Exploration study of indigenous civet (*Paradoxorus hermaphroditus*) bacteria: isolates characterization and molecular identification

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ABSTRACT

Biochemistry of civet coffee fermentation in the digestive tract of civets until now is not fully known. This study aims to isolation, characterization and identification of bacteria molecularly from the civet's digestive tract. Analysis of bacteria begins with the civet surgery to obtain fluid from three parts of the civet's digestive tract. Isolation of bacteria conducted by pour plate method using nutrient agar media. Single and different colonies (shape, colour, margin, elevation, size) were separated and purified by streaking method. The pure isolate was further characterized biochemically. The biochemical characteristics of the bacteria observed were Gram staining, catalase, protease and pectinase activity. Identification of the bacteria was carried out molecularly by identifying the 16S rRNA gene. The results of molecular identification showed that the bacteria from the civet were identified as Alcaligenes faecalis strain LHW 1749, Ochrobactrum anthropi, Stenotrophomonas sp. 262 and Bacillus sp.

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INTRODUCTION

Kopi Luwak or civet coffee is one of the rare and most expensive coffees in the world. This is due to its unique production process and its special taste. Civet coffee is produced from coffee that has been eaten by the civet animal (*Paradoxorus hermaphroditus)* and then released back when the civet poops. The coffee that comes out with civet droppings is still intact but without the cherry skin. The coffee is then cleaned, washed, dried in the sun, separated from the husk and roasted like regular coffee. Coffee experts consider that there are two reasons why civet coffee has such a special taste: the selection of the civets and their digestion. The civets reliably select the best coffee cherries naturally, that is, the coffee beans they consume are of high quality. In addition, in the civet's digestive process, coffee beans absorb acids and enzymes in their digestion. Then there is fermentation, which gives civet coffee its distinctive taste (Smith, 2014; Muzaifa and Hasni, 2016; Muzaifa et al., 2019).

With exorbitant prices, there are those who are looking for ways to produce civet coffee at low prices. Civet coffee farms have sprung up in Southeast Asia, including Indonesia, and have drawn protests from animal rights activists. The civet is locked in a cage so that it is not free, and forced to eat coffee cherries. Due to such an environment, civet farming has a high mortality rate, and the civets are malnourished because they shouldn't just eat coffee cherries. The civet became sick frequently, and their fur fell out. The increasing demand for civet coffee around the world means an increase in civet farming. Coupled with deforestation, the civet population is decreasing and decreasing, even endangered. The business of 'forced farming' of civet is also believed to be counter-productive. There is a difference between coffee beans produced by wild civets and farmed civets. Natural selection does not occur, because the civet are forced to eat. Unhealthy civets don't produce the same enzymes as healthy civets, so the taste of coffee has changed (Keningar, 2016).

Artificial civet coffee production by creating coffee that has a taste similar to civet coffee is a possible opportunity to do. This can anticipate the market segment of coffee enthusiasts who want to experience the unique taste of civet coffee but feel it is too expensive to buy natural civet coffee. This at the same time can help solve problems in the production of civet coffee (Sucipto, 2010). According to Marcone (2004), the natural fermentation process in the civet stomach changes the chemical composition of coffee beans and can improve coffee quality. This is thought to occur because in addition to being at the optimal temperature, enzymes and microorganisms found in the civet digestive tract also help ferment so that the taste and aroma of civet coffee becomes very special. However, until now the biochemistry of civet coffee fermentation in the digestive tract of civets is not fully known.

Rahmawati (2017) has isolated and conventionally identified proteolytic bacteria from civet feces, these bacteria are *Bacillus* and *Proteus*. While Rahayu et al (2018) identified 9 genera of bacteria originating from civet feces from zoos, namely *Xylophilus, Caryophanon, Aeromicrobium, Exiguobacterium, Brochotrix, Alcaligenes, Alteromonas, Halomonas, Chromobacterium, Corynebacterium* and *Cellulomonas*. All of the above identifications are conventional identifications (phenotypic methods). Molecular identification of bacteria (genotypic method) from the civet's digestive tract is still rarely done.

Phenotypic methods were widely used before the development of molecular biology. This method characterizes microorganisms based on their morphological and physiological properties. The weakness of this method is related to the level of reproducibility which gives different results when repeated so that it is considered less reliable. In this method, microorganisms are characterized based on gene expression products that are highly sensitive to various environmental conditions (Pangastuti, 2006; Prakash et al., 2007). Another disadvantage of the phenotypic method is that it requires a long identification time, special equipment and expertise, highly dependent on pure culture, so that some microorganisms cannot be cultured (Woo et al., 2008; Rinanda, 2011; Buszewski et al., 2017).

Bacteria and yeast from digestive tract of civet have identified by Muzaifa et al (2019b) and Rasdiansyah and Muzaifa (2019), but molecular identification has not been carried out. Molecular biology method is currently recognized as the most accurate method and is the gold standard in identifying microorganisms to the species level with high sensitivity and reproducibility (Buszewski et al., 2017). This study aims to

identify molecularly bacteria from the civet's digestive tract.

METHOD

Sample Preparation

Analysis of bacteria originating from the civet's digestive tract begins with the civet surgery carried out by the veterinary surgery team. This surgery to obtain fluid from three parts of the civet's digestive tract consisting of the stomach, small intestine and large intestine refers to the procedure of Tilley and Smith (2005). Prior to surgery, the civet was fasted for 8 hours and injected subcutaneously with atropine sulfate as a premedicated drug at a dose of 0.02 mg/kg BW. After ten minutes given general anesthesia ketamine 10 mg/kg body weight and xilazine 1 mg/kg body weight intramuscularly. Next, an incision is made to remove the digestive tract organs. Into the stomach, physiological NaCl was added, homogenized and aspirated again using a 5 ml syringe. This liquid is put into a test tube containing a peptone solution. Further collection of fluid is carried out in the small intestine and large intestine in the same way as in the stomach. The dissected digestive tract is then returned to the abdominal cavity and sutured. The area around the operation was cleaned with 3% iodine tincture and smeared with gentamicin. Fluid collection from each part of the gastrointestinal tract was then incubated for 24 hours at 37°C.

The use of civet in this study was approved by the veterinary ethics committee of the Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh (Ref: 58/KEPH/6/2020).

Isolation and characterization

The collection of fluid from each part of the gastrointestinal tract that has been incubated is taken 1 ml and serial dilutions are carried out. The results of the selected dilution were cultured as much as 1 ml into each petri dish with Nutrient agar media, with the pour plate method and incubated at 37°C for 24 hours. Single and different colonies (shape, colour, margin, elevation, size) were separated and purified by streaking method. The pure isolate was further characterized biochemically. The characteristics of the bacteria observed were Gram staining and test of catalase production (Bell et al., 2005), protease and pectinase activity (Nespolo and Brandelli, 201; Janani et al., 2011).

Molecular identification

Identification of bacterial isolates was carried out molecularly by identifying the 16S rRNA gene. Identification with molecular techniques was chosen because it has several advantages, namely more precise, accurate and can identify all microorganisms (Ercolini, 2004; Nuroniyah and Putra, 2012). The identification stages consisted of isolation of the chromosomal DNA of each isolate, DNA amplification by PCR (Polymerase Chain Reaction), agarose gel electrophoresis from PCR results, 16S rRNA gene sequencing and construction of phylogenetic trees. DNA isolation and PCR amplification were performed simultaneously using a direct PCR Kit (KOD FX Neo, Toyobo) following the company's protocol. The primer used is a universal primer. Primer F:16F27 (AGA GTT TGA TCM TGC CTC AG) and primer R:16R1492 (TAC GGY TAC CTT GTT ACG ACT T). The sequencing process was sent to PT Genetics Science (Jakarta). The data from the sequences were then edited in BLAST with genomic data that had been registered with the National Center for Biotechnology Information (NCBI) to determine which species had the greatest homology or similarity and molecularly closest (NCBI, 2016).

RESULT AND DISCUSSIONS

A total of 5 pure and different isolates were obtained at the isolation stage. The basis for the selection was done by observing the morphological characteristics of the colonies that grew on each petri dish (NA medium). Single and different colonies were isolated and purified by streaking method. The five isolates were characterized morphologically and biochemically with the results shown in Table 1.

Colony morphology in bacteria can vary due to differential gene expression. Different types of bacteria will produce different-looking colonies, some colonies may be coloured, some colonies circular in shape and others are irregular (ATCC, 2015). Each bacterial colony has a distinctive shape, size, edge, texture, opacity and color (Christopher and Bruno, 2003). In this study, two different forms of colonies were obtained, circular and irregular. Only ICMM8 2779 isolate had an irregular shape. Based on the appearance of the margins, two different margins were obtained, undulate and entire. The colony elevation obtained was in two forms, raised and convex, while the colony size was small and moderate. Bacterial

colonies grow from single cells and consist of millions of cells. The appearance of different colonies growing on the same medium was assumed to come from different bacterial species. However, due to the large number of bacterial species that share the same colony morphology, conditions are sometimes not always true. Thus the parameters observed in colonies are only needed for the initial identification of bacterial species (Christopher and Bruno, 2003; Kshikhundo and Itumhelo, 2016).

Further analysis of the isolate cells showed that the bacterial isolates obtained were cocococobacilli and bacilli. In recent years, shape of bacteria cell has shown to play a critical role in regulating the important bacteria functions of dispersal, attachment, motility, polar differentiation, predation and cellular differentiation (Cabeen and Jacobs-Wagner, 2005).

Based on Table 1, the result of Gram staining analysis showed that all bacterial isolates were Gram-negative group, except isolate 4011. The differentiation of bacteria into either Gram-positive and Gram-negative group is fundamental to most bacterial identification systems. Gram staining is a differential staining procedure that can distinguish the types of bacteria based on the reactions that occur in the cell wall structure during the staining procedure. Gram-positive bacteria are bacteria that retain crystal violet dye during the Gram staining process so that it will turn blue or purple under a microscope. On the other hand, Gram-negative bacteria will be red. This difference is due to differences in the structure of their cell wall and cell membrane permeability. The peptidoglycan layer found in the cell wall layer of Gram-positive bacteria is thicker (50-90% of cell wall) than that of gram-negative bacteria (10% of cell wall) (Thairu et al., 2014). Gram-negative and positive bacteria are commonly found in the gastrointestinal tract. Gram positive bacteria are included in the phylum Firmicutes while Gram negative are the phylum Bacteroides (Ley et al., 2008). Isolated Gram-negative bacteria were more dominant in this study. It is suspected that the media used is suitable for the growth of Gram-negative bacteria.

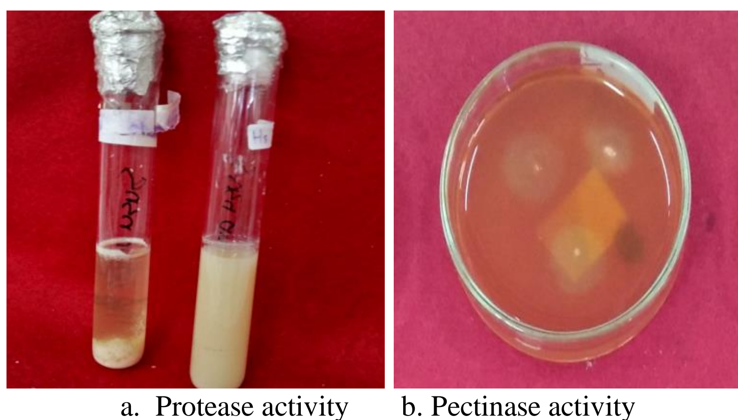
Table 1 Morphological and biochemical characteristics of isolates

Characteristics		Isolate code				
		4011	2779	2780	2781	2782
Morphological	Shape	Circular	Irregular	Circular	Circular	Circular
- Colony	Color	Yellowish white	Yellowish white	Yellowish white	Light yellow	Light yellow
-	Margin	Entire	Undulate	Entire	Entire	Entire
	Elevation	Raised	Raised	Raised	Convex	convex
	Size	Moderate	Moderate	Small	Small	Small
- Cell	Shape	Bacilli	Cocobacilli	Bacilli	Bacilli	Bacilli
Biochemical	Gram	+	-	-	-	-
	Catalase	+	+	+	+	+
	Protease	+	+	-	+	-
	Pectinase	+	-	+	-	+

a (positive)

b (negative)

Figure 1 Catalase activity



a. Protease activity b. Pectinase activity

Figure 2 Protease and pectinase activity

Table 2 Molecular identification of bactria from civet

Isolate	Accesion number (NCBI)	Homology	Similarity
ICMM5 4011	MN945364	*Bacillus* sp	100.00%
ICMM8 2779	MN945406	*Alcagelines faecalis* strain HX2016003	100.00%
ICMM9 2780	MN945430	*Ochrobactrum anthropi*	100.00%
ICMM10 2781	MN967318	*Stenotrophomas* sp. 262	100.00%
ICMM11 2782	MN960316	*Stenotrophomas* sp. 262	99.91%

Enzyme-based tests play a crucial part in the identification of bacteria. Catalase test aims to determine the ability of bacteria to degrade hydrogen peroxide through enzyme production catalase. The reaction can be proven by the rapid formation of bubbles (MacFaddin, 2000; Reiner, 2016). All isolates in this study had catalase activity. The catalase activity was indicated by the appearance of bubbles as shown in Figure 1a.

The ability of bacteria to degrade pectin and protein showed different results. Isolates ICMM8 2779 and ICMM10 2781 had the ability to degrade proteins, while isolates ICMM9 2780 and ICMM11 2782 were only able to degrade pectin. The specific activity of enzymes is important to know because the activity of these enzymes can produce certain compounds. The activity of these enzymes can produce certain compounds. Bacteria that are able to break down pectin or protein, apart from being able to decompose sugar, are also able to utilize pectin or protein as a substrate (Nespolo and Brandelli, 2010; Cheriguene et al., 2006; Moulay et al., 2006). Pectin decomposition will produce a number of simple sugar compounds while the results of protein decomposition are a number of peptides (Benen et al., 2000; Farah, 2012; Anisa and Girish, 2014). The activity of protease and pectinase enzymes was indicated by the appearance of a clear zone as shown in Figure 2.

Table 2 showed the results of bacteria identification from the digestive tract of civet. Molecular bacterial results were obtained by homology to 16S rRNA sequences of other bacteria registered with the National Center for Biotechnology Information (NCBI). All isolates have been registered with NCBI and have obtained accession numbers. Based on Table 2, the identified bacteria consisted of *Bacillus sp, Alcaligenes faecalis* strain HX2016003, *Ochrobactrum anthropi* and *Stenotrophomonas sp*. 262 with a similarity of 99.91-100%. The variety of bacterial genera obtained in this study consisting of *Bacillus, Ochrobactrum*, *Alcaligenes* and *Stenotrophomonas*, looks less than the variation of bacterial genera that have been reported by Suhandono et al [35]. In this study, the genera obtained included *Enterobactericeae, Bacillus, Pseudomonas, Pantoea, Escherichia, Ochrobactrum* and *Kocuria*. The presence of these bacterial isolates spread in the stomach, small intestine and large intestine. *Enterobacteriaceae* is the dominant bacteria in the digestive tract of civet. The use of isolation media used is thought to affect the number of genera obtained. In the study, Suhandono et al (2016) used a different isolation

medium, Luria-Bertani (LB) broth media. LB media is a nutrient-rich medium consisting of 10 g tryptone, 5 g yeast extract and 10 g NaCl, with this composition certainly very supportive and accelerating the growth of many bacterial species (Sezonov et al., 2007).

The *Bacillus* strains obtained in this study was *Bacillus sp* similar to Suhandono et al (2016). *Ochrobactrum* was obtained in both studies while *Stenotrophomonas* was only obtained in this study. According to composition of the BLAST results, *Stenotrophomonas* has similarities with *Pseudomonas.* The bacterial isolates obtained in this study generally colonize the gastrointestinal tract of animals and humans, including *Alcaligenes*. This is in accordance with Obata et al (Obata et al., 2010) which states that *Alcaligenes* is one of the indigenous bacteria of the gastrointestinal tract.

Scientific information about bacteria isolated from the civet's digestive tract is still very limited. In several previous research results, researchers generally used civet feces as a source of civet bacteria with various identification results, including *Pantoea vagans* and *Enterobacter sacchari* (Hoang et al., 2015), Bacillus and Proteus (Rahmawati , 2017) and *Xylophilus, Caryophanon, Aeromicrobium, Exiguobacterium, Brochotrix, Alcaligenes, Alteromonas, Halomonas, Chromobacterium, Corynebacterium* and *Cellulomonas* (Rahayu et al., 2018)]. Based on the level of similarity that reaches 99-100%, it can be seen that there are no new species found in this study. Stackebrandt and Gobel (1994) stated that bacteria can be considered a new species if they have less than 97% homology of the 16S rRNA gene sequence. However, this result is the first study to report the presence of Stenotrophomonas bacteria in the digestive tract of civet. The presence of stenotropomas in the civet's digestive tract is not known whether it is a native (resident/ autochthonous) or transient (allochthonous) species.

CONCLUSION

A total of 5 pure and different isolates were obtained at the isolation stage of bacteria from the digestive tract of civet. They have various morphological and biochemical characteristics. The results of molecular identification showed that the bacteria from the civet were identified as *Alcaligenes faecalis* strain LHW 1749 (isolate 2779), *Ochrobactrum anthropic* (isolate 2781), *Stenotrophomonas* sp. 262 (isolates 2781 dan 2782) and *Bacillus* sp (isolate 4011) with a similarity of 99.91-100%. The presence of *Stenotrophomonas* bacteria in the digestive tract of civet is the first report.

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