CHARACTERIZATION OF SOIL *ACTINOMYCETES* FROM MALINO PINE FOREST RHIZOSPHERE OF SOUTH SULAWESI

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Abstract: *Actinomycetes* are a group of Gram-positive bacteria that produce active compounds with a wide distribution range in nature especially in soil. The purpose of this study was to isolate *actinomycetes* from the rhizosphere soil of the Malino pine forest, South Sulawesi. This research is a descriptive qualitative study of 15 *actinomycetes* isolated from the rhizosphere soil of the Pine Forest. These 15 isolates have been successfully identified to the genus level. *Actinomycetes* were isolated by direct dilution method and further morphological identification was carried out. *Actinomycetes* were isolated on yeast malt agar (YMA) medium. The growing isolates showed colony colours brown, grey, and white. Produces yellow, brown, and beige colour pigments. Based on the characterization carried out, all isolates were identified into the genus *Streptomyces.*
Keywords: *Actinomycetes*; rhizosphere soils; pine forests

Abstrak: *Aktinomisetes* merupakan kelompok bakteri gram positif yang menghasilkan senyawa aktif dengan rentang distribusi yang luas di alam, terutama di tanah. Tujuan penelitian ini untuk mengisolasi *aktinomisetes* dari tanah rizosfer hutan pinus Malino Sulawesi Selatan. Penelitian ini merupakan penelitian kualitatif deskiptif terhadap 15 isolat *aktinomisetes* yang diisolasi dari tanah rizosfer Hutan Pinus Malino Sulawesi Selatan. 15 isolat ini telah berhasil didentifikasi sampai tingkat genus. *Aktinomisetes* diisolasi dengan metode pengenceran secara langsung dan dilakukan identifikasi morfologi lebih lanjut. *Aktinomisetes* diisolasi pada media yeast malt agar (YMA). Isolat yang tumbuh memperlihatkan warna koloni coklat, abu-abu dan putih. Menghasilkan pigmen warna kuning, coklat dan krem. Berdasarkan karakterisasi yang dilakukan keseluruhan isolat teridentifikasi ke dalam genus *Streptomyces.*
Kata kunci: *Aktinomisetes*; tanah rizosfer; hutan pinus

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Introduction

Actinomycetes are soil microorganisms that resemble bacteria and fungi and possess the characteristics of these two groups of microorganisms. *Actinomycetes* are often said to be the missing link of evolution between bacteria and fungi. However, the characteristics of bacteria are more visible in *actinomycetes* (Johns, 2017). *Actinomycetes* can be recognized by their distinctive morphological structure from the mycelium. Based on the content of sugar polymers, amino sugars, and several other amino acids that make up the cell walls, *actinomycetes* into a group of Gram-positive bacteria and are characterized by high GC (Gunanin Cytosin Nitrogen Base) content. In general, the GC content of *actinomycetes* ranges from 51 % to more than 70 %, although there are several types of *actinomycetes* contain GC less than 50 % (Budiyanto and Muhtadi, 2012; Ventura et al., 2007). Among Gram-positive bacteria, *Actinomycetes* appear the enormous morphological differentiation with branched hyphae and specialized spore structures (Kim & Garson, 2005; Prescott et al., 1993).

Actinomycetes are the most abundant microorganisms in soil and survive in a variety of other ecosystem habitats such as aquatic ecosystems, including sediments and are found in the deep sea (Bawazir & Shantaram, 2018). The types of *Actinomycetes* that are abundant in the soil are *Actinobacteria* and *Streptomyces*. The abundance of *Actinobacteria* in the soil reaches 10^6 to 10^9 cells per gram of soil, while the genus *Streptomyces* dominates the soil microbial population and makes up 95 % of the *actinomycetes* group in the soil (Barka et al., 2016). Heterogeneous physical characteristics of soil, abundant nutrient availability and other abiotic factors make the soil an ideal ecosystem for microbial growth (Lemanceau et al., 2017). In soil, *actinomycetes* compete with other microbes and are able to withstand unfavourable environmental conditions (Salaria et al., 2017).

The abundance and types of *actinomycetes* present in the soil are influenced by geographic location and soil physicochemical characteristics such as temperature, soil type, pH, salinity, soil organic compounds, cultivation, aeration, and soil moisture (Zanane et al., 2018). *Actinomycetes* grow well on dry and alkaline soil (Aeny et al., 2018). The pine forest is one of the areas where it is possible to find *actinomycetes* in abundance. This is due to the high evapotranspiration value of pine compared to other tree species, which is around 64.5 % of the total rainfall, consequently, it can reduce the amount of water contained in the soil (Indrajaya & Handayani, 2008).

The Malino Pine Forest area in Gowa Regency, South Sulawesi, is a striking ecological environmental target for *actinomycetes* exploration. Apart from the tropical climate and stable rainfall which averages between 135 days and 160 days per year, information about the biodiversity especially the microbes is also restricted. Until the day, no information has been obtained regarding the diversity of microorganisms, including *actinomycetes* from this area. Therefore, a study was conducted to determine the characteristics of *actinomycetes* from rhizosphere soil from the Malino Pine Forest in Gowa Regency, South Sulawesi, which provides information on the diversity of biological resources in the pine forests particularly Malino pine forests.

Methods

Sampling and Preparation of Samples

Samples were taken from 5 locations representing the Malino Pine Forest area in Gowa Regency, South Sulawesi. 5 points were determined in each location; the Patappang (Lembanna) area with coordinates 119 ° 54 '14 "East Longitude and 5 ° 14' 36" South Latitude, Malino (Pendidikan Street) 119 ° 51 '59 "East Longitude and 5 ° 15 '9 "South Latitude, Gantarang with coordinates 119 ° 51' 33" East Longitude and 5 ° 13 '54 "South Latitude, Lombosang with coordinates 119 ° 51' 6" East Longitude and 5 ° 15 '23 "South Latitude, and Resort Taman Wisata Alam (TWA) Malino with coordinates 119 ° 52 '15 "East Longitude and 5 ° 14' 38" South Latitude.

Samples were taken from the ground near the roots of pine trees by digging at a depth of 10-15 cm from the ground. It is estimated that 10 grams of soil are taken and placed in sterile plastic clips, closed tightly transported to the laboratory, and stored at 4° C before isolating *actinomycetes*. Measurement of environmental parameters such as temperature, humidity, and soil pH was carried out using a soil thermometer and soil tester. The sample was dried for 7 days (Nurkanto, 2010) and filtered to separate large dirt and homogenized sample (Susilowati et al., 2007).

Isolation of *Actinomycetes*

One gram of rhizosphere soil was suspended in 10 mL of sterile water, homogenized for 2 minutes, and left for 1 minute. Furthermore, 1 mL of the suspension was serially diluted to 10^{-5} dilution. Soil suspensions from the 10^{-4} and 10^{-5} dilution series were spread on Humic Acid Vitamin Agar (HVA) media (contain 0.1 % Humic Acid; 0.002 % $CaCO_3$; 0.001 % $FeSO_4$; 0.171 % KCl; 0.005 % $MgSO_4$. $7H_2O$; 0.05 % Na_2HPO_4; 2 % bacterial agar, and vitamin B; pH 7) and incubated at room temperature for 7-14 days.

Morphological Identification

The growing *actinomycetes* colonies were taken and transferred one by one to yeast malt agar (YMA) media. Incubation was carried out at room temperature for 1-14 days to give the isolates a chance to grow completely. Identification up to the genus level was carried out by macroscopic and microscopic observation. Macroscopic identification was carried out by observing the colony colour and the resulting pigment. Microscopic identification was carried out by observing the arrangement of the spore chains under a microscope (Miyadoh, 1997; Holt et al. 1994; Nurkanto, 2007).

Results and Discussion

Environmental Parameters of Malino Pine Forest

Samples were taken from five locations of Malino Pine Forest, South Sulawesi. The sampling process is carried out aseptically at a depth of 10-15 cm because it greatly affects the presence of microorganisms. Soil depth affects the population of microorganisms. The population of microorganisms including *actinomycetes* is mostly found at soil depths of 10-15 cm (Bhattarai, 2015). Algafari (2014) added that at the depth of the soil 10 cm is recommended for the isolation of *actinomycetes* because in its oxygen and nutrients for the growth of *actinomycetes* are available in abundant quantities. Other factors that affect the presence of microorganisms are chemical and physical include moisture, pH, and soil temperature (Table 1).

Table 1. The average of environment parameter measurement in Malino pine forest

Location	The average of environment parameter measurement		
	Moisture (%)	pH	Temperature (°C)
Patappang (Lembanna)	1	6.9	24.8
Malino (Pendidikan street)	1	6.9	26
Resort TWA Malino	1	6.8	24.6
Lombosang	1	6.9	25
Gantarang	1	6.9	25.2

The diversity and types of *actinomycetes* are greatly dependent on chemical, physical, and biological factors in the environment. To determine the effect of these factors, environmental parameters are measured. The results showed that the average humidity at the five sampling locations was 1 %, while the average pH in Patappang (Lembanna), Malino (Pendidikan Street), Lombosang, and Gantarang was 6.9. the average pH at the Taman Wisata Resort. Alam (TWA) Malino which is 6.8. Furthermore, the temperature obtained at five locations ranged from 24.6 °C – 26 °C. The pH, temperature, and humidity values in the malino pine forest showed the optimum for the growth of *actinomycetes*.

Determination of soil type and environmental conditions is veritably important in the isolation of *actinomycetes* because their growth is influenced by soil conditions such as geographical location and biotic factors (temperature, soil type, depth, soil pH, the content of organic compounds, aeration and humidity) (Samar et al., 2018). The measurement of pH range at the sampling location (pH 6.8-6.9) supports the optimal growth of the genus *Streptomyces*. This is correlated with the characterization result which showed that all genera found at the sampling location refer to the genus *Streptomyces*. As mentioned by Zanane et al., (2018) that the pH of *Streptomyces* growth is range from 6.5 to 8.0.

The abundance of *actinomycetes* populations is various infertile soils with humidity ranging from 0.67 % to 0.89 %. However, *actinomycetes* can survive and grow at low humidity (0.67 %) and grow optimally at 0.89 % humidity (Zenova et al., 2007). As for the physical factors, the suitable temperature for the growth of *actinomycetes* is 25 °C to 30 °C (Adriani & Tulak, 2013). *Actinomycetes* usually grow at a soil depth of 11-15 cm above the soil surface. At this depth, there is a combination of pH and water content that optimal for *actinomycetes* growth (Miyadoh & Otoguro 2004).

According to Kanti (2005), waterlogged soils are not suitable for the growth of *actinomycetes*. However, soils in dry or semi-dry areas can maintain a fairly large population of *actinomycetes*. This is due to the resistance of spores to drought. *Actinomycetes* can grow with a large population in dry areas. The drier the soil sample and the less water content, the more live *actinomycetes* particularly the genus *Streptomyces* (Zenova et al., 2007). Soil sampling in this study was conducted in Malino Pine Forest, South Sulawesi. The Perhutani Forest Resources Development Center stated that the loss of water from rainfall due to the interception process from pine forests was the highest, namely 15.7 % compared to other forests, agathis (14.7 %) and puspa (13.7 %) (Indrajaya & Handayani, 2008). This reduces the amount of water content in the soil causes the soil to become drier and allowing for the growth of *actinomycetes*.

Isolation and Morphological Characterization of *Actinomycetes*

It was found that 15 *actinomycetes* colonies with different characteristics on YMA media were shown in table 2. The observed macroscopic characteristics were colony colour and pigment. Based on these characteristics, all isolates were identified in the genus *Streptomyces*.

Table 2. Macroscopic and microscopic characteristic of *actinomycetes* from Malino pine forest

Isolates	Macroscopic		Microscopic (spore arrangement)	Identified genus
	Colony colour	Pigment		
PTG 4	White	Beige	*Flexuous*	*Streptomyces*
MLN 1	Brown	Yellow	*Open spirals*	*Streptomyces*
MLN 2	Gray	Beige	*Flexuous*	*Streptomyces*
GNT 1	Brown	Yellow	*Flexuous*	*Streptomyces*
GNT 2	Brown	Yellow	*Flexuous*	*Streptomyces*
GNT 3	Brown	Yellow	*Straight*	*Streptomyces*
GNT 5	Gray	Beige	*Flexuous*	*Streptomyces*
LMG 1	White	Beige	*Flexuous*	*Streptomyces*
LMG 2	Brown	Brown	*Open loops primitive spirals hooks*	*Streptomyces*
LMG 4	Brown	Brown	*Flexuous*	*Streptomyces*
LMG 5	Brown	Yellow	*Flexuous*	*Streptomyces*
RWM 1	Brown	Yellow	*Flexuous*	*Streptomyces*
RWM 4.1	White	Beige	*Open loops primitive spirals hooks*	*Streptomyces*
RWM 4.2	Brown	Yellow	*Flexuous*	*Streptomyces*
RWM 5	Brown	Yellow	*Monoverticillate with spiral*	*Streptomyces*

Based on the result, *actinomycetes* isolates that grew on Humic Acid Vitamine Agar (HVA) media were presumed. The isolation method used was the spread method. The suspected isolates obtained were purified on Yeast Malt Agar (YMA) media. *Actinomycetes* are a type of soil bacteria with a resistant spore to heat, dryness, and chemicals. In nutrient-poor media such as HVA, *actinomycetes* grow for a long time, which is about 1-2 weeks due to their complex life cycle. The introduction of colonies to the media should be relatively easy and simple. *Actinomycetes* can be distinguished from other bacteria easily based on their colony shape. The selection results based on the diversity of colony, 15 pure isolates of *actinomycetes* were obtained with various colony morphologies, air mycelium, and are able to sporulate at the age of 7-14 days. Four isolates were obtained from the Gantarang, 4 isolates from Lombosang, 4 isolates from Resort TWA Malino, while the other 2 isolates were obtained from Malino (Pendidikan Street), while the remaining 1isolate was isolated from Patappang (Lembanna). Based on the observation of colony characteristics, all the isolates obtained showed characteristics refer to the genus *Streptomyces*.

In different with other bacteria, *actinomycetes* colonies are not formed from a uniform collection of single cells but in branched filamentous masses form. *Actinomycetes* colonies that grew in YMA media in this study were vegetative mycelium with powdery air mycelium, grew widely attached to the media and the whole unit was easy to take with loop wire. Initially, the surface of the *Streptomyces* colony was relatively smooth. Gradually with the development of aerial hyphae floccose, granular, powdery, or velvety characteristics was formed. *Streptomyces* filaments and spores are slight and no more than 1 µm in diameter. Spores are formed from filament fragmentation and arranged in straight, wavy, or helical chains (Hasani et al., 2014). Filaments allow efficient use of nutrients and allow Streptomyces to colonize substrates better than other unicellular microorganisms. When the colony becomes old and its nutrients are depleted, filamentous branches develop from the surface of the colony and produce aerial mycelium that grows vertically. (Olanrewaju & Babalola, 2019).
All the fifteen *actinomycetes* isolates are showed the genus *Streptomyces*. This indicates that the *Streptomyces* group is the dominant *actinomycetes* group in the Malino pine forest. According to (Hasani *et al*., 2014), *Streptomyces* are the most abundant microorganism in the soil and makeup 40 % of the total bacteria in the soil. This is due to the filamentous structure of *streptomyces* cause condensation of soil texture, and protect them from wind and eradication of rain.

Another distinctive characteristic of the 15 isolates from this study is the distinctive earthy odour that only belongs to the genus *Streptomyces*. Akbar *et al*., (2017) stated that this is because the main habitat of *Streptomyces* is on the ground. *Streptomyces* compose approximately 70 % of microorganisms in the soil and can be isolated in various environments, even from an unusual environment. According to Zhi et al., (2016) this soil odour is caused by *Streptomyces* producing a metabolite called Geosmin. This compound is a sesquiterpenoid component consisting of carbon, oxygen, and hydrogen. Acetic acid, acetaldehyde, ethanol, isobutanol, and isobutyl acetate have been identified as odour compounds also produced by *Streptomyces*.

Macroscopic Observation

Macroscopic observations on the surface of the medium showed that 15 *actinomycetes* isolates had morphological characteristics powdery air mycelium, flat and convex colony surfaces, and vegetative mycelium which was firmly attached to the media surface as shown in Figure 1.

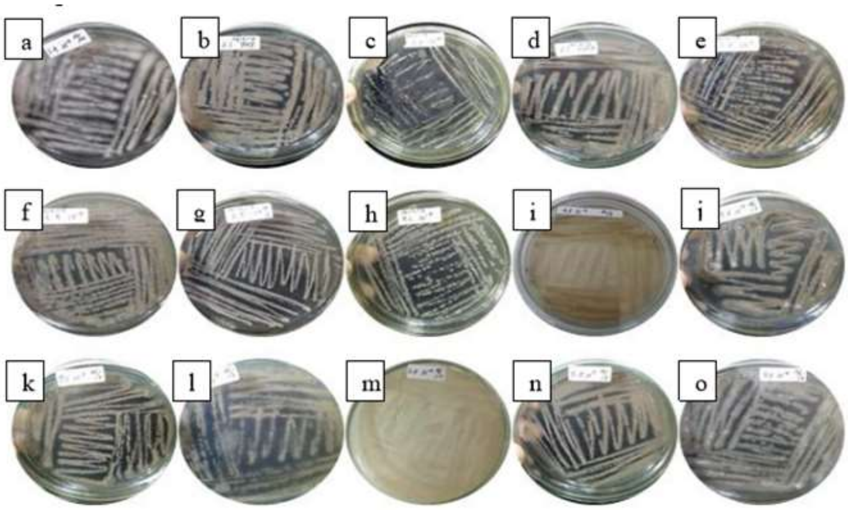

Figure 1. Morphology of *Actinomycetes* colony from Malino pine forest. (a) PTG 1, (b) MLN 1, (c) MLN 2, (d) GNT 1, (e) GNT 2, (f) GNT 3, (g) GNT 5, (h) LMG 1, (i) LMG 2, (j) LMG 4, (k) LMG 5, (l) RWM 1, (m) RWM 4.1, (n) RWM 4.2, (o) RWM 5.

According to Ambarwati et al., (2011), the characteristics of the genus *Streptomycetes* are involved vegetative hyphae and air mycelium. Vegetative hyphae will produce broad branched mycelium which rarely has fragments. The air mycelium in the adult phase form three too many spores (more than 50 spores). Meanwhile, Korn Wendisch & Kutzner (1992) stated that some species of *Streptomyces* have short spore chains in the vegetative mycelium. Spores are nonmotile. The colonies that appear are relatively smooth, but then there is a development that reveals the air mycelium in the form of floccose, powder grains, or velvet.

The colours of the vegetative mycelium and air mycelium of the 15 isolates obtained can be grouped into 3 different groups. they are brown, white, and grey and turn to black over time. The colours in the vegetative and air mycelium are caused by the formation of special metabolites called pigments. The pigmentation of *actinomycetes* varies according to the type of *actinomycetes* obtained. Many *Streptomyces* produce pigments in their vegetative mycelia or around agar media (Bennett et al., 2018). Pigmentation can range from white or grey to yellow, orange, lavender, blue and green, so it is often referred to as a colour wheel. Some pigments consist of 2 to 3 compounds, while others can consist of 7 to 10 or even 15 compounds (Abdulla et al., 2008).

Approximately 200 *actinomycetes* pigments have been identified with available data regarding chemical properties and then divided into several products such as acyclic compounds, aromatic compounds, quinones, oxygen-containing heterocyclic compounds, nitrogen-containing heterocyclic compounds, cidromycin. Each type of pigment can provide a specific colour and can be used to classify *Streptomyces*. A greenish pigment which may indicate viridomycin is produced by *Streptomyces* with air mycelia grey, pink, and yellow-greenish. Pigments associated with antibiotics of the type rhodomycin, griseorodin-rubromycin, and lithmosidin produced by *Streptomyces* with air mycelia grey and pink (Salvameenal et al., 2009). Sathi et al., (2002) identified 4-hydroxynitrobenzene in brown and cream-coloured *Streptomyces* pigments, *Streptomyces* with brownish pigments reported to have an antimicrobial activity that inhibits the growth of pathogenic bacteria (Azimi et al, 2014). Prashanti et al (2015) also reported that the yellow pigment produced by *Streptomyces griseoaurantiacus* JUACT 01 contains carotenoids with anticancer activity.

Morphological characters shown in the formation of pigments in the media by *actinomycetes* are used to identify genera or species. According to the pigment, formation is influenced by media pH, aeration, growth temperature, carbon, and nitrogen sources. Pigments produced by *actinomycetes* in the media are usually various, such as blue, purple, red, yellow, brown, green, and black. these pigments may dissolve in the media or remain on the mycelia. (Anandan et al., 2016). The same *Actinomycetes* species can produce different pigment colours depending on the growth medium, possibly due to the result of hydrogen ion concentration (Akilandeswari & Pradeep, 2017).

Microscopic Observation

This study obtained 5 types of spores arrangement of *Streptomyces* isolates based on microscopic observation. They are flexuous arrangement as seen in Figure 2, loops primitive spirals hooks as shown in Figure 3, open spirals, straight, and monoverticillate with spirals as shown in Figure 4. Bergeys Manual of Determinative Bacteriology noted that the genus *Streptomyces* has 10 types of spore chain arrangement is straight, flexuous, fascicled, monoverticillate no spiral, open loops primitive spirals hooks, open spiral, closed spiral, monoverticillate with spiral, biverticillate no spiral, and biverticillate with spiral (Bergey & Holt 1994).

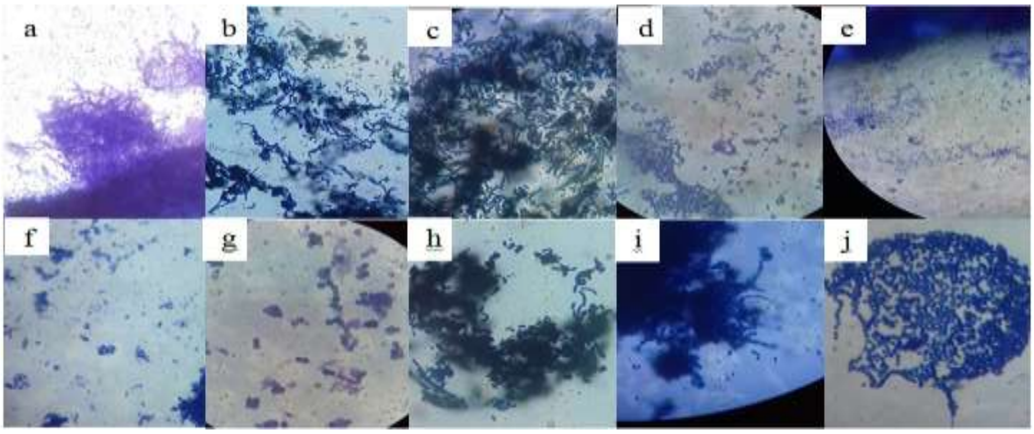

Figure 2. Spore arrangement *Flexous* of Genus *Streptomyces* from Malino pine forest, 400x magnification. (a) MLN 2, (b) GNT 1, (c) GNT 2, (d) GNT 5, (e) PTG 4, (f) LMG 1, (g) LMG 4, (h) LMG 5, (i) RWM 1, (j) RWM 4.2

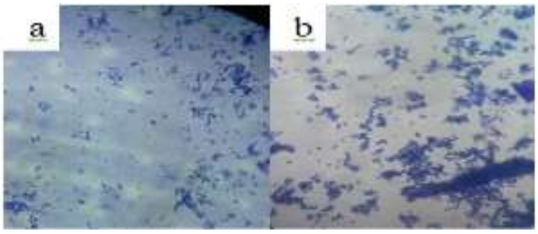

Figure 3. Spore arrangement *Open Loops Primitive Spirals Hooks* Genus *Streptomyces* from Malino pine forest, 400x magnification. (a) LMG 2, (b) RWM 4.1

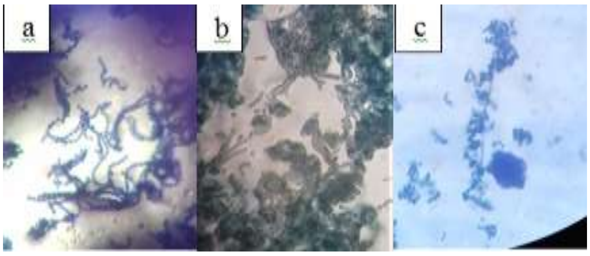

Figure 4. Spore arrangement *Open Spirals, Straight* and *Monoverticillata with Spiral* Genus *Streptomyces* from Malino pine forest. 400x magnification. (a) MLN 1, (b) GNT 3, (c) RWM 5

Observations using a 400x microscope magnification showed that 15 *actinomycetes* isolate obtained from the rhizosphere soil of the Malino Pine Forest of South Sulawesi generally had spores in the form of streptococci, include long elliptical chains in aerial hyphae, tight and dense mycelium. The results are in accordance with Armaida's (2016) statement that *Streptomyces* is a Gram-positive bacteria and streptococci arranged in a spiral, measuring about 0.8 - 2 μm. The spore chains form the air mycelium are rarely undergo fragmentation but the spores are formed from the segmentation of hyphae. Krieg and Holt (1994) explained that *Streptomyces* contains L-DAP and glycine as the characteristics of cell walls to differentiate it from other groups of *actinomycetes*. A stable filament and air mycelium with long spore chains usually come from the genus *Streptomyces* and *Streptoverticillium.* The genus *Streptomyces* also has chain-shaped spores. The results of the observations showed that the spores of 15 *actinomycetes* genus *Streptomyces* isolates were arranged in the coiled or twisted form, short and long strands that curled at one end.

Conclusion

A total of 15 isolates were isolated from the rhizosphere soil of the Malino Pine Forest of South Sulawesi. All isolates produced pigments and have aerial mycelium. The colony colour of isolated *actinomycetes* is white, brown, and grey. The spore chain arrangement of *actinomycetes* isolates are flexuous, open loops primitive spirals hooks, open spirals, straight and monoverticillate with spiral chain arrangement. Based on the morphological characteristics of these isolates, they were indicated that all the isolates belonged to the genus *Streptomyces.*

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