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Isolation and identification of caffeine-degrading bacteria from soil, coffee pulp waste and excreted coffee bean in Luwak feces

TOTO ISWANTO[1,3], MAYA SHOVITRI[2], ALI ALTWAY[3], TRI WIDJAJA[1,♥], DINIHARI INDAH KUSUMAWATI[4], PUSPITA LISDIYANTI[4]

[1]Biochemical Technology Laboratory, Department of Chemical Engineering, Faculty of Industrial Technology, Institut Teknologi Sepuluh Nopember. Jl. Raya ITS, Sukolilo, Surabaya 60111, East Java, Indonesia. Tel. +62-31-5946240, ♥email: triw@chem-eng.its.ac.id
[2]Microbiology and Biotechnology Laboratory, Department of Biology, Faculty of Science, Institut Teknologi Sepuluh Nopember. Jl. Raya ITS, Sukolilo, Surabaya 60111, East Java, Indonesia
[3]Heat and Mass Transport Laboratory, Department of Chemical Engineering, Faculty of Industrial Technology, Institut Teknologi Sepuluh Nopember, Jl. Raya ITS, Sukolilo, Surabaya 60111, East Java, Indonesia
[4]Research Center for Biotechnology, Indonesian Institute of Sciences. Jl. Raya Bogor Km 46, Cibinong, Bogor 16911, West Java, Indonesia

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Abstract. *Iswanto T, Shovitri M, Altway A, Widjaja T, Kusumawati DI, Lisdiyanti P. 2019. Isolation and identification of caffeine-degrading bacteria from soil, coffee pulp waste and excreted coffee bean in Luwak feces. Biodiversitas 20: 1580-1587.* The present study deals with the isolation and identification of caffeine-degrading bacteria obtained from the caffeine contaminated environment or caffeinated wastes. These bacteria are useful for various biotechnological applications especially in increasing the potential utilization of caffeinated wastes and producing the high-value chemicals. The suspected caffeine-degrading bacteria have been isolated from the soil of coffee plantation area, coffee pulp waste, and the excreted coffee bean in fresh feces of *Luwak* (*Paradoxurus hermaphroditus* or Asian Palm Civet) by growing them on the caffeinated agar medium (CAM) containing basal salt medium (M9) and caffeine as a sole source of carbon and nitrogen. CAM-supplemented with 1.5 to 10 g L^{-1} of caffeine has been used for screening of the potential bacteria which able to grow in high caffeine concentration. Molecular identification based on 16S rRNA gene sequence was performed to identify the selected bacteria. The result revealed that there were 11 and 3 strains of 12 selected bacteria which could grow on the CAM-supplemented with caffeine up to 7 and 10 g L^{-1}, respectively. Based on 16S rRNA gene sequence and phylogenetic analysis, those bacteria were from 5 Gram-negative species, namely *Pseudomonas japonica* (4/12), *Methylobacterium populi* (5/12), *Raoultella ornithinolytica* (1/12), *Klebsiella quasipneumoniae* (1/12), and *Stenotrophomonas chelatiphaga* (1/12). Further investigations to determine their metabolic pathway, enzyme, and growth kinetics in the caffeinated medium may provide insights into its possible utilization for scientific or other applications.

Keywords: 16S rRNA gene sequence, bacteria, caffeine, isolation, identification, phylogenetic analysis

INTRODUCTION

Indonesia is the top fourth largest coffee-producing country in the world after Brazil, Vietnam, and Colombia (Szenthe 2018). However, the presence of caffeine in its residual products such as coffee leaves, pulp, and husk have become the main limiting factor in its utilization, because of its role as anti-nutritional factor and inhibitor of microbial growth during the biological processes (Raj and Dhala 1965; Gokulakrishnan et al. 2005). Caffeine was regarded as toxic to microorganisms until early 1970 when Kurtzman Jr. and Schwimmer (1971) found that there were specific bacteria and fungi which were able to grow in the caffeinated medium. Since that, a number of developments have been carried out on utilizing caffeine as a sole source of carbon and nitrogen for isolation of new strain of caffeine-degrading bacteria or fungi.

In the last few years, the use of caffeine-degrading bacteria is a great demand as it is more advantageous than physical and chemical treatments which require special materials and expensive equipment (Gokulakrishnan and Gummadi 2006). That bacteria can be utilized for several biotechnological applications such as environmental remediation by reducing the caffeine level which is released into soil and water from the caffeinated wastes such as coffee pulp and husks and increase the biotechnological potential of these carbohydrate- and protein-rich wastes for animal feed, mushroom cultivation, and production of biogas, ethanol, enzymes and organic acids (Pandey et al. 2000; Dash and Gummadi 2006a). Moreover, some bacterial strains can produce high-value chemicals which resulted as metabolite products of caffeine degradation for pharmaceutical preparations, cosmetic, and human and animal nutrition such as methylxanthine, uric acid, methyluric acids, and 8-oxomethylxanthines (Mohanty 2013; Summers et al. 2015).

Due to those advantages, various efforts to find new bacterial strains have been conducted. Caffeine-degrading bacteria can be isolated from the caffeine contaminated environment or caffeinated waste (from tea and coffee) which was easily obtained in Indonesia. However, the reports of this study in Indonesia are still rarely found. To the best of our knowledge, there is only one report which successfully isolated and identified 14 caffeine-degrading bacteria from coffee pulp waste in Malangsari and Jampit coffee plantation, Indonesia. One of the bacteria was

Pseudomonas monteilii KRM9 which was reported to have the highest caffeine degradation activity (Arimurti et al. 2017). Since Indonesia is a country with high biodiversity in the world (Butler 2016), its bacterial diversity was still interesting to be explored. Therefore, the aims of this study were to isolate and identify the potential bacteria from the various sources in Indonesia such as coffee pulp waste, soil from coffee plantation area, and excreted coffee bean in fresh feces of Asian Palm Civet which can grow in growth medium supplemented with high caffeine concentration. The identities of isolated bacteria were established via analysis of the 16S rRNA gene sequence and phylogeny.

MATERIALS AND METHODS

Bacterial isolation and purification

Samples of coffee pulp wastes, soil from the coffee plantation area, and excreted coffee bean in fresh feces of *Luwak* (*Paradoxurus hermaphroditus* or Asian Palm Civet) were collected from Situbondo, Dampit, and Kediri in East Java, Indonesia.

The isolation of caffeine-degrading bacteria was conducted using methods described by Babu et al. (2005) using an enriched-caffeine liquid medium (CLM). It was prepared by dissolving basal salt medium (M9) which consisted of 6.4 g of $Na_2HPO_4.7H_2O$, 1.5 g of KH_2PO_4, 0.25 g of NaCl, and 0.5 g of NH_4Cl into 1 L of distilled water (Sambrook et al. 1989) and supplemented with 0.3 g of caffeine as a sole source of carbon and nitrogen. The pH of CLM was adjusted to 7.2 using 0.5 N NaOH.

For enrichment culture, 1 g of each sample was introduced into a 250 mL Erlenmeyer flask containing 100 mL of CLM. All cultures were incubated in a benchtop incubator shaker at 32ºC and 120 rpm for 72 h. One mL of liquid from each culture was serially diluted using sterile distilled water from 10^{-1} to 10^{-7} folds. Then, 0.1 mL of an aliquot of 10^{-5} to 10^{-7} was spread on caffeinated agar medium (CAM) with 0.3 g L^{-1} of caffeine and incubated at 32ºC for 72 h. A single colony which has distinct shape and color grown on CAM were further purified on the fresh CAM until a pure culture was obtained. In these steps, CAM was made by addition of 18% of pure agar to CLM as same as the enrichment medium and poured it into the sterile petri dishes.

Screening of the potential isolates

The screening was performed by streaking the pure cultures on CAM with higher caffeine concentration stepwise from 1.5, 3, 5, 7, and 10 g L^{-1} to figure out their growing ability on the higher caffeine concentration.

In brief, every single colony was transferred to the fresh CAM containing 1.5 g L^{-1} of caffeine and incubated at 32ºC for 7 days. The growing bacterial colony was then transferred again into fresh CAM with 3 g L^{-1} of caffeine concentration and incubated again at the same condition. These steps were repeated until caffeine concentration in CAM was 10 g L^{-1}. The isolates capable of growing on CAM with high caffeine concentration were selected for molecular identification analysis.

Molecular identification of the potential isolates

Identification of selected isolates was conducted using colony PCR based on 16S rRNA gene**.** An overnight single colony of each isolate grown on nutrient agar for a day was picked up into a sterile PCR tube and heated in a microwave for 2 minutes. This process could extract DNA from the cell. The 15 µL of sterile nuclease-free water was put in that tube and vortexed for a moment until a homogeneous mixture, called DNA template. Its quality and quantity were examined by BioSpec-nano micro-volume UV-Vis spectrophotometer (Shimadzu).

For the DNA amplification, the PCR reagent was prepared following the recommendation of GoTaq® Green Master Mix protocol (Promega Corporation, USA) with universal eubacterial primers 27F (5'-AGAGTTTGATCC TGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTAC GACTT-3'). The PCR reagent was incubated in a PCR machine to generate amplicons of approximately 1500 base pairs (bp) in size, as the listed conditions in Table 1.

After the PCR amplification, the amplicons were confirmed using 1% agarose gel electrophoresis in TEA buffer pH 8.0 (40 mM Tris-Acetate, 1 mM EDTA) solution at 100 Volt for 20 min and stained with ethidium bromide solution and observed under UV transilluminator. Further, the qualified amplicons were sequenced at the 1st BASE Laboratories in Malaysia.

The obtained 16S rRNA gene sequences have been analyzed using BioEdit program (Hall 1999) and initially identified using EzBioCloud's Identify service in EzBioCloud website: https://www.ezbiocloud.net (Yoon et al. 2017) to determine their similarity with validly published taxa name and type strains. All identified isolates and their 16S rRNA gene sequences were deposited in Indonesian Culture Collection (InaCC) and the GenBank of National Center for Biotechnology Information (NCBI), respectively. Their designation code/strain name in InaCC and accession numbers were recorded in Table 3.

Phylogenetic analysis

Multiple alignments of all 16S rRNA gene sequences were performed using ClustalW included in the software of Molecular Evolutionary Genetics Analysis version X (MEGA-X) (Kumar et al. 2018). All valid reference sequence with the accession number of bacterial type strains were obtained from EzBioCloud, list of prokaryotic names with standing in nomenclature (LPSN), and NCBI website. The unrooted phylogenetic tree was constructed by MEGA-X using a neighbor-joining statistical method (Saitou and Nei 1987) and the Kimura 2-parameter model (Kimura 1980) to quantify sequence similarity among the strains. The complete deletion was chosen for gaps in data treatment. *Aquifex pyrophilus* strain KOL5A^T and *Hydrogenivirga okinawensis* strain LS12-2^T were used as out-group in constructing the phylogenetic tree.

Table 1. Operating conditions of the PCR amplification

Treatment	Temperature	Time
Pre denaturation	96°C	5 min
Subsequently followed by 35 cycles of;		
Denaturation	96°C	30 s
Annealing	55°C	30 s
Elongation	72°C	1 min
Extention	72°C	7 min
Stand-by	4°C	∞

RESULTS AND DISCUSSION

Caffeine-degrading bacteria can be isolated using a specific medium containing caffeine as a sole source of carbon and nitrogen. As the preliminary investigation, it can become an easy way before subjecting to the advanced identification of its enzyme or metabolites. Some studies have used caffeine in the range of 0.1-2.5 g L^{-1} as the initial concentration (Mazzafera et al. 1996; Babu et al. 2005; Gokulakrishnan and Gummadi 2006; Yu et al. 2008, 2009). The presence of 0.1% caffeine in the growth medium has been reported to inhibit protein synthesis (Ibrahim et al. 2014), while concentration above 2.5 mg mL^{-1} could inhibit the growth of many bacterial species (Gokulakrishnan et al. 2005). In addition, caffeine could induce mutagenic effects which inhibit ultraviolet wound repair and DNA synthesis in *Escherichia coli* (Sandlie et al. 1980). In this study, we conducted isolation, screening, and identification of selected strains to utilize caffeine from 1.5 to 10% g L^{-1}.

Isolation and screening of caffeine-degrading bacteria

In this study, the bacterial colonies were isolated aerobically and tested to grow on CAM in the several concentrations of caffeine. In the initial process of isolation using CAM-supplemented with 0.3 g L^{-1} of caffeine, 62 colonies of bacteria were selected; 16 from each coffee pulp waste in Situbondo and Dampit, 5 from the soil of coffee plantation area in Situbondo, and 11, 5, and 9 from coffee bean in feces of *Luwak* which were obtained from Situbondo, Dampit, and Kediri, respectively. In the screening process, all selected isolates could grow on CAM-supplemented with 1.5 g L^{-1} of caffeine after 2-4 days incubation. However, only 12 isolates could grow on CAM contained 3 and 5 g L^{-1} of caffeine and less of which for caffeine concentration of 7 to 10 g L^{-1} after 3-7 days incubation, as shown in Figure 1 and Table 2.

Figure 1 shows the morphological appearance of the isolates. Using the naked eye, general shape and chromogenesis of isolates were determined. Isolates C9, C49, C52, and C57 have small colonies, creamy white to pale yellow, circular, flat, opaque and slightly spreading edge. Other characteristics; small colonies, pink pigment, opaque, convex, and round was observed in C20, C22, C25, C29, and C39. Isolate C28 has large and small colonies with a slightly undulate edge, white pale, and umbonate elevation. C32 has small colonies, pale yellow, flat, and opaque. On the other hand, C56A has small colonies with slightly irregular shape, white pale, and raised elevation. These isolates were chosen for the molecular identification analysis.

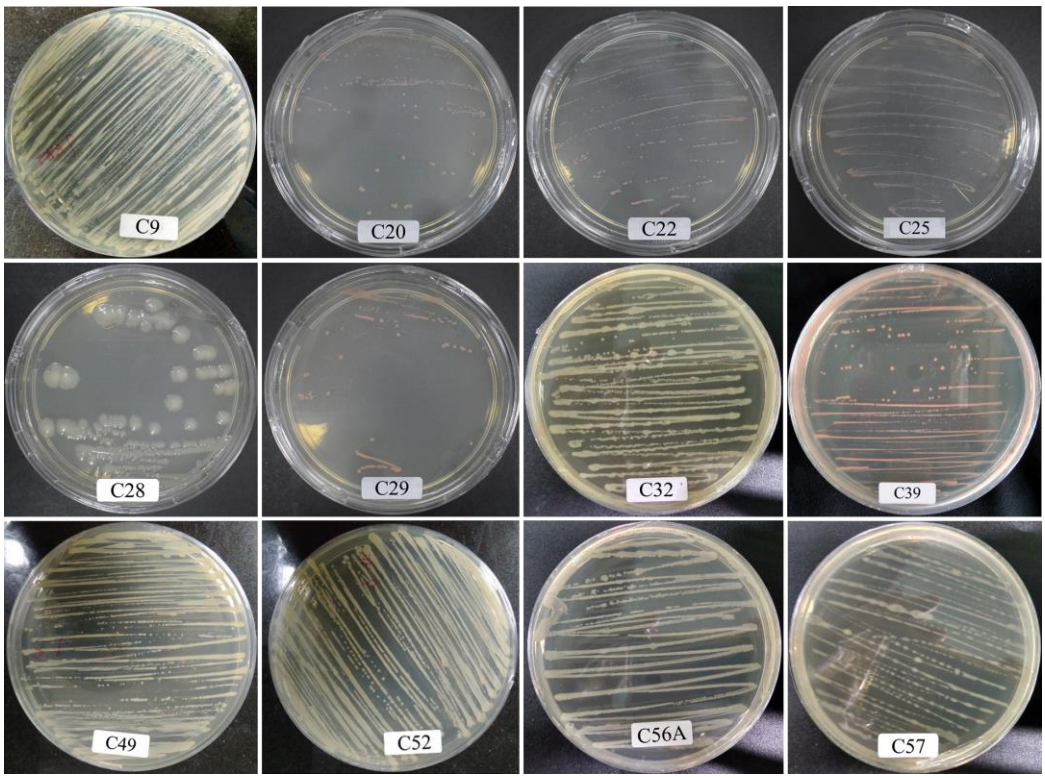

Figure 1. Twelve bacterial isolates which were able to grow on the caffeinated agar medium

Table 2. Isolated bacteria from the various sources in East Java, Indonesia

Sample	Designation	Ability to grow on CAM with initial caffeine concentration of				
		1.5 (g L^{-1})	3 (g L^{-1})	5 (g L^{-1})	7 (g L^{-1})	10 (g L^{-1})
Coffee pulp waste[1]	C39	+	+	+	+	-
Coffee pulp waste[2]	C9	+	+	+	+	-
Soil from coffee plantation area[1]	C20	+	+	+	+	-
Coffee bean in feces of *Luwak*[1]	C22	+	+	+	+	-
	C25	+	+	+	+	-
	C28	+	+	+	+	+
	C29	+	+	+	+	-
	C32	+	+	+	-	-
Coffee bean in feces of *Luwak*[2]	C49	+	+	+	+	-
	C52	+	+	+	+	-
Coffee bean in feces of *Luwak*[3]	C56A	+	+	+	+	+
	C57	+	+	+	+	+

Note: [1]: obtained from Situbondo; [2]: obtained from Dampit; [3]: obtained from Kediri; "+": growing; "-": not growing

The data in Table 2 indicates that the isolates have different ability to grow on CAM with various concentrations of caffeine. They have shown good potential due to their ability to tolerate caffeine which requires further investigation regarding their optimal growth condition and caffeine-degrading efficiency. In other studies, *Pseudomonas* sp. GSC 1182 had been tested to grow on various concentrations of caffeine ranging from 0.05 to 20 g L^{-1}. The result showed that its maximum growth rate was obtained in medium containing 2.5 g L^{-1} of initial caffeine concentration. That strain was able to completely consume caffeine within 48 h when the initial concentration was up to 4 g L^{-1} and only 50% of caffeine in 72 h when the initial substrate concentration was 5-10 g L^{-1} (Gokulakrishnan and Gummadi 2006). Yu et al. (2015) also reported that *Pseudomonas* sp. CES can degrade caffeine with the concentration of 9.0 g L^{-1}. Another one, *Pseudomonas* sp. NCIM 5235, isolated from the soil of coffee plantation area, was reported to degrade high concentration of caffeine (6.4 g L^{-1}) in 24 h (Dash and Gummadi 2007). It was maintained on caffeine associated sucrose (CAS) agar medium and subcultured once a week to prevent the loss of its caffeine-degrading ability. Under optimal growth condition in the bioreactor, this strain was found to degrade 100% caffeine with initial caffeine concentration of 20 g L^{-1} (Gummadi and Santhosh 2010). This was the best strain withstanding higher concentrations of caffeine reported so far.

Molecular identification based on 16S rRNA gene

Amplification process using the primers 27F and 1492R was aimed to get the complete 16S rRNA gene, the size around 1400-1500 bp, for yielding high similarity with the valid taxa name in the GenBank.

The size of all amplicons was analyzed and resulted in approximately 1,500 bp in the agarose gel, as shown in Figure 2. The exact number of base pairs would be confirmed after nucleotide sequencing. On the upstream and downstream region of sequence, several nucleotides was deleted due to the presence of an irregular peak in the chromatogram. Their final size was used for checking identification via EzBioCloud's Identify service (Table 3). The identification result revealed that some isolates were from the genus of *Klebsiella, Methylobacterium, Pseudomonas, Raoultella,* and *Stenotrophomonas.* All listed taxa in Table 4 are reported as Gram-negative and aerobic bacteria except *K. quasipneumoniae* and *R. ornithinolytica* which are a facultative anaerobe. The evolutionary mapping of isolates is shown in phylogenetic trees in Figure 3.

The phylogenetic tree based on the 16S rRNA gene sequences shows that strains C9, C49, C52, and C57 have an apparent relationship with bacteria belonging to the *Pseudomonas* assemblage and formed a stable phylogenetic position, close to the strain *P. japonica* NBRC 103040^T (BBIR01000146) with 76% bootstrap value and >98% similarity (Figure 3a and Table 3). Those 4 strains formed new cluster in the genus *Pseudomonas*. The position of strains C20, C22, C25, C29, and C39 showed close to *Methylobacterium populi* type strain BJ001^T (CP001029) with 99% bootstrap value, which was isolated from internal poplar tissues and able to utilize methane as carbon and energy (Aken et al. 2004). The sequence similarity of strain C32 was 98.25% close to strain belonging to species *S. chelatiphaga* DSM 21508^T (LDJK01000058), isolated from municipal sewage sludge and reported equivalent with *S. chelatiphaga* sp. nov. type strain LPM-5^T = VKM B-2486 = CCUG 57178 (Kaparullina et al. 2009).

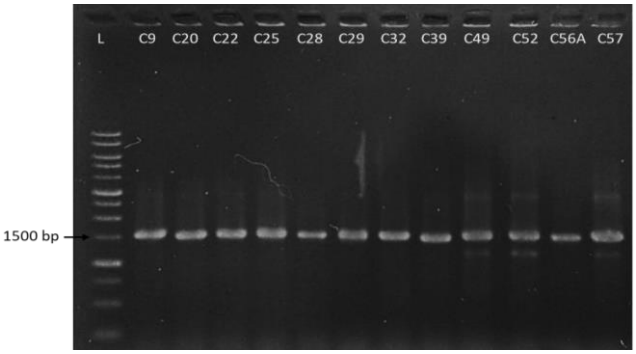

Figure 2. PCR products electrophoresed of the isolates on 1% agarose gel. The molecular size was approximately 1,500 bp. L is the DNA ladder with 1 kb DNA marker

Table 3. Results for the identification of isolates based on 16S rRNA gene sequence

Designation (strain code in InaCC)	Accession number	Top-hit taxon (Accession number)	Top-hit strain	Sequence similarity (%)	Sequence size (bp)
C9 (InaCC B1340)	MK336722	*Pseudomonas japonica* (BBIR01000146)	NBRC 103040^T	98.89	1423
C20 (InaCC B1341)	MK336723	*Methylobacterium populi* (CP001029)	BJ001^T	99.78	1368
C22 (InaCC B1342)	MK336724	*Methylobacterium populi* (CP001029)	BJ001^T	99.85	1367
C25 (InaCC B1343)	MK336725	*Methylobacterium populi* (CP001029)	BJ001^T	99.85	1367
C28 (InaCC B1344)	MK336726	*Raoultella ornithinolytica* (AJ251467)	JCM 6096^T	98.38	1422
C29 (InaCC B1345)	MK336727	*Methylobacterium populi* (CP001029)	BJ001^T	99.90	1020
C32 (InaCC B1346)	MK336728	*Stenotrophomonas chelatiphaga* (LDJK01000058)	DSM 21508^T	98.25	1430
C39 (InaCC B1347)	MK336729	*Methylobacterium populi* (CP001029)	BJ001^T	99.63	1355
C49 (InaCC B1348)	MK336730	*Pseudomonas japonica* (BBIR01000146)	NBRC 103040^T	98.00	1417
C52 (InaCC B1350)	MK336732	*Pseudomonas japonica* (BBIR01000146)	NBRC 103040^T	98.59	1419
C56A (InaCC B1359)	MK336733	*Klebsiella quasipneumoniae* (HG933296)	01A030^T	99.86	1418
C57 (InaCC B1351)	MK336734	*Pseudomonas japonica* (BBIR01000146)	NBRC 103040^T	98.80	1416

Table 4. The current reported caffeine-degrading bacteria

Name of isolate	Source of isolate	Region	Reference
Bacillus coagulans	Soil	Not reported	Kurtzman Jr. and Schwimmer (1971)
P. putida strain 40	Soil	California	Woolfolk (1975)
P. putida C1	Soil	Germany	Blecher and Lingens (1977)
P. putida C3024	Garden soil	Netherlands	Middelhoven and Bakker (1982)
P. putida WS	Soil	Germany	Glück and Lingens (1987)
Pseudomonas. sp. No. 6	Soil	Japan	Asano et al. (1993)
P. putida No. 352	Soil	Japan	Asano et al. (1993)
Serratia marcescens	Soil under coffee tree	Brazil	Mazzafera et al. (1996)
P. putida ATCC 700097	Wastewater streams	California	Ogunseitan (1996)
Klebsiella and *Rhodococcus*	Soil	India	Madyastha and Sridhar (1998)
P. putida (8 strains)	Soil under coffee tree	Brazil	Yamaoka-Yano and Mazzafera (1998)
P. fluorescens	Soil under coffee tree	Brazil	Yamaoka-Yano and Mazzafera (1998)
Coryneform (4 strains)	Soil under coffee tree	Brazil	Yamaoka-Yano and Mazzafera (1998)
Acinetobacter sp. (3 strains)	Soil under coffee tree	Brazil	Yamaoka-Yano and Mazzafera (1998)
Flavobacterium sp. (2 strains)	Soil under coffee tree	Brazil	Yamaoka-Yano and Mazzafera (1998)
Moraxella sp.	Soil under coffee tree	Brazil	Yamaoka-Yano and Mazzafera (1998)
P. putida IF-3 and its mutants	Soil	Japan	Yoshinao et al. (1996)
P. putida L	Soil under coffee tree	Brazil	Yamaoka-Yano and Mazzafera (1999)
P. putida KD6	Not reported	Not reported	Sideso et al. (2001)
P. alcaligenes CFR 1708	Soil of coffee plantation	India	Babu et al. (2005)
Alcaligenes fecalis T1	Not reported	India	Babu et al. (2005)
Acetobacter sp. T3	Not reported	India	Babu et al. (2005)
Alcaligenes sp.CF8	Surface water of lake	Canada	Mohapatra et al. (2006)
P. putida NCIM 5235	Soil of coffee plantation	India	Dash and Gummadi (2006b)
Pseudomonas sp. GSC 1182	Soil of coffee plantation	India	Gokulakrishnan and Gummadi (2006)
Pseudomonas sp. CBB1	Soil	Iowa	Yu et al. (2008)
P. putida CBB5	Soil	Iowa	Yu et al. (2009)
P. stutzeri Gr 21 ZF	Soil	Lebanon	Zakia et al. (2013)
P. pseudoalcaligenes TPS8	Soil of tea plantation	Iran	Ashengroph and Ababaf (2013)
Pseudomonas sp. CES	Coffee waste	Iowa	Yu et al. (2015)
P. monteilii KRM9	Coffee pulp waste	Indonesia	Arimurti et al. (2017)
P. putida CT25	Soil of tea garden	China	Ma et al. (2018)
P. japonica C9	Coffee pulp waste	Indonesia	In this study
P. japonica (3 strains)	Coffee bean in *Luwak* feces	Indonesia	In this study
M. populi C39	Coffee pulp waste	Indonesia	In this study
M. populi C20	Soil of coffee plantation	Indonesia	In this study
M. populi (3 strains)	Coffee bean in *Luwak* feces	Indonesia	In this study
K. quasipneumoniae	Coffee bean in *Luwak* feces	Indonesia	In this study
R. ornithinolytica C28	Coffee bean in *Luwak* feces	Indonesia	In this study
S. chelatiphaga C32	Coffee bean in *Luwak* feces	Indonesia	In this study

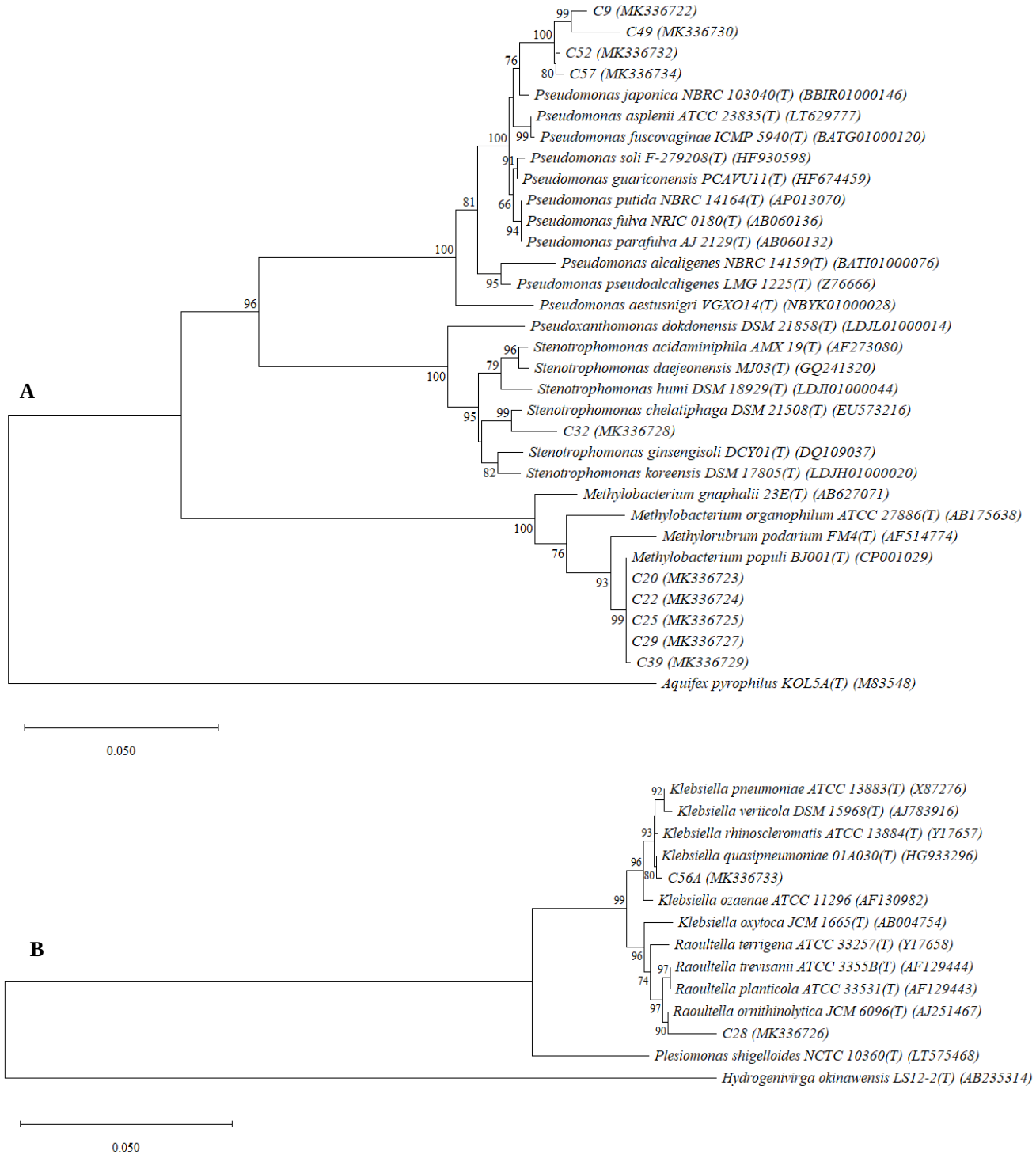

Figure 3. The phylogenetic tree based on the partial sequence of the 16S rRNA gene was constructed using the Neighbor-Joining method in MEGA-X with 1000 of bootstrap replicates that shows the evolutionary relationship between isolates and other species from GeneBank. The bootstrap value is shown at the branch indicates levels of confidence. Bootstrap values lower than 60% are not shown. The scale shows substitution on each nucleotide. (T): type of strain. The accession number for the 16S rRNA gene sequence is written after the strain name in parenthesis.

The strains C28 and C56A were identified from the cluster belonging to *Klebsiella* and obtained in the samples of coffee bean in *Luwak* feces from Situbondo and Kediri, respectively. This was not surprising because the previous study of Madyastha et al. (1999) has also found that a mixed culture of *Klebsiella* and *Rhodococcus* could utilize

and convert caffeine to 1,3,7-trimethyl uric acid. In the phylogenetic tree (see Figure 3b), strain C28 was placed in the cluster of *Raoultella* with 90% bootstrap value and 98.38% similarity, close to the type stain *R. ornithinolytica* JCM 6096^T (AJ251467) which has a basonym as *Klebsiella ornithinolytica* (Drancourt et al. 2001). The previous study has reported that *K. ornithinolytica* was confirmed from the same cluster with *K. planticola* due to the high sequence similarity which has a species-specific nucleotides within a 165-bp stretch of their 16S rDNA sequences (Boye and Hansen 2003). The sequence similarity and bootstrap of strain 56A were 99.86% and 80%, respectively, close to *K. quasipneumoniae* 01A030(T) (HG933296), isolated from human blood cultures by Brisse et al. (2014).

Some bacteria and fungi were reported capable of utilizing caffeine as a source of carbon and nitrogen. However, bio-decaffeination using bacteria was more commercially applicable due to its rapid growth without requiring a wide scope of space. Moreover, bacteria are able to grow at extreme temperature and pH and easy to genetically manipulate than fungi (Dash and Gummadi 2006a). Among the reported strains of caffeine-degrading bacteria, a few of which are *Pseudomonas* sp. that were isolated from the different environment and region, as shown in Table 4.

Caffeine catabolism by bacteria occurs through two pathways namely N-demethylation with the help of N-demethylase enzyme and C8 oxidation with caffeine oxidase and xanthine oxidase enzymes (Summers et al. 2015). In this study, caffeine catabolism by the isolates was not explored. Further investigation was needed to utilize these isolated bacteria which could be applied for producing the decaffeinated coffee bean in vitro, in cooperation with xylanolytic, cellulolytic, and proteolytic bacteria that have been isolated from *Luwak* feces by other researchers (Fauzi 2008; Dewi et al. 2015). This can prevent the exploitation of *Luwak* for in vivo coffee bean fermentation through its the gastrointestinal tract.

In conclusion, bacteria that can grow in enriched caffeine medium have been isolated and identified from the soil, coffee pulp waste, and excreted coffee bean in feces of *Luwak* (Asian palm civet). Each isolate showed different ability to grow in a growth medium containing various caffeine concentrations ranging from 1.5 to 10 g L^{-1}. The potential isolates have been identified as species belonging to the genera *Klebsiella, Methylobacterium, Pseudomonas, Raoultella,* and *Stenotrophomonas* which were indicated by high similarity of 16s rRNA gene sequence and bootstrap value in phylogenetic analysis. All the isolates were deposited in InaCC. Next study regarding the catabolism pathway, optimum growth condition, and caffeine-degrading ability of each isolate are interesting topics for further application.

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