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Characterization and Identification of Caffeine-Degrading Bacteria KAJ 36

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Abstract

Caffeine could be utilized by caffeine-degrading bacteria as a source of carbon and nitrogen. These bacteria have the potential as an agent of decaffeinating coffee. The objective of this research was to characterize and identify the caffeine-degrading bacteria KAJ 36 that was isolated from the pulp waste of *Coffea arabica*. The isolate-characterization was performed based on its growth and caffeine degradation activity.in a medium M9 added with 10 g/L caffeine for 7 days. The isolate identification was conducted using 16S rRNA sequence analysis and biochemical analysis. The result indicated that caffein-degrading bacteria were able to grow and degrade the caffein up to 86% activity on M9 medium added 10 g/L caffeine for 7 days incubation. Based on 16S rRNA, the isolated KAJ 36 had sequences of 99% homology with *Pseudomonas monteilii*. Biochemical analysis showed that this isolation on the specific medium was a gram-negative, oxidative, positive enzymatic activity (oxidase, catalase, urease) and reduce nitrate. Our results showed that the isolated KAJ 36 was capable of producing fluorescent pigment. This bacterium was unable to produce indole or utilize sucrose and lactose. Based on the cultural characteristics, we found that the isolated KAJ 36 could be grown in a medium with pH 5, 7, and 9, and in the environment with up to 37°C. This evidence suggested that *P. monteilii* KAJ 36 can be used as a potential degradation agent of caffeine and recommend for future research in the bacterial based on the degradation of caffeine-contained coffee.

Keywords: Caffeine, caffeine-degrading bacteria, coffee, *Pseudomonas monteilii*

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is alkaloid purine compound that belongs to the group of methylxanthine derivatives. Caffeine is abundant in nature and produced by several types of plants such as coffee (Gaascht *et al*., 2015), cacao, kola, quarana, and tea as a secondary metabolite (Heckman *et al*., 2010).

People consume coffee and tea for their daily drinks in social interaction. High caffeine is not recommendable for pregnant women and people suffering from ulcers, osteoporosis, high blood pressure, and high cholesterol. Consuming caffeine for more than 300 mg each day is possibly associated with increasing gestational age at birth (Hoeven, 2017). Maternal caffeine intake is associated with adverse birth outcomes (Chen *et al*., 2018).

Therefore, reducing the content of caffeine in raw materials (coffee or tea) before serving as a daily drink is essential for high-risk people to caffeine. One strategy for this purpose is to utilize bacterial agents for coffee decaffeinating.

Some microbes, including bacteria and fungi, can utilize caffeine. These microbes can utilize caffeine as a source of carbon and nitrogen and produce CO_2 and NH_3 as the final results (Nayak *et al*., 2012). In the process of utilization, the concentration of caffeine decrease (Arimurti *et al*., 2018). There are several caffeine degrading microbes i.e. *Leifsonia* sp. (Ibrahim *et al*., 2016), *Paraburkholderia caffeinitolerans* (Gao *et al*., 2016), *Pseudomonas monteilii* RKM 9 (Arimurti *et al*., 2018), *Pseudomonas putida* CT25 (Ma *et al*., 2018), *Fusarium solani* (Nanjundaiah *et al*., 2016), *Aureobasidium* sp. (Ashengroph, 2017).

The isolate KAJ 36 was indigenous caffeine-degrading bacteria from the natural fermented of pulp waste of *Coffea arabica* of PTPN XII in Jampit, Bondowoso (Arimurti, 2016). These bacteria can utilize caffeine as a source of carbon and nitrogen in M9 minimal medium containing 0.1% caffeine. These bacteria need characterization and identification to get information about these bacteria's potentials as the agent of decaffeinating coffee. To understand the type of isolation and potentials to grow in a medium that contains caffeine, these isolates need characterization and identification.

MATERIALS AND METHODS

The research was conducted at the Microbiology Laboratory and Biotechnology Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, University of Jember. The DNA sequencing was carried out at the 1st Base in Malaysia.

Bacterial Growth and Caffeine Degradation Activity

The isolate KAJ 36 was caffeine-degrading bacteria collected at the Microbiology Laboratory and Biotechnology Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, University of Jember. The bacteria was isolated from the naturally fermented pulp waste of *Coffee arabica* of PTPN XII in Jampit, Bondowoso.

The growth analysis was conducted by growing the KAJ36 isolate in a medium M9 ($Na_2HPO_4.12H_20$ 15 g/L, KH_2P0_4 3 g/L, NaCl 0.5 g/L, $MgSO_4$ 0.25 g/L, NH_4Cl 1 g/L, and bacto agar 15 g/L) (Arimurti *et al.*, 2018) added 10 g/L caffeine media and incubated for 7 days. The growth of cell population was measured by spectrophotometer at an absorbance of 600 nm every 24 hours. The conversion of an absorbance value to density bacteria (cell/mL) based on a standard curve that was made earlier. The standard curve is the relation between absorbance value (OD) and the total number of cells (cell/mL) measured with haemocytometer.

The caffeine degradation activity of bacteria was done by analyzing the concentration of caffeine in a medium every 24 hours for 7 days. The concentration of caffeine was analyzed by spectrophotometer at an absorbance of 273 nm (Arimurti *et al*., 2018). The standard curve of caffeine concentration was made at range 0.2-2.0 g/L.

Morphology Characteristics and Identification of Bacteria

Morphological study of the bacteria was carried out by characterizing cell colonies, cell forms, and gram bacteria types in a nutrient agar medium for 24 hours. Physiological-biochemical characters were obtained by testing various types of biochemical tests that were following gram bacteria based on Cowan &

Steel (1993), such enzymatic activity (oxidase, catalase, and urease), hydrolysis of starch, nitrate reduction, production of indole pyruvate, haemolysis, carbohydrates (sucrose, lactose, fructose, and glucose) utilization, citrate utilization, H_2S production, gelatin liquefaction, fluorescent pigment production, MacConkey test, oxidative-fermentative test, the ability of growth at different pH (3, 5, 7, 9) using the buffer system was examined on NB medium at 30 ºC for 48 hours, the ability of growth at different temperature (4, 30, 37, 45) ºC on NA medium and cell motility.

Identification of bateria based on 16S rRNA gene suquence analysis. The whole genome DNA of this isolate was extracted using the freezing and thawing method (Fitriyah *et al*., 2013). Amplification of the 16S rRNA encoder used a using primers 27f (5'-GAGAGTTTGA TCCTGGCTCAG-3') and 1495r (5'-CTAC GGCTACCTTGT TACGA-3') (Arimurti *et al*., 2017). DNA data sequencing was carried out by aligning DNA sequences encoding 16S rRNA and controlling the phylogenetic tree model using Bioedit and MEGA 6.06 software. The results were compared to the 16S rRNA coding gene in Gene Bank using BLAST online software at NCBI GeneBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

Growth and Caffeine Degradation Activity

Caffeine is a purine alkaloid compound composed of 2 cyclic carbon rings, 4 nitrogen atoms, and 3 methyl groups. This compound can be used as a source of carbon and nitrogen by microbes to metabolize for growth. The degradation activity of bacterial caffeine KAJ 36 on caffeine media with a high concentration of 10 g/L showed a high degradation rate. This rate was defined using the degradation percentage, which reached 86.74 ± 0.1 % at 168 hours (7 days), with the remaining caffeine amount found from 10 g/L to be 1.22 g/L. The number of cell population reached from 3.38 ± 0.47 x 10^8 cells/mL at 0 hours to 35.75 ± 1.8 x 10^8 cells/mL at 168 hours, at which point the cell population continued to increase (Figure 1). This indicated that the process of catabolism of caffeine as a source of nutrients continued to occur.

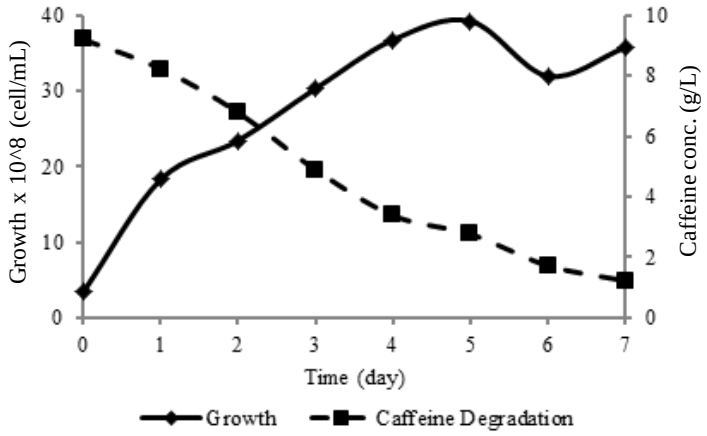
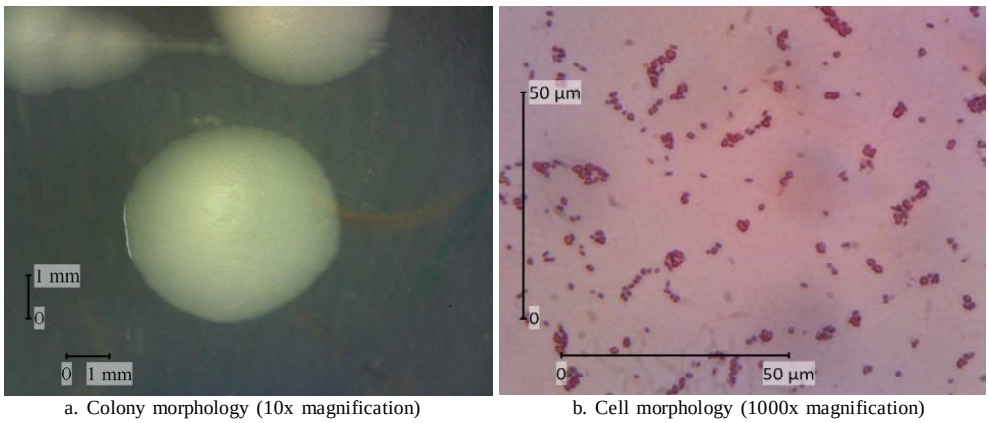


Figure 1. Growth and degradation patterns of caffeine by *P. monteilii* KAJ 36 in a medium M9 added 10 g/L caffeine for 7 days

Identification of Bacteria

Colony morphology was obtained from macroscopic characters or characteristics including the white bone colonies, a diameter of 4.72 mm at 48 hours, circular colony shape, low convex elevation, entire edge colony shape, shiny surface, and smooth/ smooth inner structure. Microscopic observation was carried out by Gram staining, and the results obtained from bacterial isolated KAJ 36 were gram-negative, bacilli, and motile (Figure 2 and Table 1).

The biochemical characteristics of isolated KAJ 36 bacteria include positive reactions to the enzymatic activity (oxidase, catalase, and urease), reduction of nitrate, glucose utilization, citrate utilization, a pigment in the form of fluorescent pigment, MacConkey test. KAJ 36 bacteria grow fertile at pH of 5, 7, and 9, also thrive at 30°C and 37°C, but at temperature of 4°C, the bacteria encounter a little growth. Based on the OF test indicated that this bacteria was oxidative. This bacteria indicated negative reactions to starch hydrolysis, production of indole pyruvate,



a. Colony morphology (10x magnification) b. Cell morphology (1000x magnification)

Figure 2. The morphology of colony and cell *P. monteilii* KAJ 36 on a medium nutrient agar for 24 hours incubation

Table 1. Characteristics of physiology-biochemistry of bacteria KAJ 36

Characteristic	Result	Characteristic	Result
Oxidase	+	Gelatin liquefaction	-
Catalase	+	Fluorescent pigment production	+
Urease	+	MacConkey test	+
Hydrolysis of starch	-	OF test	oxidative
Nitrate reduction	+	Growth at pH 3	-
Production of indole pyruvate	-	Growth at pH 5	+
Haemolysis	-	Growth at pH 7	+
Sucrose utilization	-	Growth at pH 9	+
Lactose utilization	-	Growth at 4°C	+
Fructose utilization	-	Growth at 30°C	+
Glucose utilization	+	Growth at 37°C	+
Citrate utilization	+	Growth at 45°C	-
H_2S production	-	Motile	+

*) + : positive; - : negative.

haemolysis, carbohydrates (sucrose, lactose, and fructose) utilization, H_2S production, gelatin liquefaction, and not growing at pH of 3 and temperature of 45°C (Table 1).

Bacterial identity was determined through molecular analysis of DNA sequences, specifically those that encode 16S rRNA. Results were compared with GenBank and showed similarities between *Pseudomonas plecoglossicida* NBRC 103162, *P. taiwanensis* BCRC 17751, and *P. monteilii* CIP 104883 with an identity value of 99% and 100% query cover. The isolated KAJ 36 has proximity to *Pseudomonas monteilii* CIP 10488 with a similarity value of 99.93% (Figure 3). The comparison of some biochemical characteristics of KAJ 36 with the type strain of *P. plecoglossicida* (Nishimori *et al*., 2000), *P. taiwanensis* (Wang *et al*., 2010), and *P. monteilii* (Elomari *et al.*, 1997) showed on Table 2. Based on the biochemical analysis, it indicated that the biochemical characteristic of KAJ 36 which was the same as *P. monteilii*. KAJ 36 also had catalase and urease activity was able to utilize glucose and citrate but not able to utilize sucrose, had fluorescent pigment, did not have starch hydrolysis capacity, an inability to produce indole, a lack of capability to lyse blood cells (not pathogens), could not melt gelatine, growing occurring at 10°C and not 45°C.

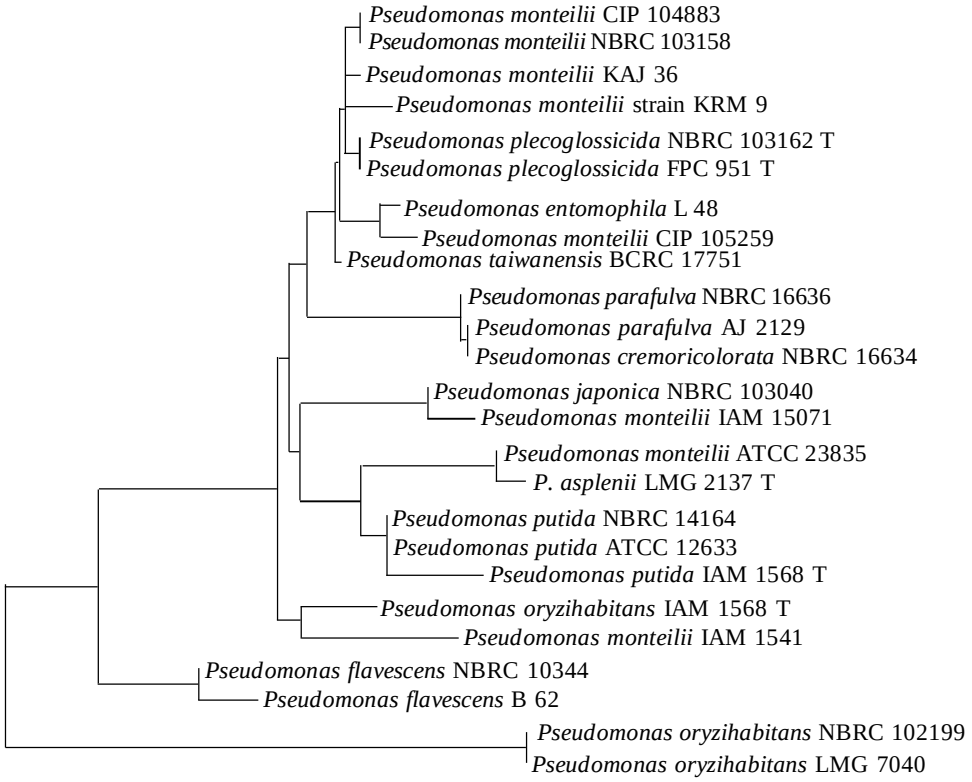


Figure 3. Tree of phylogeny between DNA sequences encoding 16S rRNA *P. monteilii* KAJ 36 and other bacteria using MEGA software with neighbor-joining tree method

Table 2. Comparison of between biochemical characteristics of *P. monteilii* KAJ 36 and other bacteria

Characteristics	*P. taiwanensis*[1]	*P. plecoglossicida* [2]	*P. monteilii*[3]	*P. m.* KAJ 36
Cell shape	Rod	Rod	Rod	Rod
Catalase	+	+	+	+
Urease	-	v	+	+
Hydrolysis of starch	v	-	-	-
Nitrate reduction	-	+	-	+
Production of indole pyruvate	-	v	-	-
Haemolysis	-	+	-	-
Sucrose utilization	v	-	-	-
Lactose utilization	v	-	v	-
Fructose utilization	+	+	+	-
Glucose utilization	+	+	+	+
Citrate utilization	v	+	+	+
Gelatin liquefaction	-	-	-	-
Fluorescent pigment production	+	+	+	+
Growth at 10 °C	+	-	+	+
Growth at 41 °C	+	-	-	-
Motile	+	+	+	+

+ : positive; - : negative; v : no data.
[1]) Wang *et al*, 2010
[2]) Nishimori *et al*., 2000
[3]) Elomari *et al*., 1997

P. monteilii isolated from the soil samples has antifungal properties, particularly through the production of volatile organic compounds (VOC). VOCs can inhibit the development of generative spores and *Fusarium oxysporum* hyphae (Dharni *et al*., 2014). Species *P. monteilii* first reported as caffeine degrading bacteria was KRM9 (NCBI number: KY. 319030.1) (Arimurti *et al*., 2018). These were isolated naturally fermented pulp waste of *C. canephora* and could degrade around 99.26 ± 0.01% for 24 hours in the medium M9 added 1 g/L caffeine.

Bacteria, *P. monteilii* KAJ 36, can degrade caffeine in laboratory scale by using caffeine as the source of carbon and nitrogen on a medium. There is also a need to conduct further study to know the potentials of these bacteria as the agent of decaffeinated coffee on the coffee fermentation process.

CONCLUSIONS

The bacteria KAJ 36 can utilize caffeine and have the caffeine degradation activity reached 86.74% for 7 days in the medium M9 added 10 g/L caffeine. These bacteria had the highest potential as caffeine degrading bacteria from the naturally fermented pulp waste of *Coffea arabica*. These bacteria were identified as *Pseudomonas monteilii*.

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