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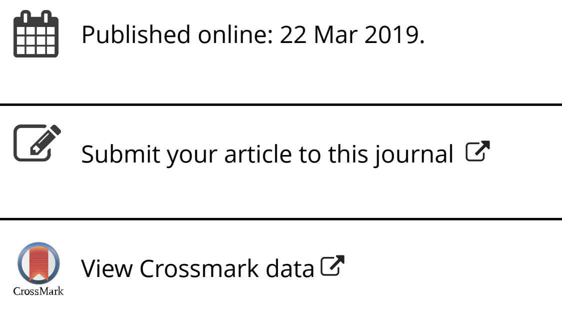

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Isolation and identification of caffeine-degrading bacteria from coffee plantation area

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ABSTRACT
Decaffeination of food and beverage products is in high demand. In this study, a caffeine-degrading bacterium *Burkholderia* spp. was isolated from coffee plantation area of Chiang Mai province of Thailand. The bacterial isolates were first identified by morphological, physiological, and biochemical tests followed by 16S rDNA analysis. The bacterial isolate of *Burkholderia* spp. showed 45.5% of caffeine degradation in caffeine containing media (2.5 g/L) after 110 h of incubation period. *Burkholderia* spp. showed only 2.6% caffeine degradation when exposed to high concentrations of caffeine containing medium (20 g/L). The growth rate of *Burkholderia* spp. declined with the increase in the caffeine concentration, which indicated the inhibiting effect of caffeine at very high concentrations. The maximum growth rate of 0.053 h^{-1} was observed at 2.5 g/L of caffeine. Overall due to high caffeine tolerance and biodegradation of caffeine, *Burkholderia* spp. can be effectively used to degrade caffeine from agro-industrial wastes targeted for value added food applications and environmental remediation.

KEYWORDS
Decaffeination; caffeine tolerance; growth rate; biodegradation; *Burkholderia* spp

1. Introduction

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid and one of the most widely consumed pharmacologically active substances. Naturally, it is found in more than 60 plant species including coffee, cocoa, tea, kola nut, and guarana berries (Ashihara and Crozier 2001; Yu et al. 2014). Caffeine is incorporated into various food and beverage products like ice creams, pastries, sweets, carbonated beverages, and energy drinks (Algharrawi, Summers, and Subramanian 2017). Some of the food supplements for weight loss and sports performance contain caffeine along with *p*-synephrine (EFSA, 2015). Caffeine is also widely used in pharmaceutical preparations as central nervous system, cardiac, cerebral, and respiratory stimulant (Buerge et al. 2003). Various painkillers (such as aspirin and acetaminophen) are used in combination with caffeine for migraine headache treatment (Shapiro 2007).

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Coffee is one of the major plantation crops and the world's second most tradable commodities after oil. Coffee processing produces huge amount of waste that is hazardous in terms of its removal and has negative impact on health of humans, animals, as well as the environment (Nanjundaiah, Mutturi, and Bhatt 2017). The caffeine containing agro-industrial waste is discarded into the environment that contaminates the surrounding soil and water as caffeine cannot be easily hydrolyzed and degraded in nature (Mazzafera 2002; Pandey et al. 2000). In the human body, 3% of the whole intake of caffeine is found in urine, thus polluting the soil and water (Bolignano et al. 2007). Caffeine was found as one of the most frequent compounds with high concentration in groundwater samples in Europe during a survey on the occurrence of selected polar organic persistent pollutants in groundwater (Loos et al. 2010).

The conventional methods used for caffeine removal such as supercritical fluid and solvent extraction are expensive and involve hazards. The biodegradation of caffeine by microorganisms and enzymes is more specific, eco-friendly, sustainable, and relatively a low-cost strategy to overcome the problems associated with conventional methods (Ibrahim et al. 2016). The caffeine-degrading bacterial species have been isolated from several sources during the past few decades, which can be used in decaffeination of food products (Mohapatra et al. 2006). The biodegradation of caffeine in coffee pulp can be an alternative approach to utilize it as valuable nutritional source. The bio-decaffeination is found as an effective and alternative method to remove caffeine from caffeine contaminating solid or liquid wastes or fresh water by using caffeine-tolerant microbes (bacteria, yeast, fungi). The caffeine degrading microorganisms are explored in naturally occurring microflora from the caffeine containing waste (Ibrahim et al. 2016). Bacterial strains belonging to *Serratia* and *Pseudomonas* and fungi species of *Aspergillus, Penicillium, Stemphylium, Rhizopus*, and *Phanerochaete* are reported to be capable of degrading caffeine (Gokulakrishnan, Chandraraj, and Gummadi 2007). The bacterial caffeine degradation exhibits two major metabolic pathways: N-demethylation and C-8 oxidation. Both pathways degrade caffeine into ammonia and carbon dioxide to harvest energy (Summers et al. 2015). The ability of bacteria to grow on caffeine as sole carbon and nitrogen source has been known since many decades. During demethylation, caffeine is sequentially N-demethylated to form xanthine and finally xanthine enters purine catabolic pathway (Summers et al. 2015; Yu et al. 2014). Whereas C-8 oxidation converts caffeine into 1,3,7-trimethyluric acid, which eventually enters the uric acid homologous metabolic pathway (Mohanty et al. 2012; Yu et al. 2008). The high concentration of caffeine restricts the bacterial growth; however, *Pseudomonas* spp. was found capable of growing in the medium containing >5 g/L of caffeine (Dash and Gummadi 2007).

This study aims to isolate the high caffeine-tolerable and caffeine-degrading bacteria from coffee plantation soil area of Chiang Mai province of Thailand

with potential for future applications in food decaffeination and environmental remediation of caffeine contamination.

2. Material and methods

2.1. *Chemicals and media*

Pure caffeine (>99%) used for caffeine degradation experiments was purchased from Sigma-Aldrich, USA. The bacterial growth media including nutrient agar and nutrient broth were obtained from Himedia, India. For screening and isolation of caffeine-tolerant bacteria, caffeine-containing media was prepared with mineral solution from Difco, USA (7.5 g/L $Na_2HPO_4.2H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 0.5 g/L $MgSO_4.7H_2O$, and 0.015 g/L $CaCl_2$ with pH 7 ± 0.1) as mentioned by Ashengroph and Ababaf (2013). Solid screening medium (SSM) for isolating the caffeine-tolerant bacteria was made by mixing mineral solution with 2.5 g/L of caffeine and agar (2.7% w/v, Himedia, India) and autoclaved at 121°C for 15 min. Solid purifying medium (SPM) was prepared using SSM but, with different concentration of caffeine (2, 3, 4, and 5 g/L). All other chemicals used were of analytical grade.

2.2. *Isolation of caffeine-degrading bacteria*

Soil samples were collected under arabica coffee trees growing at plantation area in Chiang Mai province of Thailand at various depths (0, 15, and 30 cm) of the soil from ten different locations. After soil samples collection, aseptic condition was maintained throughout the bacterial isolation procedure. The collected soil samples were stored at low temperature (4°C) in the dark until further use (Gokulakrishnan, Chandraraj, and Gummadi 2007). The caffeine-degrading bacteria were isolated from soil, purified, and amplified by the method as described by Gao et al. (2016), with slight modifications. The soil samples (10 g) were added into 100 mL of distilled water for 30 min and subjected to shaking at 100 rpm (Velp Scientifica, Europe) for 5 min, followed by centrifugation (Camloc Leicester, England) at 940 ×*g* for 15 min at room temperature (25°C). The supernatant was collected into a 250-mL conical flask containing 100 mL mineral solution added with 5 g/L caffeine. After 72 h enrichment, the microbial solution obtained by filtration (Whatman filter paper No. 1, GE Healthcare UK) was diluted to 10^3–10^6 times with distilled water and 0.2 mL of the solution for each sample was spread over SSM surface in petri dishes. After 72 h of incubation at 30°C, fast-growing single bacterial colonies were picked and transferred to SPMs by streak plating method and incubated for 48 h. For further purification, streak plating of the colonies was repeated on the same media and caffeine-tolerant bacterial colonies were observed under microscope for morphological examination.

The colonies with similar morphology were picked again from SPMs as inoculum and transferred to the tubes containing 10 mL nutrient broth with 1 g/L caffeine (each colony for one tube) for amplification. The tubes were further incubated for 24 h at 30°C. The bacterial culture was then added to sterile aqueous glycerol (60%, v/v) to maintain the bacterial stock (50%, v/v) in glycerol, and stored at −70°C for further tests.

2.3. *Identification of caffeine-degrading bacteria*

2.3.1. *Morphological, physiological, and biochemical tests*

Different morphological (cell size, shape, colony surface, and color), physiological, and biochemical tests (Glucose fermentation, lactose fermentation, Methyl Red Voges Proskauer (MRVP), citrate utilization, oxidase test, starch hydrolysis, indole and hydrogen sulfide production tests, gelatin hydrolysis, urease, nitrate reduction, and arginine dihydrolase tests) were carried out for the identification of caffeine-degrading bacteria as described in Bergey's manual of determinative bacteriology (Buchanan and Gibbons 1974).

2.3.2. *Identification of caffeine-degrading bacteria by amplification of 16S rDNA*

The caffeine-degrading bacterial solution (0.5 mL) was centrifuged (Camloc Leicester, England) at 4000 rpm for 5 min, washed with phosphate buffer solution and suspended in distilled water (0.1 mL). DNA templates for polymerase chain reaction (PCR) amplification were prepared by using "Genomic DNA mini kit (blood/culture cell)" (Geneaid Biotech Ltd., Taiwan). DNA coding for 16S rDNA regions was amplified by means of PCR with Taq polymerase, as described by Yamada et al. (2000) and Katsura et al. (2001). A PCR product for sequencing 16S rDNA regions was prepared by using the two primers; 20F (5′-GAG TTT GAT CCT GGC TCA G-3′, positions 9–27 on 16S rDNA by *Escherichia coli* numbering system and 1500R (5′-GTT ACC TTG TTA CGA CTT-3′, position 1509–1492 on 16S rDNA by the *E. coli* numbering system (Brosius et al. 1981). The PCR amplification was carried out by DNA Engine Dyad® Thermal Cycler (Bio-Rad Laboratories). The 100 µL of reaction mixture containing 15–20 ng of template DNA, 2.0 µmol of each of the two primers, 2.5 units of Taq polymerase, 2.0 mM $MgCl_2$, 0.2 mM dNTP, and 10 µL of 10xTaq buffer at pH 8.8 maintained by 750 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$, and 0.1% Tween 20. The PCR amplification was programmed to carry out an initial denaturation step at 94°C for 3 min, 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 2 min, followed by a final extension step at 72°C for 3 min. The PCR product was analyzed by 0.8% (w/v) agarose gel electrophoresis and purified with a gene pH low TM Gel/PCR Kit (Geneaid Biotech Ltd.). The purified PCR product was stored at −20°C for sequencing.

Direct sequencing of the single-banded and purified PCR products (ca. 1500 bases, on 16S rDNA by the *E. coli* numbering system; Brosius et al. 1981) was carried out. Then, sequencing of the purified PCR products was performed on an ABI Prism® 3730XL DNA Sequence (Applied Biosystems, Foster City, CA, USA) by sequencing service provider (Macrogen Inc., Korea). The two primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) or 800R (5′-TAC CAG GGT ATC TAA TCC-3′) and 518F (5′-CCA GCA GCC GCG GTA ATA CG-3′) or 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) for single-strand 16S rDNA sequencing, and four primers of 27F, 518F, 800R, and 1492R for double-strand 16S rDNA sequencing were used (Anzai, Kudo, and Oyaizu 1997; Lane 1991).

The nucleotide sequences obtained from all primers were assembled using Cap contig assembly program, an accessory application in BioEdit (Biological sequence alignment editor) Program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The identification of phylogenetic neighbors was initially carried out by the BLASTN (Altschul et al., 1997) program against the database containing strains with validly published prokaryotic names (Kim et al. 2012). The top 30 sequences with the highest scores were selected for the calculation of pairwise sequence similarity using global alignment algorithm (Myers and Miller 1988), which was implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al. 2012). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987).

2.4. *Caffeine tolerance of isolated bacterial strain*

Nutrient broth with different concentrations of caffeine (0, 2.5, 5, 10, 15, and 20 g/L) was prepared and the isolated bacterial culture (1 mL, of 10^8 CFU/mL) was added to each medium. The bacterial samples were incubated in a rotary shaker (150 rpm) at 30°C. The bacterial growth was measured at $A_{600\ nm}$ with UV-visible spectrophotometer (Shimadzu, Kyoto, Japan) at different time intervals (0–110 h) (Ibrahim et al. 2016). The cell mass concentration was determined at same time intervals (0–110 h) following the method of Ashengroph and Ababaf (2013). Briefly, the samples (2 mL) were collected at each time interval and centrifuged (Camloc Leicester, England) at 5000 rpm for 10 min. The cell pellets were washed with distilled water twice and recentrifuged. Finally, the pellets were dried at 75°C for 36 h and weighed. The specific growth rate (μ) in exponential phase was calculated by using Eq. (1) (Gokulakrishnan and Gummadi 2006).

$$\mu = \frac{\ln\left(\frac{X_2}{X_1}\right)}{(t_2 - t_1)} \quad (1)$$

where X_1 and X_2 are the cell dry weight obtained at time t_1 and t_2, respectively. All experiments were performed in triplicates under identical conditions.

2.5. *Degradation of caffeine*

The absorbance of pure caffeine standard (Sigma-Aldrich) aqueous solutions (1–10 mg/mL) was measured by using UV-visible spectrophotometer (Shimadzu) in the range of $A_{200-500\ nm}$ (Belay et al. 2008). The peak absorption of the samples was found at maximum wavelength $A_{275\ nm}$. This maximum wavelength (λ_{max} = 275) was used to measure the concentration of caffeine in media following the method of Sinija and Mishra (2009) with slight modifications.

The caffeine biodegradation was determined by following the method of Ashengroph and Ababaf (2013) with some modifications. The nutrient broth (100 mL) was prepared with different concentration of caffeine (2.5, 5, 10, 15, and 20 g/L) and 1 mL of seed culture (10^8 CFU/mL) was added followed by incubation at 150 rpm and 30°C for 5 d. The nutrient broth supplemented with different caffeine concentrations without isolated bacterial strain was kept as control. After incubation, the residue level of caffeine in caffeine-supplemented media was monitored by using UV-visible spectrophotometer (Shimadzu). The absorbance of media was measured at $A_{275\ nm}$ at different time intervals and concentration of caffeine was calculated by using the caffeine calibration curve (1–10 mg/L).

2.6. *Statistical analysis*

All the experiments were performed in triplicates and expressed as mean values ± standard deviation. Statistical testing was conducting by using SPSS statistical software (SPSS 23.0, IBM, Armonk, NY, USA). ANOVA and Tukey's HSD test were carried out to determine the significant differences ($p < 0.05$) between the means.

3. Results and discussion

3.1. *Isolation of caffeine-degrading bacteria*

A selective screening method based on enrichment technique and ability of bacterial isolates to grow in caffeine media was used for the selection of target bacteria. Fast-growing single colonies were selected from SSM media for further purification and incubated on SPMs (containing 2, 3, 4, and 5 g/L caffeine concentration). The bacterial colonies were found similar to each other when observed under microscope; therefore, bacterial colonies only from SPM (5 g/L caffeine) were selected as high caffeine-tolerant bacteria.

3.2. *Identification of caffeine-tolerant bacteria*

Isolated bacterial strain was identified by morphological, physiological, biochemical properties, and genetic profiling (16S rDNA gene sequence analysis).

3.2.1. *Morphological, physiological, and biochemical tests*

Morphologically, the isolated strain was identified as gram-negative bacilli-type with the size of 0.8–2 µm and without any immediate genus indication. The colonies were observed round, smooth, moist, and creamy white. In the physiological and biochemical tests, the bacterial isolate showed positive results for citrate utilization, oxidase activity, nitrate reduction, and arginine dihydrolase test. The bacterial isolate was able to utilize citrate and arginine as carbon and nitrogen source. Glucose fermentation, lactose fermentation, MRVP test, starch hydrolysis, indole production, hydrogen sulfide production, gelatin hydrolysis, and urease test indicated negative result for the isolated bacteria (Table 1).

Based on the combination of morphological, physiological, and biochemical properties, the isolated strain was now placed in the genus *Pseudomonas*. Isolated strain was gram-negative bacilli type with positive oxidase test indicating the ability of producing cytochrome oxidase enzyme. Therefore, it was used to differentiate between the families of *Pseudomonadaceae* and *Enterobacteriaceae*.

3.2.2. *16S rDNA analysis gene identification of caffeine-degrading bacteria*

The nucleotide sequence of bacterial isolate was submitted to GenBank, NCBI with accession number MH094849. The 16S rDNA gene sequence analysis concluded that the caffeine-degrading bacteria isolated from the soil belonged

Table 1. Comparative biochemical characteristics of isolated bacteria with other species of *Burkholderia*.

Characteristics	Isolated bacteria	*B. zhejiangensis*[a]	*B. grimmiae*[b]
Glucose fermentation	Negative	Negative	Negative
Lactose fermentation	Negative	NT	NT
Methyl red test	Negative	Negative	Negative
Citrate utilization test	Positive	Positive	Negative
Starch hydrolysis	Negative	Negative	Negative
Oxidase	Positive	Positive	Positive
Indole production	Negative	Negative	Negative
Hydrogen sulfide production	Negative	Negative	Negative
Gelatin hydrolysis	Negative	Negative	Negative
Urease	Negative	Positive	Positive
Nitrate reduction	Positive	Negative	Positive
Arginine dihydrolase	Positive	Positive	Negative

NT = Not tested.
[a]Adopted from Lu et al. (2012); [b]Adopted from Tian et al. (2013).

to genus *Burkholderia* (Fig. 1). *Burkholderia* is a genus of *Proteobacteria* and were previously part of *Pseudomonas* (Baune et al. 2017). However, the caffeine-degrading ability of *Burkholderia* spp. has been rarely reported. The genus *Burkholderia* comprises of more than 30 species and are found in diverse ecological niches such as in contaminated soils, water, animals, and humans. Some of the species of *Burkholderia* have been exploited for biocontrol, bioremediation, and plant growth promotion. But some species have also been reported to be associated with the human infections (Coenye and Vandamme 2003). Gao et al. (2016) have isolated the *Paraburkholderia caffeinitolerans* spp. nov. as a caffeine-degrading bacterium from the tea plantation soil.

The bacterial strains belonging to the genus *Pseudomonas, Alcaligenes, Rhodococcus, Klebsiella, Serratia*, and fungal strains of *Aspergillus, Penicillium, Stemphylium, Rhizopus*, and *Phanerochaete* have been studied for caffeine degradation (Gummadi, Bhavya, and Ashok 2012). A caffeine degradation efficiency of 100% has been reported by *Aspergillus* and *Penicillium* species (Roussos et al. 1995).

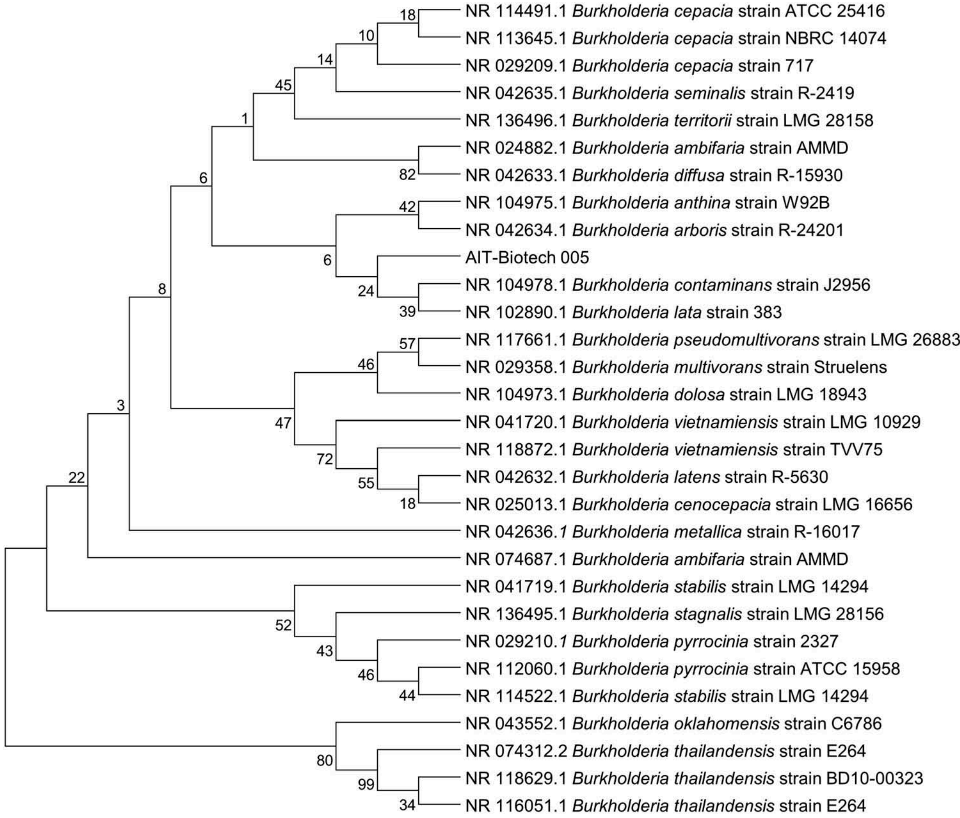


Figure 1. Neighbor-joining tree showing the phylogenetic relationship of isolated bacteria (AIT-Biotech 005) and related species of the genus *Burkholderia* based on 16S r DNA gene sequences.

3.3. *Caffeine tolerance of isolated bacteria*

The growth of *Burkholderia* spp. was observed by measuring the cell growth at different time intervals (0–110 h) after exposing to different caffeine concentrations (Fig. 2A). The maximum cell growth was observed at caffeine concentration of 2.5 g/L which then decreased with increasing concentration of caffeine. *Burkholderia* spp. was able to grow in the media with 20 g/L caffeine although the growth was very slow as compared to the lower caffeine concentrations. At the caffeine concentration of 2.5 and 5 g/L, the bacterial growth was in lag phase until 5 h of incubation followed by log phase with the abrupt increase in the growth until 30 h and then finally entered stationary phase. The growth pattern was found different at the higher caffeine concentration. The log phase started at 10 and 20 h for the 10 and 15 g/L of caffeine concentration, respectively. Similarly, the caffeine concentration of 20 g/L significantly inhibited the bacterial growth.

After 5 h of incubation, the bacterial growth at caffeine concentration of 2.5 g/L was significantly different ($p < 0.05$) from bacterial incubations at higher caffeine concentrations (5, 10, 15, and 20 g/L), whereas after 110 h of incubation, bacterial growth pattern was significantly different ($p < 0.05$) for all the caffeine concentrations.

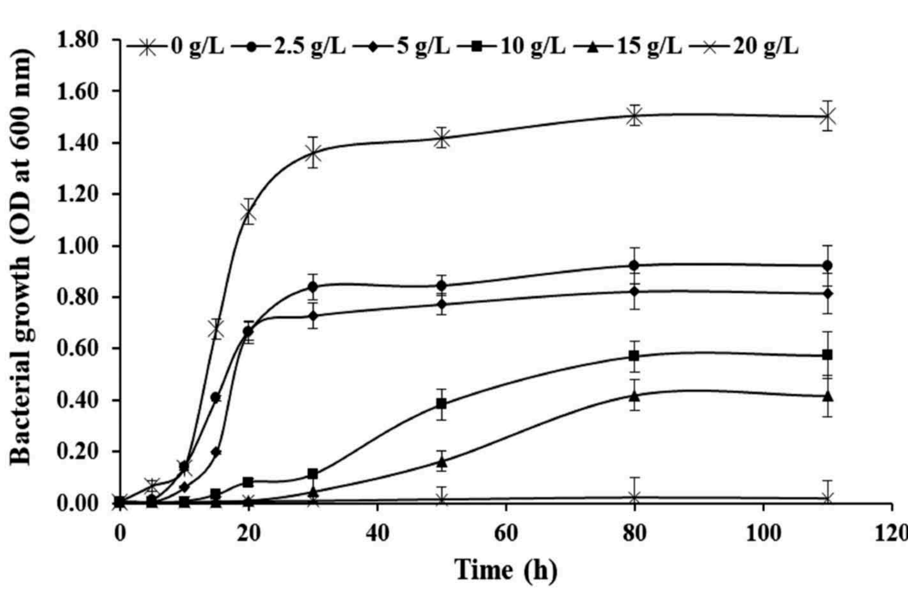
3.4. *Specific growth rate at different caffeine concentrations*

The specific growth rate of isolated *Burkholderia* spp. was determined based on growth curve as shown in Figure 2B. The growth rate of *Burkholderia* spp. declined with the increase in the caffeine concentration which indicated the inhibitory effect of caffeine at high concentrations. The maximum growth rate of 0.053 $h^{-1}$was observed at 2.5 g/L of caffeine. The specific growth rate of another caffeine-degrading bacteria *Leifsonia* spp. at caffeine concentration of 1.5 and 5 g/L was reported in the range of 0.03–0.04 h^{-1} (Ibrahim et al. 2016).

3.5. *Degradation of caffeine*

Burkholderia spp. was incubated in nutrient broth with five different caffeine concentrations (2.5, 5, 10, 15, and 20 g/L) and the residual caffeine was calculated at different time until it reached to the steady state during incubation period.

Different concentrations of caffeine in the media significantly affect caffeine degradability of bacteria. Caffeine degradability of isolated bacteria decreased with the increase in caffeine concentration. After 110 h of incubation, *Burkholderia* spp. degraded 45.5% and 43.9% of original caffeine concentration when incubated in media supplemented with 2.5 and 5 g/L caffeine concentration, respectively as shown in Figure 3. In the case of 10 and 15 g/L of caffeine-incorporated media,



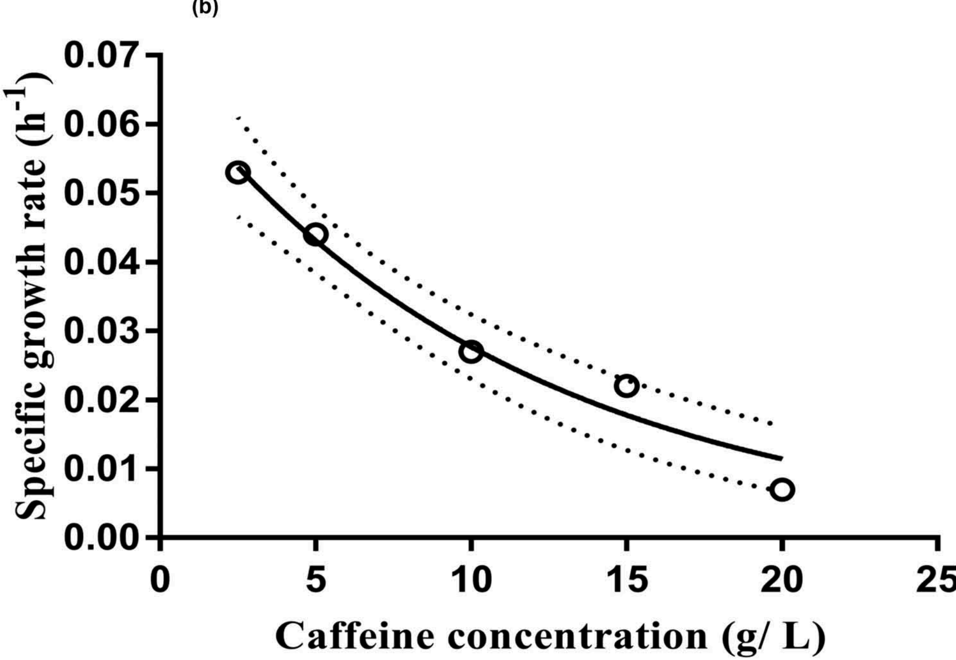


Figure 2. (a) Bacterial growth in nutrient broth ($A_{600\ nm}$) at different caffeine concentrations, (b) Specific growth rate *of Burkholderia* spp. at different caffeine concentration with nutrient broth. The dotted lines represent the 95% confidence interval of the estimates (n = 3).

30.4% and 14.7% of caffeine degradation was observed respectively, whereas, caffeine degradation was found only 2.6% in the media containing 20 g/L of caffeine. Hence, it indicated that high caffeine concentration in the media decreased the caffeine-degrading ability of *Burkholderia* spp. Sarath Babu et al. (2005) reported that high concentrations of caffeine inhibited the growth of *Pseudomonas* spp. and it could degrade 53%, 45%, and 30% of caffeine at concentrations of 2, 4, and 5 g/L caffeine, respectively. Moreover, to achieve maximum caffeine degradation, a culture requires several years of maintenance on caffeine containing medium (Sarath Babu et al. 2005). Gokulakrishnan and Gummadi (2006) reported that the growth of *Pseudomonas* spp. GSC 1182 was completely inhibited at 20 g/L of caffeine concentration.

Statistical analysis indicated that the degradation of caffeine induced by *Burkholderia* spp. was significantly higher ($p < 0.05$) at the lower caffeine concentration (2.5 and 5 g/L) compared to other high caffeine concentrations (10, 15, and 20 g/L).

According to previous studies on decaffeination by microbes, bacterial strains presented variable caffeine-degrading capability. *P. pseudoalcoligenes* TPS8 was found to remove 80.2% of caffeine from 2.5 g/L of caffeine containing substrate after 72 h of incubation and can be convenient for decaffeination in case of agro-industrial effluents (Ashengroph and Ababaf 2013). *Serratia marcscens* degraded 100% of 0.6 g/L of caffeine after 72 h by demethylation (Mazzafera, Olsson, and Sandberg 1996). *Pseudomonas alcaligenes CFTR1708* were found capable of completely degrading caffeine from solutions containing 1 g/L caffeine in 6 h. Gokulakrishnan, Chandraraj, and Gummadi (2007)

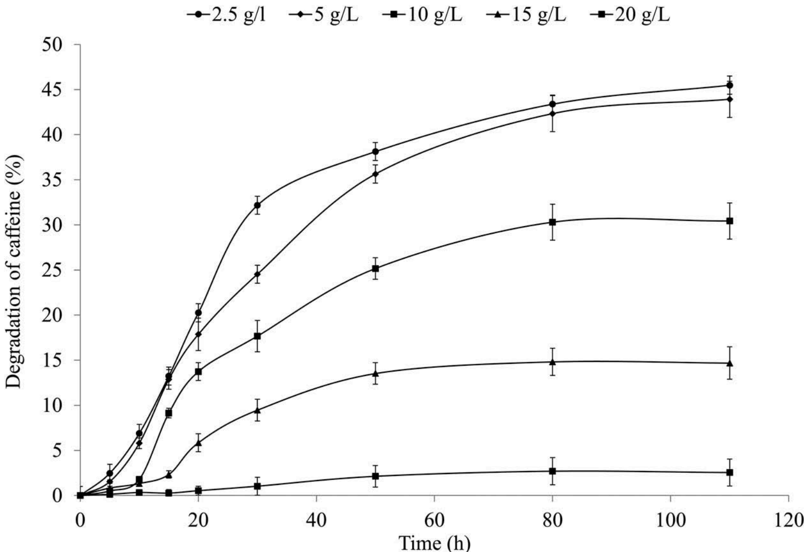


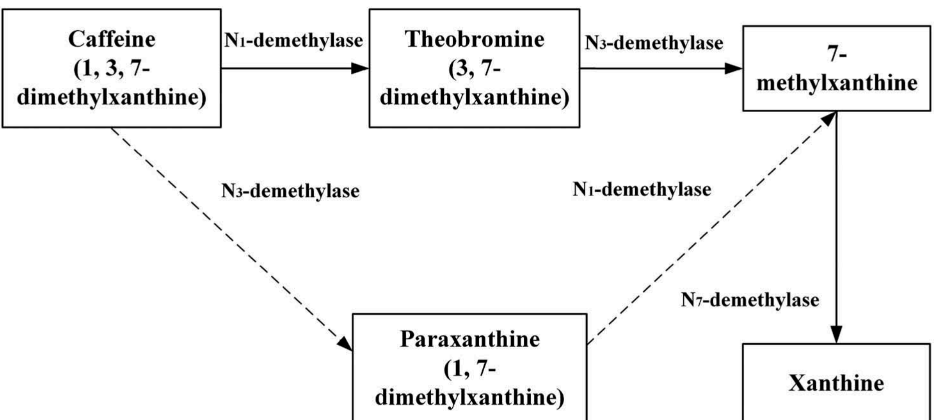
Figure 3. Degradation of caffeine by *Burkholderia* spp. at different caffeine concentrations in nutrient broth as determined by change in absorbance at $A_{275\ nm}$ (n = 3).

reported the biodegradation of caffeine by *Pseudomonas* spp. strain *GSC1182* which showed 80% degradation of caffeine in 48 h when caffeine was used as the sole carbon and nitrogen source. Different carbon sources have different effects on caffeine biodegradation (Hakil et al. 1999). Ibrahim et al. (2016) studied the effect of different carbon sources (sucrose, fructose, mannitol, maltose, dextrin, starch, lactose, galactose, and cellulose) on caffeine degradation and found that sucrose was the best carbon source for bacterial growth followed by galactose and fructose, whereas, other carbon sources only support the bacterial growth. Gokulakrishnan and Gummadi (2006) reported that caffeine degradation by *Pseudomonas* spp. was inhibited when glucose was used as carbon source and was enhanced by sucrose and lactose.

The metabolic degradation of caffeine by *Burkholderia* spp. was hypothesized based on previous reports regarding the bacterial degradation of caffeine (Fig. 4). The previous reports revealed that caffeine-degrading bacterial isolates exhibited two major metabolic pathways: N-demethylation and C-8 oxidation. The N-demethylation is found to be the most common metabolic pathway, as more than 80% of reported caffeine-degrading bacterial isolates demonstrated N-demethylation pathway. In both the pathways, bacteria degrade caffeine into ammonia and carbon dioxide to harvest energy (Summers et al. 2015). Caffeine (1,3,7-trimethylxanthine) during demethylation is sequentially N-demethylated to form xanthine. The bacterial degradation of caffeine involves successive demethylation at N-1, N-3, and N-7 terminals, and finally xanthine enters purine catabolic pathway (Summers et al. 2015; Yu et al. 2014). The second bacterial caffeine degradation pathway involves C-8 oxidation that converts caffeine into 1,3,7-trimethyluric acid, which is further degraded by a pathway homologous to the uric acid metabolic pathway (Mohanty et al. 2012; Yu et al. 2008). Further investigation is needed to identify the exact degradation mechanism and specific genes associated with caffeine degradation pathway by *Burkholderia* spp. to target for further food and environmental applications.

4. Conclusion

Caffeine-degrading bacteria was isolated from coffee plantation soil from Chiang Mai, Thailand, for targeting bio-decaffeination. The bacteria were identified as *Burkholderia* spp. by morphological, physiological, and biochemical tests, and genetic profiling (16S rDNA analysis). The bacteria can tolerate high caffeine level up to 20 g/L but where only 10% of initial caffeine can be degraded. There are very few published results about bacterial species which can tolerate such high caffeine level (20 g/L). They can grow well at media containing 2.5, 5, and 10 g/L caffeine. The isolated bacteria can degrade up to 45.5% of caffeine in media with 2.5 g/L within 120 h of incubation at 30°C with 120 rpm. The caffeine-degrading ability of bacteria was affected by the concentration of



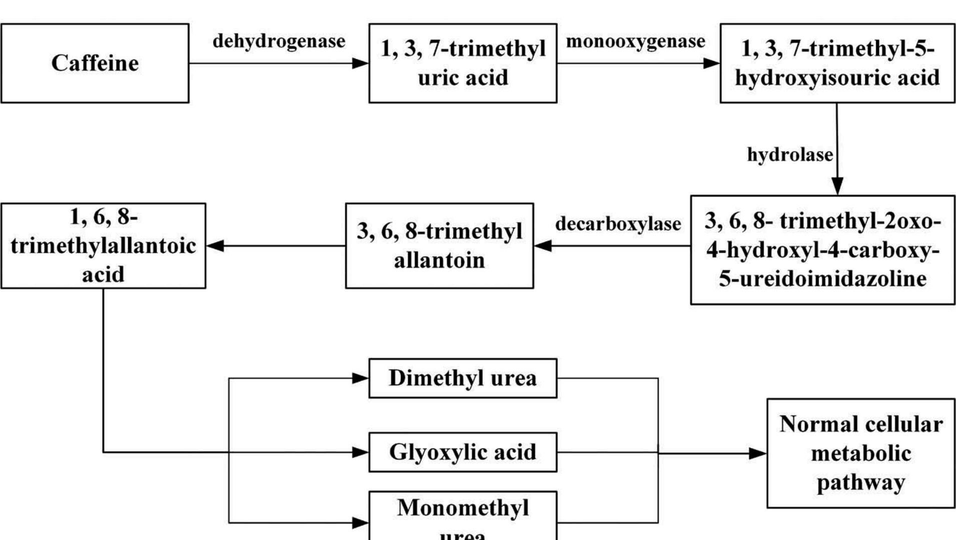


Figure 4. Proposed caffeine metabolic degradation pathways by *Burkholderia* spp. (adopted from Mazzafera 2002; Summers et al. 2015).

caffeine concentration; lower the concentration, higher the caffeine-degrading ability. Thus, the high caffeine-tolerance and -degrading ability of *Burkholderia* spp. can be efficiently targeted for further studies in bio-decaffeination for caffeine containing effluents and agro-industrial waste as well as decaffeination in food processing applications.

Conflict of interest

Authors declare no conflict of interest.

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