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Screening and identification of indigenous cellulolytic bacteria from Indonesian coffee pulp and investigation of its caffeine tolerance ability

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ABSTRACT

Aims: The objective of the research was to get the potential cellulolytic bacteria which was caffeine tolerance from Indonesian coffee pulp waste.
Methodology and results: The cellulolytic bacteria were isolated from coffee pulp wastes of *Coffea arabica* and *C. canephora*. These isolates were selected based on their cellulose hydrolysis, CMCase activity, and caffeine tolerance. The density of cellulolytic bacteria of *C. arabica* pulp waste was 4.7 ± 3.5 × 10^6 CFU/g, and that of *C. canephora* pulp waste was 1.5 ± 1.5 × 10^6 CFU/g. Among 61 cellulolytic bacterial isolates, 24 isolates formed clear zones on CMC medium with Gram iodine flooding. Three isolates (CRM10, CRM1, and CRM12) from *C. canephora* pulp waste had the highest cellulolytic activity. Based on the CMCase activity, it was indicated that an isolate of CRM10 showed the highest CMCase activity with 3.38 ± 0.65 U/mL. This bacteria had tolerance ability to caffeine until 0.4% on nutrient agar medium. Isolates of CRM10 had similarity to *Bacillus subtilis* based on 16S rDNA sequence.
Conclusion, significance, and impact of study: CRM10 was identified as *Bacillus subtilis* and considered as a potential isolate to degrade cellulose of coffee pulp waste that contained caffeine.
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Keywords: Cellulolytic bacteria, caffeine tolerance, coffee pulp waste

INTRODUCTION

Indonesia is the fourth largest coffee producer in the world after Brazil, Vietnam, and Colombia. The coffee production in Indonesia was amounted more than 660 thousand tons in 2015 (Wordatlas, 2017). It included robusta coffee (*Coffea canephora*) and arabica coffee (*C. arabica*).

Coffee processing industry generates about 50% waste (Menezes *et al.*, 2013) and the largest waste is coffee pulp. Best potency utilization of coffee pulp waste is used as organic fertilizer (Rojas *et al.*, 2003; Dzung *et al.*, 2013). Organic fertilizer is a source of energy and nutrients for soil microorganisms that capable of increasing the nutrients for plants growth and improving soil quality either directly or indirectly. If the chemical fertilizers were replaced by 20-30% with bacteria-enriched coffee pulp compost by *Azotobacter* sp. and *Bacillus megaterium*, it will increase the 14% plant growth. The addition of coffee pulp waste compost as fertilizer could increase the physical (bulk density, particle density and pore space) and chemical (percentage of N, P, K, and organic carbon) properties of the soil compared to the 100% chemical-fertilizer (Dzung *et al.*, 2013).

Coffee pulp waste contains 16.2 to 29.4% cellulose (Sanchez *et al.*, 1999; Silva *et al.*, 2013). Cellulose is carbohydrate macromolecules which became the largest component of the plant's lignocellulose. Chemically, cellulose is a linear polymer of 100 to 10,000 subunits of D-glucose (Bayer *et al.*, 2013). The degradation of cellulose into glucose is an important step for the utilization of coffee pulp waste.

Cellulolytic bacteria are the microorganisms that capable to hydrolyze cellulose to glucose. This bacteria has a complex enzyme which consists of endoglucanases (EC 3.2.1.4), cellobiohydrolases (1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91), and β-glucosidases (β-D-glucoside glucohydrolase; EC 3.2.1.21) (Bhalla *et al.*, 2013). Some cellulolytic bacteria have been studied and characterized from coffee pulp waste in coffee-producing areas in India and Vietnam (Divakaran and Elango, 2009; Bui, 2014). However, there were no reports yet about

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indigenous cellulolytic bacteria from Indonesian coffee pulp.

Naturally, coffee pulp contains caffeine. The coffee pulp of *C. canephora* and *C. arabica* contained caffeine are 0.12% and 0.26% respectively. Caffeine is an alkaloid purine as a secondary metabolite of the coffee plant and has an antibacterial activity. Several bacteria are intolerant to caffeine, such as *Escherichia coli*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Paracoccus yeei*, *Bacillus subtilis*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella typhimurium* (Ramanaviciene *et al.*, 2003; Dash *et al.*, 2008; Al-Janabi, 2011). The study of coffee pulp indigenous cellulolytic bacteria that tolerant to caffeine is important to get the biodegradation agent of coffee pulp waste.

MATERIALS AND METHODS

Coffee pulp sampling

The coffee pulp of *C. arabica* was collected from Jampit coffee plantation at approximately 1349 m above sea level (8°00'46.87''S and 114°08'07.19''E), whereas *C. canephora* pulp was collected from Malangsari coffee plantation at approximately 954 m above sea level (8°21'34.34''S and 113°56'43.37''E). The location of coffee plantations is in East Java Province, Java Island, Indonesia. There were two kinds of pulp: fresh pulp and decomposed pulp naturally (± 3 months). The fresh pulp was composited from the factory waste while decomposed pulp was obtained from three different random samples in in each location.

Analysis of samples content of organic carbon (OC) was done by using Walkley and Black method, the content of total nitrogen (N) was done by using Kjeldahl method and moisture content was done by using gravimetric (Alef *et al.*, 1995). It was to determine the ratio of organic carbon and total N of each sample. The data of samples were analyzed by independent sample T-Test with α 0.05.

Enumeration and isolation of cellulolytic bacteria

Enumeration of cellulolytic bacteria of coffee pulp waste was carried out according to Sutton (2011). Twenty-five gram of coffee pulp sample was added to 225 mL of physiological salts solution (0.85%) and suspended. The sample suspension was diluted from 10^{-1} to 10^{-7} and taken 0.1 mL from each dilution level. The aliquots were spread on a minimum M9 medium with 0.1% of Carboxymethylcellulose (CMC) as a carbon source in Petri dish and incubated at 30 °C for 3 days. The Minimum M9 medium consist of $Na_2HPO_4 \cdot 12H_20$ 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. Bacteria isolates that grown and formed a clear zone around the colony on the CMC medium were counted based on Total Plate Count (TPC) using colony counter. The cellulolytic bacteria density of samples was analyzed by independent sample T-Test with α 0.05.

A single colony of bacteria isolates that grew on CMC medium was futher isolated using a Nutrient Agar (NA) medium. A pure culture of the bacteria was preserved by added 200 mL of glycerol into slant culture then stored in a freezer at −20 °C.

Screening of cellulolytic bacteria base on cellulose degrading activity

The experiment was carried out according to Complete Randomized Design with isolates as treatment and 3 replications. One loop of each cellulolytic bacteria was inoculated into 10 mL of Nutrient Broth (NB) in 50 mL Erlenmeyer flash then incubated incubated at room temperature, 120 rpm for overnight. The plate of 1% CMC medium on the Petri dishes was made holes by cork borer 0.5 cm. The culture suspensions 40 µL with equal cell density [1.4 optical density (OD) at 600 nm] were filled into each hole then incubated at 30 °C for 3 days.

Cellulose degradation activity was detected by flooding Iodine solution on the agar plate for 5 min (Kasana *et al.*, 2008). A clear zone which surrounded the hole was regarded as a positive hydrolysis activity (cm). The activity reflected the cellulose degradation activity. The data was done an analysis of variance (ANOVA) with α 0.05. The bacteria isolates with the highest cellulolytic activity (largest clear zone) was selected to assay base on CMCase activity.

Screening of cellulolytic bacteria base on CMCase activity

The experiment was carried out according to Complete Randomized Design with isolates as treatment and 3 replications. One loop of each selected cellulolytic bacteria was inoculated into 10 mL Nutrient Broth (NB) in 50 mL Erlenmeyer flask and incubated at room temperature, 120 rpm for overnight. The culture suspensions 5 mL with equal cell density (1.4 OD at 600 nm) were inoculated on 100 mL M9 Broth medium contained 1% CMC + 1% yeast extract in 250 mL Erlenmeyer flask and incubated on the rotary shaker at 30 °C, 120 rpm for 3 days. The bacteria culture were centrifuged at 10,000 rpm for 10 min, the supernatant was collected as a crude enzyme and stored at −20 °C.

CMCase activity was measured by using Nelson and Somogyi method according to Singh *et. al.* (2014) with modification. The enzyme solution (100 µL) was mixed with 500 µL of 1 % (w/v) CMC dissolved in phosphate buffer (pH 6.2) and incubated at 37 °C for 2 h. The negative control was prepared by adding boiled enzyme. The solution boiled on 100 °C for 15 min, then added with 500 µL Nelson solution and centrifuged at 5.000 rpm for 1 min. The absorbance was measured at 500 nm using UV-visible spectrophotometer against a blank with D-glucose as standard. One unit (U) of cellulose activity was defined as the amount of enzyme necessary to release 1 µmol

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reducing sugar (glucose) per minute, expressed as units per milliliter.

Caffeine tolerance analysis of selected cellulolytic bacteria

The selected bacteria culture with 24 h incubation time in NB medium were made equal cell density (OD=1.4 at 600 nm). The culture 5 µL of each isolate was inoculated into 15 mL NA medium contained 0.1 %, 0.2 % and 0.4 % caffeine and into M9 medium contained 0.1 % caffeine as carbon source based on drop plate method on Petri dish and incubated at 30 °C for 48 h. Pure isolate KRM9 caffeine-degrading bacteria (GenBank: KY319030.1) was used as positive control.

Identification of cellulolytic bacteria base on 16S rDNA sequence

Bacteria's DNA was extracted according to Wilson (2003) with modification. The CTAB/NaCl buffer solution was consist of 41 g NaCl and 100 g CTAB per liter of solution. Bacterial cell biomass from agar plates was resuspended in 567 µL of TE and added with 6 µL of lysozyme (30 mg/mL) then incubated for 2 h, 37 °C. The DNA sample solution added with 30 µL 10% SDS and 3 µL proteinase K (20 mg/mL) and incubated at 45 °C for 45 min. The mix solution of 100 µL 5 M NaCl and 80 µL CTAB/NaCl (pre-heated to 60 °C) were added into DNA sample solution and it was incubated at 65 °C, 10 min. After that, 0.5 mL of chloroform:isoamyl alcohol was added to the solution (24:1) and centrifuged 10,000 rpm 4 °C for 5 min. The aqueous phase was taken and replaced and extracted with 0.5 mL of phenol:chloroform:isoamyl alcohol (25:24:1). The solution centrifuged 10,000 rpm 4 °C for 5 min and the aqueous phase was taken and replaced then extracted again with 0.5 mL PCI and the organic phase was discarded. DNA in the aqueous phase was precipitated by ethanol absolute and stored at −20 °C overnight. The precipitate added 0.5 mL ethanol 70%, centrifuged for 5 min, then resuspended in 100 µL TE and incubated at 55 °C for 10-15 min.

The 16S rDNA sequence was amplified by Polymerase Chain Reaction (PCR) using primers 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1495R (5'-CTACGGCTACCTTGTTACGA-3') (Yang *et al.*, 2015). The thermal profile consisted of 35 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 30 sec, and an elongation at 72 °C for 90 sec, followed by a final elongation at 72 °C for 5 min. The amplicon of partial 16S rDNA was purified and sequenced in First Base, Malaysia. Partial sequences of 16S rDNA were alignment with 16S rDNA reference sequence from database in Gene Bank (http://www.ncbi.nlm.nih.gov) using BLAST to identify bacteria isolate based on highest percentage of nucleotide similarity. Phylogeny tree was constructed based on a neighbor-joining algorithm using MEGA 6 for Windows.

Phenotype characterization of selected bacteria

Phenotype of bacteria were characterized consist of Gram reaction, reduction of nitrate, fermentation of carbohydrates (glucose, fructose, sucrose, lactose), production of indole, motility at 30 °C, citrate utilization, H_2S production, viability on pH (3,5,7,9) and viability on temperature (30, 35, 45, 55 °C). All tests were done using 24 h old cultures (Cappuccino and Sherman, 1996).

RESULTS AND DISCUSSION

Density of cellulolytic bacteria

The density and activity of bacteria on the substrate were influenced by the physiochemical characteristics carried by some substrates such as the organic carbon (OC), Nitrogen (N) and C/N ratio (Table 1). The bacteria require OC to construct cells and to produce energy in a metabolism process. Nitrogen helps the cells to compose protein, nucleic acid, amino acid and enzymes which are vital to the microorganism growth and activities (Tuomela *et al.*, 2000). An adequate C/N ratio to support the composition process on a substrate is 25-30. This ratio can be explained by the fact that bacteria need 30 carbon for a metabolism per nitrogen unit (Sanchez *et al.*, 1999; Sriakilam *et al.*, 2016).

The density of the cellulolytic bacteria on both decomposed *C arabica* and *C. canephora* pulp was very high, that was 10^6 CFU/g. The density of cellulolytic bacteria $4.7 \pm 3.5 \times 10^6$ CFU/g of decomposed *C. arabica* pulp was higher than $1.5 \pm 1.5 \times 10^6$ CFU/g of *C. canephora* (Table 1). This could happen because the fresh *C. arabica* pulp contained lower OC (29.43 %) compared to *C*. *canephora* (45.35%), and it contained higher nitrogen (1.67 %) than *C. canephora* did (0.48 %). The *C. arabica* pulp has lower C/N ratio (17.64) than *C*. *canephora* (93.94). This ratio provided more ideal nutrition (C and N) for growing of the cellulolytic bacteria in *C. arabica*. The lower C/N ratio, the easier the bacteria to be degraded coffee pulp. As a result, the bacteria grew faster and produced biomass or higher cell density. The C/N ratio (93.94) on fresh *C. canephora* pulp was very high. The C/N ratio more than sufficient range (≥30) make the degradation process very slow as there is an excess of degradable substrate for the microorganisms (Bernal *et al.*, 2009). This lowered its number of the cellulolytic bacteria compared to the decomposed *C. arabica* pulp.

The OC content in decomposed *C. arabica* and in *C. canephora* pulp were 16.4 ± 2.3% and 16.4 ± 2.0%, respectively even tough based on the statistic, there was no difference between those two (Table 1). This indicates that OC content of both decomposed pulp was less than of the fresh pulp (Table 1). The substrate lost OC because CO_2 was produced in the decomposition process (Cheng and Jhonson, 1998; Saha *et al.*, 2012). Furthermore, the N content of decomposed *C. arabica* pulp (0.1 ± 0.1%) was lower than on fresh pulp (1.67%) and it was significantly lower than 0.6 ± 0.1% of the decomposed pulp *C. canephora* ($p < 0.05$). This condition

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produced C/N ratio on *C. arabica* (170.1 ± 43.5%) which was higher ($p < 0.05$) than that on *C. canephora* (29.5 ± 8.7%). This occurred because C/N ratio (17.64) on fresh *C.arabica* pulp was less than 25 which showed excess nitrogen. At the composting stage, ammonia would be produced and nitrogen would be lost to evaporation (Bernal *et al.*, 2009; Xie *et al.*, 2012).

Table 1: The density of cellulolytic bacteria and physicochemical characteristics on coffee pulp.

Parameters	Pulp				
	Fresh		Decomposed		
	C. arabica	*C. canephora*	*C. arabica*	*C. canephora*	P value
Density of cellulolytic bacteria (CFU/g)	nt	nt	$4.7 \pm 3.5 \times 10^6$	$1.5 \pm 1.5 \times 10^6$	0.186
C-organic (%)	29.43	45.35	16.4 ± 2.3	16.4 ± 2.0	1
N-total(%)	1.67	0.48	0.1 ± 0.1	0.6 ± 0. 1	0.003
Ratio C/N	17.64	93.94	170.1 ± 43.5	29.5 ± 8.7	0.005
Moisture content (%)	nt	nt	65.56 ± 8.88	81.36 ± 5.29	0.057

nt, not tested

p value > 0.05 on decomposed pulp indicate do not differ significantly (α ≥0.05) according to the independent sample T-Test

The higher number of cellulolytic bacteria contained in decomposed *C. arabica* pulp was affected by its moisture content. The moisture content was approaching an ideal number of 65% and of 60% from the composting solid organic ideal number (Angel *et al.*, 2005; Yong *et al*., 2013; Sriakilam *et al.*, 2016; Qdais *et al*., 2016). Unlike the decomposed *C. arabica* pulp, decomposed *C. canephora* pulp contained 81.36% water. Too much water (> 65%) would cause oxygen depletion and losses of nutrients by leaching on the decomposed of cattle manure:chicken manure:wheat straw (1:3:6) (Wang *et al.*, 2007; Gill *et al.*, 2014). This condition could also lead to an anaerobic situation in which cellulolytic bacteria grow slower (Ryckeboer *et al*., 2003).

Cellulose degrading and CMCase activity of cellulolytic bacteria

The bacteria isolates were screened to determine their ability to hydrolyze the cellulose. The screening was based on clear zone formation around hole using iodine staining (Li and Yu, 2012; Jimenez *et al*., 2013; Venkatachalam *et al*., 2014; Riddech *et al*., 2015). Iodine assay is used to assess the cellulose activity of microorganisms. Iodine staining formed the clear zone by producing bluish-black complex as a reaction with cellulose (Kasana *et al*., 2008). There was 61 isolates origin from decomposed coffee pulp, but there are total 24 isolates (40%) that formed a clear zone, there are 15 isolates from *C. canephora* and 9 isolates from *C. arabica* (Table 2). The study from Vietnam obtain 38 bacteria isolates derived from coffee pulp, 27 isolates among them have cellulolytic activity. Fourteen isolates from the total cellulolytic bacteria isolates were derived from Indian coffee pulp. Based on the identification, those bacteria belong to Genus *Actinomycetes, Bacillus, Clostridium, Pseudomonas*, and *Streptomyces* (Divakaran and Elango, 2009; Bui, 2014).

Table 2: The cellulose degrading activity of some isolates cellulolytic bacteria on M9 medium with carboxymethylcellulose 0.1 % for 3-day incubation.

Isolates	Pulp waste sources	Hydrolysis activity (cm)
CRM1	*C. canephora*	5.2 ± 1.4 g
CRM2	*C. canephora*	3.7 ± 1.0 de
CRM3	*C. canephora*	0.4 ± 0.4 a
CAJ4	*C. arabica*	4. 0 ± 1.1 def
CAJ5	*C. arabica*	0.4 ± 0.4 a
CRM6	*C. canephora*	3.8 ± 1.1 de
CRM7	*C. canephora*	3.5 ± 1.0 de
CRM8	*C. canephora*	4.0 ± 1.1 ef
CRM9	*C. canephora*	0.4 ± 0.4 a
CRM10	*C. canephora*	5.5 ± 1.5 g
CAJ11	*C. arabica*	2.0 ± 0.6 b
CRM12	*C. canephora*	4.4 ± 1.2 f
CRM13	*C. canephora*	3.5 ± 1.0 de
CRM14	*C. canephora*	3.9 ± 1.1 def
CRM15	*C. canephora*	4.0 ± 1.1 ef
CAJ16	*C. arabica*	3.3 ± 1.0 cd
CRM17	*C. canephora*	0.4 ± 04 a
CAJ18	*C. arabica*	3.8 ± 1.0 de
CRM19	*C. canephora*	3.8 ± 1.1 de
CAJ20	*C. arabica*	0.4 ± 0.4 a
CRM21	*C. canephora*	2.9 ± 0.8 c
CAJ22	*C. arabica*	2.0 ±1.2 b
CAJ23	*C. arabica*	0.4 ± 0.4 a
CAJ24	*C. arabica*	1.8 ± 0.5 0b

The same letter within each column do not differ significantly ($p > 0.05$) according to the Duncan test.

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The cellulose degrading activity of 24 isolates is significantly different (Table 2). The cellulolytic bacteria of CRM1 and CRM10 have highest cellulose hydrolysis activity were 5.2 ± 1.4 and 5.5 ± 1.5 cm diameter, respectively. The second highest cellulose hydrolysis activity produced by CRM12 is 4.4 ± 1.2 cm diameter. The bacteria isolates with highest and second highest cellulose hydrolysis activity was origin from decomposed *C. canephora* pulp. Three isolates CRM1, CRM10, and CRM12 were assayed quantitatively based on their CMCase enzyme activity.

CMCase assay is an endoglucanase activity analysis that uses carboxymethyl dissolved cellulose (CMC) as a sole carbon of the substrate. This analysis is common to screened cellulolytic bacteria (Maki *et al*., 2009). The results showed that CRM10 is the best cellulolytic isolate with CMCase activity 3.38 ± 0.65 U/mL (Figure 1). The CRM10 of cellulolytic bacteria had the highest cellulose hydrolysis activity by clear zones (Table 2) and it had has the highest CMCase activity as well. This indicates that the qualitative test based on the clear zone provide information regarding the quantitative cellulase activity. According to Kasana *et al*. (2008), this method can be applied to screen a numerous amount of cellulolytic bacteria because it is not toxic and fast respond to analyze. According to previous research, the CMCase activity of CRM10 was higher than Tibetan pig's intestine isolate bacteria BY-2 a (2.91 U/mL) (Yang *et al.*, 2014).

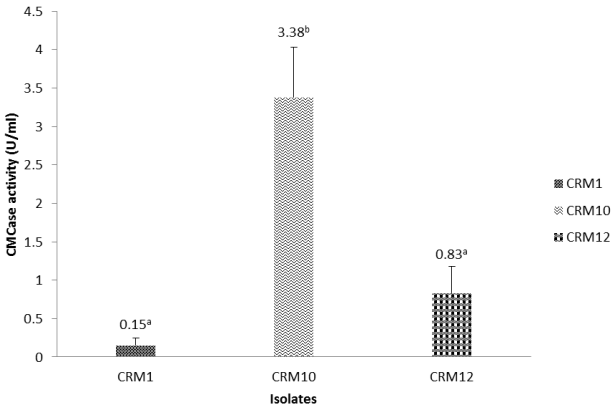

Figure 1: CMCase activity of selected cellulolytic bacteria on substrat CMC 1% for 3 days incubation. Error bars indicate standard deviation above and below the mean (n=3).

Caffeine tolerance of cellulolytic bacteria

Coffee pulp waste contains caffeine 0.12 until 0.26%, that has an antibacterial effect. This compound is capable suppress the bacteria life cycle by degrading its cell wall and block the mechanism of DNA synthesis (Sandlie *et al*., 1980, Dash *et al*., 2008). Cellulolytic bacteria that able tolerance towards caffeine is a necessary condition for coffee pulp waste degradation using bacteria.

The indigenous bacteria CRM10 obtained from this research successfully grew on NA medium contained 0.1%, 0.2% and 0.4% of caffeine, and CRM1, as another indigenous bacteria was, did not grow on NA medium plus to 0.1% and 0.2% caffeine (Table 4). This result showed that CRM10 had ability tolerant to caffeine and CRMI did not have it. The result showed that were not all of the indigenous isolates from coffee pulp waste tolerance to caffeine.

Species of cellulolytic bacterial isolate CRM10

The Isolate CRM10 has a gene of 16S rDNA 1415 bp (GenBank: KY010306.1). Based on 16S rDNA sequence alignment showed that CRM10 had 99% similarity with *Bacillus subtilis* subsp. *subtilis* NBR 13719 (Figure 2).

Table 3: Phenotype characteristics of isolate *Bacillus subtilis* CRM10.

Characteristics	Result
Gram-positive	+
Catalase	+
Reduction of nitrate	+
Acid from	
Sucrose	+
Fructose	+
Glucose	+
Lactose	+
Indol production	-
Motility	+
Citrate utilization	-
H_2S production	-
Viability on	
pH 3	-
pH 5	-
pH 7	+
pH 9	-
Viability on	
30 ^{o}C	+
35 ^{o}C	+
45 ^{o}C	+
55 ^{o}C	+

+, positive; -, negative

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Table 4: Caffeine tolerance of cellulolytic bacteria.

Isolates	Medium				
	NA	NA + Caffeine 0.1 %	NA + Caffeine 0.2 %	NA + Caffeine 0.4 %	M9 + Caffeine 0.1 %
CRM1	++	-	-	-	-
CRM10	++	++	++	+	-
CRM12	++	++	+	-	-
KRM9	++	++	++	++	++

Note : ++ = Grow well, + = grow, - = no grow after 48 h incubation on 30 °C

Some species of *Bacillus* were reported had cellulolytic activity (Li and Yu, 2012; Bhalla *et al.,* 2013). Cellulolytic bacteria that had the highest CMCase from Tibetan pig's intestine was a *B. subtilis* (Yang *et al.*, 2014). Biochemical analysis showed that the CRM10 isolate was a Gram-positive, positive catalase activity, reduced nitrates, and capable of producing acid from sucrose, fructose, glucose, and lactose. This bacterium did not produce indole and cannot utilize of citrate. Based on its cultural characteristic, isolate CRM10 only able to grow at pH 7 and can grow up to 55 °C (Table 3).

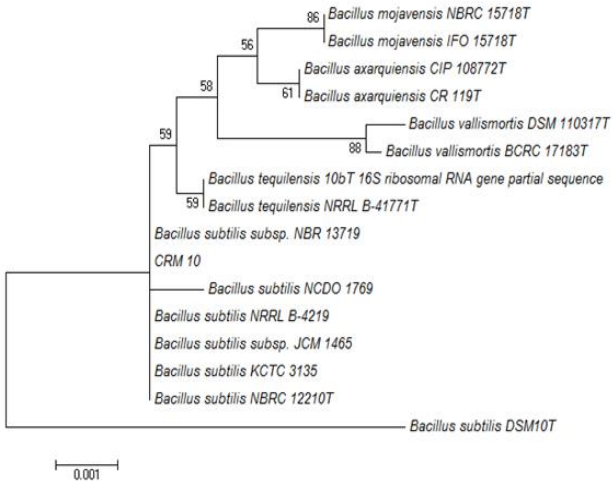

Figure 2: Phylogeny tree of the CRM10 cellulolytic bacteria and reference *Bacillus* based on Neighbor-joining algorithm.

CONCLUSION

Indigenous cellulolytic bacteria CRM10 from coffee pulp waste of *C. canephora* was identified as *Bacillus subtilis* and it had the highest potency as cellulose degrading bacteria caffeine tolerance. This bacteria has potential as an agent to composting of the coffee pulp waste.

REFERENCES

Alef, K. and Nannipieri, P. (1995). Methods in applied soil microbiology and biochemistry. Academic Press. London. **pp. 150-172.**

Al-Janabi, A. A. (2011). Potential activity of the purine compounds caffeine and aminophylline on bacteria. *Journal of Global Infectious Diseases* **3, 133-137.**

Angel, M., Zavala, L., Funamizu, N. (2005). Effect of moisture content on the composting process in a biotoilet system. *Compost Science & Utilization* **13, 208-2016.**

Bayer, E. A., Shoham, Y. and Lamed, R. (2013). Lignocellulose-decomposing bacteria and their enzyme systems. *In*: The Prokaryotes. Rosenberg, E., DeLong, E. F., Lory, S., Stackebrantdt, E. and Thompson, F. (eds.). Springer-Verlag, Berlin. **pp. 216-257.**

Bernal, M. P., Alburquerque, J. A. and Moral, R. (2009). Composting of animal manures and chemical criteria for compost maturity assessment. *Bioresource Technology* **100, 5444-5453.**

Bhalla, A., Bansal, N., Kumar, S., Bischoff, K. M. and Sani, R. K. (2013). Improved lignocellulose conversion to biofuels with thermophilic bacteria and thermostable enzymes. *Bioresource Technology* **128, 751-759.**

Bui, H. B. (2014). Isolation of cellulolytic bacteria, including actinomycetes from coffee exocarps in coffee-producing areas in Vietnam. *International Journal of Recycling of Organic Waste in Agriculture* **3, 1-8.**

Cappuccino, J. G. and Sherman, N. (1996). Microbiology: A laboratory manual. The Benjamin/Cummings Publishing Company. New York. **pp. 99-182.**

Cheng, W. and Johnson D. W. (1998). Elevated CO_2, rhizosphere processes and soil organic matter decomposition. *Plant and Soil* **202, 167-174.**

Dash, S. S., Salaja, N. S. and Gummadi, S. N. (2008). Chemotaxis of *Pseudomonas* sp. to caffeine and related methylxanthines. *Journal of Basic Microbiology* **48, 130-134.**

Divakaran, J. and Elango, R. (2009). Microorganisms consortium for effective composting of coffee pulp waste by enzymatic activities. *Global Journal of Environmental Research* **3, 92-95.**

Dzung, N. A., Dzung, T. T. and Khanh, V. T. P. (2013). Evaluation of coffee husk compost for improving soil fertility and sustainable coffee production in rural central highland of Vietnam. *Resources and Environment* **3, 77-82.**

Gill, S. S., Jana, A.M. and Shrivastav, A. (2014). Aerobic bacterial degradation of kitchen waste: A

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

review. *Journal of Microbiology, Biotechnology and Food Sciences* **3, 477-483.**

Jimenez, D. J., Korenblum, E. and van Elsas, J. P. (2013). Novel multispecies microbial consortia involved in lignocellulose ad 5-hidroxymethylfurfural bioconversion. *Applied Microbiology and Biotechnology* **98, 2789-2803.**

Kasana, R. C., Salwan, R., Dhar, H., Dutt, S. and Gulati, A. (2008). A rapid and easy method for the detection of microorganisms cellulases on agar plates using Gram's iodine. *Current Microbiology* **57, 503-507.**

Li, X. and Yu, H. Y. (2012). Purification and characterization of an organic-solvent-tolerant cellulase from a halotolerant isolate, *Bacillus* sp. L1. *Journal of Industrial Microbiology and Biotechnology* **39, 1117-1124.**

Maki, M., Leung, K. T. and Qin, W. (2009). The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *International Journal of Biological Sciences* **5, 500-516.**

Menezes, E. G., de Carmo, J. R., Menezes, A. G., Alves, J. G., Pimenta, C. J. and Queiroz, F. (2013). Use of different extracts of coffee pulp for the production of bioethanol. *Applied Biochemistry and Biotechnology* **169, 673- 687.**

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

Qdais, H. A. and Al-Widyan, M. (2016). Evaluating composting and co-composting kinetics of various agro-industrial wastes. *International Journal of Recycling of Organic Waste in Agriculture* **5, 273-280.**

Ramanaviciene, A., Mostovojus, V., Bachmatova, I. and Ramanavicius, A. (2003). Anti-bacterial effect of caffeine on *Escherichia coli* and *Pseudomonas fluorescens*. *Acta Medica Lituanica* **10, 185-188.**

Ryckeboer, J., Mergaert, J., Vaes, K., Klammer, S., De Clerco, D., Coosemans, J., Insam, H. and Swings, J. (2003). A survey of bacteria and fungi occurring during composting and self-heating processes. *Annals of Microbiology* **53, 349-410.**

Riddech, N., Saharm, N., Chaisawang, C., Pongtongmee, P., Boonchern, S., Sarin, P. and Phibunwatthanawong, T. (2015). Multifunctional cellulolytic activities from *Streptomyces osmaniensis* for agricultural and enzyme industry *Malaysian Journal of Microbiology* **12, 85-90.**

Rojas, J. B. U., Verreth, J. A. J., Amato, A. and Huisman, E. A. (2003). Biological treatments affect the chemical composition of coffee pulp. *Bioresource Technology* **89, 267-274.**

Saha, N., Mukherjee, D. Sen, S., Sarkar, A., Bhattachharaya, K. K., Mukhopadyay, N. And Patra, P. K. (2012). Application of highly efficient lignocellulolytic fungi in composting of paddy straw amended poultry droppings for the production of humus rich compost. *Compost Science and Utilization* **20, 239-244.**

Sanchez, G., Olguin, E. J. and Mercado, G. (1999). Accelerated coffee pulp composting. *Biodegradation* **10, 35-41.**

Sandlie, I., Solberg, K. and Kleppe, K. (1980). The effect of caffeine on cell growth and metabolism of thymidine in *Escherichia coli*. *Mutation Research* **73, 29-41.**

Silva, C. F., Schwan, R. F., Dias, E. S. and Wheals, A. E. (2000). Microbial diversity during maturation and natural processing of coffee cherries of *Coffea arabica* in Brazil. *International Journal of Food Microbiology* **60, 251-260.**

Silva, C. F., Vilela, D. M., de Souza, C. C., Duarte, W. F., Dias, D. R. and Schwan, R. F. (2013). Evaluation of a potential starter culture for enhance the quality of coffee fermentation. *World Journal of Microbiology and Biotechnology* **29, 235-247.**

Singh, S. and Moholkar, V. S. (2014). Optimization of carboxymethyl cellulose production from *Bacillus amyloliquefaciens* SS35. *3 Biotech* **4, 411-424.**

Sriakilam, C., Saseetharan, M. K. and Jeyapriya, S. P. (2016). Studies on aerobic compost from the solid waste generated in Coimbatore City, Tamilnadu, India. *Nature Environment and Pollution Technology* **15, 393-400.**

Sutton, S. (2011). Accuracy of plate counts. *Journal of Validation Technology* **17, 42-46.**

Tuomela, M., Vikman, M., Hatakka, A. and Itavaara, M. (2000). Biodegradation of lignin in a compost environment: A review. *Bioresource Technology* **72, 169-183.**

Venkatachalam, S., Sivaprakash, M., Gowdaman, V. and Prabagaran, S. R. (2014). Bioprospecting of cellulase producing extramesophilic bacterial isolates from India. *British Microbiology Research Journal* **4, 138-150.**

Yang, W., Meng, F., Peng, J., Han, P., Fang, F., Ma, L. and Cao, B. (2014). Isolation and identification of a cellulolytic bacterium from the Tibetan pig's intestine and investigation of its cellulase production. *Electronic Journal of Biotechnology* **17, 262-267.**

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.**

Yong, C., Xiaoli, W. and Shuaixia, L. (2013). Analysis of the factors influencing the sludge aerobic microbial compost using orthogonal mode. *Information Technology Journal* **12, 4715-4719.**

Wang, W., Wang, X., Liu, J., Ishii, M., Igarashi, Y. and Cui, Z. (2007). Effect of oxygen concentration on the composting process and maturity. *Compost Science and Utilization* **15, 184-190.**

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

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

Wilson, K. (2003). Preparation of genomic DNA from bacteria. *In*: Current Protocols in Molecular Biology. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (eds.). John Wiley and Sons, New Jersey. **Suplement 56.**

Xie, K., Jia, X., Xu, P., Huang, X., Gu, W., Zhang, F., Yang, S. and Tang, S. (2012). The addition of modified attapulgite reduces the emission of nitrous oxide and ammonia from aerobically composted chicken manure. *Journal of the Air and Waste Management Association* **62, 1174-1181.**

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