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Production of methlxanthines by metabolically engineered E. coli

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PRODUCTION OF METHLXANTHINES BY METABOLICALLY ENGINEERED *E. COLI*

by

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of the requirements for the Doctor of Philosophy
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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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has been approved by the Examining Committee for
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To Sheima for unconditional love and support

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ABSTRACT

Methylxanthines are natural and synthetic compounds found in many foods, drinks, pharmaceuticals, and cosmetics. Aside from caffeine, production of many methylxanthines is currently performed by chemical synthesis. This process utilizes many chemicals, multiple reactions, and different reaction conditions, making it complicated, environmentally dissatisfactory, and expensive, especially for monomethylxanthines and paraxanthine. In this work, we developed a novel biocatalytic platform for the production of methylxanthines from economic feedstocks; bench scale production of three different methlxanthines, theobromine, 3 and 7-methylxanthines has been demonstrated. The biocatalytic process used in this work operates at 30 ^{O}C and atmospheric pressure, and is environmentally friendly. The biocatalyst was *E. coli* BL21(DE3) engineered with *ndmA/D* or *ndmB/D* genes combinations. These modifications enabled specific N_1 and N_3- demethylation of caffeine, theophylline and theobromine to theobromine & paraxanthine, 3-methylxanthine and 7-methylxanthine respectively. This common production platform consists of uniform fermentation conditions with a specific metabolically engineered strain, uniform induction of specific enzymes for methylxanthine production, uniform recovery and preparation of biocatalyst for reaction and uniform recovery of pure products.

Many *E. coli* BL21(DE3) strains metabolically engineered with single and/or multiple ndmA/D or 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 to produce the higher value methylxanthines. Strain pDdA had the highest activity for the production of 3-methylxanthine from theophylline; strain pAD1dDD had the highest

activity for the production of theobromine from caffeine, and strain pBD2dDB had the highest activity for the production of 7-methylxanthine from theobromine. Each of these strains were used to find the optimum amount of cells required to achieve complete conversion of substrates to product(s) within two hours. It was found that 15 mg/mL resting cells concentration of pDdA strain was required to completely N-demethylate 1 mM theophylline to 3-methylxanthine (81% conversion) and 1-methylxanthine (13%). Also, 15 mg/mL resting cells concentration of pAD1dDD strain was required to completely convert 1 mM caffeine to theobromine (98.5% conversion) and paraxanthine (1.5%). The optimum concentration of pBD2dDB strain to achieve 100% conversion of 0.5 mM theobromine to 7-methylxanthine was 5 mg/mL. Moreover, coffee post -brew waste was used as a source of caffeine, which was completely utilized by 25 mg/mL resting cells pAD1dDD strain to theobromine by a conversion of 97%.

The cell growth of each specific strain was studied using different growth media, including Luria-Bertani Broth, Terrific Broth, and Super Broth. In all cases, super broth was found to be the best medium to produce the highest amount of cell paste. The amount of cell paste produced from 100 mL Super broth medium after 14-16 hour of growth was found to be 0.9, 0.9, and 1.5 g for pDdA, pAD1dDD, and pBD2dDB strains respectively. Subsequently, each reaction was scaled up to produce 100-300 mg pure methylxanthines products, and therefore cell growth was also scaled up (1-4 L) to produce adequate amount of biocatalyst to carry out these larger scale reactions. 1.3 L reaction volume was used to produce 3-methylxanthine (81%conversion) from 1 mM theophylline catalyzed by 15 mg/mL pDdA strain. 2 L reaction volume was used to produce theobromine (98.5% conversion) from 1 mM caffeine catalyzed by 15 mg/mL pAd1dDD strain. 2 L

reaction volume was used to produce 7-methylxanthine (100% conversion) from 0.5 mM theobromine catalyzed by pBD2dDB strain. All reactions were carried out at 30 ^{o}C and 250 rpm shaker speed, and the reaction medium was 50 mM potassium phosphate buffer (pH=7). All methylxanthines products were separated by preparative chromatography with high recovery, and each product solution was collected in bottles. Products were purified by drying at 120-140 C for 4 hours and 100, 255, and 127 mg 3-methylxanthine, theobromine, and 7-methylxanthine were recovered. Also, 178 mg theobromine was produced form post brew coffee waste from 1.16 L reaction catalyzed by 25 mg/mL pAD1dDD strain.

Purity of the isolated methylxanthine products was comparable to authentic commercially standards with no contaminant peaks, as observed by HPLC, LC-MS and NMR.

PUBLIC ABSTRACT

Methylxanthines are a group of compounds that can be used in the treatment of heart, lung, and kidney diseases. Currently, they are produced by difficult and environmentally unfriendly chemical methods. In this work, a new method exploits the use of genetically engineered *E. coli* bacteria to produce high value methylxanthines from economic feedstocks such as caffeine. This bio-based process introduces the use of low cost raw material to produce high value methylxanthines at room conditions (30 ^{o}C and atmospheric pressure), in one single reaction, with no negative effect on the environment. The feasibility of this production method was approved by producing and separating pure 106 mg 3-methylxanthine from theophylline (1,3-dimethylxanthine), 255 mg theobromine (3,7-dimethylxanthine) from caffeine, and 178 mg 7-methylxanthine from theobromine (3,7-dimethylxanthine).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Methylxanthines

Methylxanthines (MX) are purine alkaloids, which are secondary metabolites derived from purine nucleotides (Anaya, Cruz-Ortega et al. 2006). They are structurally comprised of a xanthine ring with methyl group(s) attached to nitrogen atoms at locations 1, 3, and/ or 7 positions (Figure 1.1). Caffeine (1, 3, 7-trimethylxanthine) is a trimethylxanthine while dimethylxanthines include theophylline (1, 3-dimethylxanthine), theobromine (3, 7-dimethylxanthine), and paraxanthine (1, 7-dimethylxanthine). Monomethylxanthines are 1-methylxanthine, 3-methylxanthine, and 7-methylxanthine. Traditionally, caffeine, theobromine, and theophylline are the most common methylxanthines in plants.

Caffeine, theophylline, theobromine, and paraxanthine are naturally occurring methylxanthines. Caffeine is the most abundant, followed by theobromine. Other methylxanthines, which are intermediates of caffeine metabolism and/or caffeine biosynthesis can be found in nature in small amounts. These methylxanthines are theophylline (1,3-dimethylxanthine), paraxanthine (1,7-dimethylxanthine), 1-methylxanthine, 3-methylxanthine, and 7-methylxanthine (Ashihara and Suzuki 2004).

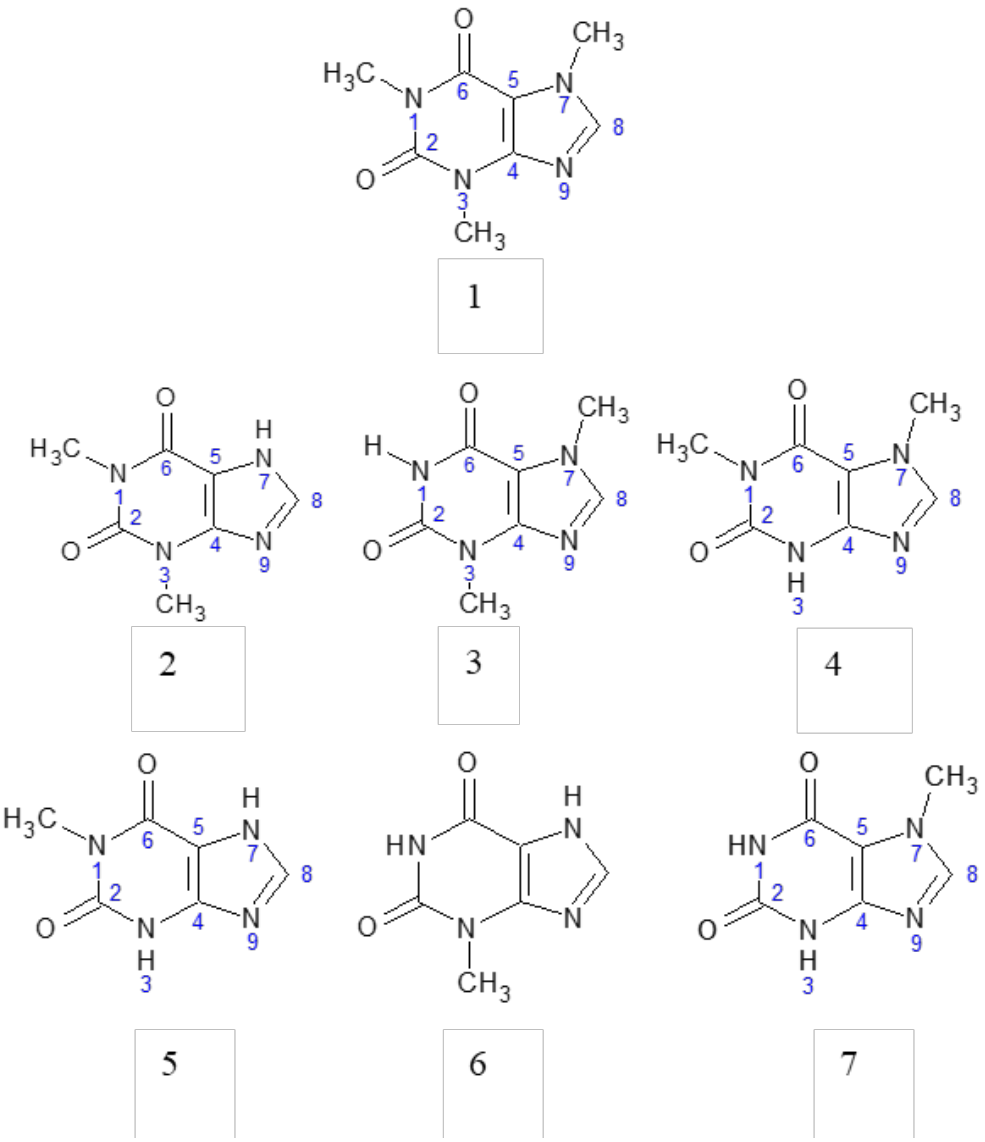


Figure 1.1 Methylxanthines (1) caffeine (1,3,7-trimethylxanthine), (2) theophylline (1,3- dimethylxanthine), (3) theobromine (3,7-dimethylxanthine), (4) paraxanthine (1,7-dimethylxanthine), (5) 1-methylxanthine, (6) 3-methylxanthine, (7) 7-methylxanthine

Natural Distribution of Methylxanthines

Caffeine, theophylline, and theobromine, are naturally occurring purine alkaloids. They occur in many plant species. For example, caffeine is present in coffee, theophylline in tea, and theobromine in cacao (Gummadi and Santhosh, 2006; Gummadi and Santhosh, 2006; Suzuki and Waller, 1984). Methylxanthines have been found in about 100 species representing 13 orders of plants kingdom (Ashihara and Crozier 1999, Ashihara and Suzuki 2004). Table 1.1 summarizes number of plants in which these major alkaloids present. Some of these are coffee (*Coffea* species), cupuacu (*Theobroma* granduflorum), and guarana (*Paullinia* cupana) (Ashihara and Crozier 1999). In these plants, caffeine is the most abundant purine alkaloid. Other purine alkaloids, including theobromine, theophylline, theacrine, and libertine also exist in other species. The ecological role of purine alkaloids in plants has been explained by two theories: (i) chemical defense theory and (ii) allelopathic or autotoxic theory. In the "chemical defense" theory, it is suggested that high amounts of caffeine is produced in new tissues of plants and fruits to protect them from insect larvae and other predators (Nathanson 1984, Hollingsworth, Armstrong et al. 2002). In the "allelopathic or autotoxic theory", it is postulated that seed coats and other plant-matter degradation release caffeine to soil to inhibit germination of other seeds to provide a selective advantage (Bolton and Null 1981, Suzuki and Waller 1984, Waller 1989).

Table 1.1 Distribution of purine alkaloids in plants. Source: (Anaya, Cruz-Ortega et al. 2006)

Latin Name	**Family Name**	**Common Name**	**Major Alkaloid**
Coffea Arabica	Rubiaceae	Arabica coffee	Caffeine
Coffea canephora	Rubiaceae	Robusta coffee	Caffeine
Coffea liberica	Rubiaceae		Theacrine, libertine
Coffea dewevrei	Rubiaceae		Theacrine, libertine
Camellia sinensis	Theaceae	Tea	Caffeine
Camellia assamica	Theaceae	Assam tea	Caffeine
Camellia assamica var *Kucha*	Theaceae	Kucha	Theacrine
Cammelia taliensis	Theaceae		Caffeine
Cammelia irrawadiensis	Theaceae		Theobromine
Camellia ptilophylla	Theaceae	Cocoa tea	Theobromine
Theobroma cacao	Sterculiaceae	Cacao (cocoa)	Theobromine
Theobroma grandiflorum	Sterculiaceae	Cupu	Liberine
Paulinia cupana	Sapindaceae	Guaraná	Caffeine
Cola sp.	Sterculiaceae	Cola	Caffeine
Citrus sp.	Rutaceae		Caffeine
Ilex paraguariensis	Aquifoliaceae	Yerba mate	Caffeine

Methylxanthines in Food and Drinks

It is well known that caffeine is one of the major components in human diet in the form of daily drinks like coffee, tea, and chocolate. Also, theobromine and theophylline can be found in chocolates and tea respectively (Ashihara and Crozier 1999, Anaya, Cruz-Ortega et al. 2006, Dash and Gummadi 2006) . Table 1.2 lists caffeine content in several known foods, beverages, and drugs.

Table 1.2 Concentration of caffeine in various common beverages and drugs. Source: (Barone and Roberts 1996, Carrillo and Benitez 2000, Heckman, Weil et al. 2010, Gopishetty, Louie et al. 2011)

Source of Caffeine	**mg/serving**	**mg/oz**
Coffee		
Decaffeinated (10 oz)	4-15	0.4-1.5
Instant (10 oz)	9-216	0.9-21.6
Plain, brewed (10 oz)	128-250	12.8-25
Espresso (5 oz)	150-450	30-90
Tea		
Tea, brewed (10 oz)	80-120	8-12
Iced Tea (12 oz)	65-75	5.4-6.3
Green (8 oz)	30-50	3.8-6.3
Black (8 oz)	25-110	3.1-13.8
Yerba Mate (8 oz)	65-130	8-16
Chocolate		
Hot cocoa (5 oz)	4	0.8
Chocolate milk (6 oz)	4	0.7
Milk chocolate (1.5 oz)	9	6
Chocolate bar (1.5 oz)	30	20
Soft Drinks		
Coca Cola (12 oz)	35	2.9
Pepsi (12 oz)	40	3.3
Dr. Pepper (12 oz)	40	3.3
Mountain Dew (12 oz)	55	4.6
Energy Drinks		
Monster (16 oz)	160	10
Rockstar (16 oz)	160	10
Red Bull (8.3 oz)	80	9.6
Amp (8.3 oz)	75	8.9
Drugs		
Anacin (2 tablets)	32	N/A
Exedrin (1 tablet)	65	N/A

Historical Science of Natural Methylxanthines

The usefulness of coffee in treating asthma was first reported in the nineteenth century (Salter 1860). The alerting effects of caffeine was found by Samuel Hahnemann (Hahnemann 1803). Caffeine was identified in coffee by Friedrich Ferdinand Runge (1795-1865) (Weinberg and Bealer 2001). The chemistry of caffeine was first explained by Fischer (Fischer 1881, Fischer 1881) in which he found that caffeine had same heterocyclic skeleton as uric acid. In 1841, theobromine was identified and its name was driven from the cocoa plant "*Theobroma*" which means "the food of gods"(Weinberg and Bealer 2001). As a minor compound in tea, theophylline was identified (Kossel 1888, Kossel 1889) in 1888, which was later introduced as a diuretic (Minkowski 1902) and in the treatment of asthma (Schultze-Werninghaus and Meier-Sydow 1982). Paraxanthine is not a main compound in plants; it is the primary metabolite of caffeine degradation in humans (Guerreiro, Toulorge et al. 2008).

Methylxanthines Effect on Human Health

In the field of pharmaceuticals, purine alkaloids are known as xanthine derivatives. Natural and synthetic xanthine derivatives have antagonistic activity based on binding to adenosine receptors A_1 and A_2. These compounds inhibit or stimulate adenylate cyclase activity. Also, they behave as non-selective phosphodiesterase inhibitors (Daly, Bruns et al. 1981, Gummadi, Bhavya et al. 2012). In addition to that, they have many effects as component of pharmaceutical preparations (Daly, Butts-Lamb et al. 1983, Schwabe, Ukena et al. 1985, Schwabe, Ukena et al. 1985, Essayan 2001, Lowinson 2005). Caffeine is the world's number one used drug for mood-altering(Lowinson 2005). It is known to be used as neurological, cardiac, and respiratory

stimulant (Gokulakrishnan, Chandraraj et al. 2005, Dash and Gummadi 2006). In addition to that, caffeine is used as an analgesic in asthma, headache, cough, and cold medicines (Stavric 1988, Stavric 1988, Daly 2007). As a potent bronchodilator, theophylline is used to control asthma, treat neonatal apnea, and relieve bronchial spasms (Stavric 1988, Stavric 1988, Mazzafera 2002). The severity of the physiological reactions of theobromine is lower than those of caffeine; the major use of theobromine is as a diuretic, miocardiac stimulant, or vasodilator (Stavric 1988). 7-Methylxanthine has been observed to have the ability to improve the quality of sclera collagen, and was suggested for the treatment or prevention of axial myopia, glaucoma, and macular degeneration (Trier, Olsen et al. 1999). Caffeine and theobromine were reported to be toxic to both human and animals. For example, it was demonstrated that exposure to caffeine was related to poorer neuromuscular development and significant increases in breech presentation of fetuses; theobromine is demonstrated to be toxic to dogs, rats, and rabbits (Eteng, Eyong et al. 1997). Caffeine dosages greater than 500 mg/day could result in anxiety, headaches, irritability, insomnia, anorexia, lightheadedness, and tremulousness (Barceloux 2012). Theobromine is toxic to human at dosages higher than 250 mg (Baggott, Childs et al. 2013). Also, theobromine levels at 300 mg/kg and higher were deadly to dogs (Gans, Korson et al. 1980). Moreover, It was found that feeding cocoa meal containing 1.7 mg % theobromine was toxic to laying hens (Black and Barron 1943).

1-Methylxanthine has been investigated for its antioxidant properties (Lee 2000). Sveltan, a mixture of 1.5, 0.07, 1.4, 0.6 g/L of caffeine, paraxanthine, theobromine, and 7-methylxanthine respectively, has been marketed as a natural skincare and slimming

agent (Libragen 2016). Paraxanthine (PX), like other methylxanthines, is a psychoactive nervous system stimulant. Its main use is due to its functions as an adenosine receptor antagonist and phosphodiesterase inhibitor. PX is found to be the best repressor of hepatocellular connective tissue growth factor expression, and also the least toxic (Gressner, Lahme et al. 2009). In another study, paraxanthine was found to have potential for the protection of dopaminergic neurons, the main source of dopamine (DA) in the brain. Death of these neurons cause Parkinson's Disease (Guerreiro, Toulorge et al. 2008). Moreover, paraxanthine was found to have effect on temperature/heart-rate curve in frogs, and very strong anti-thyroid activity where it was able to neutralize the effect of 5000-fold concentration thyroxine (Mann and Porter 1945).

Biosynthesis of Methylxanthines

Purine alkaloids, including methylxanthines, are synthesized from purine nucleotides, the precursors of nucleic acids (Stryer 1995, Ashihara and Crozier 1999, Henderson and Paterson 2014). The cellular resources that provide purine nucleotides are purine salvage and *de novo* biosynthesis (Ashihara and Crozier 1999). Synthesis of purine nucleotides by *de novo* is done on a ribose phosphate molecule by incorporating parts of many different smaller molecules, including aspartate, glycine, glutamine, a single carbon unit from formyltetrahydrofolate, and CO_2 (Shemin and Rittenberg 1947, Sonne, Buchanan et al. 1948, Anderson and Gibbs 1962, Stryer 1995). The actual final product of de novo purine nucleotide biosynthesis is inosine 5'-monophospphate (IMP) (Figure 1.2). It is the precursor of adenosine (AMP), guanosine (GMP), and xanthosine 5'-monophosphates (XMP), which are the initial components used in the biosynthesis of

purine alkaloids. Biosynthesis of methylxanthines is the first step in the biosynthesis of purine alkaloids where AMP, IMP, XMP, and GMP are converted to xanthosine.

Figure 1.3 depicts the pathways of the biosynthesis of purine alkaloids. In coffee and tea plants, caffeine biosynthesis is accomplished by converting xanthosine to caffeine by multiple N-methylation steps. N-methyltransferase enzymes catalyze the N-methylation where S-adenosyl-L-methionine is the donor of the methyl groups. 7-Methylxanthosine synthase catalyzes the conversion of 7-methylxanthosine to 7-methylxanthine (Negishi, Ozawa et al. 1985, Mizuno, Kato et al. 2003). 7-Methylxanthonsine is converted to 7-methylxanhtine by hydrolytic cleavage of the purine ring from ribose by methylxanthine nucleosidase (Negishi, Ozawa et al. 1988). Methylation of 7-methylxanthine at nitrogen atom N_3 forms theobromine, which is further N-methylated at N_1 position to produce caffeine (Suzuki and Takahashi 1975, Roberts and Wallert 1979, Baumann, Koetz et al. 1983, Fujimori, Suzuki et al. 1991). Secondary biosynthetic pathways of caffeine are also known, and these include the transient formation of 7-methyl-XMP, paraxanthine, xanthine, 3-methylxanthine, and theophylline (Ashihara and Crozier 1999).

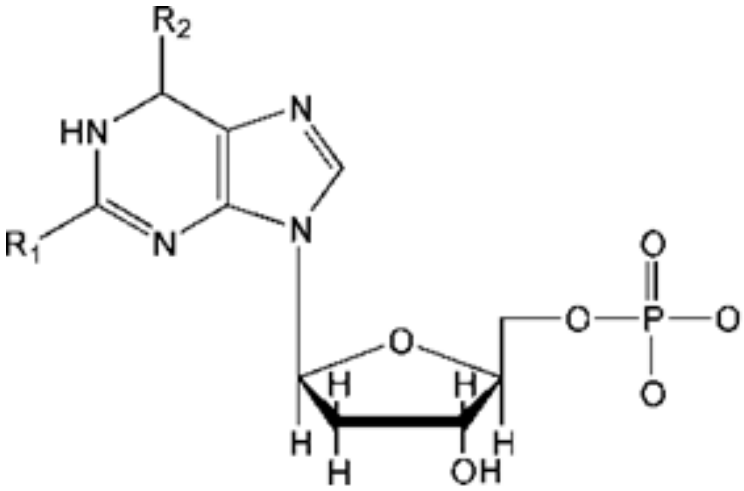


Figure 1.2 Structure of the four precursors for purine alkaloid biosynthesis [adenosine 5'-monophosphates (AMP), guanosine 5'-monophosphates (GMP), inosine 5'-monophospphate (IMP), and xanthosine 5'-monophosphates (XMP)] AMP: R_1 = H, R_2 = NH_2; GMP: R_1 = NH_2, R_2 =O; IMP: R_1 = H, R_2 =O; XMP: R_1 = R_2 = O

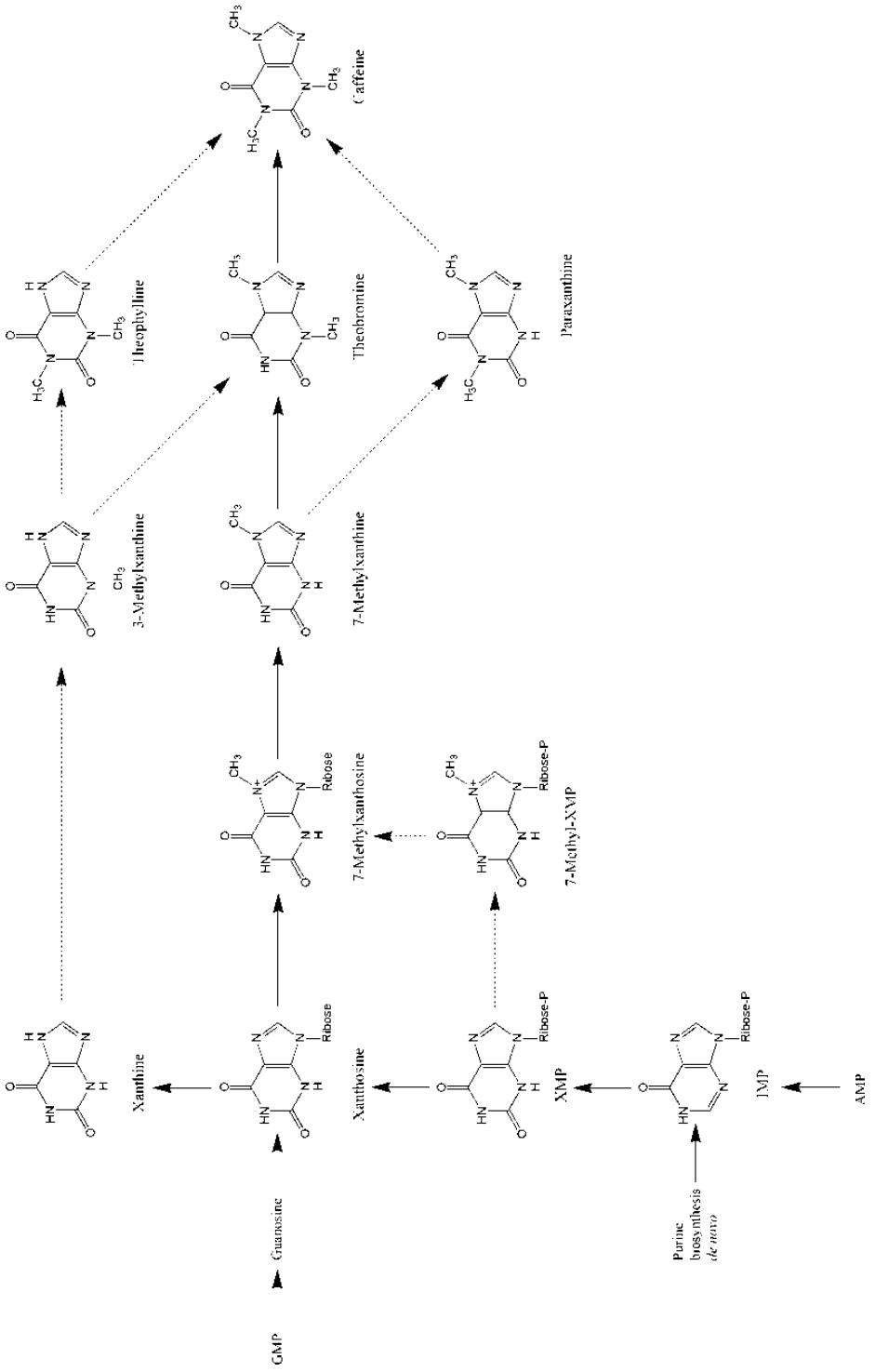


Figure 1.3 Caffeine biosynthesis in plants. Source: (Ashihara and Crozier 1999)

Catabolism of Methylxanthines in Plants

Caffeine catabolism in plants is carried out by two pathways: (i) N-demethylation to lower methylxanthines is the major pathway (ii) oxidation to the corresponding methyluric acids (Petermann and Baumann 1983, Suzuki and Waller 1984, Ashihara, Monteiro et al. 1996, Mazzafera 2004). In the N-demethylation pathway, caffeine is sequentially N-demethylated to theophylline (Ashihara and Crozier 1999), which is then converted 3-methylxanthine. 3-Methylxanthine is further N-demethylated to xanthine, which is oxidized to uric acid. This acid enters the uric acid cycle (purine catabolic cycle) where allantoin, allantoic acid, and carbon dioxide and ammonia are the breakdown products (Anaya, Cruz-Ortega et al. 2006). In the oxidation pathway, caffeine is oxidized to 1,3,7-trimethyluric acid (Petermann and Baumann 1983), which is converted to theacrine. Finally, theacrine is converted to methylliberine and liberine. Figure 1.4 shows the catabolism of caffeine by N-demethylation and oxidation pathways.

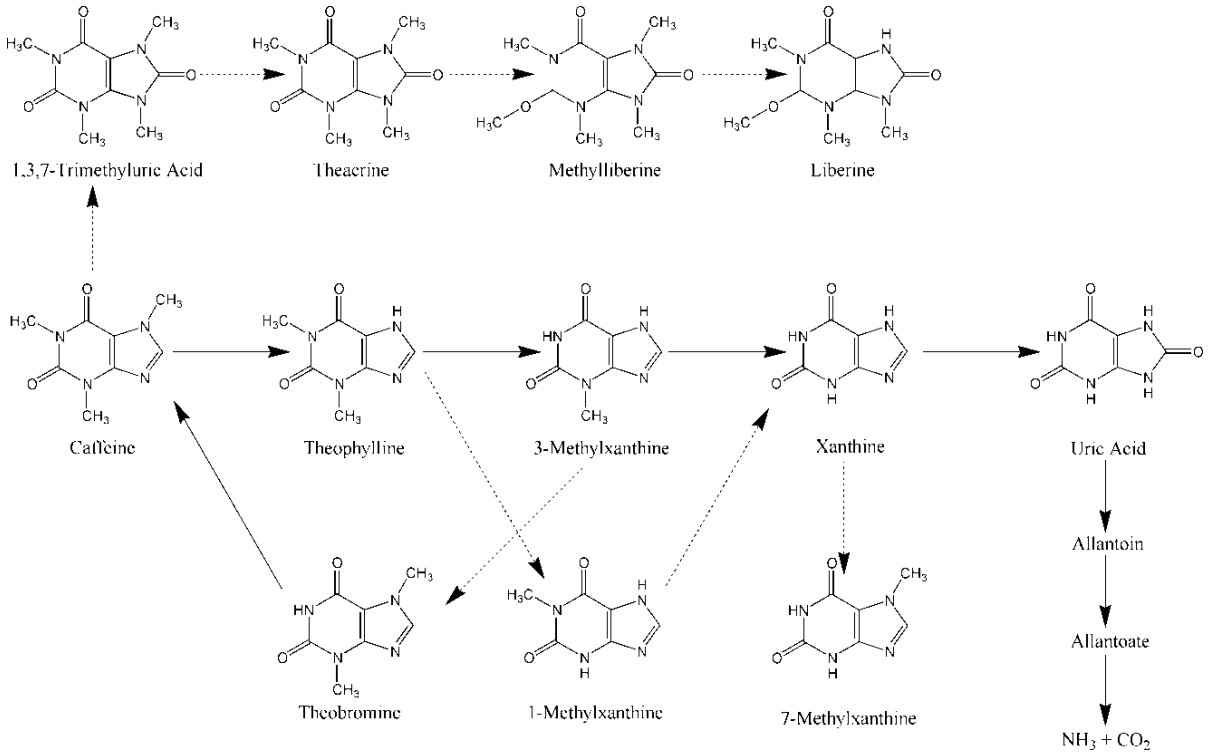


Figure 1.4 Catabolism of caffeine in coffee and tea plants. Solid lines indicate the major degradative routes while dashed lines represent alternative degradation steps (Mazzafera 2004, Anaya, Cruz-Ortega et al. 2006)

Metabolism of Caffeine in Mammals

Caffeine is metabolized in mammals by cytochrome P450 enzymes, primarily CYP1A1 and CYP1A2 (Grant, Tang et al. 1983, Campbell, Grant et al. 1987, Butler, Iwasaki et al. 1989, Berthou, Flinois et al. 1991, Eugster, Probst et al. 1993, Tassaneeyakul, Birkett et al. 1994). Also, other cytochrome P450 enzymes, such as CYP2E1 and CYP2A6 are involved in the metabolism of caffeine and its derivatives (Carrillo and Benitez 2000). In humans, 80% of caffeine is N-demethylated to paraxanthine, 10% to theobromine, 4% to theophylline, and the rest excreted as trimethyluric acid and 6-amino-5-[*N*-formylmethylamino]-1,3-dimethyluracil(Lelo, Miners et al. 1986). Paraxanthine is the major metabolite of the caffeine by N_3-demethylation in humans and rabbits. However, theophylline is the major product of N_7-demethylation of caffeine in monkeys. Caffeine is metabolized in equal rates to all dimethylxanthines in mice and rats (Berthou, Guillois et al. 1992). Figure 1.5 shows the major pathways of caffeine metabolism in humans in which caffeine undergoes sequential N-demethylation to form paraxanthine in the first step, which is further converted to 1-methylxanthine. Finally, 1-methylxanthine is oxidized in to 1-methyluric acids (Lelo, Miners et al. 1986, Carrillo and Benitez 2000). Caffeine also is degraded by other N-demethylation pathways resulting in the formation of monomethylxanthines from dimethylxanthine such as 7-methylxanthine from paraxanthine, and 1- and 3-methylxanthne from theophylline, and 3- and 7-methylxanthine from theobromine. Moreover, all methylxanthines might be oxidized to produce the corresponding

methyluric acids or acetylated to the corresponding methyluracils (Grant, Tang et al. 1983, Tang-Liu, Williams et al. 1983, Lelo, Miners et al. 1986).

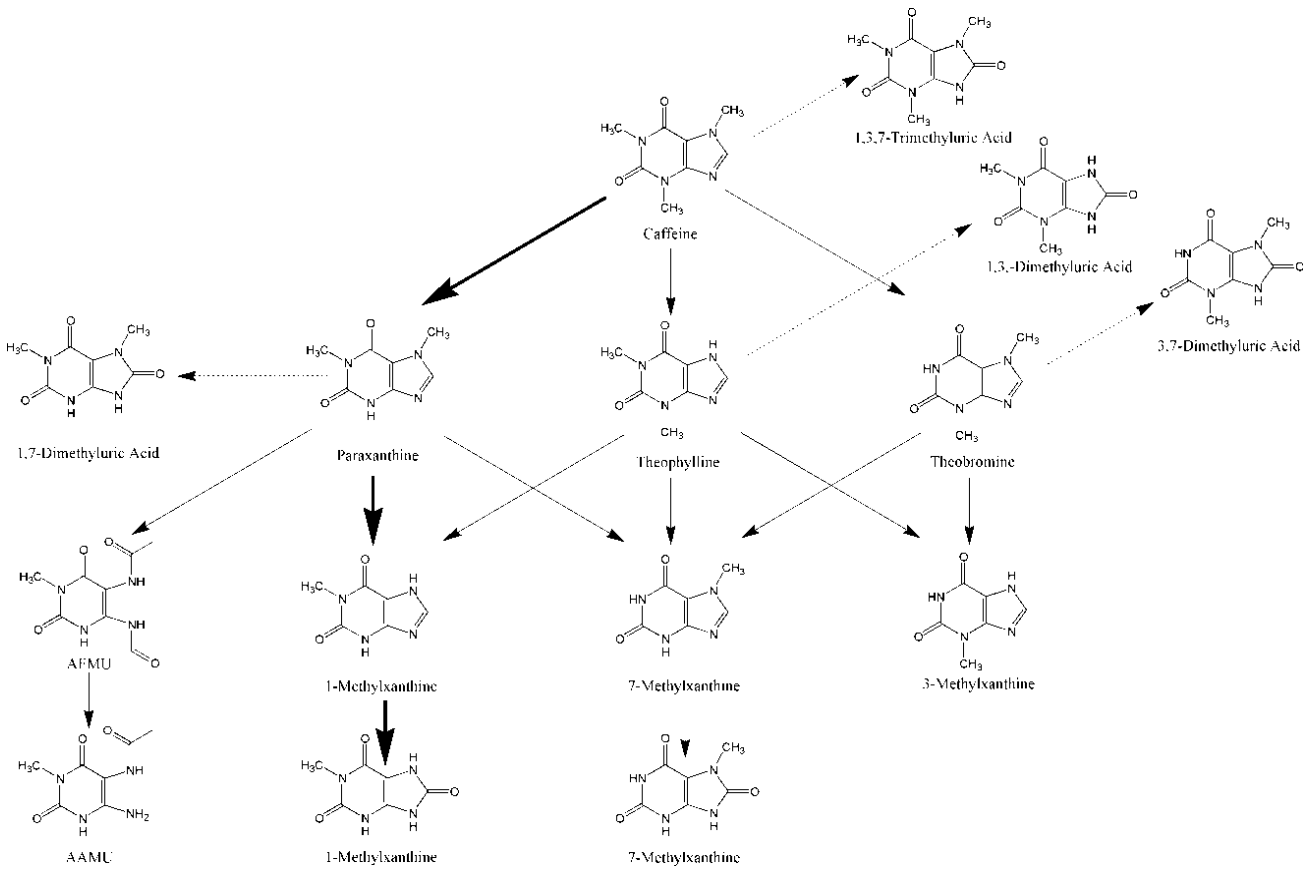


Figure 1.5 Catabolism of caffeine s in humans. Source: (Lelo, Miners et al. 1986, Carrillo and Benitez 2000)

Metabolism of Caffeine in Microorganisms

Caffeine is a ubiquitous naturally occurring purine base and it enters the soil from different sources such as caffeine containing plant parts and coffee waste. Therefore, microorganisms, in addition to plants, are exposed to caffeine. Thus, it is expected that some organisms might have developed the ability to metabolize caffeine as a source for carbon and nitrogen. Many researchers have been interested in studying caffeine degradation in different organisms (Dash and Gummadi 2006, Dash and Gummadi 2007, Yu, Kale et al. 2008, Yu, Louie et al. 2009, Gopishetty, Louie et al. 2011, Summers, Louie et al. 2012).

Yeast cells degrade caffeine by cytochrome P450 enzymes system in which same N-demethylation pathway occur as in humans (Schwimmer, Kurtzman et al. 1971, Sauer, Kappeli et al. 1982). Several strains of filamentous fungi have been grown on caffeine as source of carbon and nitrogen, in which theophylline is the first metabolite formed, which is further N-demethylated to 3-methylxanthine (Hakil, Denis et al. 1998). However, no monomethylxanthines degradation has been reported in these organisms. Also, no methyluric acids of theophylline or monomethyl xanthines have been reported.

Caffeine degradation in bacteria has been well studied for several decades (Mazzafera 2004, Gummadi and Santhosh 2006, Gokulakrishnan, Chandraraj et al. 2007, Summers, Louie et al. 2011). The most prominent bacteria that grow on caffeine as sole source of carbon and nitrogen is the genus *Pseudomonas*; however, *Serratia*, *Klebsiella*, and *Rhodococcus* species also grow on caffeine (Woolfolk 1975, Mazzafera, Olsson et al. 1996, Madyastha and Sridhar 1999, Gummadi and Santhosh 2006, Yu, Kale et al. 2008). In general, caffeine degradation occurs in bacteria by one of the two pathways, N-

demethylation or C-8 oxidation, followed by ring cleavage to assimilate carbon and nitrogen (Dash and Gummadi 2006).

Bacterial Degradation of Methylxanthine by C-8 Oxidation

Xanthine derivatives are oxidized at the C-8 position followed by ring cleavage to form the corresponding allantoin (Madyastha and Sridhar 1999, Dash and Gummadi 2006). Several enzymes are involved in the C-oxidation pathway. A flavin-containing caffeine oxidase, which was isolated from a mixed culture of *Klebsiella* sp. and *Rhodococcus* sp. (Summers, Subramanian et al. 2011). Electrophoretic analysis showed that caffeine oxidase was composed of single subunit with a molecular weight of 85 kDa. This enzyme also oxidized to a lower extent theobromine, theophylline, 7-methylxanthine, and xanthine. Another caffeine oxidase (single subunit, 65 kDa) from *Alcaligenes* sp. CF8 was also characterized (Mohapatra, Harris et al. 2006). This enzyme was active on theophylline and theobromine, but not on xanthine. Oxygen was the final electron acceptor in both caffeine oxidases; however in the case of *Alcaligenes* sp. CF8 hydrogen peroxide formation was reported. A three-subunit (71, 66, and 62 kDa) xanthine oxidase was isolated form *Pseudomonas putida* L. Its activity was primarily observed on theophylline, 3-methylxanhtine, and 7-methylxanthne; activity on xanthine, paraxanthine and theobromine was very low. However no activity was observed on caffeine (Summers 2011).

In their studies with *Pseudomonas* sp. CBB1, Yu *et. al.* found a novel caffeine dehydrogenase enzyme capable of degrading caffeine to trimethyluric acid (Yu *et al*., 2008). This enzyme was a heterotrimer consisting of three subunits (90, 40, and 20 kDa).

This enzyme was also active on theobromine and at a lower level on theophylline, but was not active on 3-methylxanthine, 7-methylxanthine, or xanthine. Methylxanthines oxidation reaction by caffeine dehydrogenase was stoichiometric and hydrolytic with water being the source of oxygen.

Mohanty *et. al.* provided the first complete report of biochemical and genetic analysis of caffeine degradation by C-8 oxidation (Mohanty, Yu et al. 2012). They fully characterized the gene encoding caffeine dehydrogenase, and proposed a complete pathway for C-8 oxidation in *Pseudomonas* sp. (Figure 1.6). The first reaction of the proposed pathway is the hydrolytic oxidation of caffeine to 1,3,7- trimethyluric acid (TMU), catalyzed by a novel caffeine dehydrogenase (Cdh) (Yu, Kale et al. 2008). Then, hydroxylation of TMU to 1,3,7-trimethyl-5-hydroxyisourate (TM-HIU) catalyzed by a novel NADH-dependent trimethyluric acid monooxygenase (TmuM). Eventually, TM-HIU enzymatically converted to 3,6,8-trimethylallantoin (TMA).

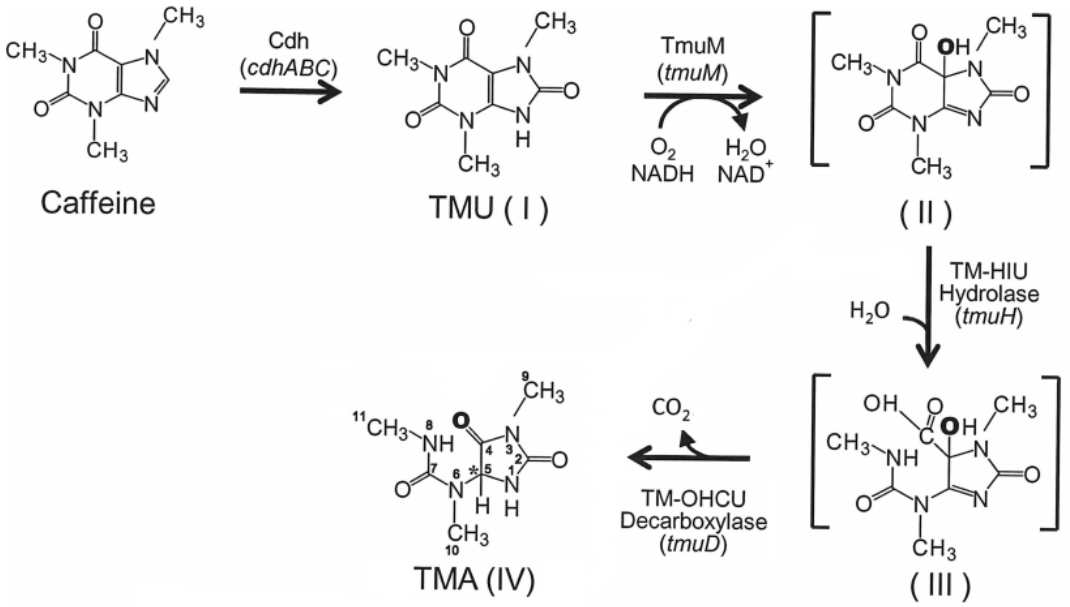


Figure 1.6 Caffeine transformation pathway in *Pseudomonas* sp. strain CBB1. Caffeine dehydrogenase (Cdh) oxidizes caffeine to TMU (I), which is oxidized to TM-HIU (II) by trimethyluric acid monooxygenase. The gene representation is in italics. Metabolites: I, 1,3,7- trimethyluric acid (TMU); II, 1,3,7-trimethyl-5-hydroxyisourate (TM-HIU); III, 3,6,8-trimethyl-2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (TMOHCU); IV, 3,6,8-trimethylallantoin (TMA). Source: (Mohanty, Yu et al. 2012)

N-demethylation of Methylxanthines by Bacteria

Degradation of methylxanthines by N-demethylation has been studied by many researchers and it appears to be the predominant pathway for caffeine degradation (Woolfolk 1975, Vogels and Van der Drift 1976, Mazzafera, Olsson et al. 1996, Chappe, Mettey et al. 1998, Hakil, Denis et al. 1998, Mazzafera 2004, Dash and Gummadi 2006, Yu, Louie et al. 2009, Gopishetty, Louie et al. 2011). Like the case in humans,

methylxanthine degradation in bacteria occurs by sequential N-demethylation in which all three methyl groups are sequentially removed from caffeine. In this pathway, the methyl group is initially removed from N3 or N7 to produce paraxanthine or theobromine. In the next step, both paraxanthine and theobromine are converted to 7-methylxanthine, which is further N-demethylated to xanthine. Xanthine is further oxidized by xanthine oxidase or dehydrogenase to form uric acid. Finally, uric acid is degraded to form carbon dioxide and ammonia (Woolfolk and Downard 1977, Summers, Subramanian et al. 2011). Also, it was found that oxidation of caffeine and other downstream N-demethylated metabolites occur in some types of bacteria, but the methyluric acids formed were not further metabolized (Blecher and Lingens 1977, Yamaoka-Yano and Mazzafera 1999, Mazzafera 2002). In some studies, only partial characterization of N-demethylation enzymes were achieved, due to high instability of these enzymes (Gokulakrishnan, Chandraraj et al. 2005, Beltrán, Leask et al. 2006). A single enzyme in *P. putida* strain 40 was found to be able to remove all the methyl groups of caffeine resulting in xanthine and methanol (Woolfolk 1975). However, more studies indicated that N-demethylation enzyme consists of more than one monooxygenase catalyzing caffeine N-demethylation (Sideso, Marvier et al. 2001, Dash and Gummadi 2007). Instead of xanthine, formation of aldehyde was observed during caffeine degradation by *Pseudomonas putida* C1(Blecher and Lingens 1977). Sideso *et. al.* (2001) reported two proteins (43.5 and 36.6 kDa) in *Pseudomonas putida* KD6 involved in caffeine metabolism. Asano et. al. (1994) reported caffeine and theobromine N-demethylases consisted of six identical subunits (41 kDa) in *Pseudomonas putida* No.352. Gummadi *et al.* (2012) reviewed the physiology, biochemistry, large scale, and

applications of microbial caffeine degradation. They explained that nutrient concentration, such as caffeine concentration, and culture conditions, such as the pH, are critical physiological factors that affect caffeine degradation. The extensive elaboration of the N-demethylation pathway and the enzymes and genes involved was explained by Summers *et. al*., which is reviewed later.

Chemical Synthesis of Methylxanthine

The first reported chemical synthesis of purine alkaloids was in 1882 by Fischer (Fischer 1924); he coined the word "purine" (McGuigan 1921). Later he was awarded the Nobel Prize in Chemistry in 1902 for his work on sugar and purine synthesis. In his work on purine synthesis, Fisher was able to chemically synthesize caffeine, theobromine, and theophylline (Ahluwalia 2012) in a reaction sequence starting from uric acid (Figure 1.7). Also, purine alkaloids, including methylxanthines, can be synthesized by traditional Traube synthesis (Traube 1900) in which methylated urea and ethyl cyanoacetate are used as starting materials. For example, N-methyl urea was reacted with ethyl cyanoacetate to produce theobromine as shown in Figure 1.8.

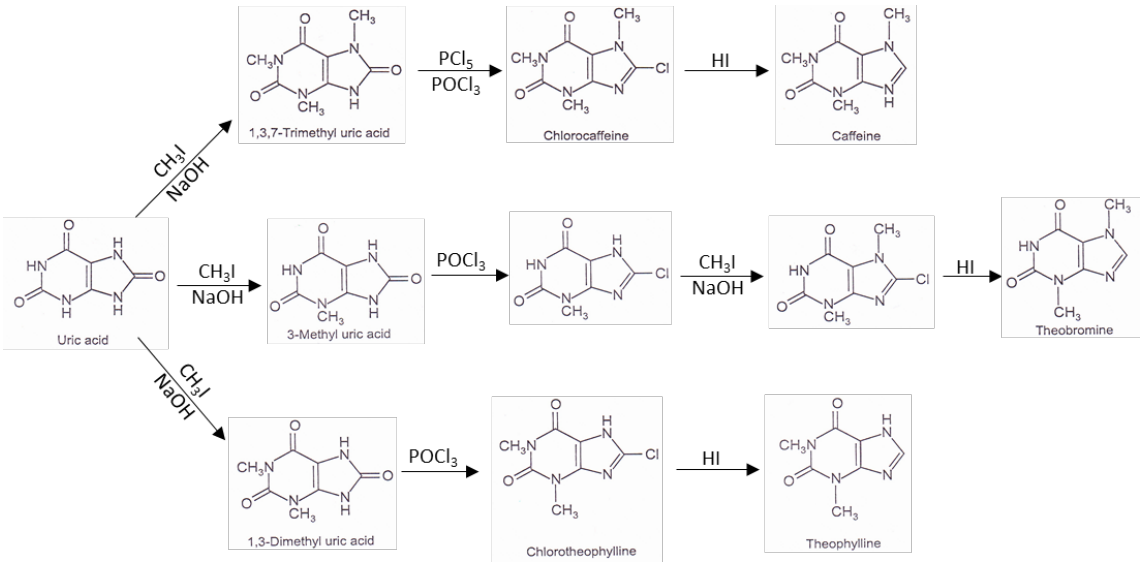


Figure 1.7 Reaction schemes for the synthesis of caffeine, theobromine, and theophylline from uric acid. Source: (Ahluwalia 2012)

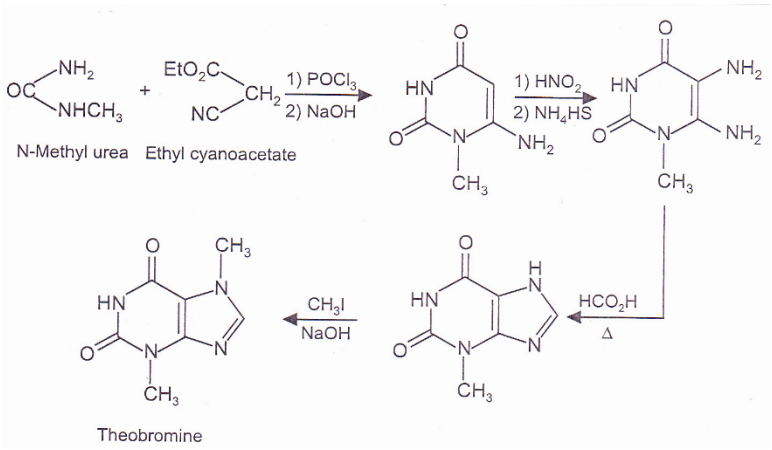


Figure 1.8 Reaction scheme for theobromine production by Traube synthesis. Source: (Ahluwalia 2012)

Moreover, many purine alkaloids are traditionally produced via Traube synthesis in which the cyclization of 4,5-diaminopyrimidines with formic acid, dithioformic acid, or other carboxylic acids is used (Traube 1900, Yoneda, Higuchi et al. 1978). Imidazoles are also used for the production of purines (Lister 2009). Mann and Porter (1945) synthesized 1,7-dialkylxanthines by two methods. In the first method which was essentially Traube synthesis, primidine was used as a starting material while iminazole was used in the second method (Mann and Porter 1945). Fumio Yoneda *et al.* (1978) synthesized xanthine derivatives by treating 6-amino-5-benzylideneaminouracil with diethyl azodicarboxylate (Fumio, Masatsugu et al. 1978). Hutzenlaub and Pfleiderer (1979) were able to synthesize 7-methylxanthine and paraxanthine starting from 6-amino-1-benzyl-5-bromouracil in a long procedure (Hutzenlaub and Pfleiderer 1979).

Zavialov *et. al.*, developed a practical method describing the synthesis of 1- and 1,3- substituted xanthines (Figure 1.9) by reacting an imidazole precursor with carbamates in the presence of a suitable base (Zavialov, Dahanukar et al. 2004). The reaction was carried out under inert conditions using solvents such as tetrahydrofuran, bis(2-methoxyethyl) ether, and toluene. About seven steps of synthesis were needed to get the targeted methylxanthine. Allwood *et. al*. developed Zavialov's method to produce a highly efficient synthesis route to *N*-functionalized derivatives of xanthine (Figure 1.10) by orthogonal safety-catch protection strategy using cyclocondensation of aminoimidazole with methyl-2-phenylthioethyl carbamates (Allwood, Cannan et al. 2007).

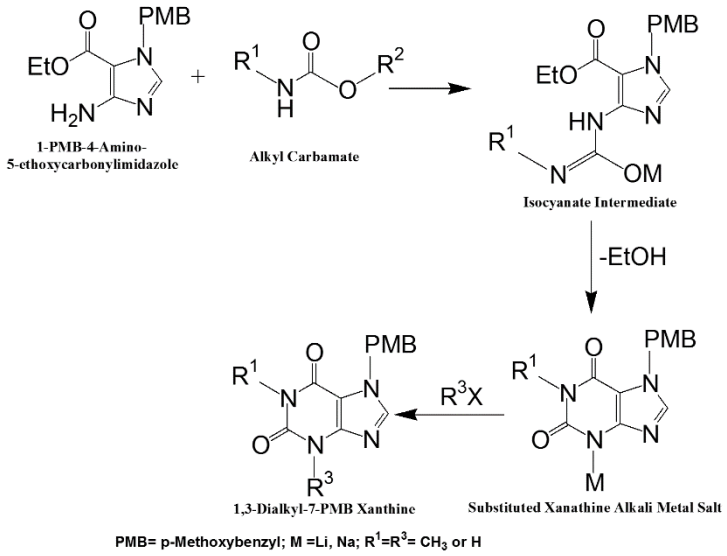


Figure 1.9 Reaction scheme for 1- and 1,3- substituted xanthines synthesis developed by Zavialov *et. al.* Source: (Zavialov, Dahanukar et al. 2004)

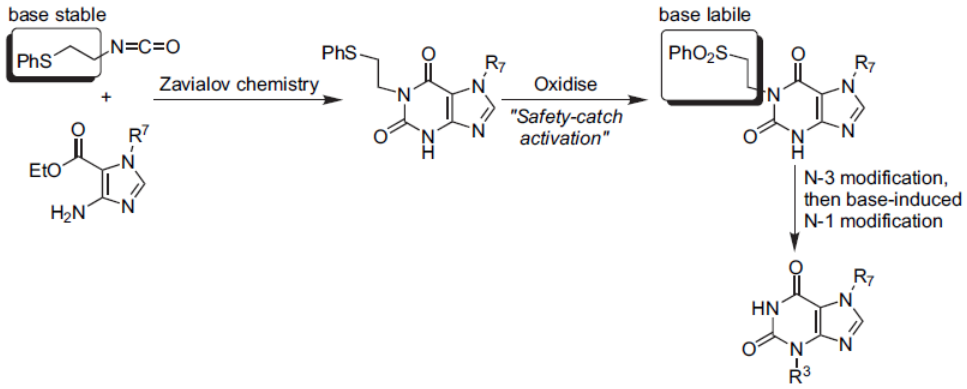


Figure 1.10 Reaction scheme for the synthesis of 1,3,7- substituted xanthines developed by Alwood *et. al.* Source: (Allwood, Cannan et al. 2007)

Traditionally, synthetic methods for the production of purine alkaloids utilize many undesirable chemicals, solvents, and harsh reaction conditions, and involve multiple steps. Besides the product of interest, several undesired byproducts are

produced. Therefore, it is complicated and expensive. For synthesis of specific alkylxanthines, protection and deprotection steps are needed, and the overall yield from starting material such as an imidazole precursor is highly variable, i.e., 65% to 90%. At each step of the reaction, the intermediate needed to be purified before the next synthetic step. Three different solvents reportedly gave different yields (Zavialov, Dahanukar et al. 2004).

Liu *et al.* synthesized novel substituted xanthines by 46 routes (Liu, Reddy et al. 2010). In one of the routes, xanthine analogs containing substituents at the N_1, N_3, and N_7 atoms were produced by treating 1,3-dialkyl-5,6-diaminouracils with triethylorthoformate. Yamada et al. (1972) synthesized purine ring from formamide in one step at high temperature (160-200 ^{o}C). The reaction time was 10-30 hours and the overall yield was 14.4-20.5% based on formamide fed to the reaction vessel (YAMADA and OKAMOTO 1972). Black *et. al.* (2011) produced 1-methylxanthine in six step sequence starting with the condensation of urea derivative and ethyl cyanoacetate or diethyl malonate. However, the overall yield reported in this study was only 20% (Black and Gatto 1989). Bobranski *et. al.* (1948) produced caffeine and theophylline by modifying Fischer synthesis in which they started from urea and cyanoacetic acid (Bobranski and Synowiedski 1948); overall yield of theophylline was 14.3%.

Methylxanthine Degradation by *Psudomonas putida* CBB5

Recently, Professor Subramanian group in the Chemical & Biochemical Engineering Department, University of Iowa, achieved extensive progress in the area of

methylxanthine degradation (Yu, Kale et al. 2008, Yu, Louie et al. 2009, Summers, Louie et al. 2012, Summers, Gopishetty et al. 2014, Summers, Mohanty et al. 2015). All the genes involved in the N-demethylation steps, starting from caffeine were fully characterized. This is the first report of complete characterization of bacterial N-demethylases and genes that enable bacteria to live on caffeine.

Yu *et. al.* (2009) isolated *Psudomonas putida* CBB5 from soil in Coralville, Iowa, using the caffeine enrichment technique. It was found that CBB5 was capable of degrading caffeine and its derivatives, such as theophylline, theobromine, paraxanthine, 3-methylxanthine, and 7-methylxanthine. According to the metabolite analysis in the growth media, it was confirmed that CBB5 N-demethylated theophylline to 1- and 3-methylxantine by NAD(P)H-dependent reactions; 1- and 3-methylxanthines were further N-demethylated to xanthine. CBB5 was also able to N-demethylate caffeine primarily to theobromine (90%) and paraxanthine (10%). Both theobromine and paraxanthine were further N-demethylated to 7-methylxanrhine, which was finally N-demethylated to xanthine. Additionally, CBB5 metabolized theobromine, paraxanthine, and 7-methylxanthine by the same pathway. Further, it was shown for the first time that CBB5 was capable of oxidizing theophylline and 1- and 3-methylxanthine to the corresponding methyluric acids. A broad-substrate range xanthine-oxidizing enzyme was responsible for the formation of methyluric acids; however, these acids were not metabolized further. N-demethylation pathways of both, caffeine and theophylline, converged at *xanthine via* different methylxanthine intermediates, and xanthine was ultimately oxidized to uric acid. This was the first report of theophylline N-demethylation and co-expression of distinct pathways for caffeine and theophylline degradation in bacteria. Moreover, two pathways

(Figure 1.11) for the degrading of caffeine, theophylline, and other xanthines by CBB5 were fully characterized in terms of metabolites, enzymes and genes.

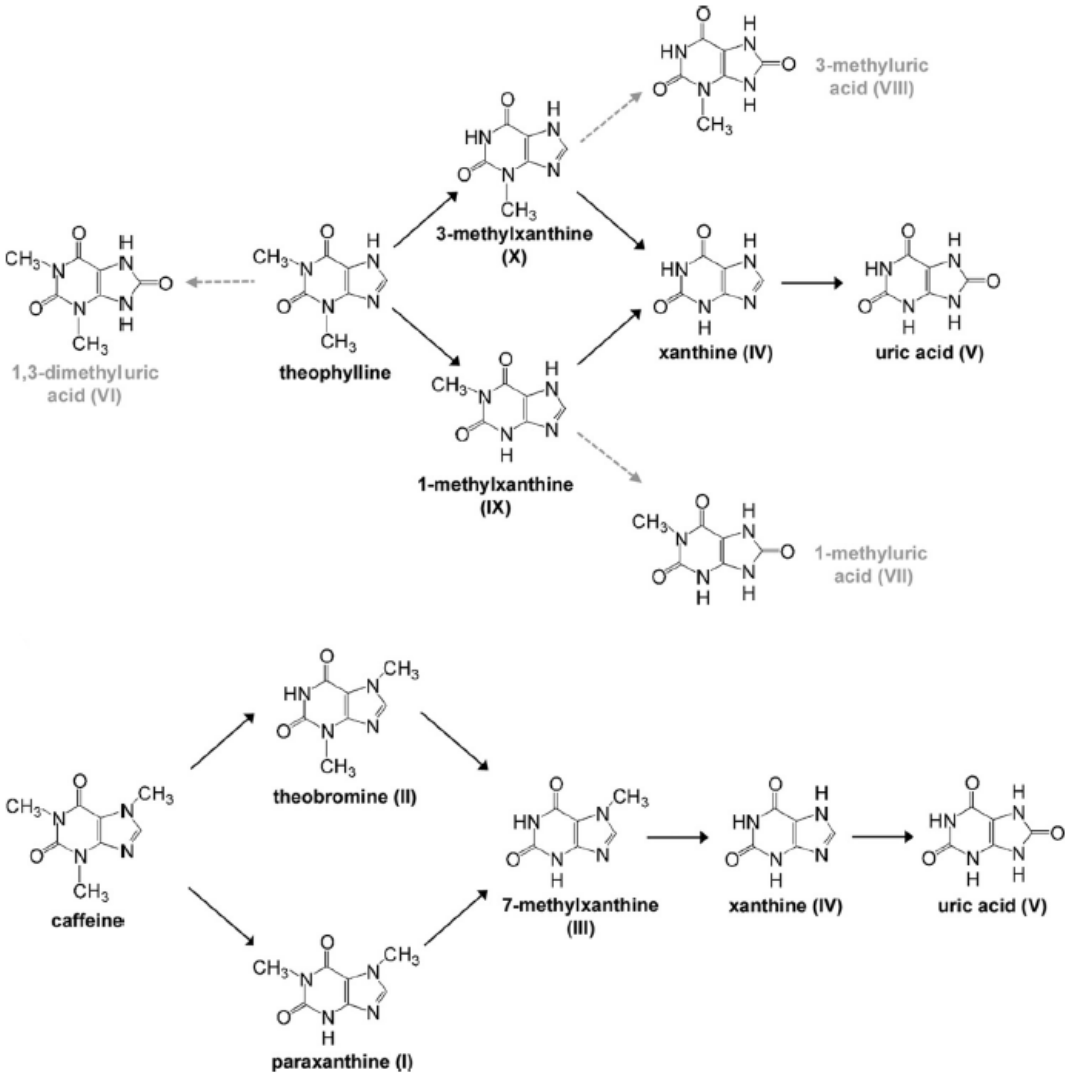


Figure 1.11 N-demethylation pathways for degradation of theophylline and caffeine by *P. putida* CBB5. The dashed arrows indicate fortuitous oxidation of theophylline and 1- and 3-methylxanthines to 1,3-dimethyluric acid and 1- and 3-methyluric acids. Source: (Yu, Louie et al. 2009)

Summers *et. al.* (2011) investigated the enzymes responsible for the N-demethylation of naturally occurring purine alkaloids such as caffeine, paraxanthine, theobromine, and theophylline. They demonstrated the first description on purification and biochemical characterization of soluble methylxanthine N-demethylase (Ndm) from CBB5 (Table 1.3). It was found that this holoenzyme consisted of two components, a reductase with NADH-cytochrome c activity (Ccr) and a two-subunit N-demethylase component (Ndm). Ndm activity was dependent on NAD(P)H oxidation, which is catalyzed by Ccr. Therefore, Ccr transferred the electrons from NAD(P)H to Ndm, and Ndm catalyzed an oxygen-dependent N-demethylation of methylxanthines to xanthine. Moreover, based on analysis of the N-terminal protein sequences of the Ndm subunits as well as distinct UV/visible absorption spectrum, it was predicted that Ndm is a Rieske [2Fe-2S] domain-containing, non-heme iron oxygenase.

Table 1.3 Purification of methylxanthine N-demethylase from Pseudomonas putida CBB5. Source: (Summers, Louie et al. 2011)

Purification step	Total protein (mg)	Total activity (mU)	Specific Activity (mU/mg)	Purification (fold)	Yield (%)
Cell extract	2361	5053	2.1	1	100
DEAE Sepharose	4802	1767	3.7	1.7	35
Phenyl Sepharose HP	37.5	962	25.6	12	19
Q Sepharose	7.0	388	55.4	26	7.7

Furthermore, Summers *et. al.* (2012) reported the first complete characterization of bacterial N-demethylase genes that allow bacteria to live on caffeine. It was found that NdmA and NdmB are two independent Rieske non heme iron monooxygenases that catalyze N- demethylation of caffeine, and other related purine alkaloids, at N_1 and N_3 locations respectively (Figure 1.12). Both enzymes used NdmD, a redox center-dense reductase, for transfer of electrons from NADH. NdmD was characterized as a novel reductase that has one Rieske [2Fe-2S] cluster, one plant-type [2Fe-2S], and one flavin mononucleotide (FMN).

Moreover, *Summers et al.* cloned ndmA, ndmB, and ndmD as His_6 fusion proteins, in *E. coli*, and purified them by using a Ni-NTA column. Full stoichiometry of the reactions catalyzed by NdmA-His6 and His6-NdmD including demethylation of caffeine to theobromine, theophylline to 3-methylxanthine, paraxanthine to 7-methylxanthine, and 1-methylxanthine to xanthine was established. Similarly, NdmB-His6 and His6-NdmD stoichiometry of theobromine to 7-methylxanthine, 3-methylxanthine to xanthine, caffeine to paraxanthine, and theophylline to 1-methylxanthine was established. In addition, biochemical activity for N_7- specific N-demethylase, NdmC was confirmed as catalyzing 7-methyl xanthine to xanthine. During all of the N-demethylation reactions, one molecule of formaldehyde is formed from each of the methyl groups removed. Also, they were able to separate NdmA, NdmB, and NdmD, and characterize the genes encoding these enzymes. By cloning NdmA and NdmB in *E. coli*, they were characterized as Rieske Oxygenases that catalyze specific N_1- and N_3- demethylation of methylxanthines. All the kinetic parameters catalyzed by these

two enzymes were also established (Table 1.4). Currently, the three-dimensional structures of these proteins are under study.

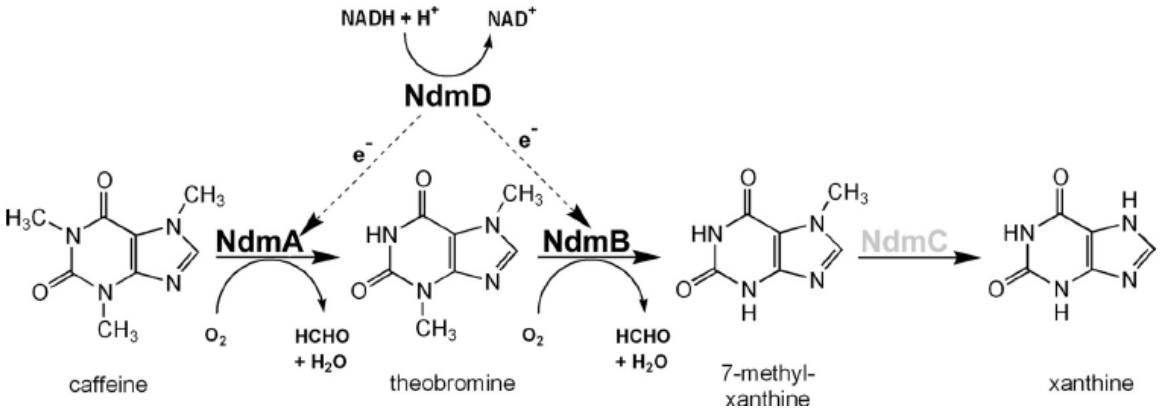


Figure 1.12 Proposed sequential *N*-demethylation of caffeine by *Pseudomonas putida* CBB5. Source:(Summers, Louie et al. 2012)

Table 1.4 Kinetic parameters of N-demethylases, NdmA and NdmB. Source: (Summers, Louie et al. 2012)

Enzyme	**Substrate**	**Product**	***Km*** **(μM)**	***k*cat** **(min^{-1})**	***k*cat/*Km*** **(min^{-1}. μM^{-1})**
	Caffeine	Theophylline	37±8	190±10	5.1±1.2
	Theophylline	3-Methylxanthine	9.1±1.7	83±1.7	9.1±1.7
	Paraxanthine	7-Methylxanthine	53±20	130±10	2.5±0.8
	Theobromine		>500	NA	NA
	1-Methylxanthine	Xanthine	270±50	16±1	0.06±0.01
	3-Methylxanthine		>500	NA	NA
	7-Methylxanthine		>500	NA	NA
	Caffeine	Paraxanthine	42±9	0.23±0.03	0.006±0.001
	Theophylline	1-Methylxanthine	170±50	0.27±0.03	0.016±0.005
	Paraxanthine		>500	NA	NA
	Theobromine	7-Methylxanthine	25±5	46±1.9	1.8±0.4
	1-Methylxanthine		>500	NA	NA
	3-Methylxanthine	Xanthine	22±5	32±1.5	1.4±0.3
	7-Methylxanthine		>500	NA	NA

Previous Attempts at Biocatalytic Production of Methylxanthines

Although N-demethylation genes and enzymes were just recently characterized (Summers, Louie et al. 2012), few prior attempts were made to use wild type bacteria as a biocatalyst to produce methylxanthines. Gluck *et. al.* (1979) isolated caffeine degrading bacteria, identified as *Pseudomonas putida* WS, which was able to grow on only caffeine as the source of carbon and nitrogen. A mixture of theobromine and uncharacterized hetroxanthine (unknown methylxanthine) accumulated in resting cells experiments. Maximum yield was 50% based on amount of caffeine degraded. However, the study did not specify what hetroxanthine was. Moreover, the reaction time was about 14 days using resting cells, 28 hours during growth on GC medium (1 g glucose/L plus caffeine mineral salts medium) in 10 liter fermentor, and 44 hours during growth on NB+C medium (8 g nutrient broth + 1 g caffeine per liter deionized water) in 10 liter fermentor (Glück and Lingens 1987). In the 10 liter fermentor experiment, both theobromine and hetroxanthine reached their maximum concentrations then declined due to their degradation by the cells. Also, the yield reported was in the tank and assumed, based on the maximum amount of products during the reaction. In addition to that, no further separation and purification of the products were achieved.

Asano et al. (1993) used caffeine degrading *Pseudomonas putida* No.352 to produce 20 g/L theobromine from caffeine by adding Zn^{2+} ions to inhibit further theobromine degradation (Asano, Komeda et al. 1993). However, the reaction time was 48 hours. In addition, the bacterial strain used in this study was wild type. Also, addition of metal ions has the potential to complicate separation and could cause adverse effect on humans if they remained as contaminant in the final product. In another study, they

indicated that sequential N-demethylation of caffeine to 7-methylxanthine by two enzymes, caffeine demethylase and theobromine demethylase were inhibited by Zn^{2+} ions; however 7-methylxanthine production was not demonstrated (Asano, Komeda et al. 1994).

Retnadhas and Gummadi (2014) used *Pseudomonas sp.* to produce theobromine from caffeine similar to the method described previously by Asano et al. (1933). However, they used Co^{2+} to inhibit theobromine degradation and produced 1g/L theobromine (Retnadhas and Gummadi 2014). McKeague et al. (2016) engineered *Sacchearomyces cerevisiae* for the biosynthesis of methylxanthines. In 300 mL fermentor, they were able to produce only 270 μg/L caffeine, 61 μg/L theophylline, and 3700 μg/L 3-methylxanthine (McKeague, Wang et al. 2016).

Despite these biocatalytic methods to produce methylxanthines, they are still produced by chemical synthesis, which as mentioned earlier, is complicated and expensive. Therefore, there is need to develop new biocatalytic methods for the production of methylxanthines, specially paraxanthine and monomethylxanthines, which are of high value.

Retail price of various natural methylxanthines obtained from the Sigma-Aldrich website are listed in Table 1.5. Caffeine is produced principally by extraction from plant matter (coffee decaffeination), and is available in bulk quantities (up to 25 kg). The remaining compounds are primarily produced through chemical synthesis. The maximum quantity of 1-, 3-, and 7-methylxanthines available is 1 g, while the maximum available quantity of paraxanthine is only 0.5 g. For uniformity, retail price of the largest available quantity is listed.

Table 1.5 Prices of various natural methylxanthines. Source: Sigma-Aldrich website (https://www.sigmaaldrich.com/united-states.html)

Compound	**Catalog Number**	**Quantity (g)**	**Price (US$)**	**Price per unit mass (US$/g)**
Caffeine	W222402-25KG	25000	$ 1,049.00	$ 0.04
Theophylline	T1633-1KG	1000	$ 186.00	$ 0.19
Theobromine	T4500-100G	100	$ 71.00	$ 0.71
Paraxanthine	D5385-500MG	0.5	$ 577.00	$ 1,154.00
1-Methylxanthine	69720-1G	1	$ 845.00	$ 845.00
3-Methylxanthine	222526-1G	1	$ 234.00	$ 234.00
7-Methylxanthine	69723-1G	1	$ 466.50	$ 466.50

Metabolically Engineered *E. coli* for Production of Methylxanthines

Degradation of caffeine and theophylline *via* sequential N-demethylation by *Pseudomonas putida* CBB5 has been reviewed earlier. CBB5 contains five novel N-demethylase genes, *ndm*A, *ndm*B, *ndm*C, *ndm*D, and *ndm*E, which are responsible for caffeine degradation (Summers, Gopishetty et al. 2014).

In order to create new routes for production of high value methylxanthines via metabolically engineered bacteria, *E. coli* strains were created with various combinations of *ndm* genes. Three compatible expression vectors were used as vehicles to mobilize *ndm* genes *A, B* and *D* into *E. coli* to carry out conversion of caffeine and theophylline to specific methylxanthines. These expression vectors are pET-32a(+), pET-28a(+), and pACYCDuet-1 (Figure 1.10). Single and multiple copies of *ndm* genes were

incorporated into these vectors, which were transformed into *E. coli* BL21(DE3), a well-known Gram-negative bacterium (Figure 1.13). The rate limiting steps for the N-demethylase reactions have not been established; hence, a molecular biology approach by introducing multiple copies of *ndm* genes to create a libaray of hosts was the strategy. Subsequently, this library would be screened to select the best host for production of specific methylxanthines. Therefore, several *E. coli* strains were metabolically engineered for production of specific methylxanthine using different combinations of *ndmA, B and D genes* (Table 1.6).

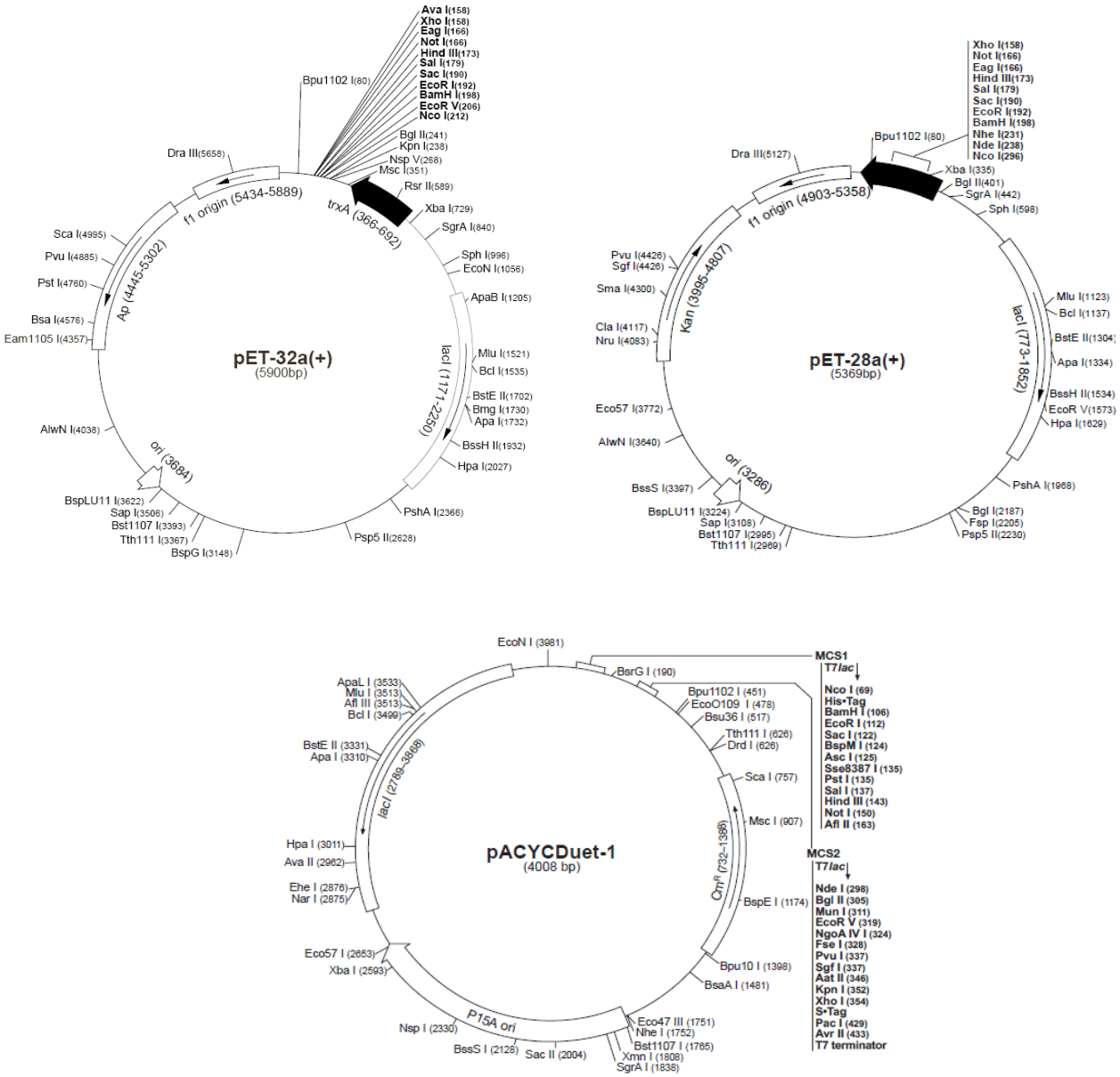


Figure 1.13 Vectors maps for expression vectors (pET-32a(+) , pET-28a(+), and pACYCDuet-1) used to carry the N-demethylase genes. Source: http://www.emdmillipore.com

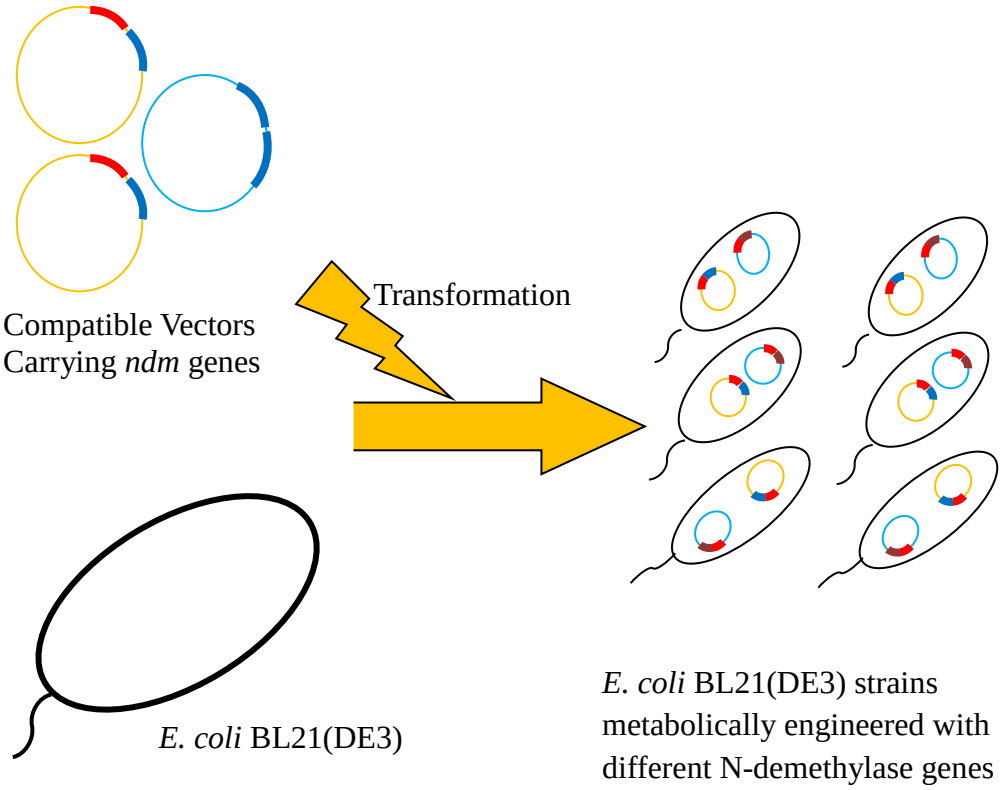


Figure 1.14 Transforming of N-demethylase genes in to *E. coli*

Table 1.6 List of *E. coli* BL21(DE3) strains engineered with N-demethylase genes (*ndmA, ndmB,* and *ndmD*)

Strain	**Plasmids**	**Gene Organization**	**Expected Ndm Rxn**
pAD1	pAD1	*ndmA*-rbs1-*ndmD*	Caff -> TB, TP -> 3MX
pAD1dAA	pAD1, dAA	*ndmA*-rbs1-*ndmD, ndmA, ndmA*	Caff -> TB, TP -> 3MX
pAD1dDA	pAD1, dDA	*ndmA*-rbs1-*ndmD, ndmD, ndmA*	Caff -> TB, TP -> 3MX
pAD1dDD	pAD1, dDD	*ndmA*-rbs1-*ndmD, ndmD, ndmD*	Caff -> TB, TP -> 3MX
pAD1dAB	pAD1, dAB	*ndmA*-rbs1-*ndmD, ndmA, ndmB*	Caff -> 7MX, TB -> 7MX TP -> Xan
pAD1dBB	pAD1, dBB	*ndmA*-rbs1-*ndmD, ndmB, ndmB*	Caff -> 7MX, TB -> 7MX TP -> Xan
pAD1dDB	pAD1, dDB	*ndmA*-rbs1-*ndmD, ndmD, ndmB*	Caff -> 7MX, TB -> 7MX TP -> Xan
dDA	dDA	*ndmD, ndmA*	Caff -> TB, TP -> 3MX
dDB	dDB	*ndmD, ndmB*	Caff -> PX, TP -> 1MX, TB -> 7MX
pBD1	pBD1	*ndmB*-rbs1-*ndmD*	Caff -> PX, TP -> 1MX, TB -> 7MX
pBD2	pBD2	*ndmB*-rbs2-*ndmD*	Caff -> PX, TP -> 1MX, TB -> 7MX
pBD1dAA	pBD2, dAA	*ndmB*-rbs2-*ndmD, ndmA, ndmA*	Caff -> 7MX, TB -> 7MX, TP -> Xan
pBD2dDA	pBD2, dDA	*ndmB*-rbs2-*ndmD, ndmD, ndmA*	Caff -> 7MX, TB -> 7MX, TP -> Xan
pBD2dDD	pBD2, dDD	*ndmB*-rbs2-*ndmD, ndmD, ndmD*	Caff -> PX, TP -> 1MX, TB -> 7MX
pBD2dAB	pBD2, dAB	*ndmB*-rbs2-*ndmD, ndmA, ndmB*	Caff -> 7MX, TB -> 7MX, TP -> Xan
pBD2dBB	pBD2, dBB	*ndmB*-rbs2-*ndmD, ndmB, ndmB*	Caff -> PX, TP -> 1MX, TB -> 7MX
pBD2dDB	pBD2, dDB	*ndmB*-rbs2-*ndmD, ndmD, ndmB*	Caff -> PX, TP -> 1MX, TB -> 7MX

Objectives

The aim of this research is to use these metabolically engineered *E. coli* strains as biocatalysts to produce methylxanthines from economic feedstocks such as caffeine, theobromine and theophylline. The specific goals of this research are:

1. Screening for activity from a library of metabolically engineered *E. coli* strains to produce high value methylxanthines from lower value feedstocks. This includes identifying and selecting clones of *ndmA,B and D* gene combinations that are most active for production of theobromine/paraxanthine, 3-methyl xanthine and 7-methyl xanthine from caffeine, theophylline and theobromine and/or caffeine respectively.
2. Achieving maximum conversion of feedstocks to products
3. Testing different growth media and finding the optimum media for higher cell growth and specific methyl xanthine production.
4. Demonstration at bench-scale, production of theobromine and paraxanthine from caffeine, 3-methylxanthine and 1-methylxanthine from theophylline, and 7-methylxanthine from theobromine and caffeine via:
    1. Scale-up cell growth at the bench scale to 1-4 L.
    2. Proportionate scale-up the N-demethylation reactions using resting cells, under optimized conditions, and demonstration of production of specific products listed above.
    3. Separation and purification of product(s).

4. Testing purity of product(s) by various analytical methods including LC-MS, NMR
5. Calculation of total conversion, and yield of the final products relative to feedstock on a mole to mole basis

Under the first task, a library of metabolically engineered *E, coli* was screened for specific feedstock utilization and specific product formation. The best 3-5 strains were chosen for growth optimization and reproducibility. Then from the initially selected 3-5 metabolically engineered strains for each product, the best strain was chosen for further work. A seed bank of one hundred vials of each optimized host of metabolically engineered *E. coli* strain capable of producing a specific methyl xanthine was prepared and stored at -80 ^{o}C for future use and strain transfer to industry partners. The second task focused on finding the amount of cells required to achieve complete conversion of substrate to the designated products. Under the third task, different growth media were used to determine the best growth conditions, and the best media that yielded highest amount of cells and activity was chosen for bench-scale production of each product. Finally, the biocatalytic process for production was scaled-up to produce a about 300 mg each product. This includes, scaling-up both cell growth and reaction. After that, the individual products was separated, purified, structurally characterized and compared with commercial standard.

The broader interest of this research is to generate a new common platform for biocatalytic production of high value methylxanthines. The common platform includes (i) optimized growth media for maximum growth of different engineered hosts (ii)

uniform reaction conditions for maximum utilization of the best feedstock for conversion to specific methyl xanthine (iii) common separation and recovery platform using preparatory HPLC of the methyl xanthine products with high purity. The vision of this common biocatalytic process is shown in Figure 1.12. Overall, this research proposes development of a novel process for the production of methylxanthine from caffeine, theophylline, and/or theobromine, where any of these substrates can be converted to desired products by one single and specific N-demethylation reaction using metabolically engineered *E. coli* as a catalyst.

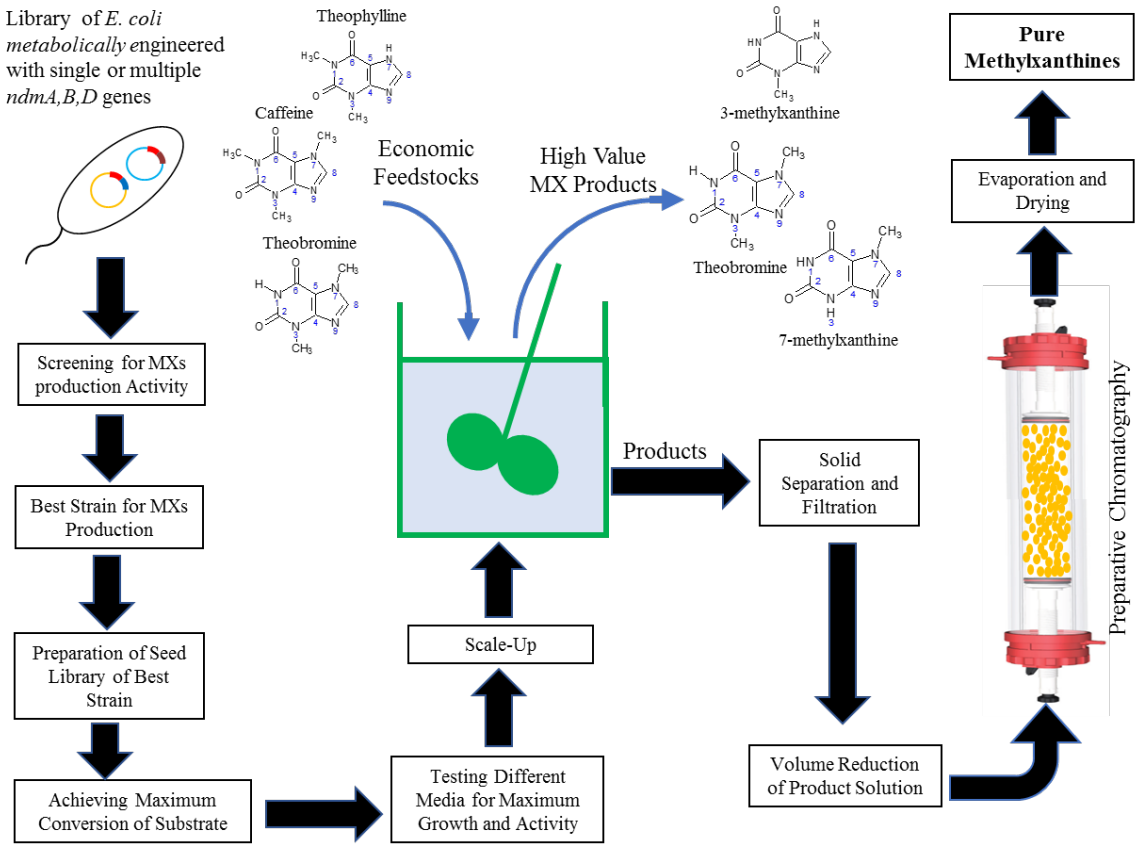


Figure 1.15 Common platform for the production of high value methylxanthines (MXs) from economical feed stocks catalyzed by metabolically engineered *E. coli*

CHAPTER 2: MATERIALS AND METHODS

Chemicals and Reagents

Caffeine, theophylline, theobromine, 1-methylxanthtine, 3-methylxanthine, 7-methylxanthine (7MX), paraxanthine (PX), and xanthine, and potassium phosphate mono basic and dibasic were purchased from Sigma-Aldrich (St. Louis, MO). Luria-Bertani Broth (LB), Terrific Broth (TB), and Difco Select APS™ Super Broth (SB) dehydrated media were obtained from Becton Dickinson and Company (Sparks, MD). Isopropyl β-D-thiogalactopyranoside (IPTG) was obtained from RPI Corp. (Mt. Prospect., IL). Ferric Chloride (FeCl3.6H2O), acetic acid glacial (ACS Certified), and glycerol were purchased from Fisher Scientific (Waltham, MA). Chloramphenicol was obtained from Research Products International Corp (Mt. Prospect, IL). HPLC columns (Hypersil BDS C18; Particle Size: 5µm; 125 mm x 4.6mm and 21.1 mm x 250 mm) were purchased from Fisher Scientific (Hanover Park, IL).

Plasmid Construction

Dr. Ryan Summers cloned *Ndm* genes in to *E. coli* expression plasmids. Since this thesis is based on using the metabolically engineered strains for production of methyl xanthines, the procedures for strain construction, in which the author of this thesis participated in some of the work, are presented in detail. All plasmids and strains used in this work are listed in Table 2.1. The pACYCDuet-1 vector backbone, which has a low-to-medium copy number of 10-12, was used for plasmids dAA, dA, dDD, dDA, dDB, and dBB. Plasmid dA was created by adding a single copy of *ndmA* in a manner that removed the second multiple cloning site. Plasmids pAD1 and pET28-His-ndmD contain

the pET32a(+) and pET28a(+) vector backbones, respectively, which have a copy number of approximately 40. All genes are under the control of the strong T7 promoter for induction with IPTG. In the case of plasmid pAD1, the genes *ndmA* and *ndmD* are under the control of a single T7 promoter with a short synthetic ribosomal binding site between the two genes to promote translation of *ndmD*.

The *ndmA* gene was amplified by polymerase chain reaction (PCR) from *P. putida* CBB5 genomic DNA (gDNA) with three sets of primers: ndmA-F-NcoI (5′GCAAGGTCCATGGAGCAGGCGATCATCAATGATGA-3′) and ndmA-R-KpnI (5′-CCTCCGGGTACCTTATATGTAGCTCCTATCGCTT-3′) produced insert 1, ndmA-F-NcoI and ndmA-R-BamHI (5′-CCTCCGGGATCCTTATATGTAGCTCCTATCGCTT-3′) produced insert 2, and ndmA-F-NdeI (5′-GCACGGCATATGGAGCAGGCGATCATCAATGATGA-3′) and ndmA-R-KpnI produced insert 3. Insert 1 was cloned into the pACYCDuet-1 plasmid at the NcoI and KpnI sites, resulting in plasmid dA. This plasmid contained only one copy of *ndmA* controlled by the T7 promoter. Insert 2 was also cloned into the pACYCDuet-1 plasmid using the NcoI and BamHI sites, resulting in plasmid dA0. Plasmid dA0 contained one copy of *ndmA* and a second, empty multiple cloning site. Insert 3 was cloned into the second multiple cloning site of plasmid dA0 at the NdeI and KpnI sites, resulting in plasmid dAA.

In a similar fashion, the *ndmD* gene was also amplified from *P. putida* CBB5 gDNA by PCR using two sets of primers: ndmD-F-NcoI (5′-GTGAGATCCATGGACAAACTTGACGTCAACCAGTGG-3′) and ndmD-R-BamHI

(5′-GGGACGGGGATCCTCACAGATCGAGAACGATTTTTTTGGA-3′) produced insert 4, and ndmD-F-NdeI (5′-GTGAGATCATATGAACAAACTTGACGTCAACCAGTGG-3′) and ndmD-R-KpnI (5′-GGGACGGGGTACCTCACAGATCGAGAACGATTTTTTTGGA-3′) produced insert 5. Insert 4 was cloned into the pACYCDuet-1 plasmid at the NcoI and BamHI sites, resulting in plasmid dD0, which contained one copy of *ndmD* and a second, empty multiple cloning site. Insert 5 was cloned into the empty multiple cloning site at the NdeI and KpnI sites of dD0, resulting in plasmid dDD. Insert 3 (containing *ndmA*) was also cloned into the NdeI and KpnI sites of dD0, yielding plasmid dDA. DNA sequencing of all plasmids confirmed that PCR amplification and cloning procedures did not introduce any mutations into the gene sequences.

Table 2.1 Plasmids and strains used in this study

Name	Characteristics	Source
Plasmids		
pAD1	AmpR, T7 promoter, ndmA, ndmD, rbsAD1, F1 origin	
pBD2	AmpR, T7 promoter, ndmB, ndmD, rbsBD2, F1 origin	
pACYCDuet-1	Expression vector, CmR, 2 T7 promoters, p15A origin	Novagen
dA	pACYCDuet-1 with one copy of ndmA	This study
dAA	pACYCDuet-1 with two copies of ndmA	This study
dDD	pACYCDuet-1 with two copies of ndmD	This study
dDA	pACYCDuet-1 with one copy of ndmD and on copy of ndmA	This study
dBB	pACYCDuet-1 with two copies of ndmB	This study
dDD	pACYCDuet-1 with two copies of ndmD	This study
dDB	pACYCDuet-1 with one copy of ndmD and on copy of ndmB	This study
pDCAF2	pSB1C3 with ndmA, ndmB, and ndmD, origin	This Study
***E. coli* strains**		
E. coli BL21(DE3)	F- ompT hsdSB (rB-mB-) gal dcm (DE3)	Invitrogen
E. coli pAD1*	BL21(DE3) pAD1	(R. Summers et al., 2014)
E. coli pAD1dDD	BL21(DE3) pAD1 dDD	This study
E. coli pAD1dDA	BL21(DE3) pAD1 dDA	This study
E. coli pAD1dAA	BL21(DE3) pAD1 dAA	This study
E. coli dDA	BL21(DE3) dDA	This study
E. coli pDdAA	BL21(DE3) pET28-His-ndmD dAA	This study
E. coli pDdA	BL21(DE3) pET28-His-ndmD dA	This study
E. coli pBD2	BL21(DE3) pBD2	This study
E. coli dDB	BL21(DE3) dDB	This study
E. coli pBD2,dDD	BL21(DE3) pBD2dDD	This study
E. coli pBD2,dBB	BL21(DE3) pBD2dBB	This study
E. coli pBD2,dDB	BL21(DE3) pBD2dDB	This study
E. coli pDCAF2	BL21(DE3) pDCAF2	This study

*Strain pAD1 was originally named *E. coli* strain RMS1 in a previous publication (Summers, Gopishetty et al. 2014).

Strain Construction

E. coli BL21(DE3) is the parent strain for all bacterial strains used. A list of all strains used in this study is given in Table 2.1. Plasmids dDD, dDA, and dAA were transformed into strain pAD1, which already contained plasmid pAD1 (Summers, Mohanty et al. 2015), yielding strains pAD1dDD, pAD1dDA, and pAD1dAA, respectively. Plasmids dAA and dA were transformed into strain pHisD, which already contained pET28-His-ndmD (Summers, Louie et al. 2012), resulting in strains pDdAA and pDdA, respectively. Plasmids dDD, dDB, and dBB were transformed into strain pBD2, which already contained plasmid pBD2, yielding strains pbD2dDD, pBD2dDB, and pBD2dBB respectively. Plasmid dDB was transformed in to E. coli BL21(DE3) yielding strain dDB. Transformants were recovered on LB agar containing appropriate antibiotics at the following levels: 34 μg/mL chloramphenicol, 100 μg/mL ampicillin and 30 μg/mL kanamycin.

Cell Growth and Protein Expression

E. coli strains were grown in Laura Broth (LB), Terrific Broth (TB), or Super Broth (SB) medium with appropriate antibiotic at 37^{o}C in a shaker-incubator at 250 rpm. Concentrations of antibiotics 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 (OD_{600}). Upon reaching an OD_{600} of ~ 0.5, Ferric chloride ($FeCl_3 \cdot 6H_2O$) was added (0.02 mM final concentration) and temperature was lowered to 18^{o}C. This was done to maximize the soluble expression of cloned proteins. When the OD reached (0.8-1), Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added (0.2 mM final concentration) to induce expression of *ndmA, B* and *ndmD* depending on the strain

used. The IPTG concentration of 0.2 mM was previously optimized to get most soluble expression of these proteins. (Summers, Louie et al. 2012). Cells were harvested after (14-16) hours of induction by centrifugation at 10,000 x g for 15-20 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 re-suspended in 50 mM KPi buffer prior to activity assays, and this cell suspension was designated resting cells. Resting cells don't grow and carry out minimal metabolic activity since they are swimming in buffer devoid of any nutrients. Therefore, it is important to minimize the time of resting cells to a couple of hours in order to to avoid any drop in the desired biocatalytic activity. Same growth procedure was followed at all levels including scale-up. Appropriate volumes are noted in various chapters dealing with specific strains.

Preparation of Metabolically Engineered *E. coli* Seed Bank

Two 250 mL flasks (100 mL growth media and 100 mL 50% glycerol solution) were autoclaved at 121°C for 20 min along with 50-100 cryovials, plastic trays to hold the cryovials, pipette tips, forceps, and an empty 250 mL flask. *E. coli* strain of interest was used to inoculate 100 mL Laura Broth (LB) medium with appropriate antibiotic, then left on shaker at 37°C and 250 rpm to grow over night. Concentrations of antibiotics used were 34, 30, and 100 µg/mL for chloramphenicol, kanamycin, and ampicillin respectively. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}). After 14 hours of growth, cell culture purity was confirmed by microscopic inspection. After that, 50 mL of each of cell culture and 50% sterile glycerol solution were mixed in the empty sterile 250 mL flask so the final concentration of glycerol in the cell suspension was 25%. Cell suspension (1.0 mL) was dispensed into each cryovial, and

the seed vials were capped, labeled, and stored in a cryovial freezer box at -80°C. Later, a seed vial was randomly selected and used to inoculate a growth medium for further studies.

SDS-PAGE Analysis of Enzyme Expression

In addition to the whole cells enzymatic assays, protein expression was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Cell culture samples were taken right before induction (pre-induction) and before harvesting (post-induction) for cell disruption, total protein measurement, and gel analysis. Molecular weight of the protein bands of interest were determined by comparison to molecular weight standards. The genetically engineered E. coli with the N-demethylases genes was grown in shake flasks as previously described for SDS-PAGE analysis.

Materials for SDS-PAGE Analysis and Protein Assay

Potassium phosphate monobasic, potassium phosphate dibasic, and DL-dithiothreitol (DTT) was purchased from Sigma, St. Louis, MO. Bovine serum albumin (BSA) were received from Pierce, Thermo Fisher Scientific, Rockford, IL. Bradford protein assay reagent was obtained from Bio-Rad, Hercules, CA. The following materials were purchased from Invitrogen, Carlsbad, CA: 10% Bis-Tris gel (10 well, 30 μL comb), NuPage 4x lithium dodecylsulfate (LDS) sample buffer, SeeBlue Plus2 pre-stained standard, NuPage MOPS SDS running buffer, and SimplyBlue safe stain.

Method for SDS-PAGE Analysis

First, approximately 20 mL of cell culture (of each of pre- and post-induction samples) were centrifuged at 10,000 g fro 15 minutes at 4 ^{o}C to separate the cells. Then, the cells were washed twice with 50 mM cold potassium phosphate buffer (KPi) (pH=7.5). After that, the cells were re-suspended in KPi so that the final volume was 10 mL. Subsequently, cell disruption was accomplished by applying shear stress on the re-suspended cells by SLM Aminco French Pressure Cell Press.

Disrupted cells solutions were collected in 50 mL centrifuge tubes and centrifuged at 30,000 rpm at 4 ^{o}C for 20 minutes. Then, the soluble proteins (supernatants) were carefully separated from the insoluble proteins (pellets) in 15 mL tubes and an equal volume of KPi was added to the corresponding insoluble pellets. Total protein concentrations were determined by Bradford assay using BSA as standard. Then, soluble and insoluble fractions were mixed with 4X LDS loading buffer and 1 M DTT (70:25:5, v/v) and loaded onto 10% Bis-Tris gels. 10 mg of protein was loaded in the well. Constant 200 volt was subjected on the gel for 50 min. Upon completion, gels were washed three times in 100 mL deionized water and stained with GelCode Blue Safe Protein Stain.

Assays for Methylxanthines Production

Other than where noted, reactions were carried out in 2 mL micro centrifuge tubes with 1 mL total reaction volume containing appropriate substrate and cells in 50 mM potassium phosphate buffer (pH=7.5) as the reaction medium. The resting cells were obtained from a cells suspension that was prepared previously by suspending the cells

pellet (after harvesting, washing twice in KPi, and weighed) in KPi so the total volume was 10 mL. A VWR® symphony™ Incubating Microplate Shaker was used to carry out the reactions at 30 °C and 400 rpm. 100 µL Samples were taken periodically for HPLC analysis, and concentrations of substrate and product(s) were calculated using standard plots created for each compound of interest.

Preparing Samples for HPLC Analysis

100 uL Reaction mixture sample was mixed with 100 uL acetonitrile (ACN) (in a 0.5 mL centrifuge tube) to stop the reaction, placed on ice for few minutes, then centrifuged at 13000 rpm by Eppendorf Centrifuge (Model 5417) for 10 minutes. After that, the supernatant was carefully removed to another empty 0.5 mL centrifuge tube. Then, 1 mL syringe used to filter the sample using 0.2 µm filter and the sample was collected in another empty 0.5 mL centrifuge tube. Then, the collected sample was dispensed in to a plastic insert which was placed in to an HPLC vial; the vial capped and placed in the HPLC auto-injection rack for analysis.

HPLC Analysis

Identification and quantification of TP, 3MX, 1MX, Caffeine, TB, and 7MX were conducted on Shimadzu LC-10AD HPLC Prominence System equipped with a photodiode array detector. A Hypersil BDS C18 column (4.6 by 125 mm) was used as the stationary phase. Methanol-water-acetic acid system was used as the mobile phase with a flow rate of 0.5 mL/min. The molecules resolved by the C18 column passed through the photodiode array detector, in which UV-visible absorption spectra were recorded. 1 µL of the sample in the HPLC vial was injected in to the column and the HPLC chromatogram

was recorded. Methylxanthines (substrates and products) concentrations were calculated by comparing the peak area with those of authentic standards. Standard curves for all substrates and products used in this study are shown in Figure 2.1. Different concentrations of each of the methylxanthines ranging from 0.01 to 1 mM were used. Each standard curve was represented by a linear algebraic equation relates peak are and concentration. During HPLC analysis, the peak area of the substrate and product(s) were recorded by EZStart software, and the concentrations were found from the linear equation.

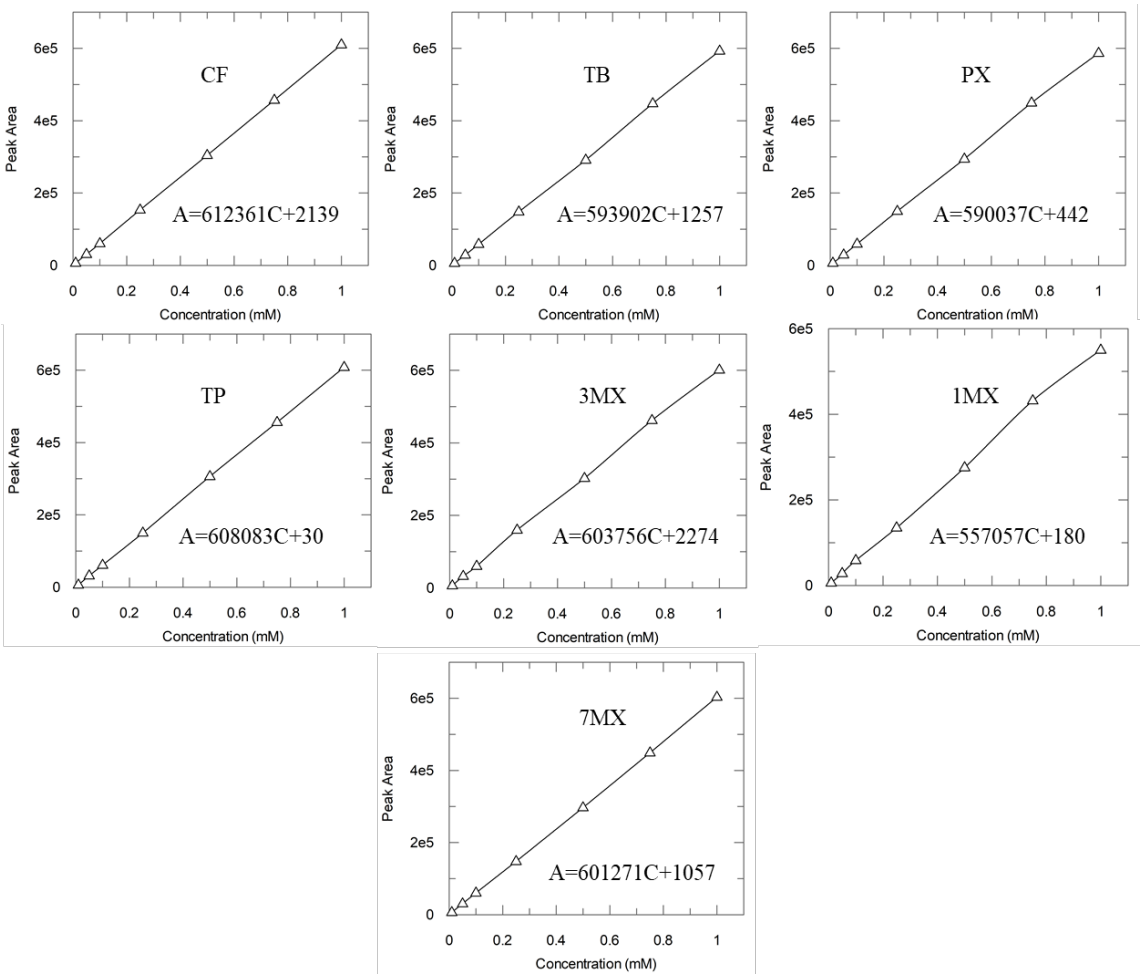


Figure 2.1 Standard curves for methylxanthines use in this study (peak area (A) vs. concentration (C))

Scaled-Up Reactions

Reactions for product isolation were carried out after growth of the metabolically engineered stains in 2.8 L flask. These large-scale reactions were carried out in an Excella E24 Incubator Shaker (Eppendorf, Hamburg, Germany) at 30 °C and 250 rpm. After all substrate was consumed, the post-reaction mixture was centrifuged at 10,000 x g to separate the supernatant (products) from the cells. After that, the supernatant was filtered under vacuum by 0.2 µm filter to ensure removing all microparticles from the reaction product(s) for HPLC.

Preparatory HPLC Method and Product Isolation

Purification of 3MX, 1MX, TB, PX, and 7MX was carried out with preparatory-scale. The same HPLC system described above was used to separate the product(s). A Hypersil BDS C18 column of 21.2 mm diameter and 25 cm length was used as the stationary phase. Methanol-water-acetic acid 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. Retention times of methylxanthines produced in this work are shown in Table 2.2. 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 the product(s) solution(s) collected in a bottle(s). The solution(s) were concentrated by vacuum drying using Buchi Rotovap R114. The bath temperature was 60-70 °C.

Table 2.2 Retention times of methylxanthines eluted from preparatory HPLC column

Component	Retention time (minutes)	Solvent (methanol: water: acetic acid)
Theobromine	102	5:95:0.5
3-methylxanthine	116	
1-methylxanthine	135	
7-methylxanthine	102	

Freeze Drying

Virtis Genesis 35EL freeze dryer (SP Scientific, Stone Ridge, NY) was used to dry the 3MX and 1MX solutions at a vacuum of 90 torr. The solutions were frozen to -80 °C, then dried overnight in the freeze dryer. At the end of the drying process, the product powder was quantitatively collected and weighed.

Oven Drying

TB, PX, and 7MX solutions were dried in Fisher Scientific Isotemp Oven, Model 203 FS. The drying of product(s) solution(s) were poured in to aluminum and then placed inside the oven. The drying temperature was ~140 ^{o}C. The drying process was monitored until the majority of the liquid had evaporated. Then 250 mL DI water added to the solution, mixed, and placed again in the oven at 140 ^{o}C; this process was repeated twice. At the end of the drying process, the product powder was collected and weighed.

Analytical Procedures

Testing MX product(s) purity was carried out by HPLC, LC-MS, and/or NMR. Same HPLC system described above (HPLC Analysis) was used to test MX product (purity). Also, purity of products 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. Each 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.

CHAPTER 3: PRODUCTION OF 3-METHYLXANTHINE USING METABOLICALLY ENGINEERED *E. COLI*

Introduction

As it was reviewed in Chapter One, chemical synthesis of methylxanthines, including 3-methylxanthine (3MX) is complicated and expensive. The current synthesis method of 3MX is unknown, and no details are available on either the chemical production procedure or the exact yield. Sigma Aldrich technical service did not reveal any information on the chemical synthesis of 3-methylxanthine. They claimed another company supplied it, and they cannot reveal the source. Sigma-Aldrich provides only 'reagent size, up to 1 g of 3-methyl xanthine with a price tag of $234 /g. In this chapter, a biocatalytic approach for production of 3-methyl xanthine using metabolically engineered *E. coli* is detailed; the feed stock for this bio-process is theophylline. The reaction is carried out at atmospheric pressure and 30^{o}C, and the reaction medium is water. No organic solvents are used or intermediates produced in this process. Undesirable waste is not generated in the entire process. Therefore, the whole production process in this work is environmentally friendly.

The present work is the first report on the biological production of 3-methylxanthine from theophylline. The biocatalyst is *E. coli* engineered with N-demethylases genes *nDmA* and *ndmD* (Figure 3.1). The genes *ndmA* and *ndmD* were introduced into *E. coli* at different gene dosages, and the resultant strains were screened for 3MX production (for procedural details, see Chapter Two and also Figure 1.12). The strain containing the optimum gene dosage and the displaying the highest 3MX

production was chosen for further study, including small-scale production of 3MX to dispatch to clients. *NdmA* produced 1MX as a minor product because of non-specific N-demethylation at the N_3-position. 1MX was not fully characterized since this is not the best method to produce this fine chemical. The biocatalytic approach used here operates at ambient temperature and pressure and is environmentally friendly. In contrast, chemical synthesis of methylxanthines uses several chemicals, multiple reactions, and non-ambient reaction conditions.

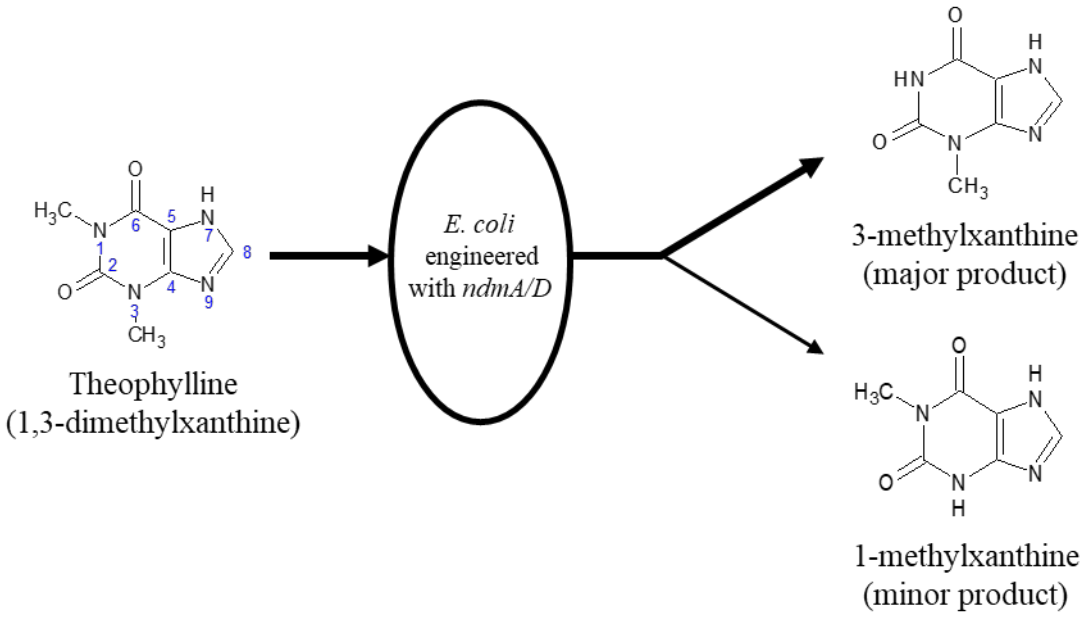


Figure 3.1 Reaction scheme for the biocatalytic N-demethylation of caffeine by *E. coli* engineered with NdmA and NdmD genes

Initial Screening of Growth and 3MX Production by Metabolically Engineered *E. coli*

All plasmids and strains used in this work are listed in Table 1.6 (Chapter 1), and plasmid maps are provided in Figure 1.10 (Chapter 1). First, the conversion of TP to 3MX using a strain of *E. coli* that contained plasmid pAD1 (Summers, Mohanty et al. 2015) was tested. This strain contained one copy of each of NdmA and NdmD genes on the same pET-32a(+) vector. Resting cells (OD_{600} = 2.5) consumed approximately 30% of the 1 mM TP, presented initially in the reaction mixture, to 3MX and 1MX over one hour, after which the reaction essentially stopped (Figures 3.2, 3.2, and 3.3). In order to increase cells activity, plasmids dAA, dDD, and dDA (Table 3.1) were added to the strain carrying pAD1, resulting in three new strains pAD1dAA, pAD1dDD, and pAD1dDA. These new strains allowed us to test the effect of different levels of *ndmA* and *ndmD* copy numbers on 3MX production (see Table 3.1 for approximate gene copy numbers of each strain). Addition of *ndmA* only (strain pAD1dAA) had very little effect on activity (Figure 3.2). Increasing the copy number of both genes (strain pAD1dDA) greatly increased the activity over strain pAD1dAA, with more than 99% of theophylline consumed. However, increasing the *ndmD* gene copy number only (strain pAD1dDD) resulted in complete conversion (100%) of TP within two hours (Figure 3.2). Strain pAD1dDD, which contained one *ndmA* copy and three copies of *ndmD* gene, exhibited a slightly higher activity than did strain pAD1dDA, which had consumed about 90% of TP in two hours, suggesting that plasmid pAD1 provided a sufficient *ndmA* gene dosage. These results also indicated that the reaction was limited by the amount of soluble NdmD produced inside the cells, since the activity increased with increasing *ndmD* copy number and solubility, although NdmD expression as inclusion body also increased.

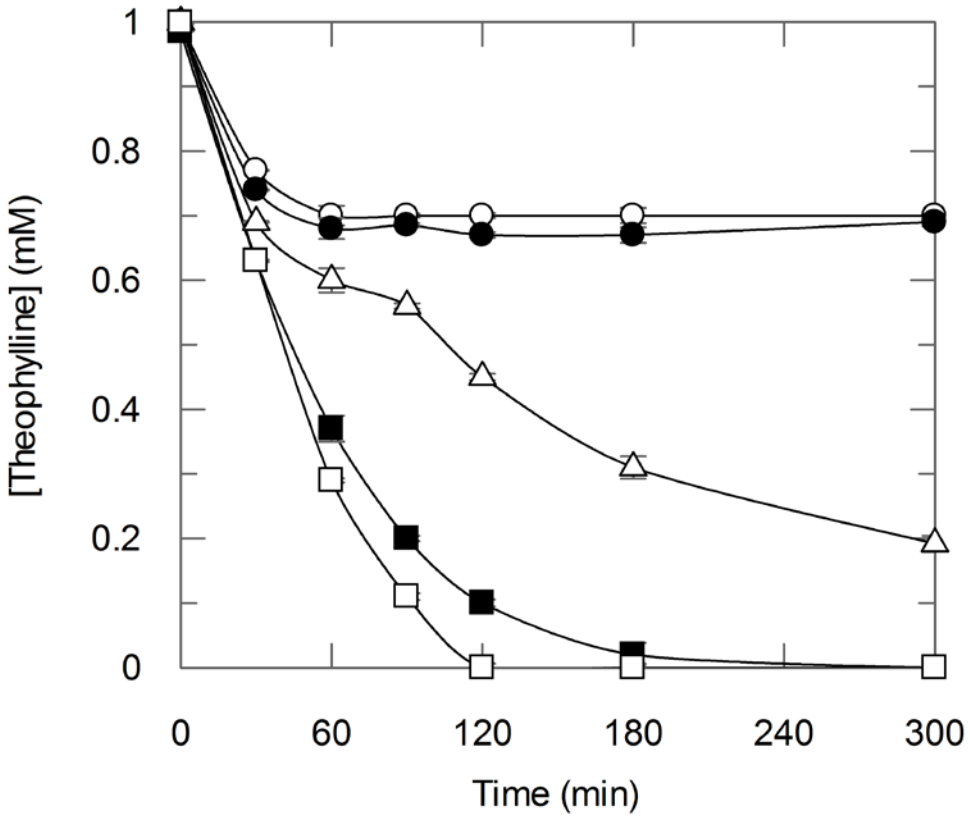


Figure 3.2 Degradation of TP by metabolically engineered *E. coli* resting cells. ○, strain pAD1; ●, strain pAD1dAA; △, strain dDA; ■, strain pAD1dDA; □, strain pAD1dDD. Cells (OD_{600} = 2.5) were incubated with 1 mM TP in 50 mM KP_i buffer at 30°C with 400 rpm shaking, and metabolites were quantified via HPLC

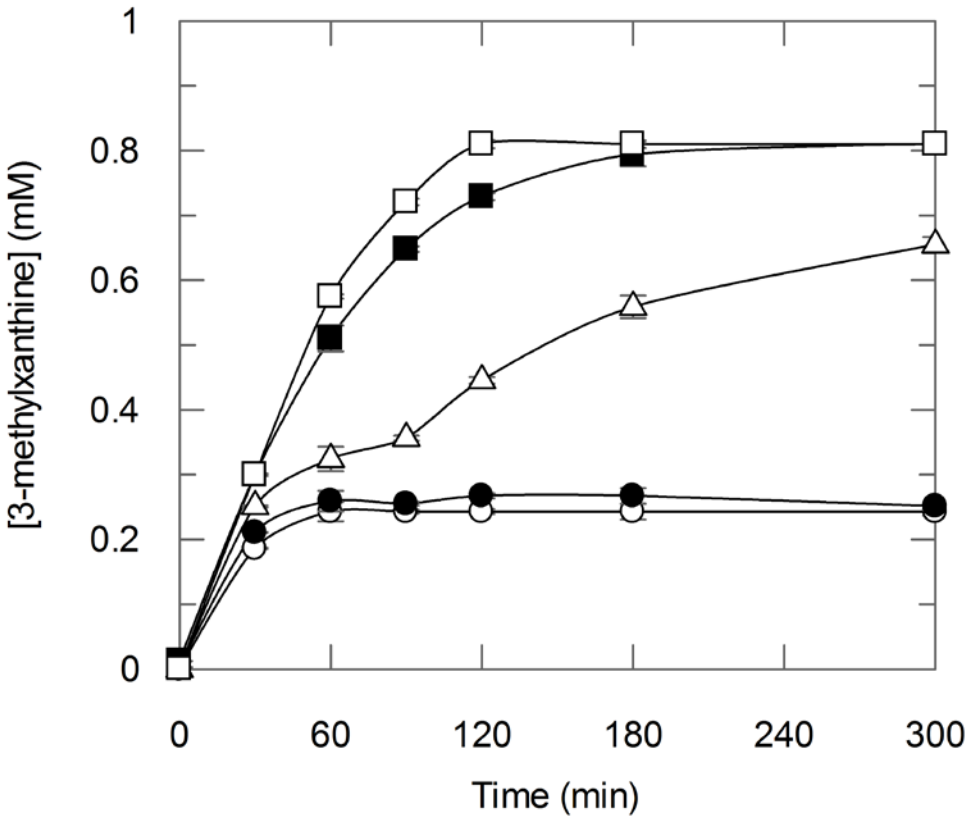


Figure 3.3 Formation of 3MX by metabolically engineered *E. coli* resting cells. ○, strain pAD1; ●, strain pAD1dAA; △, strain dDA; ■, strain pAD1dDA; □, strain pAD1dDD. Cells (OD_{600} = 2.5) were incubated with 1 mM TP in 50 mM KP_i buffer at 30°C with 400 rpm shaking, and metabolites were quantified via HPLC

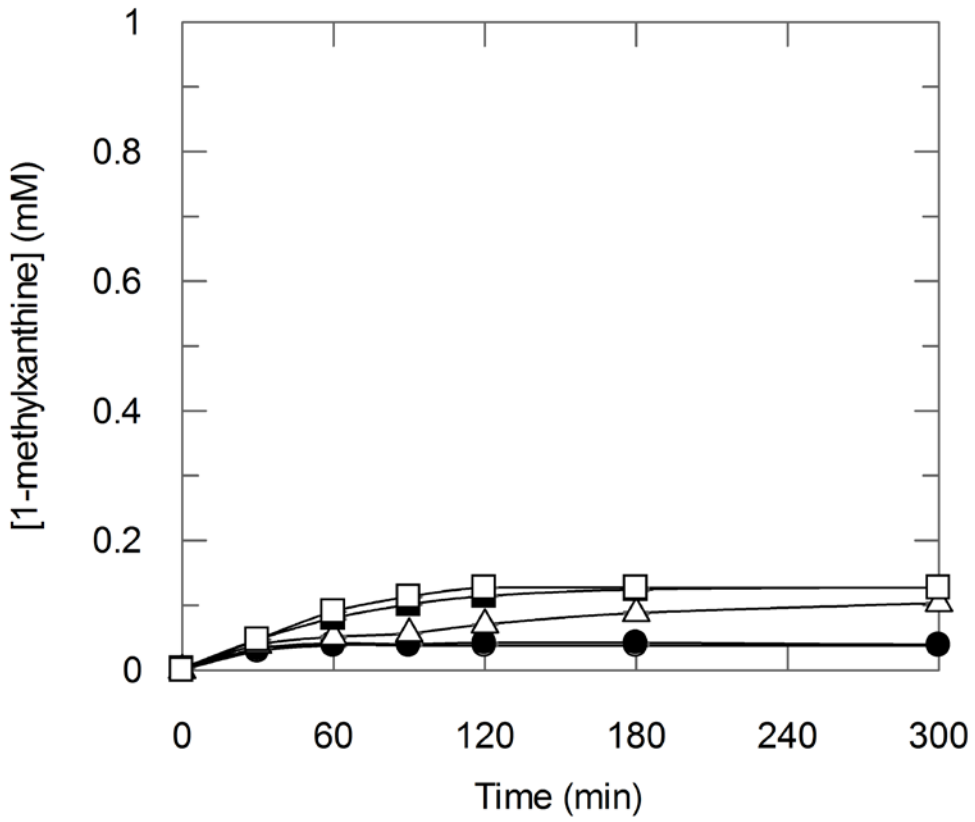


Figure 3.4 Formation of 1MX by metabolically engineered *E. coli* resting cells. ○, strain pAD1; ●, strain pAD1dAA; △, strain dDA; ■, strain pAD1dDA; □, strain pAD1dDD. Cells (OD_{600} = 2.5) were incubated with 1 mM TP in 50 mM KP_i buffer at 30°C with 400 rpm shaking, and metabolites were quantified via HPLC

Table 3.1 Estimated copy number of *ndmA* and *ndmD* genes in strains used in this study

Strain	Approximate gene copy number*		*ndmD* : *ndmA* ratio
	ndmA	ndmD	
pAD1	40	40	1.0
pAD1dAA	60	40	0.67
pAD1dDA	50	50	1.0
pAD1dDD	40	60	1.5
dDA	10	10	1.0
pDdAA	20	40	2.0
pDdA	10	40	4.0

* Approximate gene copy number was estimated based on approximate copy number of the plasmid (40 for pAD1 and pET28-His-ndmD, 10 for dA, dAA, dDD, and dDA) and number of genes in each plasmid. This value was calculated as $C_i = \sum N_{ij} P_{ij}$, 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 (*ndmA* or *ndmD*), and *j* = plasmid backbone (pET or pACYCDuet-1)

In the case of plasmid pAD1, the *ndmD* gene is separated from the T7 promoter by approximately 1.1 kb of sequence containing the *ndmA* ribosomal binding site and gene, followed by a short synthetic ribosomal binding site of unknown strength just before the *ndmD* gene (Figure 3.5). SDS-PAGE of strain pAD1 (Figure 3.6) showed a strong band of soluble *NdmA*, but very little *NdmD* (soluble or insoluble). In contrast, strain pAD1dDD had relatively strong soluble and insoluble NdmD bands. Based on activity and electrophoretic analysis, it was concluded that plasmid pAD1 clearly did not produce sufficient soluble intracellular NdmD. Hence this strain was not the best to take forward for scale-up. This was further confirmed using resting cells (OD_{600} = 2.5) of an *E. coli* strain containing only plasmid dDA. This strain consumed 0.8 mM TP over 300 min (Figure 3.2) and produced 0.65 mM 3MX. Plasmid dDA is based on the

pACYCDuet-1 backbone (Figure 1.10), giving a plasmid (and gene) copy number approximately 4-fold lower than that of pAD1 (Table 3.1). In spite of the lower overall gene dosage, activity was much higher in strain dDA (80% TP conversion) than in strains pAD1 (30% TP conversion) and pAD1dAA (70% TP conversion). Methods to increase expression of *ndmD* from plasmid pAD1 only may be achieved by using a known strong ribosomal binding site and/or a second T7 promoter between *ndmA* and *ndmD*.

Figure 3.5 Maps of plasmids in strains used to produce 3MX from TP. ori_pBR322, pBR322 origin of replication; ori_P15A, P15A origin of replication; Amp-R, ampicillin resistance gene, Kan-R, kanamycin resistance gene, CAT, chloramphenicol resistance gene, ndmA, N_1-demethylase gene; ndmD, *N*-demethylase reductase gene; His-ndmD, N-terminal His_6-tagged *N*-demethylase reductase gene; T7, T7 promoter. Plasmids beginning with "p" use pET backbones, plasmids beginning with "d" use the pACYCDuet-1 plasmid backbone

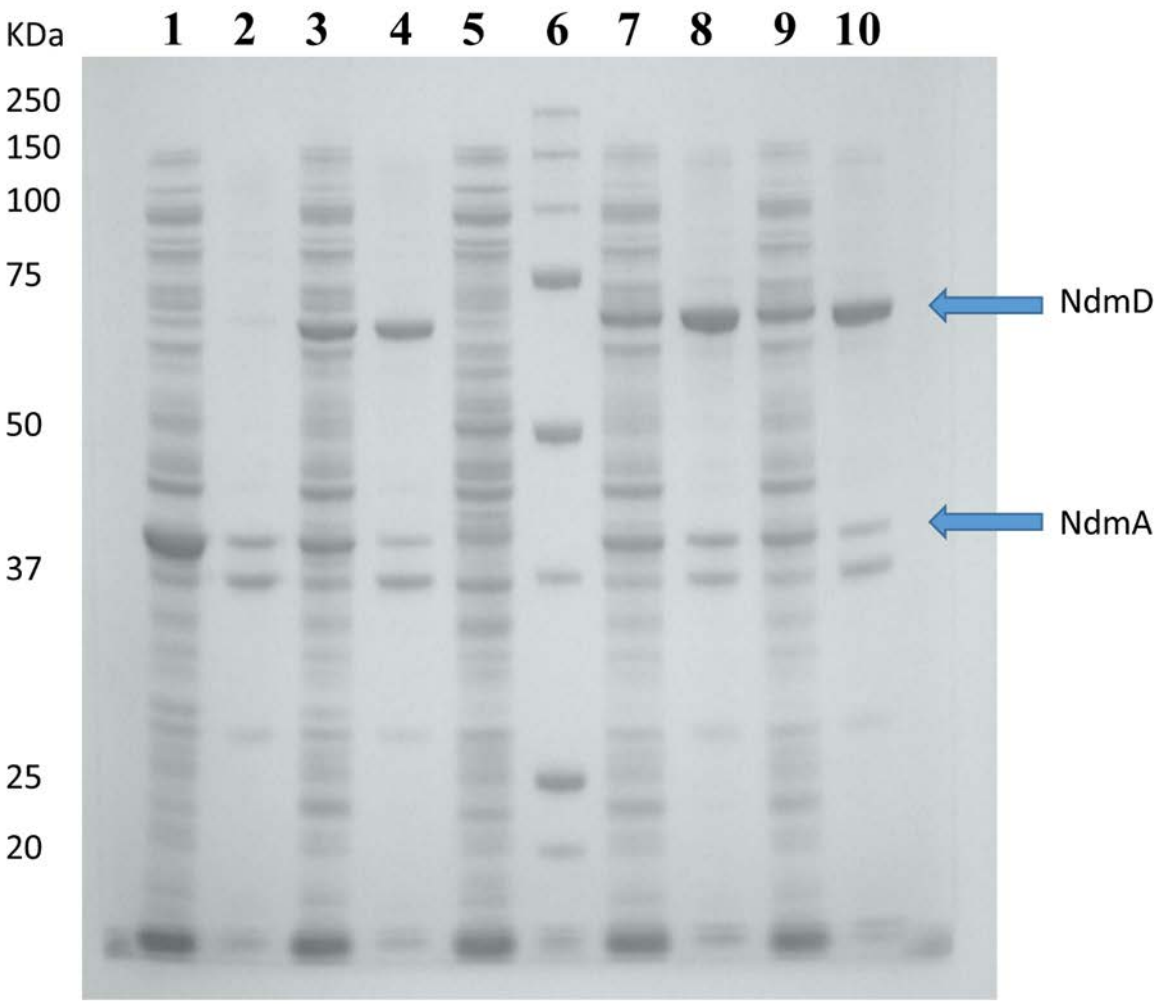


Figure 3.6 SDS-PAGE analysis of *ndmA* and *ndmD* expression in metabolically engineered strains of *E. coli*. A total of 10 µg protein was loaded into each well. Molecular weights of markers (in kDa) are shown to the left of the gel. Blue arrows indicate NdmA and NdmD protein bands. Lane 1, pAD1 soluble fraction; lane 2, pAD1 insoluble fraction; lane 3, pDdA soluble fraction; lane 4, pDdA insoluble fraction; lane 5, BL21(DE3) soluble fraction (negative control); lane 6, molecular weight standard; lane 7, pAD1DD soluble fraction; lane 8, pAD1DD insoluble fraction; lane 9, pDdAA soluble fraction; lane 10, pDdAA insoluble fraction

In order to increase intracellular levels of NdmD, a plasmid containing the *ndmD* gene placed immediately downstream of the T7 promoter and ribosomal binding site in pET28a(+) (Summers, Louie et al. 2012) was used (Table 3.1). A compatible plasmid (pACYCDuet-1) containing one or two copies of *ndmA* (plasmids dA and dAA, respectively) was then added to a strain of *E. coli* harboring pET28-His-ndmD. This resulted in strains with a low (pDdA) or medium (pDdAA) *ndmA* gene dosage, based on estimated copy number and number of genes in each plasmid. The activity and protein expression levels of these two strains were then compared with strain pAD1dDD, which had the highest *ndmD* dosage of the three plasmid constructs (Table 3.1). Each of the strains pDdA, pDdAA, and pAD1dDD was grown 100 mL LB (Table 3.2) when gene expression was induced by adding IPTG (0.1 mM final concentration) as described in Chapter 2. The final OD_{600} upon harvesting was 6.2, 6.3, and 6.7 and the wet cells weight collected after harvesting was 0.75, 0.84, and 0.8 g for strain pDdA, pDdAA, and pAD1dDD respectively (Table 3.2). SDS-PAGE revealed that soluble (active) protein expression is about the same for NdmA and NdmD among the three strains (Figure 3.6). 30 mg of each wet cell paste was used to test the conversion of TP to 3MX by resuspending in KP_i buffer to a final reaction volume of 1 mL so the cell concentration was 30 mg/mL and initial TP concentration of 4 mM. After 90 minutes of the reaction time, TP was reduced 56, 51, and 43% by suspensions of pDdA, pDdAA, and pAD1dDD, respectively. Approximately 84, 82, and 81% of the consumed TP was converted to 3MX in strains pDdA, pDdAA, and pAD1dDD, respectively, with the remaining TP forming 1MX (Table 3.2). Based on these results, strain pDdA was chosen for further studies due to the highest yield of 3MX from TP. Clearly, the additional gene dosage of *ndmA*

(pDdAA) did not improve 3MX yield, relative to single gene dose (pDdA). Therefore, the activity of the cells was proven to be independent of the *ndmA* gene dosage and highly dependent on the *ndmD* gene dosage and soluble expression of this protein in each *E. coli* strain.

Table 3.2 Comparison of growth and activity of resting cell suspensions of strains pDdA, pDdAA, and pAD1dDD. Concentrations of TP, 3MX, and 1MX after 90 min are reported as means with standard deviations of triplicate reactions [Reaction conditions: 4 mM initial theophylline, 30 mg/mL wet cells, temperature 30 ^{o}C, and microplate shaker speed 400 rpm]

Strain	Cell growth		Reaction		
	Final OD_{600}	Final cell mass (g)	Final [TP] (mM)	Final [3MX] (mM)	Final [1MX] (mM)
pDdA	6.2	0.75	1.52 ± 0.27	2.25 ± 0.31	0.42 ± 0.05
pDdAA	6.3	0.84	1.70 ± 0.83	1.84 ± 0.74	0.40 ± 0.15
pAD1dDD	6.7	0.81	2.03 ± 0.54	1.59 ± 0.55	0.37 ± 0.10

Comparison of Growth Media

Further work was carried out with pDdA strain to study the effect of culture medium on cell growth and activity by growing strain pDdA in Luria-Bertani Lennox (LB) and super broth (SB) media. The choice of LB, a well-known media used in molecular biology, and SB, a rich nutrient medium, allows investigating the cell growth and the amount of wet cells produced. It is important to produce large amount of cells which leads to higher production of 3MX. The cells in each of the 100 mL medium grew to OD_{600} of 4.75 and 7.5 in LB and SB respectively and after harvesting the cells the wet

cells weight was 0.6 and 0.9 g respectively (Table 3.3). Therefore, SB produced approximately 50% more cells than LB did.

Table 3.3 Comparison of growth of strain pDdA grown in LB and SB [100 mL of each medium was used]

Media	Cell growth	
	Final OD_{600}	Final cell mass (g)
LB	4.75	0.6
SB	7.5	0.9

After washing the Cells from growth media in 50 mM potassium phosphate buffer (pH=7.5), they were resuspended to 15 mg/mL, and the initial TP concentration in activity assays was lowered to 1 mM. The goal here was to achieve complete conversion of TP to 3MX and 1 MX, which would facilitate downstream purification and product recovery. TP was completely consumed in SB-grown cells within 90 min (Figure 3.7). After two hours, nearly all of the TP was consumed in both reactions (Figure 3.8). 3MX yield from theophylline was 81-83%; 12-13% of TP was converted to 1MX. Because the cells are capable of performing both N_1- and N_3-demethylations on both TP and also 1- and 3MX, very minor amount of xanthine was also formed from the monomethyl xanthine products. These results demonstrate that the media composition had no significant effect on NdmA activity and product ratio. Given the complete conversion of TP achieved in shorter time and 50% more biocatalyst harvested from SB, this medium was chosen for the production of 3MX to supply clients.

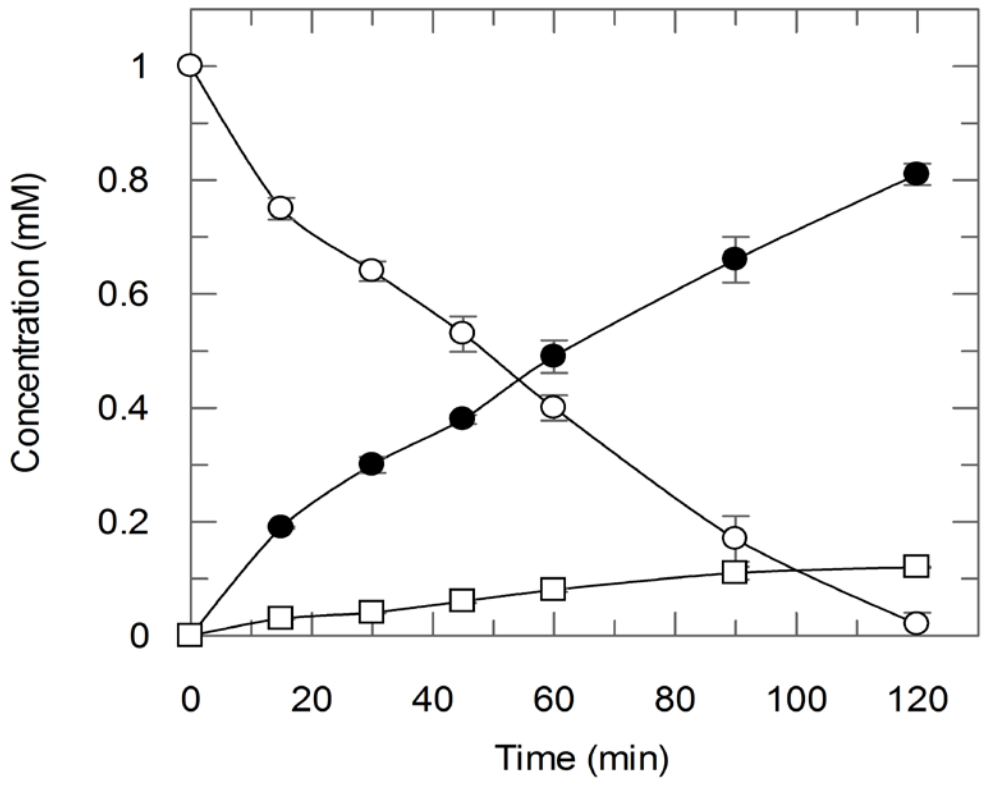


Figure 3.7 Production of 3-methylxanthines from TP by strain pDdA grown in LB. 1 mM TP (○) was converted to 0.81 mM 3MX (●) and 0.13 1MX (□) within 120 min by 15 mg/mL of resting cells. [Reaction conditions: 1 mM initial theophylline, 15 mg/mL wet cells, temperature 30 °C, and microplate shaker speed 400 rpm]. Concentrations reported are means with standard deviations of triplicate results

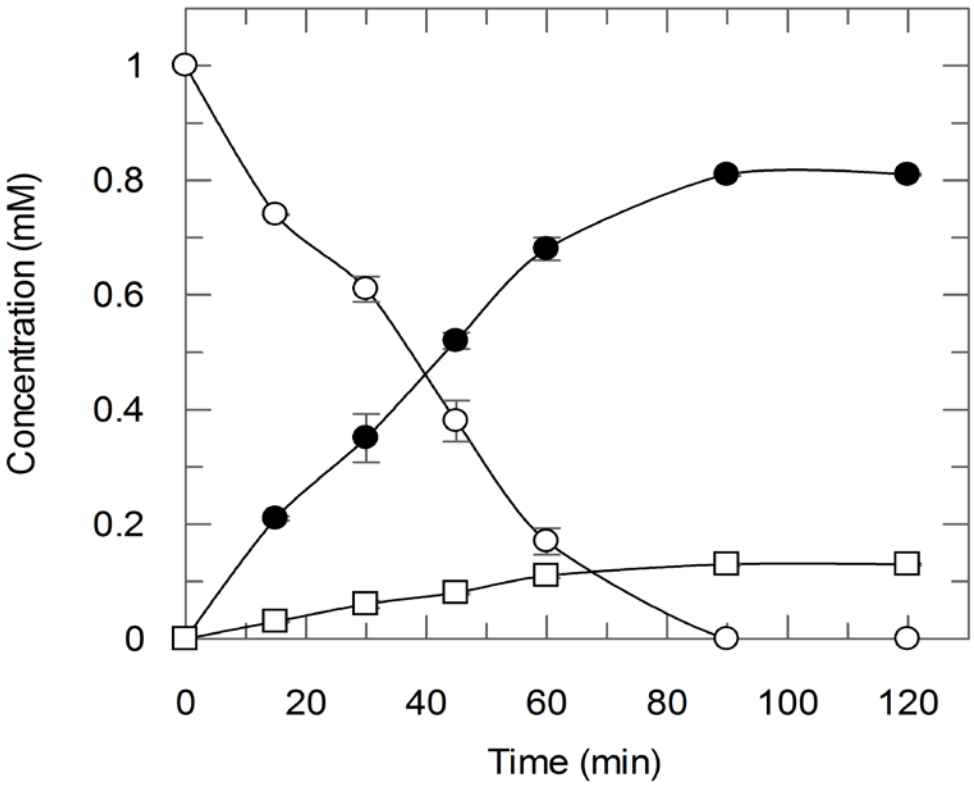


Figure 3.8 Production of 3-methylxanthines from TP by strain pDdA grown in SB. 1 mM TP (○) was converted to 0.81 3MX (●) and 0.13 1MX (□) within 90 min by 15 mg/mL of resting cells. [Reaction conditions: 1 mM initial theophylline, 15 mg/mL wet cells, temperature 30 ºC, and microplate shaker speed 400 rpm]. Concentrations reported are means with standard deviations of triplicate results

Although yield of 3MX is high, minor production of 1MX decreased the overall yield of 3MX. The slight N_3-demethylation of TP by NdmA to form 1MX is surprising and in contrast with previous findings that purified NdmA is highly specific for the N_1 methyl group of caffeine and TP (Summers, Louie et al. 2012). We therefore tested the activity of strain pDdA on caffeine and observed a slight (<2%) N_3-demethylation activity to form paraxanthine (1,7-dimethylxanthine, data not shown). The enzyme in the previously reported work was expressed in *E. coli* BL21(DE3) with a C-terminal hexahistidine (His_6) tag for facile purification and the purified enzyme produced only 3MX from TP. 1MX was shown to be produced from TP by the N_3-demethylase NdmB-His. The present study utilizes NdmA expressed in the same microbial chassis without the His_6 tag, and the reaction is carried out *in vivo*. It is unclear whether performing the reaction *in vivo*, elimination of the His_6 tag from NdmA, enzyme expression level, and/or enzyme solubility (Summers, Seffernick et al. 2013) are involved in the slight change in specificity to form 1MX. In the *in vitro* studies, the minimum amount of enzyme was used in order to determine the kinetics (Summers, Louie et al. 2012), and the paraxanthine and 1MX products may have been below the detection limit. However, the reduction in enzyme expression level (comparing strains pAD1 and dDA vs. strain pDdA) in this work did not result in a lower ratio of products. Clearly, an *in vitro* approach to produce 3MX would not be economical, as it would require addition of external NADH, which is expensive. It should be noted, however, that addition of a His_6 tag has been implicated in changing substrate specificity of thioesterase I in *E. coli* due to a slight change in active site geometry (Lee, Su et al. 1999). The reason for the discrepancy between NdmA and NdmA-His_6 is currently under investigation in a

separate study. The original strain of *P. putida* CBB5 produced approximately twice as much 3MX as 1MX (Yu, Louie et al. 2009), however, the 1MX production in this strain, besides slight specificity of NdmA at N_3-position, can mostly be attributed to NdmB (Summers, Louie et al. 2012). Future work to reduce the N_3-demethylation activity of NdmA via mutation should create a more efficient process for production of 3MX, while simultaneously simplifying downstream recovery of 3MX.

Larger Scale Reaction, Preparative Chromatography, and Purification of 3MX

The reaction conditions for conversion of TP to 3MX were optimized by evaluating different concentrations of cells (5, 10, 15, 30, and 60 mg wet cells/mL) and initial substrate concentration (1, 2, and 4 mM TP). Figure 3.9 A represents the use of wet cells concentrations of 5,10, and 15 mg with initial TP concentration of 1 mM. TP consumed by 60, 78, and 98% by 5, 10, and 15 mg wet cells/mL respectively after ninety minutes of the reaction time. Figure 3.9 B and C represent the use of wet cells concentrations of 15, 30, and 60 mg with initial TP concentration of 2 mM (middle row) and 4 mM (lower row). With initial TP of 2 mM, it was consumed 50% and 100% in ninety minutes by 15 and 30 mg wet cells/ mL respectively while 100% TP conversion was achieved in one hour when the cells concentration was 60 mg/mL (Figure 3.9.B). When TP presented in the reaction was 4 mM, its conversion was 25, 62, and 100% with cells concentrations of 15, 30, and 60 mg/mL respectively (Figure 3.9.C). In all these reactions theophylline was converted to 3MX (81-85%) and 1MX (12-15%).

It is clear from the data presented in Figure 3.9 that a reaction containing 1 mM TP and 15 mg/mL resting cells provides linear conversion of TP to 3MX (Figure 3.10). At these reaction conditions, the product concentration and reaction volume suited the prep HPLC column capacity for complete product recovery. Hence this condition was chosen for scale-up.

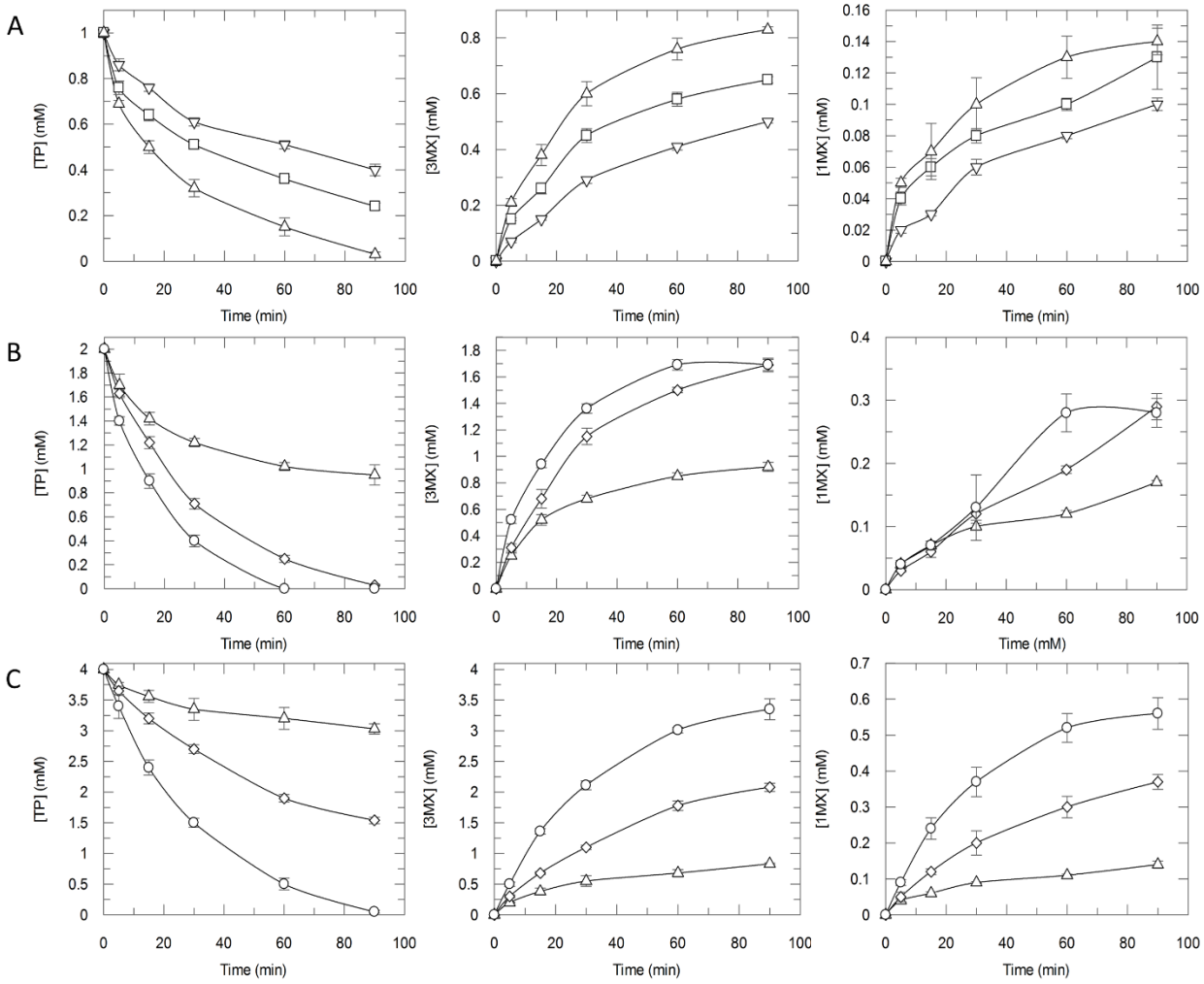


Figure 3.9 Effect of cell and substrate concentrations on 3MX production by *E. coli* pDdA. Resting cell assays were performed using 5 (▽), 10 (□), 15 (△), 30 (◇), and 60 (○) mg/mL wet cells. TP concentrations were 1 mM (A), 2 mM (B), and 4 mM (C). Concentrations of TP (left), 3MX (middle), and 1MX (right) are shown as means with standard deviations of triplicate reactions

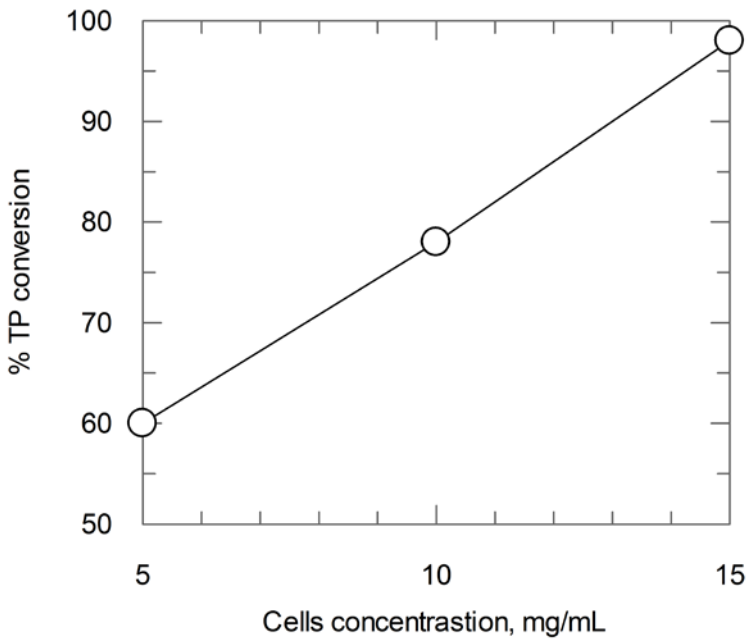


Figure 3.10 Conversion vs. wet cells concentration at 1 mM initial theophylline

Production of 3MX was scaled up by growing the pDdA strain in SB media in four 2.8 L Fernbach flasks, resulting in a total yield of 20 g wet cells. The cell yield was sufficient to perform a 1.3 L reaction with an initial TP concentration of 1 mM at 15 mg/mL resting cell suspension. Initial analysis by HPLC showed complete consumption of TP after two hours of reaction time with formation of 0.81 and 0.13 mM 3MX and 1MX, respectively (Figure 3.11). The products were separated by preparative chromatography (Figure 3.12). Resolution of 3MX (retention time of 116 min) and 1MX (retention time of 136 min) was sufficient to collect each of the two products separately. The two products were crystallized through evaporation and freeze-drying, resulting in 106 mg 3MX and a minor amount of 1MX. Because the very small amount of 1MX

produced could not be collected from the walls of the freeze dryer tray, 1MX was not further characterized.

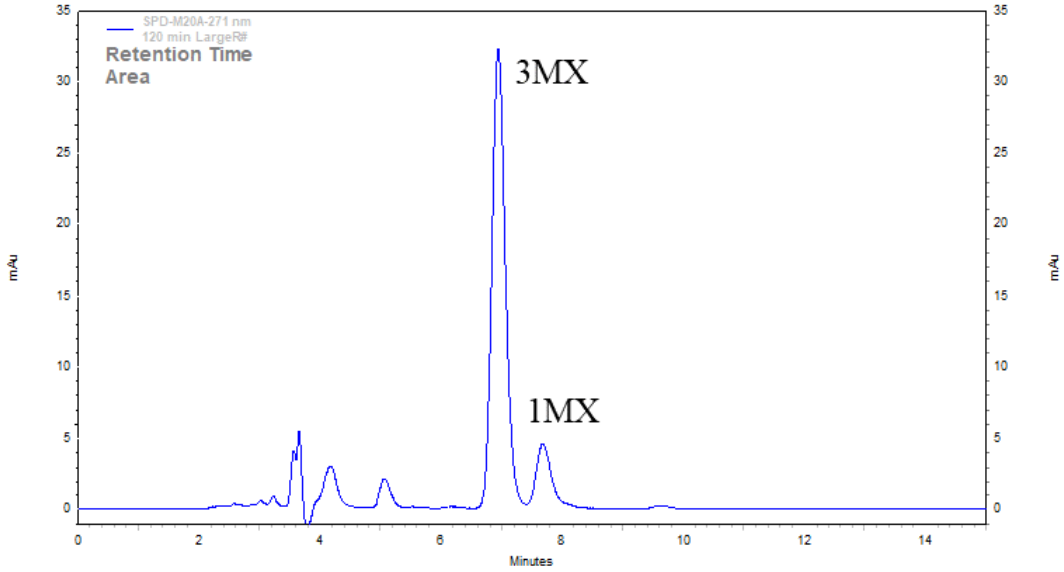


Figure 3.11 HPLC chromatograms for the 1.3 L reaction after 2 hours [Injection volume 1 µL, mobile phase: 0.5 mL/min: methanol-water-acetic acid (5:95:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 4.6 mm x 125 mm]

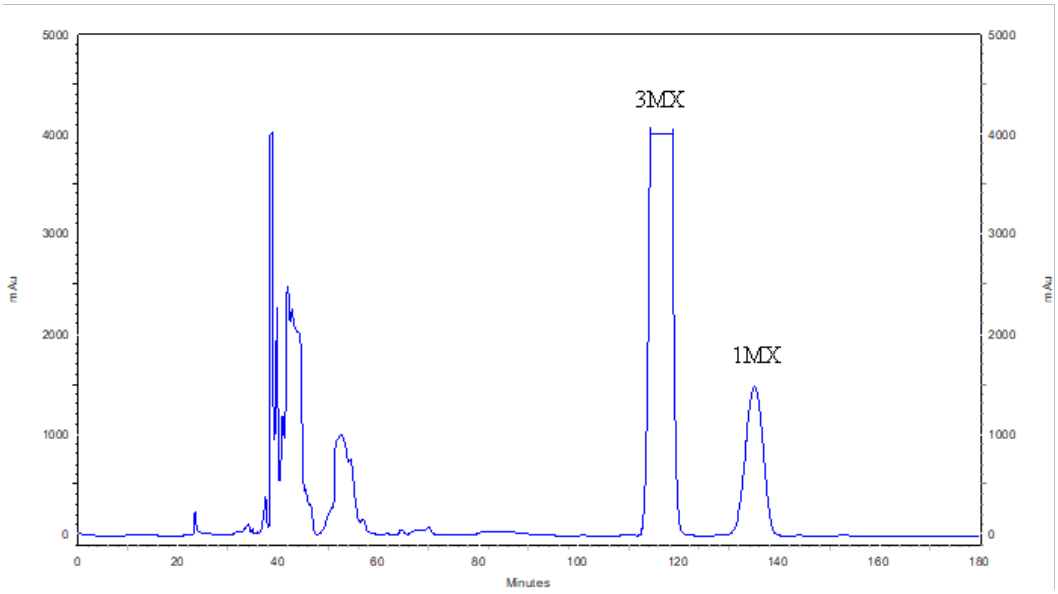


Figure 3.12 Separation of 3MX and 1MX by preparative chromatography. Retention times of 3MX and 1MX are 116 and 135 min, respectively

The theoretical amount of 3MX produced in the reaction was 175 mg (~81% mole to mole conversion from theophylline); however, 36% of the post-reaction mixture was used to optimize the preparative chromatographic separation of 3MX and 1MX. Therefore, a total of 111 mg 3MX (64% of the post-reaction mixture) was loaded onto the column for purification and recovery. The resulting 106 mg pure 3MX recovered indicates very little loss during separation with a purification yield of 95.5%. Improving the selectivity of NdmA so that it only produces 3MX from TP would further increase the yield.

Because the *ndm* genes have only recently been discovered (Summers, Louie et al. 2012, Summers, Seffernick et al. 2013), previous attempts to produce methylxanthines through a biocatalytic route have focused primarily on metabolism and enzymology studies for conversion of caffeine to theobromine. Research has shown that addition of

certain divalent metal ions, such as Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} have a strong inhibitory effect on degradation of theobromine accumulated from caffeine in whole cells of *Pseudomonas putida* (Asano, Komeda et al. 1993, Dash and Gummadi 2007). However, there are no known specific inhibitors to stop the reaction at the desired, high-value methylxanthines such as paraxanthine and 1-, 3-, and 7-methylxanthine. Also, this approach would not be optimal for methylxanthine production, as the wild type *P. putida* strains (CBB5 and others) have lower growth rates and cannot produce the same amount of enzyme (hence, catalytic activity) as can *E. coli* expressing the recombinant *ndm* genes. Jin *et al*. (Jin, Bhuiya et al. 2014) cloned genes from the caffeine biosynthetic pathway of coffee and tea into *Saccharomyces cerevisiae*. The resulting strain produced a very low level (0.38 mg/L) of caffeine when exogenous xanthosine was fed. Without addition of xanthosine, no caffeine was detected. Besides the low production level, use of plant genes restricts the possible products to 7-methylxanthine, theobromine, and caffeine, which are the metabolites of the caffeine biosynthetic pathway. Caffeine is mostly produced during the decaffeination of coffee beans (Udayasankar, Manohar et al. 1986, Ramalakshmi and Raghavan 1999). Theobromine and theophylline are mostly produced synthetically (Bobranski and Synowiedski 1948, Hu, Wan et al. 2003), although extraction from plants is possible (Saldaña, Mohamed et al. 1999). Thus, further strain optimization and protein engineering will be required before use of plant-based genes can be used in a microbial system to produce high value methylxanthines.

Analytical Characterization of Biologically Produced 3MX

The purity of both 3MX and 1MX was analyzed by analytical HPLC using appropriate authentic standards. The retention time of the biologically produced products and authentic standards were identical (Figure 3.13). The High Resolution LC-MS spectrum of biologically produced and standard 3MX matched very well (Figure 3.14) and corresponded to the published spectrum (spectrum). LC/MS was recorded on ESI positive mode; distinct M+1 ion peak at 167.0569 m/z was observed both in the standard (Figure 3.14 A) and the biologically produced 3MX (Figure 3.14 B).

The ^{1}H NMR spectrum of biologically produced and standard 3-methyl xanthine also matched very well (Figure 3.15). ^{1}H NMR was recorded on a Bruker 500 MHz spectrophotomer using DMSO-d_6 as solvent. Standard 3-methylxantine showed presence of peaks at δ 13.48 (s, 1H) and 11.07 (s, 1H) corresponding to –NH proton, and peaks at δ 8.01 and 3.3 corresponding to –C=H (s, 1H) and –CH_3 group (s, 3H). The biologically produced 3-methylxanthine also showed peaks at δ 13.48 (s, 1H) and 11.07 (s, 1H) corresponding to –NH proton, and peaks at δ 8.0 and 3.3 corresponding to –C=H (s, 1H) and –CH_3 group (s, 3H).

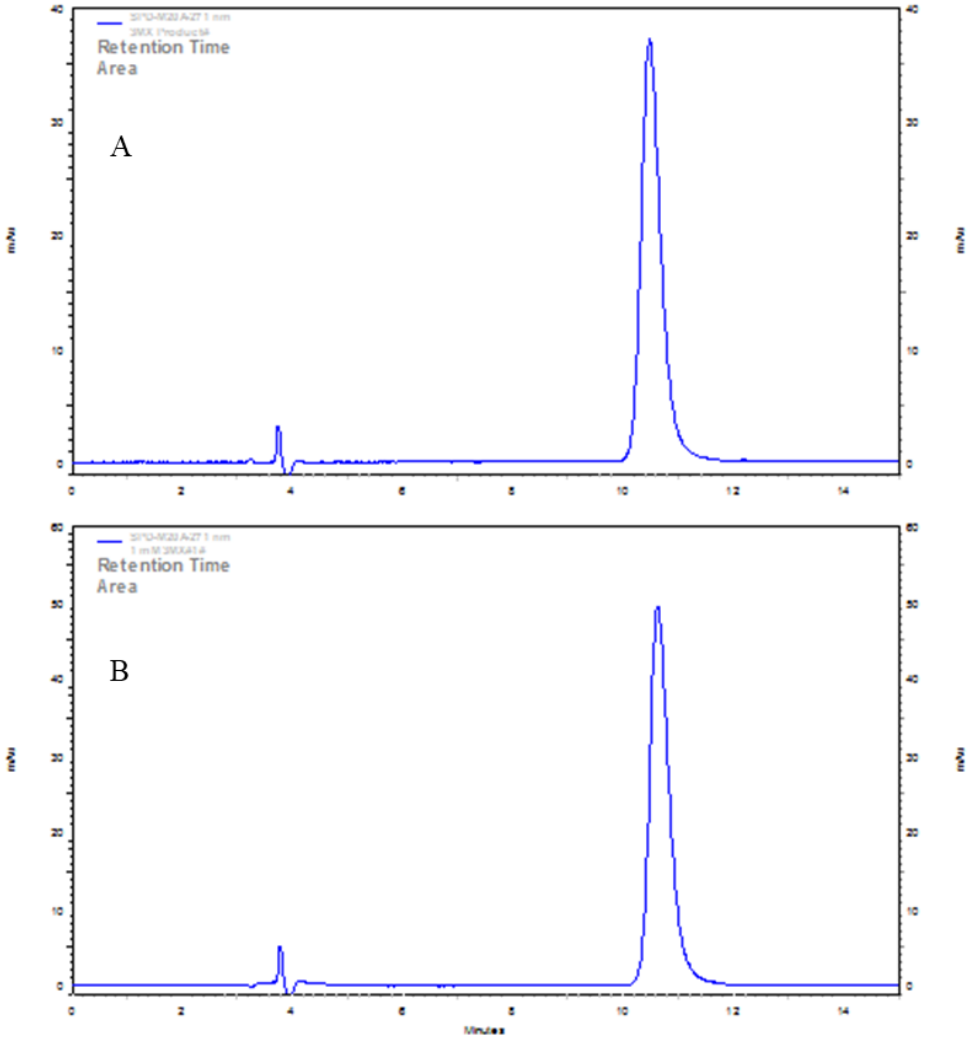
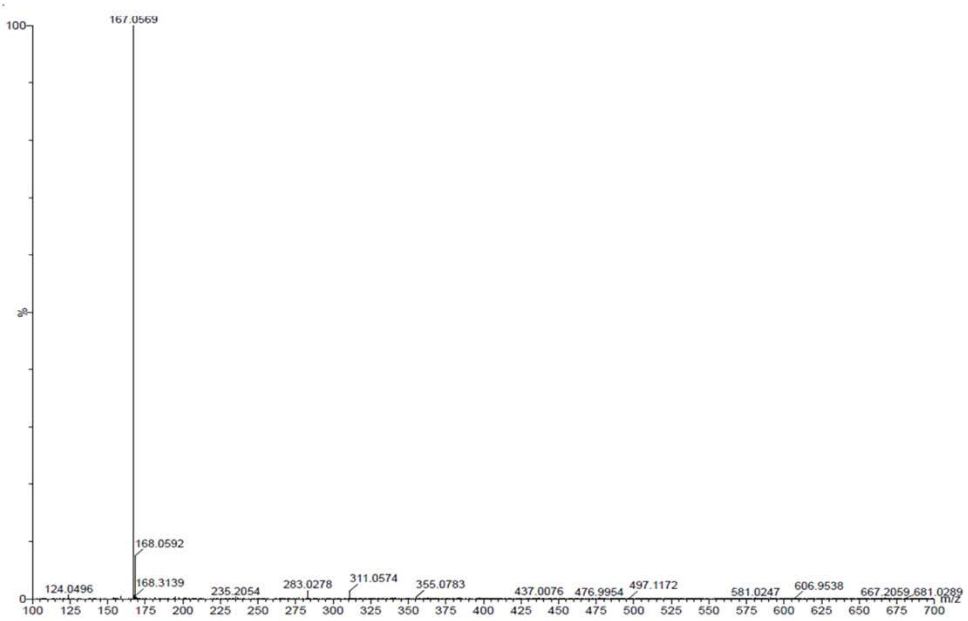


Figure 3.13 HPLC chromatograms for the 3MX (A) produced in this work and (B) standard from Sigma Aldrich [Injection volume 1 µL, mobile phase: methanol-water-acetic acid (5:95:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 4.6 mm x 125 mm]



A

B

Figure 3.14 LC-MS spectra of 3MX samples. (A) Spectrum of 3MX standard purchased from Sigma-Aldrich. (B) 3MX produced in this work. Inset to (B): Purified, crystallized 3MX produced in this work

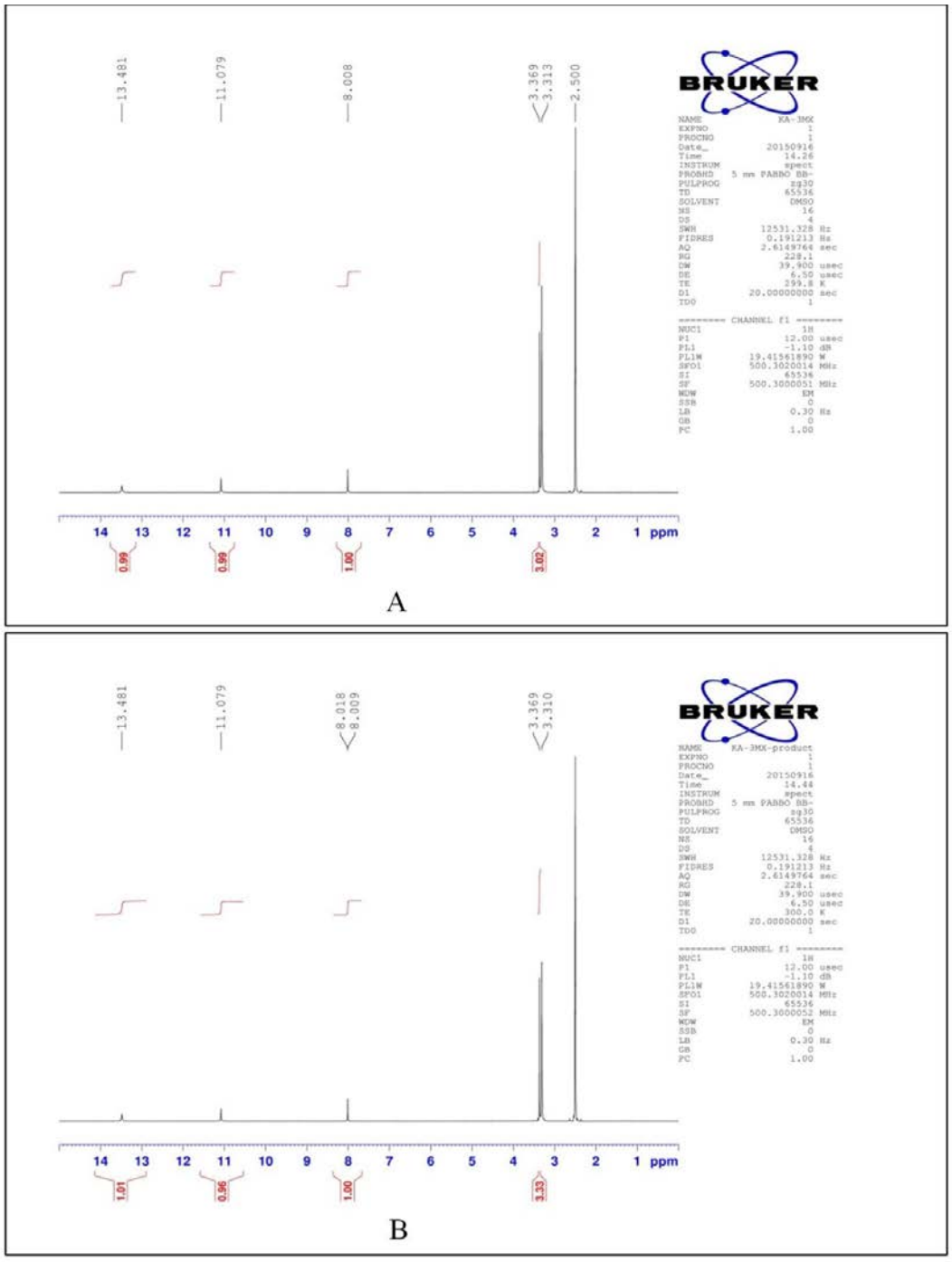


Figure 3.15 NMR of 3-methylxanthine. (A) NMR of 3MX standard obtained from Sigma Aldrich. (B) NMR of biologically produced and purified 3MX sample produced in this work

CHAPTER 4: PRODUCTION OF THEOBROMINE AND PARAXANTHINE USING METABOLICALLY ENGINEERED *E. COLI*

Introduction

Theobromine is produced by both natural and synthetic methods. The conventional natural methods use the extraction of theobromine from cocoa beans by solvents, water, or supercritical fluids (Brennecke 1996, Li and Hartland 1996, Mehr, Biswal et al. 1996, Mohamed, Saldaña et al. 2002, Saldaña, Zetzl et al. 2002, Hanamura, Chikauchi et al. 2009). These processes are expensive, non-specific, and involve the use of high operating pressures and toxic organic solvents (Gokulakrishnan, Chandraraj et al. 2005) . The first reported chemical synthesis of theobromine was in 1882 by Fischer (Fischer 1924). Like other methylxanthines, theobromine and paraxanthine could also be synthesized by traditional Traube synthesis (Traube 1900), which is used to produce purine alkaloids through the cyclization of 4,5-diaminopyrimidines with formic acid, dithioformic acid, or other carboxylic acids. Theobromine can also be chemically synthesized from 3-methylxanthine via methylation by dimethylsulfate as a methylating agent (Christ 2008). This method is unpractical because 3-methylxanthine is much more expensive than theobromine (Algharrawi, Summers et al. 2015) and dimethylsulfate could have toxic effects on the skin and mucous membranes (Littler and McConnell 1955). These environmentally unfavorable chemical methods utilize several chemicals, multiple reactions, and harsh reaction conditions (Hutzenlaub and Pfleiderer 1979).

An alternative theobromine production process that is environmentally favorable is via biocatalysis. Caffeine is an abundantly available feedstock from agricultural commodity, coffee, which can be converted to the higher value non-toxic theobromine.

In this chapter, we describe a method using metabolically engineered *E. coli* with ndmA and ndmD genes, for direct conversion of caffeine (Figure 4.1) to theobromine (major product (98.5 % conversion)) and paraxanthine (minor product (1.5 % conversion)). Different copy numbers of these genes were incorporated into *E. coli* using two compatible vectors to produce five metabolically engineered *E. coli* strains. These metabolically engineered strains were screened for production of theobromine and paraxanthine from caffeine. Theobromine is the major product due to highly selective N_1-demethylation caffeine by NdmA. Paraxanthine is a minor product due to a slight promiscuity of NdmA for N3-demethylation. The optimal strain with the highest conversion of caffeine was chosen for the bio-catalytic conversion of caffeine to theobromine. Yield of paraxanthine was very low (1.5%), hence it was not subjected to analytical characterization. This new bioprocess involves preparation of the biocatalyst, the N-demethylation reaction of caffeine to theobromine and recovery of highly pure theobromine (79% molar yield). The conversion of caffeine to theobromine (and paraxanthine) occurs at ambient temperature and pressure, and is environmentally friendly, unlike the chemical process.

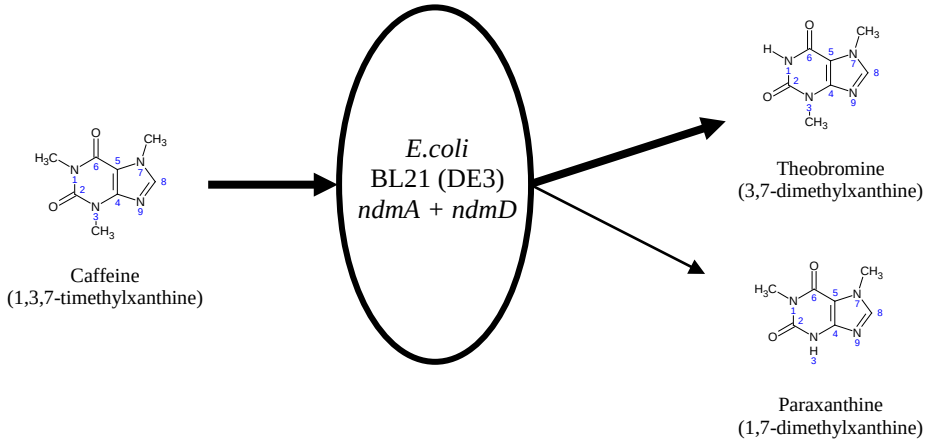


Figure 4.1 Schematic representation for the biocatalytic N-demethylation of caffeine to theobromine (major product) and paraxanthine (minor product) by *E. coli* BL21(DE3) genetically engineered with N-demethylation genes of *Pseudomonas putida* CBB5 *ndmA* and *ndmB*

Initial Screening of Theobromine and Paraxanthine Production by Metabolically Engineered *E. coli*

Five strains of *E. coli* that were previously engineered for production of 3-methylxanthine from theophylline were screened for production of theobromine and paraxanthine from caffeine. Theses strains are pAD1, dDA, pAD1dAA, pAD1dDA, and pAD1dDD. The genes carried by these strains and the genes copy numbers are shown in Table 1.6 and Table 3.1 respectively. Strains pAD1(copy number of each of ndmA and ndmD is 40) and dDA (copy number of each of ndmA and ndmD is 10), both of which harbored a single plasmid, exhibited nearly same conversion of caffeine to the products. Strain pAD1 converted 54% of the 1 mM caffeine presented initially in the reaction while strain dDA converted 56%. (Table 4.1). Addition of plasmid dAA to the pAD1 strain

resulted in strain pAD1dAA, which demonstrated 75% conversion of caffeine, which is nearly 40% increase in caffeine conversion to TB and PX. This is different than our previous observations when theophylline was used as a substrate, where activity of pAD1 and pAD1dAA strains were roughly the same while dDA activity was higher (Algharrawi, Summers et al. 2015). Given the unpredictability of how much soluble NdmA and NdmD are expressed with each plasmid, 'optimizing' the activity obtained with each construct appeared to be the best approach to get the best strain and high product yields. Specifically, increasing the estimated *ndmD* gene copy number increased activity, indicating that soluble expression of *ndmD* influenced activity to a greater level than did *ndmA* expression. After two hours, strain pAD1dDD achieved 89% conversion of caffeine and produced the most theobromine (0.87 mM) and paraxanthine (0.01mM) (Table 4.1), and was selected for further work to produce and purify TB from caffeine.

Table 4.1 Concentrations of methylxanthines in resting cell assays by 5 metabolically engineered *E. coli* strains [Reaction time was two hours. Initial concentration of caffeine was 1 mM. Reaction volume was 1 mL with 10 mg of wet cells in each case. Products were analyzed by HPLC as described in Chapter 2]

Strain	**Concentrations (mM)**		
	Caffeine	**Theobromine**	**Paraxanthine**
pAD1	0.46 ± 0.020	0.53 ± 0.006	0.006 ± 0.000
dDA	0.44 ± 0.019	0.54 ± 0.006	0.006 ± 0.000
pAD1dAA	0.25 ± 0.003	0.74 ± 0.012	0.010 ± 0.000
pAD1dDA	0.22 ± 0.003	0.77 ± 0.007	0.009 ± 0.001
pAD1dDD	0.11 ± 0.005	0.87 ± 0.008	0.011 ± 0.001

Optimization of Reaction and Growth Conditions for Production of Theobromine

Achieving complete conversion of caffeine to theobromine and paraxanthine was an important step to facilitate downstream purification and product recovery. In order to determine the concentration of resting cells required to accomplish complete conversion of 1 mM caffeine to theobromine and paraxanthine, three different concentrations (10, 15, and 20 mg/mL) were tested. As expected, increasing the concentration of resting cells in the assay increased the rate of caffeine consumption. At a cell concentration of 10 mg/mL, 90% of the caffeine was consumed within two hours. Caffeine was completely consumed in the same period of time with 15 mg/mL whereas at 20 mg/mL, complete conversion was achieved in 90 minutes (Figure 4.2). The majority of caffeine was consumed in the first hour of the reaction, with 81, 91, and 98% of caffeine consumed by 10, 15, and 20 mg/mL resting cells, respectively. In all cases, theobromine was the major product and paraxanthine was the minor product. Considering two hours as a reasonable reaction time for complete conversion of caffeine to theobromine and paraxanthine, a resting cell concentration of 15 mg/mL was chosen for production and recovery of the products.

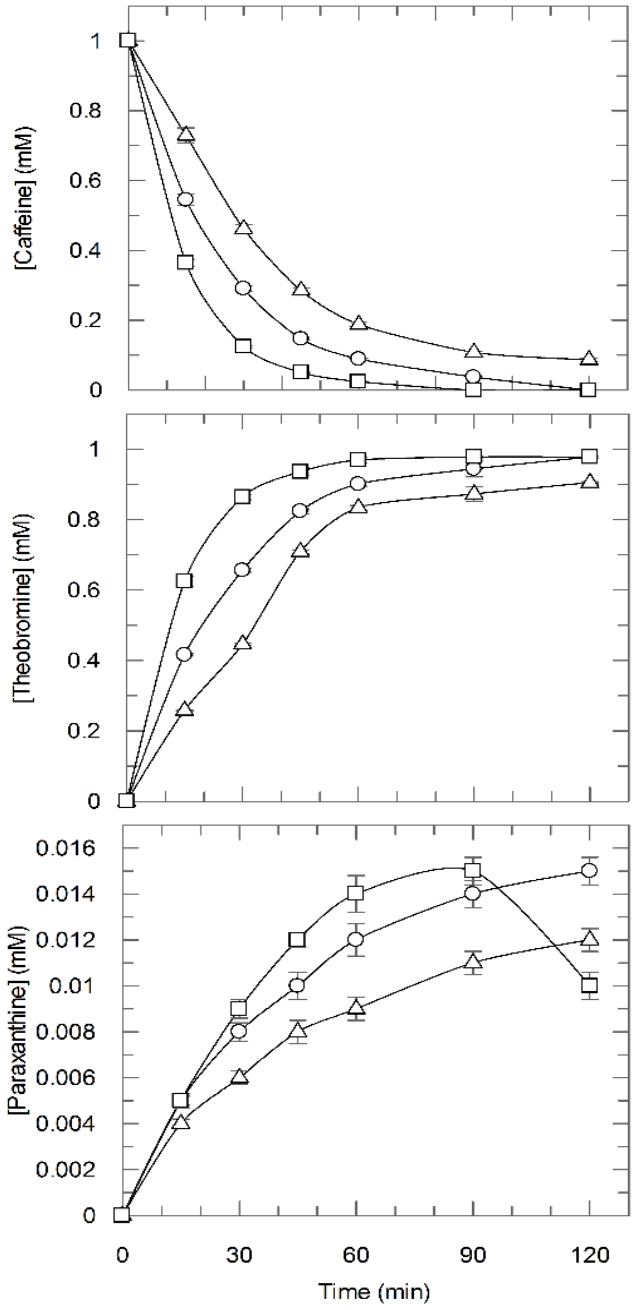


Figure 4.2 Studying complete conversion of caffeine and formation of theobromine and paraxanthine by pAD1dDD strain using different wet cells concentrations (△, 10; ○,15; □, 20 mg wet cells/mL of reaction volume). Reaction conditions are: temperature 30 C, micro plate shaker speed 400 rpm, initial caffeine concentration 1 mM. (a) Caffeine (b)Theobromine (c)Paraxanthine

We also studied the effect of three different growth media, Twice Luria Broth (2XLB), Terrific Broth (TrB), and Super Broth (SB), on the growth and productivity of theobromine using the strain pAD1dDD. These media were chosen due to the difference in their composition which is an important factor that affect cell growth and the amount of biocatalyst produced. Table 4.2 shows the wet weight of cells harvested from different growth media employed, and the substrate and products found in the 1 mL reaction mixture after two hours of reaction time. Reactions were done as described in the methods (Chapter 2) using resting cell suspensions. The 2XLB medium produced the lowest amount of cells, while the TB and SB media resulted in about 10% and 27% higher cell mass under uniform growth conditions, respectively. In terms of resting cell activity, the amount of theobromine and paraxanthine produced by the cells harvested from each medium was about the same (Table 4.2). SDS-PAGE analysis revealed that active soluble protein expression is about the same for NdmA and NdmD in the pAD1dDD strain whether it is grown in 2XLB, TrB, and SB (Figure 4.3). Although judging the soluble expression of NdmA and NdmD using SDS-PAGE is not the best criteria, growth yield and conversion of caffeine to theobromine (and paraxanthine) were the basis of selection of SB as growth media for theobromine production and recovery. In all cases, the conversion of caffeine to theobromine and paraxanthine was about 98.5% and 1.5% respectively.

Table 4.2 Comparison of growth and activity of resting cell suspension of strain pAD1dDD grown in 2XLB, TB and SB [Concentrations of caffeine, theobromine, and paraxanthine after 2 h are reported as means with standard deviations of triplicate reactions. Reaction time was 2 hours with initial concentration of caffeine at 1 mM and 15 mg/mL wet cells concentration was used in each reaction]

Media	Final OD_{600}	Final wet cell mass (g)	Concentrations (mM)		
			Caffeine	Theobromine	Paraxanthine
2XLB	6.20	0.75	0.000 ± 0.000	0.978 ± 0.003	0.013 ± 0.001
TB	7.15	0.81	0.000 ± 0.000	0.968 ± 0.005	0.014 ± 0.001
SB	8.22	0.95	0.000 ± 0.000	0.984 ± 0.026	0.014 ± 0.001

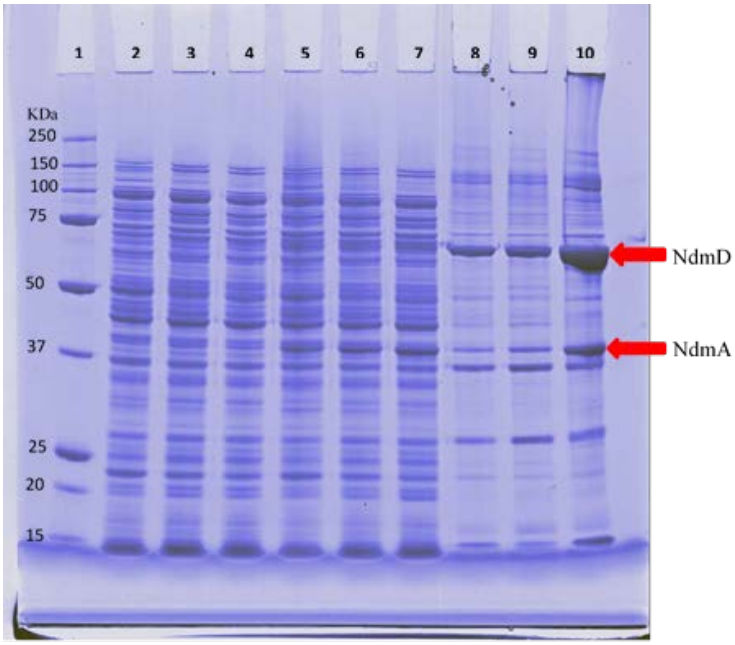


Figure 4.3 SDS-PAGE analysis of *ndmA* and *ndmD* expression in metabolically engineered strain of *E. coli* (pAD1dDD) grown in 2XLB, TrB, and SB. A total of 10 µg protein was loaded into each well. Molecular weights of markers (in kDa) are shown to the left of the gel. Red arrows indicate NdmA and NdmD protein bands. Lane 1, molecular weight standard; lane 2, 2XLB pre-induction soluble fraction; lane 3, TrB pre-induction soluble fraction; lane 4, SB pre-induction soluble fraction; lane 5, 2XLB post-induction soluble fraction; lane 6, TrB post-induction soluble fraction; lane 7, SB post-induction soluble fraction; lane 8, 2XLB post-induction insoluble fraction; lane 9, TrB post-induction insoluble fraction; lane 10, SB post-induction insoluble fraction

Attempted Methods to Increase Paraxanthine (PX) Production

The minor production of paraxanthine from caffeine observed using the different metabolically engineered strains is due to slight promiscuity of the NdmA towards the N_3-position on the caffeine molecule. Because paraxanthine is the most valuable methylxanthine (Algharrawi, Summers et al. 2015), production of this compound is very desirable. Direct *N*-demethylation of caffeine at the N_3-position to give high yield of PX does not occur appreciably with NdmB (Summers, Louie et al. 2012). We attempted several reaction techniques to increase the amount of paraxanthine to achieve at least greater than 10%. Addition of 1 mM glucose to the reaction mixture reduced the reaction time for complete conversion to 60 min, again with 1.5% yield of paraxanthine (Figure 4.4). However, the strategy of glucose addition complicates further downstream purification by introducing an additional component that must be removed. The increased reaction rate observed upon addition of glucose is most likely due to regeneration of NADH due to glucose metabolism. To further increase the amount of products produced, resting cells were also used as biocatalyst in multiple rounds, with activity decreasing over each cycle (Fig. 4.5). This recycling did not increase the inherent selectivity of NdmA for N_3-demethylation of caffeine and subsequent production of PX. Thus, under all conditions, the proportion of paraxanthine produced from caffeine was low, and constant at about 1.5%. A third method to increase the amount of paraxanthine was attempted by spiking additional caffeine into the reaction mixture periodically as noted below. After one hour, caffeine was added to the resting cell reaction mixture to a concentration of 2.7 mM, and the reaction was allowed to proceed for an additional three hours (Figure 4.6). The final concentration of caffeine was 0.3 mM, while theobromine

and paraxanthine levels were at 1.6 and 0.027 mM. Thus, caffeine spiking resulted in doubling the quantity both products, but not the molar ratio.

The amount of paraxanthine produced from caffeine by NdmA is limited by the thermodynamics of enzyme-substrate binding and orientation of the caffeine molecule in the active site (Summers, Louie et al. 2012). It is highly likely that more sophisticated directed evolution and/or isolating bacteria with a caffeine N_3-demethylase from caffeine-enriched soil would result in a favorable biological method for production of paraxanthine. Given the low yield of paraxanthine, the focus of the rest of this work was on production of theobromine.

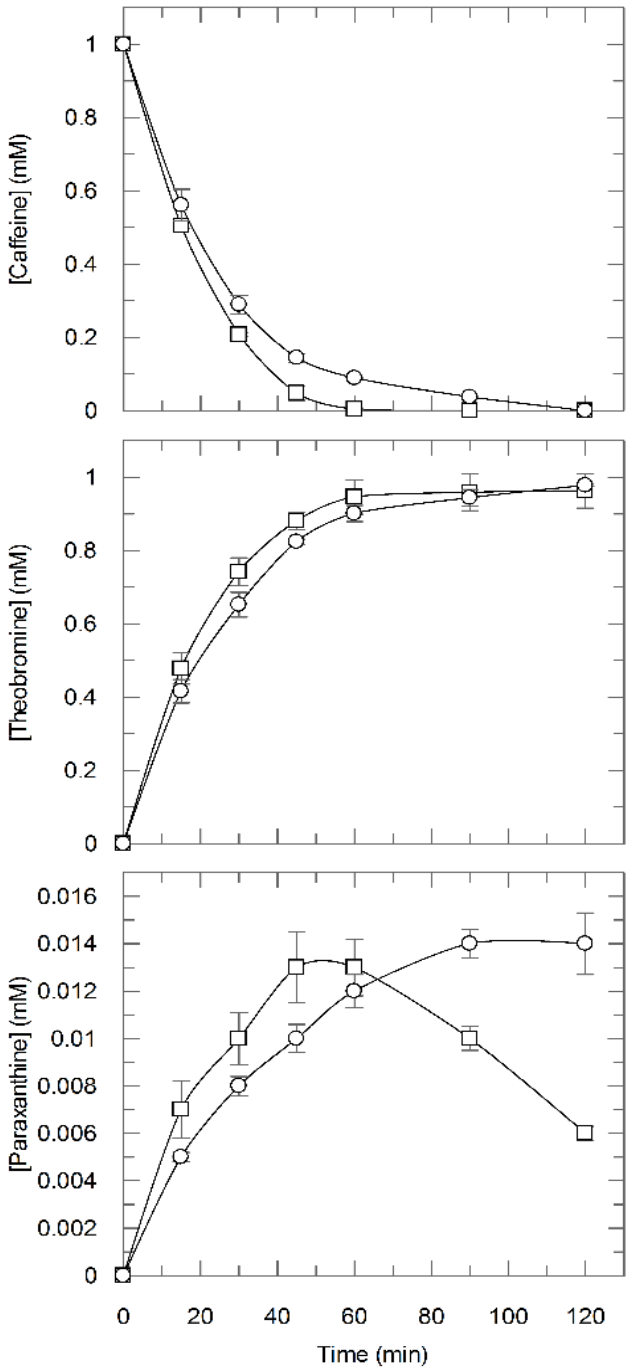


Figure 4.4 Effect of adding glucose to the conversion of caffeine to theobromine and paraxanthine (○,without glucose; □, with glucose) by resting cells of pAD1dDD (a) caffeine consumption (b)theobromine formation (c) paraxanthine formation [Reaction conditions are: wet cells concentration 15 mg/mL, initial caffeine concentration 1 mM, temperature 30 ^{o}C, microshaker speed 400 rpm]

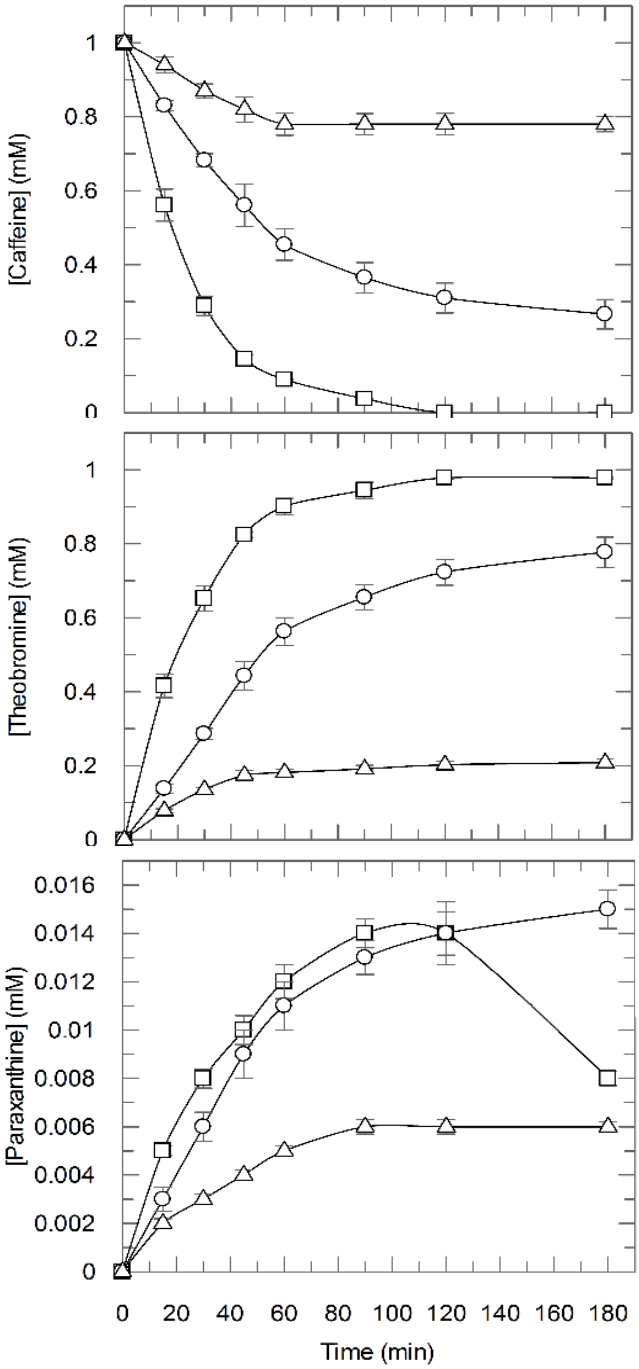


Figure 4.5 Effect of Biocatalyst reuse on caffeine consumption and theobromine and paraxanthine formation (a) caffeine consumption (b)theobromine formation (c) paraxanthine formation (□, first cycle; ○, second cycle; △, third cycle) [Reaction conditions are: wet cells concentration 15 mg/mL, initial caffeine concentration 1 mM, temperature 30 ^{o}C, microshaker speed 400 rpm]

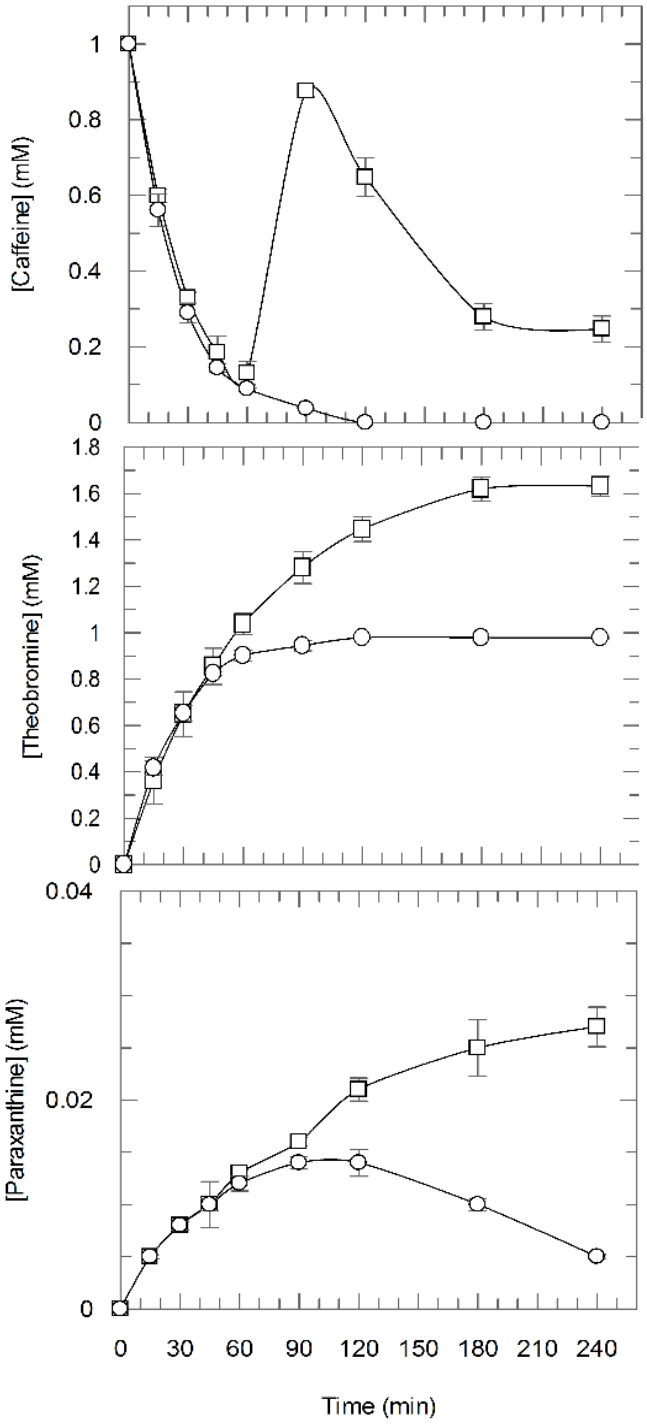


Figure 4.6 Effect of caffeine spiking on caffeine consumption and theobromine and paraxanthine formation (a) caffeine consumption (b) theobromine formation. (c) paraxanthine formation (□, with spiking; ○, no spiking) [Reaction conditions are: wet cells concentration 15 mg/mL, initial caffeine concentration 1 mM, temperature 30 ^{o}C, microshaker speed 400 rpm]

Scale-Up in Fernbach Flask, Larger Reaction and Recovery of Theobromine

The production and recovery of theobromine using strain pAD1dDD was scaled up in order to demonstrate the feasibility of the biological approach. Strain pAD1dDD was grown in SB medium in four 2.8 L Fernbach flasks (4 L growth medium total). Harvesting the cell culture after overnight growth yielded 30.2 g wet cells. This amount of cells was adequate to perform a 2 L biocatalytic reaction with the established condition of initial caffeine concentration of 1 mM and resting cell suspension of 15 mg/mL. These reaction conditions were chosen to ensure the production and recovery of about 300 mg theobromine. However, substrate concentration, consumption and product formation in the enzymatic N-demethylation reaction are proportional with the amount of biocatalyst used (Algharrawi, Summers et al. 2015).

The reaction was carried out at a temperature of 30 ^{o}C and a shaker speed of 250 rpm. Initial HPLC analysis showed that caffeine was completely consumed within two hours (Figure 4.7). Theobromine produced during that period of time was 0.983 mM (177 mg/L). The 1.95 L post-reaction supernatant, which included theobromine was then evaporated under vacuum to reduce the volume in to 1 L. Subsequently, theobromine was separated by preparative chromatography (Figure 4.8) in which theobromine retention time was 102 minutes. 1.1 L theobromine solution was collected from the preparative column in a bottle.

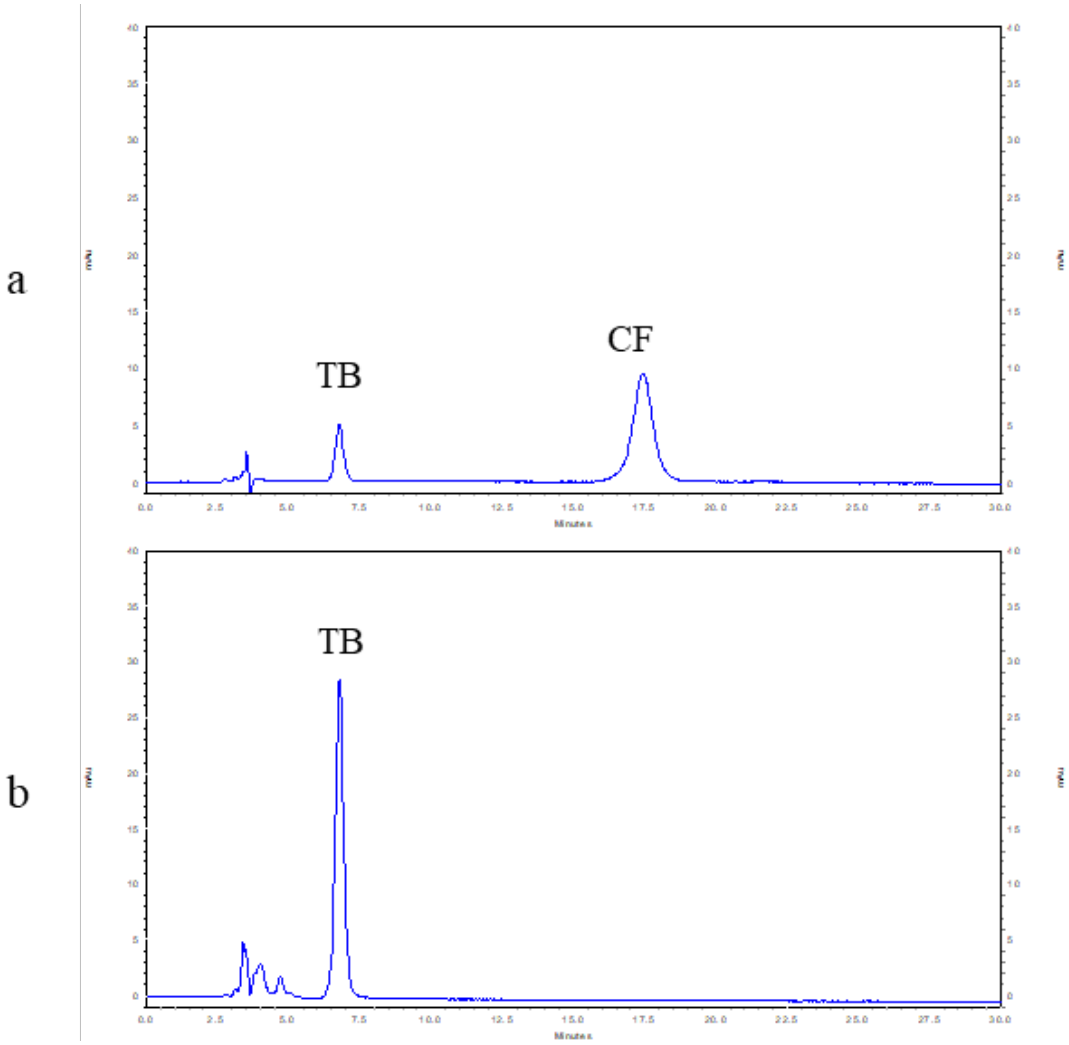


Figure 4.7 HPLC chromatograms for the 2 L reaction showing caffeine (CF) and theobromine (TB) peaks (a) after 5 min (b) after 2 hours of reaction

The 98.3% conversion of caffeine to theobromine in the 2 L reaction resulted in production of 354 mg of theobromine. However, 10% of the post-reaction mixture was used for the optimization of product separation *via* preparative chromatography. As a result, the amount of theobromine loaded to the column for purification and recovery was 318 mg. Upon drying, 255 mg pure theobromine powder was obtained. This indicates that theobromine yield after separation and purification based on the amount of theobromine injected into the column was 80%. To the best of our knowledge, this is the first report that describes the highest conversion (98.5%) of caffeine to theobromine catalyzed by metabolically engineered *E. coli*, with a final recovery yield of 80%. Thus, the total theoretical molar yield of theobromine from caffeine though the reaction and recovery processes is about 79%.

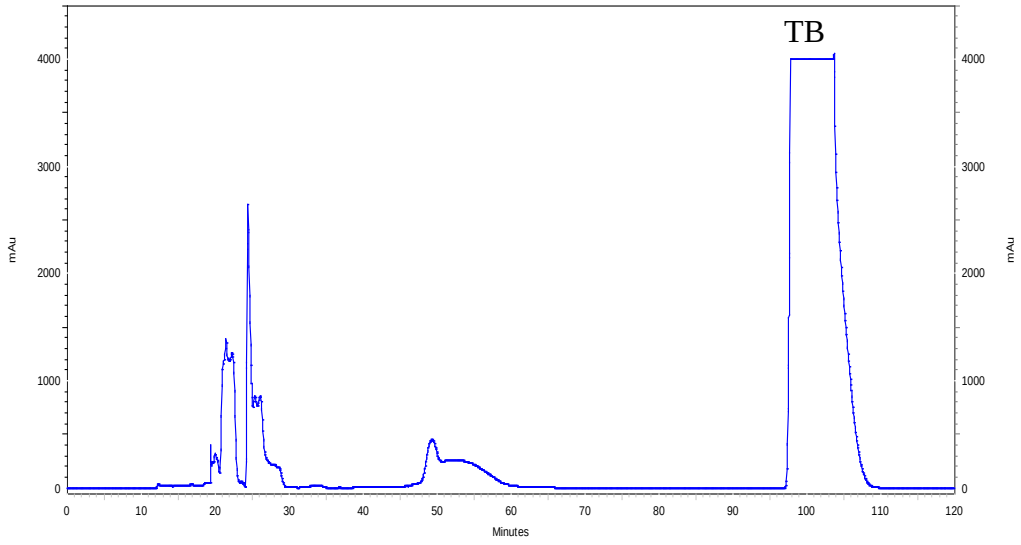


Figure 4.8 Separation of theobromine (TB) by preparative chromatography. Retention time of theobromine is 102 minutes [Injection volume 25 mL, mobile phase: methanol-water-acetic acid (5:95:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 21.2 mm x 250 mm]

In a recent attempt to produce theobromine from caffeine, Retnadhas *et. al.* investigated the use of metal ions, such as Co^{2+}, Cu^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}, to accumulate theobromine from caffeine degradation by inhibiting N_3-demethylation of theobromine in induced cells of *Pseudomonas sp.* (Retnadhas and Gummadi 2014). They found that by adding Co^{2+}, theobromine accumulated up to 1.08 ± 0.10 g/L from 3.2 g/L caffeine, which resulted in a 33.75 ± 3% molar conversion in eight hours. The molar conversion we report in this study is three times higher (98.5%) and achieved in two hours without the addition of metal ions to the reaction mixture. The 1 mM Co^{2+} or other metal ions added to the fermentor to stop further degradation of theobromine, could pose problems in terms of recovery of the product without contaminating metal ions. Also, disposal of metal waste may be an issue. The bacteria used by Retnadhas *et. al.* (Retnadhas and Gummadi 2014) (*Pseudomonas sp.*) is a wild type strain with very little information on the gene(s) involved. In the present study, a metabolically engineered *E. coli* strain with well characterized genes and with appropriate gene-copy numbers was used in order to achieve a rapid growth and high yield of theobromine. Finally, study by Retnadhas *et. al.* (Retnadhas and Gummadi 2014) did not include separation or purification of theobromine; hence final yield after recovery and purity of the product is not documented. Asano *et al.* reported a 92% yield of theobromine over 48 h from 25 g/L caffeine using the wild-type *P. putida* No. 352 with 1 mM Zn^{2+} to inhibit the further degradation of theobromine (Asano, Komeda et al. 1993). Again, the reported yield represents in the reaction tank; recovery process is not described, and the final yield and purity of theobromine has not been reported.

Analytical Characterization of Biologically Produced Theobromine

Analytical HPLC was used to assess the purity of the biologically produced theobromine powder by comparing with an authentic standard. Both samples eluted at the same retention time from the analytical HPLC column. The High-Resolution LC-MS spectrum of the authentic and biologically produced theobromine was identical. ESI positive mode was used to record the LC/MS results, and the M+1 ion peak at 181.072 m/z was recognized for both authentic standard and biologically produced theobromine (Figure 4.9).

The ^{1}H NMR spectrum for each of the authentic standard and the biologically produced theobromine also matched very well (Figure 4.10). A Bruker 500 MHz spectrophotomer with a DMSO-d_6 as solvent was used to record the ^{1}H NMR spectrum. The authentic theobromine standard showed presence of a peak at δ 11.098 (s, 1H) corresponding to –NH proton, and peaks at δ 7.973 and 3.847 corresponding to –C=H (s, 1H) and –CH_3 group (s, 3H). The biologically produced theobromine also showed a peak at δ 11.097 (s, 1H) corresponding to –NH proton, and peaks at δ 7.973 and 3.848 corresponding to –C=H (s, 1H) and –CH_3 group (s, 3H).

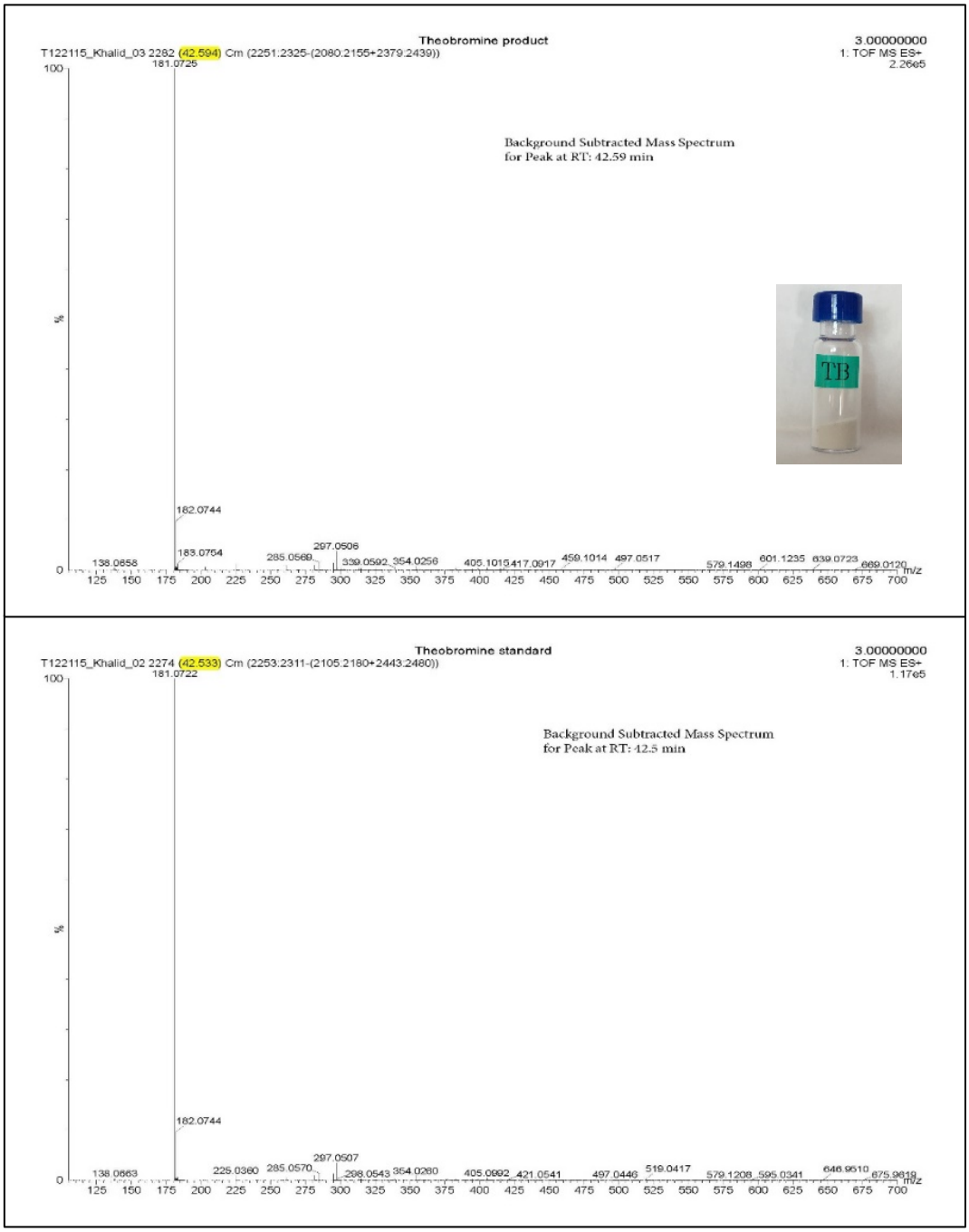


Figure 4.9 LC-MS spectrum of theobromine (TB). (a) LC-MS of biologically produced and purified throbromine sample produced (in set) in this work. (b) LC-MS of threobromine standard obtained from Sigma Aldrich

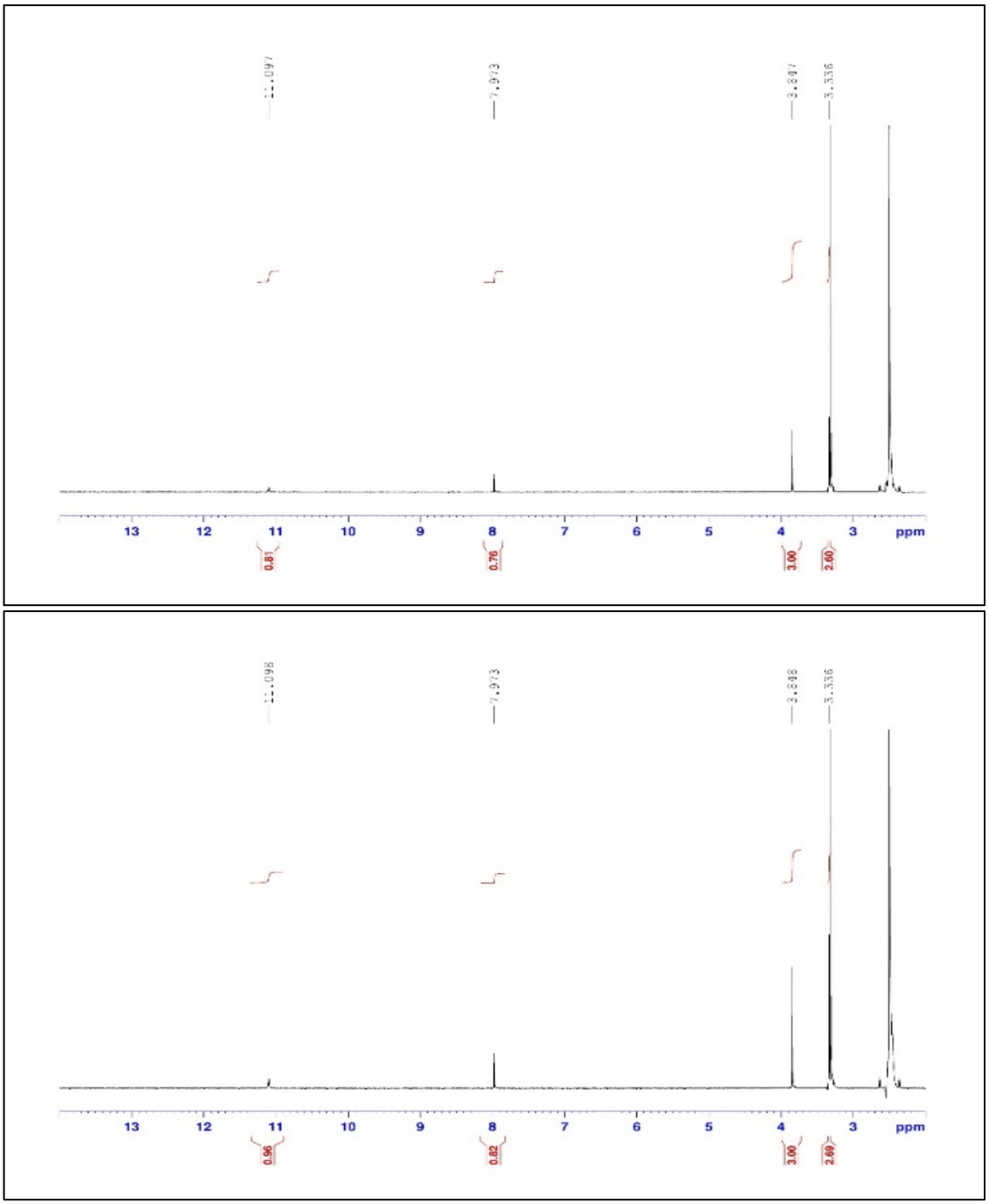


Figure 4.10 NMR of theobromine (TB) (a) NMR of biologically produced and purified theobromine produced in this work. (b) NMR of theobromine standard obtained from Sigma Aldrich

Production of Theobromine from Post-Brew Coffee Waste

Caffeine is a major pharmacological substance consumed by the population. It is found in many common drinks and beverages such as coffee, tea, and coke. Coffee is one of the most consumed beverages around the world. The world production of coffee in 2016/2017 was 156.6 million 60 kg bags (USDA 2017). In 2014, the annual coffee consumption in United States was 23.8 million (60 kg bag), which is 4.5 kg per capita (ICO 2017). Coffee pulp represents 40% of the wet processed coffee, and it is rich in many compounds such as caffeine, carbohydrates, proteins, and minerals (Bressani 1979). The waste-water produce from coffee industry has high concentrations of pollutants such as caffeine and tannins, which results in disposal problems. This work attempts to demonstrate metabolically engineered *E. coli* to convert caffeine in post-brew coffee waste to theobromine. Conceivably, other forms of caffeine-waste could also be used to produce not only theobromine, but also other methylxanthines of higher value.

The aim of this work was to determine if post-brew coffee waste can be used as a feedstock for the production of theobromine. This included the following steps:

1. Determine the amount of caffeine in coffee waste.
2. Achieve complete conversion of caffeine to theobromine (by pAD1dDD strain).
3. Scale up cell growth and reaction
4. Separate and purify theobromine.

Determination the Amount of Caffeine in Post-Brew Coffee Waste

One kilogram of wet coffee waste was collected from Starbucks in Iowa City. It was dried in the oven for 6 hours at 120 ^{o}C. Subsequently, 300 g of the dried coffee was added to 3 L distilled water (DI) and heated at 100 ^{o}C for 15 minutes. The solution was filtered; final volume after heating and filtration was 1500 mL. The pH of the coffee extract was 7.46; hence pH adjustment was not necessary for theobromine production. Concentration of caffeine, as determined by HPLC was 2.8 mM (Figure 4.11) with the amount of caffeine in the extracted solution being 816 mg. Thus, the amount of caffeine in coffee waste was 2.72 mg caffeine/g dried coffee waste (816 mg caffeine per 300 g dried coffee waste [or 0.27%]).

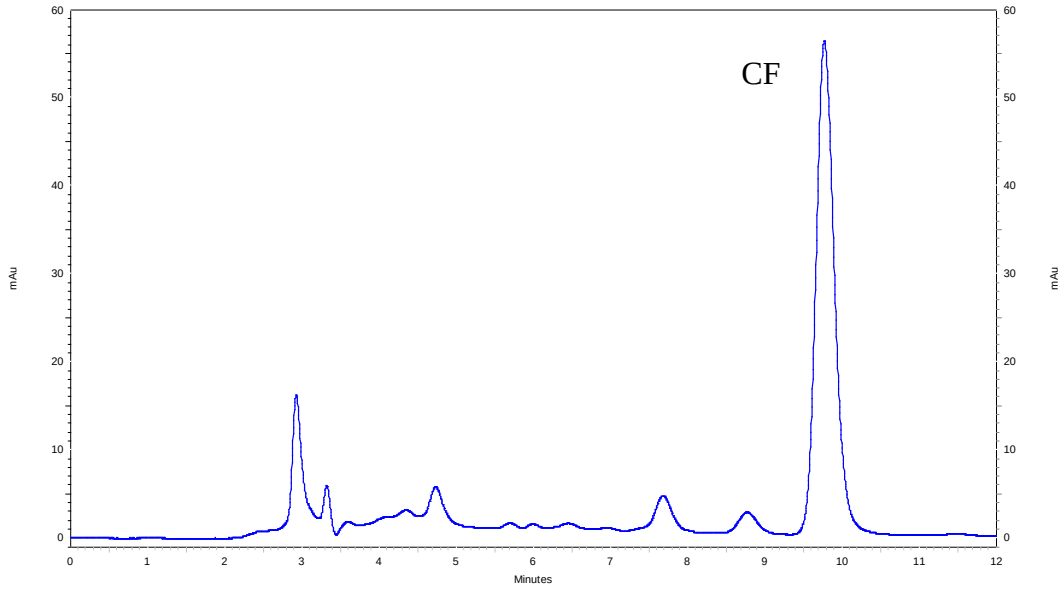


Figure 4.11 Chromatogram shown caffeine (CF) peak in coffee waste [Injection volume 1 µL, mobile phase: methanol-water-acetic acid (20:80:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 4.6 mm x 125 mm (5 µm)]

Complete Conversion of Caffeine in Post-Brew Coffee Waste by pAD1dDD Strain

Initial step was to determine the wet weight of resting cells required to accomplish the complete conversion of 1 mM caffeine in post-brew coffee waste. Three different resting cell concentrations were tested for conversion of caffeine in the waste (15, 20, and 25 mg wet cells/mL). The reaction volume in each case was 1 mL and the reaction conditions were 30 ^{o}C and 400 rpm micro-plate shaker speed. Caffeine conversion was 77, 89, and 100 % by resting cells concentrations of 15, 20, 25 mg/mL respectively (Figure 4.12). However, when a resting cells concentration of 15 mg/ mL was used, about 70% of caffeine presented in the reaction was consumed rapidly in the first thirty minutes, then caffeine consumption became slower and did not change much in the last hour of the reaction. Same behavior was observed with resting cells concentrations of 20 and 25 mg/mL, however, with 20 mg/mL conversion of caffeine exceeded 80% in the first half hour then remain stable at about 85% while 100% caffeine conversion was achieved in thirty minutes when the resting cells concentration was 25 mg/mL (Figure 4.12). This means that the activity of the cell suspension was high during the first thirty minutes, after which it significantly dropped. Similarly, theobromine production was high in the first half hour of the reaction time then it became slow. After thirty minutes, TB concentration reached 0.55, 0.67, and 0.95 mM by 15, 20, and 25 mg/mL resting cells concentration respectively, and theobromine concentrations at the end of the reaction reached to 0.76, 0.87, and 0.98 mM respectively.

By comparison, 100% caffeine conversion to theobromine in post-brew coffee waste was achieved by 25 mg/mL resting cells (about 67% more) vs. 15 mg/mL to achieve complete conversion of pure caffeine to TB. This suggests that some of the

constituents present in the coffee waste might be partially inhibiting the enzymatic N-demethylation activity. Thus, 25 mg/mL of resting cells concentration was used in the mention volume reaction to produce ~ 150 mg theobromine.

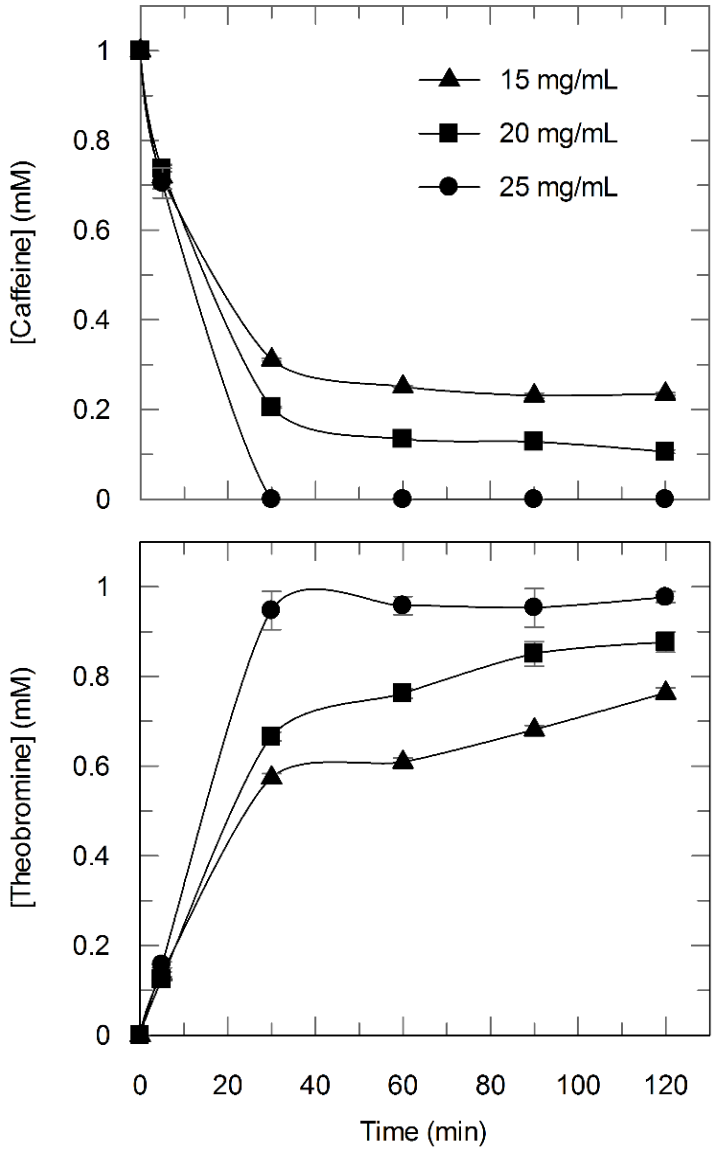


Figure 4.12 Caffeine consumption and theobromine formation by different concentrations of resting cells in post-brew coffee waste (▲, 15; ■, 20; ●, 25 mg/mL) [Reaction conditions are: initial caffeine concentration 1 mM, temperature 30 ^{o}C, microshaker speed 400 rpm]

Scale-Up of Post-Brew Coffee Waste Extract for Production of Theobromine Using Resting Cells of pAD1dDD

Four 2.8 L flasks, each containing 1 L Super Broth medium was used to grow the cells overnight for 14-16 hours. Amount of cells obtained was 29 g wet weight. This was adequate, based on the preliminary experiment above, to carry out a reaction of 1.16 L of post-brew coffee waste containing 1 mM caffeine and with 25 mg/mL of resting cells of pAD1dDD strain. The reaction conditions were as before, 30 ^{o}C and 250 rpm. At the end of the reaction (Figure 4.13) all caffeine in the waste solution was consumed with concomitant production of theobromine (0.97 mM; 175 mg/L). Thus, the theoretical amount of theobromine produced from the coffee waste was 203 mg. For recovery of the product, solids were removed by centrifugation and the clear supernatant was further filtered by 0.2 µm filter for HPLC separation. The resulting micro-filtered solution (1.06 L) was analyzed by HPLC. Theobromine peak in the supernatant eluted at 4.6 min as shown in Figure 4.14. which was identical of that of authentic standard (Figure 4.16 b).

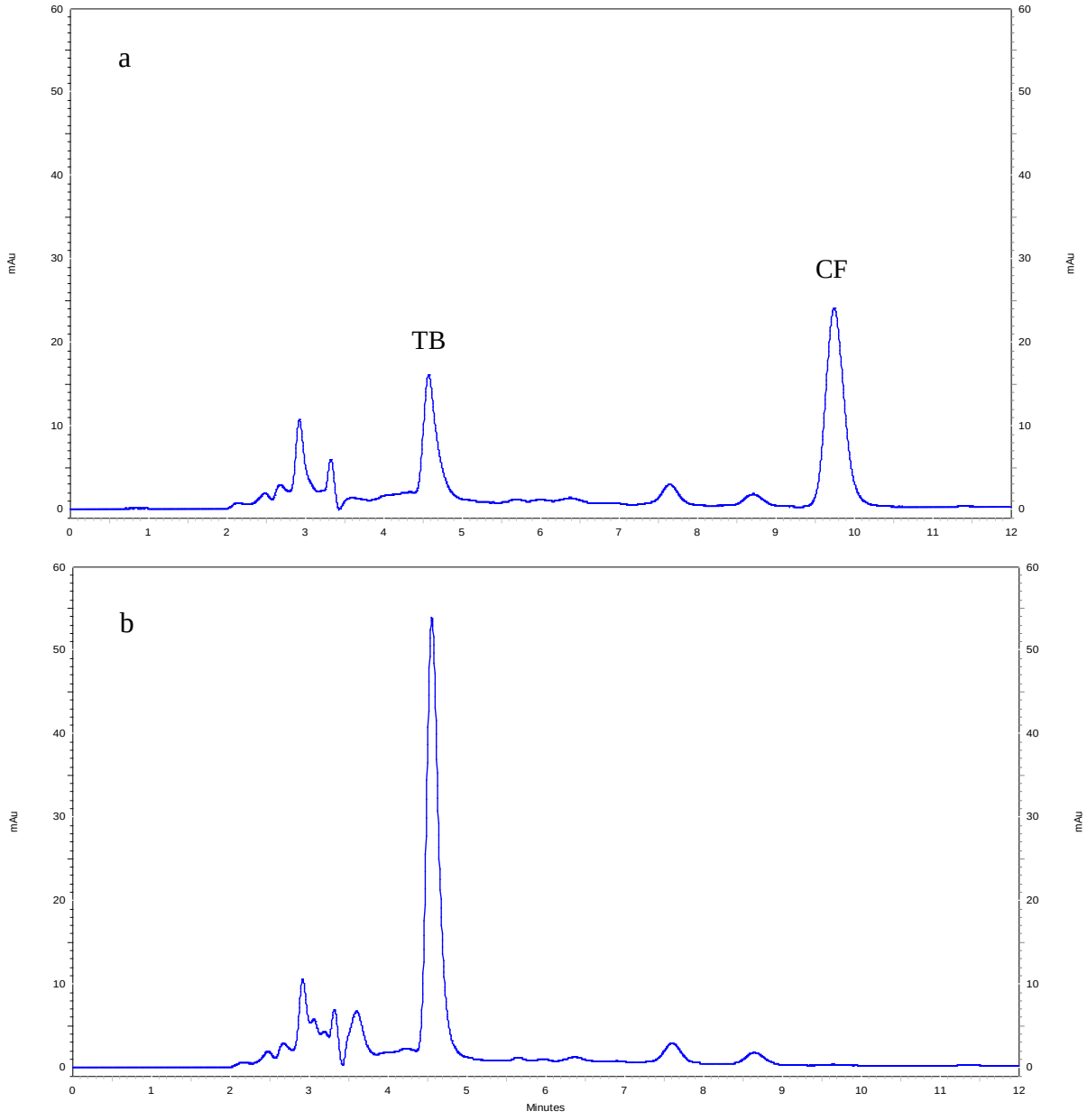


Figure 4.13 HPLC chromatograms shown theobromine (TB) and caffeine (CF) peaks: (a) After (a) 5 minutes and (b) 60 minutes of the beginning of the reaction [Injection volume 1 µL, mobile phase: methanol-water-acetic acid (20:80:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 4.6 mm x 125 mm (5 µm)]

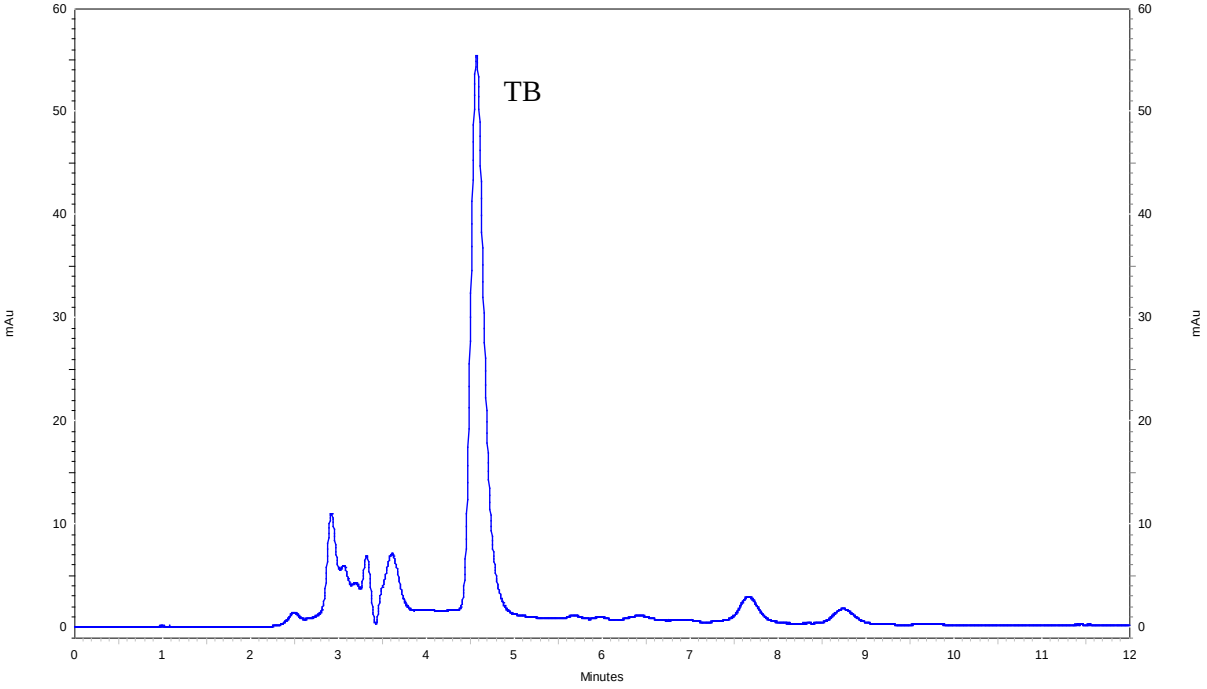


Figure 4.14 HPLC chromatogram, for the reaction supernatant after removing the cells and filtration, shown theobromine peak [Injection volume 1 µL, mobile phase: methanol-water-acetic acid (20:80:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 4.6 mm x 125 mm (5 µm)]

Separation and Purification Theobromine

The 1.06 L was injected in to the preparative chromatography column and theobromine peaks were collected. Theobromine retention time was 142 minutes as shown in the chromatogram in Figure 4.15. The total amount of theobromine solution collected was 1.2 L, which was then concentrated to 300 mL by evaporation at 60-70 ^{o}C under vacuum. The concentrated theobromine solution was dried at 140 ^{o} C in the oven. Theobromine powder was then scraped, collected and quantitated. The amount of

theobromine recovered after purification was 178 mg. Based on the on caffeine fed to the reaction (1 mM caffeine in 1.16 L reaction volume), theobromine total yield was 79%.

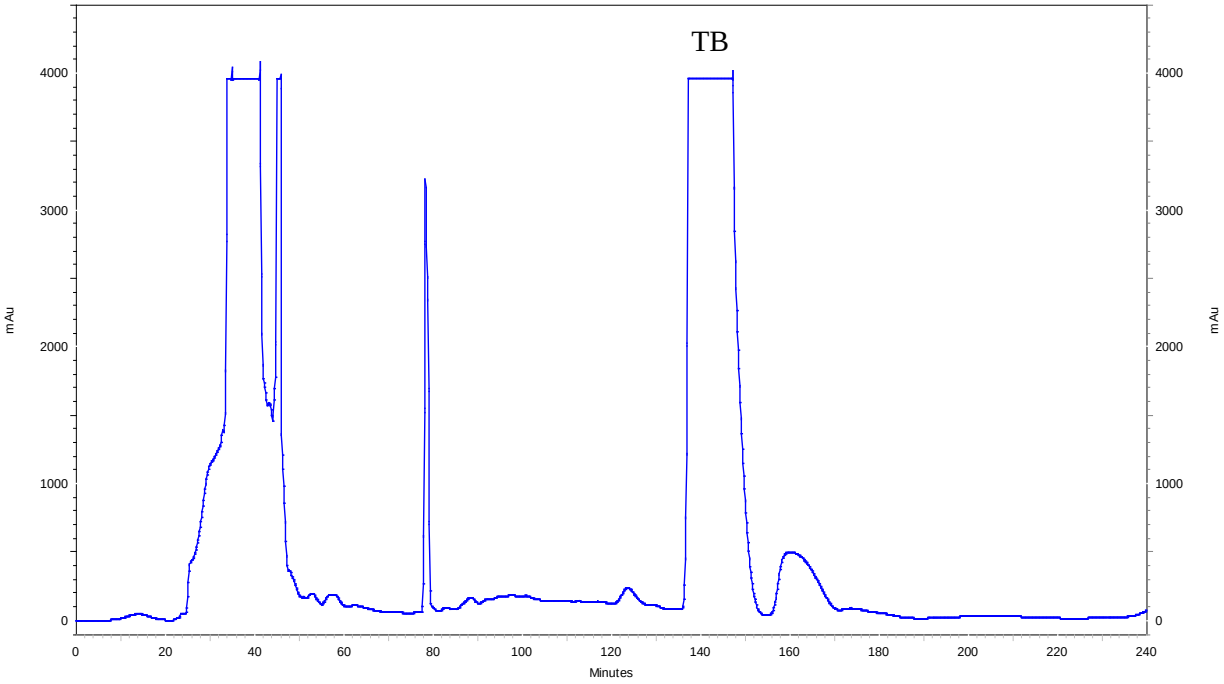


Figure 4.15 Separation of theobromine (TB) by preparative chromatography. Retention time of theobromine is 142 minutes [Injection volume 25 mL, mobile phase: methanol-water-acetic acid (5:95:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 21.2 mm x 250 mm (5 µm)]

Testing Theobromine Purity by HPLC

To ensure Theobromine purity, the biologically produced TB powder was used to prepare 1 mM solution. Then, 1 µL of that solution was injected in to the HPLC, and compared with the authentic standard from Sigma Aldrich. The biologically produced theobromine eluted at the same retention time (4.6 min.) as authentic theobromine

(Figure 4.16). Therefore, theobromine produced from post-brew coffee waste is highly pure. No further characterization of theobromine has been done.

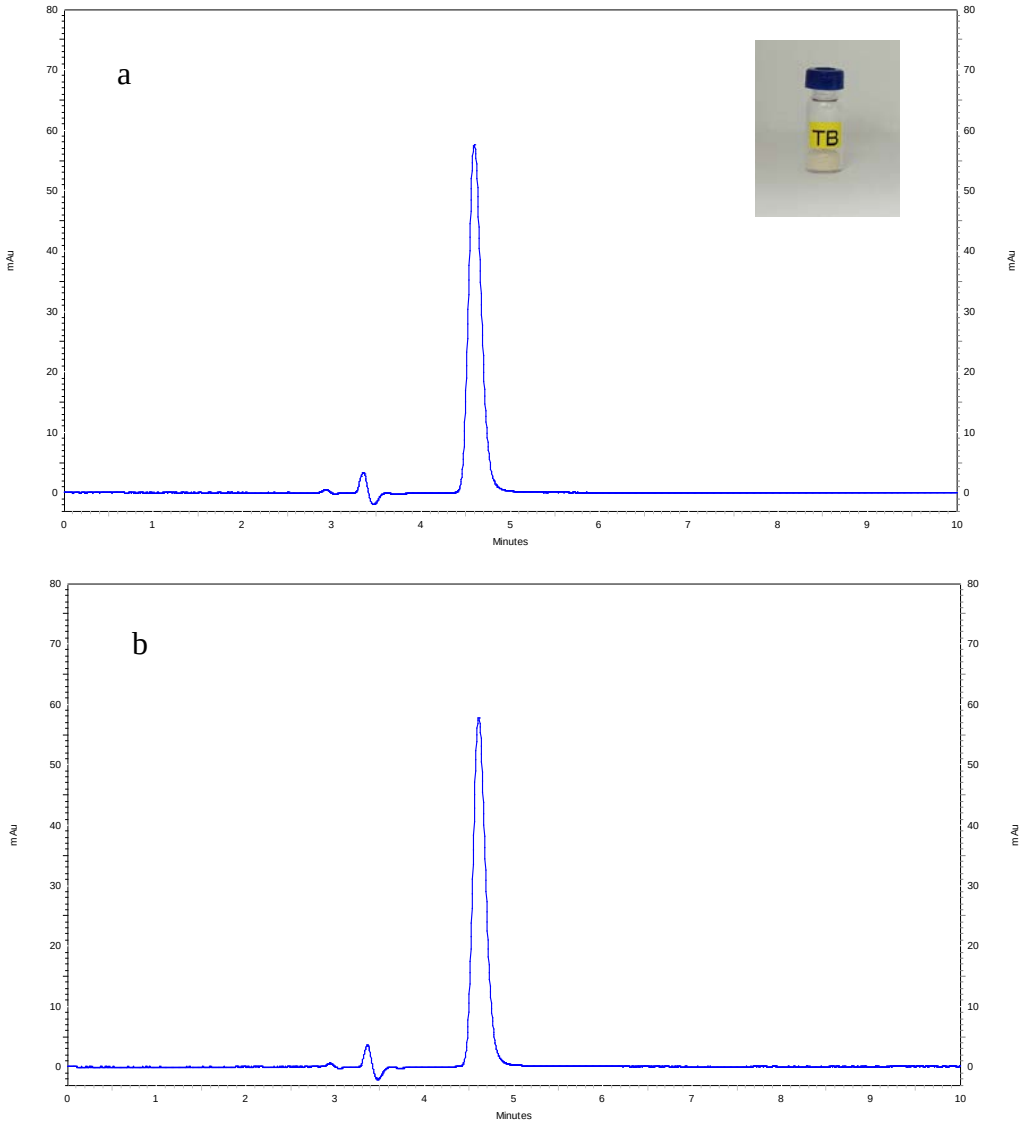


Figure 4.16 HPLC chromatograms for 1 mM theobromine (TB) (a) biologically produced in this work and (b) standard from Sigma Aldrich [Injection volume 1 µL, mobile phase: 0.5 mL/min: methanol-water-acetic acid (20:80:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 4.6 mm x 125 mm]

This work demonstrates that coffee-waste like post-brew coffee cake can be used as a feed stock for production of methylxanthines using metabolically engineered *E. Coli*. The activity of NdmA and D used to produce theobromine was only minimally compromised by other components in the waste. The final product was as authentic as the one produced from pure caffeine and commercial standard. This work demonstrates the flexibility of metabolically engineered cells in terms of feedstock use, pure economical starting chemical like caffeine or coffee-rich waste.

Acknowledgements

The author of this thesis would like to thank USP Labs. for their partial support of PX project. This compound was of interest to the company as a nutraceutical.

CHAPTER 5: PRODUCTION OF 7-METHYLXANTHINE USING METABOLICALLY ENGINEERED *E. COLI*

Introduction

7-Methylxanthine (7MX) which has a methyl group attached to N_7 of the xanthine ring has been proven to have a therapeutic effect on the development of form-deprivation myopia in pigmented rabbits (Nie, Huo et al. 2012). Trier et. al., studied the biochemical and ultrastructural changes in rabbit sclera after treatment with 7-MX (Trier, Olsen et al. 1999). Similar study was also conducted on guinea pigs (Cui, Trier et al. 2011). In another study, Trier et. al., found that 7-methylxanthine reduces eye elongation and myopia progression in childhood myopia (Trier, Ribel-Madsen et al. 2008). Although not validated by any publication, nutraceutical interest on this molecule has been expressed by at least two companies. 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 (Traube 1900) or purine synthesis by Fischer (Fischer 1924). Additionally, there is no recorded research on the bio-catalytic 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. VB Medicare has specifically contacted Professor Subramanian about biocatalytic production of 7MX. This company has already signed a research license agreement with The University of Iowa Research Foundation (UIRF) for the two patent applications filed for the production of 7 MX (Subramanian, Louie et al. 2015, Subramanian 2016)

This work aims to use *E. coli* engineered with NdmA and NdmD genes to directly produce 7-methylxanthine from theobromine in one single reaction (Figure 5.1). The N_3-demethylation reaction 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 O_2 per methyl group removed, resulting in the production of formaldehyde and water (Summers, Mohanty et al. 2015).

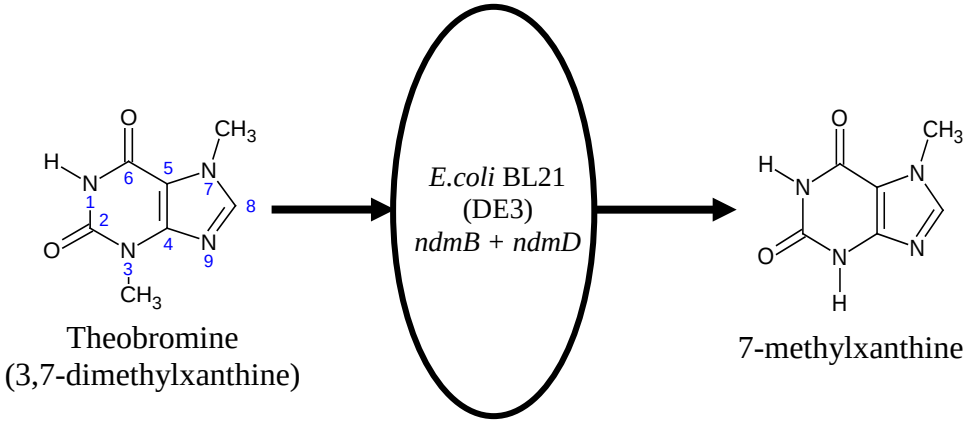


Figure 5.1 Schematic representation for the biocatalytic N-demethylation of theobromine to 7-methylxanthine by *E. coli* BL21(DE3) genetically engineered with N-demethylation genes of *Pseudomonas putida* CBB5 *ndmB* and *ndmB*

This work is the first report on the biocatalytic production of 7MX (This work has not been published due to licensing agreement with VB Medicare Pvt. Ltd). 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.

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 1.6 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 (Figure 1.10).

7-MX screening of the above strains were carried out in 1 mL reactions at 30 ^{o}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 (Figure 5.2). 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 (dDD), 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 (Figure 5.2).

Each of the above three strains were tested for N-demethylation of theobromine to 7-methylxantine 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 (Figure 5.2). 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. SDS-PAGE of strain pBDdDB (Figure 5.3) revealed a clear band for both NdmD and B in the soluble fraction, but the insoluble fraction did not show any band, which means most the expressed enzymes by pBDdDB strain ended up in the soluble active fraction. Consequently, pBDdDB strain was able to consume all caffeine (100%) within the shortest time (90 minutes) and therefore this strain was chosen to produce 7MX

Table 5.1 Estimated copy number of *ndmA* and *ndmD* genes in strains used in this study

Strain	Approximate gene copy number*		*ndmD*:*ndmA* ratio
	ndmA	ndmD	
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 $Ci=NijPij$, 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)

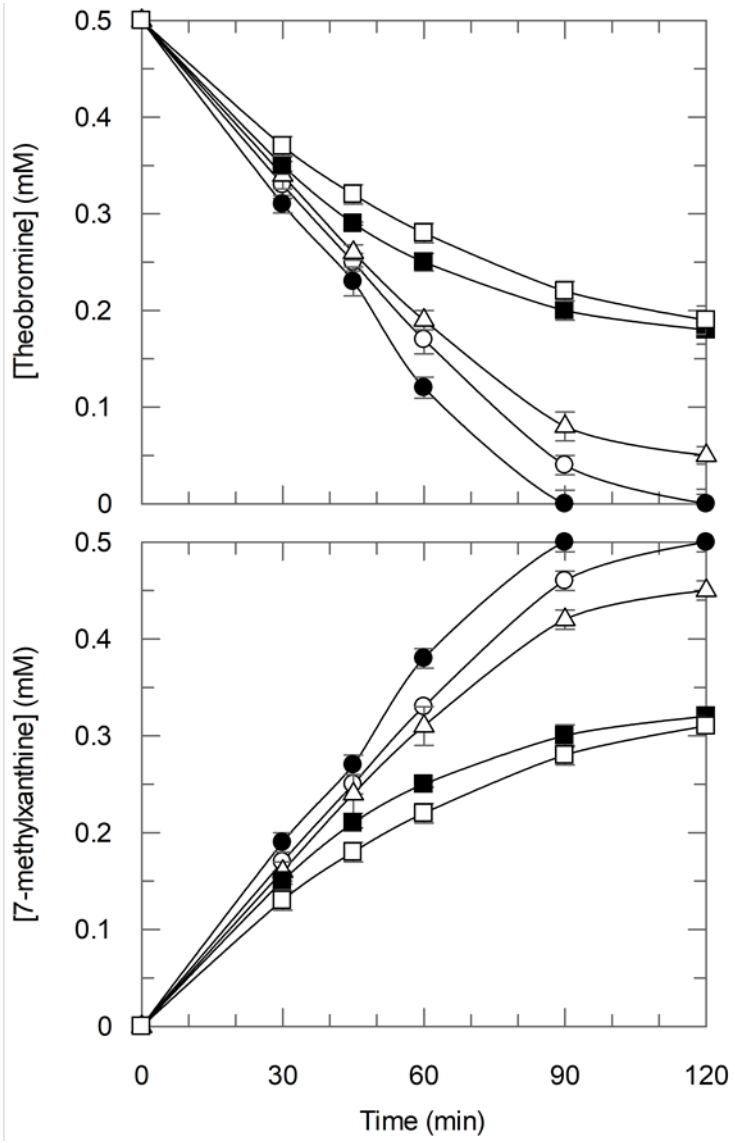


Figure 5.2 (a) Consumption of theobromine and (b) formation of 7-methylxanthine by metabolically engineered *E. coli* resting cells (□, strain pBD ; ■, strain dDB; △, strain pBDdBB; ○, pBDdDD; ●, strain pBDdDD) [initial theobromine concentration 0.5 mM, wet cells weight 5 mg/mL, temperature 30 ºC, microplate shaking 400 rpm]

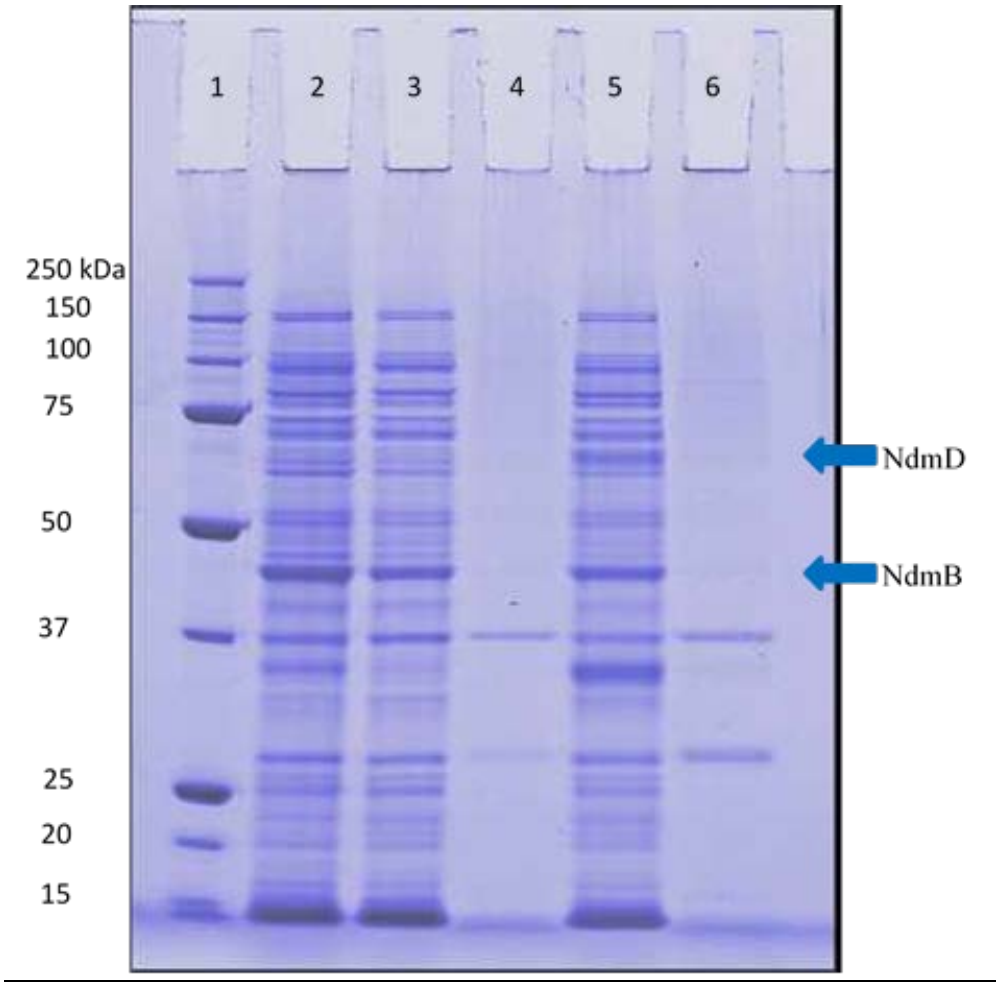


Figure 5.3 SDS-PAGE analysis of *ndmB* and *ndmD* expression in metabolically engineered strains of *E. coli* pBD2dDB strain. A total of 10 µg protein was loaded into each well. Molecular weights of markers (in kDa) are shown to the left of the gel. Blue arrows indicate NdmB and NdmD protein bands. Lane 1, molecular weight standard; lane 2, *E. coli* BL21(DE3) soluble fraction (negative control); lane 3, pre-induction soluble fraction; lane 4, pre-induction insoluble fraction; lane 5, post-induction soluble fraction; lane 6, post-induction insoluble fraction

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 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. Figure 5.4 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 5.2). Therefore, the highest activity was achieved by at 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.

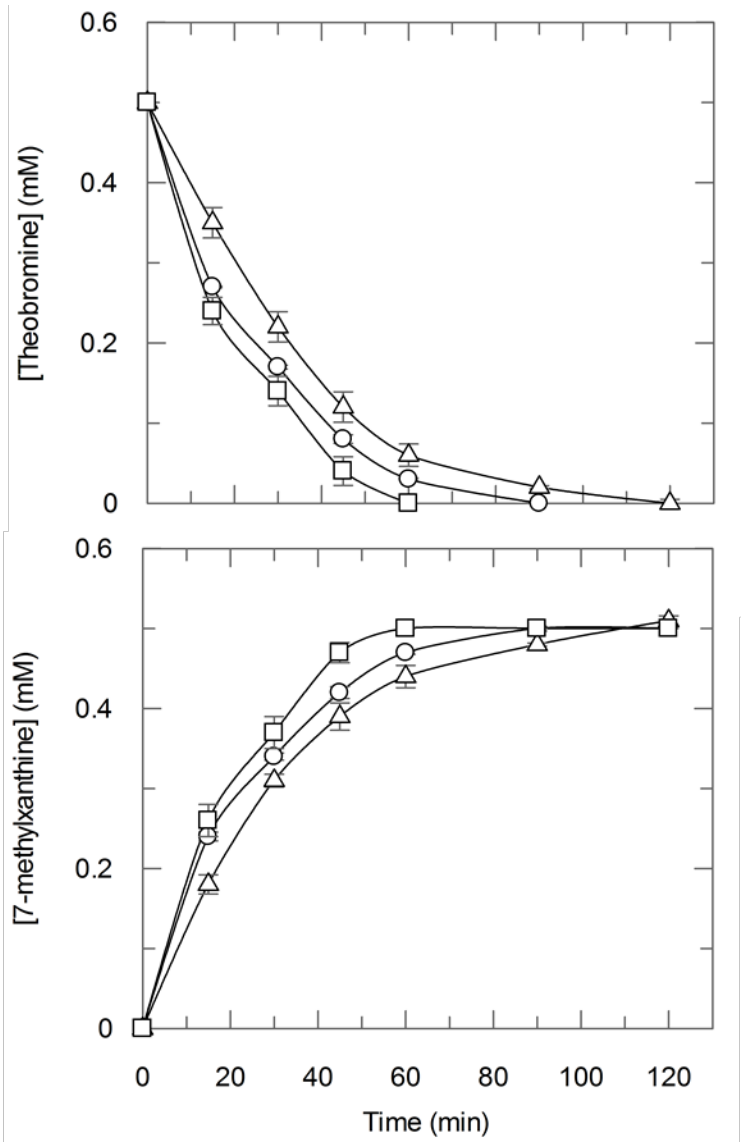


Figure 5.4 Theobromine consumption and 7-methylxanthine formation by different concentrations of metabolically engineered *E. coli pBDdDB* strain (△, 5 mg/mL; ○, 10 mg/mL; □, 15 mg/mL) [initial theobromine concentration 0.5 mM, temperature 30 C, microplate shaking 400 rpm]

Table 5.2 Cell activities at different cells concentrations (pBDdDB strain)

Wet cell concentration (mg/mL)	Time (min)	7-methylxanthine (mmole/mL)	Cells Activity (mmole 7MX/mg cells.min) * 107
5	120	0.0005	8.3
10	90	0.0005	5.6
15	60	0.0005	5.6

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 (OD_{600}) 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 was 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. Also, the amount of wet cells produced by pBDdDB strain in SB is higher than the amount of cells produced by pDdA strain (0.9 g,

Chapter 3) and pADdDD strain (0.95 g, Chapter 4). Strain pBDdDB had highest growth in SB medium. The activity of the cells harvested from each medium were also tested. Figure 5.5 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 'healther cells' in the rich SB, Thus, Super Broth medium (SB) was considered as the growth medium for further work.

Figure 5.6 shows the N-demethylation reaction of 0.5 mM theobromine to 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.

a

a

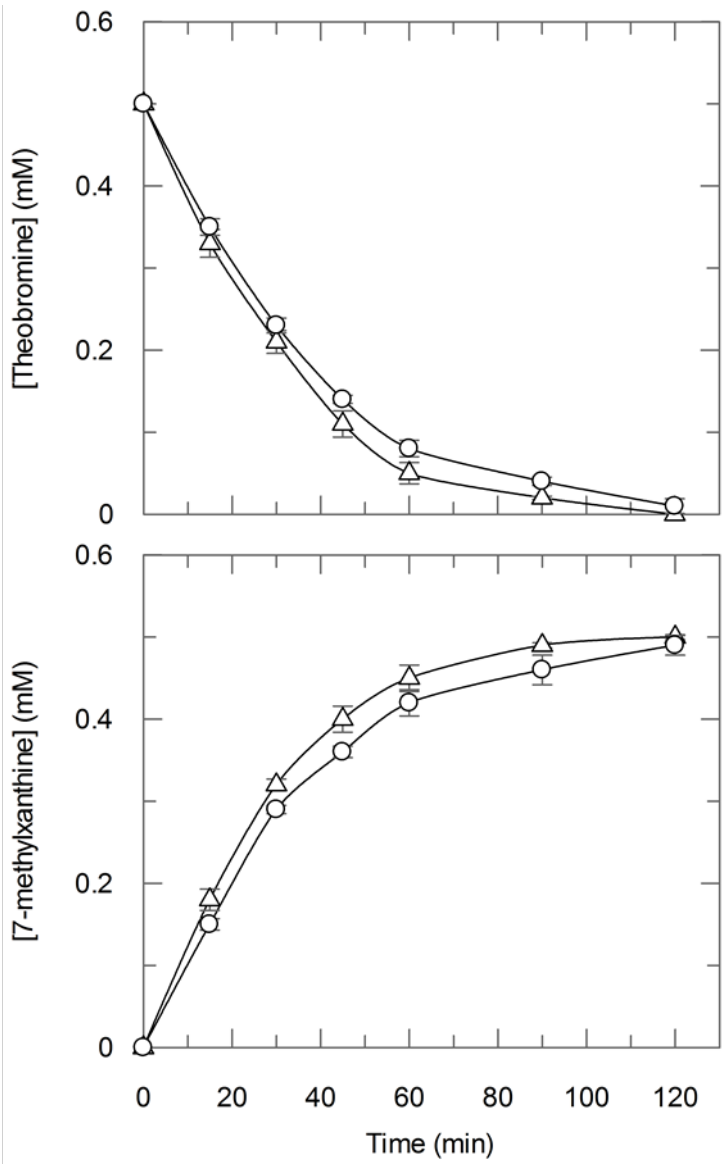


Figure 5.5 Theobromine consumption and 7-methylxanthine formation by *coli* pBDdDB strain grown in Lauria Broth (△) and Super Broth (○) [initial theobromine concentration 0.5 mM, temperature 30 ^{o}C, microplate shaking 400 rpm]

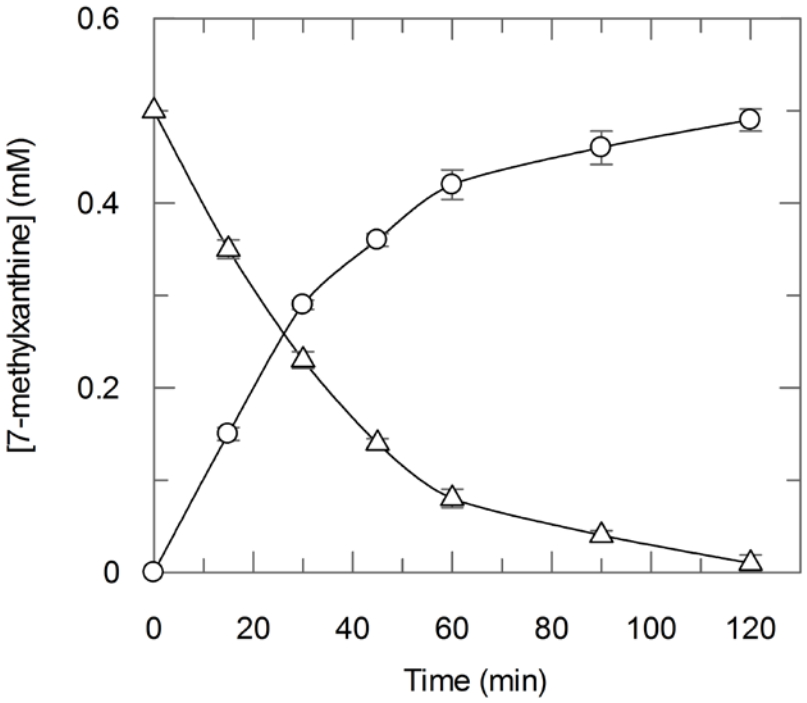


Figure 5.6 Theobromine consumption (△) and 7-methylxanthine production (○) by 5 mg/mL *E. coli* pBDdDB strain grown in Super Broth [initial theobromine concentration 0.5 mM, temperature 30 C, microplate shaking 400 rpm]

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 pDBdDB overnight (14-16 hr). When the cells were harvested, the optical density at 600 nm (OD_{600}) 9.26. The wet cells were stored at 4 ^{o}C 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 ^{o}C, and shaker speed of 250 rpm. After two hours of reaction, all theobromine was converted to 7-MX (100% conversion). Figure 5.7 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.

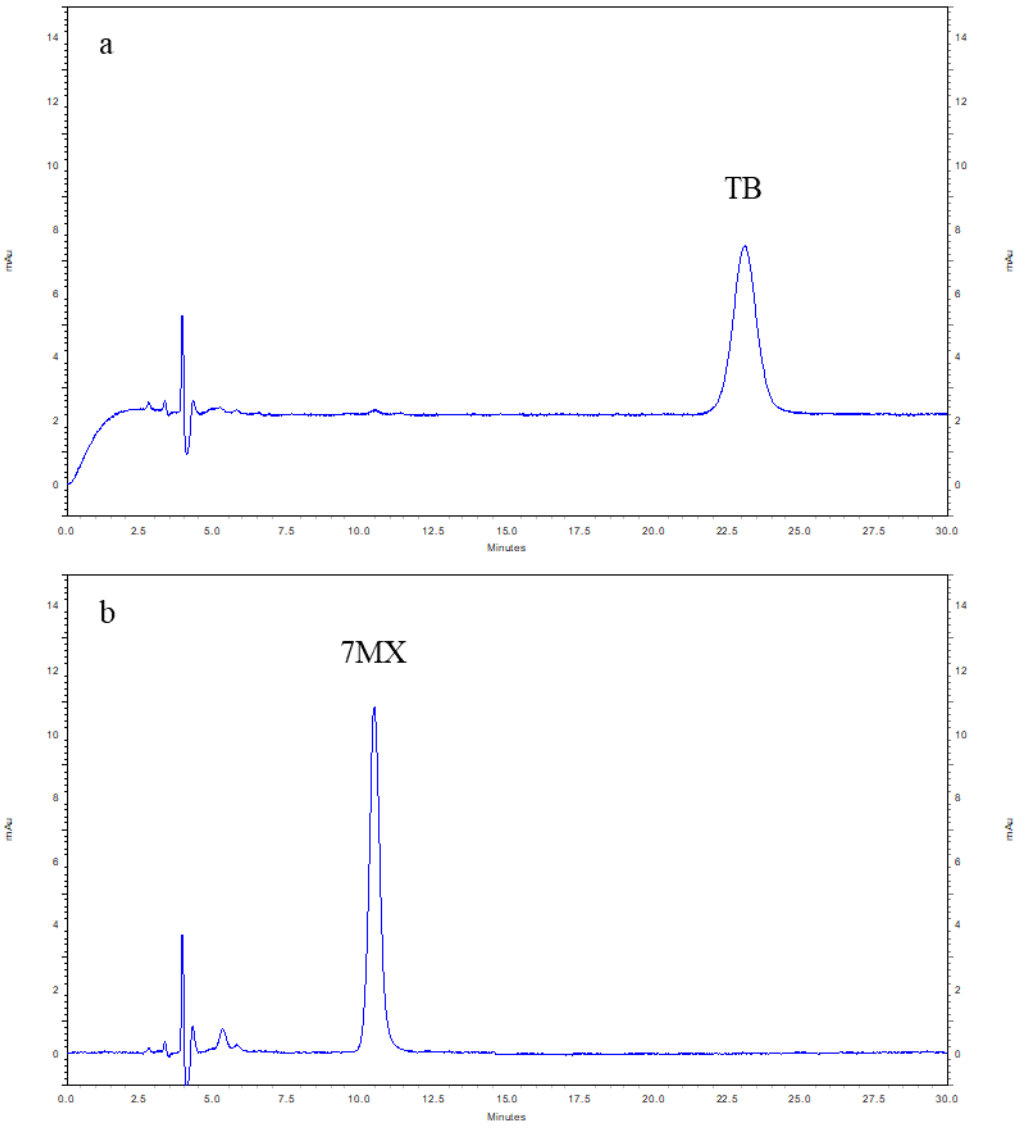


Figure 5.7 HPLC chromatograms for the 1.96 reaction at (a) 0 hour and (b) 2 hours [Injection volume 1 µL, mobile phase: methanol-water-acetic acid (5:95:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 21.2 mm x 250 mm]

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. Same procedure, previously described for methyl xanthine separation (Chapter 2), was used to separate 7MX by injecting 25 mL supernatant volume to the column. 7MX eluted at a retention time of 104 minute as it is shown in Figure 5.8. 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 ^{o}C for four hours to ensure removal of methanol (B.P. 65 ^{o}C), water (B.P. 100 ^{o}C), and acetic acid (B.P. 118 ^{o}C). The resultant 7MX powder in the tray was collected, weighed (127 mg) and stored in a vial (Figure 5.9).

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.

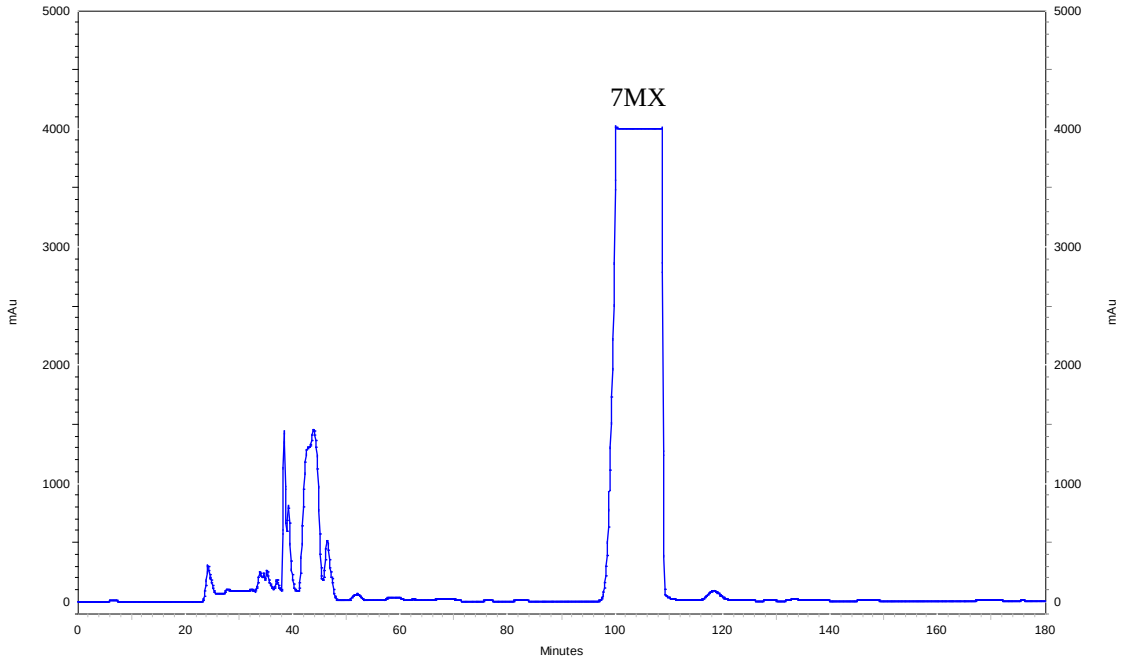


Figure 5.8 Separation of 7-methylxanthine (7MX)by preparative chromatography. Retention time of 7MX is 102 minutes [Injection volume 25 mL, mobile phase: methanol-water-acetic acid (5:95:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 21.2 mm x 250 mm]

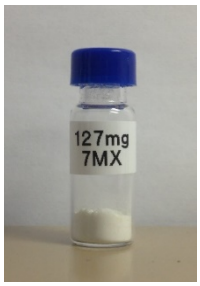


Figure 5.9 127 mg biologically produced 7-methylxanthine powder from theobromine

Analytical Characterization of 7-methylxanthine

The purity of 7MX was initially confirmed by analytical HPLC using appropriate authentic standards. The retention time of the biologically produced product (Figure 5.10) and authentic standards were identical. The High-Resolution LC-MS spectrum of biologically produced and standard 7MX were identical (Figure 5.11). 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. The ^{1}H NMR spectrum of biologically produced and standard 7-methyl xanthine also matched very well (Figure 5.12). ^{1}H NMR was recorded on a Bruker 500 MHz spectrophotomer using DMSO-d_6 as solvent. Standard 7-methylxantine showed presence of peaks at δ 11.46 (s, 1H) and 10.82 (s, 1H) corresponding to –NH proton, and peaks at δ 7.87 and 3.81 corresponding to –C=H (s, 1H) and –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 –NH proton, and peaks at δ 7.87 and 3.81 corresponding to –C=H (s, 1H) and –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.

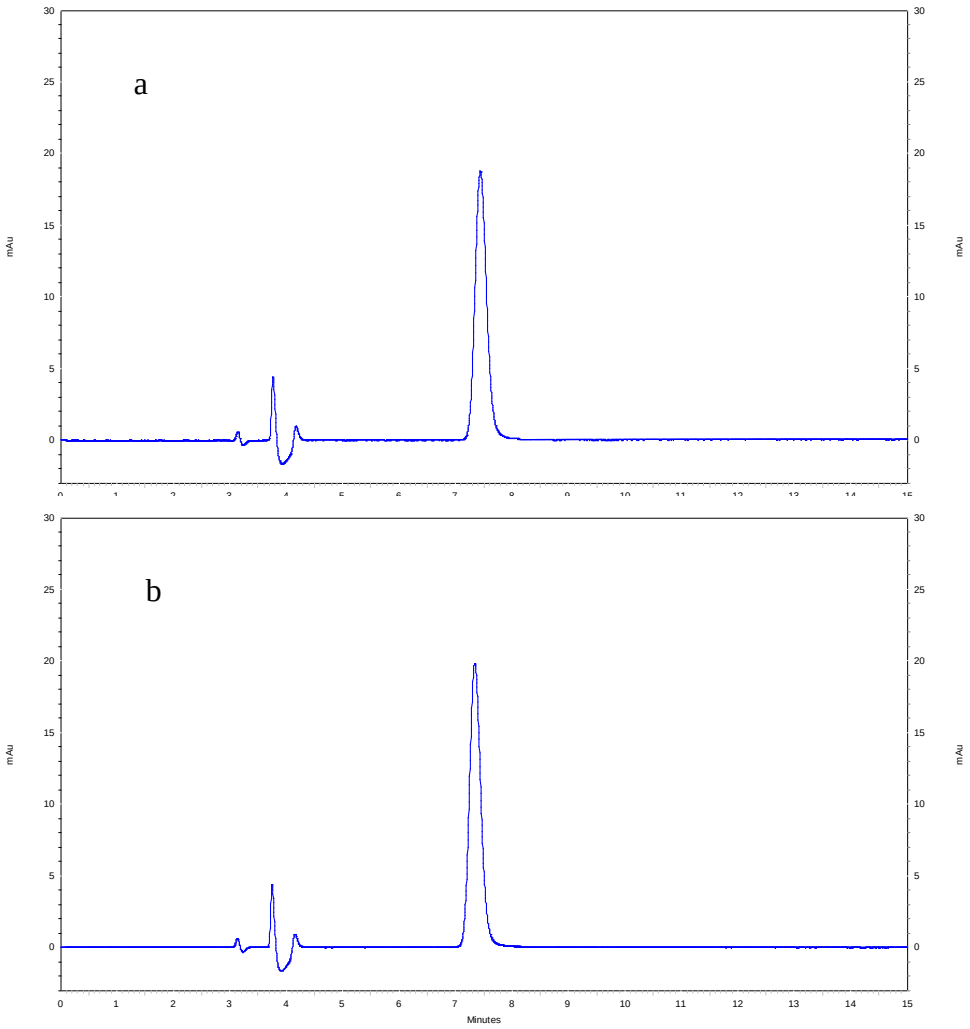


Figure 5.10 HPLC chromatograms for 0.5 mM 7-methylxanthine (7MX) (a) biologically produced in this work (b) standard from Sigma Aldrich [Injection volume 1 µL, mobile phase: 0.5 mL/min: methanol-water-acetic acid (5:95:0.5, vol/vol/vol), stationary phase: Hypersil BDS C18 column of 4.6 mm x 125 mm]

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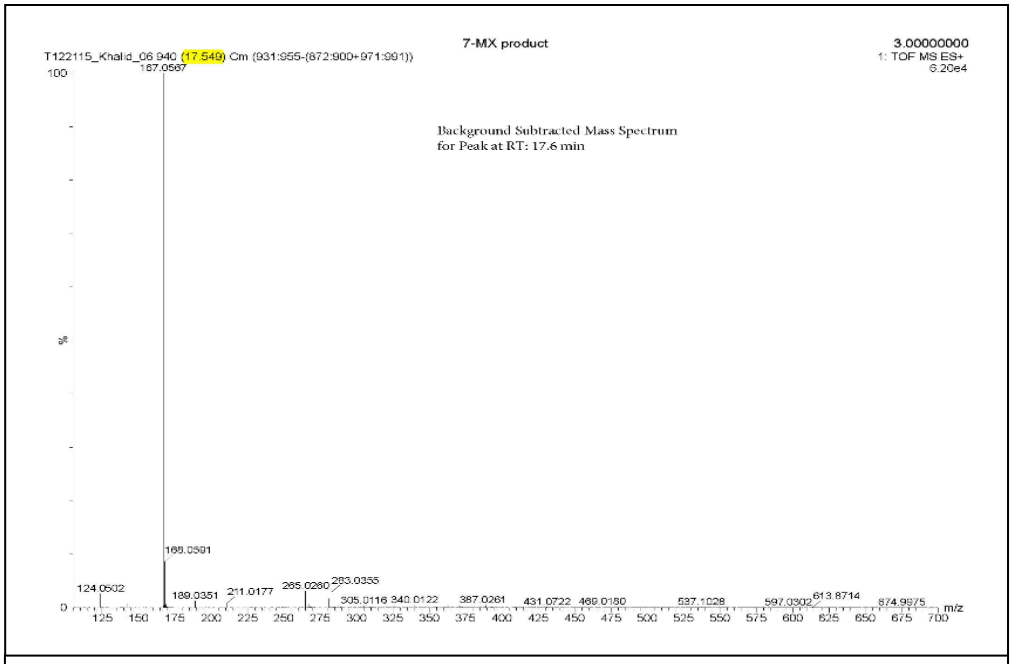

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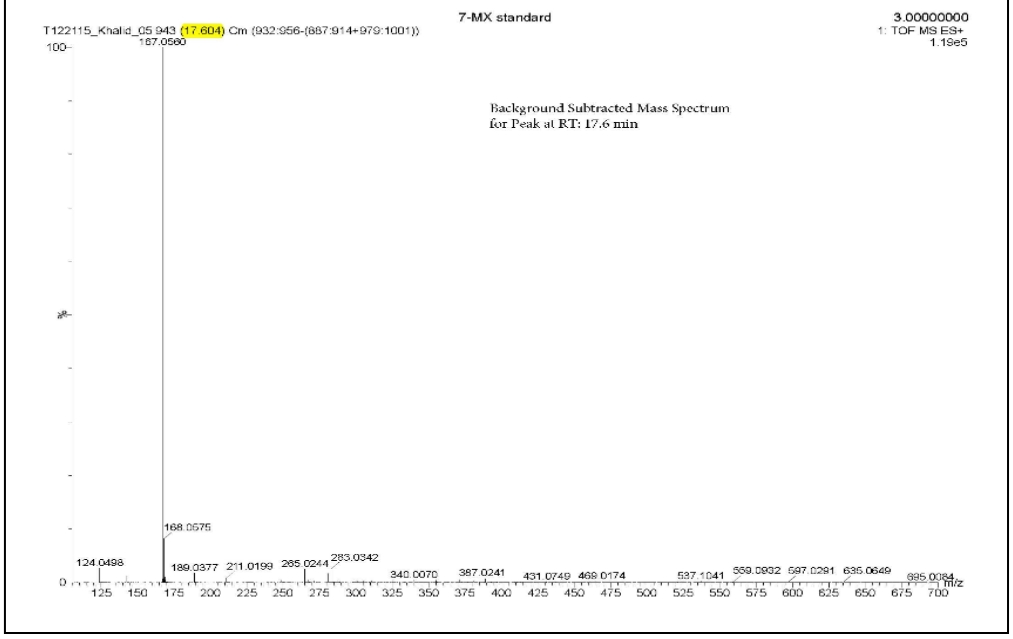
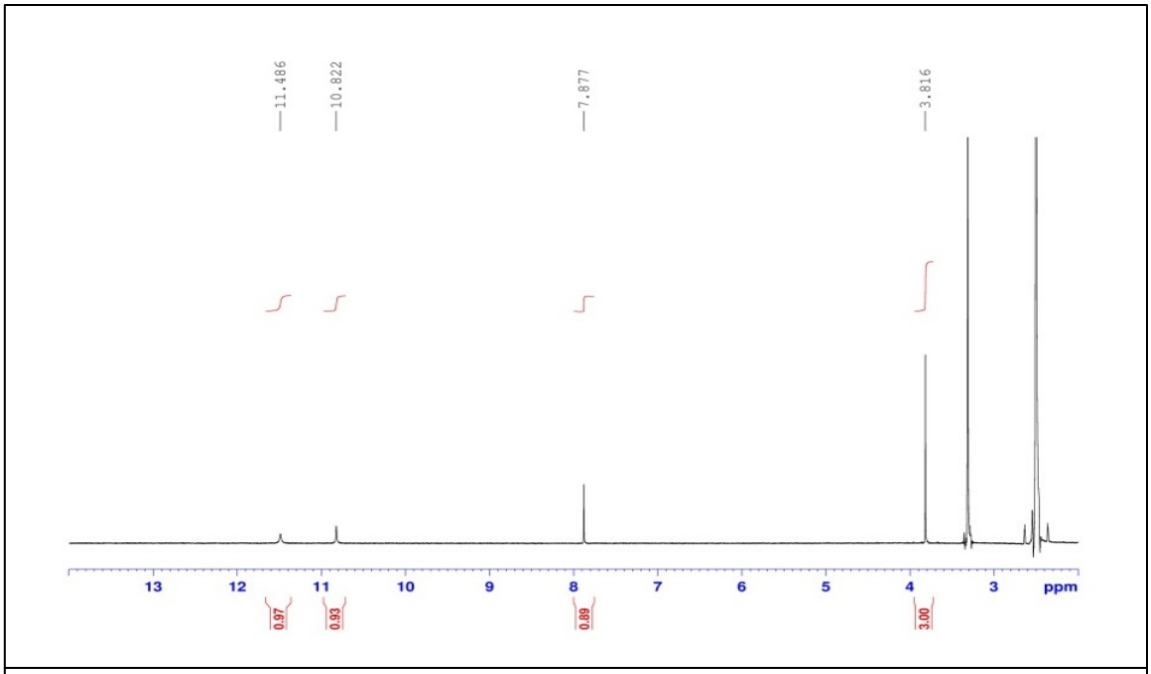


Figure 5.11 LC-MS spectrum of 7-methylxanthine (7MX). (a) LC-MS of biologically produced and purified 7-methylxanthine sample produced in this work. (b) LC-MS of 7-methylxanthine standard obtained from Sigma Aldrich

a



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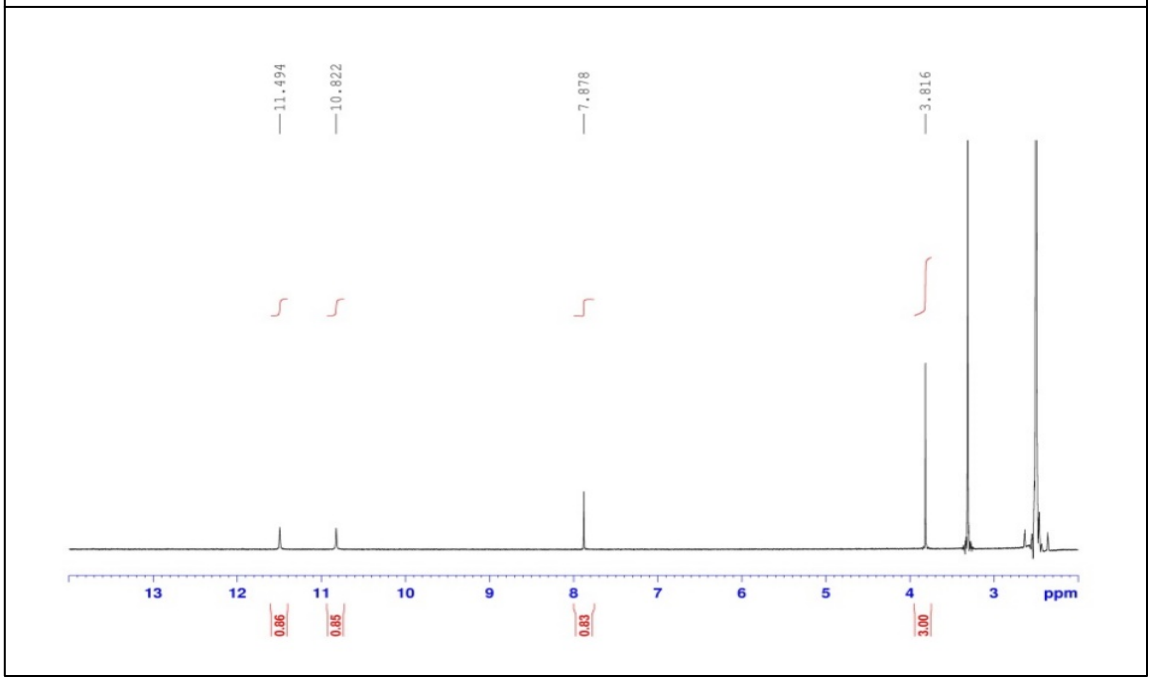

Figure 5.12 NMR of 7-methylxanthine (7MX) (a) NMR of biologically produced and purified 7-methylxanthine produced in this work. (b) NMR of 7-methylxanthine standard obtained from Sigma Aldrich

Addendum

Subsequent to completion of this work, VB Medicare requested additional work on production of 7-MX from caffeine. Based on information provided by the company, caffeine is a more reliable feedstock with favorable cost than theobromine. For conversion of caffeine to 7-MX, three genes, i.e., *ndmA*, *B*, and *D* are required. As filed in the disclosure (UIRF #2016-145), the required strains were constructed by Dr. Sujit Mohanty and tested by Khalid Algharrawi to confirm caffeine to 7-MX conversion. The strain was sent to VB Medicare for further in-house validation and scale-up. The details of this work have been filed in the Invention Disclosure to the University of Iowa Research Foundation (URIF#2016-145). For confidentiality reasons based on the licensing agreement between UIRF and VB Medicare, this work, funded by VB Medicare, has not been included.

Acknowledgements

The author of this thesis would like to thank VB Medicare Private Limited for their partial support of 7MX work. This compound is of interest to the company as a pharmaceutical and nutraceutical.

CHAPTER 6: SUMMARY OF COMPLETED RESEARCH AND SUGGESTIONS FOR FUTURE WORK

Common Biocatalytic Platform for the Production of Methylxanthine by Metabolically Engineered *E. coli*

A novel biocatalytic platform for the production of methylxanthines from economic feedstocks has been developed; bench scale production of three different methylxanthines has been demonstrated. The biocatalyst *E. coli* BL21(DE3) engineered with ndmA/D or ndmB/D genes combinations enabled specific N-demethylation at N_1 and N_3- of caffeine, theophylline and theobromine to produce theobromine & paraxanthine, 3-methylxanthine and 7-methylxanthine respectively. This production common platform consists of uniform fermentation conditions with a specific strain, uniform induction of specific enzymes for methylxanthine production, uniform recovery and preparation of biocatalyst for reaction and uniform recovery of pure products (Figure 6.1). The market value of products is significantly higher than the feedstocks; hence there is commercial interest in licensing of the metabolically engineered strains for production of specific products. The biocatalytic process used in this work operates at ambient temperature and pressure and is environmentally friendly. In contrast, chemical synthesis of methylxanthines uses several chemicals, multiple reactions, high temperature, and releases environmentally unfriendly waste.

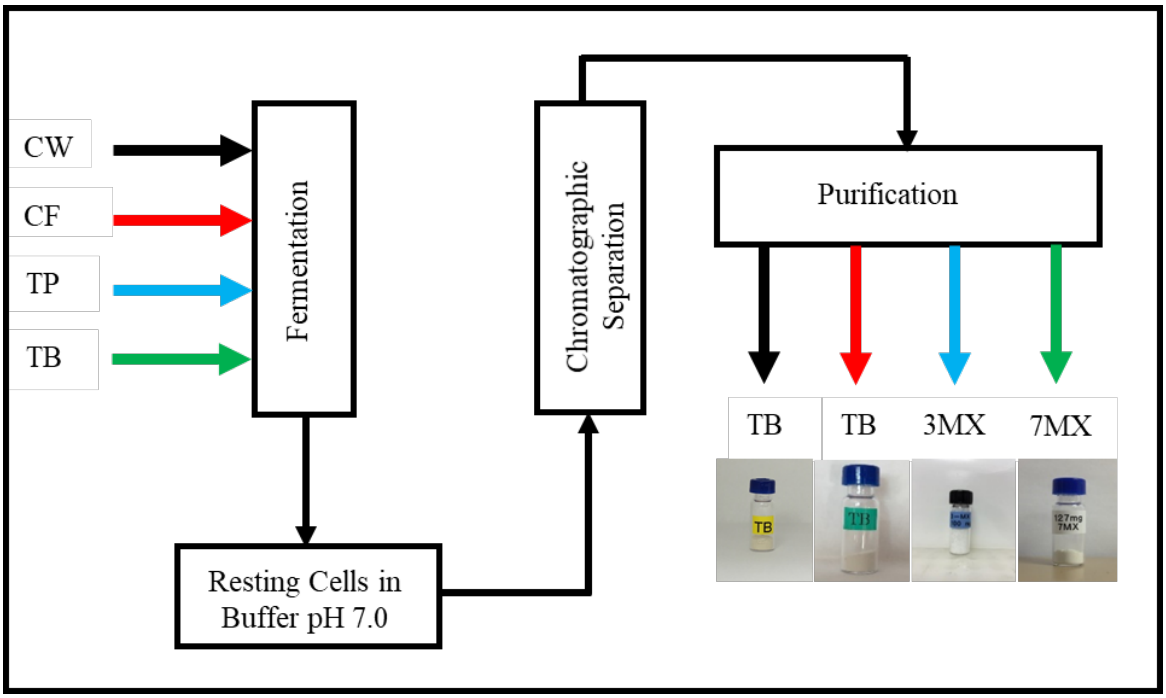

Figure 6.1 Process flow diagram for biological production of methylxanthine (CW, coffee waste; CF, caffeine; TP, theophylline; TB, theobromine; 3MX, 3-methylxanthine; 7MX, 7-methylxanthine)

The initial focus was to produce 3MX from TP using *E. coli* BL21 (DE3) pDdA strain, which was engineered with one copy of each of *ndmA* and *ndmD* genes. A full conversion of 1 mM TP to 3MX (81% conversion) and 1MX (13% conversion) catalyzed by 15 mg/mL pDdA was achieved at 30 ^{o}C and atmospheric pressure. The reaction was scaled up to produce 106 mg highly pure 3MX powder. Biocatalytically-produced 3MX, besides having a reagent market, has larger pharmaceutical applications (Daly, Butts-Lamb et al. 1983), and commercial interest as a nutraceutical (unpublished, personal communication between Professor Subramanian and two different nutraceutical

companies). This is the first report on the biocatalytic production of 3MX by metabolically engineered *E. coli*.

Second, theobromine (TB) was produced from caffeine using E. coli BL21(DE3) pAD1dDD strain, which was engineered with one copy of ndmA gene and three copies of ndmD genes. A full conversion of 1 mM caffeine to TB (98.5% conversion) and PX (1.5% conversion) catalyzed by 15 mg/mL pAD1dDD strain was achieved at 30 ^{o}C and atmospheric pressure. The reaction was scaled up to produce 255 mg highly pure TB powder. This is the first report of theobromine production via a metabolically engineered strain with highest yield and purity compared to other chemical and biological methods. During production of TB, highly valuable PX was also produced in low amounts (1.5% conversion of caffeine to PX). This was due to slight non-specificity of the enzyme in conversion of caffeine to TB. Due to low yield of PX, this process was not considered a viable method of production even though PX is a very high value compound. This work was partly funded by UPS Labs. The same metabolically engineered strain was used to demonstrate the conversion of coffee post-brew waste to produce TB. A 97% conversion of 1 mM caffeine in post-brew coffee waste to TB catalyzed by 25 mg/mL pAD1dDD strain was achieved at 30 ^{o}C and atmospheric pressure. The required resting cells concentration (25 mg/mL) to achieve complete conversion of 1 mM caffeine in post-brew coffee waste was higher than what was required (15 mg/mL) for complete conversion of pure caffeine to TB and PX. The reaction was scaled up to produce 178 mg highly pure TB powder. This is the first report of theobromine production via a metabolically engineered strain, from post-brew coffee waste.

Third, 7MX was produced from TB using *E. coli* BL21(DE3) pBD2dDB strain, which was engineered with two copies of each of ndmB and ndmD genes. A full conversion of 0.5 mM TB to 7MX catalyzed by 5 mg/mL pBD2dDB strain was achieved at 30 ^{o}C and atmospheric pressure. The reaction was scaled up to produce 127 mg highly pure 7MX powder. This is the first report of the biological production of 7MX, with the highest yield. This work was partly funded by VB Medicare Pvt. Ltd. The same company has licensed the technology to commercially produce 7MX.

Suggestions for Future Work

A common platform for the biocatalytic production of caffeine to theobromine, theophylline to 3MX, and TB to 7MX, using metabolically engineered *E. coli* has been validated at bench scale. Additional work along the lines below is required for commercialization of the technology to produce high value methylxanthines.

1. Carrying 10-1000 L growth of the metabolically engineered strains to produce kg quantities methylxanthines products.
2. Conducting growth-media optimization to achieve high cell growth (OD_{600} ~ 50) for high yield of product/Kg of biocatalyst. Growth medium optimization explores using different compositions of nutrients which is one of the important factors affecting cell growth.
3. Using resting cells to demonstrate the feasibility of multi-cycles of biocatalytic N-demethylation reactions, including adding glucose as source of NADH. This will further enhance the productivity.

4. Combining fermentation of individual strains with direct production of products in the tank, with appropriate addition of feedstocks at the optimum time, would significantly reduce unit operations. Multiple cycles of catalysis and production could be directly performed with growth of the strain.
5. Given the low solubility of methylxanthine products in water at pH 7 and 100% conversion of feedstock to products, crystallization of methylxanthine products could be achieved directly in the growth tank. Subsequently, the products could be easily recovered and purified in minimum unit operations.
6. Improving PX production from caffeine by mutation of *ndmA* to get yields higher than 80% will open commercial opportunity for production of this high value nutraceutical. Alternately, another bacterium can be isolated from soil that expresses an enzyme that directly converts caffeine to PX.
7. Studying (a) the use of coffee waste from decaffeination process plants to produce higher value methylxanthines and/or (b) bioremediation of waste from caffeine decaffeination plants.
8. Exploring the use of metabolically engineered strains with *ndm* genes to use tea waste as a source of theophylline to produce 3MX.
9. Constructing new next generation improved strains of *E. coli* by using alternate promoters and expression systems (such as using different promoters, ribosomal binding sites, and different plasmid vector). The engineered strains used in the present study produce very low amounts of soluble enzymes. Thus, there is much scope for further improving productivity of high value methylxanthines

10. Inserting the N-demethylation genes directly into the host chromosome will eliminate the use of antibiotics during growth of the metabolically engineered strains. This will eliminate handling of antibiotic containing post-fermentation waste and regulatory issues associated with it. It will also eliminate the use of expensive IPTG as Ndm induction-agent during growth of bacterial strains.

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