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Assessment of Caffeine-degrading Ability on Bacterial Strains Klebsiella sp. Isolated from Feces of Asian Palm Civet (Luwak)

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Abstract. Asian palm civet (APC) known as Luwak, has an important role in producing the most expensive coffee in the world, Kopi Luwak, which is excreted from its feces. The bacterial diversity in the feces of APC was rarely explored, especially for decaffeination. In the present study, aerobic caffeine-degrading bacteria were successfully isolated from the feces of bred Luwak or Asian palm civet (Paradoxurus hermaphrodite) in Wonosobo (LW) and Lumajang (LL), Java Island-Indonesia and identified using 16s rRNA gene. After identification, they were known as Klebsiella (coded LW1 & LL1). Further, the ability of caffeine degradation was assessed using a Caffeine Agar Medium and Caffeine Liquid Medium (CLM), which were M9 mineral salt with the particular concentration of caffeine. As the additional carbon source, 5 g l 1 of sucrose was added into CLM. Residual caffeine concentration in the CLM was measured periodically using RV/UV-HPLC. LW1 and LL1 were detected to degrade 0.5 g L^{-1} of caffeine entirely in the CLM for 3 days. If 1 g L^{-1} of caffeine was introduced, only 63 and 66% of caffeine were degraded respectively for LW1 and LL1. Theobromine did not appear in the CLM, indicated that C8-oxidation is their catabolic pathway. Kinetic parameters of cell growth also have been determined using the different substrate inhibition models to find experimental kinetic data. This is the first report of caffeine-degrading bacteria isolated from the feces of Asian palm civet.

Keywords: Asian palm civet; Caffeine; Decaffeination; Inhibition kinetics; Klebsiella; Specific growth; Substrate

1. Introduction

Caffeine (1,3,7-trimethylxanthine) is the most widely used psychoactive substances in the world that easily can be found in beverage product, such as coffee, tea, caffeinated soft drink, energy drink, chocolate, and pharmaceutical preparations (Mohanty, 2013; Mohanty *et al.*, 2012; Durrant, 2002). Caffeine has a beneficial effect in increasing alertness and overcoming fatigue as long as not be consumed more than 400-700 mg/day/adult, otherwise it might influence the negative effect on health, e.g., change in sleep pattern, anxiety, palpitations, irritability, nausea, increased blood pressure, and restlessness (Schmidt and Stiber, 2008; Eteng *et al.*, 1997). For extreme and long-term consumption, caffeine potentially causes the serious health condition problems such as auditory

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hallucination, insomnia, increased risk of coronary heart disease, loss of appetite, vitamin deficiency, and several types of cancer (Van-Dam, 2008).

Caffeine residue in tea and coffee was also reported may influence several environmental problems, since it was a poisonous compound and difficult to be degraded. It can be toxic to germinating seeds, microorganisms, and marine organisms (Ibrahim *et al.*, 2014). Partial degradation of wastes remained xenobiotics (Dash and Gummadi, 2007), which if this secondary product accumulated at a certain concentration may inhibit the growth of natural soil microorganisms and then causes environmental instability. The Decaffeinated of caffeinated-beverages and waste in the environment became an important focus (Gokulakrishnan and Gummadi, 2006; Babu *et al.*, 2005), where applying a microorganism in bio-decaffeination was reported as the better alternative methods due to the environment, health, and economic point of views than other physical and chemical methods (Gokulakrishnan, Chandraraj, and Gummadi, 2005). Besides, caffeine-degrading microbes can utilize caffeine as a sole carbon and nitrogen source to produce valuable chemical and pharmaceutical by-products, such as alkylxanthines and alkyl uric acid (Summers *et al.*, 2015). For several years, caffeine-degrading bacteria had already been explored from different habitat source, mostly from the soil. They were identified mainly Pseudomonas, but several were Acetobacter, Acinetobacter, Alcaligenes, Coryneform, Flavobacterium, Klebsiella, Moraxella, Rhodococcus, and Serratia (Summers *et al.*, 2015).

The purpose of the current study is to isolate and characterize caffeine-degrading bacteria from APC feces, and to determine how effective they are in breaking down caffeine when sucrose is added as an extra carbon source. Furthermore, the different substrate inhibition models were tested to find the kinetic parameters of cell growth in resulting the best fit to the experimental kinetics data.

2. Methods

2.1. Assessment of caffeine-degrading ability using CLM

2.1.1. Preparation of induced cell

transferred into 150 ml of a solution containing caffeine (0.3 g/L) and nutrient broth (8 g/L) and incubated at 32°C on an orbital shaker at 120 rpm for 3 days. Then, the culture was transferred 10% (v/v) into 150 ml of the same fresh medium and incubated for 3 days under the same conditions. Every 6 hours, the cell growth was measured by OD_{600}. At the late log phase, the induced cells were centrifuged at 13,000 rpm for 10 min at 4°C. The cell pellet was washed twice using phosphate buffer (pH 7.0) to remove the caffeine and then suspended in fresh phosphate buffer with a ratio of 1:10 (m/v). This suspension was ready for caffeine degradation study.

2.1.2. *Caffeine degradation study*

This study was carried out by aseptically transferred 10% (v/v) of the suspension into 0 ml of a CLM with 5 g/L of sucrose and 0.5 g/L of caffeine. The culture was incubated at 32°C on an orbital shaker at 120 rpm for 3 days and periodically measured by OD_{600}. After the culture reached a late log phase, 10% (v/v) of the culture was aseptically transferred into 150 ml of the same fresh CLM but with 1.0 g/L of caffeine. The culture was again incubated and measured with the same steps. The culture was again transferred into the same medium but with a final caffeine concentration of 1.5 g/L, and same further steps were followed. Each culture was conducted in triplicate. Isolates were inoculated stepwise into 3 different caffeine concentrations from low to high.

2.1.3. Determination of residual caffeine in CLM

The residual caffeine concentration of each culture and potential produced theobromine was calculated as mentioned. Two ml of an aliquot of each culture with

different caffeine concentration was centrifuged at 13,000 rpm for 10 min. The supernatants were measured by reversed-phase UV-HPLC LC-AD20 Shimadzu Liquid Chromatograph equipped with an SPD-20A Shimadzu UV/Vis detector and a CMB-20A as a system controller. The column was a Cosmosil 5C18-MC-II reverse-phase packed column, 5mm of particle size, 4.6 mm I.D. x 150 mm (Nacalai USA), and operated at 25°C. The mobile phase system was adopted from Bispo *et al.* (2002) after modification with milli-Q water that has been applied in methanol/acetic acid/milli-Q water (20:5:75, v/v/v) system, pH 3, at a flow rate of 0.7 mL/min and detected at a wavelength of 273 nm. A method of the external standard calibration was used. The peaks of caffeine and theobromine common solution were detected at approximately 5.07 and 3.26 min, respectively (Figure 3A). The sample consisting of 0.4 mL of supernatants was diluted to 3.6 ml of milli-Q water and then introduced into an injector with a 20 ml sample loop. The percentage of residual caffeine concentration (PRCC) in the CLM and caffeine degradation was calculated as follows:

2.2. Kinetics of caffeine degradation

Models of Haldane, Edwards, Double exponential, and Luong, as listed in Table 1. The parameters were estimated using solver in Microsoft Excel with the constraint of the models formulated in equation (1):

$$\mu_{max} \leq \mu_m \leq 3\mu_{max} \; ; \; K_s > 0 \qquad (1)$$

where μ_{max} is the maximum specific growth rate which was obtained by fitting the data of the unhibited region to Monod's equation.

Table 1 Estimated parameters of various substrate inhibition models

Msodel	Equations	Estimated Parameters		R^2 value	
		LW1	LL1	LW1	LL1
Haldane	$m = m_m S/((K_s + S)(1+(S/K_i))$	m_m = 0.131; Ki = 0.472; Ks = 0.009	m_m = 0.240; Ki = 0.270; Ks = 0.120	0.869	0.967
Edwards	$m = (m_m S/K_s + S) \exp(-S/K_i)$	m_m = 0.247; Ki = 0. 764; Ks = 0.290	m_m = 0.209; Ki = 1.013; Ks = 0.326	0.974	0.964
Double exponential	$m = m_m [\exp(-S/K_i) - \exp(-S/Ks)]$	m_m = 0.299; Ki = 0.587; Ks = 0.224	m_m = 0.112; Ki = 1.000; Ks = 0.090	0.988	0.986
Loung	$m = m_m S/K_s+S(1-S/S_m)^n$	m_m = 0.2; K_s = 0.104; S_m = 5.1; n = 6	m_m = 0.102; K_s = 0.012; S_m = 6.458; n = 4.50	0.944	0.985

3. Results and Discussion

3.1. Performance of caffeine degradation

3.1.1. The growth of isolates in NB medium supplemented caffeine and CLM

The isolates have been grown in nutrient broth with 0.3 g/L of caffeine to induce and improve their resistance to caffeine as an inhibitor in the liquid phase medium. Their growth was represented by optical density at 600 nm shown in supplementary data. Generally, the culture can grow in the caffeinated NB medium. However, the culture in the non-caffeinated NB medium has better growth than in the caffeinated medium. This suggests that the presence of caffeine will hamper the growth of bacteria, although they were grown in a nutrient-rich medium such as Nutrient Broth.

The growth and caffeine-degradation ability of isolates were tested further using CLM with caffeine concentration of 0.5, 1, and 1.5 g/L and 5 g/L of sucrose as another carbon

source. The OD_{600} value revealed that the biomass of isolates decreased with increasing caffeine concentration during the incubation time. It is shown in Figure 1.

Figure 1 shows the absorbance of the culture of LW1 and LL1 in CLM during 72 h of incubation time. The curve indicates that the initial concentration of caffeine affects the growth and accumulated biomass in the media, decreasing with increasing caffeine concentration in the medium. The stationary phase of isolates when grew in 0.5, 1, and 1.5 g/L of caffeine was reached an OD_{600} of 0.55, 0.18, 0.11, respectively for LW1 and 0.65, 0.22, and 0.11, respectively for LL1. The sucrose in the medium could not increase the resistance of isolates against caffeine as an inhibitor. But it helps to increase the growth rate and final density of the isolates, especially in the medium supplemented with 0.5 g/L of caffeine.

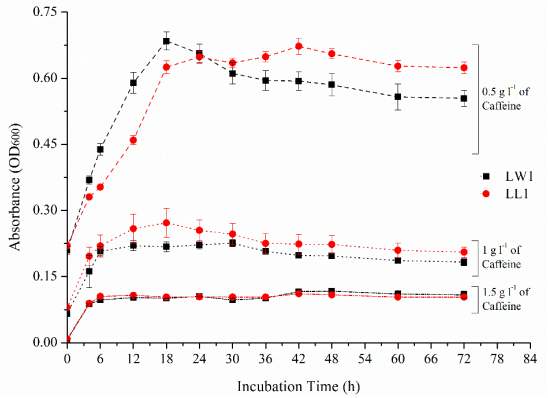

Figure 1 Effect of caffeine on the growth of the pure isolates

When the caffeine concentration was increased to 1 and 1.5 g/L, the growth of isolates had given a significantly different cell density gap and a shorter log phase time. The isolates can optimally degrade the caffeine until a caffeine concentration of 1 g/L.

In other studies, the addition of other carbon or nitrogen sources such as sucrose, glucose, and yeast nitrogen base without amino acids and ammonium sulfate (YNB) into a caffeinated medium could significantly increase the growth rate, final density, and ability to consume the same caffeine concentration. As shown in Table 2, Yu *et al.* (2009) have used M9 medium supplemented with 2.5 g of caffeine and 4 g of YNB or Soytone (as the second nitrogen source) per liter of M9 medium to grow *Pseudomonas sp.* CBB5. When it grew in the M9-caffeine medium, its stationary phase was approximately 0.4 after 72 h of incubation and consumed 92% of caffeine concentration. CBB5 can completely consume caffeine in 53 h and 20 h, respectively and growth rate and final cell density were significantly increased with OD_{600} of 1.06 and 2.43, respectively in the M9-caffeine medium supplemented by YNB or soytone. The difference in composition of vitamins was suggested to affect their growth in that medium-supplemented soytone and YNB.

3.1.2. Caffeine degradation in CLM

LW1 and LL1 strain in CLM supplemented 0.5 g/L of caffeine have shown a good caffeine degradation performance which resulted in 98.5% and 98.1% of caffeine degradation, respectively, for 48 h of incubation., They completely consumed the caffeine at 72 h, as shown in Figure 2. When caffeine concentration was increased to 1 g/L, LW1 and LL1 have degraded 63.3% and 66% of caffeine at 48 h, respectively which were not changed until 72 h. The degradation was very poor when LW1 and LL1 grew in CLM-supplemented caffeine of 1.5 g/L. LW1 and LL1 could degrade caffeine only 1% and 3%, respectively, for 48 to 72 h of incubation time.

Figure 2 shows the evolution of residual caffeine concentration during incubation for 72 h. Residual caffeine concentration in the test medium is important to determine how much concentration of caffeine that was degraded by bacteria. Residual caffeine in 48 and

72 h were 1% and ~0% for LW1 and 2% and ~0% for LL1, respectively. The degradation curve of LW1 has been slower evolution during 48 h than LL1, especially since 18 h of incubation time. It probably happened because the stock of sucrose in the medium as another carbon source was still enough until 24 h and the bacteria consumeless caffeine in the same time of sucrose consumption.

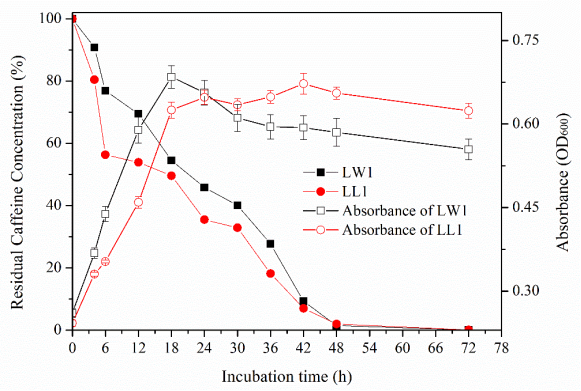

Figure 2 Profile of caffeine degradation by LW1 and LL1 in the CLM supplemented 0.5 g/L of caffeine

In Figure 3, the kinetics of caffeine degradation in LL1 culture show a faster evolution with better growth after 18 h of incubation. The growth curve after 18 h of LW1 decreased while in LL1 still increased with slow speed, relatively stationary phase until finally reduced after 42 hours. Caffeine degradation was continuing although the log phase was over at 18 hours of incubation time. After that time, the stationary phase was riched. But, the population was still metabolically active during this phase and might produce a product called secondary metabolites, which were not produced during the log phase (Stanbury, Whitaker, and Hall, 2000).

The sucrose content at 18 h and 24 h incubation time in the culture of LW1 and LL1 respectively have been measured to determine the residual sucrose in the medium. Surprisingly, the results showed that the sucrose content that time was 0.11% and 0.12% of the total 5 grams of sucrose. These data confirmed that those isolates were capable of utilizing carbon containingin the sucrose.

That isolate's ability of caffeine degradation was lower than other bacteria that have been reported. But, it was maybe changed if isolates were treated using a different medium with the optimized addition of other minerals or carbon or nitrogen sources. Table 2 shows some bacteria, isolated fromdifferent sources, that can degrade caffeine. Among the reported strain, *Pseudomonas sp.* is the most excellent caffeine-degrading bacteria. Gokulakrishnan and Gummadi (2006) have reported that *Pseudomonas sp.* GSC 1182, isolated from soil samples from the coffee plantation area, could withstand high levels of caffeine (around 20 g/L). Induced cells of this strain could degrade 10 g/L of caffeine at a maximum rate of 0.3 g/L.h. So far, this is the highest rate reported for caffeine degradation.

3.2. Isolates' catabolic pathway Estimation

Summers *et al.* (2015) stated that there are only two catabolic pathways in caffeine degradation by bacteria (C-8 oxidation and N-demethylation) which N-demethylation is the most comment pathway in over 80% of reported isolates. The main product of the first N-demethylation pathway is theobromine, with tiny amounts of paraxanthine produced by demethylase enzymes. The caffeine oxidase enzyme is largely responsible for oxidizing caffeine to methyl uric acid in the C-8 oxidation pathway. In both pathways, caffeine is broken down into carbon dioxide and ammonia for energy and cellular building blocks.

Klebsiella sp. was reported can degrade caffeine using C-8 oxidation pathway (Ibrahim *et al.*, 2014). In the C-8 Oxidase pathway, bacteria do not produce theobromine as in the N-demethylase pathway. Figure 3 shows the results of HPLC analysis of caffeine and theobromine identification from the samples at 24 and 48 hours. The results did not show theobromine as a result of the metabolism of LW1 and LL1 cultures in the CLM supplemented 0.5 g/L of caffeine. This indicated that those isolates confirm their C-8 oxidation catabolism. The same result has been found by Madyastha and Sridhar (1998), which in the culture consortium of *Klebsiella sp.* and *Rhodococcus sp.*, caffeine was directly oxidized by the caffeine oxidase at the C-8 position forming 1,3,7-trimethyluric acid. Demethylation steps were not found in this culture. This oxidative caffeine-degradation present an efficient way to produce trimethyluric acid by enzymatic degradation of caffeine (Gokulakrishnan, Chandraraj, and Gummadi, 2005).

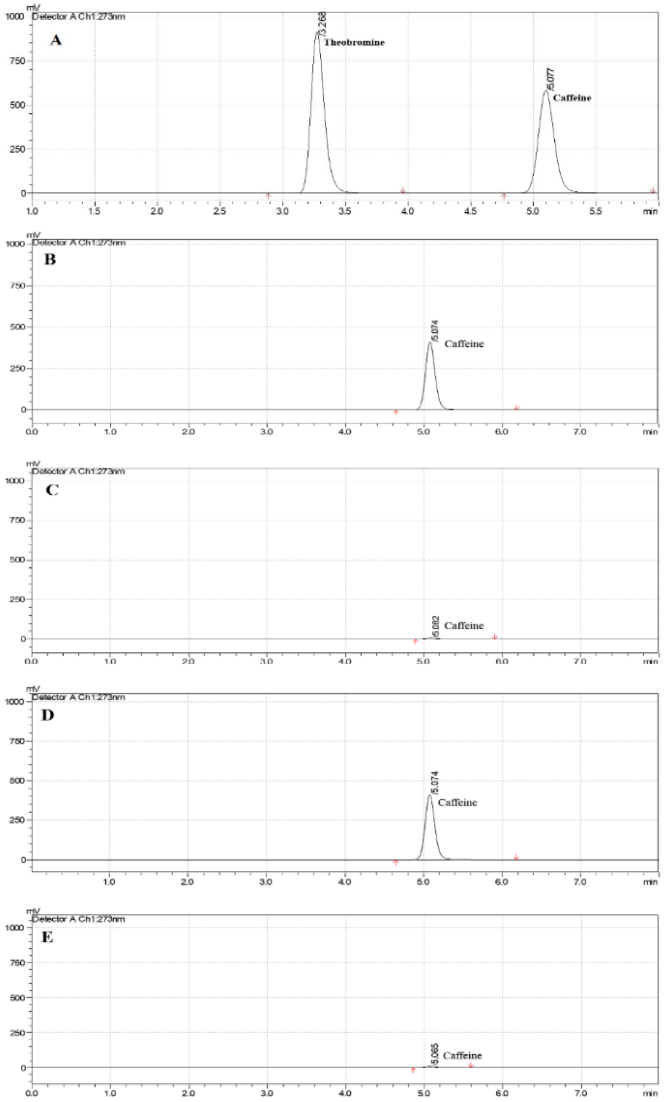

Figure 3 Chromatograms of caffeine and theobromine analysis using RV/UV HPLC. **A** for 75 ppm of each theobromine and caffeine standard solution. **B** and **C** for the culture of LW1 at the incubation time of 24 and 48 hours, respectively. **D** and **E** for the culture of LL1 at the incubation time of 24 and 48 hours, respectively. Caffeine showed a retention time of 5.07 min. Theobromine was not identified in the samples

Table 2 Comparative results of degradation performance of various caffeine-degrading bacteria

Name of Bacteria (Strain)	Isolate's sourc	Carbon or Nitrogen source	Initial Caffeine concentration (g/L)	Caffeine degradation	T (°C)	Initial pH	Reference
Mixed *Klebsiella* and *Rhodococcus*	Soil	Caffeine+glucose	2	100% in 36 h	29-30	4-5	(Madyastha and Sridhar, 1998)
Serratia marcescens	The soil under a coffee tree	Caffeine only	0.1 0.3	100% in 3 days 100% in 7 days	30	- -	(Mazzafera, Olsson, and Sandberg 1996)
Pseudomonas alcaligenes (CFR 1708)	Soil of coffee and tea garden	Caffeine only	1	100% in 24 h	30-32	7.2	(Babu *et al.*, 2005)
Pseudomonas sp. (GSC 1182)	The soil of the coffee cultivation area	Caffeine+sucrose	0.05-4 5-10	100% in 48 h 50% in 72 h	30	6	(Gokulakrishnan and Gummadi, 2006)
Pseudomonas sp. (CBB5)	Soil from Coralville	Caffeine only Caffeine+YNB Caffeine+Soyton	2.5 2.5 2.5	92% in 72 h 100% in 53 h 100% in 20 h	29	- -	(Yu *et al.*, 2009)
Pseudomonas pseudoalcaligenes (TPS8)	Soil of tea plantation	Caffeine+glucose	2.5	80.2% in 72 h	28	7	(Ashengroph and Ababaf, 2013)
Pseudomonas stutzeri (Gr 21 ZF)	Soil from Bekka Valley	Caffeine+sucrose	1.2	80.1% in 48 h	30	6	(El-Mched, Olama, and Holail, 2013)
Klebsiella sp. (LW 1)	Feces of Asian Palm Civet	Caffeine+sucrose	0.5 1 1.5	98.5% in 48 h 63% in 48 h 1% in 48 h	30	7.2	This study
Klebsiella sp. (LL 1)	Feces of Asian Palm Civet	Caffeine+sucrose	0.5 1 1.5	98.1% in 48 h 66% in 48 h 3% in 48 h	30	7.2	This study

3.3. The specific growth rate in various initial caffeine concentration

When used in larger-scale production, the kinetic parameter of isolates in the caffeinated medium is crucial. Therefore, the specific growth rate (h^{-1}) of isolates for each initial caffeine concentration was measured. It was obtained as the slope from the plot of ln cell dry weight versus time in the logarithmic phase as discussed in Section 2.4. When the isolate grew in CLM with various initial caffeine concentration from 0.15 to 5, the initial and final log phase occurred at different time of incubation, so that timing of harvesting cells during the logarithmic phase was important to be consistently set in the same time for each culture. From Figure 4, the specific growth rate increased with an increase in caffeine concentration to 0.3 g/L and slightly decreases with an increase in caffeine of 0.5 g/L. The values of LW1 and LL1 have sharply down to a caffeine concentration of 1.5 g/L and continuously decreased until 5 g/L as the obtained maximum concentration to totally inhibit the growth of isolates. Among the various models considered, the double exponential model showed the highest and most satisfied R^2 for both LW1 and LL1, which were 0.988 and 0.986, respectively as shown in Table 1. The kinetic parameters for LW1 were μ_m = 0.299, Ki = 0.587, and Ks = 0.226, while for LL1 were μ_m = 0.112, Ki = 1.00, and Ks = 0.090.

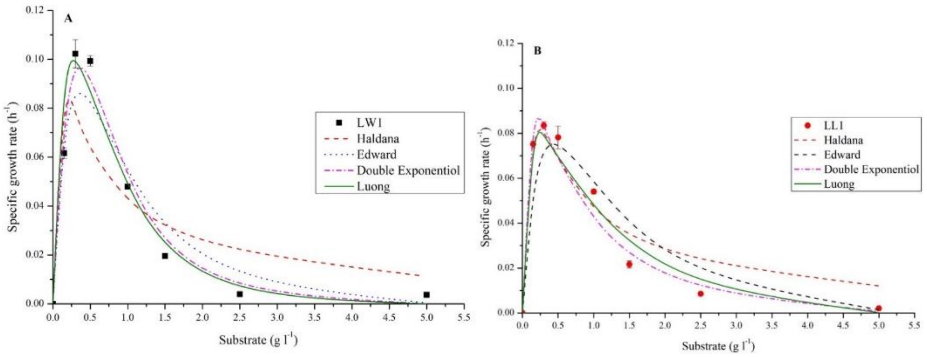

Figure 4 The plot of initial substrate concentration vs. specific growth rate (h^{-1}) for LW1, B for LL1

4. Conclusions

Aerobic bacteria, which can utilize caffeine as a carbon and nitrogen source for their catabolism, have been isolated and identified from the faces of Asian palm civet (Paradoxurus hermaphrodite). Caffeine Agar Medium with caffeine concentration of 1.5 to 10 g/L have selected 2 isolates coded LW1 and LL1 that able to grow on the medium with maximum caffeine concentration of 7 g/L and 10 g/L respectively, but their growth was poor. Those potential isolates were identified using 16s rRNA gene sequence analysis of which classified as Klebsiella sp. Both of them showed a good degradation ability in a Caffeine Liquid Medium containing caffeine of 0.5 g/L and 1 g/L. Those bacteria have potentially used C8-oxidation as their catabolic pathway. The specific growth rate of LW1 and LL1 can be probably increased by acclimatizing the strain in an enrichment medium containing a higher concentration of caffeine. Further treatment using a different medium with optimized addition of other minerals or carbon or nitrogen sources was needed to enhance their caffeine-degradation ability. Among the various models considered, the best-fitted kinetic data for LW1 and LL1 resulted by a double exponential model. The kinetic parameters for LW1 were μm = 0.299, Ki = 0.587, and Ks = 0.226, while for LL1 were μm = 0.112, Ki = 1.00, and Ks = 0.090.

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