

Key words: *Antibiotics, Antibiotic resistant pathogens, Biological resources forests*

Introduction

Infectious diseases are a serious health problem in several countries, especially in developing countries (Ministry of Health RI, 2011). One of the causes of infectious diseases is infection by bacteria (Konoralma, 2019). Bacteria are prokaryotic organisms that carry genetic information in double-

stranded circular DNA molecules. Depending on the type of bacteria, there are pathogenic and non-pathogenic bacteria. These pathogenic bacteria can cause a disease in living things (Doron and Gorbach, 2008).

Escherichia coli is a normal flora in the intestines of humans and animals. Most *E. coli* is harmless and plays an important role in the intestinal tract of healthy humans and animals. However, some strains of *E. coli* can be pathogenic such as diarrhea or diseases outside the digestive tract (CDC, 2014). One of the types of *E. coli* that can cause disease is the O157:H7 strain. *Escherichia coli* O157:H7 is often associated with outbreaks of diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) in humans (Sari *et al.,* 2021). The Centers for Disease Control and Prevention (CDC) reported that there were 8,598 cases of disease outbreaks from 49 countries caused by *Escherichia coli* O157:H7. Therefore it is necessary to carry out various treatments for this bacterial infection, one of which is the use of antibiotics (Rizky *et al.,* 2021).

Antibiotics are antimicrobial substances that can be produced by microorganisms that kill or inhibit the growth of other microbes (Byrne *et al.,* 2019). Antibiotics do not only play an important role in saving the patient's life, but play an important role in the progress of the medical world. Antibiotics can also prevent or treat infections in patients undergoing chemotherapy, diabetes, organ transplants and other chronic diseases (Ventola, 2015). Antibiotics are a type of drug that is widely used in the world. Generally, hospitals budget more than 25% for the use of these antibiotics (Atmadinata *et al*., 2012). Due to the large use of antibiotics and the increasing cases of antibiotic resistance, various steps to obtain new antibiotics are important to implement. The steps for searching for antibiotics can come from plants, animals, to bacteria. One type of bacteria that has produced many types of antibiotics is *Streptomyces* sp. (Chater, 2006).

The genus *Streptomyces* is a Gram-positive bacteria that is generally and in large quantities isolated from soil, which is its natural habitat (Goliñska and Dahm, 2011). Tropical rain forest areas are a very abundant source of biodiversity, including bacteria (Nurkanto *et al.,* 2010). Alas Purwo is a lowland forest area located on the eastern tip of Java Island. This forest area can be divided into several vegetations, namely savanna/grass meadows, bamboo forests, coastal forests, lowland natural/wild forests, and mangrove forests. Therefore, the Alas Purwo area has the potential to carry out various explorations including the search for certain bacteria (Widodo, 2016). This study aims to explore the bacteria *Streptomyces* sp. in grassland vegetation and tropical rain forests in the jungle zone in the Alas Purwo National Park Area East Java Indonesia and tested its ability to inhibit the growth of *Escherichia coli* O157:H7.

Materials and Method

The research was conducted from September 2022 to January 2023 at the Microbiology Laboratory of the Biology F. MIPA Study Program, Udayana University. Soil samples were taken at 5 location points on each vegetation by taking them at the soil surface (Fig. 1 and 2).

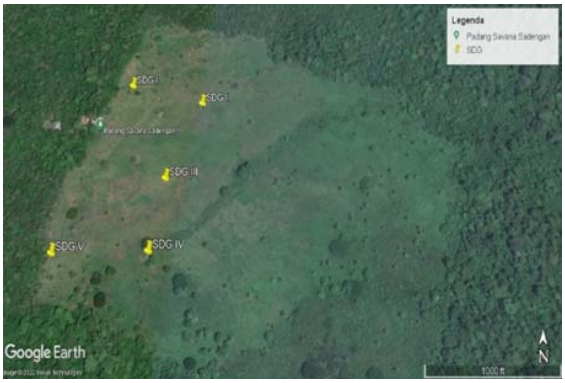

Fig. 1. The sample location in Sadengan Grasslands (*Source*: Google Earth, 2022)

Description of coordinates

SDG I : 8°39′10.23"S 114°22′21.72"T
SDG II : 8°39′11.85"S 114°22′28.64"T
SDG III : 8°39′18.47"S 114°22′26.05"T
SDG IV : 8°39′24.39"S 114°22′25.35"T
SDG V : 8°39′24.60"S 114°22′17.06"T

The soil samples obtained were measured for pH using a pH meter. Dilution of the sample up to 10-3 was then heated in the oven for 30 minutes at 40 °C. As much as 1 mL of the 10^{-2} and 10^{-3} dilutions was put into a sterile Petri dish which was then poured with 10 ml of YEMA (Yeast Extract Malt Agar) media and incubated at 28 °C for 5-7 days (Dharmawan *et al.,* 2009).

Identification of *Streptomyces* sp. by looking at the structure of the colony macroscopically, microscopically, catalase test, Gram stain and acid-fast stain using the identification book *Streptomyces* sp. from

Fig. 2. The sample location in Tropical Rain Forest (*Source*: Google Earth, 2022)

Description of coordinates

HHT I : 8°39′27.34"S 114°22′02.76"T
HHT II : 8°43′52.33"S 114°21'.30.19"T
HHT III : 8°35′07.12"S 114°21′52.32"T
HHT IV : 8°37′20.73"S 114°19′35.23"T
HHT V : 8°35′27.17"S 114°15′53.78"T

Seung and Goodfellow (2002).

Inhibition was used dual culture methode. *E. coli* O157:H7 was grown in Nutrient Broth (NB) medium for 24 hours at 37 °C. *Streptomyces* sp. grown on YEMA media for 5 days at 28 °C. After that *Streptomyces* sp. cut using a cock borer with diameter 5 mm. *E. coli* culture as much as 1 Ose was scratched on the Petri dish containing Nutrient Agar (NA) media, then holes were made in the media using a cock borer. *Streptomyces* sp. colony fragments placed in the hole, then incubated at 37 °C for 48 hours. The positive control used was *Ciprofloxacin* with a concentration of 1% and 0.1%. The antibacterial activity test on the positive control was carried out by making diffusion wells. The next process is to measure the clear zone produced around the *Streptomyces* sp. isolate and positive control were then compared. Each treatment in the antibacterial power test was repeated 3 times. Illustration of clear zone measurement can be seen in Figure 3 with the following formula (Fig. 3):

$$\text{Inhibitory Zone} = \frac{V + H + D1 + D2}{4}$$

Keterangan:

V = Vertical diameter
H = Horizontal diamters
D1 = Diagonal length 1
D2 = Diagonal length 2

The dataobtained was analyzed by Analysis of Variance (ANOVA) with SPSS software for windows version 23.0 in 2022 (Kuntari *et al*., 2014).

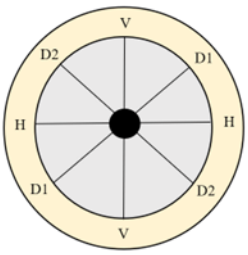

Fig. 3. Illustration of Clear Zone Measurement (*Source:* Personal documentation, 2022)

Results

Fifteen *Streptomyces* sp. isolates obtained from the Sadengan grasslands, whereas in tropical rain forest vegetation as many as 5 *Streptomyces* sp. isolates obtained with different macroscopic and microscopic morphology. Data can be seen in Table 1.

Table 1. Distribution of Streptomyces sp. at Research Locations

Nu.	Location	Number of Isolates
1	Tropical Rain Forest	5
2	Sadengan Grassland	15
Total	20	

The presence of *Streptomyces* sp. can be influenced by environmental factors such as soil pH, location altitude, and ambient temperature. Environmental factor measurement data can be seen in Table 2.

Catalase test, Gram stain, and acid fast stain 20 isolates of Streptomyces sp. found from tropical rain forests and Sadengan grassland can be seen in Table 3, Fig. 4.

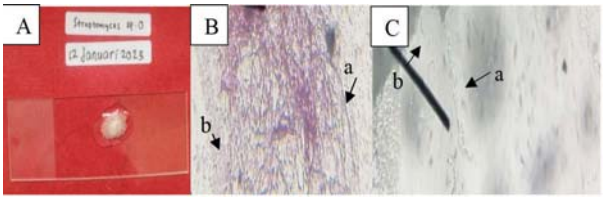

Fig. 4(A). Catalase Test (B) Gram Stain and (C) Acid Resistant Stain Results of *Streptomyces* sp. Isolates Found (*Source*: Personal documentation, 2023)

Information:
A = Hyphae
B = Conidia

Macroscopic and microscopic characteristics of *Streptomyces* sp. isolates found in Tropical Rain Forest and Sadengan Grasslands can be seen in Tables 4, 5, and Fig. 6.

Description: Determination of macroscopic and microscopic characters, following most of the patterns of Seung and Goodfellow (2002).

The results of the *Streptomyces* sp. inhibition test found from tropical rain forest and Sadengan grasslands can be seen in Table 6. The isolate with the highest inhibition was *Streptomyces* sp. O (2.725 cm), while the one with the least inhibition was *Streptomyces* sp. G (0.542 cm) and the species which has no inhibitory power, namely *Streptomyces* sp. I. Pictures of the inhibition test of *Streptomyces* sp. O, *Streptomyces* sp. G, positive control and negative control respectively can be seen in Figures 7 and 8.

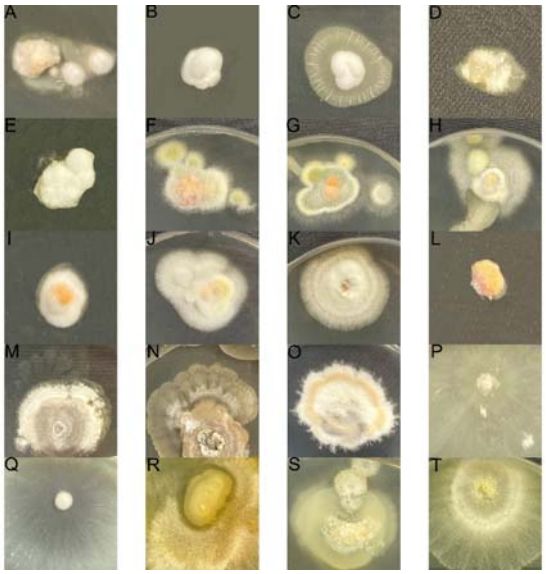

Fig. 6. Photo of *Streptomyces* sp. Found from Each Location

Table 2. pH, Altitude, and Temperature at Sample Location

Nu.	Location Point	pH		Altitude (Meter)		Temperature (ºC)	
		Tropical Rain Forest	Sadengan Grassland	Tropical Rain Forest	Sadengan Grassland	Tropical Rain Forest	Sadengan Grassland
1.	I	6.73 ± 0.058	6.53 ± 0.058	17	12	23	23
2.	II	6.7 ± 0.173	6.7 ± 0.1	21	11	27	23
3.	III	6.83 ± 0.058	6.3 ± 0.173	22	11	25	23
4.	IV	6.53 ± 0.31	6.77 ± 0.153	9	9	25	23
5.	V	6.567 ± 0.153	6.57 ± 0.058	26	5	25	23

Table 3. Results of Catalase Test, Gram Stain, and Acid Resistant Stain of Isolate Streptomyces sp.

Nu.	Species	Location	Catalase Test	Gram Stain	Acid Resistant Stain
1.	*Streptomyces* sp. A	HHT	+	+	Can't stand acid
2.	*Streptomyces* sp. B	HHT	+	+	Can't stand acid
3.	*Streptomyces* sp. C	HHT	+	+	Can't stand acid
4.	*Streptomyces* sp. D	HHT	+	+	Can't stand acid
5.	*Streptomyces* sp. E	HHT	+	+	Can't stand acid
6.	*Streptomyces* sp.F	SDG	+	+	Can't stand acid
7.	*Streptomyces* sp.G	SDG	+	+	Can't stand acid
8.	*Streptomyces* sp.H	SDG	+	+	Can't stand acid
9.	*Streptomyces* sp.I	SDG	+	+	Can't stand acid
10.	*Streptomyces* sp.J	SDG	+	+	Can't stand acid
11.	*Streptomyces* sp.K	SDG	+	+	Can't stand acid
12.	*Streptomyces* sp.L	SDG	+	+	Can't stand acid
13.	*Streptomyces* sp.M	SDG	+	+	Can't stand acid
14.	*Streptomyces* sp.N	SDG	+	+	Can't stand acid
15.	*Streptomyces* sp.O	SDG	+	+	Can't stand acid
16.	*Streptomyces* sp.P	SDG	+	+	Can't stand acid
17.	*Streptomyces* sp.Q	SDG	+	+	Can't stand acid
18.	*Streptomyces* sp.R	SDG	+	+	Can't stand acid
19.	*Streptomyces* sp.S	SDG	+	+	Can't stand acid
20.	*Streptomyces* sp.T	SDG	+	+	Can't stand acid

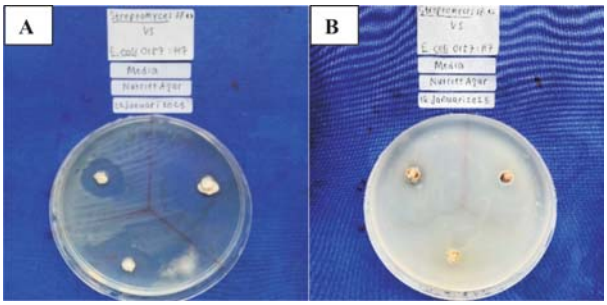

Fig. 7. (A) *Streptomyces* sp. O and (B) *Streptomyces* sp.G (*Source*: Personal documentation, 2023)

Discussion

Total 20 isolates *Streptomyces* sp. were successfully isolated from 2 vegetations. The first vegetation, namely tropical rain forest, found 5 isolates and the second vegetation, namely Sadengan grassland, found 15 isolates which can be seen in Table 1. The soil taken in this study was part of the surface. This is because *Streptomyces* sp. which is part of the Actinobacteria phylum can be found on the soil surface to a depth of more than 2 meters (Barka *et al.*, 2016). *Streptomyces* sp. in this study found more in

Table 4. The Characteristics of *Streptomyces* sp. Found in Tropical Rain Forests

Isolate Character	Tropical rain forest				
I. Macroscopic Characteristics	SP. A	SP. B	SP. C	SP. D	SP. E
a. Colony Color					
White	■	■	■		■
Yellowish green					
Orange white					
Yellowish white			■	■	
Reddish yellow					
Gray white					
Mix of white, yellow, red					
Mix of white, green, yellow					
A mix of green, yellow, white, orange					
Gray					
b. Colony Surface					
Convex	■				
Wavy		■	■		■
Flat					
Irregular			■	■	
c. Colony Outskirts					
Stringy	■		■		
Wavy		■			■
Flat					
d. Colony Form					
Round	■				
Oval					
Irregular	■	■	■	■	■
II. Microscopic Characteristics					
a. Hyphae					
Branched		■	■		
Non-Sept	■	■			■
Spiral					
b. Conidia					
Cluster			■		
Chain	■	■		■	■

grassland vegetation when compared to tropical rain forests. This is because even though the soil samples were taken from the surface, grass roots were also found in the Sadengan grassland. Actinomycetes including *Streptomyces* sp. commonly found in grassy soil (Rahayu, 2011).

The growth of Actinomycetes bacteria such as *Streptomyces* sp. greatly influenced by environmental factors such as pH, soil altitude, to temperature. *Streptomyces* sp. can grow at pH 6-9 with optimum growth at a pH close to neutral (Fitri *et al.*, 2021). The soil pH range in this study was 6.3 – 6.83 (Table 2).

Table 5. The Characteristics of *Streptomyces* sp. Found at Sadengan Grasslands

Isolate Character	Sadengan Grassland														
I. Macroscopic Characteristics	SP. F	SP. G	SP. H	SP. I	SP. J	SP. K	SP. L	SP. M	SP. N	SP. O	SP. P	SP. Q	SP. R	SP. S	SP. T
a. Colony Color															
White						■					■				
Yellowish green				■											
Orange white					■								■		
Yellowish white							■		■					■	
Reddish yellow								■		■					
Gray white															
Mix of white, yellow, red	■														
Mix of white, green, yellow			■												■
A mix of green, yellow, white, orange		■													
Gray								■							
b. Colony Surface															
Convex		■				■					■				
Wavy			■		■										
Flat										■		■			
Irregular							■	■		■			■	■	
c. Colony Outskirts															
Stringy		■				■		■	■	■	■			■	
Wavy			■		■								■		
Flat				■			■				■	■			
d. Colony Form															
Round			■												
Oval				■							■				
Irregular	■	■			■	■	■	■	■	■		■	■	■	■
II. Microscopic Characteristics															
a. Hyphae															
Branched		■	■	■	■	■	■	■	■	■	■	■	■	■	
Non-Sept		■	■	■	■				■						
Spiral														■	■
b. Conidia															
Cluster	■	■				■		■		■	■	■			
Chain			■	■	■		■		■				■	■	■

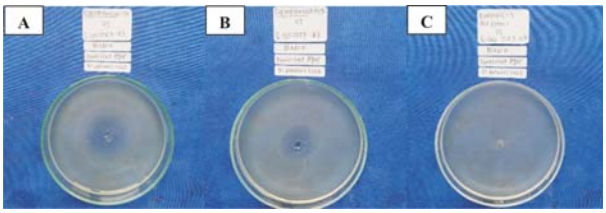

Fig. 8. Positive and Negative Controls (*Source*: Personal documentation, 2023)
Information
(A) Positive Control Ciprofloxacin 1%
(B) Positive Control Ciprofloxacin 0.1%
(C) Negative Control of Sterile Water

These results represent the pH range of *Streptomyces* sp bacteria can grow.

Measurement of the height of the sampling location in this study shows that tropical rain forest vegetation tends to be taller than Sadengan grassland (Table 2). This is in accordance with the discovery of *Streptomyces* sp. which is more in Sadengan grasslands (15 isolates) when compared to tropical rain forests (5 isolates). The height of the soil is one of the factors that influence the presence of soil microbes including *Streptomyces* sp. This is because the height of the soil will affect soil properties such as: the content of TN (total nitrogen), TOC (total organic carbon), water content, C/N (mass ratio of carbon and nitrogen), urease which will decrease with increasing soil height. (Tang *et al.,* 2020). This C/N mass ratio plays a very important role in fulfilling soil nutrients. Water content plays a role in microbial respiration, increasing the water content (moisture) will be directly proportional to respiration (Bian *et al.,* 2022). Urease is an enzyme that functions in degrading urea in soil which will be a source of N in the form of ammonium so that it can be beneficial for plant and microbial growth (Cordero *et al.,* 2019).

Table 6. The results of the Inhibitory Zone Test of *Streptomyces* sp. against *Escherichia coli* O157:H7

Nu.	Species	Location	Average(cm)
1	*Streptomyces* sp.A	HHT	1,067±0,241^{abc}
2	*Streptomyces* sp.B	HHT	0,817±0,102^{ab}
3	*Streptomyces* sp.C	HHT	1,447±2,497^{abc}
4	*Streptomyces* sp.D	HHT	0,833±0,038^{ab}
5	*Streptomyces* sp.E	HHT	1,983±0,237^{bc}
6	*Streptomyces* sp.F	SDG	1,817±2,506^{bc}
7	*Streptomyces* sp.G	SDG	0,542±0,496^{ab}
8	*Streptomyces* sp.H	SDG	0,8±0,152^{ab}
9	*Streptomyces* sp.I	SDG	0± 0,00^{a}
10	*Streptomyces* sp.J	SDG	2,217±0,795^{bc}
11	*Streptomyces* sp.K	SDG	2,058±1,617^{bc}
12	*Streptomyces* sp.L	SDG	0,75±0,129^{ab}
13	*Streptomyces* sp.M	SDG	0,825±0,175^{ab}
14	Streptomyces sp.N	SDG	2,033±0,863^{bc}
15	*Streptomyces* sp.O	SDG	2,725±0,826^{c}
16	*Streptomyces* sp.P	SDG	0,958±0,080^{ab}
17	*Streptomyces* sp.Q	SDG	0,883±0,014^{ab}
18	*Streptomyces* sp.R	SDG	0,9±0,025^{ab}
19	*Streptomyces* sp.S	SDG	0,853±0,52^{ab}
20	*Streptomyces* sp.T	SDG	1,892±0,648^{bc}
21	*Ciprofloxacin* 1%	CTL (+)	2,15±0,00^{bc}
22	*Ciprofloxacin* 0,1%	CTL (+)	1,3±0.00^{abc}
23	Sterile Water	CTL (-)	0±0,00^{a}

Note: Treatments with the same letter show no significant difference in data, otherwise treatments with different letters show significantly different data α 5 with an average of 3 repetitions. *Streptomyces* sp.O isolate had the greatest inhibition.

In general, *Streptomyces* sp. can grow in a temperature range of 25º – 35 ºC with an optimal temperature range of 28º – 30 ºC. The environmental temperature of the sampling locations in tropical rain forest and Sadengan grasslands has a range of 23º – 27 ºC (Table 2), although *Streptomyces* sp. can still grow (Gunjal and Bhagat, 2021; Sivalingam *et al.,* 2019). Bacterial growth temperature can vary depending on the type. Temperature will affect the growth rate of bacteria, the speed of enzyme formation and the speed of enzyme inactivation. All bacteria have optimal, minimum and maximum temperatures for their growth (Suriani *et al*., 2013).

Isolate suspected of *Streptomyces* sp. confirmatory tests were carried out with catalase test, Gram staining, and acid fast staining. The catalase test is a biochemical test to determine whether a bacterial isolate can produce the catalase enzyme (Khatoon *et al.,* 2022). The catalase test in this study also showed *Streptomyces* sp. isolates. found in both vegetation, namely tropical rain forest and Sadengan meadows, produced bubbles (catalase positive) which can be seen in Table 3. Isolates suspected to be *Streptomyces* sp. After the catalase test was performed, it was followed by Gram staining. Gram staining serves to determine the type of Gram and bacterial morphology (Thairu *et al.,* 2014). Bacteria with positive Gram properties will turn purple after staining. Gram staining of isolates suspected to be *Streptomyces* sp. in this study produced a purple color which means the isolate is a type of Gram positive (+). The next stage of identification with Ziehl-Neelsen (ZN) acid fast staining. Acid-fast staining in the identification process in this study was not only used to determine

the morphology of bacteria, it was also used to differentiate between *Streptomyces* sp. isolates. and Nocardia (Putri *et al*., 2021). Acid-resistant bacteria in this staining method will produce a pink color, while non-AFB bacteria (acid-resistant bacteria) will display a blue or green color (Misnarliah *et al.*, 2021). Acid fast staining in this study showed that the isolates found were not acid fast (Table 2). This negative result was caused by the cell walls of *Streptomyces* sp. which is composed of L-ADP and Glycine (Kawuri, 2016).

Identification in this study was carried out macroscopically and microscopically. *Streptomyces*sp. found in this study had white, yellowish-green, orange-white, yellowish-white, reddish-yellow, grayish-white pigment colonies, mixed (white, yellow, red), mixed (white, green, yellow), mixed (green, yellow, white, orange), and gray which can be seen in Tables 4 and 5. The macroscopic characters observed were: color, surface, margin, and colony shape. Meanwhile, the microscopic characters observed were hyphae and conidia. Hyphae form of *Streptomyces* sp. isolate. found in this study were branched and non-septate (Tables 4 and 5). Microscopically the mycelium of *Streptomyces* sp. can be branched or unbranched, straight, to spiral. The mycelium itself is a part composed of many hyphae (Kuarniati *et al.,* 2019). Conidia are spores formed at the ends of hyphae which are called conidiophores (Hafsan, 2011). According to Raharini *et al.* (2012) conidia on *Streptomyces* sp. form a chain to cluster. The results of microscopic observations of conidia from *Streptomyces* sp. isolates obtained in this study also has a round chain and cluster shape (Tables 4 and 5).

Measurement of inhibition in this study was carried out using calipers with the measurement method shown in Figure 3. Based on the results of the inhibition test obtained in this study, not all species have inhibition. The highest inhibition in this study was *Streptomyces* sp.O (2.725 cm), while the one with the least inhibition was *Streptomyces* sp.G (0.542 cm), and the one with no inhibition was *Streptomyces* sp.I can be seen in Table 6. The difference in the results of the inhibition test for each isolate found could be due to the type of media used during the test being unable to provide maximum nutrition to *Streptomyces* sp. This will cause the production of secondary metabolites to not be optimal. Shepherd *et al.* (2010) also explained that the medium for the growth of *Streptomyces* sp. varies greatly for each species.

All *Streptomyces* sp. isolates which were found to have varying inhibition. Antibiotics are one of the secondary metabolites produced by *Streptomyces* sp. (Sharma *et al*., 2014). Secondary metabolites in the form of antibiotics are produced by Streptomyces sp. varies greatly. This variation is one of the factors for the difference in inhibition produced by each species of *Streptomyces* sp. in this study (Harir *et al.,* 2018). The positive controls (Fig. 8 A and B) in this study were Ciprofloxacin 1% and 0.1% with inhibition results of 2.15 cm and 1.3 cm. Ciprofloxacin is a type of fluroquinolone antibiotic that inhibits bacterial growth bactericidal (Thai *et al*., 2022). Sterile water as a negative control (Figure 8 C) in this study did not have any inhibition on the growth of *E. coli* O157:H7 bacteria. This is in line with the research of Wahyuni and Karim (2020) that the negative control of sterile water has no antibacterial activity.

Conclusion

1. A total of 20 *Streptomyces* sp. which was successfully isolated from 2 different vegetations. Sadengan grassland vegetation obtained 15 isolates of *Streptomyces* sp. while 5 isolates were isolated from tropical rain forest.
2. The highest inhibition zone was produced by *Streptomyces* sp. O (2.725 cm), while the one with the least inhibition was *Streptomyces* sp.G (0.542 cm) and the others vary.

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