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CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC BACTERIA FROM FLESH FRUIT OF ARECACEAE FAMILY AGAINST ANTIBIOTIC RESISTANT BACTERIA *Escherichia coli*

KARAKTERISASI DAN AKTIVITAS ANTIBAKTERI BAKTERI ENDOFIT ASAL DAGING BUAH FAMILY ARECACEAE DALAM MENGHAMBAT BAKTERI RESISTEN ANTIBIOTIK *Escherichia coli*

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ABSTRACT

Bacteria can symbiotic mutualism and synergize with plants in the tissue and do not cause harm to plants. On the other hand, bacteria resistance provides an opportunity to search for antibacterials from natural material without threatening plant biodiversity. The purpose of this study was to characterize and test the antibacterial activity of endophytic bacteria from the fruit of the Arecaceae family against antibiotic-resistant bacteria Escherichia coli. The method used was direct culture on the growth of media in the laboratory by first sterilizing the surface of the flesh of the samples, such as Aren (Arenga pinnata), Coconut (Cocos nucifera), and Sago (Metroxylon sagu). The pure isolates obtained were characterized according to Bergeys Manual for Identification by performing Gram stain, catalase, and coagulase test. The results obtained were five isolates with codes A1, A2, K, S1, and S2 with variation in morphology colonies. Gram staining, catalase, and coagulase verification of the five isolates were groups of Gram-positive cocci, catalase-positive, and coagulase-negative bacteria. Based on tests carried out on the characters of the endophytic bacteria, it was obtained a close relationship with the genus Staphylococcus. The results of the inhibitory activity test showed that Staphylococcus sp. *K with the highest criteria of 43.26 mm and Staphylococcus* sp. *A1 with the lowest inhibition criteria with a value of 24.73 mm. Both are categorized as very strong inhibition criteria.*

Keywords: *Endophytic bacteria, Arecaceae, antibiotic-resistant, Escherichia coli*

ABSTRAK

Bakteri dapat bersimbiosis mutualisme dan bersinergi dengan tumbuhan di dalam jaringan serta tidak menimbulkan kerugian bagi inang tumbuhan. Disisi lain kasus resistensi bakteri memberi peluang terhadap pencarian antibakteri dari bahan alam tanpa mengancam keanekaragaman hayati tumbuhan. Tujuan penelitian ini yaitu mengkarakterisasi dan menguji aktivitas antibakteri bakteri endofit asal daging buah family Arecaceae dalam menghambat bakteri resisten antibiotik Escherichia coli. Metode yang digunakan adalah penanaman langsung (direct culture) pada media pertumbuhan di laboratorium dengan terlebih dahulu melakukan sterilisasi permukaan pada daging buah sampel yaitu Aren (Arenga pinnata), Kelapa (Cocos nucifera), dan Sagu (Metroxylon sagoe). Isolat murni yang diperoleh dikarakterisasi mengacu pada Bergeys Manual for Identification dengan melakukan pewarnaan Gram, uji katalase dan koagulase. Hasil penelitian memperoleh sebanyak lima (5) isolat dengan kode A1, A2, K, S1 dan S2 dengan variasi morfologi koloni. Pewarnaan Gram, katalase dan koagulase menunjukkan kelima isolat merupakan kelompok bakteri Gram positif kokus, katalase positif dan koagulase negatif. Berdasarkan pengujian yang dilakukan terhadap karakter kelima bakteri endofit diperoleh kedekatan kekerabatan dengan genus Staphylococcus. Hasil uji aktivitas penghambatan bahwa Staphylococcus sp. *K dengan kriteria hambat tertinggi sebesar 43,26 mm dan Staphylococcus* sp. *A1 dengan kriteria hambat terendah dengan sebesar 24,73 mm. Keduanya dengan kriteria hambat pada kategori sangat kuat.*

Kata kunci: *Bakteri endofit, Arecaceae, resistensi antibiotik, Escherichia coli*

INTRODUCTION

Diarrhea is one of the diseases with high morbidity in Aceh every year. Morbidity and mortality become the assessment indicator of public health status. Diarrhea handled by health facilities in Aceh Province in 2016 was about 71% [1]. In 2017 this disease was about 58% [2]. There was a decrease in diarrhea in 2018, and it was about 53.83% [3].

In the effort to reduce the percentage of diarrhea morbidity from the patients who come to health facilities, they are recommended to take antibiotics. Then, it needs public awareness in consuming the antibiotics as recommended by the health departments. However, in reality, some people diagnosed with diarrhea ignore to complete treatment until the antibiotics given are consumed. It is because they feel healthy by consuming one or several times of antibiotics. This causes the possibility of bacterial resistance in the patient's body that can cause infection by antibiotic-resistant bacteria.

Several species of the palm family include Aren (*Arenga pinnata*), Pinang (*Areca catechu*), Siwalan (*Borassus flabellifer*), Gebang (*Corypa utan*), Kelapa (*Cocos nucifera)* as the medicine. Aceh also has limitless biological wealth, not only the plants but also includes the microorganisms. They are the most vulnerable natural resources that live synergistically with the plants [4].

Furthermore, the synergism of life between the microorganisms in plant tissues and plants as their hosts has been widely studied. The interaction of microorganism life in plant tissue is also known as the endophytic microbes [5]. Endophytic bacteria are able to produce bioactive compounds similar to the host plants [6]. There is still limited information regarding the use of endophytic bacterias as antibacterial agents [7].

Furthermore, the previous research did an isolated endophytic bacteria of sago palm plant (*Metroxylon sago* Robbt.), and it is known to have a strong inhibitory activity of 12 mm obstructing *E. coli* bacteria. The 16S rDNA molecular analysis results showed that the endophytic bacteria were from the *Enterobacter ludwigii* species strain of EN-119 [8]. However, there is no further information regarding the ability of endophytic bacteria of the Arecaceae family that inhibiting multidrug-resistant *E. coli* bacteria. Therefore, the exploration of new antibiotics from local endophytic bacteria can be a safe alternative that can be offered to treat multidrug-resistant of *E. coli* infections.

The characterization is very important to be done in determining the similarity of morphological, physiological, and even other genetic characteristics of microbes based on tests that support previously identified microbes. The characterization is also the basis for determining the variety of living things at various levels so that the exploration of microbes for various human uses can be carried out. While the antibacterial activity test was carried out to obtain the best-isolated antibacterial, which could be a solution to the problem of antibiotic resistance. As a result, the endophytic bacteria were chosen to reduce the use of natural resources such as plant body parts to preserve biological wealth.

Based on the description above, the researcher is interested in characterizing and testing the antibacterial activity of endophytic bacteria from the fruit of the Arecaceae family against antibiotic-resistant *E. coli* bacteria.

METHODS

The research was conducted from September 2019 to January 2020 at the Microbiology laboratory, Faculty of Science and Technology, Ar-Raniry Islamic State University of Banda Aceh. The selection of *Arecaceae* family plants is based on the local potential of Aceh. These plants include Coconut var. Dalam Lampanah (*C. nucifera*), Aren (*A. pinnata*), Rumbia/sago (*M. sago*). In addition, the multidrug-resistant of isolated *E. coli* bacteria were obtained from the microbiology laboratory of the Biology Study Program, Faculty of Science and Technology, UIN Ar-Raniry. *E. coli* is known as the resistant antibiotics of cefotaxime, tetracycline, amoxicyclin, and gentamicin isolated from the cow intestine.

Isolation of endophytic bacteria. This stage has applied the direct plant method [7]. The samples were selected from ripe fruit. Each fruit flesh was first washed with the water to separate it from any impurity material that might still be attached. The flesh of each fruit was cut by 1x1 cm, then the surface was sterilized with 70% ethanol for 1 minute, 2% sodium hypochlorite (NaOCl) for 1 minute, and rinsed again with 70% ethanol for 30 seconds. Then, it was cleaned with sterile distilled water three times. The rinsed water was used as a control; it also spread on Nutrient Agar (NA) media. If there was no bacterial growth in the control of the last rinse of the distilled water, then the bacterial growth in the inoculant cup really came from the endophytes fruit flesh.

Furthermore, the pulp was dried with sterile filter paper and placed 2-3 pieces in a petri dish containing NA media at a distance and then incubated at 32 ºC for 48 hours. The colonies growing with different morphology were marked and observed.

The bacterial colonies were first noted for morphological differences, including shape, size, color, elevation, and the edge of the colony. Then, other different colonies were purified on fresh NA medium. After that, it was incubated again at a temperature of 32 ºC for 24-48 hours. The purification was carried out several times until the pure cultures were obtained.

Refreshment of test bacteria. At this stage, the pure cultures of multidrug-resistant of *E. coli* bacteria were streaked on Eosin Methylene Blue Agar (EMBA) media to observe their growth morphologically to ensure their suitability. The separated green metallic colonies on EMBA media were *E. coli* bacteria. After that, it suspended 1-2 oses of *E. coli* colonies into Nutrient Broth (NB) media and incubated for 450 minutes (±7.5 hours). Then, after the test, bacteria were in the mid-log phase, where the cell count was 5.90 x 10^8 CFU/ml as a working culture. The colonies were also grown on slanted EMBA media to be used as the stock cultures [9].

Antibacterial activity test. The selection of endophytic microbes was carried out by applying the disk diffusion method [10]. Then, the sterile cotton buds were dipped in the test of bacteria suspension and then streaked on NA medium. Furthermore, 10 L of endophytic bacterial suspension was dripped onto sterile disc paper. The paper disc containing the bacterial suspension was transferred aseptically into a sterile petri dish for a while until the suspension was absorbed to prevent excessive seepage of the growth media. Then, the disc paper was placed in the petri dish containing NA media, and it was incubated at 32 ºC for 24-48 hours. The negative control used disc paper which was dripped with sterile distilled water, while the positive control used 10 µl (1000 ppm) *chloramphenicol*. The inhibitory activity was measured by observing the clear zone formed, which could be measured with a caliper.

Characterization of endophytic bacteria. Next, the characterization of endophytic bacteria was done by selecting the isolates with the best antibacterial activity in inhibiting *E. coli*. The characterization process was carried out by identifying the cell morphology of Gram staining. Then the characterization process was carried out, including catalase and coagulase tests based on Gram staining. Finally, the characterization process was continued to the genus level by using the Bergeys Manual for Identification [11].

RESULTS AND DISCUSSION

Based on Table 1, it could be seen that the isolated colonies were varied from the observation of morphology colonies. These differences included the color of the colonies, namely cream, white, and clear white. Then, the colony shape was round and irregular. The surface elevation of the colonies tended to be flat and convex. The elevation of the colonies had five differences, namely curly, split, undulate, intact, and wavy. The results of observations of morphology colony also explained that the variation of endophytic bacteria obtained was at the species level within the same genus.

Moreover, endophytic bacteria could be found in the fruit flesh. This study found five bacterial isolates (A1, A2, K, S1, and S2) from three types of fruit, namely Aren, Kelapa var. Dalam Lampanah, and Sago. Those plants on the earth could be the host of one or more endophytic microbes [12].

This result was also relevant to the results of other studies that mentioned bacteria that live in a mutualistic symbiosis in plant tissues with a tendency to provide benefits to their host. The endophytic bacteria could be found in plant parts with access through roots, stems, cotyledons, and flowers. These bacteria enter through this access and spread throughout the plant body, reside in cells, intercellular spaces, or spread through vascular vessels. After interacting in their capacity, one of the advantages provided by endophytic bacteria was that they produce a variety of natural metabolite products that could serve as a source of medicine or antagonists for other cells that were also present at the same time. In its growth, the endophytic bacteria needed internal nutritional support in the organs. Here, the fruit provided the opportunity for the availability of nutrients for bacterial growth [13].

Bacterial cells generally had three shapes: round (*cocci*), rods (*bacilli*), and spiral. Cells showed a shape that varies permanently or changes (involution) depending on environmental influences. It could be in the form of unfavorable environmental influences (pathogenic stress) [14]. The five differences in colony morphology explained that the endophytic bacteria obtained were different at the species level. The five isolates of endophytic bacteria were found to have a variety of cell morphology variations.

Table 1. The characterization of colony morphology in endophytes bacteria in fruit flesh of Arecaceae family

No.	Isolate code	Size	Color	Colony Form	Elevation	Shape
1	A1	Big	Cream	Irregular	Even	Curly
2	A2	Small	White	Round	Even	Split
3	K	Big	White	Irregular	Even	Uneven
4	S1	Mid	Clear white	Round	Convex	Intact
5	S2	Big	Clear white	Irregular	Convex	Wavy

A1 and A2 = isolate of Aren fruit plants; K = isolate of Coconut fruit; S1 dan S2 = isolate of Sagu fruit

Table 2. The average zone of inhibition of endophytic bacteria isolates in inhibiting multidrug-resistant of *Escherichia coli*

No.	Isolate code	The inhibitory zone (mm)	The average of inhibitory zone control of chloramphenicol (mm)	The average of inhibitory in water control (mm)	Inhibitory Criteria
1	A1	24.73	8.00	0.00	Very strong
2	A2	35.93	8.00	0.00	Very strong
3	K	43.26	8.00	0.00	Very strong
4	S1	30.93	8.00	0.00	Very strong
5	S2	35.74	8.00	0.00	Very strong

A1 dan A2 isolate of Aren fruit plants; K = isolate of Cocunut fruit; S1 dan S2 = isolate of Sagu fruit

The variation in the number of endophytic bacteria depended on the type and age of the plant, soil structure, geographical distribution, and time of sampling. The variation in the visible form could be a permanent condition or an abnormality that could change at any time if environmental conditions were no longer under pathogen stress or the influence of other environmental factors [15]. In this study, the researcher used ripe fruit as the samples. In addition, the soil structure and geographical distribution were typical of Aceh Province, especially the sample of Kelapa var. Dalam Lampanah. It was a New Leading Varieties (VUB) plant that had been released by Balitbangtan in the session on the Release of Plant Varieties, which was held by the Directorate of Seeds, Directorate General of Plantations on 21st April 2017.

Another reason that was assumed to underlie the distribution of endophytic bacteria in plant organs that could cause differences in the number between one organ and another was the circulation of photosynthetic products to all parts of the plant from the leaves through the phloem transport vessels, which could be utilized by endophytic bacteria as a source of nutrition [16].

Based on the table above, it can be explained that the five isolates had great potential in inhibiting the growth of MDR of *E. coli*. The selection of endophytic bacteria with very strong inhibitory abilities was obtained in the antagonist test against the bacteria. These were one of the five isolates of endophytic bacteria which was tested with very good inhibition criteria, isolates from Coconut var. dalam Lampanah (Figure 1) was a bacterium with had highest inhibition criteria compared to the other four isolates.

Based on the table above, it can be explained that the five isolates had great potential in inhibiting the growth of antibiotic-resistant *E. coli*. The selection of endophytic bacteria had very strong inhibitory abilities that were obtained in the antagonist test against the test bacteria. These were one of the five isolates of endophytic bacteria tested with very strong inhibition criteria. Isolates from Coconut var. Dalam Lampanah (Figure 1) was a bacterium with the highest inhibition criteria compared to the other four isolates.

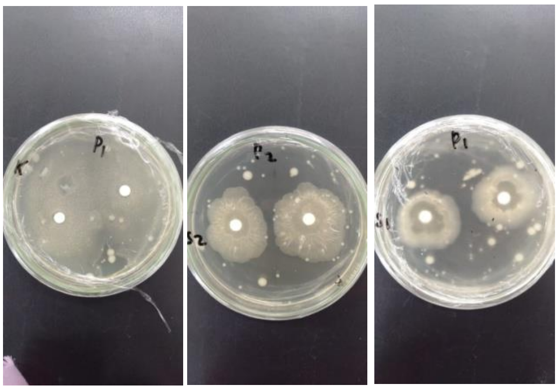

Figure 1. The inhibitory activity of isolate endophytes of *E. coli* bacteria

The other four isolates with very high inhibition criteria came from Aren fruit the isolate code A2, Sago fruit with S2 isolate code, Sago fruit with S1 isolate code, and Aren fruit with A1 isolate code. The results of this test explained that the ability of endophytic bacteria was very dominant compared to the test bacteria grown together in petri dishes *in vitro*. It was shown from the evidence of the growth of test bacteria along the growth zone of endophytic bacteria in petri dishes.

Endophytic bacteria produced secondary metabolites, which simultaneously diffused space and time to the entire surface of the growing medium in a petri dish. Each plant could contain several endophytic bacteria capable of producing biological compounds or secondary metabolites, which were thought to be the result of coevolution or genetic transfer from the host plant [17]. Moreover, the endophytic microbes produced bioactive compounds similar to those produced by the plant host tissues, so it was unnecessary to extract the plant body parts to utilize them [18].

In fact, the growth of antibiotic-resistant of *E. coli* bacteria was simultaneously inhibited, indicated by the continued growth of endophytic bacteria in the petri dish. Inhibition with a diameter of 20 mm had a very strong antibacterial potential [19]. The zone of inhibition of endophytic bacteria in this study was very strong. It provided an opportunity for endophytic bacteria to extract their secondary metabolites into new antibacterial drugs.

The inhibition zone was the activity of secondary metabolites of endophytic bacteria in inhibiting the growth of the test bacteria by interfering with bacterial metabolism, inhibiting bacterial cell wall synthesis, disrupting bacterial cell membrane permeability, inhibiting bacterial protein synthesis, and damaging the nucleic acid synthesis of test bacteria [20]. It was also proven from previous research that had obtained the endophytic bacteria of Sago palm plant (*Metroxylon sago* Robbt.) had 12 mm strong inhibitory activity against the *E. coli* bacteria [8].

The factors that affect the size of the inhibition zone were the level of sensitivity of the test organism, culture medium, and incubation condition [22]. In this study, *E. coli* was the tested bacteria with specifications resistant to more than three types of antibiotics from different groups. The formation of the inhibition zone was also influenced by environmental factors and the concentration of the test bacteria [23]. The higher the antibacterial concentration produced, the higher the inhibitory power could be. It was also indicated by the small growth of pathogenic bacteria colonies [24]. The difference in the diameter of the inhibition zone formed was assumed to be caused by the different types of antibacterial metabolites of each isolate of endophytic bacteria produced [25]. The results of this study were totally in line with other studies that used endophytic bacteria from medicinal plants as a new way to obtained antibacterial compounds without extracting them directly from these medicinal plants.

Antimicrobial resistance was currently a serious worldwide problem. This problem was a major threat to the global community. This was due to the spread of multidrug-resistant bacteria, which were known to be pathogenic. The spread of this pathogen to other bacteria could be through horizontal transfer and gene mutation. This occurred naturally but could be exacerbated by continuous exposure to antibiotics that were used wrong in their therapy, less strict supervision of antibiotic use, clinically uncontrolled antibiotic regulation in some farms, and so on.

Significant problems also arise in the health sector, one of which was the emergence of cases of resistance to bacteria [26, 27]. According to many studies, the inheritance of antibiotic resistance in bacteria was determined by chromosomes or plasmids. The bacterial cells had the ability to move the genes horizontally, which could occur in three ways, namely plasmid, phage, and transformation in which bacteria engulf the free DNA [28]. The plasmids as extra-chromosomal DNA could independently replicate, played an important role in resistance to various types of antibiotics, and spread the antibiotic-resistant genes [29]. This poses a serious problem because plasmids could cross the boundaries of various species and genera, thus allowing resistance to spread and persist in organisms not subject to antibiotics [30].

In an interesting finding, it was concluded that the increased resistant bacteria number in the human body was feared to result in the extension of the application of antibiotic therapy [31]. If antibiotic therapy took a long time, the costs involved were also not small in handling this resistance problem. Thus, finding new antibacterial drug candidates provided alternative therapies, especially multidrug-resistant of *E. coli.*

Based on Table 3, it could be explained that five bacterial isolates had the potential to inhibit the bacteria growth that was included in the group of Gram-positive bacteria. Then, the Gram-positive bacteria (Figure 2) could bind to the first crystal violet dye applied to the cells. These cells bound the dye without fading after being washed by the alcohol. So that the second dye was no longer bound by the cell. This was related to differences in the cell wall structure owned by bacteria, which consisted of a thick peptidoglycan structure for Gram-positive bacteria.

The Gram stain made the cells possible to see the shape clearly under a microscope. In Table 3, it could be seen that all the cells observed were cocci (round) in shape. The five Gram-positive cocci bacteria isolates were continued with more specific identification tests to indicated the bacterial genus.

Table 3. The identification result of endophytes bacteria from fruit flesh of Arecaceae family

No.	Isolate code	Gram Types	Cell Formation	Catalase	Koagulase	Genus
1	A1	Positive	coccus	+	-	*Staphylococcus* sp. 1
2	A2	Positive	coccus	+	-	*Staphylococcus* sp. 2
3	K	Positive	coccus	+	-	*Staphylococcus* sp. 3
4	S1	Positive	coccus	+	-	*Staphylococcus* sp. 4
5	S2	Positive	coccus	+	-	*Staphylococcus* sp. 5

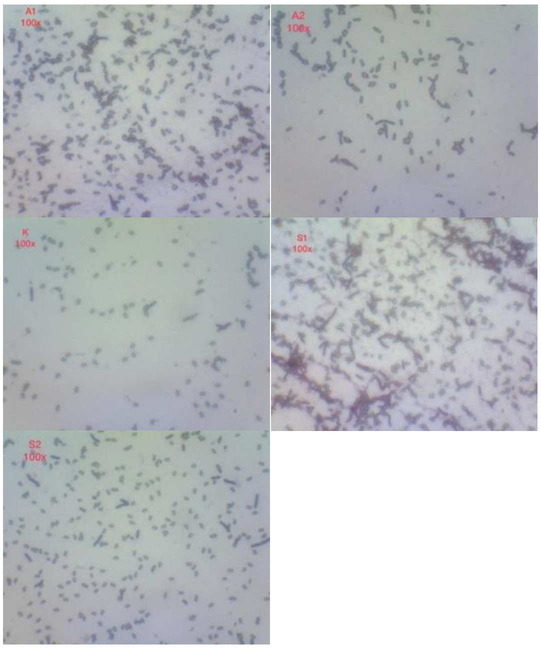

Figure 2. The endophytes morphology of cell a) A1 isolate; b) A2 isolate; c) K isolate; d) S1 isolate, and e) S2 isolate in zoom using microscope 100x

The catalase test results showed that each isolate was able to produce O_2 gas in the tube after the inactivation of enzymes in the cells. While the coagulase test showed a negative result that indicated a tendency that these bacteria were normal flora of fruit flesh and not a group of pathogenic bacteria. For instance, the non-pathogenic *Staphylococcus* groups were *S. saprophyticus* and *S. albus* [32]. Moreover, the bacteria belonging to the *Staphylococcu*s genus had the following characteristics: live singly, in pairs or irregular groups, cell diameter 0.5-1.5, Gram-positive, non-motile, and did not form spores. The colonies were white, cream, and some were even yellow or orange. Generally, the catalase was positive, and oxidases was negative [33].

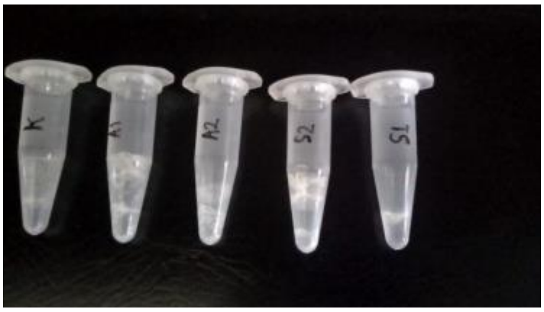

Figure 3. The result of positive catalase

Based on some characteristics of the identification results above, the final assumption was that the genus of Gram-positive cocci isolated by endophytes from the fruit flesh of the Arecaceae family was *Staphylococcus* sp. These bacteria tend to be present as endophytes in the three pulp samples used, namely Aren, Coconut, and Sago, as normal flora. This study was in line with other studies, which found that one of the endophytic bacteria in rambutan fruit flesh was the genus that belongs to *Staphylococcus* [34].

In addition, another study found *S. epidermidis* as one of the endophytic bacterial species in clove flowers (*Syzygium aromaticum* L.). In this case, the negative coagulase properties mean that the bacteria were not pathogenic but rather the normal flora in the area [35].

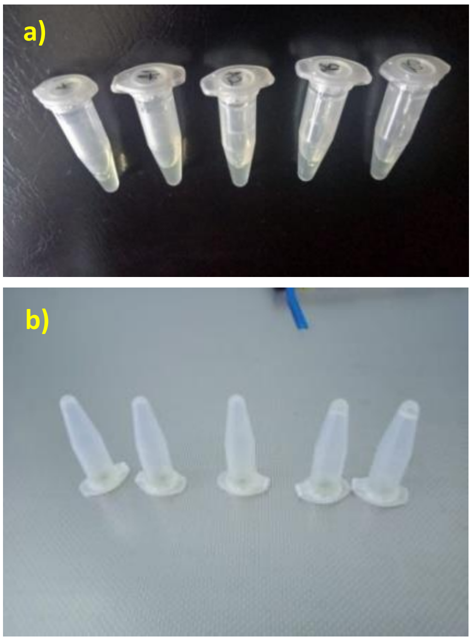

Figure 4. a). The process of coagulase, negative result; b). the process of the Eppendorf tube that was reversed to confirm the presence/no clumping

CONCLUSION

There were five isolates of endophytic bacteria found. The antibacterial activity of endophytic bacteria in the flesh of Aren, Coconut, and Rumbia on the inhibition criteria was very strong against multidrug-resistant *E. coli*. The largest inhibition zone was K isolate from Coconut of 43.26 mm, and the lowest was A1 isolate from Aren fruit pulp of 24.73 mm. The five selected bacteria were closely related to the *Staphylococcus* genus. Therefore, the findings of this study could be recommended as a new antibacterial drug against multidrug-resistant *E. coli*.

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