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ISOLATION AND IDENTIFICATION OF ENDOPHYTIC BACTERIA FROM KUMIS KUCING LEAVES (*Orthosiphon aristatus* Benth.)

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ABSTRACT

Endophytic bacteria are in symbiosis with plants and have the potential to produce secondary metabolites similar to their host. Kumis kucing (Orthosiphon aristatus) is a traditional medicinal plant that has been proven to have many pharmacological activities, including antiviral and antibacterial. This study aims to isolate and characterize endophytic bacteria from kumis kucing leaves. Endophytic bacteria were isolated from kumis kucing leaf by spreading method on Trypticase Soy Agar (TSA) media and incubated for 24 hours at room temperature. Colonies that grew with bacterial morphology were inoculated on TSA media to obtain pure cultures. Pure cultures of isolates were identified through Gram staining, 16S rRNA gene sequencing, and bacterial growth curves. There were eight isolates with bacterial morphology, which were identified by Gram staining. The staining results showed that all were classified as Gram-negative with rod and coccus shapes. The 16S rRNA gene sequencing identified three bacterial isolates : Acinetobacter schindleri, Pantoea agglomerans, and Pseudomonas lurida. The three bacteria have different time to reach stationary phases in order to produce their secondary metabolites.

Keywords: *Acinetobacter schindleri, endophytic bacteria, kumis kucing leaves, Pantoea agglomerans, Pseudomonas lurida*

INTRODUCTION

Endophytes are microorganisms that live in symbiosis with plants without causing diseases in these plants. Endophytic bacteria living inside plants have the potential to produce secondary metabolites similar to those produced by their host [1]. The ability of endophytic bacteria to produce secondary metabolites presents a great opportunity for development as medicinal substances. In addition to shorter production times and higher quantities, there is no need to harvest or extract plant parts extensively, which may take years to grow [2]. Indonesia has abundant endophytic potential, but research in this area is still limited.

Orthosiphon aristatus Benth, commonly known as "*kumis kucing*", is a traditional medicinal plant that the Indonesian community has empirically utilized. Some of the secondary metabolites that are found in kumis kucing leaves include flavonoids such as rutin, quercetin, and kaempferol, tannins, polyphenols, saponins, monoterpenoids, sesquiterpenoids, and quinones [3]. The presence and concentration of these secondary metabolites can vary depending on factors such as plant species, environmental conditions, and extraction methods. A study by Sivakumar & Jeganathan showed the presence of various phytochemical compounds in the water extract of kumis kucing leaves, which possess strong pharmacological and biological properties [4]. Many studies have been conducted on the biological and pharmacological activities of kumis kucing leaves, including diuretic [5, 6], analgesic and antipyretic [7], antioxidant [8], anti-colon cancer [9], antiviral [10], and antibacterial activities [11].

The multitude benefits of kumis kucing leaves and the potential prescence of endophytic bacteria contained in them are the background of this research. In the future, if endophytic bacteria can be isolated and have the same secondary metabolite content and activity as kumis kucing leaves, if the secondary metabolites produced by endophytic bacteria isolated from kumis kucing leaves exhibit similar content and activity, a faster production process can be developed compared to direct extraction from kumis kucing leaves which can only be harvested 2-3 months after planting [12].

METHODS

Isolation of endophytic bacteria. A total of 2 grams of freshly sorted kumis kucing leaves were washed with running water. Then, disinfection was carried out by soaking kumis kucing leaves in 70% ethanol for 1 minute, followed by 2.5% sodium hypochlorite for 4 minutes, then 70% ethanol for 30 seconds, and finally rinsed with sterile distilled water. The rinsing process was repeated three times. The last rinse water was used as a microbial testing sample using the pour plate method with Trypticase Soy Agar (TSA) medium and incubated at 25°C. After 48 hours, the growth of microorganisms was observed.

A total of 6 mL of 0.9% NaCl was added to the disinfected leaves. Then, the leaves were crushed using a mortar to obtain tissue extract. The extract was incubated at room temperature for 3 hours to allow the release of endophytic microorganisms from the host tissue. The tissue extract was diluted 10^{-1} and 10^{-2}, and 100 µL was taken from each dilution to be inoculated onto TSA media using the streak method. Incubation was carried out for 48 hours at room temperature. Several colonies were selected based on their morphological differences (color, size, and shape). The selected colonies are then subcultured on TSA media using the streak plate method and incubated at room temperature for 24 hours. This process was repeated until pure culture was obtained.

The disinfected leaves were mixed with 6 mL of 0.9% NaCl solution and crushed using a sterile mortar and pestle. The tissue extract was then incubated at room temperature for 3 hours to allow the release of endophytic microorganisms from the host tissue. The tissue extract was diluted 10^{-1} and 10^{-2} in 0.9% NaCl solution. A total of 100µl was inoculated using the streak plate method on TSA media in petri dishes. Incubation was carried out at room temperature for 48 hours. Colonies were selected based on their morphology (color, size, and shape). The selected colonies were then subcultured on TSA media using the streak plate method at room temperature for 24 hours, repeated until pure isolates were obtained [13].

Gram staining. The bacterial specimen on glass slides was stained with crystal violet for 30 seconds. The stain was removed by washing it with distilled water, then covering it with an iodine solution for 60 second, and rinsing it again with 96% ethanol and distilled water. The specimens were then stained with fuchsin for 30 seconds, rinsed with distilled water, and air-dried. The preparations were observed under a microscope at 40x magnification.

Molecular identification (16S rRNA). The endophytic bacterial isolates were molecularly identified using the 16S rRNA gene analysis. Single colonies from each selected isolate were sequenced using universal primers listed in Table 1 to determine their base sequence [14]. The sequencing results are aligned using BLAST.

Phylogenetic tree construct. A total of 10 sequences with the same genus from each isolate were aligned to construct a phylogenetic tree. Phylogenetic tree analysis was carried out using MEGA 11 software and evaluated using maximum likelihood 100 bootstrap.

Bacterial growth curve. A 10% (v/v) liquid culture of endophytic bacterial isolates was inoculated into an Erlenmeyer containing Trypticase Soy Broth (TSB) and incubated for 24 hours at room temperature. Measurements were taken at 0 hours, starting from the time of inoculation. Absorbance was measured at 2-hour intervals for 24 hours using a UV-Vis spectrophotometer at a wavelength of 600 nm. The absorbance values were calculated using a standard bacterial growth curve equation to determine the number of colony. A bacterial growth curve was constructed correlating time and colony count.

RESULTS AND DISCUSSION

Isolation of endophytic bacteria. The isolation of endophytic bacteria from kumis kucing leaves is carried out by first disinfecting the leaves to eliminate bacteria from the environment using ethanol and sodium hypochlorite. Ethanol is used as a disinfectant because it can kill bacteria through two mechanisms: protein denaturation and dissolution of lipid membranes [15]. The antimicrobial effect of sodium hypochlorite is not well-known, but in water containing active chlorine, hypochlorous acid which is a strong oxidizing agent, is formed. Hypochlorous acid irreversibly oxidizes sulfur-containing compounds present in essential enzymes, disrupting bacterial cell metabolism [16].

Pure culture isolates were obtained from bacteria growing on Trypticase Soy Agar (TSA) media. TSA is a non-selective media containing agar, casein peptone, soy peptone, and sodium chloride to support the growth of various microorganisms [17]. The results of endophytic bacterial isolates formed from the 10^{-1} dilution showed six bacterial colonies, and from the 10^{-2} dilution, there were two bacterial colonies with different visible morphologies. The selected colonies were then purified through subculture using streak plate method and repeated to obtain bacteriaha with a single colony. Eight endophytic bacterial isolates were obtained from the purification process.

Table 1. Primer for 16S rRNA analysis

Sequencing primer	PCR primer
F 5’ (GGA TTA GAT ACC CTG GTA) 3’	F 5’ (AGA GTT TGA TCM TGG CTC AG) 3’
R 5’ (CCG TCA ATT CMT TTR AGT TT) 3’	R 5’ (TAC GGY TAC CTT ACG ACT T) 3’

Gram staining. Table 2 showed the result of the Gram staining. Out of the eight isolates, all bacteria were gram-negative, with two being coccus in shape and the remaining six being rod-shaped. From the Gram staining results, four different endophytic bacterial isolates were identified, namely Isolate 3, 6, 7, and 8, which were further subjected to molecular identification.

Molecular identification. Molecular identification was performed on the four endophytic bacterial isolates by amplifying them using 16S rRNA primers. The results of bacterial identification using 16S rRNA gene sequencing showed that Isolate 6 was identified as *Acinetobacter schindleri* with a percent identity of 99.85% (Figure 1), Isolate 7 as *Pantoea agglomerans* with a percent identity of 99.57% (Figure 2), and Isolate 8 as *Pseudomonas lurida* with a percent identity of 99.42% (Figure 3). Isolate 3 could not be identified as a bacterial species as it did not yield any results when amplified using the 16S rRNA gene.

Genus *Acinetobacter* are classified as gram-negative, non-fermentative, and aerobic bacteria. These bacteria are widely distributed in various habitats such as soil, water, and dry environments [18]. *Acinetobacter* is a genus of bacteria that includes some endophytic that has been identified as a potential plant growth-promoting. The secondary metabolites produced by *Acinetobacter* have potential antioxidant, antimicrobial, and anticancer properties [19].

Pantoea agglomerans is a bacterium commonly found in environmental and agricultural settings, often isolated from plants, soil, water, and food. This bacterium is frequently associated with various plants, acting as both an epiphytic and endophytic bacterium. *Pantoea agglomerans* has also been extensively used as a biological agent to control the growth of fungi and bacteria pathogen in plants [20].

Table 2. Result of bacterial morphology and gram staining

Bacterial isolate	Colony color	Shape	Gram staining
1	Clear white	Rod	negative
2	Bright yellow	Rod	negative
3	Rose-colored	Coccus	negative
4	Bright yellow	Rod	negative
5	Clear white	Rod	negative
6	Clear white	Coccus	negative
7	Bright yellow	Rod	negative
8	Clear white	Rod	negative

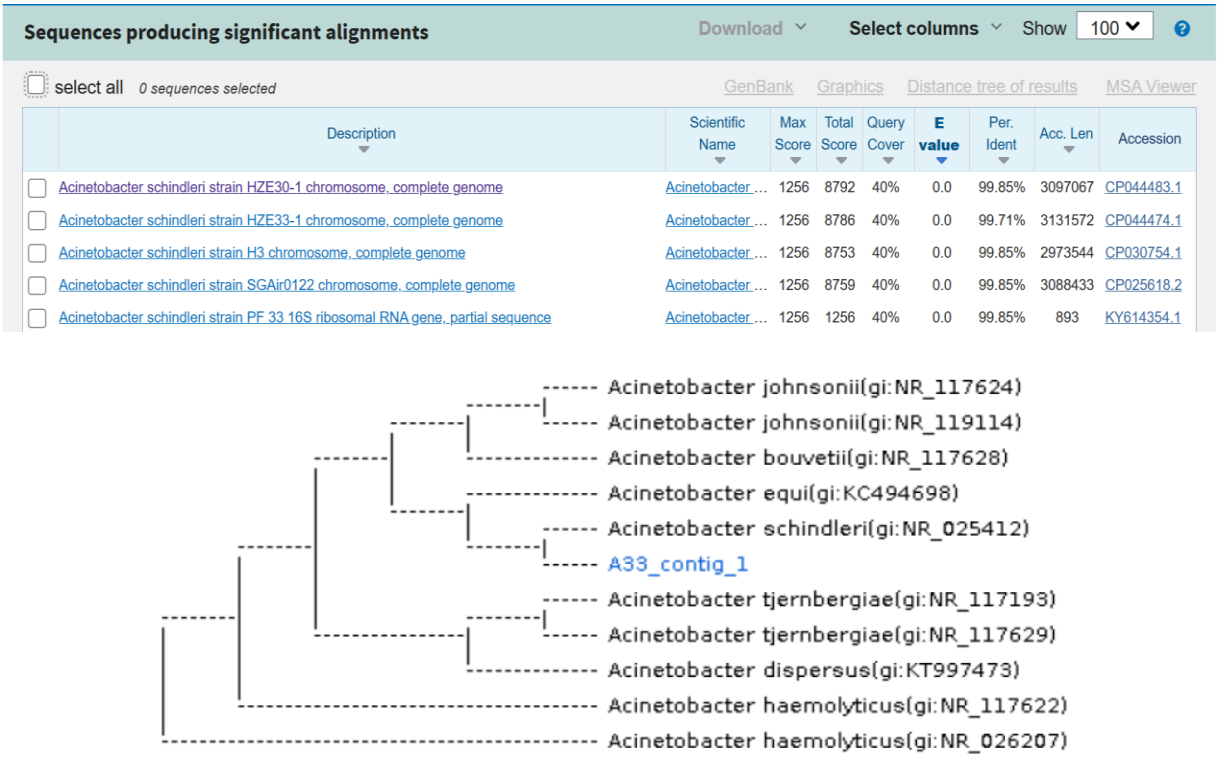

Figure 1. Percent identity and phylogenetic tree results from Isolate 6

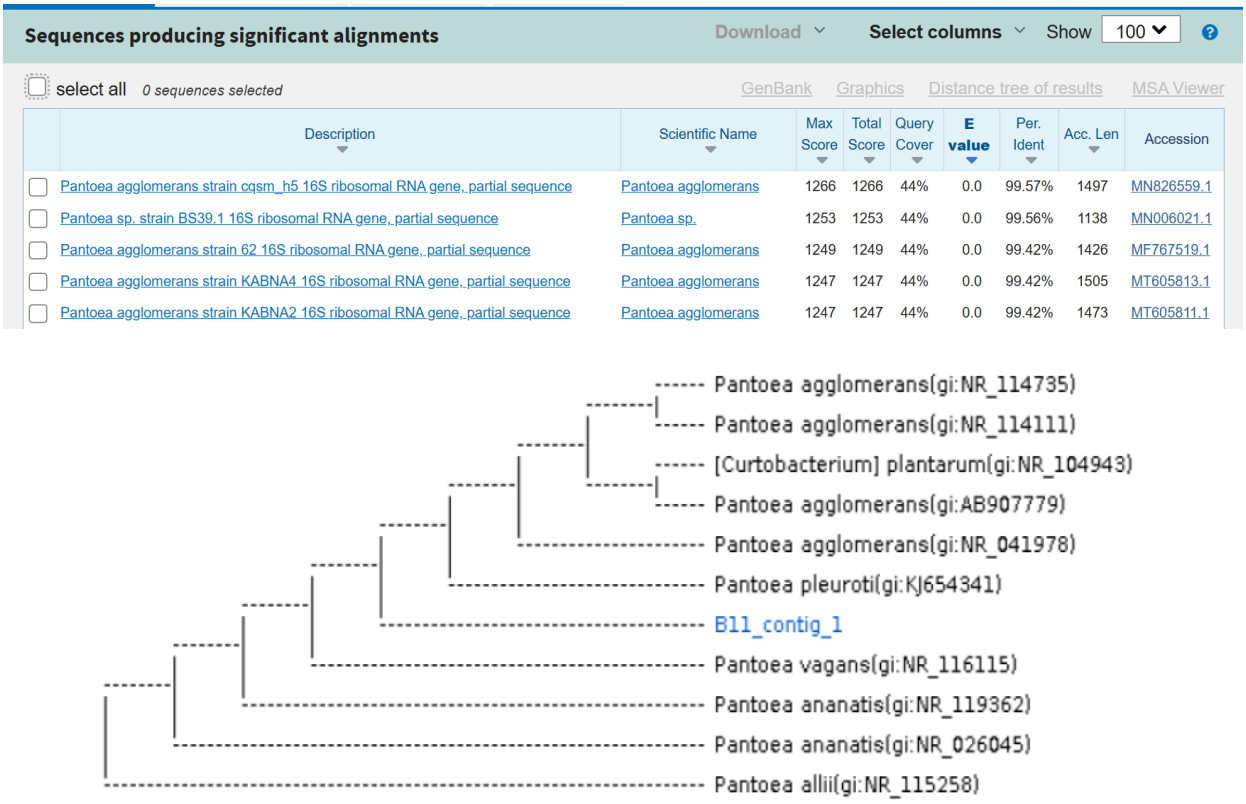

Figure 2. Percent identity and phylogenetic tree results from Isolate 7

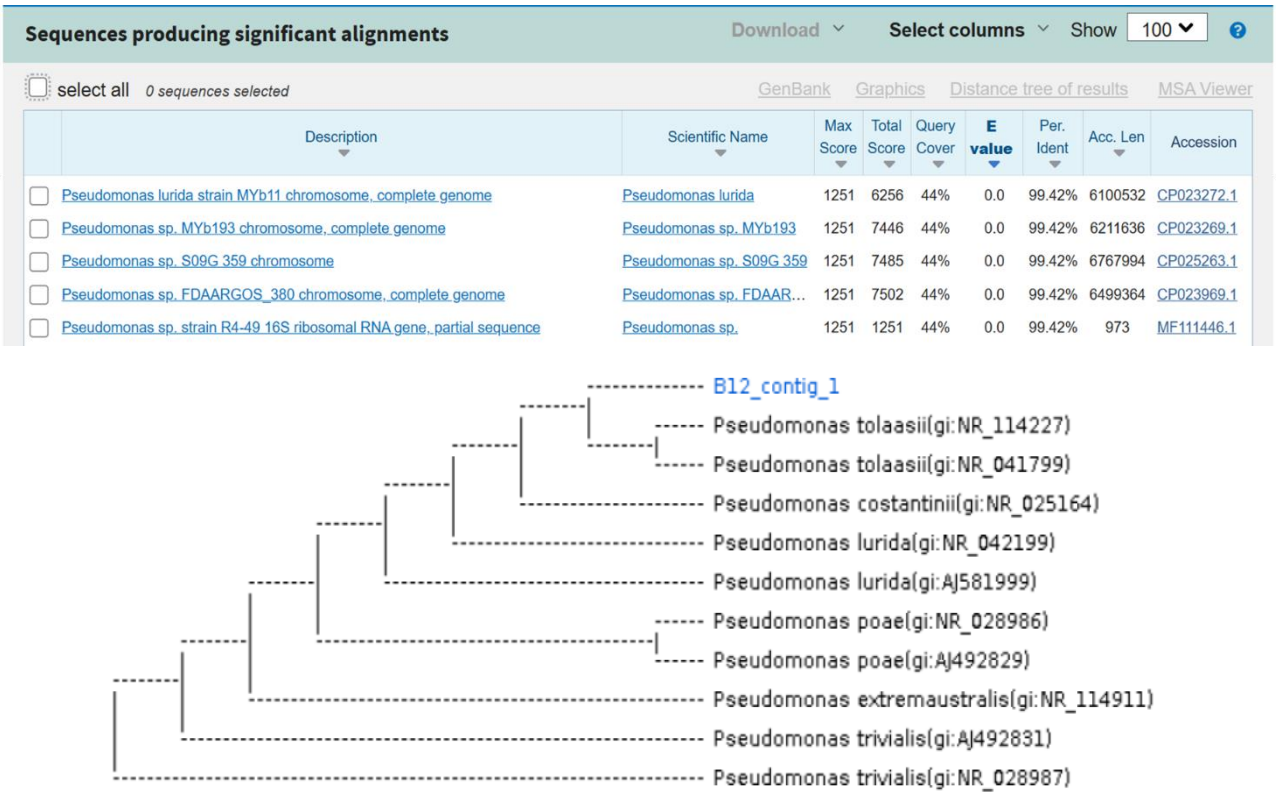

Figure 3. Percent identity and phylogenetic tree results from Isolate 8

Pseudomonas lurida belongs to the group of gram-negative bacteria, has a rod-shaped morphology, and is non-spore forming. *Pseudomonas* species are commonly found in plants, soil, and water [21]. *Pseudomonas lurida* is an endophytic bacterium that has been studied for its potential in bioaugmentation, particularly plant growth promotion through the production of growth-promoting substances, induction of systemic resistance, phosphate solubilization, and inhibition of pathogens [22].

Bacterial growth curve. The bacterial growth curve is a curve that illustrates the growth phases of a bacterium. The purpose of creating a growth curve is to determine the stationary phase in which secondary metabolites are produced. During the stationary phase, the number of bacterial cells remains constant, indicating that the number of bacteria that die is equal to the number of bacteria that grow. When the nutrients in the media begin to deplete, competition among bacteria for survival occurs [23]. During this phase, secondary metabolites are produced, which can benefit both the bacteria and its host plant. Each bacterium has a different time to reach the stationary phase. Based on Figure 4, *Acinetobacter schindleri* reaches the stationary phase in 20 hours, *Pantoea agglomerans* reaches the stationary phase in 14 hours, and *Pseudomonas lurida* reaches the stationary phase in 20 hours. Secondary metabolites are typically produced through the modification of primary metabolite synthases and are formed during the end or near the stationary phase of growth. The formation of secondary metabolites is regulated by nutrients, growth rate, and the carbon source. The regulation of secondary metabolites is conditional to the nutrients in the media, requiring a high carbon-to-nitrogen or carbon-to-iron ratio. Microorganisms have sophisticated molecular networks that can sense environmental stimuli and regulate the expression of secondary metabolites depending on environmental conditions such as the type and especially the carbon source [24].

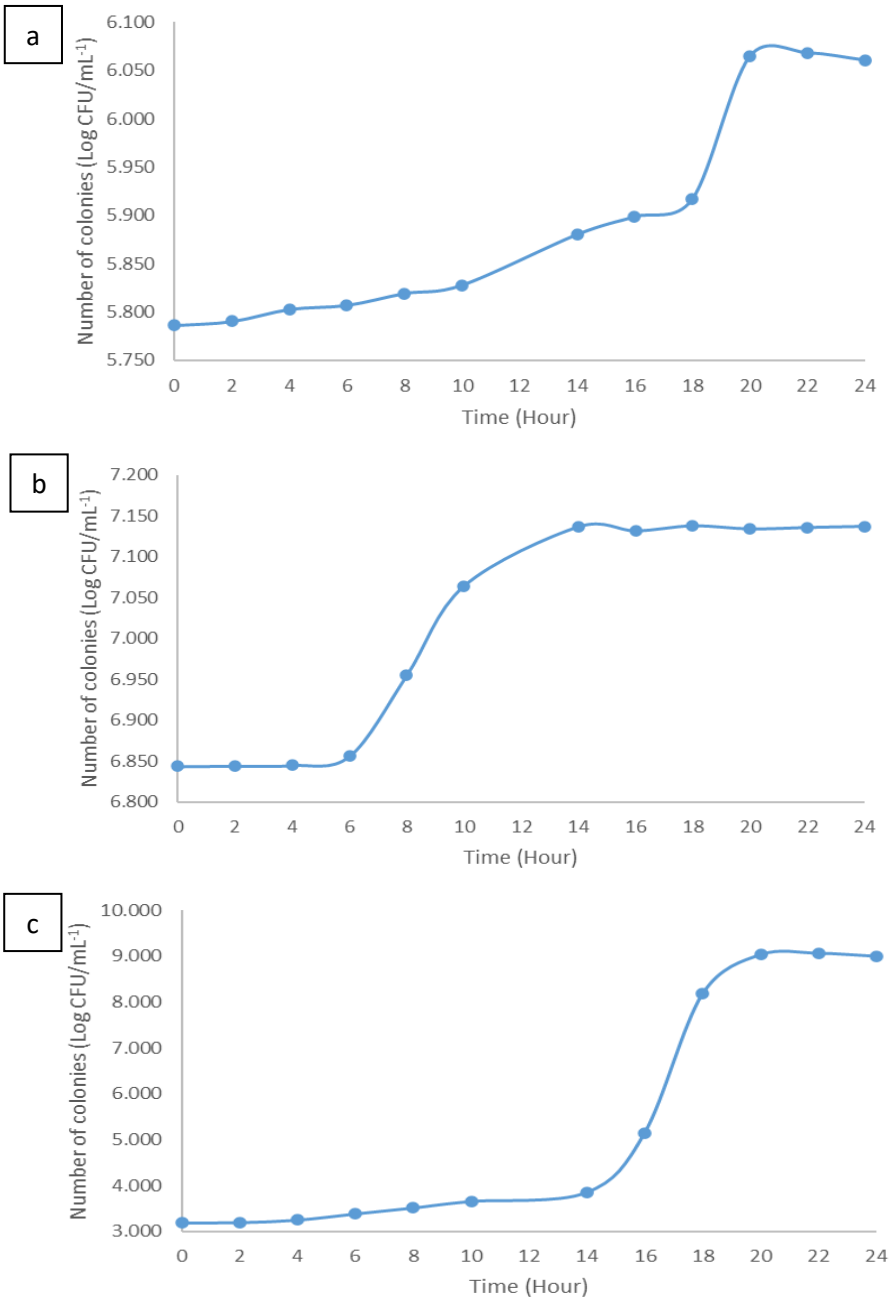

Figure 4. Bacterial growth curve (a) *Acinetobacter schindleri* (b) *Pantoea agglomerans* (c) *Pseudomonas lurida*

CONCLUSION

The endophytic bacteria found in kumis kucing leaves (*Orthosiphon aristatus* Benth.) are *Acinetobacter schindleri*, *Pantoea agglomerans*, and *Pseudomonas lurida*. These three bacteria have different times to reach the stationary phase in order to produce their secondary metabolites.

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