Biocatalytic Production of 7-Methylxanthine by a Caffeine-Degrading Escherichia coli Strain

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Abstract

7-Methylxanthine, a derivative of caffeine (1,3,7-trimethylxanthine), is a high-value compound that has multiple medical applications, particularly with respect to eye health. Here, we demonstrate the biocatalytic production of 7-methylxanthine from caffeine using *Escherichia coli* strain MBM019, which was constructed for production of paraxanthine (1,7-dimethylxanthine). The mutant *N*-demethylase NdmA4, which was previously shown to catalyze N_3-demethylation of caffeine to produce paraxanthine, also retains N_1-demethylation activity toward paraxanthine. This work demonstrates that whole cell biocatalysts containing NdmA4 are more active toward paraxanthine than caffeine. We used four serial resting cell assays, with spent cells exchanged for fresh cells between each round, to produce 2,120 μM 7-methylxanthine and 552 μM paraxanthine from 4,331 μM caffeine. The purified 7-methylxanthine and paraxanthine were then isolated *via* preparatory-scale HPLC, resulting in 177.3 mg 7-methylxanthine and 48.1 mg paraxanthine at high purity. This is the first reported strain genetically optimized for the biosynthetic production of 7-methylxanthine from caffeine.

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Abstract:

7-Methylxanthine, a derivative of caffeine (1,3,7-trimethylxanthine), is a high-value compound that has multiple medical applications, particularly with respect to eye health. Here, we demonstrate the biocatalytic production of 7-methylxanthine from caffeine using*Escherichia coli* strain MBM019, which was constructed for production of paraxanthine (1,7-dimethylxanthine). The mutant$N$ -demethylase NdmA4, which was previously shown to catalyzeN_3-demethylation of caffeine to produce paraxanthine, also retains N_1-demethylation activity toward paraxanthine. This work demonstrates that whole cell biocatalysts containing NdmA4 are more active toward paraxanthine than caffeine. We used four serial resting cell assays, with spent cells exchanged for fresh cells between each round, to produce 2,120 μM 7-methylxanthine and 552 μM paraxanthine from 4,331 μM caffeine. The purified 7-methylxanthine and paraxanthine were then isolated *via*preparatory-scale HPLC, resulting in 177.3 mg 7-methylxanthine and 48.1 mg paraxanthine at high purity. This is the first reported strain genetically optimized for the biosynthetic production of 7-methylxanthine from caffeine.

Keywords: 7-Methylxanthine, Caffeine, Biocatalysis,N -demethylase

7-Methylxanthine, a purine alkaloid, is a derivative of the well-known compound caffeine (1,3,7-trimethylxanthine). Like caffeine, 7-methylxanthine is an adenosine receptor antagonist and is capable

of crossing the blood-brain barrier (Hung et al., 2018). These features make 7-methylxanthine and other methylxanthines attractive as scaffolds for the design and synthesis of more complex compounds (Nivedita Singh, 2018), like N-heterocyclic carbenes (Valdés et al., 2018; Zhang et al., 2015), that can be used for applications such as fine-tuning the potency and specificity of receptor antagonism for use in cancer and neurodegenerative therapies (Malki et al., 2006; Rogozin et al., 2006). While many of the specific health benefits commonly assigned to caffeine could arguably belong instead to one or more of its derivatives, the health benefits of 7-methylxanthine are much more clearly defined. Most notably, 7-methylxanthine has been shown to treat and prevent myopia progression (Cui et al., 2011; Nie et al., 2012). While the exact mechanisms of action have not yet been fully characterized, oral application of 7-methylxanthine has been observed to strengthen the sclera (Cui et al., 2011), reducing the potential for axial elongation and even showing a moderate decline in the rate of axial elongation in myopic children (Trier et al., 2008). Additionally, methylxanthine derivatives, such as 1,3-dipropyl-7-methylxanthine, have been found to increase the sensitivity of lung carcinoma cell lines through modification to cell cycle checkpoints and by inducing apoptotic responses (Malki et al., 2006). Toxicity studies of 7-methylxanthine have concluded that the compound is nontoxic. Even at concentrations as high as 2,000 mg/kg body weight, 7-methylxanthine induces no observable changes in behavior and is considered safe for long-term, chronic use (Singh et al., 2019; Singh et al., 2020).

7-Methylxanthine is not found in nature as frequently as other methylxanthines, such as caffeine, theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine), but it can still be found in low concentrations in plants as an intermediate during the synthesis of caffeine (Maureen McKeague, 2016). 7-Methylxanthine has also been generated in *Escherichia coli* through the *N* -demethylation of theobromine using *N* -demethylase genes isolated and characterized from *Pseudomonas putida* CBB5 (K. H. R. Algharrawi & Subramanian, 2020). Other studies have reported that varying combinations of the *ndmABCDE* genes discovered in *P. putida*CBB5 could be used to produce 3-methylxanthine from theophylline (K. H. Algharrawi et al., 2015) and theobromine from caffeine (K. H. Algharrawi et al., 2017).

We have recently constructed *E. coli* strain MBM019 to*N*- demethylate caffeine primarily to paraxanthine (1,7-dimethylxanthine), a caffeine metabolic intermediate (Mock et al., In Press 2022) with the potential to treat and prevent Parkinson's Disease (Janitschke et al., 2021; Victorino et al., 2021). This engineered bacterium harbors a mutant *N* -demethylase gene,*ndmA4* (Kim et al., 2019; Mills et al., 2021), originally designed for the sole purpose of shifting the primary *N* -demethylation product from theobromine to paraxanthine (Mills et al., 2021). Strain MBM019 also overexpresses the *ndmDP1* gene as well as two formaldehyde-degrading genes, *frmA* and *frmB* (Fig S1). NdmDP1 is a truncated version of NdmD, a reductase required for*N* -demethylation through the transfer of electrons from NADH (R. Summers et al., 2014; R. M. Summers et al., 2012), that provides a higher *N* -demethylation activity than the wild-type NdmD (Mock et al., In Press 2022). The FrmAB enzymes are native to *E. coli* and catalyze the NAD^+-dependent degradation of formaldehyde, a by-product of *N* -demethylation (Mock et al., In Press 2022), thus generating an NADH/NAD^+ recycle system within the cell. In our study to produce paraxanthine from caffeine, we also observed the generation of a small amount of 7-methylxanthine (Mock et al., In Press 2022). Here, we describe an alternate pathway to 7-methylxanthine via paraxanthine by nearly complete conversion of caffeine using strain MBM019 (Fig 1), establishing a biosynthetic process for consuming an environmental contaminant and sequentially producing two high-value compounds.

Production of 7-methylxanthine from caffeine by strain MBM019 was optimized in a 15 mL resting cell assay reaction in which the reaction supernatant was recycled three times with fresh cells for a total of four rounds of reaction (Fig 2, Table S1 & Fig S2). Each reaction was carried out at the previously-optimized conditions of a cell OD_{600} of 50 and initial caffeine concentration of 5 mM (Mock et al., In Press 2022). After the first round of reaction, 1,686 ± 121 µM caffeine was consumed, resulting in 906 ± 26 µM paraxanthine and 350 ± 19 µM 7-methylxanthine (Table S1). The purpose of multiple reactions using fresh cells with reused supernatant stems from the observation that the reaction slowed greatly after five hours and ultimately plateaued with 3,344 ± 29 µM caffeine remaining (Fig 2 Round 1 & Table S1). We then hypothesized that the addition of fresh cells would further increase conversion of caffeine to paraxanthine and 7-methylxanthine. Thus, the reaction supernatant was recycled with fresh cells (OD_{600} = 50), constituting the second reaction

and resulting in consumption of an additional 1,862 ± 20 μM caffeine. The concentration of paraxanthine increased slightly to 1,033 ± 7 μM, while the concentration of 7-methylxanthine increased to 1,426 ± 36 μM. Both caffeine and paraxanthine decreased in further rounds of recycled reactions as 7-methylxanthine continued to increase (Fig 2 & Table S1). After four cycles, the reaction mixture contained 231 ± 10 μM caffeine, 274 ± 4 μM paraxanthine, and 2,614 ± 21 μM 7-methylxanthine (Fig 2 & Table S1).

After process optimization was complete, the reaction was scaled-up for purification purposes. Strain MBM019 was grown in 4 L LB media for use in one cycle of a 640 mL reaction with the optimized conditions determined from the 15 mL reactions. Spent cells were removed and freshly grown cells were added to a final OD_{600} of 50 between each of the four cycles, resulting in a final supernatant volume of 580 mL after removing cells from the fourth cycle. After four large-scale reaction cycles, caffeine was degraded to a final concentration of 669 μM with concomitant production of 552 μM paraxanthine and 2,120 μM 7-methylxanthine. Overall, 86.6 mol% of caffeine was consumed. Conversion to 7-methylxanthine accounted for 42.6 mol% of caffeine and conversion to paraxanthine accounted for 11.0 mol%, representing a total of 53.6 mol% of the converted caffeine. The remaining 33.0 mol% of unaccounted product may have been converted to other compounds, such as 1-methylxanthine. This theory is supported by the presence of several unconfirmed peaks seen in the HPLC chromatograph (Fig S2) (Mock et al., In Press 2022), as well as observed enzyme promiscuity that has been previously characterized in Ndm enzymes when reacted *in vivo* (Mock et al., 2021). Products were isolated from the reaction supernatant using two rounds of preparatory-scale HPLC (Fig S3). HPLC purification resulted in separation efficiencies of 92.73% for 7-methylxanthine and 98.73% for paraxanthine (Table S2). Following purification, the compounds were dried to a powder and collected, resulting in the recovery of 177.3 mg 7-methylxanthine and 48.1 mg of paraxanthine (Table S2 & Fig S4).

7-Methylxanthine and paraxanthine purity was analyzed by HPLC using authentic standards and the retention times were confirmed to be the same (Fig S4). ^{1}H-NMR was also used to confirm the identity of the biologically produced 7-methylxanthine and paraxanthine (Fig S5). The presence of proton peaks correlating with 7-methylxanthine were confirmed at δ 11.43 (1H) and δ 10.86 (1H) corresponding to –NH, δ 7.88 (1H) corresponding to -C=CH, δ 3.82 (3H) corresponding to the $–CH_3$ group. The presence of proton peaks correlating with paraxanthine were confirmed at δ 11.83 (1H) corresponding to –NH, δ 7.92 (1H) corresponding to -C=CH, δ 3.86 (3H) and δ 3.18 (3H) corresponding to both $–CH_3$ groups. Peaks δ 3.32 and δ 2.50 in both chromatograms are water and DMSO, respectively. There is a very small additional peak observed in the 7-methylxanthine chromatograph just below δ 2 that is believed to be residual acetic acid.

During the four-cycle production reaction, we observed a maximum paraxanthine concentration of approximately 1 mM, after which 7-methylxanthine concentration began to increase rapidly (Fig. 2). This plateau in paraxanthine concentration was surprising, as the NdmA4 mutant was designed for N_3-demethylation of caffeine to paraxanthine, and a single round of reaction yielded paraxanthine as the major product. Data from the multi-cycle reaction has led us to hypothesize that NdmA4 is more active toward paraxanthine than caffeine. Indeed, a small-scale MBM019 resting cell assay converted 516.6 ± 49.6 μM paraxanthine to 459.1 ± 27.2 μM 7-methylxanthine over five hours, compared with conversion of 345.7 ± 19.3 μM caffeine to 204.9 ± 6.0 μM paraxanthine and 73.4 ± 7.6 μM 7-methylxanthine over the same time period (Fig. 3). Use of baffled flasks to improve oxygenation in the resting cell assay yielded no improvement (data not shown), indicating that oxygen is not limiting. The wild-type NdmA is an N_1-demethylase capable of fully converting 1 mM caffeine to theobromine in 90 minutes, and NdmA4 has retained that N_1-demethylation activity toward paraxanthine, albeit at a lower rate, resulting in a single enzyme capable of producing 7-methylxanthine from caffeine. Further enzyme optimization via mutagenesis and the subsequent study of enzyme kinetics will be required to generate an enzyme with improved activity toward caffeine and yield of 7-methylxanthine.

In summary, we have demonstrated the ability to produce 7-methylxanthine from caffeine through four serial resting cell reactions using the previously optimized *E. coli* MBM019 strain, followed by HPLC purification of the paraxanthine (minor product) and 7-methylxanthine (major product) generated. The identity of these products was further confirmed by ^{1}H-NMR. This study also demonstrated that cells expressing the NdmA4

mutant enzyme are more active toward paraxanthine than caffeine. To our knowledge, this is the first report of the biocatalytic production of 7-methylxanthine from caffeine.

Methods:

Cell Growth, Protein Expression and Resting Cell Assays

The *E. coli* strain MBM019 was grown in LB medium, and gene expression was induced as previously described by Mock *et al* . (Mock et al., In Press 2022; Mock et al., 2021). For more details, please reference the Supplemental Material. The cells were then harvested by centrifugation at 10,000 x *g* for 10 min at 4 and washed twice in ice cold 50 mM potassium phosphate (KP_i) prior to assay. For reactions designated for product isolation, four 2.8 L Fernbach flasks each containing 1 L LB medium was used for cell growth. After washing, cells were resuspended in 10 mL of ice cold 50 mM KP_i. Reactions for supernatant recycling optimization with fresh cells were carried out in triplicate at an OD_{600} of 50, starting substrate concentration of 5 mM, and at a volume of 15 mL. Subsequent reaction substrate concentrations were dependent on the extent of the previous reaction. Reactions for the comparison of caffeine and paraxanthine as substrates were carried out in triplicate at an OD_{600} of 5, substrate concentration of 1 mM, and at a volume of 2 mL. A single large-scale reaction for purification was conducted at the maximum volume possible while still retaining the required OD_{600} of 50 for large-scale reactions with a caffeine concentration of 5 mM.

HPLC Separation

A detailed description of separation methods is provided in the Supplemental Material. Briefly, the harvested supernatant was filtered through a 0.2 μm filter, and methanol (MeOH) was added to the supernatant to reduce changes in MeOH concentration within the system during purification. The first round of HPLC purification was designated for the removal of caffeine and for the crude separation and collection of paraxanthine and 7-methylxanthine using a 15% MeOH mobile phase (Fig S3). The collected solutions were concentrated via rotary evaporation at 70 and 200-220 mbar. A second round of HPLC purification with 5% MeOH as mobile phase was required for complete purification of both the 7-methylxanthine and the paraxanthine solutions. Both purified solutions were concentrated by rotary evaporation and then dried to a powder (Fig S4 & S5).

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Conflict of interests: The authors declare no conflicts of interest.

Data availability statement: The authors confirm that the data supporting the findings of this study are available within the article and the supporting information.

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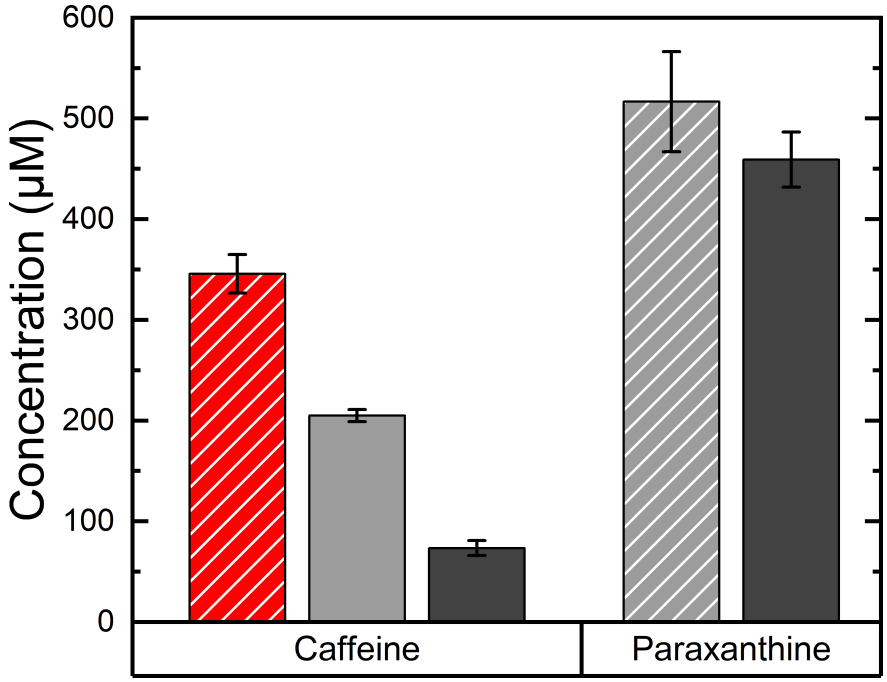
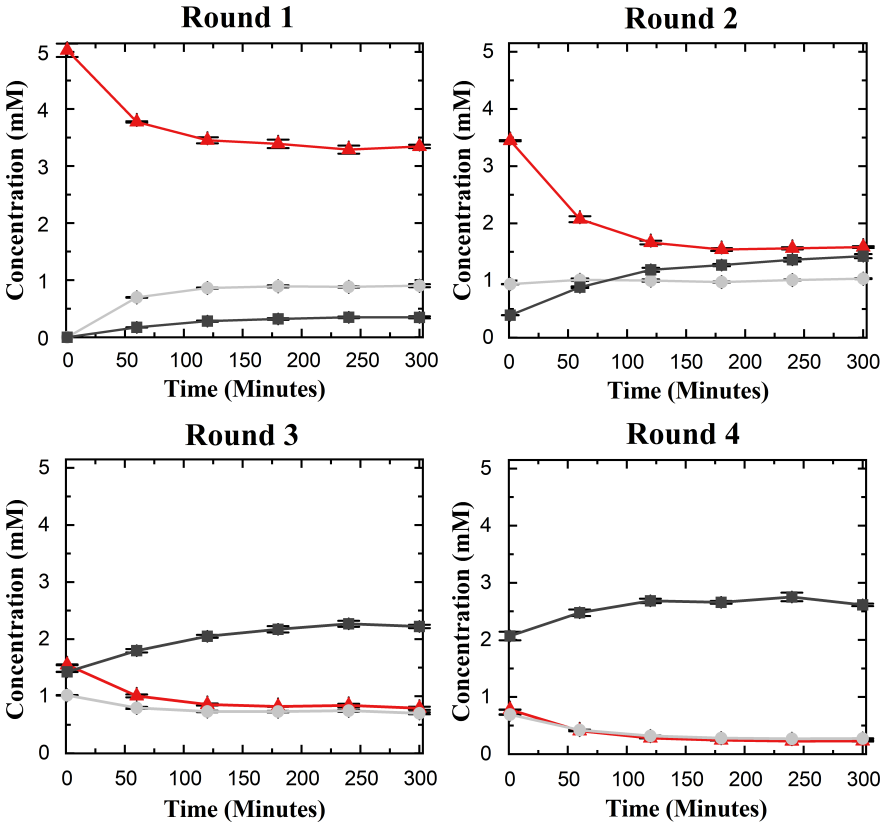
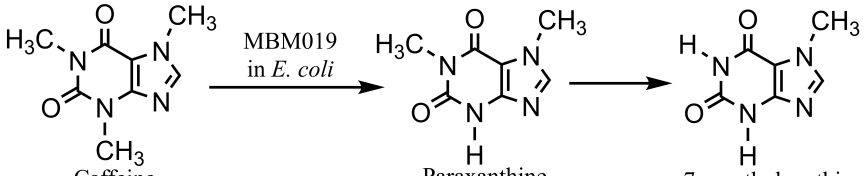
Figure 1. Sequential production of paraxanthine and 7-methylxanthine from caffeine by E. coli strain MBM019. Price per gram of each compound is based on the lowest retail values found from Sigma Aldrich (March 2022).

Figure 2. *N* -demethylation of caffeine (red; $^{)}toparaxanthine(lightgrey;)and7-methylxanthine(darkgrey;)byE.\ colistrainMBM019.Thesupernatantfrom Round1wasmixedwithfreshMBM019cellstoco methylxanthinewasachievedattheendofRound4.Meanconcentrationsandstandarddeviationsoftriplicateresultsareshown.$

Figure 3. Direct comparison of conversion of caffeine (red) to paraxanthine (light grey) and paraxanthine to 7-methylxanthine (dark grey) by the genetically engineered *E. coli* strain MBM019. Hatching indicates the concentration of substrate consumed. Solid coloring indicates the concentration of product generated. Substrates for each reaction are also listed at the bottom of the graph. Reactions were conducted at a 2 mL volume in 50 mM KP_i with cells at an OD_{600} of 5 and substrate concentrations of 1 mM. Reaction conditions were set to 37, 200 rpm for 5 hours. Mean concentrations and standard deviations of triplicate results are shown from the conclusion of a five-hour resting cell assay.







Caffeine
(1,3,7 - trimeythlxanthine)
$0.046/g

Paraxanthine
(1,7 - dimethylxanthine)
$1,480.00/g

7 - methylxanthine
$528.00/g