J. Microbiol. Biotechnol. (2018), 28(7), 1147–1155
https://doi.org/10.4014/jmb.1801.01043

jmb

Different Catabolism Pathways Triggered by Various Methylxanthines in Caffeine-Tolerant Bacterium *Pseudomonas putida* CT25 Isolated from Tea Garden Soil

Yi-Xiao Ma[1], Xiao-Han Wu[1], Hui-Shi Wu[1,2], Zhan-Bo Dong[3], Jian-Hui Ye[1], Xin-Qiang Zheng[1], Yue-Rong Liang[1], and Jian-Liang Lu[1]*

[1]*Zhejiang University Tea Research Institute, Hangzhou 310058, P.R. China*
[2]*Chongqing Vocational College of Transportation, Chongqing 402247, P.R. China*
[3]*Wenzhou Vocational College of Science and Technology, Wenzhou 325006, P.R. China*

Received: January 24, 2018
Revised: April 28, 2018
Accepted: May 1, 2018

First published online
May 11, 2018

**Corresponding author*
Phone: +86-571-88982704;
Fax: +86-571-88982704;
E-mail: jllu@zju.edu.cn

pISSN 1017-7825, eISSN 1738-8872

The degradation efficiency and catabolism pathways of the different methylxanthines (MXs) in isolated caffeine-tolerant strain *Pseudomonas putida* CT25 were comprehensively studied. The results showed that the degradation efficiency of various MXs varied with the number and position of the methyl groups on the molecule (*i.e.*, xanthine > 7-methylxanthine ≈ theobromine > caffeine > theophylline > 1-methylxanthine). Multiple MX catabolism pathways coexisted in strain CT25, and a different pathway would be triggered by various MXs. Demethylation dominated in the degradation of N-7-methylated MXs (such as 7-methylxanthine, theobromine, and caffeine), where C-8 oxidation was the major pathway in the catabolism of 1-methylxanthine, whereas demethylation and C-8 oxidation are likely both involved in the degradation of theophylline. Enzymes responsible for MX degradation were located inside the cell. Both cell culture and cell-free enzyme assays revealed that N-1 demethylation might be a rate-limiting step for the catabolism of the MXs. Surprisingly, accumulation of uric acid was observed in a cell-free reaction system, which might be attributed to the lack of activity of uricase, a cytochrome *c*-coupled membrane integral enzyme.

Keywords: *Pseudomonas*, methylxanthines, degradation efficiency, metabolic pathway, uric acid accumulation

Introduction

Methylxanthines (MXs) are the characteristic secondary metabolites of many plant species, including tea, coffee, cocoa, and yerba máte [1], which can be artificially synthesized. Caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine) are naturally occurring MXs [2]. Caffeine and related MXs can be used as analgesics, anti-inflammatories, antitussives, behavioral stimulants, diuretics, and lipolytics [3]. In humans, however, excessive consumption of caffeine and related MXs may cause sleep deprivation [4], cardiac arrhythmias [5], and osteoporosis [6]. Furthermore, without proper waste disposal, MXs can enter water and soil systems, causing environmental pollution [7].

Attempts have been made to limit the harmful effects of MXs in humans and the environment by limiting their content in products. For example, caffeine can be partially removed from tea and tea extracts through processes including hot water treatment [8], supercritical CO_2 extraction [9], column chromatography [10], and counter-current chromatography [11]. However, prior to large-scale application of these technologies, some optimizations should be further made, such as improving the decaffeination efficiency, decreasing the loss of tea ingredients, and lowering the costs. Another process involves decaffeination through microbial fermentation or digestion with cell-free enzymes. This bio-decaffeination process might be a

promising approach because of its high specificity, eco-friendliness, and cost-effectiveness [12, 13].

The genus *Pseudomonas* is well known for its ability to use caffeine as a sole source of carbon and nitrogen [14, 15], and it can be considered as a viable tool for bio-decaffeination, production of caffeine derivatives, and environmental remediation [2, 16]. Studies have shown that *N*-demethylation and oxidation pathways are involved in the catabolism of caffeine in *Pseudomonas* [14–19]. In the *N*-demethylation pathway, methyl groups of caffeine are sequentially removed from the N1, N3, and N7 positions and the formed xanthine is then degraded into uric acid and utilized further, in which demethylation is the rate-limiting step [15, 17]. Recently, genes (*ndmA, ndmB, ndmC, ndmD,* and *ndmE*) responsible for the entire *N*-demethylation pathway were isolated from *Pseudomonas putida* CBB5 [17, 20]. In the oxidation pathway, caffeine was directly oxidized at the C-8 position to form 1,3,7-trimethyluric acid, which was further oxidized to 3,6,8-trimethylallantoin. This pathway has been observed in both mixed cultures [21] and bacterial isolates [14, 19]. Furthermore, genes associated with the oxidation of caffeine (*cdhABC*) and trimethyluric acid (*tmuDHM*) have been identified in *Pseudomonas* sp. strain CBB1 [12, 19].

In a previous study, we have isolated a high-caffeine-tolerant bacterial strain from tea garden soil, *Pseudomonas putida* CT25, which can survive on the medium with 20 g/l caffeine [22]. In this study, the catabolism pathway and degradation efficiency of strain CT25 towards caffeine and related MXs were investigated.

Materials and Methods

Bacterial Strain

Pseudomonas putida CT25 used in this study was isolated from the soil of a tea garden and preserved in our laboratory. Before any testing, the preserved strain was activated on a solid medium with 2.5 g/l caffeine as described in a previous paper [22].

Chemicals

Caffeine was purchased from Wako Pure Chemical Industries, Ltd (Japan). Theobromine, theophylline, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, xanthine, uric acid, 1,3-dimethyluric acid, and 1,3,7-trimethyluric acid were purchased from Sigma-Aldrich (China). 1-Methyluric acid and 3-methyluric acid were obtained from TRC (Canada). Yeast extract was purchased from Oxoid (UK). Peptone was ordered from Shanghai Sangon Biotechnology Co., Ltd (China). High-performance liquid chromatography (HPLC)-grade methanol, acetonitrile, and acetic acid were purchased from Tedia (USA). Lastly, the ultrapure water used throughout the test was prepared with an EASY Pure II Water System (Barnstead Int., USA).

Culture Media

Luria-Bertani (LB) liquid medium was prepared using 1.0% peptone, 0.5% yeast extract, and 1.0% NaCl, and the medium acidity was adjusted to pH 7.0. LB solid medium was obtained by the addition of 1.5% agar. LB solid medium supplemented with 2.5 g/l caffeine was used to amplify the strain CT25 for guaranteeing the bacterial purity. The mineral salts medium contained 0.37 g/l KCl, 0.21 g/l $MgSO_4$, 0.71 g/l $Fe_2(SO_4)$, 0.21 g/l $Na_2HPO_4{\cdot}12H_2O$, 0.08 g/l $CaCl_2$, 8.5 g/l NaCl, 15 μg/l $ZnSO_4{\cdot}7H_2O$, 12 μg/l $NaMoO_4{\cdot}2H_2O$, 11 μg/l $MnSO_4$, 10 μg/l $CuSO_4$, and 10 μg/l H_3BO_4, and its acidity was adjusted to pH 7.0. Centrifugation was performed at 4°C and 2,500 ×*g* for 10 min to remove the particles of the medium. The supernatant was collected and used for the preparation of different MX-containing culture media. All the media were autoclaved (Panasonic, Japan) at 121°C for 30 min before use.

Detection of Bacterial Growth and Caffeine Degradation

The strain CT25 was transferred to LB liquid medium supplemented with 2.5 g/l caffeine and incubated for 16 h at 28°C and 150 rpm in a shaking incubator (Taicang Experimental Instruments Factory, China). These bacteria were harvested by centrifugation at 4°C and 900 ×*g* for 5 min and washed twice with PBS buffer (pH 7.4). The strain CT25 (5.0 ml) was inoculated into 500 ml of mineral salts medium supplemented with 2.5 g/l caffeine, and then incubated at 28°C and 150 rpm. Sampling (1.0 ml) was performed at 0, 6, 12, 18, 24, 28, 30, 32, 34, 38, 42, 46, 50, 54, 56, 58, 60, 62, and 68 h, and the optical density at 600 nm (OD_{600}) of the samples was recorded in a spectrophotometer (Unico Instruments Co., Ltd, China). Caffeine residuum in the medium was analyzed after centrifugation at 13,800 ×*g* and 4°C for 10 min and proper dilution with ethanol. The test was carried out in triplicate.

Test for Degradation Efficiency and Pathway of Different MXs

The activated strain CT25 (0.25 ml) was transferred into 25 ml of mineral salts medium supplemented with 100 mg/l caffeine (roughly equal to 0.515 mM), theobromine (0.556 mM), theophylline (0.556 mM), and xanthine (0.658 mM), respectively. Incubation was carried out at 150 rpm and 28°C, and sampling was performed at 0, 3, 6, 9, 12, and 24 h. The residuum of MXs was analyzed in the samples after centrifugation at 13,800 ×*g* and proper dilution with ethanol.

To confirm the degradation efficiency of the MXs and monitor their catabolites, a relatively high initial level of the MXs was further tested, since the concentration of catabolites was quite low in the incubated media supplemented with ~0.5 mM MXs. The activated strain CT25 (0.25 ml) was also inoculated in 25 ml of mineral salts medium supplemented with 5 mM different MXs (caffeine, theobromine, theophylline, 7-methylxanthine, and 1-methylxanthine, respectively), and incubated as above. Sampling

was carried out at 0, 3, 6, 15, 30, 50, and 72 h. The substrate and its catabolites were analyzed by HPLC in each sample after centrifugation at 13,800 ×*g* and proper dilution with ethanol.

Evaluation of the Enzyme Activity Related to MX Degradation

Enzyme location test. The activated strain CT25 was inoculated into 250 ml of mineral salts medium supplemented with 2.5 g/l caffeine and incubated at 28°C and 150 rpm for 48 h, and the cells and culture medium were separated by centrifugation at 2,900 ×*g* and 4°C for 10 min. After being washed with PBS buffer (pH 7.4) twice, the cells were ground into powder with liquid nitrogen and extracted immediately with 10.0 ml of PBS buffer (pH 7.4) for 30 min on ice. The supernatant containing intracellular enzymes was obtained by centrifugation at 13,800 ×*g* and 4°C for 10 min. The culture medium after centrifugation was mixed with the same volume of pre-cooled acetone and placed at −20°C for 15 min. The precipitate was obtained by centrifugation at 2,900 ×*g* for 10 min, washed twice with acetone, and finally dried in air. The extracellular enzymes were obtained through dissolving the precipitate into 10.0 ml of PBS buffer (pH 7.4). When the enzyme activity of caffeine degradation was assayed, the enzyme extract (0.5 ml) was mixed with 0.5 ml of caffeine solution (0.515 mM) and incubated at 28°C for 1 h. The reaction was terminated by heating the mixture at 90°C for 10 min. As a control, the enzyme was inactivated through heat treatment (90°C, 10 min), and then mixed with caffeine solution and incubated at 28°C for 1 h. After that, the mixture was centrifuged at 13,800 ×*g* and 4°C for 10 min and the supernatant was collected for analysis of caffeine by HPLC.

Enzyme-substrate specificity test. The extracted intracellular enzymes (0.5 ml) were mixed with 0.5 ml of different MX solutions (caffeine, theobromine, theophylline, and 7-methylxanthine, each at for ~0.5 mM), respectively, and reacted at 28°C. After 1 h, the mixture was heated at 90°C for 10 min to terminate the reaction. In the control group, prior to incubation at 28°C for 1 h, the enzyme was heated at 90°C for 10 min and mixed with different MX solutions. After incubation, the sample was centrifuged at 13,800 ×*g* and 4°C for 10 min and the supernatant was obtained. The substrate and catabolites in all supernatants were monitored by HPLC.

Analysis of the MXs and Their Catabolites

The MXs and their catabolites were analyzed with a model LC-20A HPLC system (Shimadzu Co., Japan) equipped with a Zorbax 5 µm TC-C18 column (250 × 4.6 mm; Agilent Technologies Inc., USA). The analysis conditions were as follows: mobile phase A, acetonitrile/acetic acid/water (3/0.5/96.5 (v/v/v)); mobile phase B, acetonitrile/acetic acid/water (30/0.5/69.5 (v/v/v)); gradient elution, mobile phase B increasing linearly from 20% to 45% in the first 20 min, then back to 20% in the next 2 min, and holding for another 3 min; flow rate, 1 ml/min; injection volume, 10 µl; oven temperature, 33°C; detecting wavelength, 280 nm. The MXs and their catabolites were identified and quantified by comparisons with the retention times and peak areas of authentic standards.

Results and Discussion

Growth and Caffeine Catabolism of CT25

When strain CT25 was inoculated in mineral salts medium supplemented with 2.5 g/l caffeine, the strain growth (OD_{600} value) was unobvious within the first 28 h, gradually increased in the next 10 h, and then sharply boosted after incubation for 38–56 h (Fig. 1A), indicating that the duration of 38–56 h might be the logarithmic growth period of the strain. It appears that the bacterium might require a long period to adapt to the high levels of caffeine and to initiate gene expression and enzyme synthesis associated with caffeine degradation [23]. The caffeine residuum in the medium was in contrast to the strain growth, especially as it decreased sharply at the logarithmic growth period and almost disappeared after 58 h.

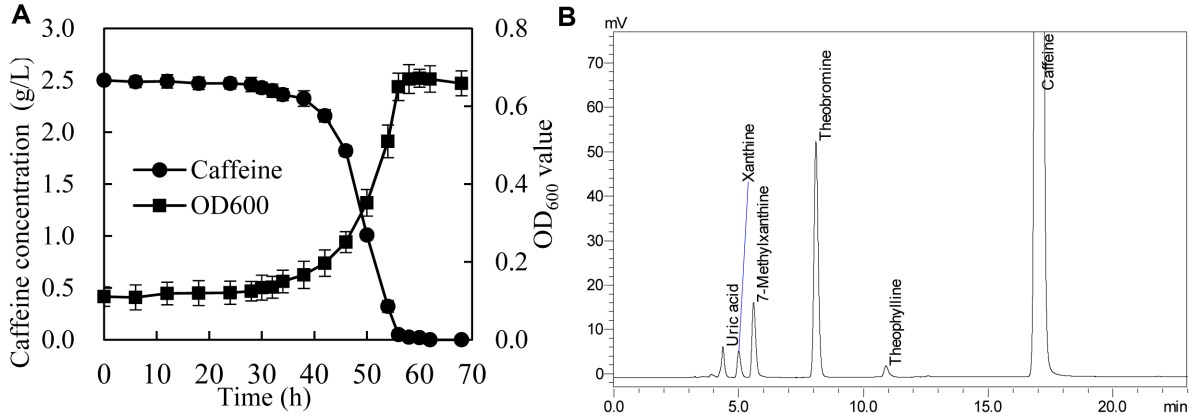

Fig. 1. (**A**) Changes of caffeine concentration and OD_{600} values in caffeine-containing (2.5 g/l) medium inoculated with *Pseudomonas putida* CT25, and (**B**) HPLC spectrum of catabolites in the medium after incubation for 38 h.

According to the HPLC analysis of the cultured medium sampled at different times, several catabolites with low concentration could be monitored after incubation for 38 h (Fig. 1B). These compounds were identified as uric acid, xanthine, 7-methylxanthine, theobromine, and theophylline (trace), respectively, after being compared with the authentic references. The results showed that theobromine and 7-methylxanthine might be the major catabolites, and theophylline might be the minor one. Interestingly, theophylline was first reported as an intermediate during the bacterial degradation of caffeine. This indicated that CT25 might utilize the caffeine through multiple *N*-demethylation patterns in some cases. Although caffeine-degrading bacteria could metabolize caffeine through *N*-demethylation and C-8 oxidation pathways, only theobromine and 7-methylxanthine were mainly detected [1, 15, 24].

Degradation Efficiency of Different MXs in Cell Culture

Strain CT25 could utilize various MXs as a sole source of carbon and nitrogen. To compare the difference in degradation efficiency of the different MXs, CT25 was incubated in mineral salts medium supplemented with various MXs. The results showed that xanthine, theobromine, caffeine, and theophylline were completely degraded within 3, 6, 9, and 24 h, respectively, when low level concentrations (100 mg/l) of these compounds was used (Fig. 2A). It was clear that CT25 could efficiently utilize xanthine and theobromine, but showed difficulty in degrading theophylline. Although an adaptation period was also observed in the medium supplemented with 100 mg/l caffeine, it was obviously shorter than that in the medium with a high level of caffeine (2.5 g/l). Interestingly, the degradation of theophylline was much more effective than that of caffeine in a short incubation time, but became ineffective after 6 h, indicating that catabolites of theophylline might accumulate and inhibit cell growth. These results supported the conclusion that the degradation efficiency of the MXs varied with the number and position of the methyl groups on the molecule and thus MXs with N-1 methylation were difficult to be metabolized.

The effect of methylation position on the degradation efficiency of MXs was also tested in a growth assay that used all MXs at a relatively high level (5 mM) and included 1-methyl- and 7-methylxanthine as additional supplements to the minimal salts medium. The results showed that CT25 could thoroughly degrade 5 mM 7-methylxanthine and theobromine within 15 h, and fully decompose the caffeine and theophylline in 30 and 50 h, respectively. However, the strain could not completely metabolize 1-methylxanthine within 72 h (Fig. 2B). This was consistent with the data obtained from incubation with 100 mg/l MXs, although relatively high level MXs (5 mM) would require a longer time to be thoroughly degraded. The results further confirmed that MXs with the N-1 methyl group could not be easily decomposed, especially those without the N-7 methyl group, indicating that N-1 demethylation might be a rate-limiting step for the metabolization of MXs. Thus, strain CT25 might be efficiently applied in the decaffeination of tea and related products, as well as in remedying soils and water bodies contaminated with N-7-methylated MXs.

Metabolic Pathways of the Various MXs

The cultured media supplemented with different initial MXs (5 mM) were monitored by HPLC, and the detectable catabolites were summarized (Table 1). Although the

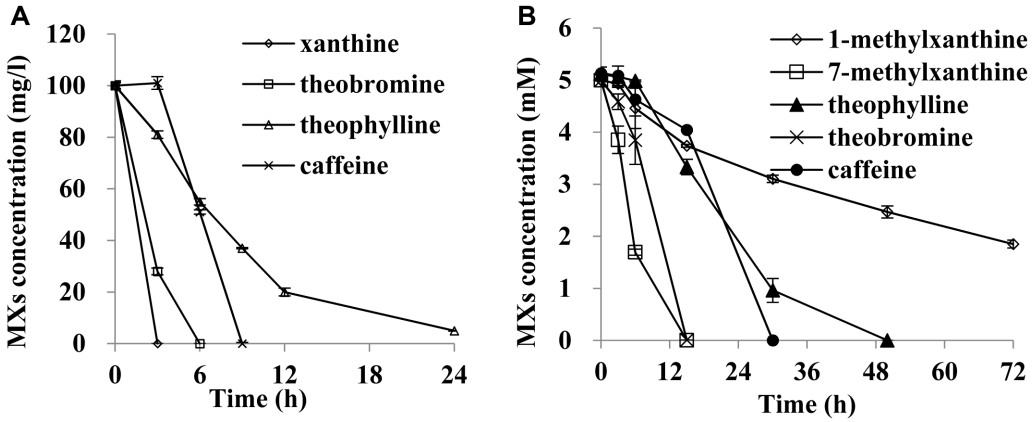

Fig. 2. Residuum of methylxanthines (MXs) in media inoculated with *P. putida* CT25 and supplemented with various MXs at two levels.

(**A**) 100 mg/l for each MX (*i.e.*, 0.658 mM for xanthine, 0.556 mM for theobromine or theophylline, and 0.515 mM for caffeine); (**B**) 5 mM for each MX.

Table 1. Catabolites of the different methylxanthines.

Substrate	Detectable catabolites in the medium
7-Methylxanthine	Uric acid[3h], xanthine[3h]
Theobromine	Uric acid[3h], xanthine[3h], 7-methylxanthine[3h], 3-methylxanthine[3h]
Caffeine	Uric acid[6h], xanthine[6h], 7-methylxanthine[3h], theobromine[3h], theophylline[15h] (very faint)
1-Methylxanthine	Uric acid[3h], xanthine[3h], 1-methyl uric acid[3h] (accumulation)
Theophylline	Uric acid[6h], xanthine[6h], 3-methylxanthine[6h], 1-methylxanthine[3h], 3-Methyluric acid[6h], 1,3-dimethyluric acid[6h], 1-methyluric acid[3h] (accumulation)

Strain CT25 was inoculated into medium supplemented with various methylxanthines (5 mM), respectively. Sampling was conducted after incubation for 0, 3, 6, 15, 30, 50, and 72 h, and HPLC was used to monitor the catabolites in the samples after centrifugation at 13,800 ×*g* and proper dilution with ethanol. The first time point at which each catabolite was detected during incubation is given in superscript font.

emergence order of these catabolites was difficult to clearly identify during incubation, metabolic pathways of the MXs could be hypothesized according to the composition of the catabolites. For caffeine, theobromine and 7-methylxanthine, degradation seemed to follow a similar demethylation pathway: caffeine → theobromine → 7-methylxanthine → xanthine → uric acid. It is important to note that faint N-7 demethylation might also occur during the decomposition of caffeine and theobromine (Fig. 3). C-8 oxidation might be a dominant pathway for 1-methylxanthine degradation because accumulated 1-methyluric acid was observed in the cultured medium. Oxidation and demethylation might be both involved in the degradation of theophylline, as monomethylxanthine, xanthine, uric acid, 3-methyluric acid, and 1,3-dimethyluric acid could be detected in the medium besides 1-methyluric acid accumulation. Many previous reports showed that various *P. putida* strains could degrade and utilize caffeine and related MXs mainly through the *N*-demethylation or C-8 oxidation metabolic pathways, which usually were controlled by several relevant operons [14, 15, 17, 20]. This present study confirmed that multiple pathways associated with MX catabolism coexisted

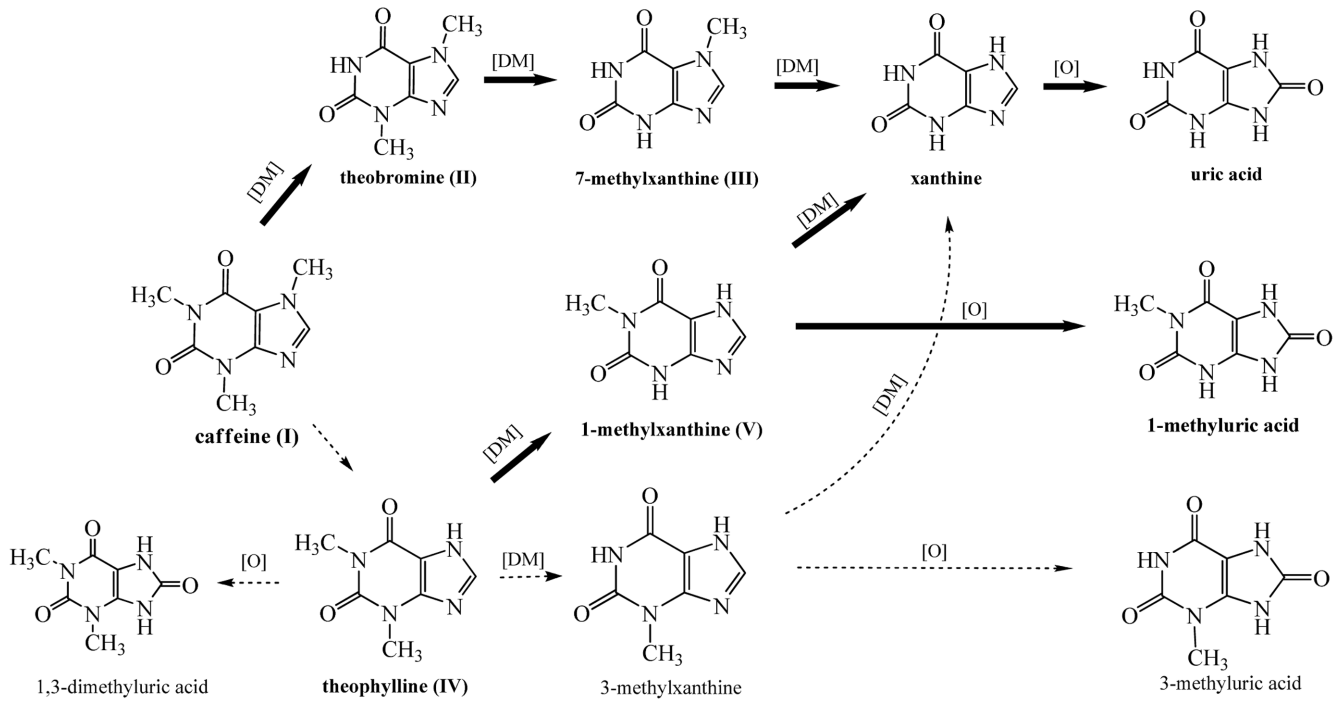

Fig. 3. Proposed methylxanthine (MX) metabolic pathways in *P. putida* CT25.
Compounds I–V represent the tested MXs. The broad dark arrows indicate the main metabolic pathway and the dashed arrows indicate the faint metabolic pathway. [DM] represents the demethylation step and [O] represents the oxidation step.

in the caffeine-tolerant bacterium CT25, but some were dominant and some were incidental. It was clearly observed that different pathways would be triggered by various MXs.

Interestingly, the demethylation pathway would dominate during the degradation of MXs with N-7 methylation, whereas C-8 oxidation would prevail during the decomposition of MXs with N-1 but without the N-7 methyl group (Fig. 3). This indicated that the methyl group at the N-7 position might be necessary for effective N-1 demethylation of the MXs by influencing substrate-enzyme interaction or impacting on inducer-operon activation. It should be noted that most of the catabolites occurred at low levels in the cultured media, except 1-methyluric acid. Two possibilities might exist: (i) that most of the initial MXs were transported into the bacterial cell before degradation, but 1-methylxanthine and theophylline were not; (ii) that all the initial MXs and their catabolites were transported but most of the catabolites were rapidly utilized further inside the cell, except 1-methyluric acid.

Localization and Substrate Specificity of the Enzymes

In order to determine whether influx of the substrates or efflux of the enzymes would happen before degradation of the MXs, intracellular and extracellular crude enzymes were prepared and mixed with caffeine. After incubation at 28°C for 1 h, the caffeine degradation efficiency mediated by the intracellular and extracellular enzymes was 44.34% and 1.50%, respectively, indicating that the catalytic activity had been mainly detected in the intracellular proteins instead of extracellular proteins. This was in accordance with a previous report [23]. The enzymes responsible for caffeine degradation are located inside the cell, and the substrates and catabolites are transported if necessary. Thus, the accumulation of 1-methyluric acid in the cultured medium seemed to be transported owing to its lack of decomposability.

Intracellular crude enzymes were then mixed with different MXs and incubated at 28°C for 1 h. The efficiency for degradation of 7-methylxanthine and theobromine was significantly higher than that of theophylline and caffeine (Table 2), consistent with the cell culture experiments. However, nearly similar catabolic efficiency for caffeine and theophylline was observed in the reaction mediated by the extracted enzyme, which was not consistent with the findings in the cell culture where the efficiency for degradation of theophylline was significantly lower than that of caffeine. The catabolites of theophylline in cell culture might be toxic to the cells, which would further impact on the utilization of theophylline through an inverse feedback mode.

Catabolite Composition of the MXs in Cell-Free System

As mentioned in reference to the cell culture experiments, most of the MX catabolites (except 1-methyluric acid) were present at low levels in the medium, and because they would finally be converted into utilizable molecules for supporting cell growth, it was difficult to identify the proportion of the catabolites. Therefore, various MXs were mixed with intracellular crude enzymes, and the catabolites were monitored in a cell-free reaction system by HPLC and their ratios were calculated according to mole-to-mole conversions (Fig. 4). When 7-methylxanthine was used as a reaction substrate, xanthine and uric acid were the main catabolites, and estimated as 6% and 60% of the initial substrate, respectively (Fig. 4A). After reaction, 64% of the initial theobromine was degraded into 7-methylxanthine (34%), 3-methylxanthine (1%), and uric acid (29%), and 36% initial substrate remained (Fig. 4B). For caffeine, the detected 7-methylxanthine and uric acid were estimated as 26% and 18% of the initial substrate, and the substrate remained at around 56%. Unlike the cell culture test, theobromine was undetectable (Fig. 4C). Thus, N-3 demethylation activity of the intracellular crude enzymes appeared to be higher than N-7 demethylation since similar efficiencies for the degradation of 7-methylxanthine and theobromine were observed. N-1 demethylation might have performed very slowly as the degraded amount of caffeine (44%) was much lower than that of 7-methylxanthine (66%)

Table 2. Substrate specificity of the extracted intracellular enzymes.

Substrate	Concentration in control group (M)	Concentration in reaction group (μM)	Degradation efficiency (%)
7-Methylxanthine	245.67 ± 3.59[a]	85.13 ± 1.44[b]	65.35 ± 0.08[a]
Theobromine	259.11 ± 4.40[a]	91.66 ± 2.81[b]	64.63 ± 0.49[a]
Caffeine	228.58 ± 2.01[b]	124.41 ± 2.60[a]	45.57 ± 0.66[b]
Theophylline	217.80 ± 2.55[b]	119.43 ± 8.98[a]	45.14 ± 4.76[b]

In the reaction group, the extracted intracellular enzymes (0.5 ml) were mixed with 0.5 ml of various methylxanthines (MXs) (~0.5 mM) and incubated at 28°C for 1 h, and then heated at 90°C to terminate the reaction. In the control group, the enzymes were denatured at 90°C for 10 min and then mixed with various MXs followed by similar incubation. The data are the mean ± standard deviation of triplicate tests, and significant difference ($p < 0.05$) is indicated by different letters in a same column.

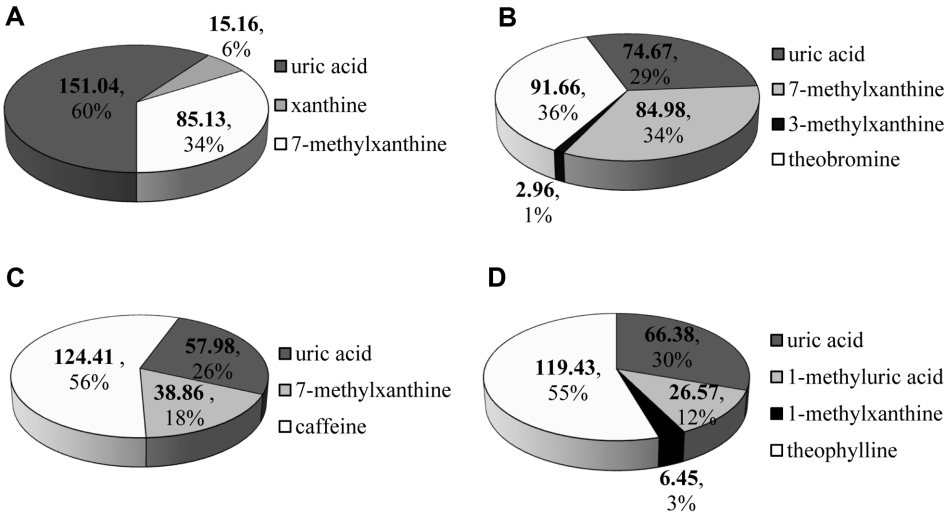

Fig. 4. Proportion of the methylxanthine catabolites and remaining substrate in the cell-free reaction system.
(**A**) 7-Methylxanthine; (**B**) theobromine; (**C**) caffeine; and (**D**) theophylline. Data in bold font indicate the concentration (mM) of the substrate and products; data in regular font indicate the molar proportion (%).

and theobromine (64%). Meanwhile, uric acid was easily produced from xanthine catabolism, but difficultly catabolized further because accumulation of uric acid was observed during the degradation of 7-methylxanthine, theobromine, and caffeine. The catabolic process of MXs with N-7 methylation could be described as follows: caffeine $\xrightarrow{\text{quite slow}}$ theobromine $\xrightarrow{\text{quite fast}}$ 7-methylxanthine $\xrightarrow{\text{slow}}$ xanthine $\xrightarrow{\text{quite fast}}$ uric acid (accumulation). After the reaction of theophylline, uric acid (30%), 1-methyluric acid (12%), and 1-methylxanthine (3%) were mostly observed, and more than half of the initial substrate remained (Fig. 4D). The accumulation of uric acid and 1-methyluric acid indicated that oxidation and sequential demethylation might simultaneously take effect during the catabolization of theophylline. The process could be described as follows: theophylline $\xrightarrow{\text{quite fast}}$ 1-methylxanthine $\xrightarrow{\text{fast}}$ 1-methyluric acid (accumulation) or theophylline $\xrightarrow{\text{quite fast}}$ 1-methylxanthine $\xrightarrow{\text{slow}}$ xanthine $\xrightarrow{\text{quite fast}}$ uric acid (accumulation).

It is worth mentioning that accumulation of uric acid was observed in all cell-free reaction systems, which was not consistent with the results of the cell culture experiments. This phenomenon might be due to lack of uricase activity in the cell-free enzyme extract. Uricase catalyzes the first reaction in the three-reaction catabolic pathway from uric acid to allantoin. Only one type of uricase usually exists as a cofactor-free enzyme in the peroxisome of eukaryotes [25, 26], whereas three distinctive types of uricase are present in organisms without peroxisomes [27]: the first type is a soluble cofactor-free enzyme similar to that in eukaryotes and encoded by the *uox* gene in most monoderm prokaryotes [28]; the second type is a soluble flavin-dependent enzyme and encoded by the *hpxO* [29] or *hpyO* gene [30] in some diderm prokaryotes; and the third type is a cytochrome *c*-coupled membrane integral enzyme and encoded by gene *puuD* only in diderm prokaryotes [27]. According to our genome sequencing data, uricase in CT25 belongs to the third type (data not shown). Absence of the membrane-associated cytochrome *c* coupling in the cell-free reaction system might lead to a lower activity of uricase and accumulation of uric acid, whereas sufficient cytochrome *c* and a proper enzyme structure could support the normal oxidation and non-accumulation of uric acid in cell culture. The exact mechanism behind the product difference between the cell culture and cell-free systems should be investigated further. Although multiple catabolism pathways could be triggered by various MXs in caffeine-tolerant bacterium strain CT25, considering the possible differences of the final products, the application of bacteria or cell-free enzymes in MX degradation needs to be carefully pretested.

Acknowledgments

This study was funded by the National Natural Science Foundation of China (31170644). We would like to thank Curt Anthony Polito for assisting with the editing of this paper.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Dash SS, Gummadi SN. 2006. Catabolic pathways and biotechnological applications of microbial caffeine degradation. *Biotechnol. Lett.* **28:** 1993-2002.
2. Algharrawi KHR, Summers RM, Gopishetty S, Subramanian M. 2015. Direct conversion of theophylline to 3-methylxanthine by metabolically engineered *E. coli*. *Microb. Cell Fact.* **14:** 203.
3. Daly JW. 2007. Caffeine analogs: biomedical impact. *Cell. Mol. Life. Sci.* **64:** 2153-2169.
4. Jha PK, Bouâouda H, Gourmelen S, Dumont S, Fuchs F, Goumon Y, *et al*. 2017. Sleep deprivation and caffeine treatment potentiate photic resetting of the master circadian clock in a diurnal rodent. *J. Neurosci.* **37:** 4343-4358.
5. Zheng JF, Zhao W, Xu K, Chen QM, Chen YY, Shen YL, *et al*. 2017. Interaction among hERG channel blockers is a potential mechanism of death in caffeine overdose. *Eur. J. Pharmacol.* **800:** 23-33.
6. Yu Q, Liu ZH, Lei T, Tang ZH. 2016. Subjective evaluation of the frequency of coffee intake and relationship to osteoporosis in Chinese men. *J. Health Popul. Nutr.* **35:** 24.
7. Yu CL, Summers RM, Li Y, Mohanty SK, Subramanian M, Pope RM. 2015. Rapid identification and quantitative validation of a caffeine-degrading pathway in *Pseudomonas* sp. CES. *J. Proteome Res.* **14:** 95-106.
8. Liang HL, Liang YR, Dong JJ, Lu JL, Xu HR, Wang H. 2007. Decaffeination of fresh green tea leaf (*Camellia sinensis*) by hot water treatment. *Food Chem.* **101:** 1451-1456.
9. Park HS, Choi HK, Lee SJ, Park KW, Choi SG, Kim KH. 2007. Effect of mass transfer on the removal of caffeine from green tea by supercritical carbon dioxide. *J. Supercrit. Fluids.* **42:** 205-211.
10. Lu JL, Wu MY, Yang XL, Dong ZB, Ye JH, Borthakur D, *et al*. 2010. Decaffeination of tea extracts by using poly (acrylamide-co-ethylene glycol dimethylacrylate) as adsorbent. *J. Food Eng.* **97:** 555-562.
11. Oliveira JCS, Lima LS, Medrado HHS, David JM, Do Vale AE, David JP, *et al*. 2015. Isolation of methylxantines from cacao beans (*Theobroma cacao*) by counter-current chromatography (CCC). *J. Liq. Chromatogr. Relat. Technol.* **38:** 1448-1451.
12. Summers RM, Mohanty SK, Gopishetty S, Subramanian M. 2015. Genetic characterization of caffeine degradation by bacteria and its potential applications. *Microb. Biotechnol.* **8:** 369-378.
13. Ibrahim S, Shukor MY, Syed MA, Johari WLW, Shamaan NA, Sabullah MK, *et al*. 2016. Enhanced caffeine degradation by immobilised cells of *Leifsonia* sp. strain SIU. *J. Gen. Appl. Microbiol.* **62:** 18-24.
14. Yu CL, Kale Y, Gopishetty S, Louie TM, Subramanian M. 2008. A novel caffeine dehydrogenase in *Pseudomonas* sp. strain CBB1 oxidizes caffeine to trimethyluric acid. *J. Bacteriol.* **190:** 772-776.
15. Yu CL, Louie TM, Summers R, Kale Y, Gopishetty S, Subramanian M. 2009. Two distinct pathways for metabolism of theophylline and caffeine are coexpressed in *Pseudomonas putida* CBB5. *J. Bacteriol.* **191:** 4624-4632.
16. Summers RM, Gopishetty S, Mohanty SK, Subramanian M. 2014. New genetic insights to consider coffee waste as feedstock for fuel, feed, and chemicals. *Cent. Eur. J. Chem.* **12:** 1271-1279.
17. Summers RM, Louie TM, Yu CL, Subramanian M. 2011. Characterization of a broad-specificity non-haem iron *N*-demethylase from *Pseudomonas putida* CBB5 capable of utilizing several purine alkaloids as sole carbon and nitrogen source. *Microbiology* **157:** 583-592.
18. Summers RM, Louie TM, Yu CL, Gakhar L, Louie KC, Subramanian M. 2012. Novel, highly specific *N*-demethylases enable bacteria to live on caffeine and related purine alkaloids. *J. Bacteriol.* **194:** 2041-2049.
19. Mohanty SK, Yu CL, Das S, Louie TM, Gakhar L, Subramanian M. 2012. Delineation of the caffeine C-8 oxidation pathway in *Pseudomonas* sp. strain CBB1 via characterization of a new trimethyluric acid monooxygenase and genes involved in trimethyluric acid metabolism. *J. Bacteriol.* **194:** 3872-3882.
20. Summers RM, Seffernick JL, Quandt EM, Yu CL, Barrick JE, Subramanian MV. 2013. Caffeine junkie: an unprecedented glutathione *S*-transferase-dependent oxygenase required for caffeine degradation by *Pseudomonas putida* CBB5. *J. Bacteriol.* **195:** 3933-3939.
21. Madyastha KM, Sridhar GR. 1998. A novel pathway for the metabolism of caffeine by a mixed culture consortium. *Biochem. Biophys. Res. Commun.* **249:** 178-181.
22. Fan FY, Xu Y, Liang YR, Zheng XQ, Borthakur D, Lu JL. 2011. Isolation and characterization of high caffeine-tolerant bacterium strains from the soil of tea garden. *Afr. J. Microbiol. Res.* **5:** 2278-2286.
23. Dash SS, Gummadi SN. 2008. Inducible nature of the enzymes involved in catabolism of caffeine and related methylxanthines. *J. Basic Microbiol*. **48:** 227-233.
24. Yu CL, Brooks S, Li Y, Subramanian M, Summers R, Pope M. 2017. Rapid proteomics to prospect and validate novel bacterial metabolism induced by environmental burden. *Methods Enzymol.* **586**: 379-411.
25. Wu XW, Lee CC, Muzny DM, Caskey CT. 1989. Urate oxidase: primary structure and evolutionary implications. *Proc. Natl. Acad. Sci. USA* **86:** 9412-9416.
26. Wei DH, Huang XQ, Qiao Y, Rao JJ, Wang L, Liao F, *et al*. 2017. Catalytic mechanisms for cofactor-free oxidase-catalyzed reactions: reaction pathways of uricase-catalyzed oxidation

and hydration of uric acid. *ACS Catal.* **7:** 4623-4636.
27. Doniselli N, Monzeglio E, Palù AD, Merli A, Percudani R. 2015. The identification of an integral membrane, cytochrome *c* urate oxidase completes the catalytic repertoire of a therapeutic enzyme. *Sci. Rep.* **5:** 13798.
28. Beedkar SD, Khobragade CN, Bodade RG, Vinchurkar AS. 2012. Comparative structural modeling and docking studies of uricase: possible implication in enzyme supplementation therapy for hyperuricemic disorders. *Comput. Biol. Med.* **42**: 657-666.
29. Hicks KA, O'Leary SE, Begley TP, Ealick SE. 2013. Structural and mechanistic studies of HpxO, a novel flavin adenine dinucleotide-dependent urate oxidase from *Klebsiella pneumoniae*. *Biochemistry* **52:** 477-487.
30. Michiel M, Perchat N, Perret A, Tricot S, Papeil A, Besnard M, *et al*. 2012. Microbial urate catabolism: characterization of HpyO, a non-homologous isofunctional isoform of the flavoprotein urate hydroxylase HpxO. *Environ. Microbiol. Rep.* **4:** 642-647.