JMB Papers in Press. First Published online May 11, 2018
DOI: 10.4014/jmb.1801.01043

Manuscript Number: JMB18-01043

Title: Different Catabolism Pathways Triggered by Various Methylxanthines in Caffeine-tolerant Bacterium Pseudomonas putida CT25 Isolated from Tea Garden Soil

Article Type: Research article

Keywords: Pseudomonas, methylxanthines, degradation efficiency, metabolic pathway, uric acid accumulation
Different Catabolism Pathways Triggered by Various Methylxanthines in Caffeine-tolerant Bacterium *Pseudomonas putida* CT25 Isolated from Tea Garden Soil

Yi-Xiao Ma¹, Xiao-Han Wu¹, Hui-Shi Wu¹,², Zhan-Bo Dong³, Jian-Hui Ye¹, Xin-Qiang Zheng¹, Yue-Rong Liang¹, and Jian-Liang Lu¹*

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

*Corresponding author

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

Running title: Ma et al., different catabolism pathways triggered by various MXs
Abstract

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 degradation efficiency of various MXs varied with number and position of the methyl-groups on the molecule (*i.e.*, xanthine > 7-methylxanthine ≈ theobromine > caffeine > theophylline > 1-methylxanthine). Multiple MXs 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), C-8 oxidation was the major pathway in catabolism of 1-methylxanthine, while demethylation and C-8 oxidation are likely both involved in the degradation of theophylline. Enzymes responsible for MXs 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 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 maté [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], super-critical 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 were sequentially removed from N1, N3 and N7 positions and the formed xanthine was then degraded into uric acid and utilized further, in which demethylation was 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 (Shanghai, China). 1-Methyluric acid and 3-methyluric acid were obtained from TRC (North York, Canada). Yeast extract was purchased from Oxoid (Basingstoke, UK). Peptone was ordered from Shanghai Sangon Biotechnology Co., Ltd (Shanghai, China). High performance liquid chromatography (HPLC)-grade methanol, acetonitrile and acetic acid were purchased from Tedia (Ohio, USA). Lastly, the ultrapure water used throughout the test was prepared by an EASY Pure II Water System (Dubuque, 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$_4$)$_3$, 0.21 g/l Na$_2$HPO$_4$·12H$_2$O, 0.08 g/l CaCl$_2$, 8.5 g/l NaCl, 15 μg/l ZnSO$_4$·7H$_2$O, 12 μg/l NaMoO$_4$·2H$_2$O, 11 μg/l MnSO$_4$, 10 μg/l CuSO$_4$ and 10 μg/l H$_3$BO$_4$, and its acidity was adjusted to pH 7.0. Centrifugation was performed at 4°C and 2500 ×g for 10 min to remove the particles of the medium. The supernatant was collected and used for the preparation of different MXs-contained 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, Jiangsu, 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 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, the optical density at 600 nm (OD$_{600}$) of the samples was recorded in a spectrophotometer (Unico Instruments Co., Ltd, Shanghai, China). Caffeine residuum in the medium was analyzed after centrifugation at 13800 × g and 4°C for 10 min and proper dilution with ethanol. The test was carried out triply.

**Test for Degradation Efficiency and Pathway of Different MXs**

The activated strain CT25 (0.25 ml) was transferred into 25 ml 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, sampling was performed at 0, 3, 6, 9, 12 and 24 h. Residuum of MXs was analyzed in the samples after centrifugation at 13800 × 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 lower in the incubated media supplemented with ~0.5 mM MXs. The activated strain CT25 (0.25 ml) was also inoculated in 25 ml mineral salts medium supplemented with 5 mM different MXs (caffeine, theobromine, theophylline, 7-methylxanthine, 1-methylxanthine, respectively), and incubated as above. Sampling was carried out at 0, 3, 6, 15, 30, 50 and 72 h. Substrate and its catabolites were analyzed by HPLC in each sample after centrifugation at
Evaluation of the Enzyme Activity Related to MXs Degradation

**Enzyme location test.** The activated strain CT25 was inoculated into 250 ml mineral salts medium supplemented with 2.5 g/l caffeine and incubated at 28°C and 150 rpm for 48 h, the cells and culture medium were separated by centrifugation at 2900 × 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 PBS buffer (pH 7.4) for 30 min on the ice. Supernatant containing intracellular enzymes was obtained by centrifugation at 13800 × g and 4°C for 10 min. The culture medium after centrifugation was mixed with same volume pre-cooled acetone and placed at -20°C for 15 min, the precipitate was obtained by centrifugation at 2900 × 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 PBS buffer (pH 7.4). When enzyme activity of caffeine degradation was assayed, the enzyme extract (0.5 ml) was mixed with 0.5 ml 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 heating treatment (90°C, 10 min), then mixed with caffeine solution and incubated at 28°C for 1 h. After that, the mixture was centrifuged at 13800 × 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 different MXs solution (caffeine, theobromine, theophylline, 7-methylxanthine, each 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 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 MXs solution. After incubation, the sample was centrifuged at 13800 ×g and 4°C for 10 min and the supernatant was obtained. Substrate and catabolites in all supernatants were monitored by HPLC.

Analysis of the MXs and their Catabolites

MXs and their catabolites were analyzed with a model LC-20A HPLC system (Shimadzu Co., Kyoto, Japan) equipped with a Zorbax 5 μm TC-C18 column (250 × 4.6 mm; Agilent Technologies Inc., CA, 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 early 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. MXs and their catabolites were identified and quantified by comparisons with 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 (OD600 value) was unobvious within the first 28 h, and gradually increased in the next 10 h, 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 CT25. 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]. Caffeine residuum in medium was in contrast to the strain growth, especially, decreased sharply at the logarithmic growth period and almost disappeared after 58 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), and 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**

The CT25 could utilize various MXs as a sole source of carbon and nitrogen. To compare the difference in degradation efficiency of the different MXs, the 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 h, 6 h, 9 h 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 the 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 high level caffeine (2.5 g/l). Interestingly, degradation of theophylline was much more effective than caffeine in a short incubation time, but became ineffective after 6 h, indicating that catabolites of the theophylline might accumulate and inhibit the cell growth. These results supported the conclusion that degradation efficiency of the MXs varied with 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 supplement 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 h 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 longer time to be thoroughly degraded. The results further confirmed that MXs with N-1 methyl-group could not be easily decomposed, especially those without N-7 methyl-group, indicating that N-1 demethylation might be a rate-limiting step for metabolization of MXs. Thus, strain CT25 might be efficiently applied in decaffeination of tea and related products, as well 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 emergence order of these catabolites was difficult to clearly identify during incubation, metabolic pathways of the MXs could be hypothesized according to composition of the catabolites. For caffeine, theobromine and 7-methylxanthine, degradation seemed to follow a similar demethylation pathway: caffeine $\rightarrow$ theobromine $\rightarrow$ 7-methylxanthine $\rightarrow$ xanthine $\rightarrow$ uric acid. It is important to note that faint N-7 demethylation might also occur during 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 degradation of theophylline as mono-methylxanthine, 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 MXs catabolism coexisted 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 degradation of MXs with N-7 methylation, while C-8 oxidation would prevail during decomposition of MXs with N-1 but without N-7 methyl-group (Fig. 3). This indicated that methyl-group at 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: (a) that most of the initial MXs were transported into bacterial cell before degradation, but 1-methylxanthine and theophylline were not; (b) 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 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 located inside the cell, and the substrates and catabolites should be transported if necessary. Thus, the accumulation of 1-methyluric acid in cultured medium seemed to be transported due 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 extracted enzyme, which was not consistent with findings in the cell culture where 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 the theophylline through inverse
feedback mode.

**Catabolite Composition of the MXs in Cell-Free System**

As mentioned in reference to the cell culture experiments, most of the MXs 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, the catabolites were monitored in 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 was 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 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 **efficiency for
degradation** of 7-methylxanthine and theobromine was observed. N-1 demethylation might be
performed very slowly as the degraded amount of caffeine (44%) was much lower than that of
7-methylxanthine (66%) and theobromine (64%). Meanwhile, uric acid was easily **produced
from xanthine catabolism**, but difficultly catabolized **further** because accumulation of uric
acid was observed during degradation of 7-methylxanthine, theobromine and caffeine. The
catabolic process of MXs with N-7 methylation could be described as follows: caffeine
\[
\text{caffeine} \rightarrow \text{theobromine} \rightarrow 7\text{-methylxanthine} \rightarrow \text{xanthine} \rightarrow \text{uric acid (accumulation)}.
\]
After the reaction of theophylline, uric acid (30%), 1-methyluric acid (12%) and
1-methylxanthine (3%) were mostly observed, more than half of the initial substrate was
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:
\[
\text{theophylline} \rightarrow 1\text{-methylxanthine} \rightarrow 1\text{-methyluric acid (accumulation)}
\]
\[
\text{theophylline} \rightarrow 1\text{-methylxanthine} \rightarrow \text{xanthine} \rightarrow \text{uric acid (accumulation)}.
\]
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 peroxisome of
eukaryotes [25, 26], while three distinctive types of uricase are present in organisms without
peroxisome [27]: the first type is a soluble cofactor-free enzyme similar to that in eukaryotes
and encoded by uox gene in most of monoderm prokaryotes [28]; the second type is a soluble
flavin-dependent enzyme and encoded by hpxO [29] or hpyO gene [30] in some of diderm
prokaryotes; and the third type is a cytochrome c-coupled membrane integral enzyme and
encoded by puuD gene 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 lower activity of uricase and accumulation of uric acid, while sufficient cytochrome c and proper enzyme structure could support the normal oxidation and non-accumulation of the uric acid in cell culture. The exact mechanism on the product difference between cell culture and cell-free system should be investigated further. Although multiple catabolism pathways could be triggered by various methylxanthines in caffeine-tolerant bacterium strain CT25, considering possible differences of the final products, application of bacteria or cell-free enzymes in MXs degradation need to be carefully pretested.

Acknowledgements

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

We declare that we have no conflicts of interest in this work.
References


25. Wu XW, Lee CC, Muzny DM, Caskey CT. 1989. Urate oxidase-primary structure and
evolutionary implications. *P. Natl. Acad. Sci.* **86**: 9412-9416.


Figure legends

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

Fig. 2. Residuum of the MXs in the media inoculated with *P. putida* CT25 and supplemented with various MXs at two levels: (A) 100 mg/l for each MXs, *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 MXs.

Fig. 3. Proposed MXs metabolic pathways in *P. putida* CT25. Compounds I-V showed the tested MXs. The broad dark arrows indicated the main metabolic pathway and the dashed arrows indicated faint metabolic pathway. The label [DM] represented the demethylation step and [O] represented the oxidation step.

Fig. 4. Proportion of the MXs catabolites and remaining substrate in the cell-free reaction system. (A) 7-methylxanthine; (B) theobromine; (C) caffeine; (D) theophylline. Data in bold font indicated the concentration (mM) of the substrate and products; data in regular font indicated the molar proportion (%).
Table 1. Catabolites of the different MXs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Detectable catabolites in medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-methylxanthine</td>
<td>uric acid&lt;sup&gt;3h&lt;/sup&gt;, xanthine&lt;sup&gt;3h&lt;/sup&gt;</td>
</tr>
<tr>
<td>theobromine</td>
<td>uric acid&lt;sup&gt;3h&lt;/sup&gt;, xanthine&lt;sup&gt;3h&lt;/sup&gt;, 7-methylxanthine&lt;sup&gt;3h&lt;/sup&gt;, 3-methylxanthine&lt;sup&gt;3h&lt;/sup&gt;</td>
</tr>
<tr>
<td>caffeine</td>
<td>uric acid&lt;sup&gt;6h&lt;/sup&gt;, xanthine&lt;sup&gt;6h&lt;/sup&gt;, 7-methylxanthine&lt;sup&gt;3h&lt;/sup&gt;, theobromine&lt;sup&gt;3h&lt;/sup&gt;, theophylline&lt;sup&gt;15h&lt;/sup&gt; (very faint)</td>
</tr>
<tr>
<td>1-methylxanthine</td>
<td>uric acid&lt;sup&gt;3h&lt;/sup&gt;, xanthine&lt;sup&gt;3h&lt;/sup&gt;, 1-methyl uric acid&lt;sup&gt;3h&lt;/sup&gt; (accumulation)</td>
</tr>
<tr>
<td>theophylline</td>
<td>uric acid&lt;sup&gt;6h&lt;/sup&gt;, xanthine&lt;sup&gt;6h&lt;/sup&gt;, 3-methylxanthine&lt;sup&gt;6h&lt;/sup&gt;, 1-methylxanthine&lt;sup&gt;3h&lt;/sup&gt;, 3-methyluric acid&lt;sup&gt;6h&lt;/sup&gt;, 1,3-dimethyluric acid&lt;sup&gt;6h&lt;/sup&gt;, 1-methyluric acid&lt;sup&gt;3h&lt;/sup&gt; (accumulation)</td>
</tr>
</tbody>
</table>

Note: The strain CT25 was inoculated into the medium supplemented with various MXs (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 samples after centrifugation at 13800 ×g and proper dilution with ethanol. The first time point at which each catabolite was detected during incubation is given in superscript font.
### Table 2. Substrate specificity of the extracted intracellular enzymes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration in control group (µM)</th>
<th>Concentration in reaction group (µM)</th>
<th>Degradation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-methylxanthine</td>
<td>245.67±3.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>85.13±1.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65.35±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>theobromine</td>
<td>259.11±4.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>91.66±2.81&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.63±0.49&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>caffeine</td>
<td>228.58±2.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>124.41±2.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.57±0.66&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>theophylline</td>
<td>217.80±2.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>119.43±8.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.14±4.76&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: In the reaction group, the extracted intracellular enzymes (0.5 ml) were mixed with 0.5 ml various MXs (~0.5 mM) and incubated at 28°C for 1 h, then heated at 90°C to terminate 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 were showed as mean ± standard deviation of triplicate tests, and significant difference (p<0.05) was indicated by different letters in a same column.
Fig. 1. Ma et al.,

A

Caffeine concentration (g/L)

0.0 0.5 1.0 1.5 2.0 2.5 3.0

0 10 20 30 40 50 60 70

Time (h)

Caffeine

OD600

B

OD600 value

0 0.2 0.4 0.6 0.8

0.0 1.0 2.0 3.0 4.0

Uric acid

Xanthine

7-Methylxanthine

Theobromine

Theophylline

Caffeine
Fig. 2. Ma et al.,
Fig. 3. Ma et al.
Fig. 4. Ma et al.,

A

151.04, 60%
85.13, 34%
15.16, 6%

- uric acid
- xanthine
- 7-methylxanthine

B

91.66, 36%
74.67, 29%
84.98, 34%
2.96, 1%

- uric acid
- 7-methylxanthine
- 3-methylxanthine
- theobromine

C

124.41, 56%
57.98, 26%
38.86, 18%

- uric acid
- 7-methylxanthine
- caffeine

D

119.43, 55%
66.38, 30%
26.57, 12%
6.45, 3%

- uric acid
- 1-methyluric acid
- 1-methylxanthine
- theophylline