Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Degradation of caffeine by *Pseudomonas monteilii* KRM9

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Received 17 July 2017; Received in revised form 2 August 2017; Accepted 9 August 2017

ABSTRACT

Aims: The objective of this research was to isolate caffeine-degrading bacteria from coffee pulp waste in Indonesia and characterize their caffeine degradation activity.
Methodology and results: The caffeine-degrading bacteria were isolated from coffee pulp wastes of *Coffea arabica* and *C. canephora*. These isolates were selected based on their caffeine degradation activity. The identification and biochemical properties of the best isolate were conducted via 16S rDNA sequence analyses and by using the Microbact kit. Meanwhile, caffeine degradation activity of this bacteria was analyzed by using LC-MS/MS. The results indicated that fourteen bacterial isolates were able to degrade caffeine. The highest caffeine degradation activity was performed by isolate KRM9 at the rate of 99.26 ± 0.01%, on a caffeine medium after 24 h of incubation. Based on the 16S rDNA analyses, the KRM9 isolate was identified as *Pseudomonas monteilii*. Till present, this species has not been reported as a caffeine-degrading bacterium. However, LC-MS/MS analysis indicated that caffeine was degraded by *P. monteilii* KRM9 and theobromine was not the secondary metabolite of caffeine degradation.
Conclusion, significance and impact of study: *Pseudomonas monteilii* KRM9 was detected as a new isolate of caffeine-degrading bacteria. This bacterium can be introduced as an agent to degrade caffeine from coffee pulp waste. It is expected that further research can be conducted on the overall mechanism of caffeine degradation by *P. monteilii* KRM9.

Keywords: caffeine degradation, coffee pulp waste, *Pseudomonas monteilii* KRM9, theobromine

INTRODUCTION

Indonesia is the fourth largest coffee producer in the world, with more than 660 tons of coffee produced in 2015 (Worldatlas, 2017). The two most common coffee species produced are *Coffea canephora* and *C. arabica*. These industries generated around 50% of coffee pulp waste.

Caffeine ($C_8H_{10}N_4O_2$) is a secondary metabolite produced by coffee plants and is formed by purine nucleotides (Ashihara *et al*., 2008). Based on its structure, caffeine (1,3,7-trimetilxantin) belongs to methylxanthine in addition to theobromine (3,7 dimethylxanthine) and methyluric acid. Methylxanthine is classified as a purine alkaloid. According to a previous study by Arimurti *et al.* (2017), 0.26% caffeine was detected in *Coffea arabica* pulp, while 0.12% was detected in *C. canephora* pulp.

Caffeine in the coffee pulp waste is known to improve the quality of the waste as an organic fertilizer. However, some studies have shown that caffeine negatively impact plants growth. These plants include onions, sunflowers, and lettuces (Pincheira *et al*., 2003; Khursheed *et al*., 2009; Gomes *et al*., 2013). Therefore, the concentration of the caffeine in coffee pulp waste must be reduced before it is used as an organic fertilizer.

Many previous studies have indicated that several bacterial species have the capability to degrade caffeine, and these bacterial group usually belong to *Pseudomonas* (Middelhoven and Bakker, 1982; Yamaoka-yano and Mazzafera, 1999; Babu *et al*., 2005; Dash and Gummadi, 2006; Dash and Gummadi, 2008; Yu *et al*., 2009; Fan *et al*., 2011; Sumitha and Sivakumar, 2013) along with some other species within the genera of *Brevibacterium, Alcaligenes, Rhodococcus, Klebsiella, Stenotrophomonas, Serratia* (Mazzafera *et al*., 1996; Nayak *et al*., 2012; Sumitha and Sivakumar, 2013). These bacteria were isolated from coffee pulp in India (Babu *et al*., 2005; Nayak *et al*., 2012; Sumitha and Sivakumar, 2013), and from the soil of a tea plantation (Fan *et al*., 2011). The

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

present study, thus, was carried out to isolate caffeine-degrading bacteria from *Coffea arabica* and *C. canephora* pulp waste in Indonesia.

MATERIALS AND METHODS

Isolation of caffeine-degrading bacteria

The coffee pulp waste was taken from two coffee plantations in East Java, Indonesia. The *C. canephora* pulp was collected from Malangsari coffee plantation which is located at approximately 954 m above sea level (8°21'34.34''S and 113°56'43.37''E) whereas *C. arabica* was collected from the Jampit coffee plantation at approximately 1349 m above sea level (8°00'46.87''S and 114°08'07.19''E). The samples were carried to the laboratory at room temperature.

Twenty five (25) grams of sample was suspended in 225 mL of physiological salt solution (0.85% NaCl) in a 500 mL erlenmeyer and then diluted at 10^{-1}. The suspension was agitated inside a shaking incubator at 120 rpm for 15 min at the room temperature. Then it was left for 5-10 min until the solid phase precipitated and liquid phase was existed at the top. The sample was diluted stepwise from 10^{-1} to 10^{-7} and 0.1 mL of each suspension was inoculated onto a caffeine medium consisting of 1 g/L caffeine, and a minimum M9 medium (15 g/L $Na_2HPO_4 \cdot 12H_2O$, 3 g/L KH_2P0_4, 0.5 g/L NaCl, 0.25 g/L $MgSO_4$, 1 g/L NH_4Cl, 15 g/L bacto agar). The culture was incubated at 30 °C for 24 h. A single colony of each bacterial isolate which grew on this medium was isolated (Yu *et al*., 2009; Fan *et al*., 2011; Nayak *et al*., 2012).

Screening of caffeine-degrading bacteria based on their caffeine degradation activity

Isolates of each bacterium were inoculated into a 5 mL caffeine medium broth and incubated at the room temperature, 120 rpm for 24 h. The culture was diluted with distilled water to obtain OD 0.4 at λ 600 nm as the starter solution. Ten percent of the starter solution was grown on a 15 mL caffeine medium broth inside a shaking incubator at the room temperature, 200 rpm for 24 h. The suspension culture was centrifuged at 4 °C, 10.000 rpm for 10 min and its supernatant was collected. The concentration of the supernatant caffeine was analyzed through its absorbent at λ of 273 nm. The percentage of caffeine degradation activity was calculated by using following formula:

$$\frac{(\text{initial caffeine concentration} - \text{caffeine concentration after incubated})}{\text{initial caffeine concentration}} \times 100\%$$

Data was analyzed using the Kruskal-Wallis variance and Dunn-Bonferroni with α 0.05. Bacterial isolates which performed the highest caffeine degradation activity were selected as potential isolates.

Identification and biochemical analysis of selected caffeine-degrading bacteria

DNA of the isolates was extracted by using the CTAB method (Wilson, 2003). The 16S rDNA fragment was amplified by PCR (polymerase chain reaction) using primers 27f(5'-GAGAGTTTGATCCTGGCTCAG-3') and 1495r(5'-CTACGGCTACCTTGTTACGA-3') (Yang *et al*., 2015; Arimurti *et al*., 2017) employing 40 cycles of the following program: denaturation at 95 °C for 20 sec, annealing at 55 °C for 30 sec, and an elongation at 72 °C for 90 sec. Finally, it was followed by a final elongation at 72 °C for 5 min was performed. The amplicon of the partial 16S rRNA fragment was purified and sequenced at First Base, Malaysia. The partial sequences were aligned together in the nucleotide database of Gene Bank (http://www.ncbi.nlm.nih.gov). BLAST was used to identify the bacteria isolates based on the highest percentage of their nucleotide similarity. The phylogeny tree of the bacteria was then constructed based on a Neighbour-joining algorithm using MEGA 6 for Windows program.

The morphology of the bacterial cells (Gram stain and bacterial cell shape) was identified using a microscope with 1000 × magnification. The biochemical characteristics of the isolates were determined using the Microbact identification Kits (Oxoid-Australia) 24E (12A (12E) + 12B) system.

Growth pattern and assay of caffeine degradation activity performed by KRM9 isolate

The KRM9 isolate was cultivated into a 5 mL M9 caffeine (1 g/L) broth in a 50 mL Erlenmeyer flask and incubated at room temperature, 120 rpm for 24 h. The culture was diluted on minimal media to obtain OD 0.4 at 600 nm using a spectrophotometer as the starter solution. Ten percent (2 mL) of the starter solution was grown on a 20 mL M9 caffeine (1 g/L) medium inside a 50 mL Erlenmeyer flask placed in a shaking incubator at room temperature, 120 rpm for 30 h. Samples were taken from the culture at 6 h intervals for 30 h of incubation time. Analyses were conducted on the cell density and caffeine concentration. The experiment was replicated three times.

The optical density (OD) of the culture suspension was measured by a spectrophotometer at the optimum wavelength (λ) of 600 nm. The cell density was calculated based on the previously determined value and plotted against time in order to conclude the growth pattern. The cell density was counted by a haemocytometer at various dilutions of the culture. The caffeine concentration of the culture suspension was measured by a spectrophotometer at the optimum wavelength (λ) of 273 nm.

Analysis of caffeine degradation activity by LC-MS/MS

Caffeine degradation activity performed by KRM9 was analyzed by liquid chromatography-tandem mass spectrometry (Ogawa *et al*., 2012). The isolate was placed in a 75 mL M9 caffeine (1 g/L) broth medium at an initial

time and 48 h incubation. Caffeine (Sigma-China) and theobromine (TCI-Japan) were used as the standards.

The samples (N=3) were collected at initial time and after 48 h of incubation. Caffeine concentration was measured based on the standard curves constructed earlier.

RESULTS AND DISCUSSION

Fourteen caffeine-degrading bacterial isolates were found from two coffee plantations in Indonesia. These bacteria were collected from *C. arabica* (6 isolates) and *C. canephora* (8 isolates) pulp waste. The results indicated that all bacteria isolates had different caffeine degradation capability (Table 1). The table shows that the ability of the bacterial isolates to degrade caffeine vary in some ways. Two isolates performed only very low caffeine degradation activities. They were isolates KRM85 and KRM93. Meanwhile, isolates KRM84 and KRM107 performed 50% of caffeine degradation and the other ten isolates performed more than 80% of caffeine degradation rates. KRM9 which was isolated from *C. canephora* showed the highest potential to degrade caffeine concentration in the culture medium, with a significant decrease in caffeine degradation concentration (99.26 ± 0.01%) from 1 g/L caffeine at an initial incubation to 0.08 g/L caffeine after 30 h of incubation time.

Table 1: The caffeine-degrading activity of caffeine-degrading bacteria isolates on a caffeine medium at 24 h incubation period.

Isolates	Pulp Waste Sources	Caffeine Degradation (%)*
KAJ18	*C. arabica*	97.28 ± 0.04 ^{gh}
KAJ35	*C. arabica*	97.19 ± 1.33 ^{gh}
KAJ47	*C. arabica*	93.82 ± 0.10 ^{fg}
KAJ49	*C. arabica*	84.42 ± 7.34 ^{e}
KAJ53	*C. arabica*	98.02 ± 0.03 ^{h}
KAJ55	*C. arabica*	96.79 ± 0.05 ^{gh}
KRM7	*C. canephora*	98.27 ± 0.03 ^{h}
KRM9	*C. canephora*	99.26 ± 0.01 ^{i}
KRM84	*C. canephora*	54.51 ± 0.33 ^{d}
KRM85	*C. canephora*	1.10 ± 1.56 ^{a}
KRM86	*C. canephora*	91.84 ± 0.13 ^{fgh}
KRM88	*C. canephora*	88.50 ± 0.35 ^{f}
KRM93	*C. canephora*	12.24 ± 0.37 ^{b}
KRM107	*C. canephora*	50.14 ± 5.15 ^{c}

*Data were analyzed using the variance of Kruskal-Wallis and further analysis by Dunn-Bonferroni with α 0.05.

The 16s rDNA sequencing result of KRM9 was submitted to GenBank of NCBI (accession number K319030). According to the 16S rDNA sequence, KRM9 B1 was the most closely related to *P. monteilii* (Figure 1). The basic biochemical properties of the *P. monteilii* KRM9 were tested with the Microbact GNB 24E (12A (12E) + 12B) system (Table 2). This bacterium provided a positive reaction to lysine decarboxylase, ornithine decarboxylase, o-nitrophenyl-ß-d-galactopyranoside (ONPG), and arginine dihydrolase enzymes. KRM9 was positive for the fermentation of mannitol and sucrose. *Pseudomonas monteilii* KRM9 was positive for indol, acetoin production, and malonate inhibition. It was negative for the fermentation of glucose, xylose, inositol, rhamnose, sorbitol, lactose, arabinose, adonitol, raffinose, salicin. KRM9 was also negative for H_2S, urea hydrolysis, citrate utilization, indole pyruvate productions and gelatin liquefaction.

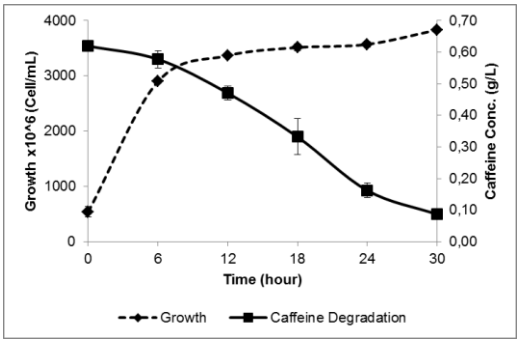

Figure 1: The growth profile and caffeine degradation of *P. monteilii* KRM9 on caffeine medium at 30 h incubation period.

In other previous studies, caffeine-degrading bacteria isolated from coffee pulp in India were identified as *Pseudomonas* sp. (Dash and Gummadi, 2008; Sumitha and Sivakumar, 2013) and *Brevibacterium* sp. (Sumitha and Sivakumar, 2013). The caffeine-degrading *P. monteilii* KRM9 isolated from this study is a novel caffeine-degrading bacterium since no caffeine-degrading activity of the species was reported before. Other studies only reported on the caffeine-degrading bacteria from the *Pseudomonas* genus of *P. putida* (Middelhoven and Bakker, 1982; Yamaoka-yano and Mazzafera, 1999; Yu *et al*., 2009; Fan *et al*., 2011) and *P. alcaligenes* (Babu *et al*., 2005).

Figure 2 depicts the bacteria growth and caffeine degradation activity of *P. monteilii* KRM9 on a medium at 30 h incubation period. It presents the increasing number of cells from (5.3 ± 0.9) × 10^8 cell/mL at the initial incubation time to (3.8 ± 0.4) × 10^9 cell/mL at the end of incubation (30 h) along with the decreasing caffeine concentration on the medium from 0.619 g/L to 0.162 g/L. *P. monteilii* KRM9 is shown to grow on a caffeine medium by utilizing caffeine as a sole carbon and nitrogen source. This process was simultaneously followed by reduction in caffeine concentration.

Results indicated that *P. monteilii* KRM9 can degrade caffeine in a medium containing caffeine within 24 h at over 99% efficiency. This finding suggested that caffeine was metabolized by *P. monteilii* KRM9. Previous findings reported of other species that have lower caffeine degrading activity. *Pseudomonas putida* C3024 was able to degrade 50% of caffeine at 30 h in a medium containing 5 g/L caffeine (Middelhoven and Bakker, 1982). *P. putida* NCIM5235 could degrade 59.9 - 21.5% of the caffeine in a

Table 2: Biochemical characterization of *Pseudomonas monteilii* KRM9.

Characteristics	Reaction	Characteristics	Reaction
Gram-negative	+	Production of indole pyruvate by deamination of tryptophan	-
Catalase	-	Gelatin liquefaction	-
Lysine decarboxylase	+	Malonate inhibition	+
Ornithine decarboxylase	+	Inositol fermentation	-
H_2S production	-	Sorbitol fermentation	-
Glucose fermentation	-	Rhamnose fermentation	-
Mannitol fermentation	+	Sucrose fermentation	+
Xylose fermentation	-	Lactose fermentation	-
Hydrolysis of o-nitrophenyl-ß-d-galactopyranoside (ONPG) by action of ß-galactosidase	+	Arabinose fermentation	-
Indole production from tryptophan	+	Adonitol fermentation	-
Urea hydrolysis	-	Raffinose fermentation	-
Acetoin production (Voges-Proskaüer reaction)	+	Salicin fermentation	-
Citrate utilization (citrate is the only source of carbon)	-	Arginine dihydrolase	+

Symbols are as follows: +: positive reaction, -: negative reaction

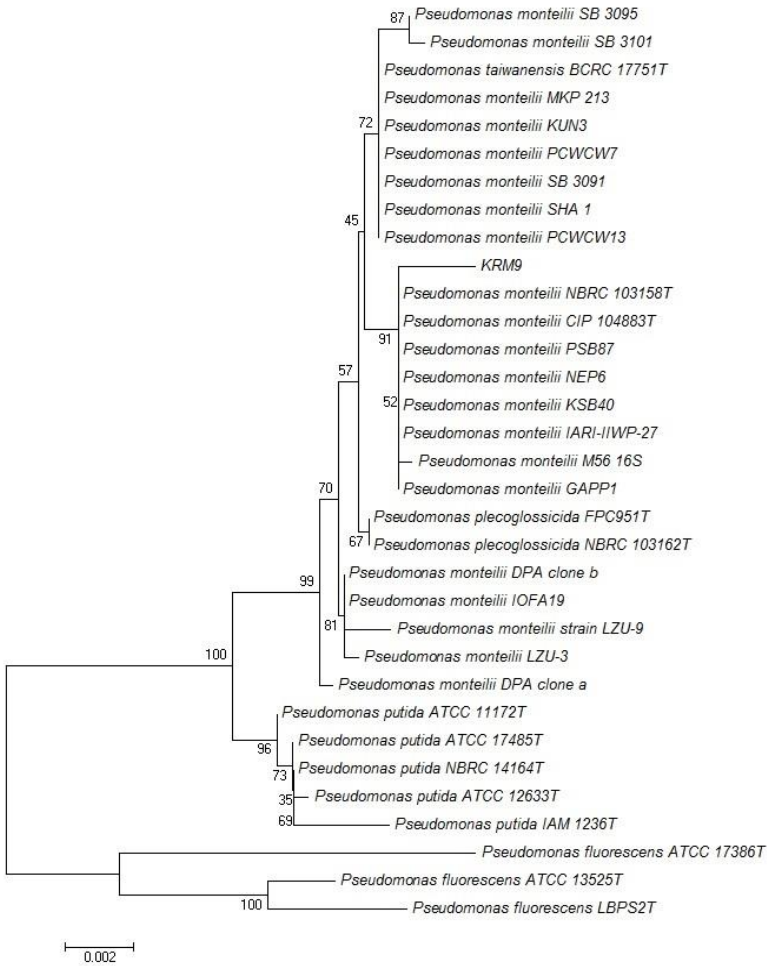

Figure 2: Phylogeny tree of *Pseudomonas monteilii* KRM9 and bacteria reference based on 16S rDNA sequence similarity using the Neighbour-joining algorithm.

medium containing 7.5 - 10 g/L caffeine (Dash and Gummadi, 2006) and this bacterium could degrade 100% of caffeine at 18 h when it was allowed to grow on a medium containing 6.4 g/L caffeine with 700 and 800 rpm aeration (Gummadi *et al*., 2009). It also could degrade 100% of caffeine on a medium contained 1 g/L caffeine after 15 h incubation (Gummadi and Devarai, 2010). Besides bacteria, another microorganism which also has the ability to degrade caffeine is *Aspergillus tamarii* V12A25. It was reported to be able to degrade 67.2% of caffeine in 48 h in a medium containing 4 g/L caffeine (Hakil *et al*., 1998).

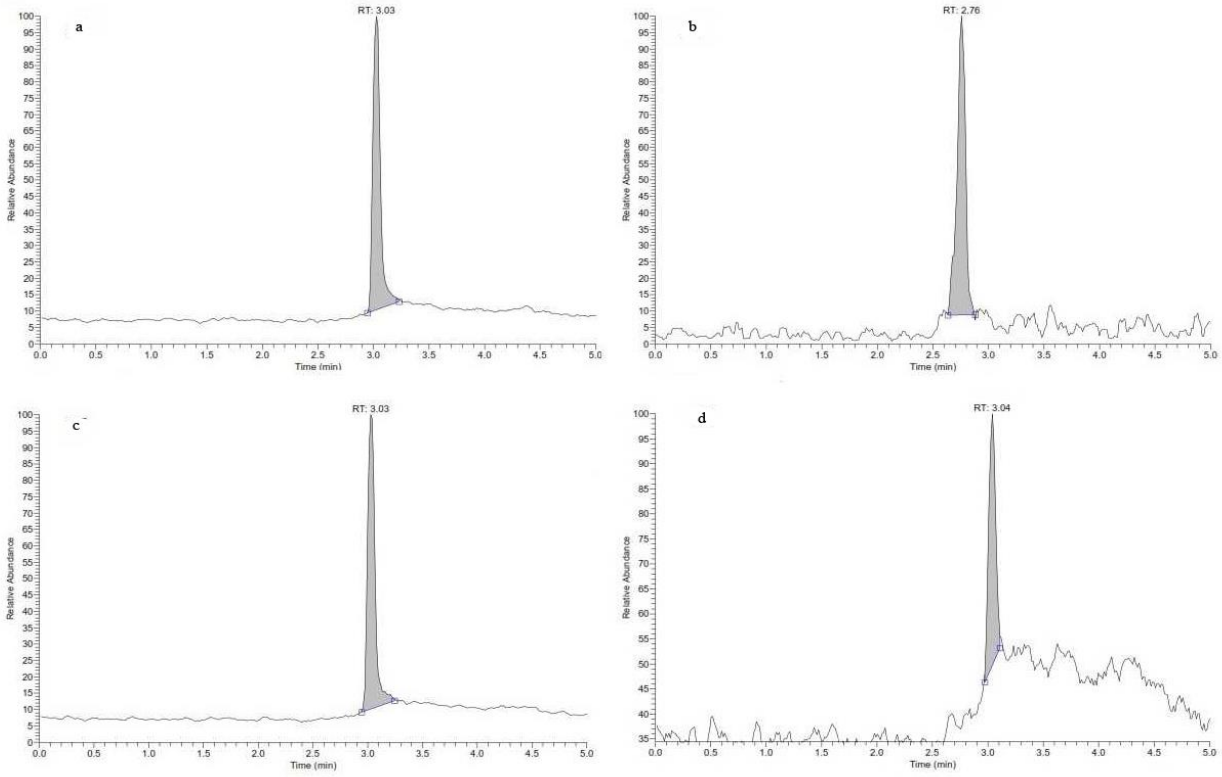

Figure 3: Chromatograms of caffeine medium inoculated *Pseudomonas monteilii* KRM9 by LC-MS/MS analysis a, standard caffeine; b, standard theobromine; c, caffeine medium at initial time; d, caffeine medium after 48 h incubation time.

There are two processes of bacterial caffeine degradation: demethylation and oxidation. In the demethylation process, caffeine is changed into theobromine or paraxanthine by a demethylation enzyme, while in the oxidation process, caffeine is changed into 1,3,7-trimethyl uric acid by an oxidative enzyme. At the end of the metabolism, those compounds produce CO_2 and NH_3 (Gummadi *et al*., 2012; Summers *et al*., 2015). LC-MS/MS was employed to analyze caffeine degradation process performed by *P. monteilii* KRM9. Caffeine and theobromine were used as the standard. The result of the LC-MS/MS analysis indicated that the retention times of caffeine and theobromine were 3.03 and 2.77 respectively (Figure 3a and 3b). The chromatogram peak showed a good resolution and the retention times were < 4 min. The result of the LC-MS/MS analysis also presented that caffeine concentration on a caffeine medium inoculated by *P. monteilii* KRM9 decreased 99,99% from 8.085.72 ± 21.50 mg/L at the initial time of incubation to 0.16 ± 0.05 mg/L at 48 h incubation (Figure 3c and 3d). The results revealed that *P. monteilii* KRM9 was able to remove caffeine from the medium. The LC-MS/MS analysis proved that theobromine was not detected on the caffeine medium on which *P. monteilii* KRM9 was inoculated at an initial time and 48 h incubation. Apparently, theobromine was not used by *P. monteilii* KRM9 as an intermediate metabolite of caffeine degradation. On the other hand, it was found that theobromine was used by *P. putida* as the major metabolite of caffeine degradation (Asano *et al*., 1993; Summers *et al*., 2015). This finding indicated that there was a possible different mechanism of caffeine degradation performed by *P. monteilii* KRM9. Base on this data, *P monteilii* KRM9 can be used as an agent to degrade coffee pulp waste. Therefore, it is necessary to conduct further research on the caffeine-degrading activity performed by *P. monteilii* KRM9. It is expected that the research can investigate the overall mechanism of caffeine degradation.

ACKNOWLEDGEMENTS

We would like to thank the Ministry of Research, Technology and Higher Education of The Republic of Indonesia who provided a research grant for the doctoral dissertation (No. 187AD / UN25.3.1 / LT / 2016).

REFERENCES

Arimurti, S., Nurani, Y., Ardyati, T. and Suharjono, S. (2017). Screening and identification of indigenous cellulolytic bacteria from Indonesian coffee pulp and investigation of its caffeine tolerance ability. *Malaysian Journal of Microbiology* **13(2), 109-116.**

Asano, Y., Komeda, T. and Yamada, H. (1993). Microbial production of theobromine from caffeine. *Bioscience, Biotechnology, and Biochemistry* **57, 1286-1289.**

Ashihara, H., Sano, H. and Crozier, A. (2008). Caffeine and related purine alkaloids: Biosynthesis, catabolism, function and genetic engineering. *Phytochemistry* **69, 841-856.**

Babu, V. R. S., Patra, S., Thakur, M. S., Karanth, N. G. and Varadaraj, M. C. (2005). Degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708. *Enzyme and Microbial Technology* **37, 617-624.**

Dash, S. S. and Gummadi, S. N. (2006). Biodegradation of caffeine by *Pseudomonas* sp. NCIM 5235. *Research Journal of Microbiology* **1, 115-123.**

Dash, S. S. and Gummadi, S. N. (2008). Inducible nature of the enzymes involved in catabolism of caffeine and related methylxanthines. *Journal of Basic Microbiology* **48, 227-233.**

Fan, F., Xu, Y., Liang, Y., Zheng, X., Borthakur, D. and Lu, J. (2011). Isolation and characterization of high caffeine-tolerant bacterium strains from the soil of tea garden. *African Journal of Microbiology Research* **5, 2278-2286.**

Gomes, T., Pereira, A. J., Ramalhosa, E., Casal, S. and Baptista, P. (2013). Effect of fresh and composted spent coffee grounds on lettuce growth, photosynthetic pigments and mineral composition. *In:* Proceedings of the VII Congreso Ibérico de Agroingenieria y Ciencias Horticolas. Associao Portuguesa de Horticultura and Sociedad Espanola de Ciencias Horticolas, Madrid, Spain. **pp. 26-29.**

Gummadi, S. N. and Devarai, S. (2010). Kinetics of growth and caffeine demethylase production of *Pseudomonas* sp. in bioreactor. *Journal of Industrial Microbiology and Biotechnology* **37, 901-908.**

Gummadi, S. N., Bhavya, B. and Ashok, N. (2012). Physiology, biochemistry and possible applications of microbial caffeine degradation. *Applied Microbiology and Biotechnology* **93, 545-554.**

Gummadi, S. N., Dash, S. S. and Devarai, S. (2009). Optimization of production of caffeine demethylase by *Pseudomonas* sp. in a bioreactor. *Journal of Industrial Microbiology and Biotechnology* **36, 713-720.**

Hakil, M. M., Denis, S., Viniegra, G. and Augur, C. (1998). Degradation and product analysis of caffeine and related dimethylxanthines by filamentous fungi. *Enzyme and Microbial Technology* **22, 355-359.**

Khursheed, T., Ansari, M. Y. K. and Shahab, D. (2009). Studies on the effect of caffeine on growth and yield parameters in *Helianthus annuus* L. variety modern. *Biology and Medicine* **1, 56-60.**

Mazzafera, P., Olsson, O. and Sandberg, G. (1996). Degradation of caffeine and related methyl xanthines by *Serratia marcescens* isolated from soil under coffee cultivation. *Microbial Ecology* **31, 199-207.**

Middelhoven, W. J. and Bakker, C. M. (1982). Degradation of caffeine by immobilized cells of *Pseudomonas putida* strain C 3024. *European Journal of Applied Microbiology and Biotechnology* **15, 214-217.**

Nayak, S., Harshitha, M. J., Maithili, Sampath, C., Anilkumar, H. S. and Rao, C. V. (2012). Isolation and characterization of caffeine-degrading bacteria from coffee pulp. *Indian Journal of Biotechnology* **11, 86-91.**

Ogawa, T., Hattori, H., Iwai, M., Ito, K., Iwakura, K., Ishii, A., Suzuki, O. and Seno, H. (2012). A rapid and simultaneous analysis of theophylline and its metabolites in human whole blood by ultra-performance liquid chromatography-tandem mass spectrometry. *Forensic Toxicology* **30, 142-148.**

Pincheira, J., López-Sáez, J. F., Carrera, P., Navarrete, M. H. and De la Torre, C. (2003). Effect of caffeine on *in vivo* processing of alkylated bases in proliferating plant cells. *Cell Biology International* **27, 837-843.**

Sumitha, J. and Sivakumar, T. (2013). Isolation and characterization of caffeine-degrading bacteria from West Karnataka, India. *International Journal of Current Microbiology and Applied Sciences* **2, 338-346.**

Summers, R. M., Mohanty, S. K. and Subramanian, M. (2015). Minireview genetic characterization of caffeine degradation by bacteria and its potential applications. *Microbial Biotechnology* **8, 369-378.**

Wilson, K. (2003). Preparation of genomic DNA from bacteria. *In:* Current Protocols in Molecular Biology 5th edn. John Wiley & Sons, New York. **Supplement 56.**

Worldatlas. (2017). Top coffee producing countries. Worldatlas http://www.worldatlas.com/articles/top-coffee-producing-countries.html. [Retrieved on 28 March 2017].

Yamaoka-yano, D. M. and Mazzafera, P. (1999). Catabolism of caffeine and purification of a xanthine oxidase responsible for methyluric acids production in *Pseudomonas putida* L. *Revista de Microbiologia* **30, 62-70.**

Yang, Q., Chen, L., Hu, X., Zhao, L., Yin, P. and Li, Q. (2015). Toxic effect of a marine bacterium on aquatic organisms and its algicidal substances against *Phaeocystis globosa*. *PLoS One* **10, 1-16.**

Yu, C. L., Louie, T. M., Summers, R., Kale, Y., Gopishetty, S. and Subramanian, M. (2009). Two distinct pathways for metabolism of theophylline and caffeine are coexpressed in *Pseudomonas putida* CBB5. *Journal of Bacteriology* **191, 4624-4632.**