Towards the microbial production of a switchable solvent by using the enzyme indolethyamine
N-methyltransferase

by

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Abstract

Switchable solvents are a novel class of chemicals that are characterized with the ability to reversibly switch specific properties with the addition/removal of a trigger. For instance, a solution of low ionic strength can become one of high ionic strength, and vice versa. Switchable solvents have potential application in industrial processes as a greener alternative for product extraction. The ability to reversibly switch properties allows these solvents to be recycled, which reduces material usage and waste generation. Moreover, the switchable reaction occurs at low temperature and pressure, which reduces energy input and operation costs. Currently, application of switchable solvents is limited by difficult chemical synthesis and/or commercial unavailability. It is therefore necessary to find alternative, cheaper means to produce switchable solvents. Microorganisms have a long history of being used and modified to produce numerous chemicals of value. With growing concerns regarding the sustainability and environmental impact of the petroleum-based industry, there has been increased interest in converting biomass using microorganisms for the production of materials and chemicals. The main goal of this research study was to investigate the microbial production of the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane through enzymatic conversion of putrescine using the enzyme indolethylamine *N*-methyltransferase (INMT).

In the first study, the growth characteristics of two common microbial hosts, *Escherichia coli* and *Saccharomyces cerevisiae* were assessed for switchable solvent tolerance and potential consumption of the switchable solvent as a nutrition source. Both microorganisms grew well in the presence of *N,N,N',N'*-tetramethyl-1,4-diaminobutane, as determined by microbial growth curves and cell viability assays. HPLC-ELSD analysis demonstrated that neither microorganism

was found to consume *N,N,N',N'*-tetramethyl-1,4-diaminobutane as a nutrition source during cultivation.

The objective of the second study was to obtain a pure isolate of INMT for characterization purposes. DNA encoding rabbit lung INMT was cloned into *E.coli* and the enzyme was successfully expressed and purified. DNA sequencing and peptide sequencing experiments demonstrated that the isolated protein is INMT. In the third study, the activity of the purified enzyme was evaluated on tryptamine, a model compound, and putrescine using a microplate assay kit, high performance liquid chromatography (HPLC) and gas chromatography (GC) analysis. The enzyme assays showed that the purified enzyme from *E. coli* has activity on both tryptamine and putrescine.

In summary, this thesis has laid down the ground work for the microbial-based production of switchable solvents. This thesis presents the first study characterizing the switchable solvent tolerance of both *E. coli* and *S. cerevisiae* as well as demonstrating that the switchable solvent is not consumed by either microorganism during growth. Additionally, this thesis has shown that the enzyme INMT was successfully cloned into *E. coli*, expressed and purified to obtain a pure isolate. Enzyme assay studies with INMT demonstrated that it has activity on tryptamine, the model compound, and putrescine, the switchable solvent precursor.

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Preface

This thesis is an original work by Emily Soon. No parts of this thesis have been previously published. The sequencing of the DNA samples in Chapter 4 was performed by the Molecular Biology Facility (MBSU), Department of Biological Sciences, University of Alberta. The in-gel protein digestion in Chapter 4 was carried out by the Alberta Proteomics and Mass Spectrometry Facility, University of Alberta. Liquid chromatography with mass spectrometry analyses of enzyme samples were conducted with the aid of the Lipid Chemistry Group at the University of Alberta.

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List of Abbreviations

ANOVA – analysis of variance

APS – ammonium persulphate

BSA – bovine serum albumin

DNA – deoxyribonucleic acid

DTT - dithiothreitol

ELSD – evaporative light scattering detector

FAME – fatty acid methyl esters

FID – flame ionization detector

GC – gas chromatography

HPLC – high performance liquid chromatography

INMT – indolethylamine *N*-methyltransferase

IPTG - isopropyl β-D-1-thiogalactopyranoside

MALDI – matrix assisted laser desorption/ionization

MS – mass spectrometry

MWCO – molecular weight cut off

ORF – open reading frame

PCA – plate count agar

PCR – polymerase chain reaction

PHA – polyhydroxyalkanoate

PLA – polylactic acid

SAH – *S*-adenosyl-L-homocysteine

SAM – *S*-adenosyl-L-methionine

TEMED - Tetramethylethylenediamine

TCA – tricarboxylic acid cycle

SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

RNA – ribonucleic acid

UV – ultraviolet

1. Introduction

1.1. Project Background

The scope of microbial biosynthesis is enormous and this fact has been taken advantage of to produce important compounds in a number of industries including the agricultural, food and pharmaceutical industries. Identification of natural producers of a target product combined with random mutagenesis and recombinant DNA technology to improve production, and advances in fermentation technology have enabled the industrial scale production of amino acids, antibiotics, vitamins and bioethanol, all of which represent huge markets (Demain, 2000; Demain, 2007; Gavrilescu and Chisti, 2005; Wendisch *et al.*, 2006). Interest in microbial fermentation has risen in recent years due to increasing concerns regarding our reliance on petroleum-based fuels and chemicals and greater demands for sustainability and renewability. There has been increasing interest to produce chemicals and materials using microorganisms rather than through chemical synthesis. This has led to increased research contributions regarding biotransformation of renewable, sustainable feedstocks into value-added products (Hatti-Kaul *et al.*, 2006; Jang *et al.*, 2012; Lee *et al.*, 2011a). Thanks to the advances in metabolic engineering sand synthetic biology technologies in recent years, it has become easier to engineer microorganisms to enhance the production of chemicals and materials as well as broadening their portfolio of products, such that even non-natural compounds can be synthesized (Na *et al.*, 2010; Prather and Martin, 2008). Furthermore, significant research has been undertaken to enable microorganisms to use non-food sources and waste streams for production of value-added products (Balat, 2011; Yazdani and Gonzalez, 2007).

Switchable solvents are a novel class of chemicals that are characterized with the ability to reversibly switch specific properties with the addition/removal of a trigger (Jessop *et al*., 2005; Jessop *et al*., 2010; Jessop *et al*., 2012b; Mercer and Jessop, 2010) For instance, an aqueous solution containing *N,N,N',N'*-tetramethyl-1,4-diaminobutane has low ionic strength. Bubbling of CO_2 into the solution raises its ionic strength. Removal of CO_2 converts the solution from its high ionic strength form to the original, low ionic strength form (Mercer and Jessop, 2010). This switchable solvent system is referred to as switchable water. Switchable solvents make excellent candidates as extraction agents since a product that is soluble in one form of the switchable solvent may be insoluble in the other form. The ability to reversibly switch properties allows these solvents to be recycled and reused for future applications, which reduces material usage and waste generation. Moreover, the switchable reaction occurs at ambient pressure and temperature, which reduces the energy input. It is clear that switchable solvents may serve as a greener alternative for industrial product extraction. Currently, application of switchable solvents is limited by difficult chemical synthesis and/or commercial unavailability. It is therefore necessary to find alternative, cheaper means to produce switchable solvents.

The basis of this research project was to investigate the bio-based production of the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane. While *N,N,N',N'*-tetramethyl-1,4-diaminobutane is not known to be produced naturally by microorganisms, we hypothesize that this compound can be biologically derived from putrescine (1,4-diaminobutane) since they are structurally similar except for the degree of substitution on the nitrogen atom (Figure 1.1). Putrescine is a natural compound produced by microorganisms, plants, and animals. It is derived from amino acid synthesis, specifically proline and arginine. Putrescine is believed to be

involved in a number of physiological reactions including cell proliferation and the synthesis of DNA, RNA and proteins (Tabor and Tabor, 1985). This compound also has industrial relevance as a component of pharmaceuticals, polymers (4,6-nylon), surfactants, and agrochemicals (Scott *et al.*, 2007). Putrescine has already been overproduced in engineered strains of *E. coli* and *Corynebacterium glutamicum* as a potential alternative to chemical synthesis, which is generally not an environmentally-friendly process (Qian *et al.*, 2009; Schneider and Wendisch, 2010). Further engineering of putrescine producing microbes may make it feasible to convert putrescine to *N,N,N',N'*-tetramethyl-1,4-diaminobutane. We have identified a candidate enzyme, indolethylamine *N*-methyltransferase (INMT), which catalyzes the methyl transfer from *S*-adenosyl-L-methionine (SAM) to indoleamines. This enzyme has broad specificity and we hypothesize that it could convert putrescine to *N,N,N',N'*-tetramethyl-1,4-diaminobutane via transmethylation reaction (Figure 1.1). In this study, INMT was cloned into an *E. coli* strain and its methylation activity was evaluated. The end vision of this research program is to engineer a microbial strain that overproduces putrescine, the precursor, and INMT, which catalyzes the formation of the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane, during fermentation in the presence of SAM. This would enable the bio-based production of a novel switchable solvent.
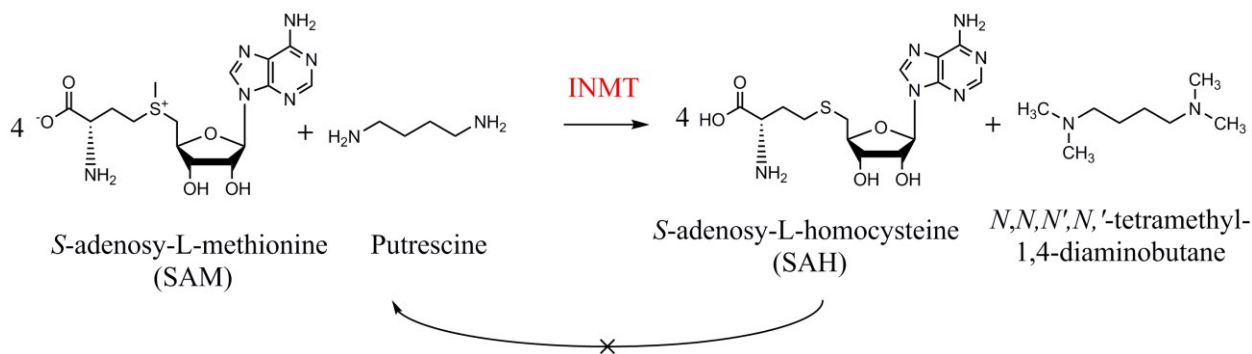
Figure 1.1. The hypothetic enzymatic conversion of putrescine to *N,N,N',N'*-tetramethyl-1,4-diaminobutane by INMT.

1.2. Objectives

1.2.1. Short-term objectives

1. To test the toxicity of the switchable solvent, *N,N,N',N'*-tetramethyl-1,4-diaminobutane, during separate cultivation of *E. coli* and *S. cerevisiae* (Chapter 3)

2. To clone INMT into an *E. coli* strain for overexpression and to purify INMT for subsequent enzymatic analysis (Chapter 4)

3. To confirm that recombinant INMT purified from *E. coli* is functional and to evaluate the ability of INMT to produce *N,N,N',N'*-tetramethyl-1,4-diaminobutane from putrescine via methylation (Chapter 5)

1.2.2. Long-term objectives of the project enabled by the program

1. To engineer a microbial strain to produce a switchable solvent by overproducing: putrescine, the precursor to a switchable solvent, and INMT, the enzyme that may catalyze the formation of *N,N,N',N'*-tetramethyl-1,4-diaminobutane from putrescine

2. To develop a technology that enables the bio-based production of switchable solvents and other industrially relevant co-products, such as ethanol, and the subsequent extraction of the co-product using switchable solvents

2. Literature Review

2.1. Microbial fermentation

Fermentation refers to the metabolic process in microorganisms that breaks down carbon into simpler compounds, such as alcohols, acids, and gases. From biotechnology perspective, fermentation refers to the process of growing microorganisms for the production of chemicals. Commercial microbial fermentation is responsible for the production of numerous chemicals important to human life, including vitamins, antibiotics, organic acids and amino acids, some of which are produced exclusively by fermentation (Chen *et al.*, 2013b; Demain, 2000; Demain, 2007; Gavrilescu and Chisti, 2005).

In recent years there have been growing concerns regarding our reliance on fossil fuels. For one, fossil fuel reserves are finite, and therefore not renewable. Another concern is that the chemical synthesis of many fine chemicals and commodity chemicals require non-renewable sources and/or require conditions that can be harmful to the environment and workers. There has been a significant influx of research conducted to develop alternative means of producing chemicals by using microorganisms, rather than through chemical methods. In addition, there has been increased interest in using renewable, waste feedstocks (e.g. wastewater, lignocellulosic biomass) as carbon sources to cultivate microorganisms for chemical production. Advancements in metabolic engineering and synthetic biology have enabled the engineering of microbes to use non-food and non-sugar based feedstocks, as well as increasing the portfolio of microbial-based products. Three of the most common microorganisms studied for these purposes are described below.

E. coli is an extensively-studied gram negative bacterium and an important model prokaryotic microorganism. *E.coli* has several characteristics that make it a good host for industrial applications. *E. coli* grows rapidly, is associated with low production costs, its metabolic and regulatory pathways are well-characterized, and there a vast number of genetic manipulation tools available for strain development. In fact, *E. coli* is often used for the production of purified enzymes and has been engineered to produce a vast repertoire of chemicals (Chen *et al.*, 2013; Atsumi and Liao, 2008).

C. glutamicum is a gram positive microorganism and most well-known for the industrial production of amino acids (Ikeda, 2003). Like *E. coli*, *C. glutamicum* is an excellent host for industrial applications. This bacterium is non-pathogenic, has few growth requirements, and grows relatively quickly. In addition, the metabolic and regulatory pathways involved in amino acid synthesis are well established (Wendisch, 2007). The elucidation of *C. glutamicum*'s complete genome has allowed for significant advances in strain development to improve industrial fermentation (Kalinowski *et al.*, 2003).

S. cerevisiae is a yeast species most commonly known for the fermentation of food and beverage products like bread, wine, and beer. *S. cerevisiae* has been extensively studied and is a key eukaryotic microorganism. There are several characteristics of *S. cerevisiae* that makes it a good candidate for industrial fermentation: it has a relatively fast doubling time, it has generally recognized as safe (GRAS) recognition, its fermentation pathways are well-characterized, and it is very amenable to genetic manipulation (Nielsen *et al*., 2013; Hong and Nielsen, 2012).

2.1.1. Biofuels

2.1.1.1. Ethanol

Ethanol is the most prominent biofuel and is produced in excess of 93 billion liters per year (Renewable Fuels Association, 2014). First generation bioethanol is derived from food sources (starch-based and sugar-based) and boasts globally well-established industries. The US alone is estimated to have produced approximately 15.5 billion liters of ethanol in 2015 (Ethanol Producer Magazine, 2015). However, concerns regarding the use of these feedstocks for fuel instead of food have prompted the development of second generation biofuels, which are derived from non-food sources. Second generation biofuels are generally derived from lignocellulose, which is comprised of lignin, cellulose, and hemicellulose. Lignocellulosic feedstocks are a sustainable alternative since they are abundant, cheap, and do not compete with food supply. These feedstocks can be residual non-food parts of crops, such as stems, leaves or husks; agricultural wastes such as woodchips and pulps; or non-food crops such as switchgrass and miscanthus (Balat *et al*., 2011). The recalcitrance of lignocellulose requires that this feedstock must undergo pretreatment (physical, chemical or biological) to free cellulose, which is broken down to glucose for ethanol fermentation. The breakdown of cellulose into simple sugars is achieved by enzymatic or acid hydrolysis.

Due to the complex nature of lignocellulosic biomass, pentose sugars such as xylose and arabinose are present in the hydrolysate and are not naturally metabolized by most fermentation microorganisms. It is therefore beneficial that ethanol-producing microorganisms be able to co-ferment mixed sugars to maximize the profitability of second generation biofuel production. To this end, there have been many research contributions aiming to develop microbial strains capable of utilizing pentose sugars for ethanol production (Hahn-Hägerdal *et al*., 2007; Jeffries

and Jin, 2004; Olsson and Hahn-Hägerdal, 1996). *S. cerevisiae* lacks the ability to utilize xylose, but can metabolize its isomer xylulose by the pentose phosphate pathway. Being one of the most prominent ethanol-producing microorganisms, *S. cerevisiae* has been engineered to metabolize xylose using two different strategies: overexpression of xylose isomerase (Brat *et al.*, 2009; Kuyper *et al.*, 2005; Madhavan *et al.*, 2009), and overexpression of both xylose reductase and xylose dehydrogenase (Ho *et al.*, 1998; Karhumaa *et al.*, 2007; Kötter and Ciriacy, 1993; Wahlbom *et al.*, 2003) to form xylulose (Figure 2.1). Both strategies require the overexpression of xylulokinase to allow xylulose to enter the phosphate pentose pathway for eventual ethanol production (Kim *et al.*, 2013).
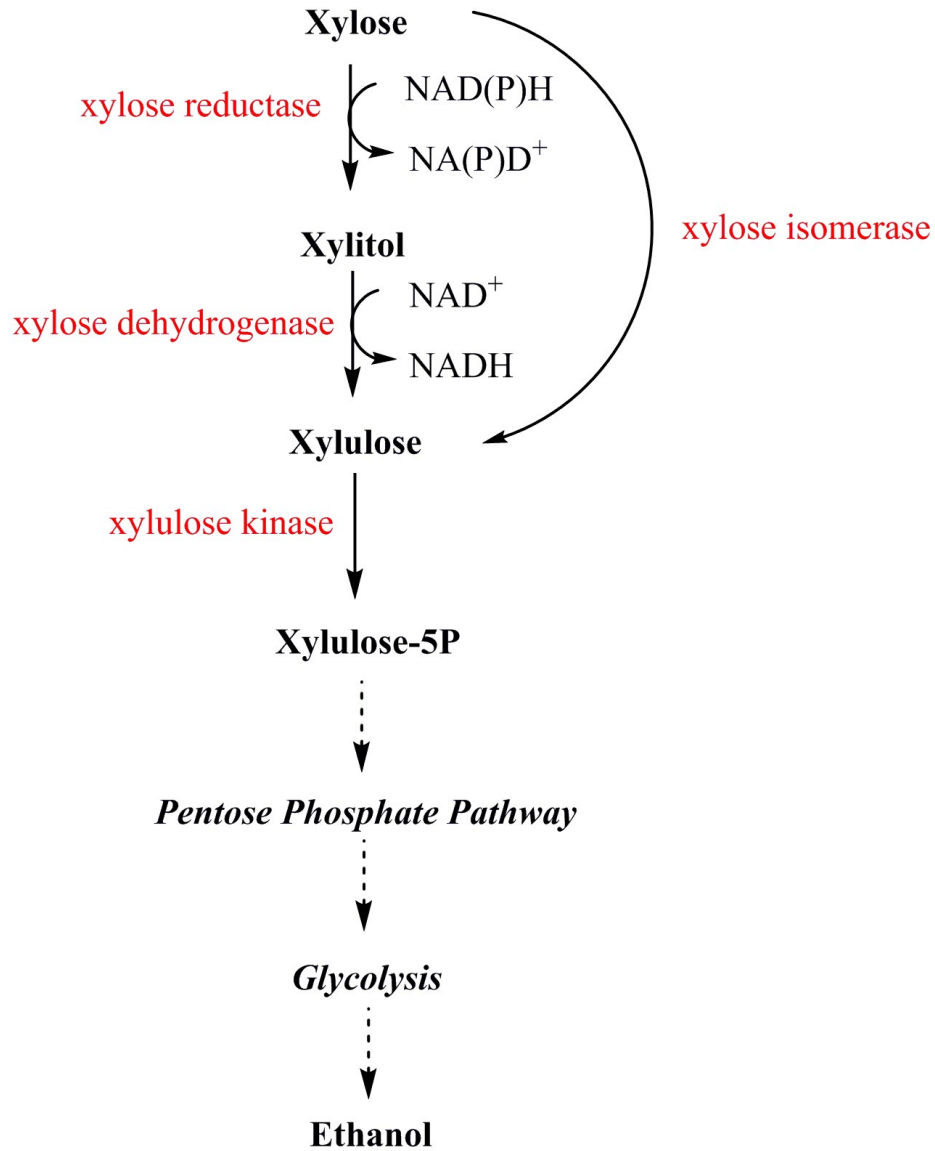
Figure 2.1. Proposed strategies for conversion of xylose metabolism for the production of ethanol in *S. cerevisiae*. Italicized words refer to general metabolic pathways.

Engineered *S. cerevisiae* strains have been examined for ethanol production using different sources of lignocellulosic biomass such as corn stover (Öhgren *et al.*, 2006), steam-treated wheat straw (Olofsson *et al.*, 2008) and steam-treated bagasse (Rudolf *et al.*, 2008).

Zymomonas mobilis has garnered interest as an ethanol-producing microorganism since it has several advantages over *S. cerevisiae*. This microorganism produces a high ethanol yield, has high tolerance to ethanol, and has the ability to ferment sugars at low pH, but is limited to a very small substrate range that includes glucose, fructose and sucrose (Rogers *et al*., 1982). Like *S. cerevisiae*, *Z. mobilis* lacks pentose-utilizing pathways and therefore has been engineered to metabolize pentose sugars for successful ethanol fermentation. Zhang *et al*. (1995) transformed two operons encoding xylose assimilation and the pentose metabolism genes into *Z. mobilis* to develop a strain capable of converting xylose to ethanol. This strain was able to grow on xylose as the sole carbon source and produced 0.44 g ethanol/g xylose (the theoretical yield is 0.51 g ethanol/g xylose). Additionally, this strain was able to co-ferment xylose and glucose to produce ethanol, achieving a 95% theoretical ethanol yield in 30 hours. It is worth noting that the sensitivity of *Z. mobilis* to inhibitors present in hemicellulose hydrolysates and its limited substrate range may prevent its commercial application for second generation biofuels production.

E. coli, unlike *S. cerevisiae* and *Z. mobilis*, is capable of metabolizing pentoses. However, ethanol is a minor product of its fermentation pathway, which also produces acetate, succinate and formate. This necessitates the use of genetic engineering to increase ethanol yields from *E. coli* in order to be a competitive producer. Expression of *Z. mobilis* pyruvate decarboxylase and alcohol dehydrogenase resulted in increased ethanol production from glucose, lactose and xylose during fed-batch fermentation (Alterthum and Ingram, 1989). The authors report 7.2%, 6.5% and 5.2% (v/v) ethanol from glucose, lactose and xylose, respectively. However, *E. coli* is characterized by lower tolerance to ethanol and other inhibitory compounds present in

hemicellulosic hydrolysates in comparison to *S. cerevisiae*. This could prove problematic for large-scale fermentation of lignocellulosic feedstocks.

2.1.1.2. Butanol

Butanol is an attractive alternative biofuel since it has a higher energy density and lower volatility in comparison to ethanol. It is also compatible with existing storage and transportation infrastructure. Butanol production in microbial fermentation was first reported by Pasteur in 1861. Solvent-producing strains of *Clostridium* spp. have been used for the industrial production of butanol, with acetone and ethanol as by-products in a process known as acetone-butanol-ethanol (ABE) fermentation (Jones and Woods, 1986). This was one of the first large-scale fermentation technologies to be developed and ranked second to ethanol fermentation in importance during the first half of the 20th century. However, with the rise of the petrochemical industry and the increased cost of feedstocks, ABE fermentation was no longer an economically competitive method to produce butanol.

Renewed interest in butanol fermentation has arisen due to increasing global warming concerns and the high price of crude oil. With advances in metabolic engineering tools, interest in microbial ABE fermentation has increased and several examples of metabolically engineered *Clostridium acetobutylicum* and *Clostridium beijerinckii* BA101 have been reported in literature (reviewed by Lee *et al.*, 2008). However, the lack of genetic manipulation tools for *Clostridium* spp. has limited further strain development, its low butanol tolerance, and its complex metabolic requirements have led to investigations of butanol production by *E. coli* and *S. cerevisiae*. Figure 2.2 refers to the general butanol synthesis pathway. Atsumi *et al.* (2008) expressed the genes involved in butanol synthesis from *C. acetobutylicum* in *E. coli* and deleted the host genes that

compete with butanol synthesis for acetyl-CoA and NADH. Butanol titers of 5.5 g/L in rich medium supplemented with 2% glycerol and 1.1 g/L in minimal (M9) medium were reported. Shen *et al*. (2011) report an engineered *E .coli* with improved butanol titers. Knockout of genes involved in mixed acid fermentation and acetate production, and the overexpression of formate dehydrogenase resulted in increased pools of acetyl-CoA and NADH for butanol production. Fermentation of this strain using a 1L chemostat with product removal by continuous gas stripping resulted in production of nearly 30 g/L in 7 days. Steen *et al*. (2008) tested the effects of butanol synthesis isozymes from different microorganisms on butanol production in an engineered *S. cerevisiae* strain. The strain with the best butanol titer (2.5 mg/L) overexpressed native thiolase and 3-hydroxybutyryl-CoA dehydrogenase from *C. beijerinckii*. Butanol yields from *S. cerevisiae* appear to be significantly lower than in *Clostridium* spp. and engineered *E. coli*. Much higher butanol yields in *S. cerevisiae* need to be achieved for it to be a competitive producer.

Acetyl-CoA

thiolase

Acetoacetyl-CoA

3-hydroxybutyryl-CoA dehydrogenase — NAD(P)H → NA(P)D$^+$

3-hydroxybutyryl-CoA

crotonase

Crotonyl-CoA

butyryl-CoA dehydrogenase — NADH → NAD$^+$

Butyryl-CoA

butryaldehyde dehydrogenase — NADH → NAD$^+$

Butryaldehyde

butanol dehydrogenase — NAD(P)H → NA(P)D$^+$
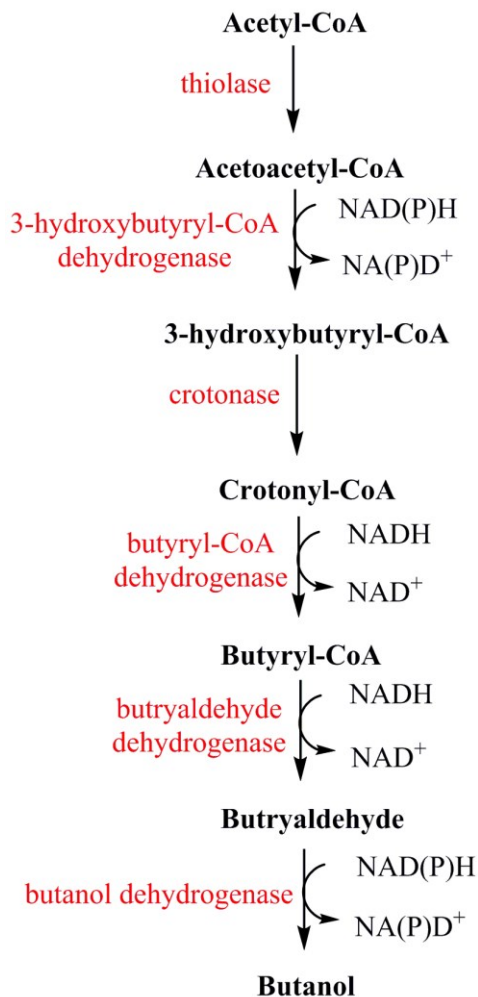
Butanol

Figure 2.2. Butanol synthesis pathway derived from acetyl-CoA.

2.1.2. Amino acids

Amino acids are important biological compounds that have numerous applications in the antibiotic, pharmaceutical and agricultural industries. Prominent examples of their applications include use in animal feed, and as food additives (i.e. monosodium glutmate (MSG) is the sodium salt of glutamic acid and is used as a flavouring enhancer). The worldwide market of amino acids is estimated at approximately $6 billion with an annual production of over 3 million tons (Demain, 2007). Production of amino acids occurs by several different methods: extraction

from protein hydrolysates, chemical synthesis, enzymatic conversion of precursors and fermentation. The majority of amino acids are produced by fermentation.

The discovery that *C. glutamicum* was a natural producer of a significant amount of glutamate pioneered the development of industrial fermentation of amino acids (Kinoshita *et al.*, 1957). Currently, approximately 1.5 million tons of glutamate is produced every year by microbial fermentation, making it the most produced amino acid (Leuchtenberger *et al.*, 2005). Cheap carbon sources like molasses or starch hydrolysates and a nitrogen source (ammonia) are generally used for commercial glutamate production.

Lysine is an essential amino acid and a nutrition requirement of animals and humans. Lysine is generally lacking in cereals and therefore an important component of animal feed. It is the most produced amino acid by fermentation after *L*-glutamate with an approximate of 850 000 tons produced annually (Demain, 2007; Leuchtenberger *et al.*, 2005). Classical strain development by random mutagenesis led to the discovery that threonine auxotrophy resulted in lysine overproduction. Determination of the complete genome sequence of *C. glutamicum*, greater understanding of this microorganism's physiology, and use of metabolic engineering tools aided the development of industrial production technology of *L*-lysine by auxotrophic *C. glutamicum* (Wittman and Becker, 2007).

Like *C. glutamicum*, *E. coli* has also been manipulated for amino acid fermentation. Some examples include L-threonine (Lee *et al.*, 2007), L-valine (Park *et al.*, 2007), L-phenylalanine (Baez-Viveros *et al.*, 2007), L-tryptophan (Zhao *et al.*, 2011) and L-tyrosine (Chavez-Bejar *et al.*, 2008).

2.1.3. Biopolymers

2.1.3.1. Polyhydroxyalkanoate

Polyhydroxyalkanoates (PHAs) are polyesters produced by a number of microorganisms in response to unfavourable growth conditions such as phosphate or nitrogen limitation (Dawes and Senior, 1973). PHAs serve as carbon and energy reserves. These biopolymers are potential candidates for plastics and have the advantages of being biodegradable and having excellent material properties (e.g. biocompatible, thermoplastic, UV stable). The commercial application of PHAs is limited by high production costs. Currently, poly(3-hydroxybutyrate) [P(3HB)] is the best characterized PHA. The general structure of PHA is as seen in Figure 2.3.
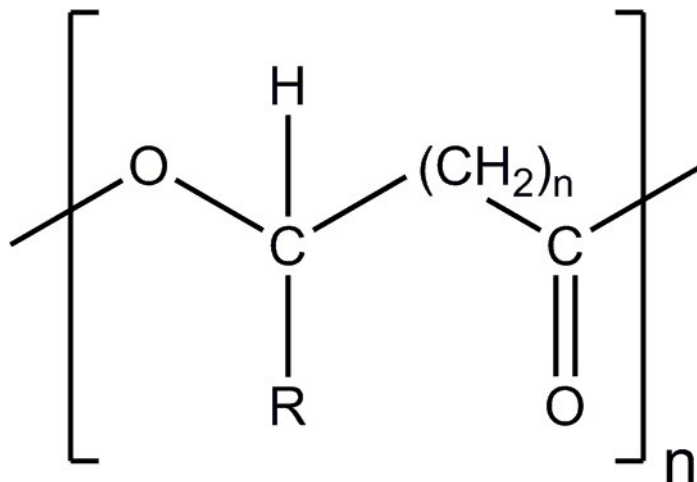
Figure 2.3. General structure of PHAs.

Microbial production of PHAs has been widely studied in recent years. Investigation of PHA production in *Ralstonia eutropha* (also known as *Cupriavidus necator*) has allowed for the elucidation of PHA biosynthesis, knowledge that has been instrumental in the development of recombinant bacterial strains for PHA synthesis. Indeed, PHA production by *E. coli* has been

heavily explored by heterologous expression of the *R. eutropha phaCAB* operon. *E. coli* is a more suitable choice for commercial PHA production since it can utilize a wider range of carbon sources, it is cheaper and easier to purify the PHAs, and it lacks PHA degradation enzymes. Several instances of PHA production by *E. coli* have been reported (Andreeßen *et al.*, 2010; Ahn *et al.*, 2001; Kusaka *et al.*, 1998; Li *et al.*, 2007)

2.1.3.2. Polylactic acid

Polylactic acid (PLA) is a biodegradable, thermoplastic derived from renewable resources and considered to be a potential alternative to petroleum-based plastics. At present, their wide-scale production is hindered by high production costs. PLAs are produced by fermentative production of lactic acids, which produces optically pure D- or L-lactic acid whereas chemical synthesis produces a racemic mixture. PLA production is followed by ring opening polymerization.

Lactic acid is produced naturally by numerous microorganisms, including *E. coli*, *C. glutamicum*, some strains of *Bacillus*, and a group of microorganisms known as lactic acid bacteria (LAB), notably *Lactobacillus*. In order to make PLA production more economically competitive, enhancing the production of lactic acid, use of alternative feedstocks for its production, and improvements to lactic acid purification technologies have been examined (Abdel-Rahman *et al.*, 2013). One report is direct production of PLA and its copolymer, poly(3-hydroxybutyrate-*co*-lactate), via a one-step fermentation strategy (Jung *et al.*, 2010; Yang *et al.*, 2010). To elaborate, *E. coli* was engineered for heterologous overexpression of propionate CoA-transferase and PHA synthase for synthesis of lactyl-CoA and incorporation of lactyl-CoA into the polymer, respectively. However, this strain required induction for expression of these genes

and succinate in the feed for proper cell growth. Further engineering of this *E. coli* strain

remedied these issues, resulting in efficient production of PLA and its copolymer with a

molecular weight of 141 000 Da at a concentration of 20 g/L (Jung and Lee, 2011).

2.1.3.3. Xanthan gum

Xanthan gum is a polysaccharide produced by *Xanthomonas campestris*. It is an important

commercial biopolymer and is one of the few microbial polymers produced at an industrial scale.

Approximately 30 0000 tons of xanthan gum are produced annually, corresponding to a value of

$408 million (Wilke, 1999; Demain, 2000). Xanthan gum has high viscosity in low

concentrations (<0.5 %) and is stable over a wide pH and temperature range (Garcia-Ochoa, *et

al.*, 2000). This biopolymer is used in a number of important food and pharmaceutical

applications such as emulsion stabilization, thickener, lubrication and temperature stability.

2.1.4. Pharmaceuticals
2.1.4.1. Antibiotics

Antibiotics are secondary metabolites with anti-microbial properties and one of the most

important fine chemicals with regards to human health. More than 100 types of antibiotics are

produced by fermentation and the estimated market value of antibiotics is 55 billion dollars and

their production exceeds 100 0000 tons annually (Demain, 2007; Ward, 1989). Production of

antibiotics is often performed by natural fungi and bacterial species such as *Penicillium

chrysogenum* and *Streptomyces griseus* during fermentation. Classical strain manipulation

methods have been used to improve antibiotic production.

2.1.4.2. Vaccines

Vaccines are biological preparations of attenuated or inactivated microorganisms that confer immunity to a specific disease. Antigens, which trigger the immune response, for bacterial vaccines are generally produced by cultivating the bacterium of interest (e.g. *Haemophilus influenza* type B, *Bordetella pertussis*). In some cases, antigens may be produced using recombinant DNA technologies. For instance, human hepatitis B antigen has been produced from recombinant yeast due to the fact that the preparation of this vaccine is restricted by the limited supply available supply. Initial recombinant strategies were performed in *S. cerevisiae* (Mcaleer *et al*., 1984). *Hansenula polymorpha*, a methylotrophic yeast, is now the common expression system for human hepatitis B angtigen (Schafer *et al*., 2001).

2.2. Switchable solvents

Switchable solvents are solvents that dramatically change their properties (e.g. polarity, hydrophobicity, ionic strength, etc.) in the presence of a trigger and change back when the trigger is removed. In many switchable solvent systems the trigger is carbon dioxide; its addition causes the initial property change and its subsequent removal changes the solvent back to its original state. The reaction of carbon dioxide with the switchable solvent results in the formation of alkylcarbonate anions, carbamate salts, or bicarbonate salts, as well as the protonation of the switchable solvent. Carbon dioxide as a trigger has the advantages of being inexpensive, easily removed and non-hazardous (Jessop *et al*., 2012b). There are a myriad of switchable solvents, but three of the main classes are: switchable polarity solvents, switchable hydrophilicity solvents and switchable water.

A number of industrial applications for switchable solvents have been envisioned and reported in literature (review by Jessop *et al.*, 2012b). The most prevalent industrial application for switchable solvents is product extraction; switchable solvents are well-suited for this application since the property switch can be controlled. To elaborate, a product may be soluble in one form of the switchable solvent, but insoluble in the other form of the switchable solvent, allowing for separation of the product from the medium or solvent. Using switchable solvents for extractions can be viewed as greener than current industrial processes, as switchable solvents can be recycled and reused, which reduces material usage and waste generation. In contrast to many industrial processes, only mild temperatures and pressures are needed for the product to be extracted by switching the solvent, which reduces the energy input and operation costs. And in some cases switchable solvents may be less volatile and less harmful to the environment than solvents used in industry.

2.2.1. Switchable polarity solvents

Switchable polarity solvents (SPS) typically exist as low polarity solvents. The introduction of atmospheric CO_2 to the switchable solvent results in the conversion of the solvent to a higher polarity form (Figure 2.4). Removal of CO_2 via purging with N_2 converts the solvent back to its original low polarity form. The initial switchable polarity solvents system was a non-ionic mixture comprised of an alcohol and an amidine or guanidine base that was reacted with atmospheric CO_2 to form an ionic liquid, specifically a 1:1 mixture of DBU (1,8-diazabicyclo-[5.4.0]-undec-7-ene) and 1-hexanol (Jessop *et al.*, 2005; Phan *et al.*, 2008b). This polarity reversal is achieved by purging the switchable solvent with an inert gas, such as N_2 or argon. Alcohol and amidine/guanidine switchable solvents are somewhat water-sensitive and must be dried before use. Although Yamada *et al.* (2007) demonstrated that amidine/primary amidine

component mixtures could serve as switchable polarity solvents with reduced water sensitivity, switchable polarity solvents still suffer from the high price of amidines and require a balanced stoichiometry in order to function. Hence, the feasibility of switchable polarity solvents that consisted of a single liquid switchable solvent was investigated (Phan *et al.*, 2008a; Blasucci *et al.,* 2009). Secondary amines such as ethylbutyamine were the first single liquid switchable polarity solvents reported (Phan *et al.*, 2008a). In addition, ethylbutylamine demonstrated decreased water sensitivity compared to DBU/alcohol switchable solvents.
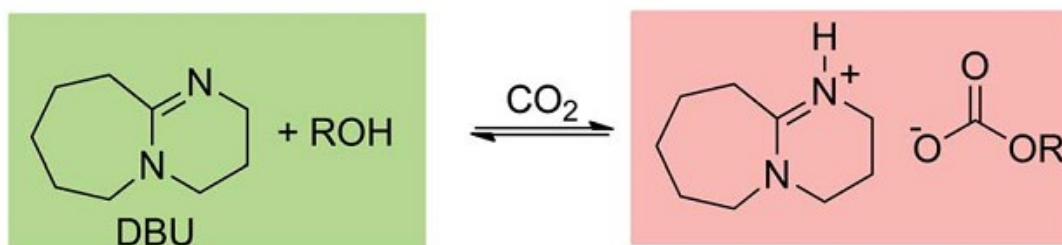
Figure 2.4. Example of a switchable solvent polarity system: 1-DBU/alcohol. Introduction of CO_2 results in an increase in polarity of the solvent system by formation of alkylcarbonate anions. Reproduced from Jessop *et al.* (2012b) with permission from The Royal Society of Chemistry.

A number of industrial applications have been identified for switchable polarity solvents and carbon capture is one of them. Switchable polarity solvents that are able to capture gases are referred to as CO_2-binding organic liquids. These organic liquids become ionic upon capture of CO_2 or other acid gases like SO_2. Release of CO_2 is achieved by heating at relatively low temperatures or purging with gas, resulting in the ionic liquid becoming non-ionic. Thus far the switchable polarity solvents that have been developed for carbon capture are two component systems of amidine/guanidine and organic alcohols (e.g. DBU/1-hexanol) and single component systems such as alkanolguanidines and alkanolamidines (Heldebrant *et al.*, 2008; Heldebrant *et*

al., 2010). These systems have been shown to bind and release CO_2 repeatedly with lower reaction energies and higher gravimetric (wt%) and volumetric (g/L) capacities in comparison to conventional amine-based carbon capture methods (Heldebrant *et al.*, 2008). These properties make switchable polarity solvents able to bind more CO_2 and thus, are more effective carbon capture agents from flue gas with the advantage of requiring a lower energy input.

Switchable polarity solvents are useful for facilitating extractions because their reversible properties allow for the target compound to be extracted when the switchable polarity solvent is in one form and then released when the solvent is converted to the other form. To elaborate, soybean oil has been extracted from soybeans using DBU/1-hexanol (Phan *et al*., 2008a), lipids extracted from the microalga *Botryococcus braunii* via DBU/alcohol (hexanol, ethanol, octanol) mixtures (Samorì *et al.*, 2010) and extraction of hemicelluloses from spruce using DBU/hexanol or DBU/butanol (Anugwom *et al.*, 2012).

Additionally, switchable polarity solvents may be utilized as reaction media. The idea is that the synthetic reaction is performed in one form of the switchable solvent and then the conversion of the solvent to its other form results in the product or catalyst precipitating due to insolubility. For instance, polymerization of styrene by an initiator in a mixture of DBU/1-propanol occurred in the low polarity form and subsequently precipitated by converting the switchable polarity solvent mixture to a higher polarity (Phan *et al*., 2008b). DBU/1-propanol was recycled for future styrene polymerization reactions by reverting the solvent mixture to its low polarity form.

2.2.2. Switchable hydrophilicity solvents

A second class of switchable solvents is characterized by having switchable hydrophilicity. Normally immiscible (or having very low miscibility) in water, these solvents become miscible in water after exposure to atmospheric CO_2 (Figure 2.5). Removal of CO_2 by heating and/or purging with inert gas is necessary to reverse the reaction for the solvent to become immiscible in water again. Carbonic acid is formed by the dissociation of CO_2 in water, resulting in the protonation of the switchable solvent and the formation of water-soluble bicarbonate salts. This reaction is responsible for the change in solvent's miscibility in water. The switchable hydrophilicity solvents that have been reported so far include the amidine *N,N,N'*-tributylpentanamide (Jessop *et al.*, 2010), tertiary amines such as cyclohexyldimethylamine (Jessop *et al.*, 2011) and bulky secondary amines (Vanderveen *et al.*, 2014).
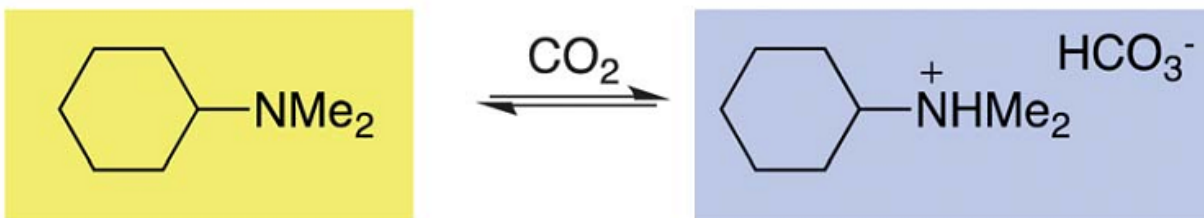
Figure 2.5. Example of a switchable hydrophilicity solvent cyclohexyldimethylamine. Introduction of CO_2 results in an increase in the hydrophilicity of the solvent system by formation of water-soluble bicarbonate salts. Reproduced from Jessop *et al.* (2012b) with permission from The Royal Society of Chemistry.

The application of switchable hydrophilicity solvents as extraction agents in industrial applications has been a main focus in literature. Switchable hydrophilicity solvents as separation

agents are a potential alternative to distillation, a process that requires the use of a volatile

solvent and high energy input. Switchable hydrophilicity solvents operate at ambient pressures

and temperatures, thus reducing energy input; they are recyclable, which reduces material

consumption and waste generation; and they are non-volatile. The tested applications of these

solvents include recycling of polystyrene foam (Jessop *et al.*, 2011), extraction of soybean oil

from soybean flakes (Jessop *et al.*, 2010; Phan *et al*., 2009), extraction of phenols from lignin

microwave pyrolysis oil (Fu *et al.*, 2014), and lipid extraction from algae (Samorì *et al.*, 2013;

Boyd *et al.,* 2012; Du *et al.,* 2013).

2.2.3. Switchable water

The switchable water class of switchable solvents describes an aqueous solution

containing and amine additive that facilitates reversible changes in the ionic strength of a

solution. In this system, a nitrogenous base (typically an amine) is added to water, creating an

aqueous solution of near zero or low ionic strength. Addition of CO_2 to the solvent system results

in an increase in the ionic strength of the aqueous solution via the formation of bicarbonate salts

and the protonation of the amine additive, while the removal of CO_2 reverts the aqueous solution

back to low ionic strength (Figure 2.6). Thus far, the model switchable water reported is an

aqueous solution of *N,N,N',N'*-tetramethyl-1,4-diaminobutane (Mercer and Jessop, 2010).
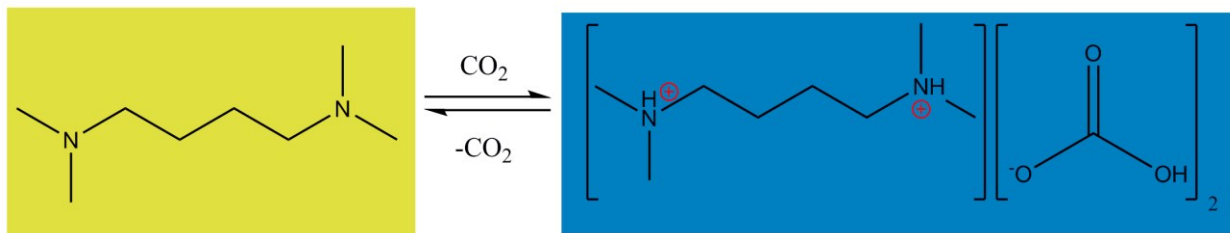
Figure 2.6. Schematic of switchable water. An aqueous solution of an amine additive, *N,N,N',N'*-tetramethyl-1,4-diaminobutane in water is switched between low (blue) and high (pink) ionic strength by the addition and removal of CO_2, respectively.

Much like switchable polarity solvents and switchable hydrophilicity solvents, switchable water has been tested for its ability to perform as an extraction agent. Specifically, switchable water acts similarly to salty water and is hypothesized to be effective in "salting out" organic contaminants out of water by raising the ionic strength of the solution. The practice of salting out is generally avoided as the salty water cannot be disposed of and removal of salt is an expensive process. Mercer and Jessop (2010) demonstrated that a green alternative to salting out was possible by using an aqueous solution of *N,N,N',N'*-tetramethyl-1,4-diaminobutane, which has switchable ionic strength. Bubbling CO_2 into the switchable water raises the ionic strength of the solution, forcing organic contaminants such as tetrahydrofuran (THF) out of solution. Reversion of the switchable water to low (or zero) ionic strength was achieved by heating and/or sparging with N_2 or air. The switchable additive *N,N,N',N'*-tetramethyl-1,4-diaminobutane was also shown to have potential application in the oilsands industry. Chen *et al.,* (2013a) discovered that aqueous solutions containing the *N,N,N',N'*-tetramethyl-1,4-diaminobutane performed as a flocculation agent and promoted clay settling and decreased turbidity of the supernatant (liberated water) in the presence of CO_2. This may allow for faster and easier recycling of water used in strip mining processes.

Switchable water has been employed for recycling of homogenous catalysts, where the catalyst is in the same phase as the reactants (Mercer *et al.,* 2012). Homogenous catalysts are often more selective and active than heterogeneous catalysts but this makes separation of the product from the catalyst difficult. One approach has been to use biphasic mixtures consisting of an aqueous and organic phase, which allows the product to be separated from the catalyst at the expense of reaction speed. The authors proposed a method for homogeneous catalysis which switches between monophasic and biphasic using switchable water. In the monophasic state, the reagents and catalysts are in the same phase, resulting in increased reaction rates. Switching to the biphasic state by CO_2 bubbling at the end of catalysis facilitates the easy separation of product and catalyst. The catalyst can be recycled by CO_2 removal. Hydroformylations of styrene have been achieved using this method (Mercer *et al*., 2012).

Another potential application of switchable water is water purification by desalination by forward osmosis (Jessop *et al*., 2013). Traditionally, seawater is purified by distillation and reverse osmosis, processes that are costly and energy-intensive. High ionic strength switchable water and salt water are separated by a forward osmosis membrane (Figure 2.7). Because the side of the membrane with the switchable water is more salty (or has higher ionic strength), the seawater will want to move to the switchable water side in order to reach equilibrium. Since the membrane is semi-permeable, only water, not salt, moves through the membrane to the switchable water side. The switchable water is converted to low ionic strength (or made less salty) by bubbling CO_2 into the solution and the additive is released as a gas, leaving pure water. The additive can be recycled for future water purification cycles. This technology is currently being commercialized by Forward Water Technologies.
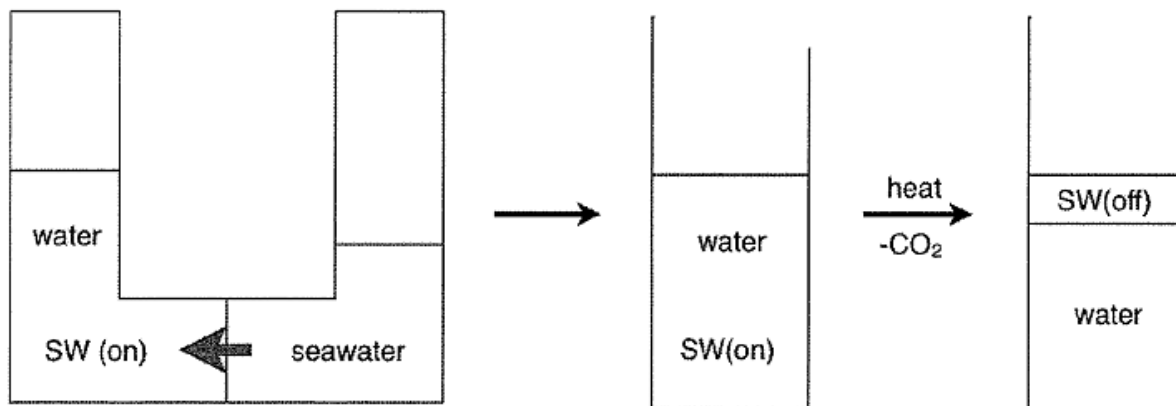
Figure 2.7. Method of water purification by separation of switchable water and seawater by a semi-permeable forward osmosis membrane. Image used in this thesis with the permission of the author (Jessop *et al.,* 2013).

2.3. Biogenic polyamines

Polyamines (putrescine, cadaverine, spermine, and spermidine) are linear compounds and contain two or more primary amino groups (Figure 2.8). Polyamines are ubiquitous to living systems and are synthesized by animals, plants, and microorganisms. These compounds are thought to have an important physiological role in cell proliferation and differentiation, though their exact function is still unclear (Pegg and McCann, 1982). Polyamines are also important in the food industry as their presence in food is indicative of spoilage and undesirable microbial activity. Ingestion of high quantities of biogenic amines in foods can result in several toxicological problems such as food poisoning, migraines and increased blood pressure. In particular, putrescine and cadaverine are known to potentiate the toxicity of other biogenic amines such as histamine (Shalaby, 1996).

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Putrescine

Cadaverine

Spermidine

Spermine
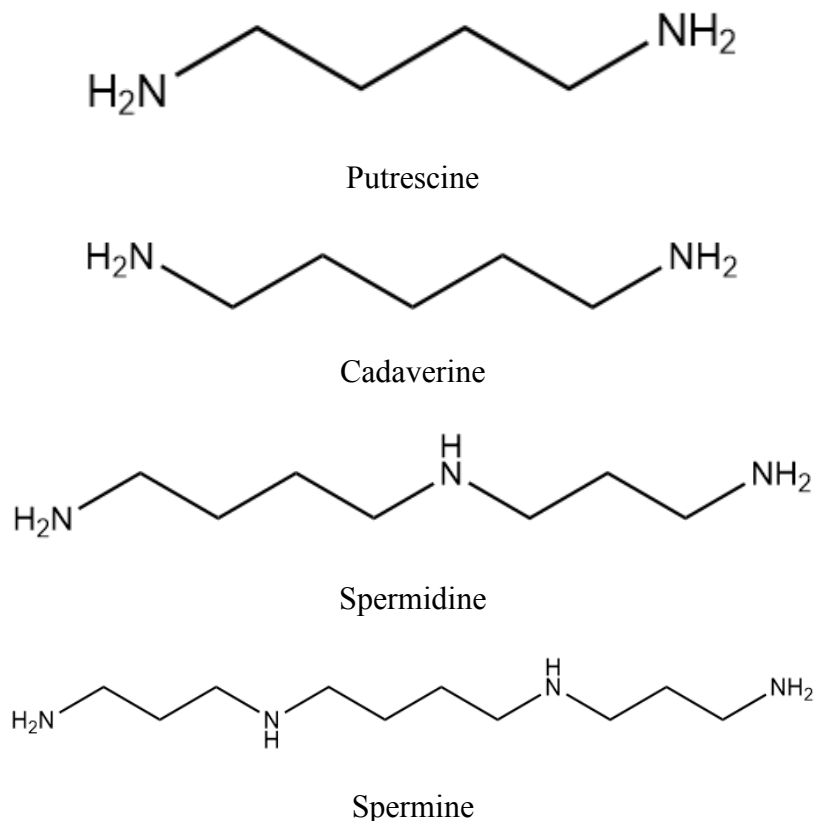
Figure 2.8. Biogenic polyamines

2.3.1. Putrescine

Putrescine, also known as 1,4-diaminobutane, is a polyamine found in all domains of life. Putrescine is an important biological compound and is implicated in a number of biological functions including cell growth, synthesis of biomolecules, and functions as a precursor to other polyamines (spermine and spermidine). However, the exact function of putrescine is not well-established. From an industrial perspective, putrescine is a component of a number of materials including polymers, surfactants, pharmaceuticals and plastics (Scott *et al.*, 2007). The most well-known commercial application of putrescine is for the synthesis of 4,6-nylon (Stanyl ®). Currently, chemical synthesis of putrescine is achieved by hydrogenation of succinonitrile,

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which is produced by combining hydrogen cyanide and acrylonitrile (Pollak *et al*., 2000). This process requires relatively harsh reaction conditions, the use of non-renewable materials, and use of expensive catalysts and is generally not an environmentally-friendly process (Lee *et al*., 2011). The feasibility of producing putrescine using biotechnology and renewable feedstocks has been investigated and will be elaborated on in the following sections.

2.3.2. Metabolic pathways of biogenic polyamines

Polyamine metabolism is derived from central metabolism. The production of putrescine occurs by the arginine decarboxylase (ADC) or ornithine decarboxylase (ODC) pathway (Figure 2.9). In the former pathway, putrescine is produced via a series of conversions starting with the decarboxylation of *L*-arginine to agmantine by arginine decarboxylase SpeA/AdiA. The subsequent conversion of agmatine to putrescine depends on the organism (Cunin *et al.*, 1986); in many bacteria, including *E. coli*, agmantinase SpeB directly hydrolyzes agmatine to putrescine and urea. In plants and some bacteria, putrescine is produced from agmatine in a two-step reaction. Agmatine deaminase AguA hydrolyzes agmatine, forming ammonia and *N*-carbamoylputrescine. The latter is hydrolyzed by *N*-carbamoylputrescine amidohydrolase AguB to produce putrescine, ammonia, and carbon dioxide. In contrast to the ADC pathway, the ODC pathway is fairly straightforward, as *L*-ornithine is directly converted to putrescine by ornithine decarboxylase SpeC.
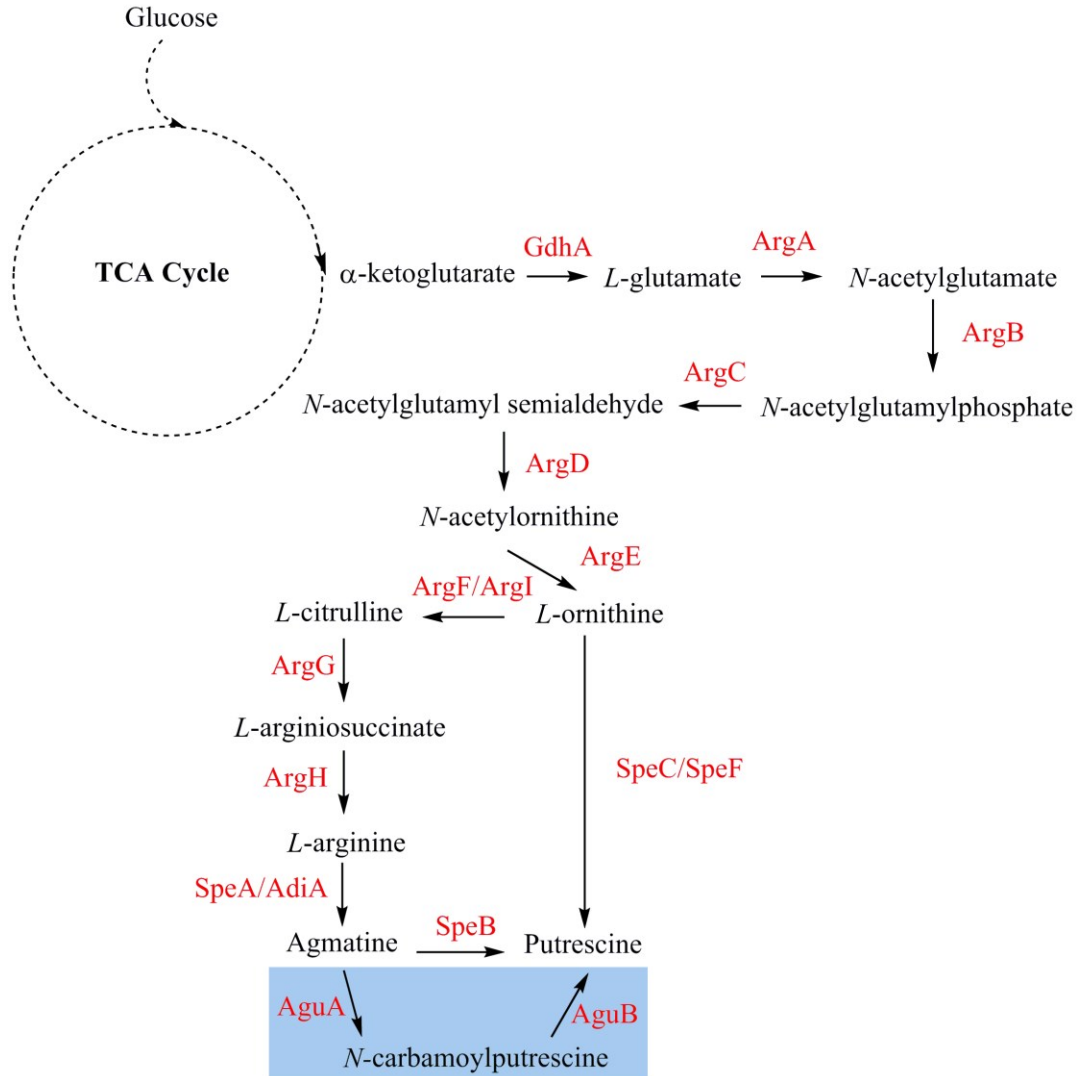
Figure 2.9. Schematic representation of the metabolic pathways involved in putrescine biosynthesis in *E. coli*, except for the reaction in the shaded area which occur in plants and some bacteria. Enzyme notation is as follows: AguA, agmatine deiminase; AguB, *N*-carbamoylputrescine amidohydrolase; ArgA, *N*-acetylglutamate synthase; ArgB, acetylglutamate kinase; ArgC, *N*-acetylglutamylphosphate reductase; ArgD, acetylornithine aminotransferase; ArgE, acetylornithine deacetylase; ArgF/ArgI, *N*-acetylornithine carbamoyltransferase; ArgG, argininosuccinate synthase; ArgH, argininosuccinate lyase; GdhA, glutamate dehydrogenase; SpeA/AdiA, arginine decarboxylase; SpeB, agmatinase; SpeC/SpeF, ornithine decarboxylase.

2.3.3. Bio-engineering of microorganisms for the production of biogenic polyamines

2.3.3.1. *E. coli*

E. coli is a common microbial catalyst of choice for industrial fermentations due to its fast growth, well-documented genetic background, and well established genetic manipulation tools. Naturally, *E. coli* was one of the first microorganisms studied for its potential to overproduce putrescine via manipulation of the ODC pathway. Two isozymes for ornithine decarboxylase exist: SpeC, the constitutive form and SpeF, the low pH inducible form. The first strategy explored for putrescine production by ornithine decarboxylase overproduction was met with minimal success. Comparison of *E. coli* strains producing normal and excessive amounts of SpeC revealed that both strains produced a similar concentration of putrescine (approximately 25 mg/L) despite the fact that the overexpression strain produced 70 times more SpeC (Kashiwagi and Igarashi, 1988). Another study also investigated the effect of ornithine decarboxylase expression on putrescine anabolism (Eppelmann, 2006). *E. coli* LJ110 overexpressing SpeC and SpeF produced 0.72 and 0.87 g/L putrescine, respectively. A significantly higher concentration of putrescine, 5.1 g/L, was obtained from *E. coli* LJ110 overexpressing SpeF during fed-batch fermentation.

More recently, Qian *et al.* (2009) reported the development of a genetically engineered *E. coli* strain for the overproduction of putrescine. A base strain was constructed by deleting several genes involved in putrescine degradation and utilization from *E. coli* strain W3110. Entry of *L*-ornithine into the ADC pathway was prevented by the knockout of *argI*, ornithine carbamoyltransferase, as this pathway results in the production of urea, which is unusable to *E. coli* since it cannot be metabolized any further. Furthermore, the genes encoding spermidine synthase (*speE*) and spermidine acetyltransferase (*speG*) were deleted to prevent the conversion

of putrescine to spermidine and *N*-acetyl-putrescine, respectively. The Puu putrescine

degradation pathway is responsible for breaking down extracellular putrescine (Kurihara *et al.*,

2005) and the genes encoding the putrescine importer (*puuP*) and glutamate-putrescine ligase

(*puuA*), the first enzyme in the Puu pathway, were deleted. In order to increase the conversion of

ornithine to putrescine, *speC* was overexpressed under the strong promoter *tac* from a low-copy

plasmid. Batch fermentation of this strain resulted in a yield of 1.2 g/L. Attempts to increase the

expression of SpeC using a higher copy plasmid resulted in the formation of inclusion bodies.

Next, to increase the pool of ornithine, the genes involved in the conversion of glutamate to

ornithine needed to be overexpressed; these genes are typically repressed by ArgR. To remove

repression, the native promoters of *argECBH* and *argD* were replaced with the strong promoter

trc. The native promoters of *speF*-*potE* and *speC* were also replaced with the *trc* promoter. The

overexpression of *potE*, a putrescine/ornithine antiporter, would increase the secretion of

putrescine. For *speC* and *speF,* this allowed for overexpression of both ornithine decarboxylases

from the chromosome. This strain produced 1.57 g/L putrescine during batch fermentation.

Lastly, RNA polymerase sigma factor S (RpoS) is a general response factor that is typically

produced in response to environmental stresses, such as UV radiation, nutrient deprivation, and

oxidative shock (Battesti *et al.*, 2011). The RNA polymerase sigma factor S is encoded by the

gene *rpoS*. Qian *et al.* (2009) hypothesized that putrescine overproduction may trigger the RpoS

response due to the stress on *E. coli*. Therefore the *rpoS* gene was deleted and batch fermentation

resulted in production of 1.68 g/L of putrescine. High density fed-batch fermentation of this

strain produced 24.2 g/L of putrescine in 32 hours.

2.3.3.2. *C. glutamicum*

C. glutamicum is a gram positive bacterium species commonly employed in industrial fermentations for producing amino acids (Hermann 2003; Hirao *et al.*, 1989), organic acids (Okino *et al.*, 2005), vitamins (Hüser *et al.*, 2005) and biopolymers (Matsumoto *et al.*, 2011). This species is an attractive microbial catalyst for industrial fermentation, particularly for amino acids, because the metabolic and regulatory pathways of amino acid synthesis are well-known. In addition, this bacterium grows quickly and has relatively few growth requirements. *C. glutamicum* is also a natural overproducer of glutamate, which feeds into putrescine biosynthesis, making it an attractive host for putrescine biosynthesis. The enzymes involved in the *C. glutamicum* putrescine metabolism are unknown and hence, Schneider and Wendisch (2010) investigated an approach to engineer a *C. glutamicum* strain capable of overproducing putrescine by heterologous expression of ADC and/or ODC genes from *E. coli*. Initial experiments revealed that the overexpression of *E. coli* ADC enzymes in wild-type *C. glutamicum* resulted in negligible production of putrescine. The authors determined that despite the overexpression of ADC pathway genes, *speA* or *adiA* (arginine decarboxylase) in combination with *speB* (agmatinase), very little putrescine was detected due to a bottleneck by both enzymes given that a substantial amount of the intermediates arginine and/or agmatine were detected. Therefore, production of putrescine by *C. glutamicum* via manipulation of the ADC pathway was not investigated further.

Heterologous expression of *E. coli* ODC genes *speC* and *speF* showed more promising results. The highest putrescine yield was obtained from an ornithine overproducing *C. glutamicum* strain. This strain was modified such that SpeC was overexpressed combined with the deletion of arginine repressor ArgR and ornithine carbamoyltransferase ArgF. During shake-

flask batch fermentation, this strain produced 6 g/L of putrescine. However, deletion of *argR* and *argF* resulted in the strain becoming auxotrophic for arginine, necessitating the need for arginine supplementation during fermentation, which is undesirable due to added production costs. Additionally, supplementation of too much arginine results in feedback inhibition of acetylglutamyl kinase *argB*, which would result in decreased productivity. To work around this issue Schneider *et al.* (2012) developed an anabolic-based metabolic plasmid addiction system for weak *argF* expression. By modifying the promoter, translational start site and ribosome binding site, *argF* could be weakly expressed, negating the need for arginine supplementation while simultaneously avoiding *argB* feedback inhibition. Fed batch fermentation of this strain produced 19 g/L of putrescine within 34 h.

Further work to improve putrescine yields from *C. glutamicum* was achieved by combinatorial strain development (Nguyen *et al.,* 2015). Putrescine production was increased by reducing oxoglutarate dehydrongease complex (ODHC) activity, allowing 2-oxoglutarate to feed into putrescine synthesis (via glutamate synthesis) rather than continue into the TCA cycle. Two approaches were taken. First, the translational start codon of *odhA* (a subunit of ODHC) was changed to decrease its synthesis, resulting in decreased ODHC activity. Second, exchange of threonine for alanine at the phosphorylation active site of OdhI (another subunit of ODHC) prevented its dissociation from ODHC. This maintained inhibition of ODHC. Following that stream of thought, increased synthesis of 2-oxoglutarate was achieved by overexpression of glyceraldehyde-3-phosphate dehydrogenase (*gapA*) and pyruvate carboxylase (*pyc*). Additional engineering of *C. glutamicum* by inhibition of *N*-acetylation of putrescine (*snaA* deletion) and overexpression of feedback resistant *N*-acetylglutamate kinase (*argB*$^{A49V/M54V}$) further improved

putrescine titers. Shake-flask fermentation of this strain resulted in a putrescine of 58.1 ± 0.2

mM, equivalent to 0.26 g putrescine per g glucose, the highest amount reported thus far.

Alternative carbon sources for putrescine production by *C. glutamicum* have been

examined (Table 1.1). Meiswinkel *et al.* (2013b) investigated glycerol utilization by *C.*

glutamicum PUT21 carrying a plasmid allowing for the overexpression of *glpF* (glycerol

facilitator), *glpK* (glycerol kinase), and *glpD* (glycerol-3-phosphate dehydrogenase) genes from

E. coli for putrescine production. When supplemented with 20 g/L of pure glycerol, the strain

produced a titer of 4.9 ± 0.9 mM (approximately 0.43 g/L). Supplementation with 10 mM

glucose (equivalent to approximately 1.8 g/L) resulted in doubled putrescine titers to a

concentration of 9.7 ± 1.4 mM (approximately 0.86 g/L). Supplementation with 20 g/L of crude

glycerol resulted in a maximum putrescine titer of 5.7 ± 0.6 mM (equivalent to 0.50 g/L). Crude

glycerol sources were obtained from commercial biodiesel plants in Europe (e.g. Tecosol GmbH,

Mannheim Bio Fuel GmbH). It is important to note that putrescine yields from crude glycerol

may vary depending on the source as the chemical composition of crude glycerol may vary

greatly between sources. Hemicellulosic wastes are becoming an attractive feedstock for

biotechnological fermentation as they are a cheap, abundant, non-food source of carbon sugars

(e.g. hexoses and pentoses). Some industrially relevant microorganisms, like *C. glutamicum* lack

the mechanisms for pentose sugar utilization and therefore require the use of metabolic

engineering for pentose utilization. *C. glutamicum* engineered to overexpress xylose isomerase

(*xylA*) from *X. campestris* and native xylulose kinase, and was tested for its ability to produce

putrescine with xylose sugars as the carbon source (Meiswinkel *et al.*, 2013a). Putrescine was

produced with a productivity of 0.03 g/L/h and a titer of approximately 15 mM (equivalent to 1.3

g/L). While alternative carbon sources from wastes are an attractive feedstock due to their

abundance and inexpensiveness, their biotransformation into putrescine by *C. glutamicum* leaves

much to be desired in terms of product concentration and productivity. Future work may be able

to improve these fermentation parameters in order to be economically competitive.

Table 2.1. Production of putrescine by different C. glutamicum and E. coli strains. Data adapted from Schneider and Wendisch (2011) and reprinted with the permission of Applied Microbiology and Biotechnology.

Microorganism	Carbon Source	Fermentation Mode	Concentration (g/L)	Yield (g/g)	Productivity (g/L/h)	Reference
E. coli	Glucose	Shake-flask	0.025	ND	ND	Kashiwagi and Igarashi (1988)
E. coli	Glucose	Fed-batch	5.1	ND	ND	Eppelman *et al.* (2006)
E. coli	Glucose	High density fed-batch	24.2	ND	0.75	Qian *et al.* (2009)
C. glutamicum	Glucose	Shake-flask	6.0	0.12	0.10	Scheider and Wendisch (2010)
C. glutamicum	Glucose	Fed-batch	18.6	0.16	0.55	Scheider *et al.* (2012)
C. glutamicum	Glucose	Shake-flask	5.1	0.26	0.21	Nguyen *et al.* (2015)
C. glutamicum	Pure glycerol	Shake-flask	0.43	0.02	ND	Meiswinkel *et al.* (2013b)
	Crude glycerol	Shake-flask	0.50	0.03	ND	
C. glutamicum	Xylose	Shake-flask	1.3	ND	0.03	Meiswinkel *et al.* (2013a)

ND - not determined

2.3.3.3. *S. cerevisiae*

Yeast, specifically *S. cerevisiae,* has long been an industrial workhorse in the food industry and is most famous for the fermentation of food products and alcohols. *S. cerevisiae* has also been used in the pharmaceutical and biotechnology industries. *S. cerevisiae* is an attractive microbial catalyst because its fermentation pathways and the process technology for scale-up are well understood, it is very amenable to genetic manipulation, and is non-pathogenic. For these reasons, *S. cerevisiae* may be a suitable microorganism for producing putrescine. Additionally, polyamine biosynthesis has been elucidated in *S cerevisiae* and putrescine production is known to occur only by the ODC pathway (Tabor and Tabor, 1985). Despite this, no attempts to produce putrescine or other polyamines by metabolic engineering of *S cerevisiae* have been presented in the literature. There may be several plausible reasons for this. One, the pathways for putrescine anabolism, catabolism, uptake and transport in *E. coli* have been studied extensively, which makes it a prime candidate for genetic engineering. In contrast, these pathways in *S. cerevisiae* are not as well characterized. Second, if we consider that putrescine is derived from amino acid metabolism then these pathways should be well understood in the microbial host. *S. cerevisiae* has not been a typical host for amino acid production.

2.3.3.4. Tolerance

When utilizing microorganisms in industrial fermentations, an important prerequisite to consider is tolerance to the desired product. Previous work has explored the tolerance of *E. coli* and *C. glutamicum* to putrescine during fermentation (Qian *et al*., 2009; Schneider and Wendisch, 2010). *E. coli* was reported to be able to tolerate 0.25M putrescine dihydrochloride (equivalent to 40 g/L) but growth was 40% lower when compared to *E. coli* grown without

putrescine. *E. coli* growth was observed at concentrations of 0.5M putrescine dihydrochloride (80 g/L), although growth was significantly slowed, and exposure to 0.6M putrescine dihydrochloride (96 g/L) resulted in immediate cell lysis. *C. glutamicum* grew well when exposed to 0.5M putrescine dihydrochloride (80 g/L), but exposure to 0.75M (120 g/L) led to reduced growth rates and biomass formation. At this concentration, Schneider and Wendisch (2010) found that *E. coli* and *S. cerevisiae* suffered even higher reductions in growth rate and biomass formation. *C. glutamicum* is able to tolerate higher concentrations of putrescine than *E. coli* or *S. cerevisiae* and appears to be more suitable for industrial fermentation for putrescine.

2.4. Analytical methods for analyzing biogenic polyamines

2.4.1. High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is an analytical chemistry method used for the separation, identification and separation of compounds in a solution. This method relies on high pressure to move a liquid mobile phase carrying the sample mixture through a column containing solid adsorbent material, also known as the stationary phase. The interaction of the each component in the mobile phase with the stationary phase differs for each compound, allowing for separation of each component. HPLC remains one of the most popular methods for analysis of biogenic amines due to its high reproducibility and sensitivity. Because most amines lack natural fluorescence or UV-Vis absorption, derivatization is often necessary for their analysis. Fluorescence is the emission of light after excitation by a specific spectrum or wavelength of light. UV detection is based on the absorption of electromagnetic radiation in the ultraviolet range (100 to 400 nm). Amines are usually derivatized using agents such as fluorescamine and *o*-phthaldialdehyde (OPA) for fluorometric detection (Benson and Hare, 1975; Kutlán and Molnár-Perl, 2003; Mafra *et al*., 1999; Undenfriend *et al*., 1972), while

benzoyl chloride, dansyl chloride and dabsyl chloride have commonly been employed for UV-Vis detection (Lin and Lai, 1980; Özdestan and Üren, 2009; Koski *et al.*, 1987; Seiler, 1970). While fluorescence and UV-Vis detection are highly sensitive, the necessity for derivatization is often accompanied by tedious sample preparation, long analysis times and risk of incomplete, or unstable derivatization, leading to inaccurate results. Analysis of underivatized amines may be preferential to avoid the mentioned challenges.

Recent developments in HPLC methodologies have allowed for the detection of underivatized amines by use of alternative detection methods. Evaporative light scattering (ELS) is a universal detector that operates by nebulizing the HPLC eluent and forms a fine mist, which is evaporated, leaving the analyte. The concentration of the analyte is determined by the intensity of the light scattering. ELS detectors enable the analysis of low volatility compounds that cannot be measured by means of optical properties. The use of ELS detectors has been reported for the detection of several underivatized biogenic amines in foods, including histamine, spermine, spermidine, tyramine, putrescine and β-phenylethylamine with good recovery and precision (Restuccia *et al.*, 2011; Spizzirri *et al.*, 2013). HPLC-MS/MS methods are also gaining popularity for detection of amines due to their high sensitivity and specificity (Gosetti *et al.*, 2013; Sirocchi *et al.*, 2014).

2.4.2. Gas Chromatography

Gas chromatography (GC) is an analytical chemistry technique that is used to separate, identify and quantify compounds that can be vaporized without decomposition. In this method, gas is used as the mobile phase. The components in the mobile phase pass through a column containing the stationary phase and the interaction of each component with the stationary phase

results in separation of each component. GC is a popular analytical method for analysis of compounds including amines due to its high sensitivity, simplicity, high resolving power, short analysis time, and low cost (Kataoka, 1996). However, GC analysis of free amines is not as commonly utilized in comparison to HPLC due to several issues with separation of low weight, free amines. These amines are difficult to analyze due their poor extractability from water, poor volatility, tendency to adsorb (due to their high polarity) and decompose on GC columns, and peak tailing. Therefore, it is often necessary to derivatize amines for GC analysis as derivatizing agents can reduce the polarity of the amino group and improve the separation, volatility, sensitivity, and detection of amines. Amines can be derivatized by a number of reactions including: acylation, alkylation, silylation, halogenation, or by forming Schiff bases, carbamates, sulfonamides, phosphonamides, thioureas, isocyanates or chiral derivatives. However, derivatization may require non-aqueous conditions and may result in the formation of unwanted derivatives and/or incomplete derivatization (Kataoka, 1996).

While derivatization enables or enhances the analysis of amines, it may not be a desirable method to use due to the drawbacks listed above. With that in mind, one approach for analyzing underivatized amines is by using specially designed GC columns. As such, a number of GC columns have been developed for this purpose, such as the Restek Rtx®-5 Amine and PoraPLOT Amine. These columns have been utilized in the literature for the analysis of underivatized amines (Ábalos *et al.*, 2001; Bonilla *et al.*, 1997; Mohnke *et al.*, 1994). These columns are base-deactivated, a process that bonds basic functional groups to the analytical surface to prevent interaction of highly basic compounds with the surface. This results in improved peak shape, response, and reproducibility during analysis of amines.

2.5. SAM-dependent methyltransferases

S-adenosyl L-methionine (SAM) is the most commonly used biological cofactor after adenosine triphosphate (ATP) and is involved in a number of important biosynthetic reactions. SAM is involved in the modification of virtually every class of biomolecules, including DNA, proteins, lipids, and small secondary metabolites via transfer of its methyl group (Struck *et al.*, 2012). This methyl transfer is catalyzed by a class of enzymes known as SAM-dependent methyltransferases, the most ubiquitous class of SAM-dependent enzymes. These enzymes have important implications in human biology due to the sheer number of biosynthetic reactions they participate in and have long been studied in biomedical research.

Interest in SAM-dependent methyltransferases also arises from its potential in biotechnological application for the production of pharmaceuticals, fine chemicals, and bulk chemicals. Production of valuable chemicals by enzymatic methylation may be preferable to the traditional method of using methyl iodide, as this method requires harsh reaction conditions. Methyl iodide exhibits moderate to high toxicity for inhalation and ingestion, and is a potential carcinogen (NIOSH, 2015). Moreover, chemical methylations often lack chemoselectivity, regioselectivity, and stereoselectivity. Hence there has been increased interest in enzymatic methylation by microbial catalysts for production of valuable chemicals. For instance, *E. coli* has been engineered for biodiesel production by utilizing a bacterial fatty acid *O*-methyltransferase (Nawabi *et al.,* 2011). This enzyme catalyzes the direct formation of fatty acid methyl esters (FAMES) from fatty acids, which may be advantageous since it bypasses the transesterification and purification steps. The authors report that increased pools of SAM, a cofactor required by fatty acid methyltransferase, resulted in increased fatty acid methyl ester formation. In another example, recombinant *E. coli* expressing catechol *O*-methlytransferase has been examined for its

production of vanillic acid, an intermediate in vanillin synthesis, during fed-batch fermentation (Li and Frost, 1998). The authors report a final concentration of 5 g/L vanillic acid after 48h of fermentation by the engineered *E. coli* strain.

2.5.1. Indolethylamine *N*-methyltransferase

Indolethylamine *N*-methyltransferase (INMT) catalyzes the *N*-methylation of tryptamine, serotonin, and other indoleamines (Figure 2.10). This enzyme belongs to a class of enzymes referred to as SAM-dependent methyltransferases. The activity of this enzyme was first reported by Axelrod (1961) in rabbit lung tissue and subsequent purification and characterization studies were described in the literature (Ansher and Jakoby, 1986; Mandel *et al.*, 1971; Porta *et al.*, 1979). Rabbit and human INMT cDNA were cloned, and its enzyme activity characterized, and sequence elucidated (Thompson and Weinshilboum, 1998; Thompson *et al.*, 1999). Characterization studies have typically used tryptamine as the prototypic methyl acceptor substrate, but INMT has been demonstrated to have broad specificity and can catalyze the methyl transfer from SAM to a range of primary, secondary, and tertiary amines. INMT is known to be inhibited by product inhibition; *N,N*-dimethyltryptamine (Thompson and Weinshilboum, 1998) and *S*-adenosyl-L-homocysteine (SAH; Lin *et al.*, 1973) are known inhibitors of INMT.

Interest in this class of methyltransferases arises from the potential of INMT to form psychoactive compounds such as *N,N*-dimethyltryptamine and *N,N*-dimethyl-5-hydroxytryptamine, and neurotoxins from the methylation of biogenic amines. However, the exact biological function of INMT remains unknown.
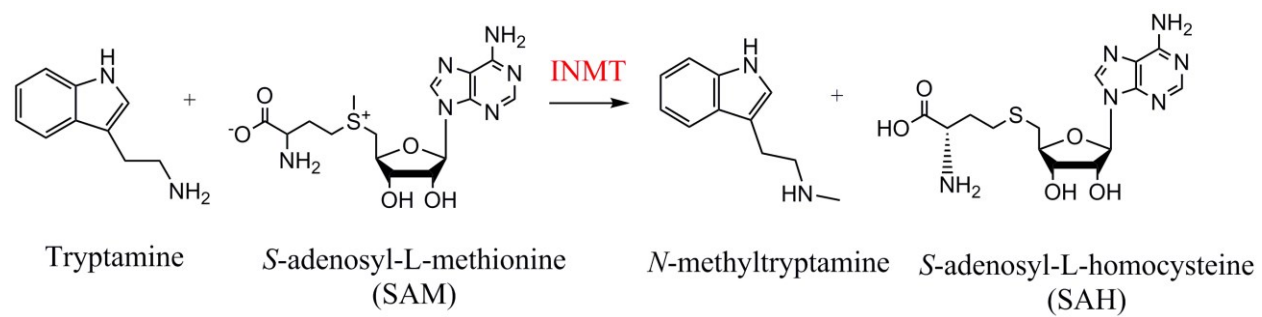
Figure 2.10. Methyl transfer from SAM to tryptamine catalyzed by the enzyme INMT.

3. Growth characteristics of microorganisms in the presence of the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane

3.1. Introduction

Microorganisms have long been utilized in fermentations for the production of chemicals in the food, pharmaceutical, and biotechnology industries. In the food industry, microorganisms have been used to ferment organic acids, alcohols, amino acids and vitamins (Bremus *et al.,* 2006; Herman, 2003; Martens *et al.,* 2002; Park *et al.,*2007; Peralta-Yahya *et al.,* 2012; Sauer *et al.,* 2008; Song and Lee, 2006). In the pharmaceutical industry, microorganisms have commonly been used to produce antibiotics, proteins such as insulin, and vaccines (Elander, 2003; Goeddel *et al.,* 1979; Johnson, 1983; Mcaleer *et al*., 1984). Recently, microorganisms have gained attention for their potential to produce biofuels and chemicals from renewable feedstocks due to increasing concerns regarding the depletion of fossil fuel reserves, our dependence on petroleum derived materials, and the contribution of fossil fuels to global warming. While microorganisms are capable of producing a wide array of products, genetic manipulation and optimization of fermentation conditions are often needed to increase the maximum yield of product in order for microbial fermentations to be economically competitive.

Metabolic engineering and synthetic biology have become valuable tools in designing microorganisms to produce chemicals and materials as it allows for production of target products in higher yields and for non-natural compounds to be produced. When engineering microorganisms for the production of chemicals by fermentation, it is necessary to consider the effects of the product on the microbes themselves. One important prerequisite for industrial fermentations is the necessity of microbes to have high tolerance to the target product. Thus far a number of studies have investigated the ability of microbial catalysts to tolerate putrescine, a

potential precursor to *N,N,N',N'*-tetramethyl-1,4-diaminobutane, during growth. Wild-type *E. coli* K12 W3110 has been found to grow well when exposed to 250 mM (40g/L) putrescine dihydrochloride (Qian *et al.*, 2009). Schneider and Wendisch (2010) reported that an engineered *C. glutamicum* grew well in presence of 500 mM (80g/L) putrescine dihydrochloride. However the effect of the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane on growth or viability of microbial catalysts remains unknown. Low tolerance of the microbial catalyst to *N,N,N',N'*-tetramethyl-1,4-diaminobutane, the intended product, would be an undesirable characteristic since it could affect fermentation performance. However, there are many other factors relating to fermentation performance, growth characteristics and economic feasibility that should also be considered.

Another important consideration regarding the effects of the target product on the microorganism is if the product is used as a nutrition source. Consumption of the end product by the microbial catalyst is undesirable, ultimately hampering the productivity and the profitability of the fermentation process. This issue may be rectified by genetic manipulation to prevent product utilization. Schneider and Wendisch (2010) reported that while *E. coli* utilized putrescine as a carbon source when exposed to a concentration of 250 mM, this was not the case for the engineered strain of *C. glutamicum*. It is currently unknown whether *E. coli* or *S. cerevisiae* can utilize *N,N,N',N'*-tetramethyl-1,4-diaminobutane as a nutrition source during cultivation.

In this study, *E. coli* and *S. cerevisiae* were grown in the presence of the switchable solvent, *N,N,N',N'*-tetramethyl-1,4-diaminobutane. The focus of this chapter was to investigate the tolerance of microbial catalysts to *N,N,N',N'*-tetramethyl-1,4-diaminobutane by exposing *E. coli* and *S. cerevisiae* to different concentrations of this chemical during cultivation and to determine which microbial catalyst would be a preferable choice for the production of the

switchable solvent during fermentation. Another focus of this chapter was to determine if the switchable solvent would be utilized as a nutrition source. This is the first study seeking to determine the growth characteristics of microbial catalysts in the presence of switchable solvents.

3.2. Materials and Methods

3.2.1. Materials

LB (Luria Bertani) and YPD broth (Difco) were purchased from Fisher Scientific (Dubuque, IA, USA). Plate count agar (PCA) was obtained from EMD Millipore (Billerica, MA, USA). *N,N,N',N'*-tetramethyl-1,4-diaminobutane was obtained from TCI America (Montgomeryville, PA, USA).

Superstart™ active dry yeast (Lallemand Ethanol Technology, Milwaukee, WI, USA) and *E. coli* BL21(DE3) pET11d-INMT (described in Chapter 4) were the microbial catalysts used in this study.

3.2.2. Preparation of *S. cerevisiae*

S. cerevisiae was supplied in active dry format. *S. cerevisiae* was activated by dissolving 0.5 g in 35 mL of preheated (37ºC) sterile double distilled (Milli-Q) water, and then incubated for 10 minutes at room temperature. The activated *S. cerevisiae* was serially diluted in YPD medium and 500 µL of culture was plated from the 10^{-4} dilutions onto YPD agar plates. This dilution resulted in single, isolated colonies. The plates were incubated at 30ºC for 24 hours. A single colony was streaked onto a fresh YPD plate and incubated at the same conditions described above. An isolated colony was used to inoculate a 5 mL culture of YPD and was

grown overnight at 30ºC with shaking at 200 rpm. The overnight culture was used to inoculate (5% v/v) a fresh 50 mL YPD culture, which was grown under the same conditions previously described until the OD_{600} reached 0.8. A glycerol stock was prepared by mixing 50% glycerol and yeast culture at a 1:1 ratio, followed by subsequent flash freezing with 70% ethanol/dry ice. The glycerol stocks were stored at -80ºC.

3.2.3. Cultivation of *S. cerevisiae* starter cultures

S. cerevisiae glycerol stocks were streaked onto YPD agar plates to isolate single colonies. The plates were incubated at 30°C for up to 72 hours. An isolated colony was inoculated from the YPD plate into 12 mL test tubes containing 5 mL YPD medium, pH 5.0 and incubated for 24 hours at 30°C with shaking at 200 rpm. 2.5 mL of the *S. cerevisiae* culture was transferred into an Erlenmeyer flask containing 50 mL YPD medium, pH 5.0 and incubated at the same conditions mentioned above for 4 hours to reach exponential phase ($OD_{600} \sim 0.4$).

3.2.4. Cultivation of *E. coli* starter cultures

E. coli glycerol stocks were streaked onto LB agar plates to isolate single colonies. LB plates were incubated at 37°C overnight. To prepare *E. coli* cultures, an isolated colony from the LB agar plate was inoculated into 12 mL test tubes containing 5 mL LB medium and incubated overnight at 37°C with shaking at 250 rpm. 2.5 mL of the overnight *E. coli* culture was transferred into an Erlenmeyer flask containing 50 mL LB medium and incubated at the same conditions mentioned above for 2 hours to reach exponential phase ($OD_{600} \sim 0.6$).

3.2.5. Switchable solvent tolerance assay

E. coli and *S. cerevisiae* starter cultures were grown as described in sections 3.2.3 and 3.2.4, respectively. For the cultivation of *E. coli,* 42 mL of LB medium were added to 125 mL Erlenmeyer flasks. A 1.25 M stock solution of *N,N,N',N'*-tetramethyl-1,4-diaminobutane, pH 7.0 was added to a final concentration of 15, 50 or 100 mM. Sterile double distilled (Milli-Q) water was added to bring the volume to 47.5 mL. The flasks were inoculated with 2.5 mL starter culture of *E. coli* in exponential phase to reflect a 5% (v/v) inoculum. For the cultivation of *S. cerevisiae*, 42 mL of YPD medium, pH 5.0, 1.25 M stock solution of *N,N,N',N'*-tetramethyl-1,4-diaminobutane, pH 5.0 (added to a final concentration of 15, 50 or 100 mM) and sterile double distilled (Milli-Q) water was added to bring the volume to 47.5 mL. The flasks were inoculated with 2.5 mL starter culture of *S. cerevisiae* in exponential phase to reflect a 5% (v/v) inoculum. The control flasks were prepared in the same manner as the *E. coli* and *S. cerevisiae* experimental flasks except sterile double distilled water was added in place of the switchable solvent . *E. coli* cultures were grown at 37ºC with shaking at 250 rpm for a total of 24 hours and *S. cerevisiae* cultures were grown at 30ºC with shaking at 200 rpm for a total of 72 hours. For the control and each concentration of the switchable solvent, *E. coli* and *S. cerevisiae* cultures were grown in triplicate.

The OD_{600} was measured in periodically in using a spectrophotometer (Ultrospec 4300 Pro, Amersham Biosciences, Mississauga, ON, Canada) to generate a growth curve and the cultures were assessed for viability and potential contamination by plating onto plate count agar (PCA). Cultures were serially diluted to obtain an appropriate number of colonies (30 to 300) on each plate. All analysis were conducted in triplicate.

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3.2.6. Quantification of *N,N,N',N'*-tetramethyl-1,4-diaminobutane

N,N,N',N'-tetramethyl-1,4-diaminobutane concentrations were determined by HPLC equipped with an evaporative light scattering detector (1200 series, Agilent Technologies, Mississauga,). Different concentrations of *N,N,N',N'*-tetramethyl-1,4-diaminobutane were prepared for standard curve preparation. The method used for separating the switchable solvent was adapted from Restuccia *et al*. (2011). The column used was Primesep 200 (SIELC Technologies, Prospect Heights, IL, USA). A gradient mobile phase consisted of A) 80:20 water:acetonitrile and B) 80:20 water:acetonitrile containing 0.15% (v/v) trifluoroacetic acid. The gradient elution scheme is defined as follows: beginning at: A:B 100:0 (v/v) and decreasing to A:B 0:100 (v/v) after 12 minutes, followed by 3 minutes of isocratic elution for 15 minutes of runtime. The composition of A:B returned to 100:0 (v/v) after 5 minutes (20 minutes runtime) and was held for 7 minutes for a total runtime of 27 minutes. The flow rate was 0.7 ml/min. The ELSD was operated with following parameters: the nebulizer gas (N_2) was maintained at 3.5 bar, gain set at 5, and the drift tube temperature set at 40ºC

3.2.7. Statistical analysis

Statistical analysis of variance of cell viability was done using one-way ANOVA with mean comparison by Tukey test with 95% confidence interval ($\alpha=0.05$, GraphPad software, La Jolla, CA, USA).

3.3. Results and Discussion

3.3.1. Metabolism of *N,N,N',N'*-tetramethyl-1,4-diaminobutane

The concentrations of *N,N,N',N'*-tetramethyl-1,4-diaminobutane were measured at the beginning and at the end of the incubation (24 hours for *E. coli* and 72 hours for *S. cerevisiae*) to determine if the switchable solvent was consumed by the microorganisms. Consumption of *N,N,N',N'*-tetramethyl-1,4-diaminobutane by either *E. coli* or *S. cerevisiae* is undesirable since it would decrease the product yield and ultimately, the profitability of the process. HPLC-ELSD analysis revealed that there was no significant difference ($p < 0.05$) in the switchable solvent concentrations at the beginning and end of incubation for both *E. coli* (Table 3.1) and *S. cerevisiae* (Table 3.2) for all experimental groups. Therefore, it can be concluded that *N,N,N',N'*-tetramethyl-1,4-diaminobutane is not utilized as a nutrition source by either *E. coli* and *S. cerevisiae*. Within the broader context of this research project, the lack of utilization of the switchable solvent by both *E. coli* and *S. cerevisiae* is a desirable trait as the engineered fermentation system would not require genetic engineering to fix the issue and the microorganism would not be consuming the intended valuable end product.

Table 3.1. Concentration of *N,N,N',N'*-tetramethyl-1,4-diaminobutane at the beginning and end of *E. coli* incubation at 37°C and 250 rpm.

	Initial concentration at 0h (mM)	Final concentration at 24h (mM)
0 mM	0 ± 0	0 ± 0
15 mM	16.3 ± 0.5	16.0 ± 0.3
50 mM	51.5 ± 1.1	51.7 ± 1.2
100 mM	105 ± 5	105 ± 3

Results where the concentration differs significantly (α=0.05) from the between time points at each concentration are marked with an asterisk (*).

Table 3.2. Concentration of *N,N,N',N'*-tetramethyl-1,4-diaminobutane at the beginning and end of *S. cerevisiae* incubation at 30°C and 200 rpm.

	Initial concentration at 0h (mM)	Final concentration at 72h (mM)
0 mM	0 ± 0	0 ± 0
15 mM	17.2 ± 0.5	17.6 ± 0.5
50 mM	49.6 ± 0.1	51.7 ± 0
100 mM	97.5 ± 3.4	101 ± 4

Results where the concentration differs significantly (α=0.05) from the between time points at each concentration are marked with an asterisk (*).

3.3.2. Cultivation of *E. coli*

The growth curve reveals that during exponential phase, *E. coli* growth is not affected by the concentration of switchable solvent (Figure 3.1). At the end of the 24 hour growth period, the OD_{600} shows a decrease among all *E. coli* treatments which suggests a decrease in cell concentration. For the control, the decrease in OD_{600} is likely due stationary phase or death due to nutrient depletion. The OD_{600} of *E. coli* grown with 15, 50, or 100 mM of *N,N,N',N'*-tetramethyl-1,4-diaminobutane is significantly ($p < 0.05$) lower than the control group, which suggests that prolonged exposure to the switchable solvent resulted in decreased cell concentration, most likely due to the toxic effects of the switchable solvent. *E. coli* culture samples were also plated onto plate count agar plates at the 8 and 24 hour time to point to assess cell viability and to check for contamination. The plate count revealed that at the 8 hour time point, there was considerable *E. coli* growth (Table 3.3) among all treatments, which supports the OD_{600} data that indicated *E. coli* grew in the presence of the switchable solvent. However, there is a significant difference ($p < 0.05$) in cell viability when comparing the *E. coli* grown with the highest concentration of switchable solvent, 100mM, to the control, suggesting that *E. coli* has decreased switchable solvent tolerance at this concentration. A look at the plate count data at 24 hours, the end of the incubation period, illustrates the toxic effect of the switchable solvent (Table 3.3). Even at the lower dose of *N,N,N',N'*-tetramethyl-1,4-diaminobutane (15 mM) a significant reduction of *E. coli* growth was observed compared to the control. At this concentration, *N,N,N',N'*-tetramethyl-1,4-diaminobutane may have led to increased cell death during the transition from exponential to stationary phase. Incubation with 50 mM and 100 mM of *N,N,N',N'*-tetramethyl-1,4-diaminobutane resulted in complete cell death as no colonies were observed. The discrepancy between the OD_{600} and plate count data at the 24 hour time point

may be explained by the fact that the latter only measures viable cells and the former simply measures the cell concentration by means of absorbance. OD_{600} does not discriminate between live and dead cells, hence the overestimation of cell growth at 24 hours. Therefore, based on the plate count data exposure to *N,N,N',N'*-tetramethyl-1,4-diaminobutane during stationary phase appears to have an inhibitory effect on the survival of *E. coli*. However, both the OD_{600} and plate count data suggest that *E. coli* is able to grow well in the presence of the switchable solvent.

The mode of *N,N,N',N'*-tetramethyl-1,4-diaminobutane toxicity in microorganisms is not currently known. Since this compound is charged under the pH conditions used in this study, it is extremely unlikely that *N,N,N',N'*-tetramethyl-1,4-diaminobutane is cell membrane permeable without the aid of transporter proteins, of which there are no reported examples. Therefore, the toxic effect of *N,N,N',N'*-tetramethyl-1,4-diaminobutane observed on *E. coli* in this study is most likely extracellular. We hypothesize that the toxicity could be attributed to a disruption of the plasma membrane, resulting in a leaky membrane, disruption of the membrane potential, and inactivation of membrane proteins. We postulate that the reason no observable effect on cell viability occured during *E. coli* exponential phase is because *E. coli* was able to repair the damage due to sufficient nutrient availability in the growth medium. However, upon onset of stationary phase where nutrients were depleted, *E. coli* was no longer able to synthesize the components needed to repair the damage caused by cell membrane disruption, which resulted in cell death for the 50 mM and 100 mM trials and a significant decrease in cell viability for the 15 mM trial. Future work will need to focus on elucidating the mechanism of toxicity of *N,N,N',N'*-tetramethyl-1,4-diaminobutane on microorganisms.
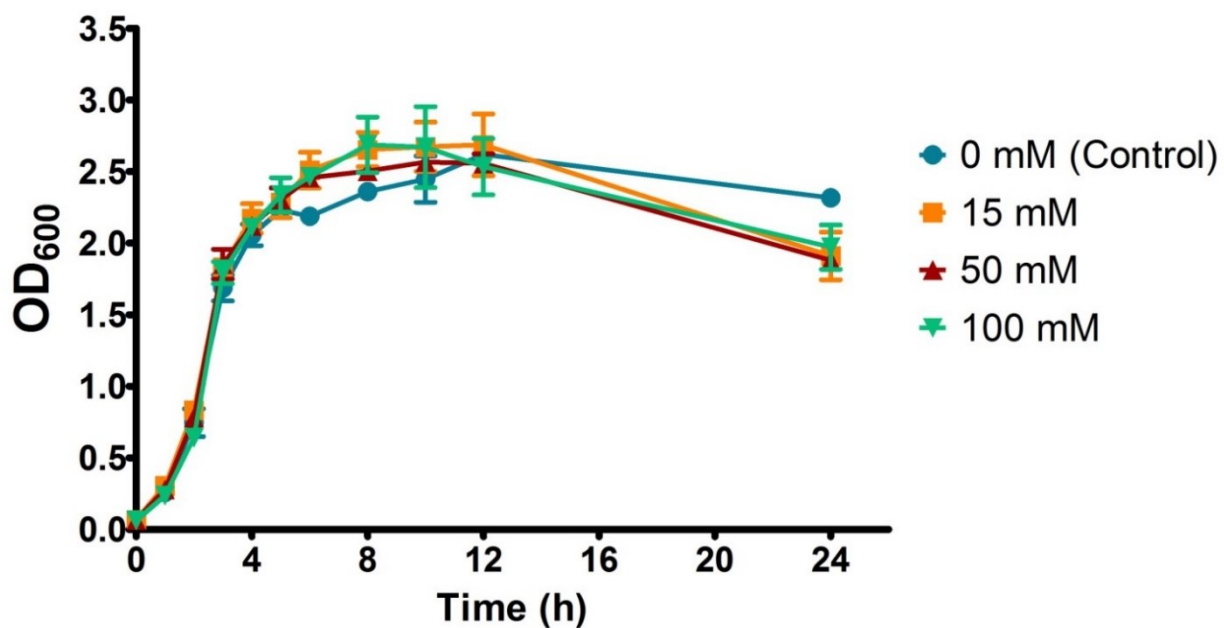
Figure 3.1. Growth curve of *E. coli* cultivated at 37ºC and 250 rpm with various concentrations of *N,N,N',N'*-tetramethyl-1,4-diaminobutane.

Table 3.3. Colony count of *E. coli* at 8h and 24h time point during cultivation at 37°C and 250 rpm.

Concentration of Switchable Solvent	CFU/mL at 8h	CFU/mL at 24h
0 mM	$2.1 \times 10^9 \pm 0.03 \times 10^9$	$5.0 \times 10^8 \pm 1.3 \times 10^8$
15 mM	$1.9 \times 10^9 \pm 0.20 \times 10^9$	$1.3 \times 10^5 \pm 0.18 \times 10^{5*}$
50 mM	$1.7 \times 10^9 \pm 0.07 \times 10^9$	NCO
100 mM	$1.4 \times 10^9 \pm 0.07 \times 10^{9*}$	NCO

NCO – no colonies observed

Results where the CFU/mL differs significantly from the control condition (0 mM) (α=0.05) are marked with an asterisk (*).

3.3.3. Cultivation of *S. cerevisiae*

Similar to the growth curve experiments with *E. coli*, *S. cerevisiae* was also cultivated in the presence and absence of the switchable solvent to determine its effect on growth. The *S. cerevisiae* growth curve revealed similar growth patterns among the control and experimental groups (Figure 3.2). An initial lag phase was observed in the first few hours of growth, followed by exponential cell growth that tapered off during the last 12 hours of cultivation. Essentially, the growth curve implies that *S. cerevisiae* growth is unaffected by concentrations of *N,N,N',N'*-tetramethyl-1,4-diaminobutane up to 100 mM during cultivation as the curves for each condition appear similar. Examination of the plate count data (Table 3.4) demonstrates that *S. cerevisiae* tolerates the switchable solvent during growth. There is no significant difference in cell viability between the control culture and the cultures grown with the switchable solvent at 24 hours and 72 hours ($p > 0.05$). In addition, the cell viability between 24 hours and 72 hours did not decrease significantly for each condition ($p > 0.05$). Considering the OD_{600} and plate count data together, it can be concluded that *S. cerevisiae* can tolerate prolonged exposure to *N,N,N',N'*-tetramethyl-1,4-diaminobutane during cultivation. Furthermore, it is possible that *S. cerevisiae* can tolerate even higher concentrations of *N,N,N',N'*-tetramethyl-1,4-diaminobutane given that no significant difference was observed in cell viability at the end of cultivation between the control and 100 mM trial. The ability of *S. cerevisiae* to tolerate prolonged exposure to the switchable solvent could be explained by the fact that *S. cerevisiae* has a more substantial barrier. Yeast cell walls consists of a thick layer (ranging from 70 to 200 nm thick) comprised of β-glucan, mannoproteins, and chitin, and accounts for approximately 25 to 33% of the dry cell weight (Klis *et al.*, 2002) whereas the cell wall of *E. coli* is composed of a thin layer of peptidoglycan covered by an outer membrane (Beveridge, 1999). The *E. coli* outer membrane is

a lipid bilayer membrane consisting of porin channels, and lipopolysaccharides on the outer face of the membrane. This could make *S. cerevisiae* more resistant to the toxic effect of the switchable solvent.
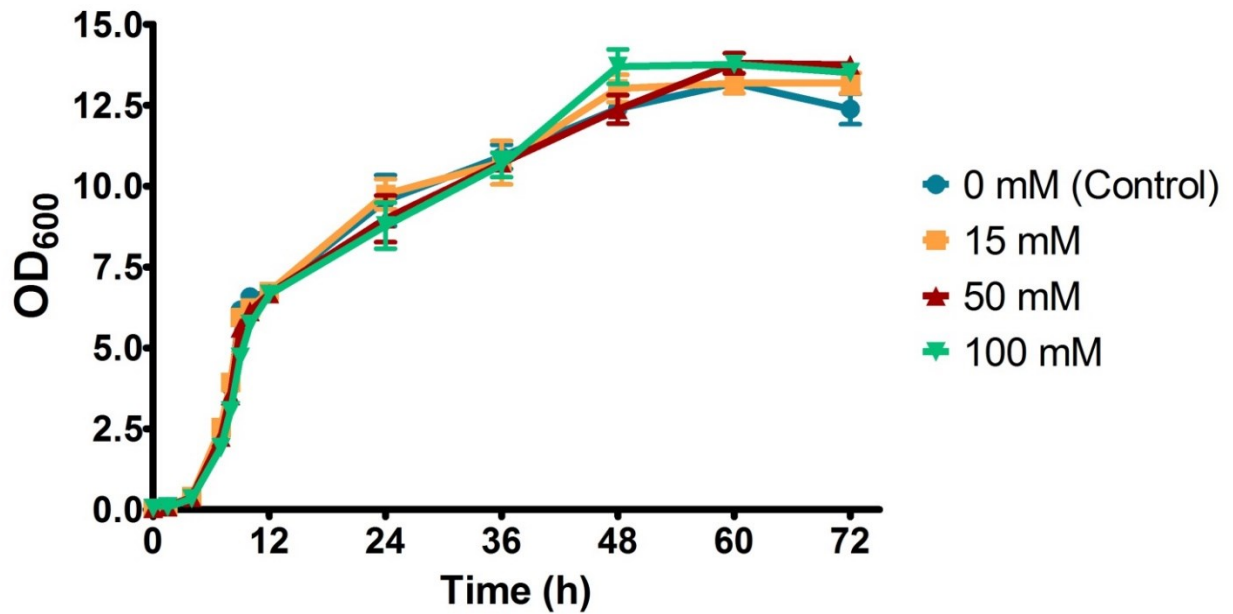
Figure 3.2. Growth curve of *S. cerevisiae* cultivated at 30ºC and 200 rpm with various concentrations of *N,N,N',N'*-tetramethyl-1,4-diaminobutane.

Table 3.4. Cell viability of *S. cerevisiae* at 24 and 72 hour time point during cultivation at 30°C and 200 rpm.

Concentration of Switchable Solvent	CFU/mL at 24h	CFU/mL at 72h
0 mM	$6.7 \times 10^7 \pm 0.99 \times 10^7$	$6.4 \times 10^7 \pm 0.12 \times 10^7$
15 mM	$6.6 \times 10^7 \pm 0.68 \times 10^7$	$6.6 \times 10^7 \pm 2.2 \times 10^7$
50 mM	$7.5 \times 10^7 \pm 1.7 \times 10^7$	$6.3 \times 10^7 \pm 0.21 \times 10^7$
100 mM	$6.0 \times 10^7 \pm 0.25 \times 10^7$	$5.8 \times 10^7 \pm 0.9 \times 10^7$

Results where the CFU/mL differs significantly from the control condition (0 mM) (α=0.05) are marked with an asterisk (*).

Considering the collective cell viability and *N,N,N',N'*-tetramethyl-1,4-diaminobutane data, it can be argued that *S. cerevisiae* is a more suitable choice for switchable solvent production. However, since *E. coli* was able to tolerate the switchable solvent during exponential phase and it is only during stationary phase where the toxic effect occurs, it is still a suitable microbial host for switchable solvent production. From a fermentation perspective, this compound is not expected to be produced in significant quantities after exponential phase, and therefore *E. coli's* susceptibility to *N,N,N',N'*-tetramethyl-1,4-diaminobutane during stationary phase can be considered a minor consequence. Although, high product tolerance is a desirable trait, there are many other factors that need to be considered in the grand scheme of things. For example, *C. acetobutylicum* is still widely used for butanol production despite its low tolerance because it produces in the highest product titers in comparison to engineered *E. coli* and *S. cerevisiae.* That considered, *E. coli* has other traits that make it an attractive host organism

because the genes relating to putrescine metabolism, uptake and degradation have been extensively studied and characterized (Tabor and Tabor, 1985; Igarashi and Kashiwagi, 1999), whereas in *S. cerevisiae*, these genes are not well characterized. Additionally, a couple of *E. coli* strains have been genetically engineered to overproduce putrescine (Eppelman, 2005; Qian *et al*., 2009). Working with *E. coli* may also make switching to a *C. glutamicum* producing strain (Schneider and Wendisch, 2010) easier as its amino acid metabolic pathways are well known as putrescine production is derived from amino acid metabolism. In addition, *C. glutamicum* is well known for being a natural overproducer of glutamate, a precursor for putrescine synthesis. In contrast, *S. cerevisiae* has not been modified to overproduce putrescine, possibly related to the fact that its putrescine metabolism genes are not well studied.

Fermentation of *E. coli* for switchable solvent production may still be feasible despite its lower tolerance if alternate fermentation strategies are used, namely removal of the switchable solvent before prolonged exposure begins to affect cell viability. There are a couple of approaches to consider. In a continuous culture, cells are grown under steady-state condition, which refers to growth at a constant rate in a constant environment. Fresh medium is continuously supplied to the bioreactor while liquid culture containing old cells, products and remaining nutrients are removed. Low product tolerance can also be overcome by simultaneous product removal. This strategy has been used for butanol production by *C. acetobutylicum* to overcome its low butanol tolerance. Some methods that have been reported in the literature include adsorption, gas stripping, pervaporation, liquid-liquid extraction and reverse osmosis (Ezeji *et al*., 2009; Qureshi *et al*., 2001).

3.4. Conclusions

In this chapter, a new study characterizing the growth characteristics of *E. coli* and *S. cerevisiae* in the presence of switchable solvents was conducted. The consumption of the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane by *E. coli* and *S. cerevisiae* was studied. Neither microbe was found to use the switchable solvent as a nutrition source during cultivation. This is desirable because it means that the microbial metabolism will not interfere with fermentation productivity and profitability by the loss of the valuable end product. Additionally, the tolerance of both microbes to *N,N,N',N'*-tetramethyl-1,4-diaminobutane was examined. This is an important consideration if these microbes are to be engineered to produce switchable solvents. Both microorganisms grew well in the presence of the switchable solvent, although *S. cerevisiae* demonstrated the ability the tolerate prolonged exposure to *N,N,N',N'*-tetramethyl-1,4-diaminobutane. *E. coli* may be still be a suitable host for the bio-based production of *N,N,N',N.'*-tetramethyl-1,4-diaminobutane. The toxic effect of the switchable solvent was observed in stationary phase, not exponential phase, which may not affect the production of the switchable solvent. Moreover, the pathways involved in putrescine synthesis, degradation, and transport have been extensively studied and characterized, which could make the engineering of *E. coli* for switchable solvent production via putrescine overproduction easier. Indeed, *E. coli* has already been reported for putrescine production (Eppelman, 2006; Qian *et al*., 2009), which is not the case for *S. cerevisiae*. In addition, low product tolerance can be further overcome by utilizing different fermentation strategies that remove the product before it accumulates to toxic levels (continuous culture, simultaneous product removal). For these reasons, *E. coli* is the microorganism used in subsequent studies presented in this thesis (Chapter 4 and 5).

4. Generation of an *E. coli* strain for expression and purification of indolethylamine *N*-methyltransferase

4.1. Introduction

Indolethylamine *N*-methyltransferase (INMT) is the enzyme responsible for the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to indoleamines, such as tryptamine, and was first reported by Axelrod (1961). The biological significance of INMT remains unresolved at present, but is speculated to play a role in the formation of psychoactive compounds and neurotoxins (Axelrod, 1961). INMT is highly expressed in rabbit, specifically lung tissues (Axelrod, 1962; Ansher and Jakoby, 1982), but has also been reported in the pasture grass *Phalaris tuberosa* (Mack and Slaytor, 1979), mice (Saavedra and Axelrod, 1972; Mavlyutov *et al*., 2012; Warner et al., 1995) and humans (Saavedra and Axelrod, 1972; Mandel et al, 1971; Bhikharidas *et al*., 1975). INMT has typically been purified from rabbit lung tissue, which remains the mostly widely studied form. Surprisingly, very few studies have used expression systems for the purification of INMT; expression systems are often used to produce high concentrations of a pure protein. In such systems, expressed proteins are generally tagged to reduce the number of purification steps and to increase the likelihood of isolating a highly pure enzyme fraction, as contaminants may affect subsequent studies such as enzyme assays. In the context of this research, the cloning of the *INMT* gene into an *E. coli* expression system would allow us to isolate high concentrations of a relatively pure enzyme.

Thompson and Weinshilboum (1998) and Thompson *et al.* (1999) examined a couple of cloning strategies for INMT. Previously, INMT studies were conducted using enzyme purified from rabbit lung tissue and therefore it was not certain if the activity attributed to the INMT isolate was due to the enzyme itself or a mixture of purified enzymes with properties similar to

INMT. By expressing INMT from an expression system, a pure source of the enzyme can be isolated, which allows for more accurate characterization of INMT. In one study, INMT was partially purified from rabbit lung tissue. The open reading frame (ORF) cDNA was amplified by PCR and cloned into a plasmid (pCR3.1) and the recombinant plasmid was used to transfect COS-1 cells (Thompson and Weinshilboum, 1998). In another study, human INMT ORF cDNA was amplified via PCR and recombined with pCR3.1 and the recombinant plasmid was used to transfect COS-1 cells (Thompson *et al*., 1999). As a result of their work, the coding DNA sequence of INMT was determined and the activity of a recombinant INMT was tested on a number of different amines, including the model compound tryptamine, *N*-methyltryptamine, serotonin, dopamine and phenylalanine. INMT purified from rabbit lung tissue has also been assayed for activity with putrescine, but its activity with this substrate was limited under the conditions examined, which was conducted in 50 mM Tris HCl at pH 8.5 with 34 µM [^{3}H] - SAM and 1 mM putrescine at 37°C (Ansher and Jakoby, 1986). Further characterization of putrescine methylation via INMT is required.

Engineered strains of *E. coli* and *C. glutamicum* have been engineered for putrescine overproduction (Eppelman, 2006; Qian et al., 2009; Schneider and Wendisch, 2011). We hypothesize that the use of a pure INMT from an expression system can catalyze the conversion of putrescine to *N,N,N',N'*-tetramethyl-1,4-diaminobutane. Since putrescine-producing strains have already been engineered, a recombinant plasmid for overexpression of INMT could be transformed into one of these strains to catalyze the formation of the switchable solvent intracellularly. The produced switchable solvent could be exported out of the cell by upregulation of a transport system or through incorporation of cell lysis during the down-stream processing.

INMT has never been expressed or purified using a bacterial expression system. Bacterial systems are popular for expressing proteins as they are one of the easiest, cheapest and quickest methods since *E. coli* grows rapidly and can produce high amounts of recombinant proteins. Bacterial expression systems are extremely versatile in that there are a vast number of vectors, *E. coli* expression strains, and purification methods, which collectively allow for the maximal expression of a number of different proteins. . While bacterial expression systems are advantageous in that they allow for fast and cheap overexpression of proteins, they are not always suitable for expression of eukaryotic proteins. Eukaryotic proteins are often post-translationally modified, while prokaryotic proteins are generally not, which means that prokaryotic cells typically lack the machinery to modify proteins post-translationally. Therefore, if eukaryotic proteins are modified post-translation, it can lead to protein misfolding and non-functional proteins in bacterial expression systems. According to the UniProt database (O97992), rabbit lung undergoes N6-succinyllysine post-translational modification although this modification occurs in prokaryotes like *E. coli* (Weinert *et al.*, 2013; Zhang *et al.*, 2011).In this study, the DNA encoding the rabbit lung INMT ORF was optimized for expression in *E. coli* and was cloned into a pET11d vector, forming the recombinant plasmid pET11d-INMT. pET11d-INMT was transformed into the *E. coli* expression strain BL21(DE3), expressed and purified to obtain a pure protein fraction. The activity of this recombinant enzyme will be tested in a future study (Chapter 5) to determine its ability to methylate putrescine and to form the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane, the desired product.

4.2. Materials and Methods

4.2.1. Materials

Competent *E. coli* strains DH5α and BL21(DE3) were purchased from New England Biolabs (Ipswich, MA, USA). The following reagents were bought from Invitrogen (Carlsbad, CA, USA): restriction enzymes *Nco*I and *Bam*HI, platinum *Pfx* DNA polymerase, dNTP set, TrackIt 1 kb plus DNA ladder and isopropyl β-D-1-thiogalactopyranoside (IPTG). QIAquick PCR Purification Kit, QIAprep Spin Miniprep Kit, and Ni-NTA agarose were obtained from Qiagen (Toronto, ON, Canada). Several different materials were purchased from Fisher Scientific (Dubuque, IA, USA), including LB Lennox broth, agar, tris and tetramethylethylenediamine (TEMED). Ampicillin and sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 40% Acrylamide/Bis Solution 19:1, ammonium persulphate (APS) and Precision Plus All Blue Prestained Standards were purchased from Bio-Rad (Mississauga, ON, Canada). Bacterial Protein Extraction Reagent (B-PER), lysozyme, DNAse I and EDTA-free protease inhibitor mini tablets were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Amicon Ultra-15 centrifugal filter units, 10 000 nominal molecular weight limit (NMWL) were purchased from EMD Millipore (Darmstadt, Germany).

A DNA fragment containing the DNA sequence for the INMT gene was synthesized and inserted into a plasmid by GeneArt (Life Technologies, Burlington, ON, Canada). The DNA sequence for INMT was optimized for codon usage in *E. coli* using the algorithm provided by GeneArt. The primers used in this study were synthesized by and ordered from Integrated DNA Technologies (IDT, Coralville, ID, USA). The DNA sequences of the primers are listed in Table 4.1.

Table 4.1. List of primers used in this study. Restriction sites are underlined.

Notation	Primer Sequence (5' to 3')
INMT-FWD	CGCTAGAT<u>CCAT GG</u>AAGGTGGTTTTACCGGTGGTGATGA
INMT-REV	CATT<u>GGATCC</u>TTAGTGGTGATGGTGATGATGGCTACCCGGTTTTTTAC
T7-FWD	TAATACGACTCACTATAGGG
T7-REV	GCTAGTTATTGCTCAGCGG

4.2.2. INMT ORF DNA

A plasmid containing the *E. coli* codon optimized rabbit lung INMT open reading frame with the stop codon removed was ordered from GeneArt (Life Technologies, Burlington, ON, Canada). The stop codon was removed to allow for the introduction of a protein tag, specifically a 6x His-tag, to the C terminus of the coding sequence by a primer during PCR. The recombinant plasmid obtained from GeneArt was transformed into *E. coli* DH5α (New England Biolabs, Ipswich, MA, USA). Briefly, a microfuge tube containing 50 µL of competent DH5α cells and 2 µL of the plasmid (approximately 0.2 µg) was incubated on ice for 30 minutes, subjected to heat shock at 42ºC for 10 seconds and followed by a 5 minute incubation on ice. 950 µL of SOC (super optimal broth) medium preheated to 37ºC was added and the microfuge tube was incubated at 37ºC at 300 rpm for 1 hour. The *E. coli* culture was plated onto LB medium plates containing 100 µg/ml ampicillin to select for transformants. The GeneArt plasmid was isolated from the transformed *E. coli* by inoculating the colonies into LB medium containing 100 µg/ml ampicillin, grown overnight at 37°C at 250 rpm, and subjected to plasmid miniprep treatment

following manufacturer's instruction (QIAprep Spin Miniprep Kit, Qiagen, Toronto, ON, Canada).

4.2.3. PCR amplification of INMT

The *E. coli* optimized INMT ORF was amplified from the purified GeneArt plasmid by polymerase chain reaction (PCR). The INMT ORF was amplified using the following PCR conditions: initial denaturation at 94°C for 60s; 30 cycles of denaturation at 94°C for 30s, primer annealing at 55°C for 30s and elongation at 72° for 60s. A *Nco*I restriction enzyme site was added to the 5' end of the coding sequence by the primer INMT-FWD. A 6x-His tag, stop codon and *Bam*HI restriction enzyme site were incorporated to the 3' end of the coding sequence by the INMT-REV. The amplified DNA was purified using QIAquick PCR Purification Kit (Qiagen, Toronto, ON, Canada) following the manufacturer's protocol.

4.2.4. Restriction analysis

Restriction digestions were carried out with 1X Buffer K, 0.01% BSA, 10 U of *Nco*I and 15U of *Bam*HI in a microfuge tube. Reactions were carried out at 37°C for 1 hour in a water bath and subsequently quenched by heating at 70°C for 15 minutes.

4.2.5. Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose and SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) in 1X TAE buffer to create a 1% (w/v) gel. DNA samples were prepared by mixing with 10X Loading Buffer (1% SDS, 50% glycerol and 0.05% bromophenol blue; Invitrogen, Carlsbad, CA, USA) and double distilled H_2O. 1kb+ TrackIt DNA Ladder (Invitrogen, Carlsbad, CA, USA) was used as a reference for DNA length. The gels were run at

120V for approximately 1 hour to separate the DNA. Agarose gels were visualized using AlphaImager HP (Alpha Innotech, San Jose, CA, USA).

4.2.6. Construction of recombinant plasmid pET11d-INMT

pET11d was the expression vector used in this study. Genes inserted into this vector are under the control of the T7/*lacO* promoter and can be expressed by addition of IPTG. pET11d also carries a selectable marker in the form of an ampicillin resistance gene.

The DNA concentrations of pET11d and purified INMT were determined using Nanodrop ND-1000 (Thermo Scientific, Waltham, MA, USA). pET11d and INMT were digested with *Nco*I and *Bam*HI and analyzed by agarose gel electrophoresis as described in section 4.2.3. and 4.2.4., respectively. Ligation of the digested fragments was performed using the Rapid DNA Ligation Kit (Thermo Scientific, Waltham, MA, USA). Briefly, the ligation reactions were set up at a molar ratio of at 3:1 of insert to vector and ligation was catalyzed by T4 DNA ligase. The ligation reaction was incubated at room temperature for 5 minutes.

4.2.7. Transformation of pET11d-INMT into E. coli DH5α and BL21(DE3)

The product of the ligation reaction was transformed into the cloning strain *E. coli* DH5α (New England Biolabs, Ipswich, MA, USA) as previously described in section 4.2.2. The transformants were grown overnight in LB medium (containing 100 µg/ml ampicillin) at 37ºC and 250 rpm and the recombinant plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen, Toronto, ON, Canada) following the manufacturer's protocol. The DNA concentration was quantified using Nanodrop ND-1000 (Thermo Scientific, Waltham, MA, USA). The purified plasmids were subjected to restriction digestion and agarose gel electrophoresis analysis as

described in section 4.2.4 and 4.2.5 to confirm that the correct recombinant plasmid was produced by the ligation reaction. The recombinant plasmids were also transformed into the expression strain *E. coli* BL21(DE3) as described above in section 4.2.2.

4.2.8. Sequencing

The recombinant plasmids transformed into *E. coli* DH5α were isolated and quantified as described previously (section 4.2.6). The INMT DNA sequence inserted into pET11d was sequenced using BigDye Terminator v3.1 Cycling Sequencing Kit (Applied Biosystems, Burlington, ON, Canada) and T7 universal primers (sequence listed in Table 4.1) with the following cycling conditions: 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 2 min for 25 cycles. The sequenced DNA was purified using ethanol precipitation and was analyzed by 3730 DNA Analyzer (Applied Biosystems, Burlington, ON, Canada) at the Molecular Biology Service Unit (Department of Biological Sciences, University of Alberta).

The sequencing output of the insert was aligned against the *E. coli* optimized INMT using CLC Sequence Viewer 6 (Boston, MA, USA) to determine the degree of sequence similarity between the two sequences and to determine if the insert was in-frame.

4.2.9. Expression of INMT

The *E. coli* BL21(DE3) expression strain containing the recombinant plasmid pET11d-INMT was grown overnight at 37ºC with shaking at 250 rpm. The overnight culture was reinoculated into fresh LB medium (containing 100 µg/ml ampicillin) and grown at the same conditions for approximately 2 hours ($OD_{600} \sim 0.6$). At this time, 1M IPTG was added to a final concentration of 0.1 mM to induce the expression of INMT. The cells were harvested after 4

hours of incubation by centrifugation (Avanti J-26 XP, Beckman Coulter, Brea, CA, USA) at 4,000 x, g for 25 minutes at 4ºC.

4.2.10. Purification of INMT

$E.\ coli$ cell lysate was prepared by treating the harvested cells with B-PER (Bacterial Protein Extraction Reagent), lysozyme and DNaseI (Thermo Fisher Scientific, Waltham, MA, USA), and then incubating at room temperature for 15 minutes. EDTA-free Protease Inhibitor tablets (Thermo Scientific, Waltham, MA, USA) were added to B-PER to inhibit any proteases that may be present in the lysate. Cleared $E.\ coli$ cell lysate was collected by centrifugation at 15,000 x g at 4ºC for 5 minutes, and then loaded onto a gravity flow column containing Ni^{2+}-NTA (nitriloacetic acid) agarose (Qiagen, Toronto, ON, Canada). Proteins with polyhistidine tracts bind tightly to transition metal ions such as Zn^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+} and can be eluted by a pH gradient, although elution with a chelator such as imidazole is a more common practice. The loaded column was incubated on ice for 1 hour with gentle shaking. After incubation, the flowthrough was collected and the column was washed twice with 4 column volumes of wash buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 20 mM imidazole, pH 8.0). 6x-His tagged INMT was eluted with 12 column volumes of elution buffer (50 mM NaH_2PO_4, 0.3 M NaCl, 250 mM imidazole, pH 8.0).

Elution fractions containing 6x-His tagged INMT were pooled and dialyzed against 0.1M Tris-HCl, pH 8.0 overnight at 4ºC. The dialyzed fraction was concentrated using Amicon Ultra-15 Centrifugal Filter Units, 10K MWCO (EMD Millipore, Darmstadt, Germany) at 4,000 x g for 25 minutes at 4ºC.

4.2.11. Bradford protein assay

Pierce™ Coomassie Plus (Bradford) Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine the concentrations of the protein fractions during purification. Briefly, 10 μL of sample was mixed with 300 μL of Coomassie Plus Assay Reagent. Protein concentration was measured using a microplate reader (Synergy MX, Biotek, Winooski, VT, USA) with absorbance set at 595 nm. Known concentrations of bovine serum albumin (BSA) were used to produce a standard curve (protein concentration vs absorbance at 595 nm). If necessary, protein samples were diluted to obtain an absorbance value within the standard curve working range.

4.2.12. SDS-PAGE

For all SDS-PAGE analyses, the gels were prepared with a 12% resolving gel and 5% stacking gel. Liquid protein samples were prepared by mixing equal amounts of sample with 2x SDS loading buffer (100 mM Tris-HCl, pH 6.8, 0.2% (w/v) bromophenol blue, 20% (w/v) glycerol, 4% (w/v) SDS and 200 mM DTT) while solid pellet samples were resuspended in 1x SDS loading buffer. All protein samples were heated to 80ºC for 3 minutes and then centrifuged for 8 minutes at 5,510 x g. Protein samples were loaded onto the SDS gel along with a protein molecular weight standard (Precision Plus All Blue Standard, Bio-Rad, Mississauga, ON, Canada). The gel was run with a voltage of 120V for 75 minutes to separate the proteins. The gel was stained with Coomassie Brilliant Blue, subsequently destained with distilled H_2O, and imaged using AlphaImager HP (Alpha Innotech, San Jose, CA, USA).

4.2.13. Protein identification

Protein gels were run as described in section 4.2.12. The band thought to be INMT (30 kDa mark) was excised and submitted to the Alberta Proteomics and Mass Spectrometry Facility (University of Alberta) for protein identification by mass spectrometry.

4.3. Results and discussion

4.3.1. Cloning of INMT into pET11d

Figure 4.1 illustrates the proposed recombinant plasmid for expression of INMT by cloning the INMT coding DNA into the vector pET11d using *Nco*I and *Bam*HI restriction enzymes. The primers used in this study were designed to add a *Nco*I restriction enzyme cut site to the 5' end of the coding sequence and a 6x His-tag, stop codon , and *Bam*HI restriction enzyme cut site to the 3' end of the coding sequence, in that order. Notably, the 6x His-tag added to the C-terminus of INMT allows for the protein to be purified by nickel affinity chromatography, which usually yields relatively pure isolates.
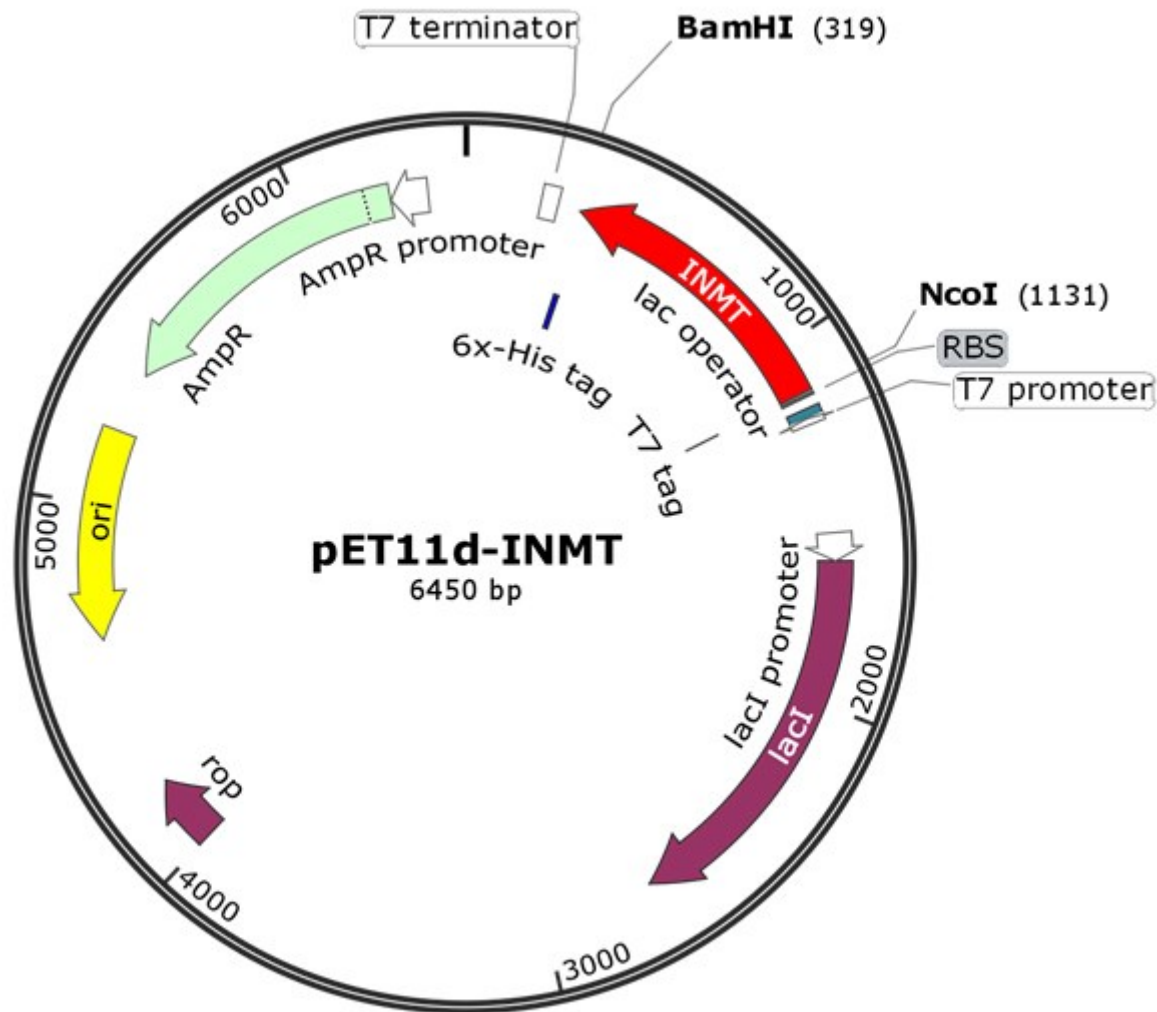
Figure 4.1. Construction of recombinant plasmid pET11d-INMT via cloning of INMT coding

DNA into the vector pET11d using the restriction enzymes *Nco*I and *Bam*HI. Ori: origin of

replication; RBS: ribosome binding site, AmpR: ampicillin resistance gene, lacI: lac repressor;

rop: repressor of primer. Image was generated by Snap Gene (Chicago, IL, US).

The DNA sequence encoding the open reading frame (ORF) of the INMT gene was

amplified by PCR and then purified. The products of the PCR reaction were analyzed by agarose

gel electrophoresis to determine if the length of the PCR products approximated the length of the

INMT ORF and to verify if contaminating fragments of DNA were present (Figure 4.2). The

DNA fragments amplified by PCR are approximately 850 bp (Figure 4.2; Lanes 1 and 2), which

corresponds to the expected size of the PCR-amplified INMT (830 bp). No bands were observed

in the negative control lane (no DNA template), indicating that no contamination had occurred

(Figure 4.2; Lane 3). The INMT ORF was therefore likely amplified by PCR.
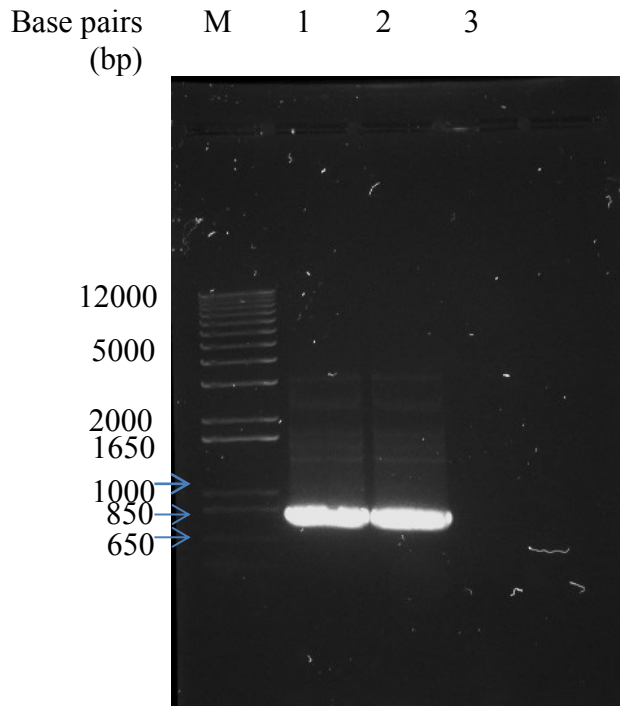
Figure 4.2. 1.0% agarose gel electrophoresis analysis of colony PCR amplification of INMT.

Lane M: TrackIt 1 kb plus DNA ladder; lane 1 and 2: PCR amplified INMT; lane 3: negative

control (no DNA).

The recombinant plasmid pET11d-INMT was constructed by ligating INMT and pET11d

with T4 DNA ligase. Both INMT and pET11d were digested with *Nco*I and *Bam*HI. The

recombinant plasmid was transformed into *E. coli* DH5α, isolated, and then subjected to

restriction enzyme digestion. The digested plasmid was analyzed using agarose gel

electrophoresis (Figure 4.3). The bands in lanes 1 and 2 that run to approximately 5600 bp are

pET11d, given that the size of pET11d is 5674 bp and that these bands migrate the same distance

as the band in lane 3 (*Nco*I and *Bam*HI digested pET11d). The bands in lane 1 and 2 that run to

approximately 850 bp are the insert, PCR-amplified INMT, which is 830 bp. These bands also

migrate the same distance as the band in lane 4 (*Nco*I and *Bam*HI digested INMT). It is therefore

likely that the recombinant plasmid contains both the vector (pET11d) and the insert (6x His-

tagged INMT ORF).
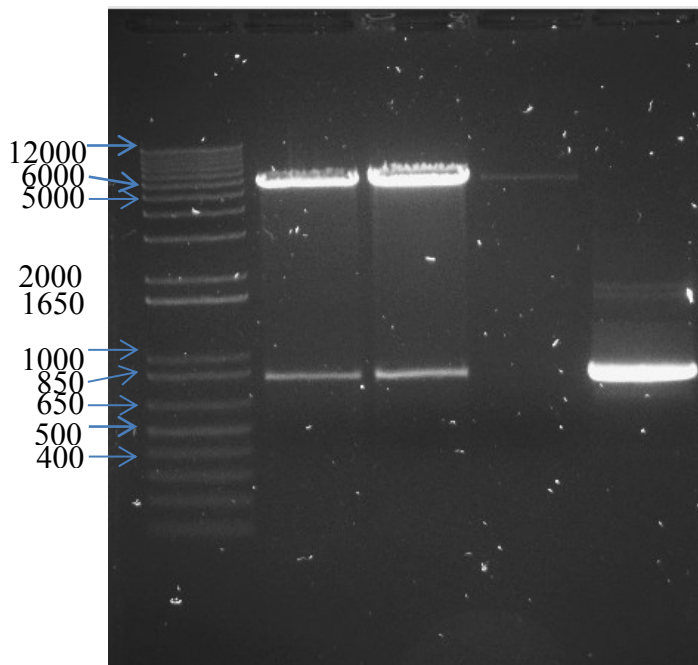
Figure 4.3. 1.0% agarose gel electrophoresis analysis of recombinant plasmid pET11d-INMT

digested with *Bam*HI and *Nco*I. Lane M: TrackIt 1 kb plus DNA ladder; lane 1 and 2: digested

recombinant pET11d-INMT from 2 transformants; lane 3: *Bam*HI and *Nco*I digested pET11d

and lane 4: *Bam*HI and *Nco*I digested INMT.

4.3.2. Sequencing analysis

In order to confirm that the recombinant plasmid had been assembled correctly and that the ORF was in-frame, the plasmid was sequenced and aligned against the *E. coli* optimized INMT coding sequence. The INMT ORF and a small portion upstream were sequenced. Figure 4.4 shows the DNA alignment of the INMT insert against the *E. coli* optimized sequence. The DNA sequence alignment revealed that the INMT ORF is in frame and its sequence conformed to the expected sequence with the addition of 6x His-tag and a stop codon at the C-terminus.

E. coli optimized INMT   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -ATGGAAGGT GGTTTTACCG 19
Sequenced INMT   ATTTTGTTTA ACTTTAAGAA GGAGATATAC CATGGAAGGT GGTTTTACCG 50

E. coli optimized INMT   GTGGTGATGA ATATCAGAAA CATTTTCTGC CTCGCGATTA TCTGAACACC 69
Sequenced INMT   GTGGTGATGA ATATCAGAAA CATTTTCTGC CTCGCGATTA TCTGAACACC 100

E. coli optimized INMT   TATTATAGCT TTCAGAGCGG TCCGAGTCCG GAAGCAGAAA TGCTGAAATT 119
Sequenced INMT   TATTATAGCT TTCAGAGCGG TCCGAGTCCG GAAGCAGAAA TGCTGAAATT 150

E. coli optimized INMT   TAACCTGGAA TGCCTGCACA AAACCTTTGG TCCGGGTGGT CTGCAGGGTG 169
Sequenced INMT   TAACCTGGAA TGCCTGCACA AAACCTTTGG TCCGGGTGGT CTGCAGGGTG 200

E. coli optimized INMT   ATACCCTGAT TGATATTGGT AGTGGTCCGA CCATTTATCA GGTTCTGGCA 219
Sequenced INMT   ATACCCTGAT TGATATTGGT AGTGGTCCGA CCATTTATCA GGTTCTGGCA 250

E. coli optimized INMT   GCATGTGAAA GCTTCAAAGA TATTACCCTG AGCGATTTTA CCGATCGTAA 269
Sequenced INMT   GCATGTGAAA GCTTCAAAGA TATTACCCTG AGCGATTTTA CCGATCGTAA 300

E. coli optimized INMT   TCGTGAAGAA CTGGCAAAAT GGCTGAAAAA AGAACCGGGT GCCTATGATT 319
Sequenced INMT   TCGTGAAGAA CTGGCAAAAT GGCTGAAAAA AGAACCGGGT GCCTATGATT 350

E. coli optimized INMT   GGACACCGGC ACTGAAATTT GCATGTGAAC TGGAAGGTAA TAGCGGTCGT 369
Sequenced INMT   GGACACCGGC ACTGAAATTT GCATGTGAAC TGGAAGGTAA TAGCGGTCGT 400

E. coli optimized INMT   TGGCAAGAAA AAGCAGAAAA ACTGCGTGCA ACCGTTAAAC GTGTTCTGAA 419
Sequenced INMT   TGGCAAGAAA AAGCAGAAAA ACTGCGTGCA ACCGTTAAAC GTGTTCTGAA 450

E. coli optimized INMT   ATGTGATGCA AATCTGAGCA ATCCGCTGAC ACCGGTTGTT CTGCCTCCGG 469
Sequenced INMT   ATGTGATGCA AATCTGAGCA ATCCGCTGAC ACCGGTTGTT CTGCCTCCGG 500

E. coli optimized INMT   CAGATTGTGT TCTGACCCTG CTGGCAATGG AATGTGCATG TTGTAGCCTG 519
Sequenced INMT   CAGATTGTGT TCTGACCCTG CTGGCAATGG AATGTGCATG TTGTAGCCTG 550

E. coli optimized INMT   GATGCATATC GTGCAGCACT GCGTAATCTG GCAAGCCTGC TGAAACCTGG 569
Sequenced INMT   GATGCATATC GTGCAGCACT GCGTAATCTG GCAAGCCTGC TGAAACCTGG 600

E. coli optimized INMT   TGGTCATCTG GTTACCACCG TTACCCTGCA GCTGAGCAGC TATATGGTTG 619
Sequenced INMT   TGGTCATCTG GTTACCACCG TTACCCTGCA GCTGAGCAGC TATATGGTTG 650

E. coli optimized INMT   GTGAACGTGA ATTTAGCTGT GTTGCACTGG AAAAAGAAGA AGTTGAACAG 669
Sequenced INMT   GTGAACGTGA ATTTAGCTGT GTTGCACTGG AAAAAGAAGA AGTTGAACAG 700

E. coli optimized INMT   GCAGTTCTGG ATGCCGGTTT TGATATTGAA CAGCTGCTGT ATAGTCCGCA 719
Sequenced INMT   GCAGTTCTGG ATGCCGGTTT TGATATTGAA CAGCTGCTGT ATAGTCCGCA 750

E. coli optimized INMT   GAGCTATAGC GCAAGCACCG CACCGAATCG TGGTGTTTGT TTTCTGGTTG 769
Sequenced INMT   GAGCTATAGC GCAAGCACCG CACCGAATCG TGGTGTTTGT TTTCTGGTTG 800

E. coli optimized INMT   CACGTAAAAA ACCGGGTAGC - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - 789
Sequenced INMT   CACGTAAAAA ACCGGGTAGC CATCATCACC ATCACCACTA AGGATCCGGC 850

                                                  └———————————┘ └————┘
                                                     6x His-tag   Stop codon

Figure 4.4. DNA sequence alignment of E. coli optimized INMT against the DNA sequence from pET11d-INMT. The alignment was performed using CLC Sequence Viewer 6.

4.3.3. Expression and purification of INMT

pET11d-INMT was transformed into *E. coli* BL21(DE3) in order to generate a strain capable of producing his-tagged INMT. The expression of INMT was induced by the addition of 0.1 mM IPTG and the enzyme was purified by nickel affinity chromatography (Figure 4.5). SDS-PAGE analysis strongly implies that INMT has been expressed and purified. The

appearance of a heavy band at 30 kDa, the reported molecular weight of INMT (Ansher and

Jakoby, 1986; Thompson and Weinshilboum, 1998), in lanes 2 and 3 implies that the

concentration of the expressed protein was increased by the addition of 0.1 mM IPTG. This

same band appeared in the eluted fraction (lane 7), dialyzed fraction (lane 8) and concentrated

protein fraction (lane 9), which suggests that INMT was purified by nickel column affinity

chromatography. These three lanes were all loaded with 1 μg of protein. Secondly, the

appearance of a clear, predominant band in lane 9 suggests that the isolated and concentrated

protein is highly pure. The appearance of a band at the 30 kDa in Lane 3 suggests that some of

the overexpressed protein formed inclusion bodies. For future reference, this issue may be

rectified by inducing with lower concentrations of IPTG or using a lower temperature during

cultivation after IPTG induction.
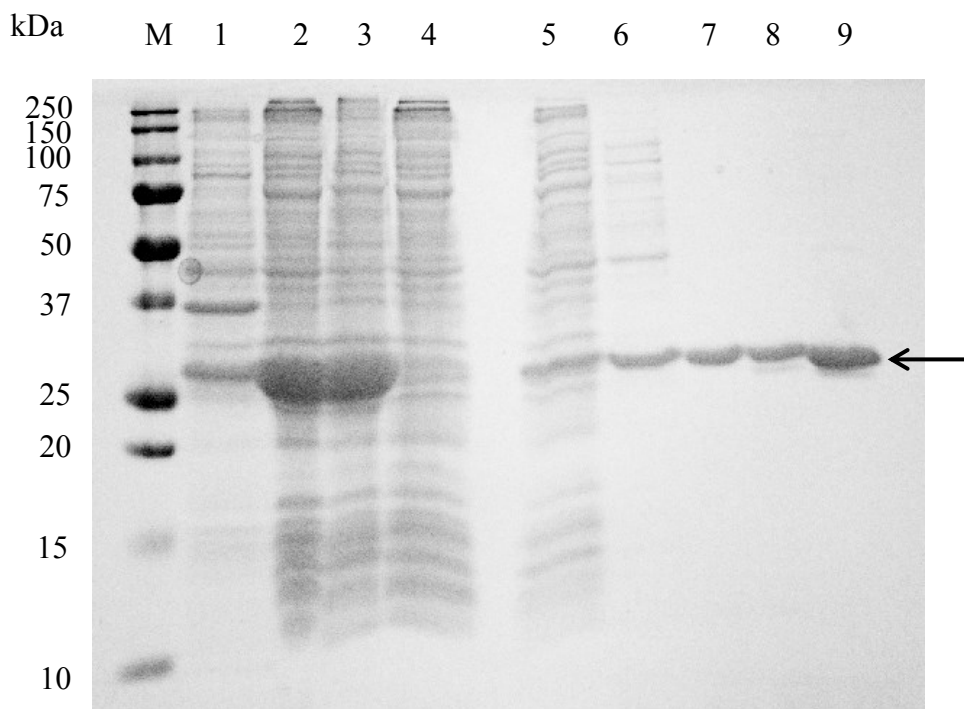
Figure 4.5. SDS-PAGE analysis of INMT during the expression and purification processes. Lane M: Precision Plus All-Blue Protein Ladder; lane 1: 0h after IPTG induction; lane 2: cell lysate (supernatant); lane 3: cell lysate (pellet); lane 4: column flow through; lane 5: wash 1; lane 6: wash 2; lane 7: pooled elution; lane 8: dialyzed protein; lane 9: concentrated protein.

In-gel protein digestion was performed to obtain a partial protein sequence and for protein identification. The protein band isolated from the SDS-PAGE was subjected to proteolytic cleavage using trypsin, which cleaves the peptide bond at the C-terminus of arginine and lysine residues. The results indicate that 25.9% of the INMT protein sequence was covered and 5 distinct peptide sequences were identified. An alignment of the peptides against the reported INMT protein sequence (Figure 4.6) demonstrates that each peptide has consensus with the INMT sequence. The low coverage % may be a result of missed cleavages as trypsin is prone to cleave at the C-terminal end of lysine sites less efficiently than at the C-terminal end of arginine sites (Olsen *et al*., 2004). The top hit reported based on the peptide sequences is INMT

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from rabbit, as expected, with a protein score of 903.7. A high protein score indicates a more confident match. In this case, considering the peptide matches, high protein score and that the DNA sequence is as expected (Figure 4.4), there is strong evidence that the expressed protein from *E. coli* BL21(DE3)-pET11dINMT is INMT.
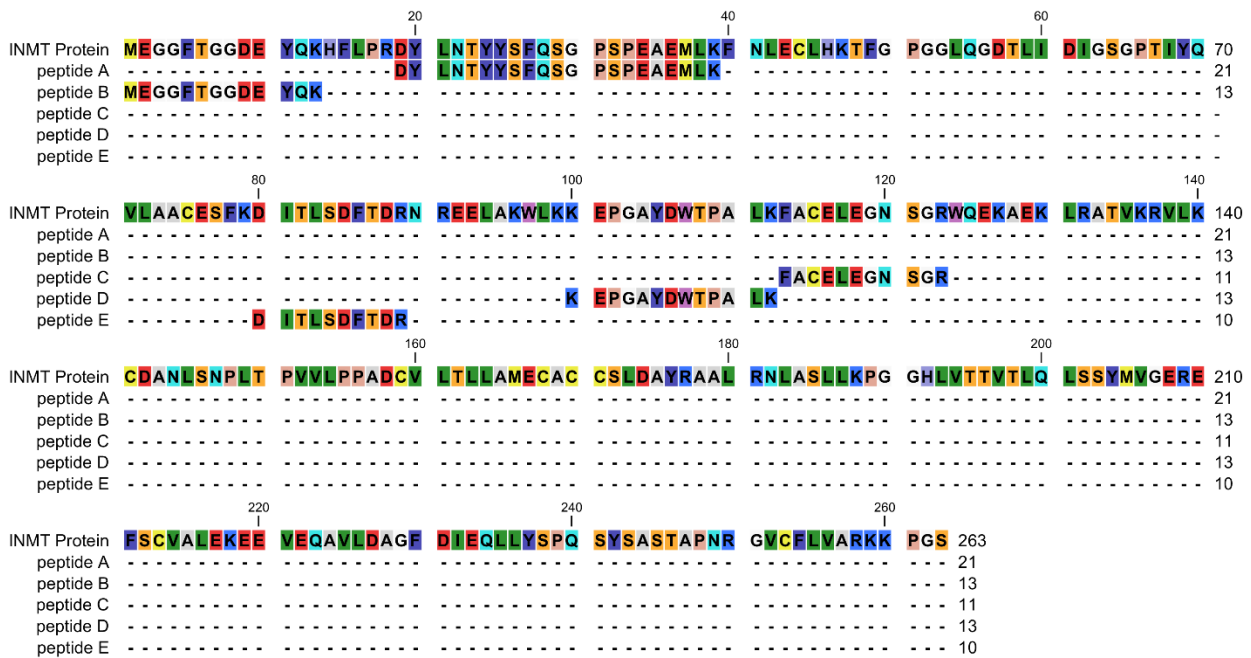
Figure 4.6. Protein sequence alignment of rabbit lung INMT and peptide fragments from in-gel tryptic digestion for protein identification.

4.4. Conclusions

This study demonstrated the feasibility of cloning rabbit lung INMT into *E. coli* BL21(DE3) to create a strain capable of inducible expression of INMT. The results confirmed that a recombinant construct consisting of INMT inserted into a pET11d plasmid was successfully assembled and transformed into *E. coli* BL21(DE3) for expression. Expression of INMT was induced by the addition of IPTG and the purification of INMT was achieved by using nickel affinity chromatography, which was made possible through the addition of a C-terminal

polyhistidine tag on INMT. SDS-PAGE analysis revealed that the expressed INMT has an approximate size of 30 kDa and that the purified protein is highly pure. Partial peptide sequencing analysis provided further evidence that the expressed protein is INMT. While we have demonstrated that INMT can be expressed and purified from *E. coli*. Determination of the methylation activity of the recombinant enzyme and its ability to form the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane will be discussed in Chapter 5.

5. Characterization of purified indolethylamine *N*-methyltransferase

5.1. Introduction

INMT catalyzes the methylation of tryptamine and other indoleamines. This enzyme is believed to play a possible role in the formation of psychoactive compounds and neurotoxins, but its exact biological significance has not been determined. Enzymes are generally functional within a narrow range of conditions; straying outside these conditions leads to decreased performance or inhibition. Enzyme activity is affected by temperature, pH, the presence of inhibitors, and substrate concentration. One of the products of INMT catalysis, *N,N*-dimethyltryptamine is reported to inhibit INMT by competitive inhibition (Ansher and Jakoby, 1986; Thompson and Weinshilboum, 1998). SAH, another product of INMT catalysis is also reported to inhibit INMT (Lin *et al.*, 1973). Typically, elucidation of INMT enzyme activity has been achieved by radiometric enzyme assays by tracking [^{3}H]-SAM or [^{14}C]-SAM (Ansher and Jakoby, 1986; Axelrod, 1962; Mandel *et al.*, 1971; Sitaram *et al.*, 1981). While these assays are very sensitive and specific, they suffer from complicated extraction and separation steps that are laborious and time-consuming. Furthermore, radioactive assays can be dangerous to work with due to potential exposure to radiation. Radioactive assays also require specific training, equipment, storage and disposal, all of which are time-consuming and costly.

Due to the difficulty in analyzing amines, few alternative assays exist to determine INMT activity or SAM-dependent methyltransferases. In general, amines lack natural fluorescence, UV absorbance, or colorimetric properties. This makes direct detection based on these properties nearly impossible without the use of derivitizing agents. Derivatizing agents such as *o*-phthaldialdehyde (OPA) and fluorescine are common for fluorometric detection (Benson and Hare, 1975; Kutlán and Molnár-Perl, 2003; Udenfriend *et al.*, 1970). Acid chlorides such as

dabsyl chloride and dansyl chloride have been used as derivatizing agents for UV-Vis detection (Lin and Lai, 1980; Seiler, 1970). It is worth noting that derivatization methods often involve laborious sample preparation and long analysis times. Additionally, derivatizing agents tend to only be reactive with one or two classes of amines (i.e. primary amines and secondary amines), which makes simultaneous analysis of putrescine (a primary amine) and *N,N,N',N'*-tetramethyl-1,4-diaminobutane (a tertiary amine) using derivatizing agents extremely difficult. Hence an alternative enzyme assay that does not require the use of derivatizing agents or radioactivity is necessary for identifying and quantifying the products of multiple INMT methylation reactions.

The construction of an *E. coli* expression strain capable of producing INMT was explored in Chapter 4. While INMT was expressed and purified from *E. coli*, the activity of this enzyme remained unknown. Therefore it was necessary to determine the activity of INMT *in vitro.* One of the objectives of this study was to develop an analytical method for the quantification of amines without the use of derivatization or radioactivity. Another objective of this study was to establish that INMT purified from a bacterial expression system had activity. The last objective was to determine the activity of INMT with putrescine relative to a model substrate, tryptamine and to determine the number of times INMT could methylate putrescine. Ultimately, we sought to determine if INMT could catalyze the formation of the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane from putrescine.

5.2. Materials and Methods

5.2.1. Materials

The SAM510 methyltransferase assay kit that was used to elucidate the activity of INMT was purchased from G-Biosciences (St. Louis, MO, USA).

Trifluoroacetic acid, putrescine, tryptamine hydrochloride, *N*-methyltryptamine, *S*-adenosyl-L-methionine dihydrochloride, 7-methyltryptamine, and *N*-methyl-1,3-diaminopropane were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and water were obtained from Fisher Scientific (Dubuque, IA, USA). *N*-methylputrescine dihydrochloride was purchased from Toronto Research Chemicals (Toronto, ON, Canada).

5.2.2. INMT enzyme assay

The production of methylated compounds (*N*-methyltryptamine, *N*-methylputrescine or *N,N,N',N'*-tetramethyl-1,4-diaminobutane) was determined in order to assess the activity of INMT. The enzyme reactions were set up in microfuge tubes that contained various concentrations of tryptamine or putrescine, *S*-adenosyl-L-methionine and 25 mM Tris-HCl, pH 8.5. The enzyme reactions were incubated at 37ºC in a water bath for four hours and then terminated with the addition of 25 µL of glacial acetic acid by pH change. The solutions used for enzyme reactions were filtered through 0.22 µm filters (Mandel Scientific, Guelph, ON, Canada) prior to analysis on HPLC or GC.

5.2.3. Analytical methods

5.2.3.1. HPLC

The HPLC column, Primesep 200 (4.6 mm x 250 mm), and guard column were purchased from SIELC Technologies (Prospect Heights, IL, USA).

Tryptamine hydrochloride, *N*-methyltryptamine, putrescine, *N*-methylputrescine dihydrochloride and *N,N,N',N*-tetramethyl-1,4-diaminobutane concentrations were determined by HPLC (1200 series, Agilent Technologies, Mississauga, ON, Canada). The method utilized for separating the amines was previously described in section 3.2.6.

Two different types of detectors were used for the detection of amines: an evaporative light scattering detector (ELSD, Agilent Technologies, Mississauga, ON, Canada) and a diode array detector (DAD, Agilent Technologies, Mississauga, ON, Canada). The former is a universal detector and the latter is a UV detector capable of measuring multiple wavelengths at once. The ELSD was operated with following parameters: the nebulizer gas (N_2) was maintained at 3.5 bar, the gain set at 5, and the drift tube temperature set at 40ºC. The DAD was set to the following wavelengths: 258 nm and 280 nm for the detection of SAM/SAH and tryptamine/*N*-methyltryptamine, respectively.

Standard solutions consisted of a combination of tryptamine hydrochloride, *N*-methyltryptamine, putrescine, *N*-methylputrescine dihydrochloride or *N,N,N',N'*-tetramethyl-1,4-diaminobutane at different concentrations to generate a standard curve.

5.2.3.2. Liquid chromatography - mass spectrometry detection (LC-MS)

Liquid chromatography with mass spectrometry detection (LC-MS) was used for compound identification for the enzyme assay samples. These experiments were performed on an Agilent 1200 LC system (Agilent Technologies, Palo Alto, CA, USA) coupled with a QStar® Elite hybrid orthogonal Q-TOF (time of flight) mass spectrometer (Applied Biosystems, Concord, ON, Canada). The column and LC conditions used are the same as described above in section 3.2.6. The mass spectrometer was operated in electrospray positive ionization mode and the conditions used were: curtain gas setting 25; gas 1 setting 40; gas 2 setting 60; declustering potential (DP) 40 V; focusing potential (FP) 150.

5.2.3.3. GC-Flame Ion Detection (FID)

Enzyme assays were also analyzed by GC (7890A, Agilent Technologies, Mississauga, ON, Canada) equipped with an autosampler (7693, Agilent Technologies, Mississauga, ON, Canada) and Rtx-5 Amine column (30 m x 0.25 mm x 0.50 µm; Restek, Brockville, ON, Canada) and flame ionization detector (FID). Samples were injected at a volume of 1 µL in split mode (10:1) at 305ºC. The oven was programmed as follows: initial temperature at 120ºC, increased at a rate of 10ºC/min until the temperature reached 280ºC. This temperature was held for 3 minutes for a total run time of 20 minutes. Helium was used as the carrier gas.

Two internal standards were selected: 7-methyltryptamine for the quantification of *N*-methyltryptamine and *N*-methyl-1,3-diaminopropane for the quantification of *N*-methylputrescine. Both compounds were chosen for as internal standards since they are not found in the sample and have structurally similarity to the compounds of interest.

5.2.4. SAM510 methyltransferase assay

The reaction scheme of the SAM510 methyltransferase assay is outlined in Figure 5.1.

Briefly, 5 µL of putrescine or tryptamine was loaded into the appropriate wells to reflect a final

concentration of 1 mM. Approximately 500 µg of enzyme was loaded into each well and the

enzyme reaction was initiated by the addition of 100 µL of a master mix that was pre-incubated

to 37°C; the master mix contained enzyme cocktail, colorimetric reagent, 0.1 M Tris-HCl, pH 8.0

buffer, and *S*-adenosyl-L-methionine. The absorbance (510 nm) was measured at 37°C every

minute using a microplate reader (Synergy Mx, BioTek, Winooski, VT, USA). The absorbance

at 510 nm vs time (min) was plotted to determine the slope (ΔA_{510nm}/min) of the linear portion of

the graph. Using the following equation (1), the rate of the enzyme reactions occurring was

calculated as follows:

$$\text{Activity (µmol/min/ml)} = \frac{\Delta A_{510}/min}{15.0\ mM^{-1}} \times \frac{total\ volume\ (ml)}{volume\ of\ enzyme\ loaded\ (ml)} \times sample\ dilution \quad (1)$$

One unit of activity is defined as the transfer of 1.0 µmol of a methyl group per minute at 37°C.
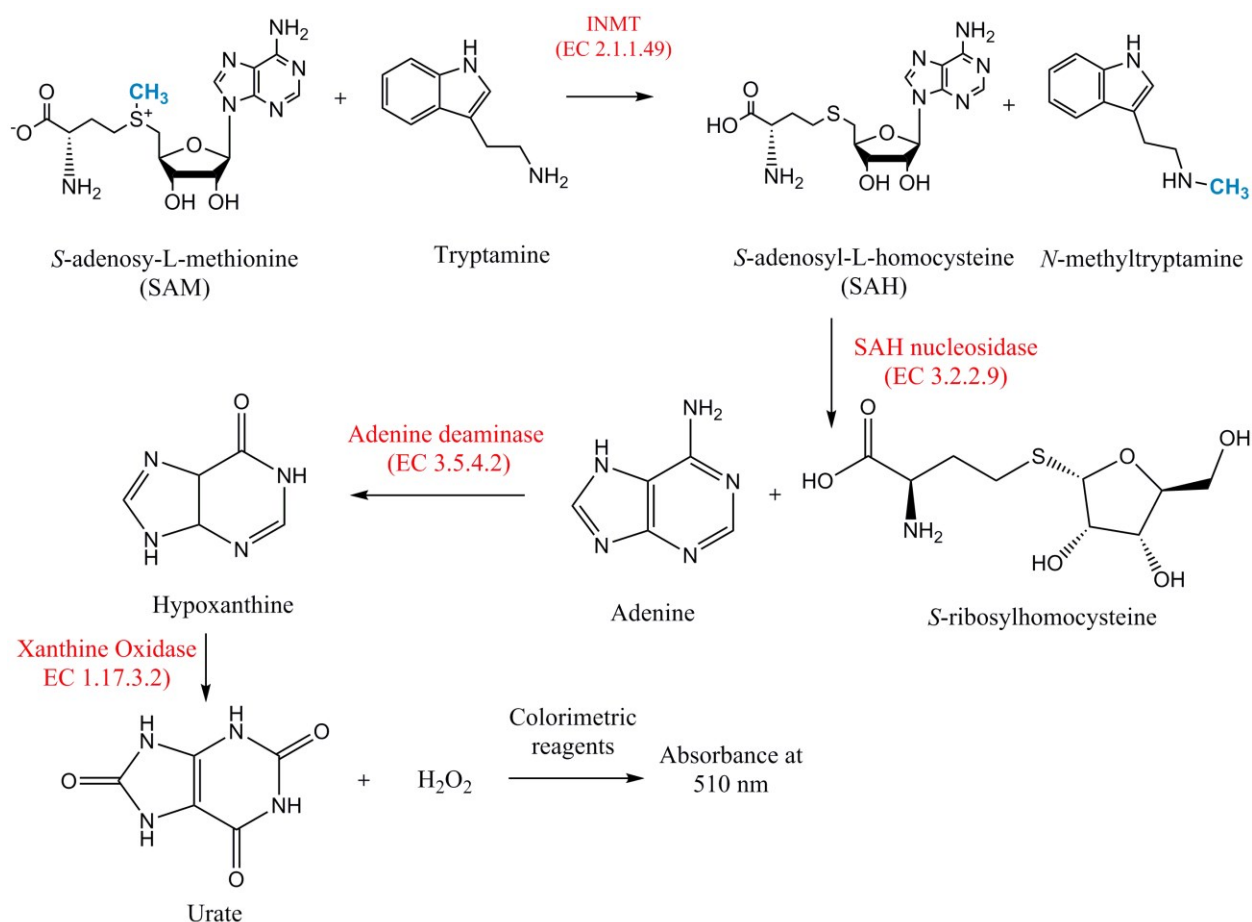
Figure 5.1. Reaction outline of SAM510 methyltransferase kit assay (G-Biosciences, SAM510 Methyltransferase Assay).

5.3. Results and Discussion

5.3.1. Analysis of INMT enzyme activity using HPLC-ELSD

Axelrod (1962) first reported a radioactive enzyme assay for determination of INMT activity. This method has largely remained the primary one for determining INMT activity (Ansher and Jakoby, 1986; Thompson and Weinshilnoum, 1998). As a starting point, initial attempts to elucidate recombinant INMT activity purified from *E. coli* were performed using the commonly reported reaction conditions in the literature, typically in 25 mM Tris HCl buffer at pH 8.5, 1 mM tryptamine, 34 µM [³H]-SAM carried out at 37° C. The analysis of the enzyme

reactions was performed with HPLC-ELSD rather than radioactivity due to health concerns associated with use of radioactive substrates. Preliminary results revealed no peaks corresponding to the methylated products, *N*-methyltryptamine and *N,N,N',N'*-tetramethyl-1,4-diaminobutane, which indicates either the purified enzyme has no activity or the amount of methylated products is below the detection limit of HPLC-ELSD.

In an attempt to determine if the absence of product peaks in the HPLC-ELSD chromatograms was due to the detection limit of the detector, the concentrations of the substrates tryptamine and SAM were increased to 50 mM each. This was also done to increase the ratio of SAM to tryptamine to 1:1. The previous ratio tested reflected a 0.34:1 ratio of SAM to tryptamine, as conducted in literature (Axelrod, 1962; Ansher and Jakoby, 1986). By increasing the ratio of SAM to tryptamine, more SAM is made available for the methyl transfer reaction. This would hypothetically translate into increased conversion of tryptamine to *N*-methyltryptamine. However, upon analyzing the enzymatic reactions using the HPLC-ELSD, no peak corresponding to *N*-methyltryptamine was observed. Again, these results suggest that either INMT purified from *E. coli* was inactive or the products were at a concentration below the detection limit of ELSD. The results do not offer insight into which possibility is more likely. Other possibilities that could explain the absence of *N*-methyltryptamine include: a 1:1 ratio of SAM to tryptamine was not sufficient to form *N*-methyltryptamine via INMT catalysis, the enzyme loading was not high enough, or the C-terminal polyhistidine tag may have interfered with protein folding, which could affect activity.

5.3.2. Enzyme assay with HPLC-UV

Since the previous experiments failed to address whether the absence of *N*-methyltryptamine and *N,N,N',N*-tetramethyl-1,4-diaminobutane was due to an issue with the enzyme itself or the analytical method, the HPLC method was modified to use UV detection in place of ELSD. UV is a much more sensitive detector than ELSD. Therefore, if methylated products were detected, it would suggest that the purified INMT has activity. While UV detection is not suitable for detection of underivatized aliphatic amines such as putrescine, it is possible to monitor the reaction of tryptamine with SAM as tryptamine and *N*-methyltryptamine absorb at 280 nm. Additionally, the appearance of the co-product *S*-adenosyl-homocysteine (SAH), which is produced from the demethylation of SAM, may also indicate that the purified INMT has activity. SAM and SAH both absorb at 258 nm, but it is possible to distinguish between the two compounds that share the same absorption due to differences in retention time.

The enzymatic reactions were set up with 10 mM tryptamine and 10 mM SAM to reflect a 1:1 ratio of methyl group acceptor to methyl group donor. HPLC-UV analyses revealed a peak corresponding to *N*-methyltryptamine (Figure 5.2A). As expected, *N*-methyltryptamine was not detected in the control without INMT (Figure 5.2B), which means that the appearance of the *N*-methyltryptamine in Figure 5.2A can be attributed to INMT enzyme activity. Identification of tryptamine and *N*-methyltryptamine was done by comparing their retention times to an amine standard solution where tryptamine elutes at 20.5 min and *N*-methyltryptamine elutes at 23 min (Figure 5.3).The detection of *N*-methyltryptamine using an UV detector but not an ELS detector confirmed two things: first, that the purified INMT from the *E. coli* expression strain has been demonstrated to have activity and second, that the products of INMT enzyme catalysis are not produced in a high enough quantity that ELSD can detect.
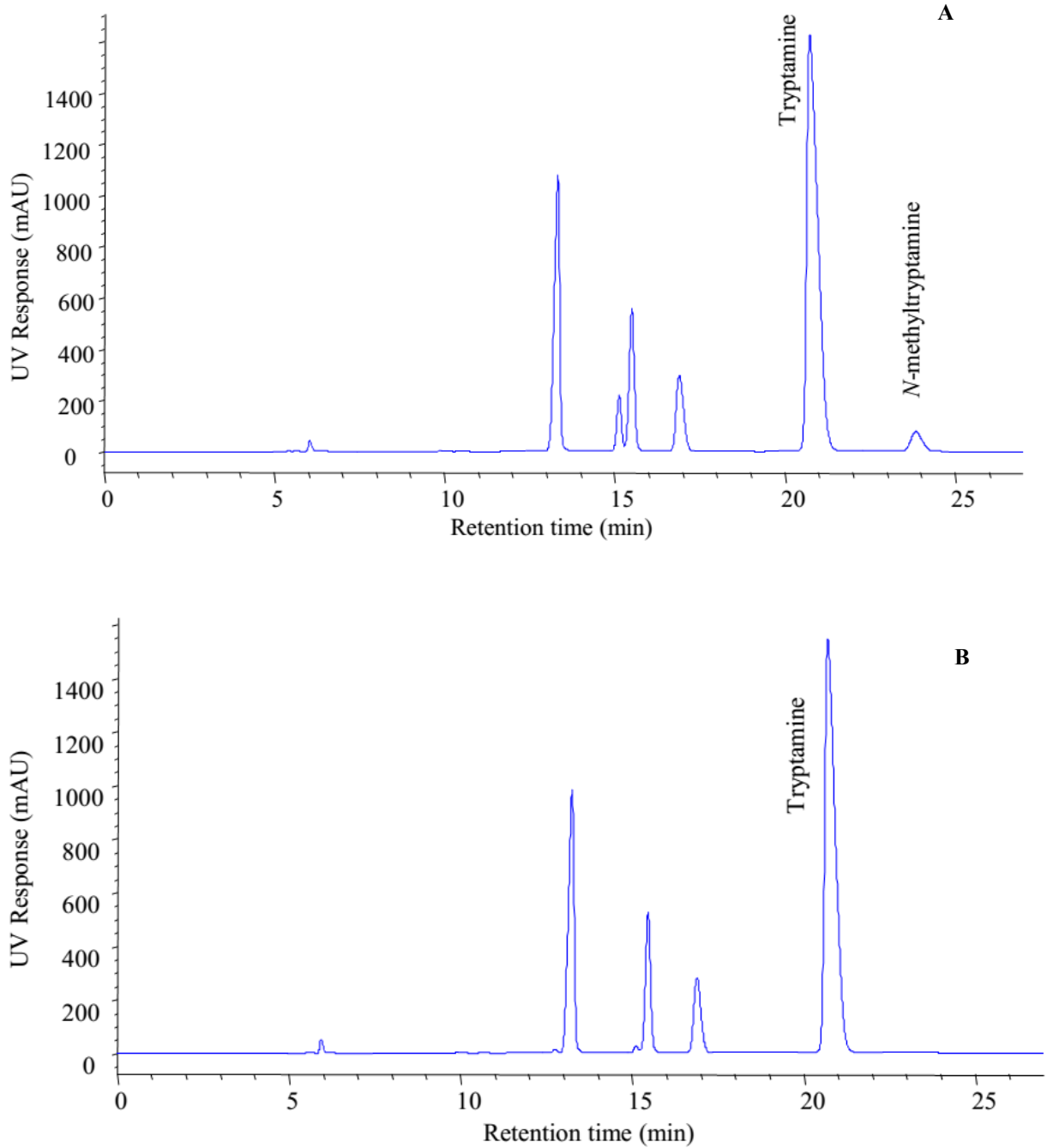
Figure 5.2. HPLC-UV chromatogram of INMT enzyme reactions carried out at 37°C for 4 hours in 25 mM Tris-HCl, pH 8.5 with A) 10 mM tryptamine and 10 mM SAM and B) control without INMT.
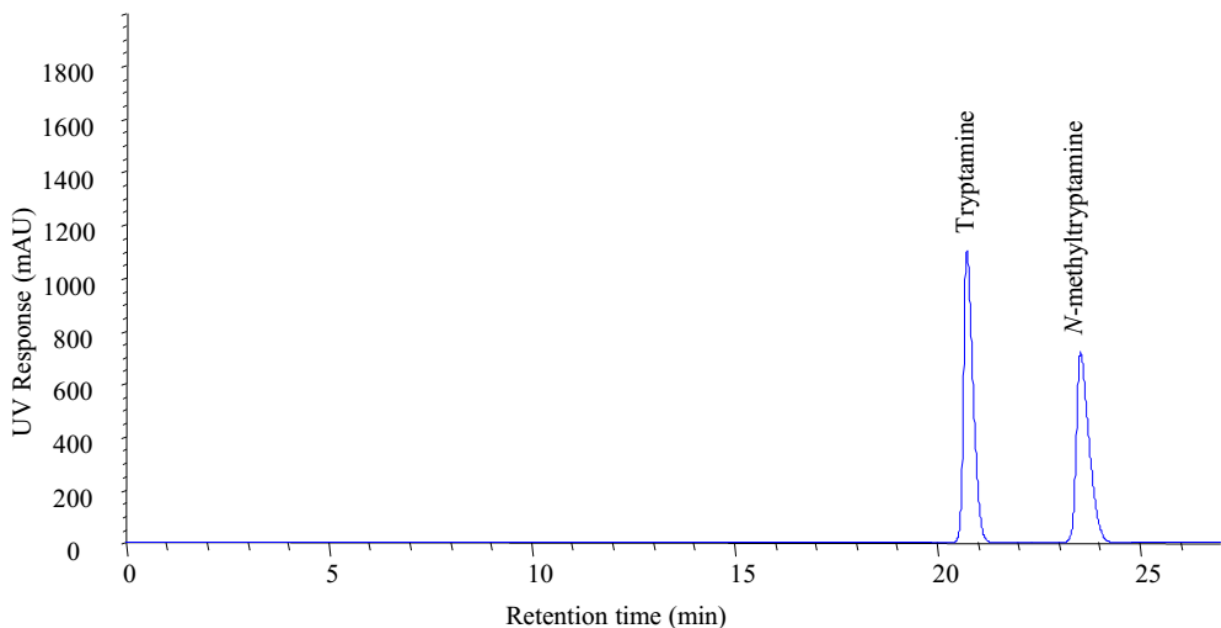
Figure 5.3. HPLC-UV chromatogram an amine standard solution containing tryptamine and *N*-methyltryptamine standards.

Four peaks eluted between the retention time period from 12 to 18 minutes. Analysis of standards suggests that SAH elutes at 15.1 minutes (Figure 5.4A), corresponding to the second eluting unknown peak in the samples. The SAM standard chromatogram reveals three peaks (Figure 5.4B). The largest peak eluting at 17 minutes is hypothesized to be SAM as it is the largest peak, the smallest peak eluting at 15.1 minutes is likely SAH, but the peak eluting at 15.5 minutes is unknown. The presence of an unknown compound and SAH in the SAM standard may be a result of SAM degradation. LC-MS analysis was conducted in section 5.3.4 (described below) to identify these unknown compounds.

Figure 5.4. HPLC-UV chromatograms (wavelength = 258 nm) of standards A) SAH and B) SAM.

5.3.3. LC-MS analysis

Enzyme assay samples were subjected to LC-MS analysis in order to provide additional evidence that the peaks thought to be tryptamine, *N*-methyltryptamine, SAM, and SAH found in the HPLC-UV chromatograms were in fact the said compounds. Compound identification by LC-MS was done by comparing the mass to cha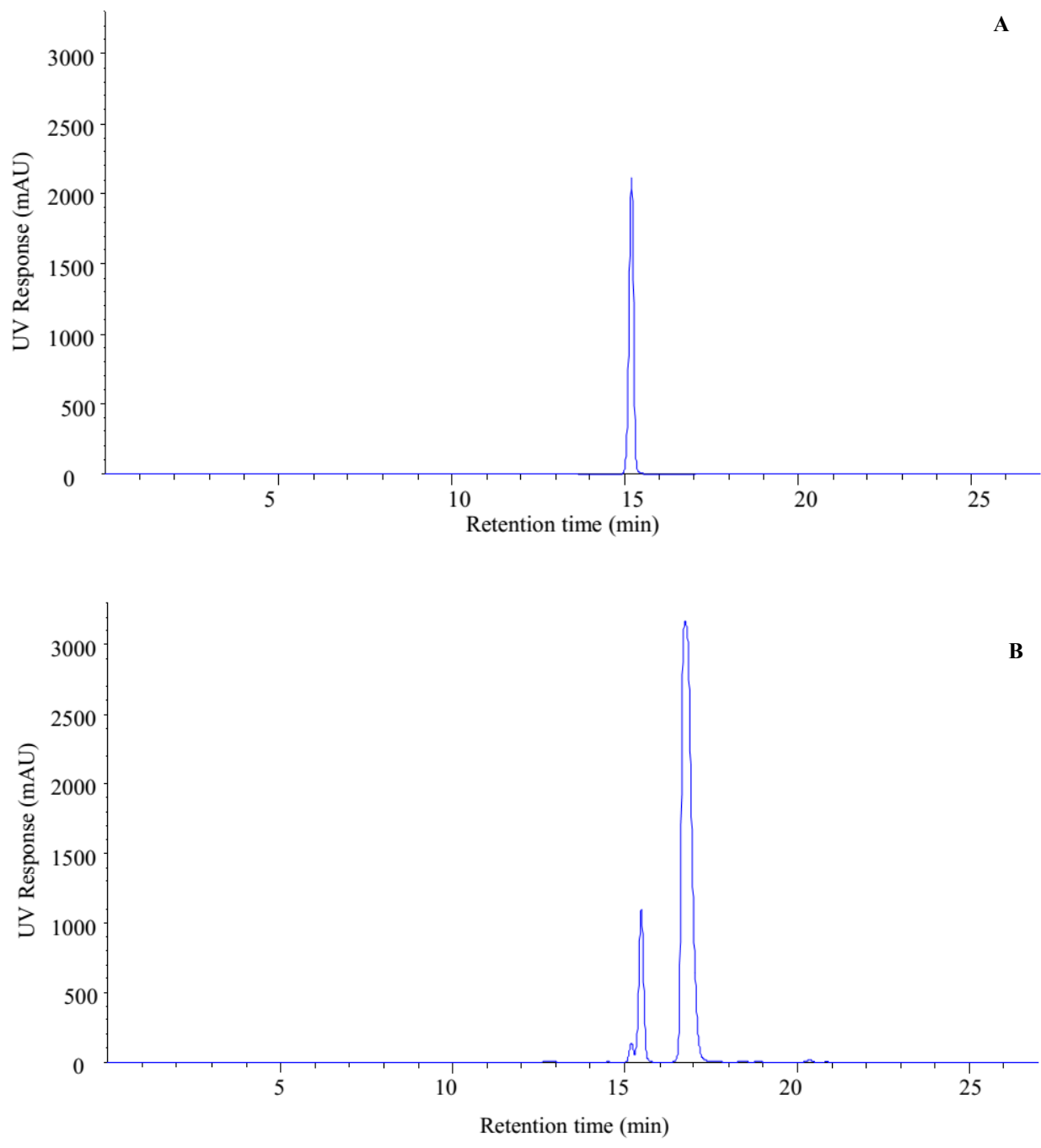rge ratio (m/z) of the protonated molecular ion ($[M+H]^+$) to the molecular weight of the compound in question. The fragmentation pattern was also used to help to determine compound identity, in some cases. The mass spectrum of tryptamine (Figure 5.5) showed two main peaks. The peak at m/z = 161.1 reflects the protonated molecular ion of tryptamine and the peak at m/z = 144.1 is a result of α cleavage of tryptamine (Chen *et al.*, 2007). Similarly, the mass spectrum for *N*-methyltryptamine (Figure 5.6) showed the protonated molecular ion (m/z = 175.1) and two additional peaks of significance at m/z= 144.1 and 132.1 that are a result of α and β cleavage, respectively (Chen *et al.*, 2007). The mass spectrum of SAH reveals a major peak at m/z = 385.1, the protonated molecular ion. No notable fragmentation pattern was observed for SAH and the observed peaks at m/z = 170.1 and 193.1 are not considered significant due to random rearrangements (Figure 5.8). The mass spectra of SAH and *N*-methyltryptamine confirmed the presence of these compounds in the enzyme samples as their protonated molecular ions ($[M+H]^+$) were observed.

LC-MS analysis was also conducted to determine the identities of the unknown compounds detected in the SAM standard by HPLC-UV. Three peaks were detected in the SAM standard (Figure 5.4B). The first peak is believed to be SAH, since its retention time matched the SAH standard (Figure 5.4A). The corresponding LC-MS peak mass spectra revealed that its fragmentation pattern matched the mass spectra of SAH standard (Figure 5.8) and is therefore SAH. The third peak in the SAM standard chromatogram is believed to be SAM itself. The

corresponding LC-MS peak mass spectrum is shown in Figure 5.7. Three main fragments were observed: m/z = 399.1 reflected the molecular ion of SAM with two additional cleavages resulted in peaks at m/z = 298.1, which yielded methylthioadenosine, and m/z = 136.1, which yielded adenosine. This compound is therefore SAM. The identity of the second peak in the SAM standard (Figure 5.4B) was unknown, but thought to be a product of SAM degradation as SAM is quite unstable (Parks and Schlenk, 1958). Analysis of the corresponding LC-MS peak revealed two major peaks: m/z = 298.1, likely the molecular ion, and m/z = 136.1. Both of the peaks were observed in the SAM mass spectra (Figure 5.7), which suggests that this compound was a result of SAM degradation. Based on the fragmentation pattern of SAM and the molecular ion charge, this compound is likely methylthioadenosine, which has been reported to be a product of SAM instability (Parks and Schlenk, 1958).

The compounds appeared to elute approximately 2 minutes earlier in the LC-MS TOF mass spectra when compared to the HPLC-UV chromatograms. This may be due to using a different instrument for analyses. However, given that the mass spectra of these compounds are almost identical to their respective standards, it is very likely we have successfully identified *N*-methyltryptamine and *S*-adenosyl-L-homocysteine, the products of INMT catalysis, as well as the unknown compounds in the SAM standard. These studies demonstrate that we have developed a HPLC-UV method for the detection of methylation of tryptamine and confirmed that the purified enzyme from an *E. coli* expression system has activity. This method was also adapted for LC-MS for product identification.

Figure 5.5. LC-MS TOF mass spectrum of ions extracted from the enzyme assay sample peak that correspond to tryptamine (t = 18.391 to 18.859 min).
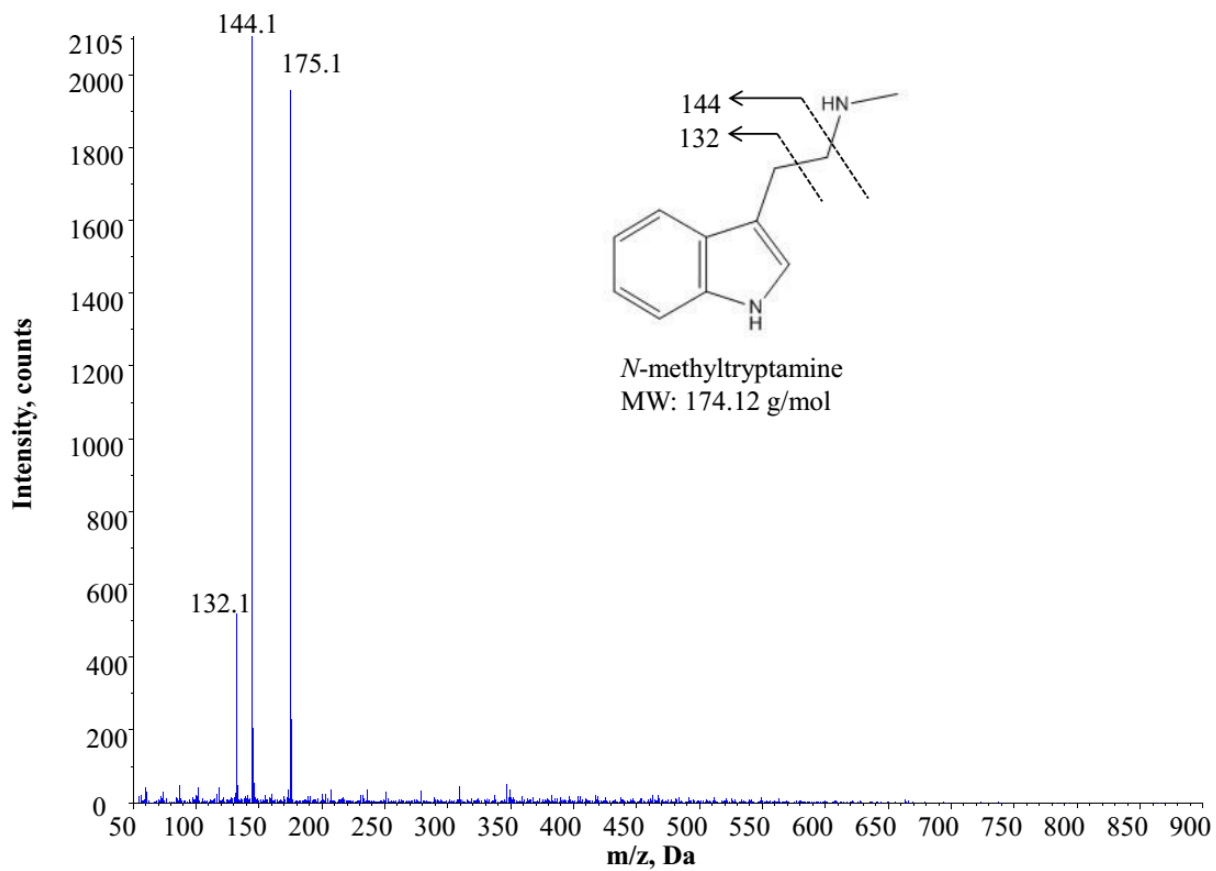
Figure 5.6. LC-MS TOF mass spectrum of ions extracted from the enzyme assay sample peak that correspond to *N*-methyltryptamine (t = 21.112 to 21.345 min).
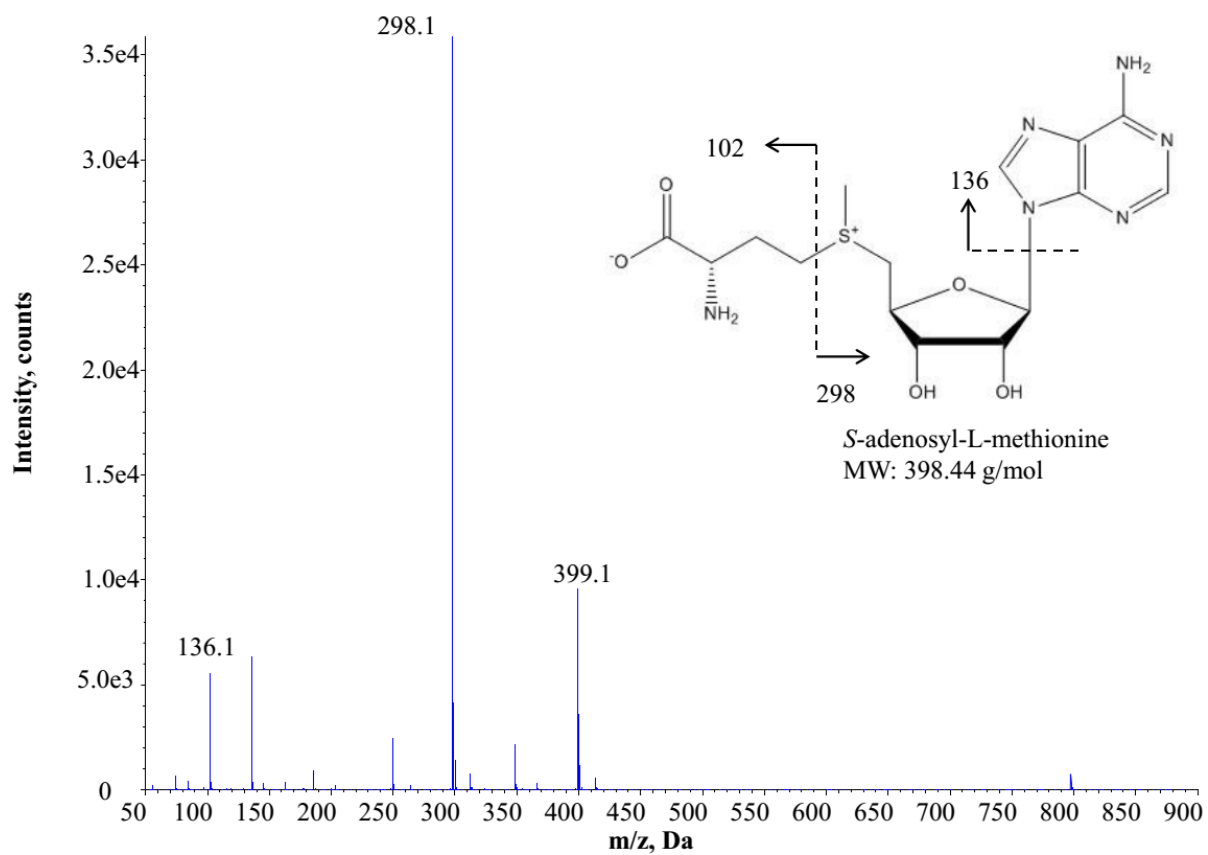
Figure 5.7. LC-MS TOF mass spectrum of ions extracted from the enzyme assay sample peak that correspond to *S*-adenosyl-L-methionine (t = 14.836 to 15.220 min).
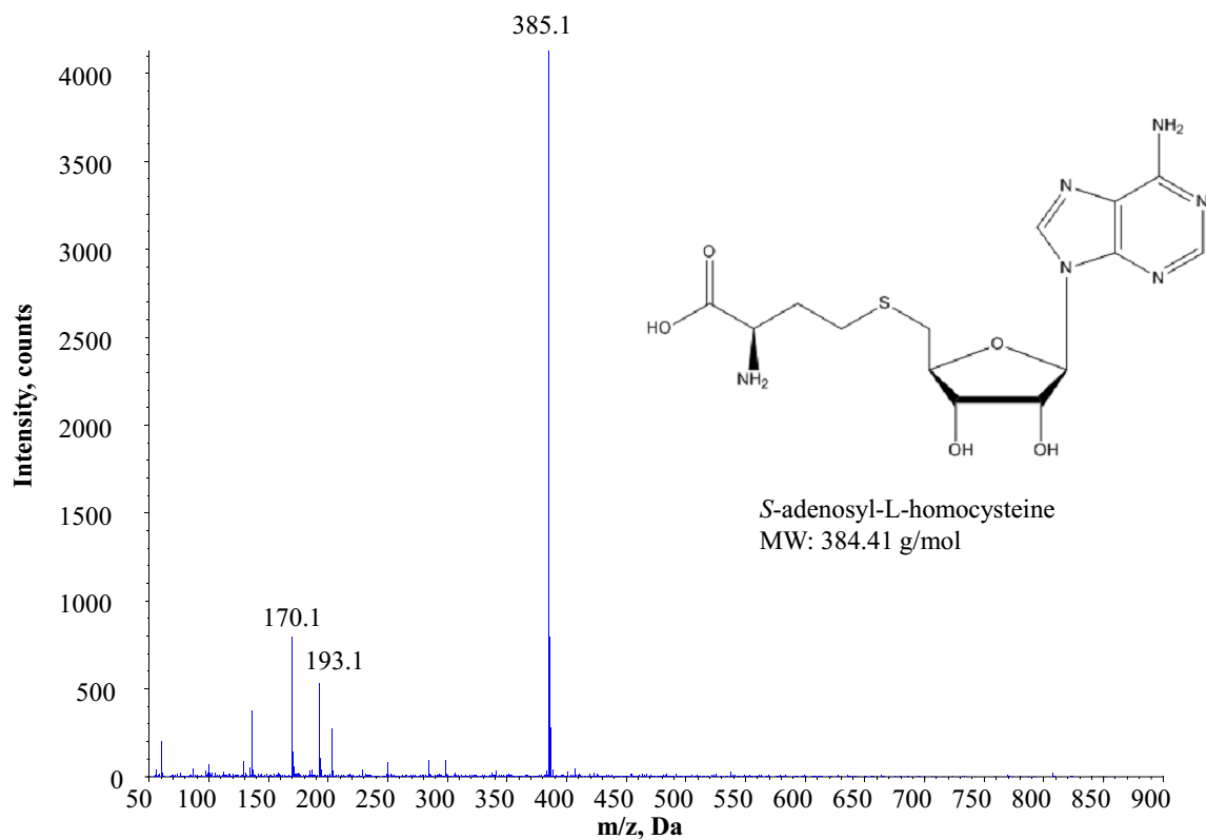
Figure 5.8. LC-MS TOF mass spectrum of ions extracted from the standard solution peak that correspond to *S*-adenosyl-L-homocysteine (t = 13.401 to 13.518 min).
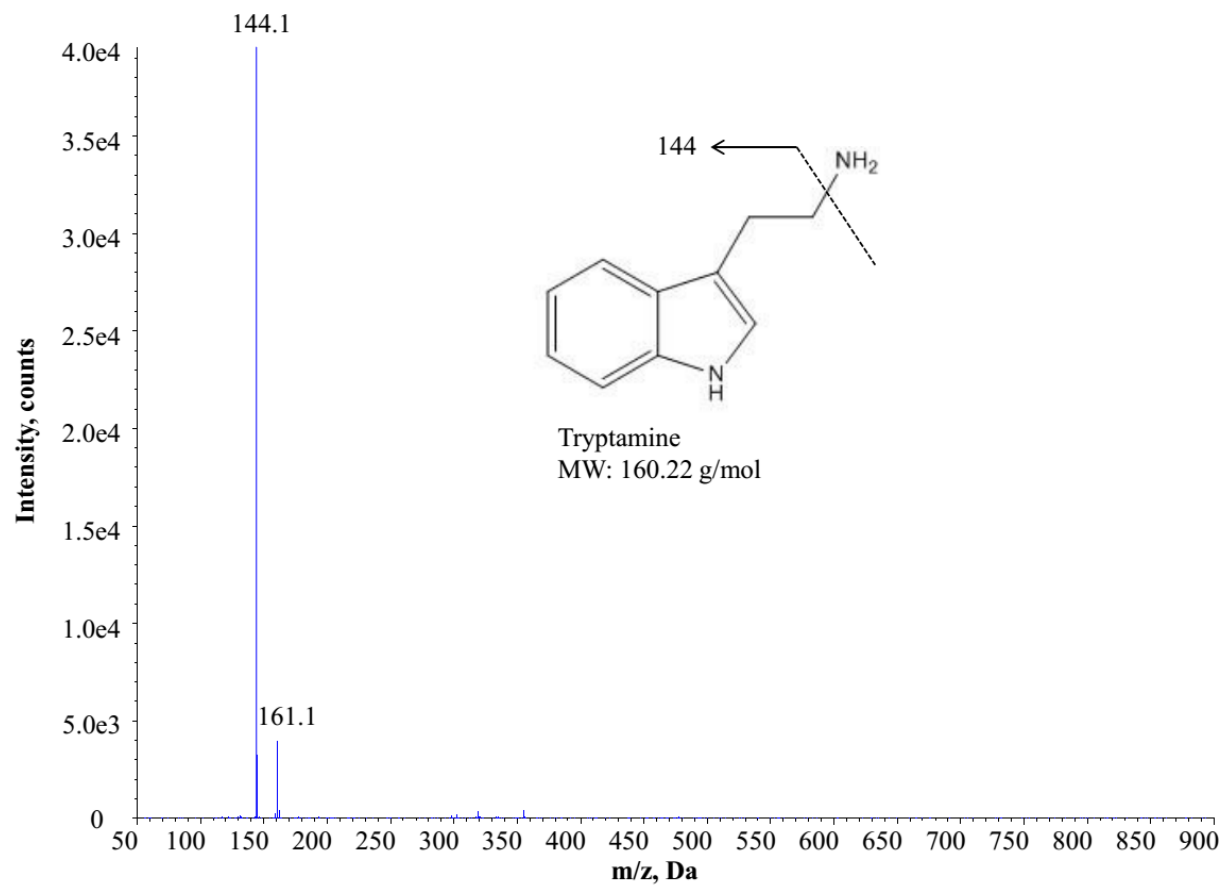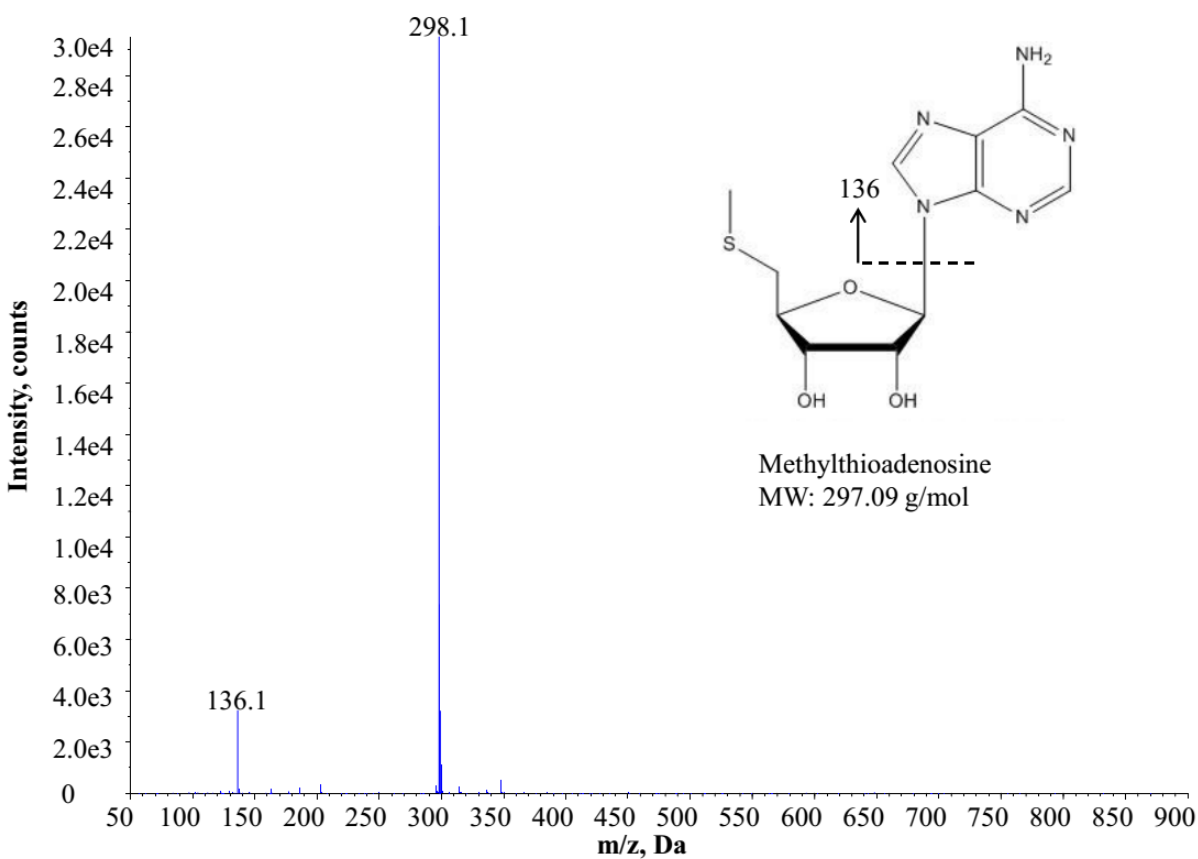
Figure 5.9. LC-MS TOF mass spectrum of ions extracted from the enzyme assay sample peak that correspond to methylthioadenosine (t = 13.685 to 14.002 min).

5.3.4. SAM510 methyltransferase enzyme assay

Recently, indirect, coupled colorimetric assays have been developed for the elucidation of SAM-dependent methyltransferases, including INMT (Dorgan *et al*., 2006; Hendricks *et al*., 2003). These assays have the benefit of providing fast, continuous monitoring of the reaction by means of absorbance. Moreover, the degradation of SAH relieves product inhibition of SAM-dependent methyltransferases. The activity of INMT was measured using a SAM510 methyltransferase assay kit (G-Biosciences, St. Louis, MO, USA). The kit is a universal SAM-

dependent methyltransferase kit, therefore it should demonstrate if the recombinant INMT purified from *E. coli* has activity on tryptamine and/or putrescine.

The activity of INMT was tested with 1 mM tryptamine and 1 mM putrescine (Figure 5.10). The measured activity of the enzymes in the SAM510 kit on tryptamine and putrescine were: 29.6 ± 4.8 nmol/min/ml and 23.9 ± 9.3 nmol/min/ml, respectively. The control without INMT (Figure 5.10C) showed that the activity due to the other components of the kit is negligible. It is worth noting that the calculated enzyme activity is not a true representation of the activity of INMT, but rather reflects the activity of the four enzymes active in the SAM510 kit (Figure 5.1). Generally, enzyme reactions do not occur with 100% efficiency and because the measured absorbance is dependent on the final amount of hydrogen peroxide produced by the four enzymatic reactions, the calculated enzyme activity is an estimation of INMT activity. Statistical analysis revealed that there was no significant difference ($p > 0.05$) in enzyme activity between either methyl group acceptors, which suggests that putrescine can serve as a methyl group acceptor. However, the INMT enzyme activity varied greatly when putrescine was tested as a methyl group acceptor. In general, the nature of microplate enzyme assays can lead to variation in results (i.e. small volumes and air bubbles), but the enzyme activity with putrescine as a substrate resulted in substantially large error bars. It may be necessary to modify the enzyme assay conditions in the future to reduce the variability when analyzing putrescine as a substrate for INMT. The results indicated that both tryptamine and putrescine acted as methyl group acceptors for INMT, and that the former is a more suitable substrate for INMT, which is expected given that tryptamine is the prototypic substrate. Because this assay monitors enzyme activity indirectly, it is difficult to pinpoint exactly why the enzyme activity with putrescine was lower. There are many possibilities for the lower activity observed with putrescine. One of the

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methylated forms of putrescine may be affecting the downstream enzyme reactions. For example, *N*-methylputrescine or *N,N*-dimethylputrescine could inhibit INMT, one of the enzymes in the kit, or the colorimetric reagent used in the assay. Another explanation is that INMT may have a lower affinity for putrescine.

While the SAM510 methyltransferase microplate enzyme assay provided evidence that INMT purified from a bacterial expression system has activity, it provided no information about which products were formed or the number of times tryptamine or putrescine were methylated. Therefore, whether INMT can methylate putrescine to form *N*-methylputrescine and *N,N,N',N'*-tetramethyl-1,4-diaminobutane still needs to be determined.
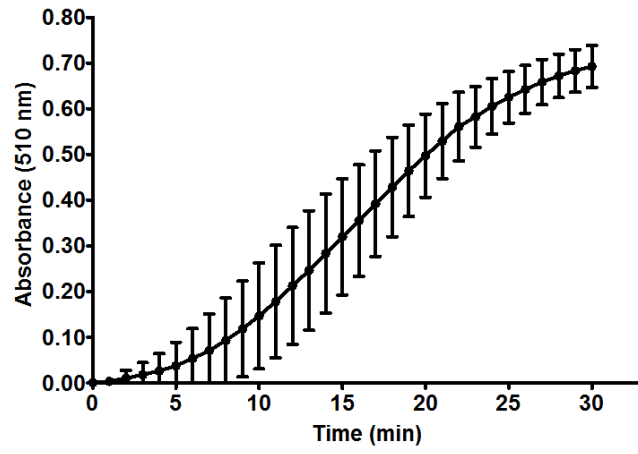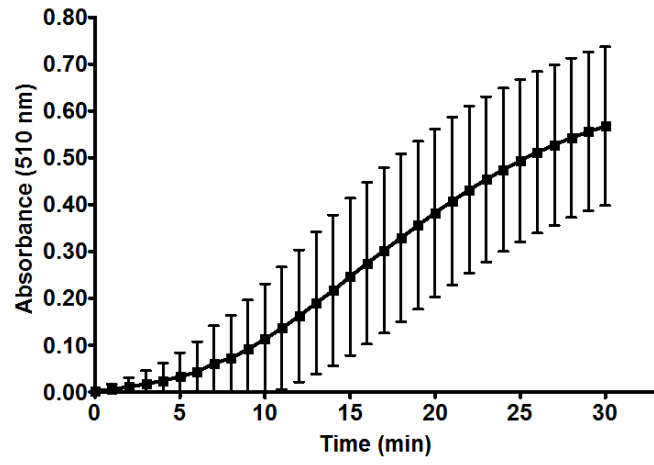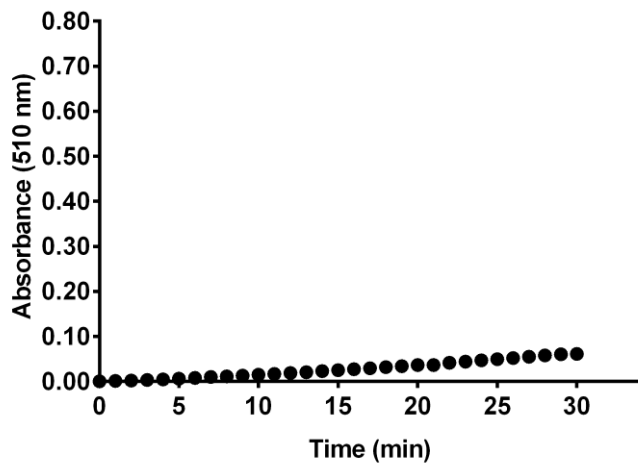
Figure 5.10. SAM510 methyltransferase enzyme assay to determine the activity of INMT with

A)1 mM tryptamine, B) 1 mM putrescine as substrates and C) control without INMT.

5.3.5. GC analysis

Previous INMT enzyme assays performed in this chapter have established that HPLC-ELSD is not sensitive enough for the detection of *N,N,N',N'*-tetramethyl-1,4-diaminobutane. It is worth considering that while *N,N,N',N'*-tetramethyl-1,4-diaminobutane may not be produced by INMT or detectable by HPLC-ELSD, it does not necessarily mean that the enzyme does not have activity on putrescine, as its formation requires four transmethylation reactions to occur. It is possible that INMT is catalyzing the formation of *N*-methylputrescine from putrescine and SAM, but product inhibition may be preventing the formation of *N,N,N',N'*-tetramethyl-1,4-diaminobutane. GC analysis of INMT enzyme assay samples was chosen as it is a highly sensitive technique and derivatization could be avoided by using a Restek Rtx®-5 column. This column is base-deactivated, which allows for analysis of amines without peak tailing and better peak shapes and resolution.

Figure 5.11 displays the structures of the internal standard, 7-methyltryptamine, and the compound of interest, *N*-methyltryptamine. Figure 5.12 displays the elution order of the amine standards. Tryptamine elutes at 12.4 min, followed by *N*-methyltryptamine and 7-methyltryptamine at 12.8 min and 13.2 min, respectively. Figure 5.13A clearly demonstrates the methylation activity of INMT with tryptamine as a substrate. The peak eluting at 12.8 minutes matches the elution time of *N*-methyltryptamine in Figure 5.12 and in addition, the control without INMT lacks the *N*-methyltryptamine peak. The amount of *N*-methyltryptamine produced by INMT was 0.24 ± 0.03 nmol. Hence, INMT catalyzed the formation of *N*-methyltrypamine.

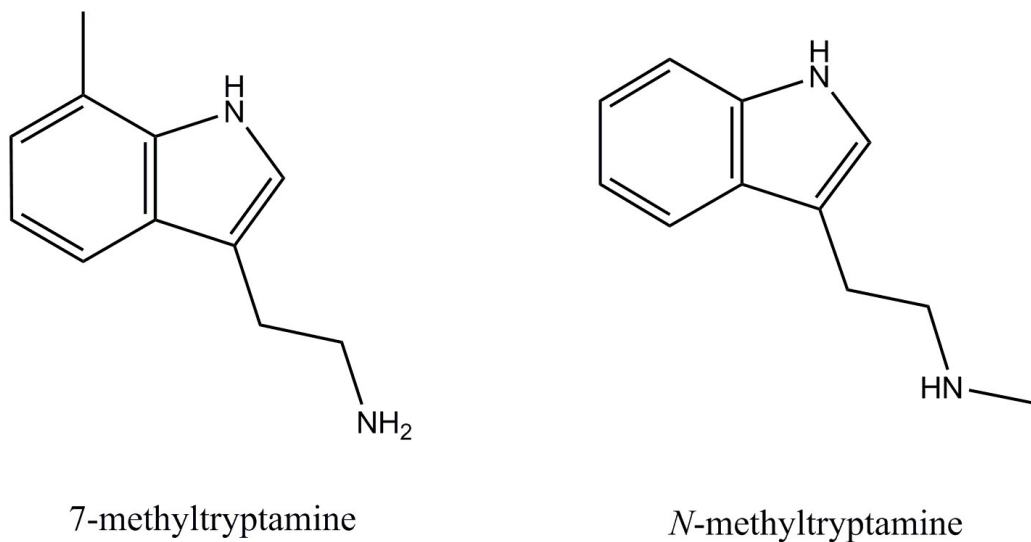7-methyltryptamine   N-methyltryptamine

Figure 5.11. Structure of 7-methyltryptamine and N-methyltryptamine.

Figure 5.12. GC-FID chromatogram of amine standards for analysis of INMT activity on tryptamine.

Figure 5.13. GC-FID chromatogram showing the A) INMT enzyme analysis with 1 mM tryptamine and 5 mM SAM carried out in 25 mM Tris-HCl buffer, pH 8.5 at 37°C and B) control without INMT.

Figure 5.14 displays the structures of the internal standard, *N*-methyl-1,3-diaminopropane , and the compound of interest, *N*-methylputrescine. Figure 5.15 displays the elution order of the amine standards. *N*-methyl-1,3-diaminopropane elutes at 2.8 min, followed by putrescine and *N*-methyltputrescine at 3.0 min and 3.4 min, respectively. *N*-methylputrescine, rather than *N,N,N',N'*-tetramethyl-1,4-diaminobutane was chosen for analysis because there is a greater chance of INMT producing *N*-methylputrescine which is formed by one methyl transfer reaction, whereas *N,N,N',N'*-tetramethyl-1,4-diaminobutane is formed by four methyl transfer reactions. *N*-methylputrescine was not detected by GC-FID analysis (Figure 5.16A), which suggests that either INMT did not methylate putrescine or that *N*-methylputrescine was below the detection limit of GC-FID. Since the SAM510 methyltransferase assay showed that INMT has activity on putrescine, the latter scenario may be more likely. There are many possibilities that could explain this observation. Compounds containing heteroatoms, such as hydrocarbons with nitrogen atoms, tend to have lower response factors and therefore may be underreported by GC-FID analysis. It is also possible that *N*-methylputrescine was produced, but the levels of SAH that accumulated resulted in the inhibition of INMT. This could explain the discrepancy between the SAM 510 and GC-FID data, as the SAM510 method relieves SAH inhibition by its degradation.

N-methylputrescine   *N*-methyl-1,3-diaminopropane

Figure 5.14. Structure of *N*-methylputrescine and *N*-methyl-1,3-diaminopropane.
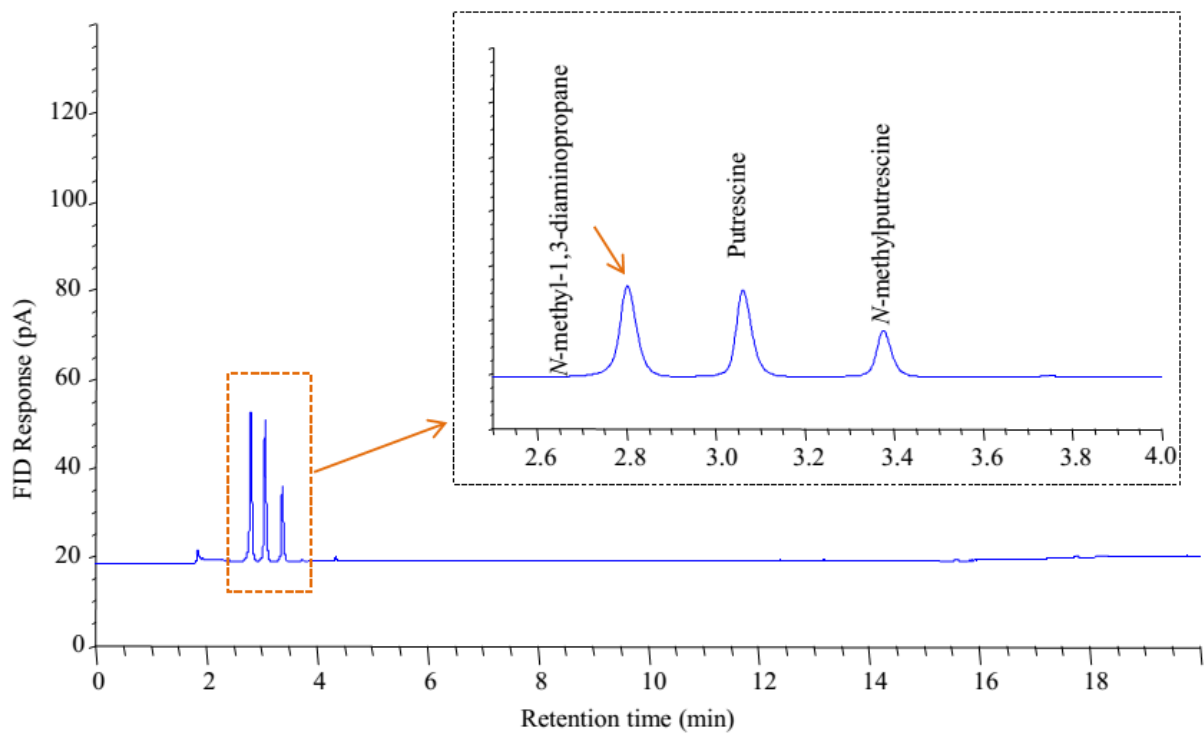
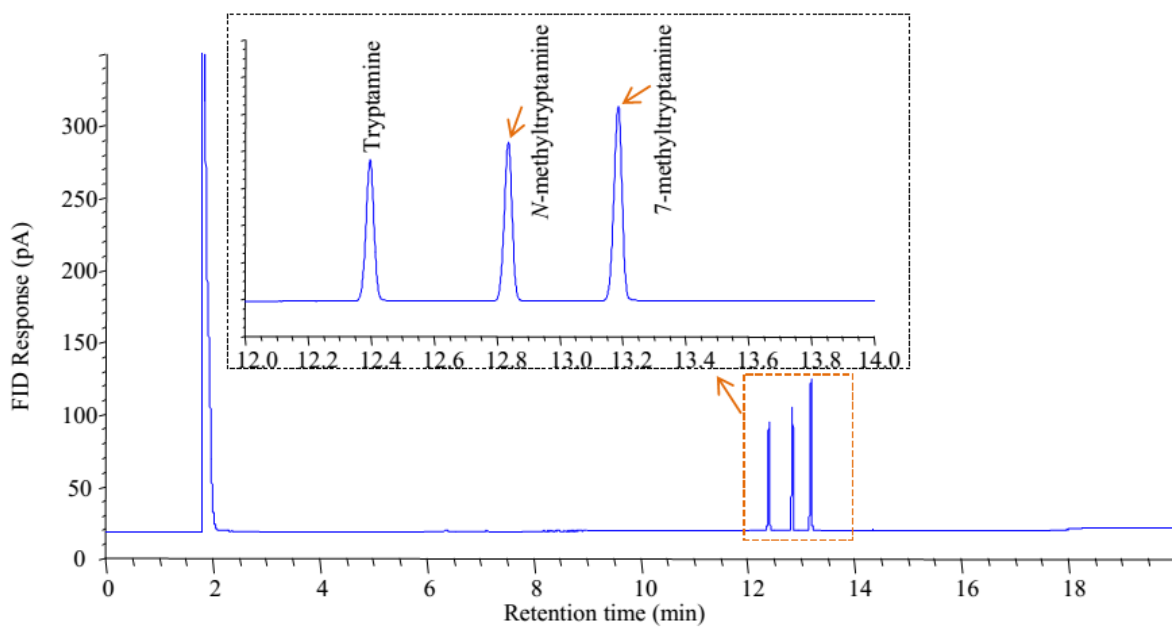**Figure 5.15.** GC-FID chromatogram of amine standards for analysis of INMT activity on putrescine.
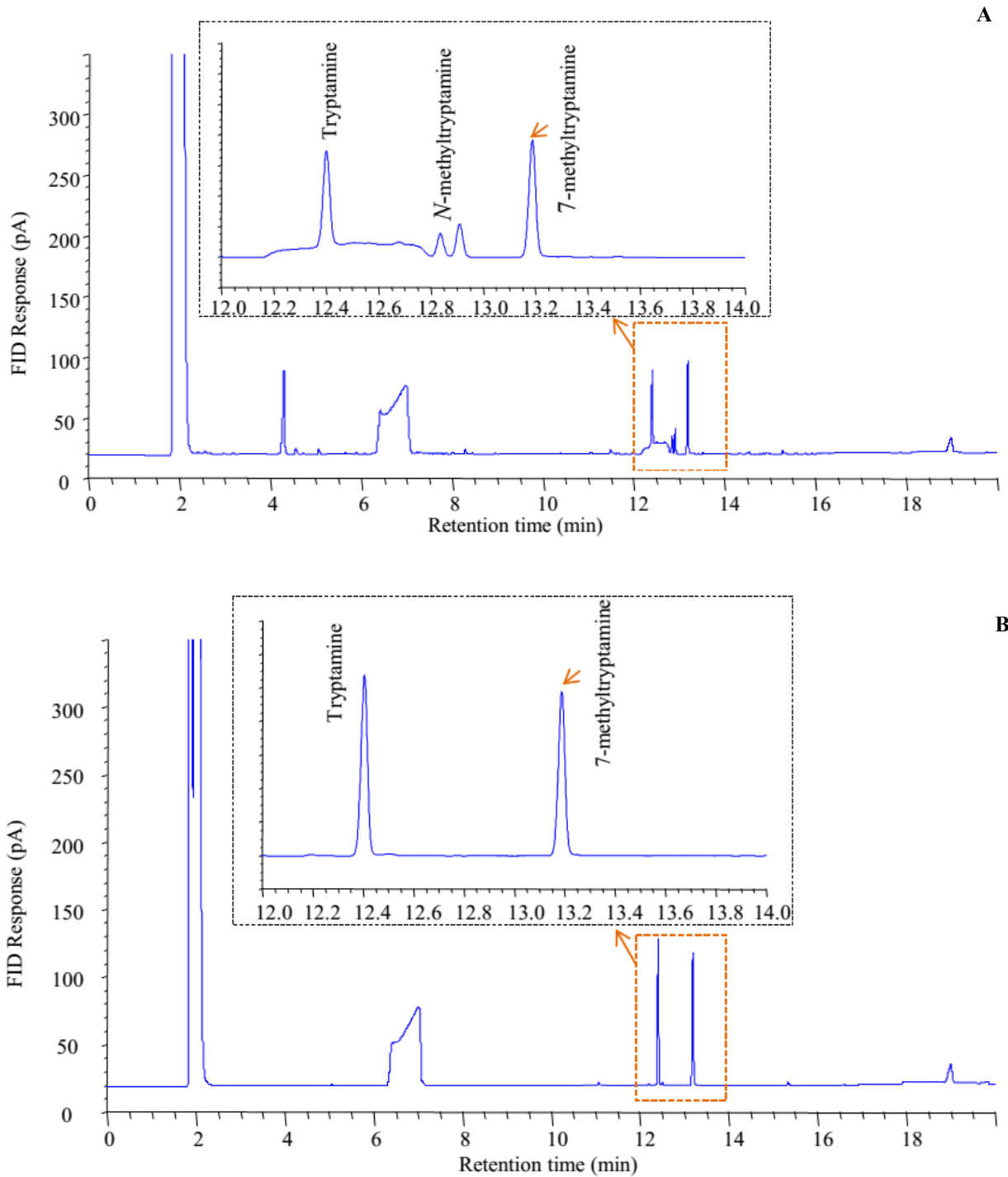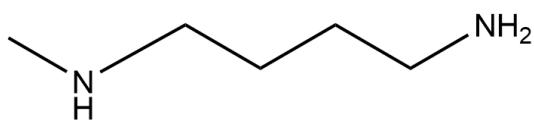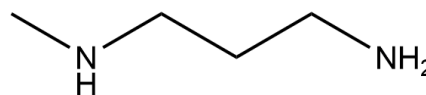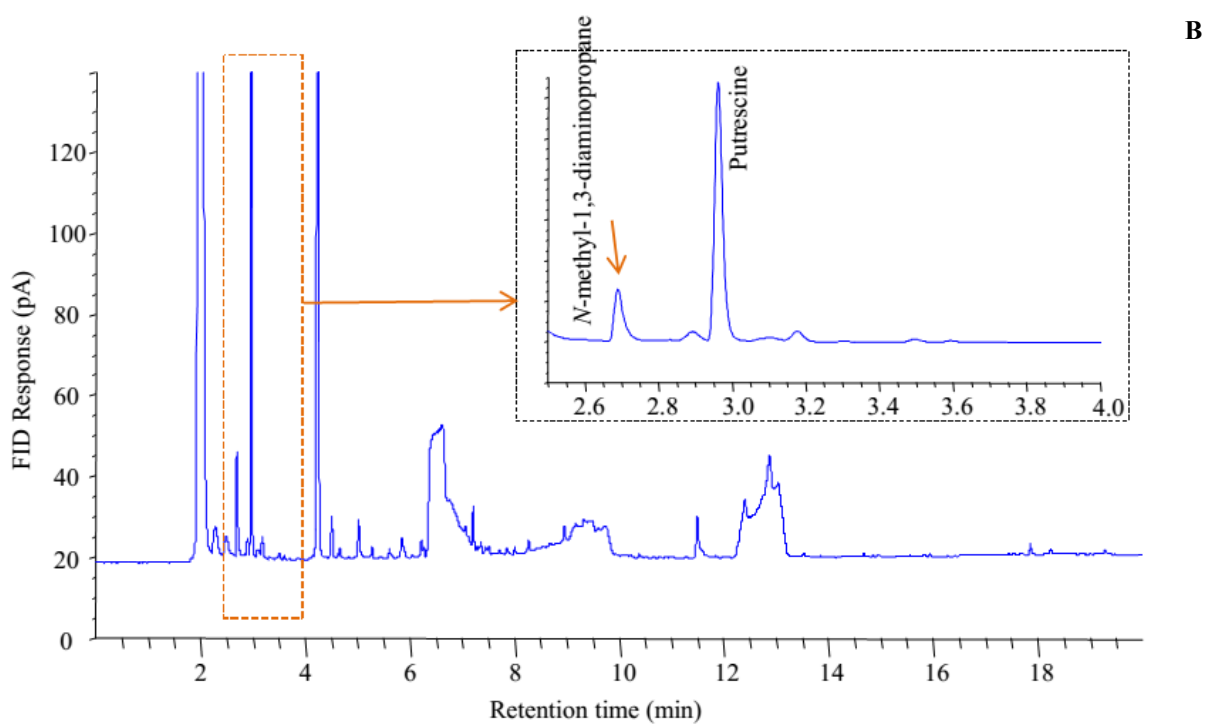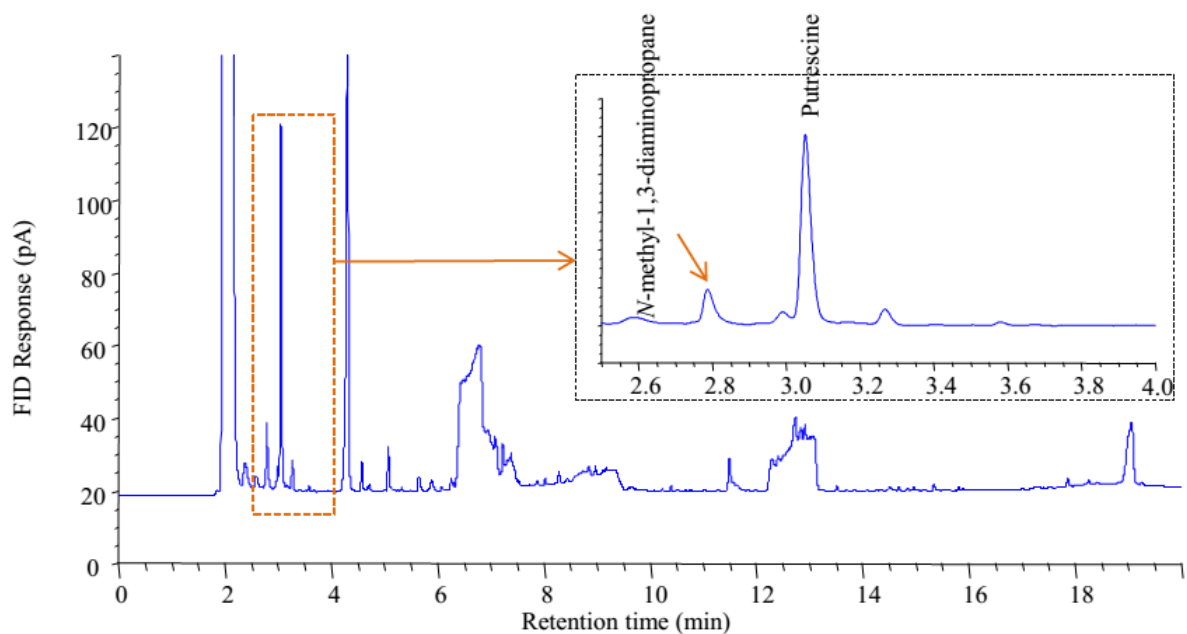
Figure 5.16. GC-FID chromatogram showing the A) INMT enzyme analysis with 5 mM tryptamine and 10 mM SAM carried out in 25 mM Tris-HCl buffer, pH 8.5 at 37°C and B) control without INMT.

5.4. Conclusions

The activity of INMT was studied in this chapter to determine if INMT expressed from a bacterial source had activity, and if putrescine could be utilized as a substrate to produce the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane. The studies conducted in this chapter revealed that rabbit lung INMT cloned into, expressed and purified from a bacterial expression host retained activity as confirmed by microplate enzyme assays. The microplate assay also showed that putrescine was able to act as a methyl group acceptor for INMT. Chromatographic analyses (HPLC and GC) also confirmed INMT enzyme activity since the enzyme was able to methylate tryptamine, the prototypic methyl group acceptor, to form *N*-methyltryptamine. Conversely, GC-FID analysis showed the methlylated form of putrescine, *N*-methylputrescine, was not produced or was produced at a concentration below the detection limit. Considering the microplate enzyme assay results, it is possible that the concentration of *N*-methylputrescine was below the detection limit.

6. Overall Discussions and Conclusions

6.1. Concluding discussion

Microorganisms are capable of producing a vast array of products, ranging from foods (e.g. bread and alcohol) to pharmaceuticals (e.g. antibiotics, vaccines) to industrial chemicals. This trait has been exploited for the development of industrial fermentation technologies and remains an important facet of modern biotechnology. In light of growing concerns about the sustainability and environmental impact of the petrochemical industries, microbial fermentations are now being seen as a viable alternative for production of chemicals. In fact, switchable solvents represent an interesting class of non-natural compounds that could be produced by microbial fermentation. Switchable solvents are unique in that they are able to reversibly switch certain properties (e.g. ionic strength) by the addition/removal of a trigger (Mercer and Jessop, 2010). This characteristic has important industrial implications as it enables more environmentally-friendly product extraction. The switchable reaction occurs at ambient pressure and temperature, which reduces energy input and therefore operation costs. Moreover, the ability to reversibly switch the solvent enables recycling and reuse of the extraction agent, which reduces material usage and waste generation. Currently, switchable solvents are limited by either commercial availability or cost, but their production by microbial fermentation may be a viable alternative.

The basis of this research project was to investigate the possibility of producing the switchable solvent, *N,N,N',N'*-tetramethyl-1,4-diaminobutane, by microbial fermentation. Since no prior work has been conducted to produce switchable solvents biologically, it was necessary to do prerequisite studies and establish proof-of-concept. First, since switchable solvents are non-natural, it is necessary to derive them from existing microbial metabolic pathways. *N,N,N',N'*-

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tetramethyl-1,4-diaminobutane may potentially be produced by modification of the biogenic amine putrescine. Reports of engineered *E. coli* and *C. glutamicum* strains for putrescine production have already been published (Qian *et al*., 2009; Schneider and Wendisch, 2010). Conversion of putrescine to *N,N,N',N'*-tetramethyl-1,4-diaminobutane could be achieved by multi-methylation of putrescine. A candidate enzyme with broad specificity, indolethylamine *N*-methyltransferase (INMT) was identified for such a purpose. Second, the candidate enzyme INMT needed to be tested for its ability to convert putrescine to *N,N,N',N'*-tetramethyl-1,4-diaminobutane. This enzyme was cloned into an *E. coli* expression system for this purpose (Chapter 4 and 5). Additionally, *E. coli* and *S. cerevisiae* were studied for their ability to tolerate *N,N,N',N'*-tetramethyl-1,4-diaminobutane during cultivation, a trait that is desirable for fermentation (Chapter 3).

In Chapter 3, *E. coli* and *S. cerevisiae* were grown with a range of 0 to 100 mM of the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane to test its toxicity and to determine if this compound could be used as a nutrition source. Neither microorganism was found to consume *N,N,N',N'*-tetramethyl-1,4-diaminobutane since its concentrations did not decrease significantly between the beginning and end of cultivation. This is a desirable trait because it will not hinder fermentation productivity. Based on the OD_{600} growth curve and the plate count data to assess cell viability, *S. cerevisiae* appeared to tolerate prolonged exposure the switchable solvent better than *E. coli* as cell viability remained unaffected during stationary phase. At the highest concentration of *N,N,N',N'*-tetramethyl-1,4-diaminobutane, 100 mM, *S. cerevisiae* did not show significant difference in cell viability when compared to the control. It is plausible that *S. cerevisiae* could tolerate even higher concentrations of the switchable solvent. In contrast, *E. coli* did not tolerate prolonged exposure to the switchable solvent as cell viability decreased

significantly towards the end of cultivation (e.g. stationary phase). However, from a fermentation perspective, the susceptibility of the *E. coli* to the switchable solvent during late stationary phase may not necessarily be problematic. *N,N,N',N'*-tetramethyl-1,4-diaminobutane is not expected to be produced during stationary phase and therefore, *E. coli* susceptibility to this compound during this phase may not be a significant factor in choosing a microbial host for production. Therefore, at this point, without knowing the levels of *in vivo* production of *N,N,N',N'*-tetramethyl-1,4-diaminobutane, there is not conclusive evidence whether *E. coli* or *S. cerevisiae* is a more suitable microbial catalyst. This work demonstrated that both *E. coli* and *S. cerevisiae* could tolerate the presence of *N,N,N',N'*-tetramethyl-1,4-diaminobutane during exponential phase. However, *E. coli* was used for the remainder of this thesis work and there are a few reasons for this. First, the pathways involved in putrescine metabolism, regulation, transport and uptake in *E. coli* have been extensively studied, more so than in *S. cerevisiae* (Igarashi and Kashigawi, 1999; Tabor and Tabor, 1985). Our belief was that this wealth of knowledge would facilitate easier strain development for putrescine overproduction. Indeed, engineered strains of *E. coli* have been reported for putrescine production (Eppelman, 2005; Qian *et al*., 2009). *C. glutamicum* has also been engineered for the same purpose (Schneider and Wendisch, 2010), but there have been no reports of an engineered *S. cerevisiae* strain for putrescine production. Second, the methylation activity of INMT on putrescine needed to be evaluated with a relatively pure enzyme. Pure proteins of interest are often obtained from expression systems. For proof-of-concept of enzymatic production of *N, N,N',N'*-tetramethyl-1,4-diaminobutane from putrescine, it may be more advantageous to work with an *E. coli* expression system since they are easy to use and recombinant proteins can be purified in high quantities in short time periods.

In Chapter 4, the DNA encoding rabbit lung INMT was cloned into the vector pET11d to create the recombinant plasmid INMT-pET11d. This recombinant plasmid was cloned into *E. coli* BL21(DE3) to allow for its overexpression by IPTG induction. The resulting overexpressed protein was designed with a C-terminal 6x-His tag, which allowed for its purification by using nickel affinity chromatography, resulting in pure protein isolate that was assayed for enzyme activity. SDS-PAGE analysis demonstrated that protein thought to be INMT was expressed from *E. coli* BL21(DE3) and purified using nickel affinity chromatography. It showed that the expressed protein was approximately 30 kDa, which corresponds to the reported size of INMT (Ansher and Jakoby, 1986; Thompson and Weinshilboum, 1998) and suggested that the expressed protein might be INMT. The identity of the protein was confirmed by partial peptide sequencing by MALDI-TOF analysis. DNA sequencing analysis also provided indirect evidence that the purified enzyme was INMT.

The methylation activity of purified INMT was examined in Chapter 5. Early attempts to determine recombinant INMT activity using commonly reported literature conditions (Ansher and Jakoby, 1986; Thompson and Weinshilboum, 1998) adapted for HPLC-ELSD analysis proved unsuccessful as no products were detected. Modification of the substrate concentrations to reflect an increase to a 1:1 ratio of SAM to tryptamine and analysis with HPLC-UV demonstrated that recombinant INMT purified from *E. coli* has low activity since levels of *N*-methyltryptamine were below the detection limit of HPLC-ELSD. Analysis of INMT activity using a universal SAM-dependent methyltransferase enzyme assay also proved that recombinant INMT had activity and showed that putrescine was able to act as methyl group acceptor. Finally, GC-FID analysis showed that *N*-methylputrescine was not detected. We hypothesize that its concentration may be lower than the detection limit of GC-FID considering that the SAM510

enzyme assay showed that tryptamine was a better substrate for INMT. In contrast, GC-FID analysis of enzyme assays carried out with tryptamine as the methyl acceptor demonstrated that the purified INMT had activity.

While, the enzymatic conversion of putrescine to the switchable solvent *N,N,N',N'*-tetramethyl-1,4-diaminobutane by INMT could not be confirmed, several of the objectives of this thesis were achieved. The achievements of these objectives have laid the ground work for future work to build upon this research project. Additionally, a couple of novel studies have been presented in this thesis work, such as the cloning and expression of INMT in a bacterial protein production system and the investigation of *E. coli* and *S. cerevisiae* tolerance to *N,N,N',N'*-tetramethyl-1,4-diaminobutane. Thus, this thesis has provided valuable insight into the engineering of a microbial strain for the production of switchable solvents.

6.2. Recommendations for future work

This thesis was the first to show that rabbit lung INMT can be expressed and purified in a bacterial expression system. Moreover, the purified enzyme demonstrated activity and that putrescine acted as a methyl group acceptor for INMT, but the number of methylation reactions it underwent could not be determined. Future studies should consider the use of enzyme engineering, which would result in modifications to the structure of enzymes to change its properties. Enzyme engineering could be used to modify the structure of INMT so that its substrate specificity is altered, its kinetic properties are enhanced, or its stability properties are improved (Bloom *et al.*, 2005). Following this stream of thought, it may be worth investigating if enzyme engineering could be applied to INMT with the goal of engineering the enzyme to have specificity for putrescine and increased catalytic activity. In addition, other enzymatic conditions

should be tested to determine if INMT methylation of putrescine can be enhanced. For example, the ratio of SAM to putrescine could be increased further to increase the likelihood of producing *N,N,N',N'*-tetramethyl-1,4-diaminobutane.

The production of *N,N,N',N'*-tetramethyl-1,4-diaminobutane from putrescine via INMT catalysis requires the transfer of four methyl groups from SAM. The demethylation of SAM forms SAH, a known inhibitor of INMT (Lin *et al.*, 1973) and could be an obstacle in the formation of *N,N,N',N'*-tetramethyl-1,4-diaminobutane. It may be wise to investigate solutions to reduce product inhibition by SAH. Overexpression of enzymes involved in SAH metabolism could alleviate product inhibition of INMT. Two potential enzymes are *S*-adenosylhomocysteine hydrolase, which is found in *S. cerevisiae* and converts SAH to homocysteine and adenine (Tehlivets *et al*., 2012), and *S*-adenosylhomocysteine nucleosidase, which is found in bacteria like *E. coli* and converts SAH to adenine and *S*-ribosylhomocysteine (Walker and Duerre, 1975; Parveen and Cornell, 2010). This approach may reduce SAH concentrations, which would alleviate product inhibition of INMT and allow for enough methyl transfer reactions to occur for the production of *N,N,N',N'*-tetramethyl-1,4-diaminobutane.

The *in vivo* production of the switchable solvent will need to be evaluated. Future studies will need to determine which microorganism is the most suitable for this purpose. The switchable solvent tolerance assay suggests that *S. cerevisiae* might be the most appropriate given its tolerance to *N,N,N',N'*-tetramethyl-1,4-diaminobutane, but other factors need to be considered. Moreover, *E. coli* and *C. glutamicum* strains have already been genetically engineered to overproduce putrescine and could also be suitable microbes for switchable solvent production, whereas that is not the case in *S. cerevisiae*.

Overall, the research conducted in this thesis has laid the ground work for the bio-based production of a novel switchable solvent using a microbial catalyst. Switchable solvents have the potential to revolutionize a number of industrial processes, including the separation of fermentation products from medium. The bio-engineering of microorganisms to produce chemicals has become an attractive alternative in lieu of chemical synthesis for environmental and economic reasons. With developments in fermentation technology and synthetic biology, the development of a fermentation system for the production of switchable solvents and other value-added products looks promising.

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