



Production of 7-methylxanthine from Theobromine by Metabolically Engineered *E. coli*

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Abstract

In this work, a novel biocatalytic process for the production of 7-methylxanthines from theobromine, an economic feedstock has been developed. Bench scale production of 7-methylxanthine has been demonstrated. The biocatalytic process used in this work operates at 30 °C and atmospheric pressure, and is environmentally friendly. The biocatalyst was *E. coli* BL21(DE3) engineered with *ndmB/D* genes combinations. These modifications enabled specific N₇- demethylation of theobromine to 7-methylxanthine. This production process consists of uniform fermentation conditions with a specific metabolically engineered strain, uniform induction of specific enzymes for 7-methylxanthine production, uniform recovery and preparation of biocatalyst for reaction and uniform recovery of pure 7-methylxanthine.

Many *E. coli* BL21(DE3) strains metabolically engineered with single and/or multiple *ndmB/D* genes were tested for catalytic activity, and the best strains which had the higher activity were chosen to carry out the N-demethylation reaction of theobromine. Strain pBD2dDB had the highest activity for the production of 7-methylxanthine from theobromine. That strain was used to find the optimum amount of cells required to achieve complete conversion of theobromine to 7-methylxanthine within two hours. It was found that the optimum concentration of pBD2dDB strain to achieve 100% conversion of 0.5 mM theobromine to 7-methylxanthine was 5 mg/mL. The cell growth of pBD2dDB strain was studied using two different growth media, (Luria-Bertani Broth and Super Broth). Super broth was found to be the best medium to produce the highest amount of cell paste (1.5 g). Subsequently, the process was scaled up in which 2 L reaction volume was used to produce 7-methylxanthine (100% conversion) from 0.5 mM theobromine catalyzed by pBD2dDB strain. The reactions was carried out at 30 °C and 250 rpm shaker speed, and the reaction medium was 50 mM potassium phosphate buffer (pH=7). 7-methylxanthines was separated by preparative chromatography with high recovery, and the product solution was collected, purified by drying at 120-140 °C for 4 hours and, recovered (127 mg). Purity of the isolated 7-methylxanthine was comparable to authentic standards with no contaminant peaks, as observed by HPLC, LC-MS, and NMR.

Keywords: 7-methylxanthine, theobromine, Biocatalyst, *E. coli*, Chromatographic separations

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1- Introduction

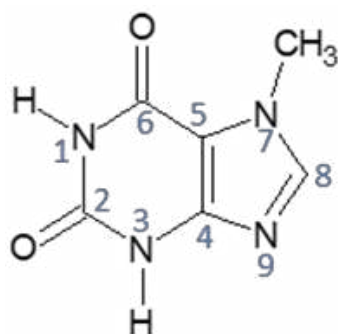


Fig. 1. Molecular structure of 7-methylxanthine

7-Methylxanthine (7MX) is one of caffeine derivatives. It, in addition to other methylated xanthines, belongs to group of compounds known as purine alkaloids.

Methylxanthines are natural and synthetic compounds found in many foods, drinks, pharmaceuticals, and cosmetics [1, 2]. 7-methylxanthine (7MX), which has a methyl group attached to N₇ of the xanthine ring (Figure 1), has been proven to have a therapeutic effect on the development of form-deprivation myopia in pigmented rabbits [3]. Trier et. al., studied the biochemical and ultrastructural changes in rabbit sclera after treatment with 7-MX [4]. Similar study was also conducted on guinea pigs [5]. In another study, Trier et. al., found that 7-methylxanthine reduces eye elongation and myopia progression in childhood myopia [6].

Aside from caffeine, production of many methylxanthines is currently performed by chemical synthesis [7, 8]. 7-Methylxanthine is currently produced only as 'retail sample' by chemical synthesis. However, no detailed information is available about the exact procedure used in the synthesis. Chemical synthesis of 7MX might follow Traube synthesis [7] or purine synthesis by Fischer [9].

Chemical synthesis of 7MX, as the other methylxanthine, utilizes many chemicals, multiple reactions, and different reaction conditions, making it complicated, environmentally dissatisfactory, and expensive.

Additionally, there is no recorded research on the biocatalytic production of 7MX using any kind of bacterium. Due to the high price of 7MX, there was no recorded market-size for 7MX; however, developing a new economical method for the production of 7MX has a potential for making this fine chemical. Recently we developed a common bioprocess for the production of 3-methylxanthine from theophylline [10] and theobromine from caffeine [11].

This work aims to use *E. coli* engineered with NdmB and NdmD genes to directly produce 7-methylxanthine from theobromine in one single reaction (Figure 2). These genes were found in *Pseudomonas Putida CBB5* that was able to degrade caffeine and its derivatives [12]. The N₃-demethylation reaction (which removes the methyl group on attached to the nitrogen atom at location 3 on the xanthine ring) is catalyzed by the enzyme NdmB. This enzyme is a Rieske [2Fe-2S] non-heme iron monooxygenase that requires a partner reductase, NdmD, to transfer electrons from NADH. The reaction requires one molecule of O₂ per methyl group removed, resulting in the production of formaldehyde and water [10].

This work is the first report on the biocatalytic production of 7-methylxanthine from theobromine by metabolically engineered *E. coli* that includes separation and purification of the product. The N-demethylases genes *ndmB* and *ndmD* were introduced into *E. coli* at different gene dosages, and the resultant strains were screened for 7-MX production. The optimum strain with the highest 7MX production was chosen for further study.

The biocatalytic approach used here operates at ambient temperature and pressure and is environmentally friendly.

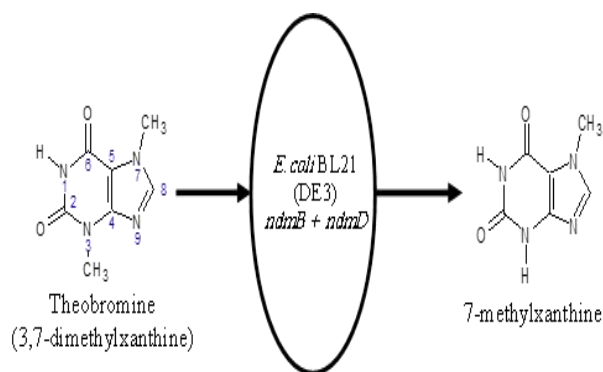


Fig. 2. Schematic representation for the biocatalytic N-demethylation of theobromine to 7-methylxanthine by *E. coli* BL21(DE3) genetically engineered with N-demethylation genes of *Pseudomonas putida* CBB5 *ndmB* and *ndmD*

2- Materials and Methods

2.1. Chemicals and Reagents

Theobromine and 7-methylxanthine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luria-Bertani Lennox (LB) and Difco Select APS™ Super Broth (SB) dehydrated media were obtained from Becton Dickinson and Company (Sparks, MD, USA). HPLC-grade methanol (J.T. Baker, Phillipsberg, NJ, USA) was used in all chromatographic studies.

2.2. Strain Construction

E. coli BL21(DE3) was used to construct all strains required as it was explained in a previous research [10, 14]. These metabolically engineered *E. coli* strains are shown in Table 1.

2.3. Cell Growth and Protein Expression

E. coli strains were grown in Super Broth (SB) or Luria Broth (LB) medium with appropriate antibiotic at 37°C with shaking speed at 250 rpm. Concentrations of antibiotic used were 34, 30 and 100 µg/mL for chloramphenicol, kanamycin and ampicillin respectively.

Cell density was monitored by measuring the optical density at 600 nm (OD₆₀₀). Upon reaching an OD₆₀₀ of ~ 0.5, Ferric chloride (FeCl₃·6H₂O) was added (0.02 mM final concentration) and temperature was lowered to 18°C. When the OD reached (0.8-1), IPTG was added (0.2 mM final concentration) to induce expression of *ndmB* and *ndmD*.

The IPTG concentration of 0.2 mM was previously determined to give optimum protein expression [12]. Cells were harvested after (14-16) hours of induction by centrifugation at 10,000 x g for 20 min at 4°C and washed twice in 50 mM cold potassium phosphate (KPi) buffer (pH 7.5). Pelleted cells (wet cells) were weighed and resuspended in 50 mM KPi buffer prior to activity assays.

2.4. Reactions for 7MX production

All reactions (unless mentioned otherwise) were carried out in 2 mL microcentrifuge tubes with 1 mL total reaction volume containing theobromine. A VWR® symphony™ Incubating Microplate Shaker was used to carry out the reaction at 30 °C and 400 rpm. 100 µL Samples were taken periodically for HPLC analysis, and concentrations of theobromine and 7-methylxanthine were calculated using appropriate standards.

Reactions for product isolation were carried out in 1.96 L total volume with 5 mg/mL cells concentration and Theobromine concentration of 0.5 mM. These large-scale reactions were carried out in an Excella E24 Incubator Shaker (Eppendorf, Hamburg, Germany) shaker at 30 °C and 250 rpm. After all theobromine was consumed, the post-reaction mixture was centrifuged at 10,000 x g to separate the supernatant (7MX) from the cells.

2.5. Preparatory HPLC methods and product isolation

Purification of 7MX was carried out with preparatory-scale HPLC using a Shimadzu LC-10AD HPLC system equipped with a photodiode array detector. A Hypersil BDS C18 column of 21.2 mm diameter and 25 cm length was used as the stationary phase. Methanol-water-acetic acid (5:95:0.5, vol/vol/vol) was used as the mobile phase with an optimized flow rate of 2.5 mL/min.

The molecules resolved by the C18 column passed through the photodiode array detector, in which UV-visible absorption spectra were recorded. This HPLC is equipped with two pumps, A and B. The isocratic method was developed to be programmed so that pump B provided the mobile phase and pump A injected 25 mL of post-reaction mixture in 10 minute periods. At the end of the preparative chromatography 750 mL 7MX solution was collected in a bottle. The solution was concentrated by vacuum drying using Buchi Rotovap R114. The bath temperature was 60-70 °C. Finally, the concentrated solution was dried at ~140 °C for four hours to ensure removal all impurities. The left 7MX powder in the tray was collected, weighed and stored in a vial.

2.6. Analytical Procedures

Identification and quantification of 7MX as conducted on the same HPLC system described above. A Hypersil BDS C18 column (4.6 by 125 mm) was used as the stationary phase. The same mobile phase was used with a flow rate of 0.5 mL/min. Purity of 3MX was confirmed by High Resolution LC-MS Facility at the University of Iowa, Department of Chemistry using a Waters Q-TOF Premier interfaced with an Acquity UPLC system.

The NMR results were obtained from the NMR facility at the Chemistry Department of the University of Iowa. The spectrum was recorded in DMSO- d_6 with a Bruker DRX 500 NMR spectrometer at 300 K. The chemical shifts were relative to DMSO- d_6 using the standard δ notation in parts per million.

3- Results and Discussion

3.1. Screening of 7-methylxanthine Production from Theobromine by Metabolically Engineered E. coli

Five metabolically engineered *E. coli* strains were tested for activity to produce 7-methylxanthine from theobromine. These are single plasmid strains, pBD, dDB and two plasmid strains, pBDdDB, pBDdDD, and pBDdDB.

Table 1 shows the number and type of genes carried on each vector in each strain.

These strains have been constructed to be incorporated with single or multiple copies of NdmB and NdmD on both pET-32a(+) and pACYCDuet-1 expression compatible vectors [2].

7-MX screening of the above strains were carried out in 1 mL reactions at 30 °C and atmospheric pressure and started with initial theobromine concentration of 0.5 mM and 5 mg/mL wet cell.

Analysis for 7-MX after two hours of reaction showed that strains pBD and dDB consumed 62% and 64% of theobromine Fig. 3, this relatively same conversion indicates that the activity of the two strains is similar when a single copy of each of *NdmB* and *NdmD* are carried by any of the two compatible vectors (pBD and dDB).

Therefore, three Duet vectors carrying two *NdmD* genes (dDD), two *NdmB* genes (dDB), and one gene of each of *NdmD* and *NdmB* (dDB) were transformed into *E. coli* carrying pBD resulting in three more strains (pBDdDD, pBDdBB, and pBDdDB). In this case, the effect of adding additional copies of *ndmB* and *ndmD* genes on the activity was observed Fig. 3.

Each of the above three strains were tested for N-demethylation of theobromine to 7-methylxanthine under the same previous reaction conditions.

After two hours of the reaction time, pBDdBB strain consumed 90% of theobromine while pBDdDD completely consumed all the 0.5 mM theobromine present in the reaction. However, pBDdDB strain was able to convert all theobromine (100% conversion) to 7MX within ninety minutes Fig. 3.

This means pBDdDB strain, which has an approximate copy number of 50 for each of *ndmB* and *ndmD* has a higher activity than pBDdDD strain which has an approximate copy number of 40 and 60 for *ndmB* and *ndmD* respectively.

Table 1. Estimated copy number of *ndmA* and *ndmD* genes in strains used in this study

Strain	Approximate gene copy number*		<i>ndmD</i> : <i>ndmB</i> ratio
	<i>ndmB</i>	<i>ndmD</i>	
pBD	40	40	1.0
pBDdDB	50	50	1.0
pBDdBB	60	40	0.67
pBDdDD	40	60	1.5
dDB	10	10	1.0

*Approximate gene copy number was estimated based on approximate copy number of the plasmid (40 for pBD, 10 for dDB, dBB, and dDD) and number of genes in each plasmid.

This value was calculated as $C_i = N_{ij}P_{ij}$, where C_i = gene copy number, N_{ij} = number of genes i on plasmid j , P_j = copy number of plasmid j backbone, i = gene (*ndmB* or *ndmD*), and j = plasmid backbone (pET or pACYCDuet-1)

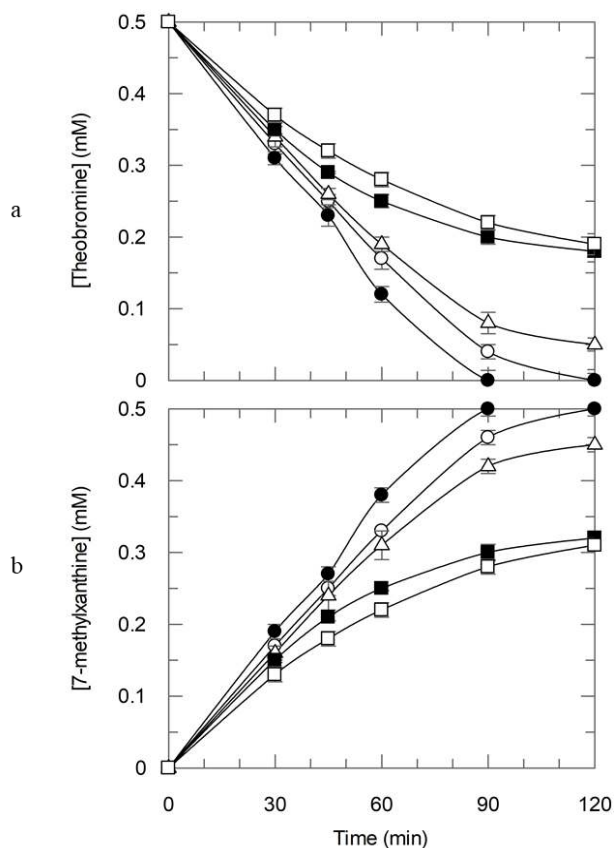


Fig. 3. (a) Consumption of theobromine and (b) formation of 7-methylxanthine by metabolically engineered *E. coli* resting cells (\square , strain pBD ; \blacksquare , strain dDB; \triangle , strain pBDdBB; \circ , pBDdDD; \bullet , strain pBDdDD) [initial theobromine concentration 0.5 mM, wet cells weight 5 mg/mL, temperature 30 °C, microplate shaking 400 rpm]

3.2. Complete Conversion of Theobromine to 7-MX by Strain pBDdDB

Strain pBDdDB was used to study theobromine consumption during the course of the N-demethylation reaction and achieving complete conversion to 7-MX. Three different wet cells concentrations were used (5, 10, and 15 mg wet cells/mL). During the two hours' reaction time, it was observed that the activity was high during the first hour of the reaction. After that, a reduction in the activity was noticed.

During the first hour of the reaction, conversion of 86%, 94%, and 100% of the 0.5 mM theobromine present initially was achieved by biocatalyst concentrations of 5, 10, and 15 mg/mL respectively. The rate of the reaction, as expected, was the highest with 15 mg wet cells/mL. The rate of the N-demethylation reaction became slower after one hour with 5 and 10 mg/mL cells and hence it took 30 and 60 minutes respectively for theobromine to be completely consumed. The reaction times for complete conversion were 60, 90, and 120 minutes for wet cells concentrations of 15, 10, and 5 mg/mL respectively. Also, based on the corresponding concentrations of 7MX produced and the time required for complete conversion for each case, the cells activities (mmole 7MX/mg cells.min) were determined.

Fig. 4 depicts theobromine consumption and 7-methylxanthine formation by the three different concentrations of pBDdDB strain. The activity for 5, 10, and 15 mg/mL resting cells concentrations were 8.3×10^{-7} , 5.6×10^{-7} , and 5.6×10^{-7} mmole 7MX / (mg wet cells.min) respectively Table 2. Therefore, the highest activity was achieved by a wet cells concentration of 5 mg/mL. This is because of the lower quantity of cells was used to produce 7MX by a complete conversion of TB within two hours. As a result, 5 mg resting cells/mL was used for further work.

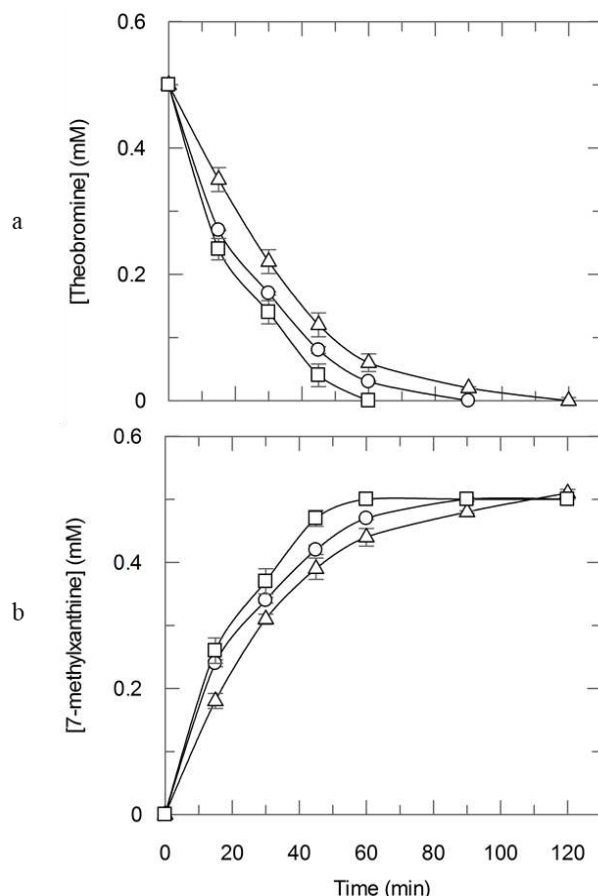


Fig. 4. Theobromine consumption and 7-methylxanthine formation by different concentrations of metabolically engineered *E. coli* pBDdDB strain (\triangle , 5 mg/mL; \circ , 10 mg/mL; \square , 15 mg/mL) [initial theobromine concentration 0.5 mM, temperature 30 C, microplate shaking 400 rpm]

Table 2. Cell activities at different cells concentrations (pBDdDB strain)

Wet cell concentration (mg/mL)	Time (min)	7-methylxanthine (mmole/mL)	Cells (mmole cells.min) * 10 ⁷	Activity (mmole 7MX/mg cells.min)
5	120	0.0005	8.3	
10	90	0.0005	5.6	
15	60	0.0005	5.6	

3.3. Cell Growth and Theobromine to 7-MX Conversion in Luria Broth and Super Broth

The growth media has an important role in cells growth since it provides the required nutrients the cells need to grow and maintain their activities. All previous reactions used to produce 7MX were using cells grown in Luria Broth (LB) medium. Luria Broth medium is considered one of the basic medium for cell growth. The purpose here is to use Super Broth (SB), a richer and more complex medium than Luria Broth, and compare the amount of cells harvested from each medium. In addition to that, activity for the cells grown in each medium was also determined.

Each media (100 mL) was inoculated with pBDdDB strain and left to grow overnight (14-16 hr). During that period, the cells grew to an optical cell density (OD600) of 5.75 and 11.61 in LB and SB respectively. After harvesting, 0.62 and 1.5 g wet cells were produced from LB and SB respectively.

The wet cells produced from SB were about 2.5 times larger the amount of cells produced from LB. This is a significant increase of the amount of wet cells harvested because SB is a richer nutrient medium and there is not much difference in cost of the media. The activity of the cells harvested from each medium was also tested.

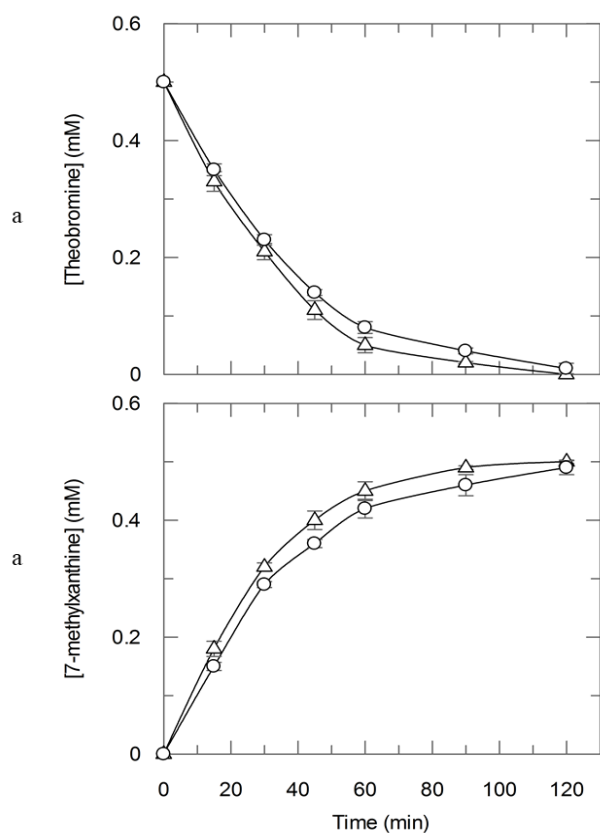


Fig. 5. Theobromine consumption and 7-methylxanthine formation by *coli* pBDdDB strain grown in Luria Broth (Δ) and Super Broth (\circ) [initial theobromine concentration 0.5 mM, temperature 30 °C, microplate shaking 400 rpm]

Fig. 5 shows TB consumption and 7MX production by the cells grown in LB and SB. Theobromine consumption and 7MX formation were a little higher by the cells grown in SB than those grown in LB. This may be due to 'healthier cells' in the rich SB, Thus, Super Broth medium (SB) was considered as the growth medium for further work.

Fig. 6 shows the N-demethylation reaction of 0.5 mM theobromine to 7-MX. The 1 mL reaction was catalyzed by 5 mg/mL strain pBDdDB grown in Super Broth. The reaction conditions were 30 C and 400 rpm micro-plate shaker speed. These conditions were used for scale up of 7-MX production.

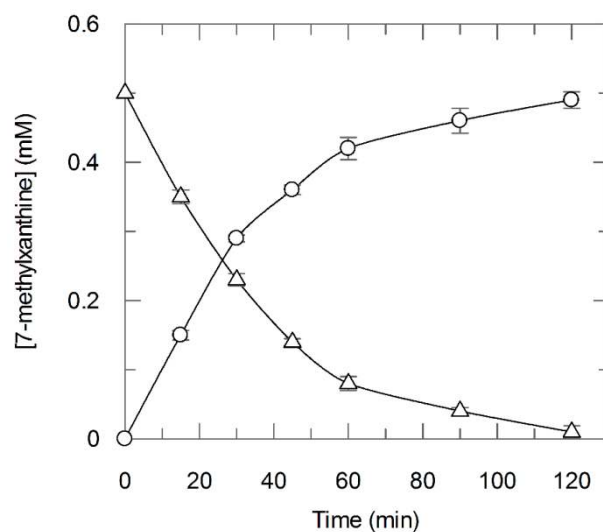


Fig. 6. Theobromine consumption (Δ) and 7-methylxanthine production (\circ) by 5 mg/mL *E. coli* pBDdDB strain grown in Super Broth [initial theobromine concentration 0.5 mM, temperature 30 C, microplate shaking 400 rpm]

3.4. Scale up Cell Growth and 7-methylxanthine Production

The purpose of scale-up was to produce 100 mg 7MX pure powder for validation of the technology at the bench scale. To produce this amount of 7MX from theobromine, cell growth had to be scaled-up first. Then, based on the amount of cells harvested, the reaction mixture was also scaled-up to produce, separate and characterize the purity of 7-MX

Super Broth (1000 mL) in 2.5 L flask was used to grow the strain pBDdDB overnight (14-16 hr). When the cells were harvested the optical density were at 600 nm (OD600) 9.26. The wet cells were stored at 4 oC until use. The amount of cells harvested was 9.8 g.

For resting cell concentration of 5 mg/mL in the reaction mixture, the amount of cell harvested was adequate to carry out a reaction of 1.96 L. The reaction conditions were 0.5 mM theobromine concentration, 5 mg/mL resting cells, temperature 30 oC, and shaker speed of 250 rpm. After two hours of reaction, all theobromine was converted to 7-MX (100% conversion).

Fig. 7 shows the HPLC chromatograms at the beginning and end of reaction in which all theobromine presented initially was consumed in two hours. Thus, 0.5 mM (83 mg/mL) 7MX was produced. Accordingly, the theoretical amount of 7MX produced in the total reaction mixture was 163 mg.

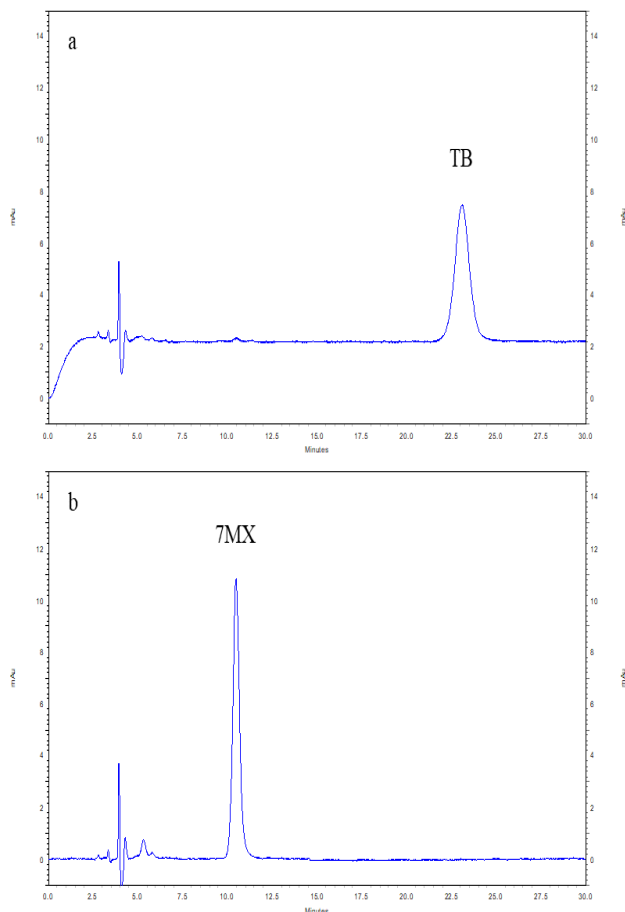


Fig. 7. HPLC chromatograms for the 1.96 reaction at (a) 0 hour and (b) 2 hours

3.5. Separation and Purification of Biocatalytically Produced 7-methylxanthine

After removing the solids from the reaction mixture by centrifugation, the post reaction supernatant volume collected was 1.9 L. This supernatant was filtered using 0.2 μm filter to completely remove any microparticles. This was done to avoid any potential contamination in the HPLC separation column.

This 1.9 L of supernatant, which contained 7MX solution, was concentrated by evaporation under vacuum to 750 mL.

After that, that amount of product solution was introduced to the chromatographic separation column to separate the product. 7MX eluted at a retention time of 104 minute as it is shown in Fig. 8. 7MX solution was collected in a bottle after each injection and the total volume of 7MX solution collected was 750 mL.

The pooled solution was dried at ~ 140 oC for four hours to ensure removal of methanol (B.P. 65 oC), water (B.P. 100 oC), and acetic acid (B.P. 118 oC).

The resultant 7MX powder in the tray was collected, weighed (127 mg) and stored in a vial Fig. 9.

The total recovery of 7-methylxanthine after chromatographic separation and purification was 78% (127 mg/163 mg), and the overall yield of 7MX based on the amount of theobromine fed to the reaction (0.5 mM in 1.96 L reaction) was 0.72 mg 7MX / mg theobromine.

The yield could be much higher with use of a larger prep scale column (and avoid repeated injections and pooling of the product).

This is the first report describing in detail, the biological production and separation of 7MX from theobromine by *E. coli*. engineered with the N-demethylation genes.

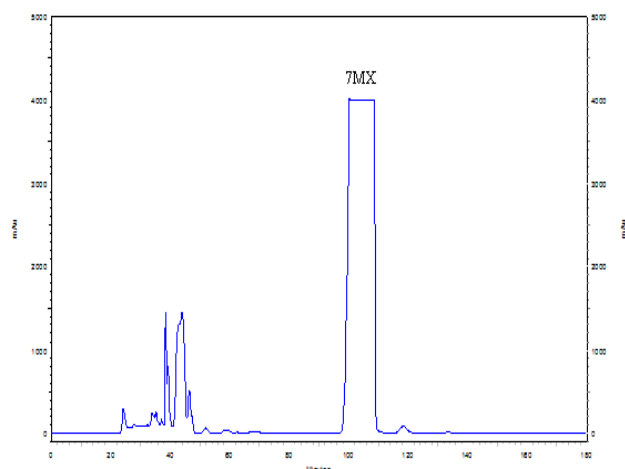


Fig. 8. Separation of 7-methylxanthine (7MX) by preparative chromatography Retention time of 7MX is 102 minutes



Fig. 9. 127 mg biologically produced 7-methylxanthine powder from theobromine

4- Analytical Characterization of-methylxanthine

The purity of 7MX was initially confirmed by analytical HPLC using appropriate authentic standards. The retention time of the biologically produced product Fig. 10 and authentic standards were identical.

The High Resolution LC-MS spectrum of biologically produced and standard 7MX was identical Fig. 11. LC/MS was recorded on ESI positive mode; distinct M+1 ion peak at 168.0591 and 168.0575 m/z were observed in the biologically produced and standard 7-methylxanthine respectively.

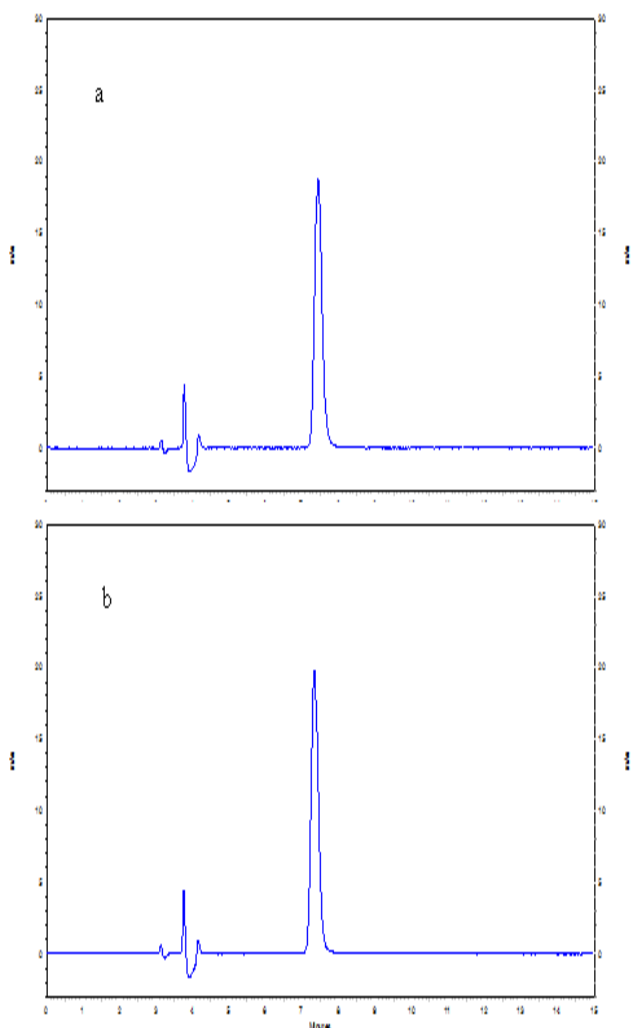


Fig. 10. HPLC chromatograms for 0.5 mM 7-methylxanthine (7MX) (a) biologically produced in this work (b) standard from Sigma Aldrich

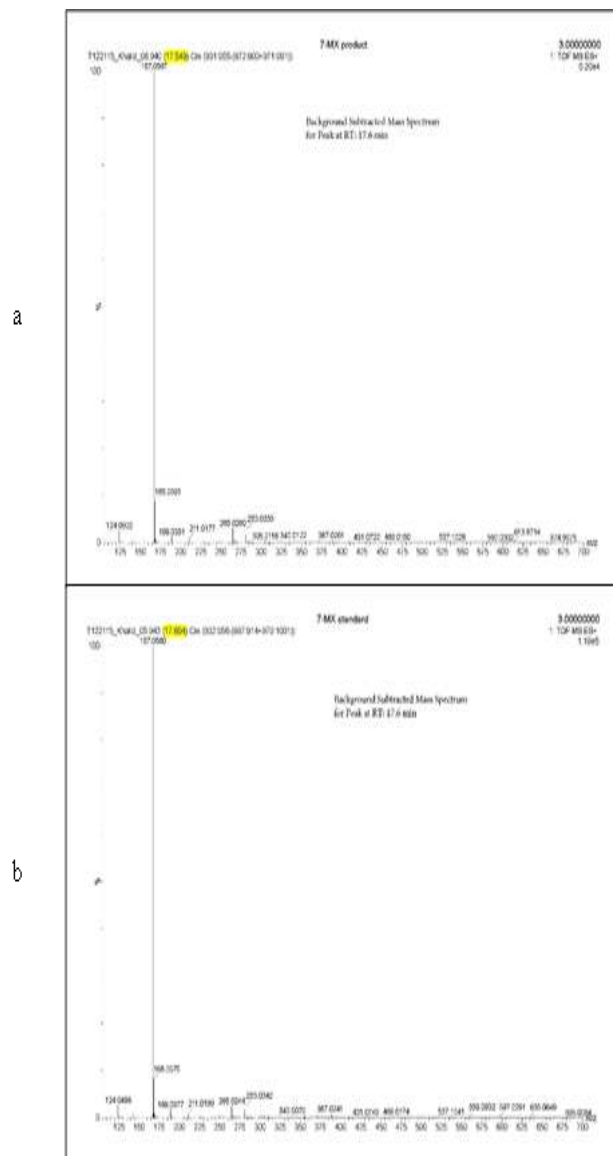


Fig. 11. LC-MS spectrum of 7-methylxanthine (7MX). (a) LC-MS of biologically produced and purified 7-methylxanthine sample produced in this work. (b) LC-MS of 7-methylxanthine standard obtained from Sigma Aldrich.

The ^1H NMR spectrum of biologically produced and standard 7-methyl xanthine also matched very well Fig. 12. ^1H NMR was recorded on a Bruker 500 MHz spectrophotometer using DMSO- d_6 as solvent. Standard 7-methylxanthine showed presence of peaks at δ 11.46 (s, 1H) and 10.82 (s, 1H) corresponding to $-\text{NH}$ proton, and peaks at δ 7.87 and 3.81 corresponding to $-\text{C}=\text{H}$ (s, 1H) and $-\text{CH}_3$ group (s, 3H).

The biologically produced 7-methylxanthine also showed peaks at δ 11.46 (s, 1H) and 10.82 (s, 1H) corresponding to $-\text{NH}$ proton, and peaks at δ 7.87 and 3.81 corresponding to $-\text{C}=\text{H}$ (s, 1H) and $-\text{CH}_3$ group (s, 3H). In conclusion, the biologically produced 7-methylxanthine in this work was highly pure and similar to the authentic standard by all analytical comparisons.

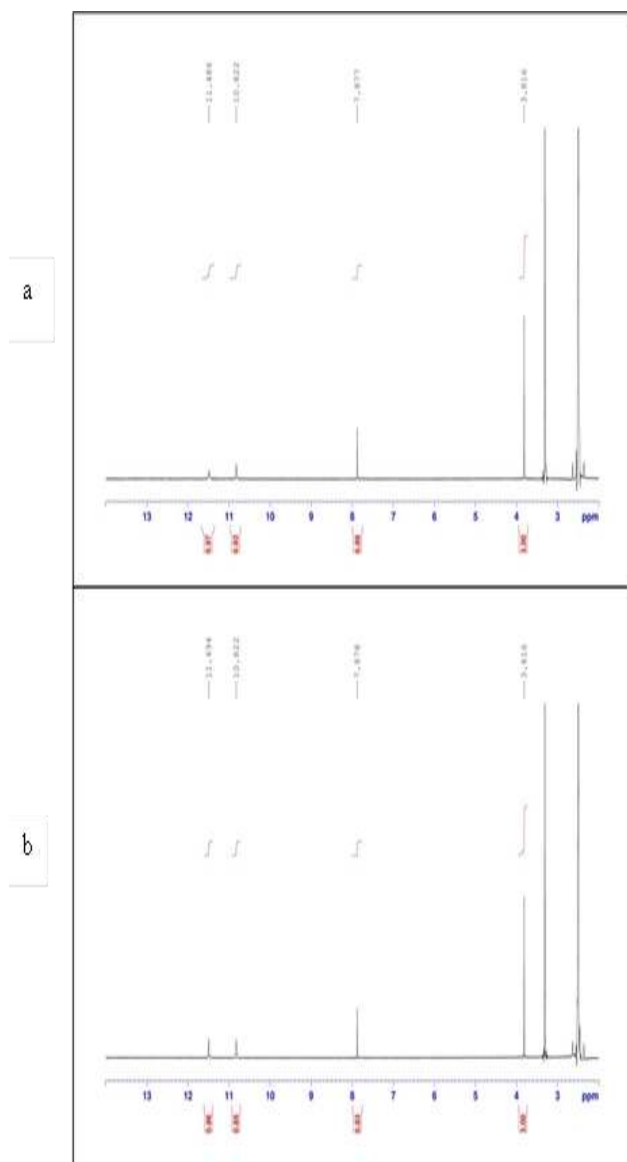


Fig. 12. NMR of 7-methylxanthine (7MX) (a) NMR of biologically produced and purified 7-methylxanthine produced in this work. (b) NMR of 7-methylxanthine standard obtained from Sigma Aldrich.

5- Conclusion

In this research, a novel biocatalytic process for the production of 7-methylxanthines from theobromine, an economic feedstock has been developed. The biocatalytic process used in this work operates at 30 °C and atmospheric pressure, and is environmentally friendly.

The biocatalyst was *E. coli* BL21(DE3) engineered with ndmB/D genes combinations. Bench scale production of 7-methylxanthines has been demonstrated, and 127 mg highly pure 7MX was produced. This is the first report for the biological production of 7-methylxanthine.

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انتاج 7-مثيل زانثين من الثيوبورومين بواسطة بكتريا القولون المعدلة وراثيا

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الخلاصة

تناولت هذه الدراسة تطوير عملية جديدة لإنتاج 7-مثيل زانثين من الثيوبورومين باستخدام بكتريا القولون BL21(DE3) المعدلة وراثيا بمورثات (*ndmB/D*) كعامل مساعد للتفاعل. الظروف التي استخدمت في عملية الانتاج هي 30 درجة مئوية عند الضغط الجوي. في البدء تم استخدام 5 سلالات من العامل المساعد وبينت النتائج ان السلالة *E. coli* BL21(DE3) pBD2dDB هي افضل عامل مساعد بتركيز (5 mg/mL) لانتاج اكبر كمية من 7-مثيل زانثين بنسبة تحول (100%) في زمن حوالي ساعتين. ايضا تم دراسة نمو العامل المساعد في نوعين من ال (media) هما (Super Broth) و (Lauria Broth) ووجد ان سلالة البكتريا pBD2dDB تنمو اكثر في ال (Super Broth) حيث وصلت كميتها الى (1.5 g). بعد ذلك تم استخدام العامل المساعد في عملية اكبر لغرض انتاج اكثر من 100 ملغرام من ال 7 مثيب زانثين. استخدم 2 لتر من سائل التفاعل يحتوي 0.5 mM من الثيوبورومين في وسط 50 mM محلول فوسفات البوتاسيوم (pH=7). التفاعل تم عند 30 درجة حرارة و 250 rpm سرعة اهتزاز الهزاز. بعد مرور حوالي ساعتين علة التفاعل تم عزل السائل الذي يحتوي على الناتج (7-مثيل زانثين) بواسطة الترشيح، ثم تم فصله بواسطة preparative chromatography حيث تم الحصول على نسبة فصل عالية. بعد ذلك تم فصل وتنقية الناتج بالتجفيف عند درجة حرارة 120-140 °C لمدة 4 ساعات. في النهاية تم الحصول على 127 ملغرام من 7-مثيل زانثين عالي النقاوة كما اثبتته فحوص ال HPLC و LC-MS و .NMR

الكلمات الدالة: 7-مثيل زانثين ، ثيوبورومين ، عامل مساعد ، بكتريا القولون ، عمليات الفصل الكروماتوغرافي