
**Design and exploitation of cadaverine
metabolic pathway for simple *N*-
heterocyclic chemical production**

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ABSTRACT

Nitrogen-heterocycles are widely found in nature as subunits of compounds from secondary metabolism (vitamins, alkaloids, hormones etc.) and are valuable building blocks to manufacture a wide range of pharmaceuticals, agrochemicals, flavours and fragrances. Currently, *N*-heterocycles are produced through unsustainable chemical processes that rely on petrochemical feedstocks and high-energy consumption. The six-membered simple *N*-heterocycle Δ^1 -piperideine (2,3,4,5-tetrahydropyridine), is a key building block for biosynthesis of some alkaloids, for example, the piperidine, quinolizidine, indolizidine and lycopodium alkaloids. Δ^1 -piperideine, also has considerable promise as a platform compound for chemical synthesis of functionalized or complex compounds with economic importance. Although the bioproduction of complex, functionalised *N*-heterocycles have been reported, bioproduction of unsubstituted simple *N*-heterocycles have not yet been achieved. Advances in metabolic engineering and synthetic biology offers an approach to develop bioprocesses for sustainable production of simple *N*-heterocycles from renewable sugar feedstocks.

Δ^1 -Piperideine can be obtained by oxidation of cadaverine using amine oxidases or transaminases, to yield 5-aminopentanal. This product is known to cyclise spontaneously into Δ^1 -piperideine. However, Δ^1 -piperideine reacts spontaneously in aqueous solution to form the multimeric products (tetrahydroanabasine, α -tripiperideine and isotripiperideine). Therefore, the suitability of putrescine oxidase from *Rhodococcus erythropolis* (PuO_{Rh}) for cell-free and whole-cell bioproduction of Δ^1 -piperideine was studied. PuO_{Rh} was characterised for the first time by monitoring oxygen concentration in a direct substrate consumption assay. Using purified PuO_{Rh} and PuO_{Rh} induced whole cells; the conversion of cadaverine to Δ^1 -piperideine was demonstrated qualitatively and quantitatively. This study indicates that PuO_{Rh} is suitable for the bioproduction of Δ^1 -piperideine. Finally, a metabolic route has been designed and proposed for whole cell bioproduction of Δ^1 -Piperideine from renewable feedstocks.

*DEDICATED TO MY WIFE, EZINNE (INSPIRE) AND MY DAUGHTERS,
CHISOROMIJE, CHIMZURUOKE AND CHIMMARANMA. My greatest joy
and source of strength is the unconditional love you have for me!*

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To my daughters, Chisoromije, Chimzuruoke and Chimmamanma, I am inspired by your brilliance and affection. You are the reason! Thank you!

Finally, to God be all the Glory!

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LIST OF ABBREVIATIONS

α -ABA	α -Aminobenzaldehyde
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ADH	Amine dehydrogenases
ADP	Adenosine dinucleotide phosphate
AL	Ammonia lyases
bp	Base pair
BSA	Bovine serum albumin
Cu^{2+}	Copper ion
D_2O	Deuterium oxide
DNS-Cl	Dansyl chloride/5-(Dimethylamino)naphthalene-1-sulfonyl chloride
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DCW	Dry cell weight
EDTA	Ethylene diamine tetracetic acid
EFl_{OX}	Oxidized form of enzyme
EFl_{RED}	Reduced form of enzyme
ESI	Electrospray ionization
EtBr_2	Ethidium bromide
EtOH	Ethanol
FAD	Flavin adenine dinucleotide
g	Gram
g/L	Gram per litre
h	Hour
HCl	Hydrochloric acid
HPLC	High-pressure liquid chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IREd	Imine reductases
K	Potassium
KCl	Potassium chloride
KDa	Kilo Dalton
KJ/mol	Kilojoules per mole
K_{cat}	Turnover number
K_{m}	Michaelis constant
$K_{\text{cat}}/K_{\text{m}}$	Catalytic efficiency of the enzyme reaction
LB	Luria Bertani
LC-MS	Liquid chromatograph – mass spectrometry
m	Meter
μm	Micrometer (micron)
min	Minutes
mg/mL	Milligram/millilitre
μL	Microliter
mL	Millilitre
mM	Millimolar
MAO	Monoamine oxidases
MgCl_2	Magnesium chloride
MgSO_4	Magnesium sulphate
MS	Mass spectrometry

MW	Molecular weight
Na	Sodium
NaCl	Sodium chloride
Na ₂ SO ₄	Sodium sulphate
NaHCO ₃	Sodium carbonate
NaOH	Sodium hydroxide
nm	Nanometre
NMR	Nuclear magnetic resonance
OD	Optical density
P	Product
PAT	Putrescine aminotransferase
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PhCN	Benzonitrile
ppm	Parts per million (chemical shift)
PuO _{Rh}	Putrescine oxidase (<i>R. erythropolis</i>)
qNMR	Quantitative nuclear magnetic resonance
S	Substrate
s	Seconds
SDS	Sodium dodecyl sulphate
TA	Transaminases
TPQ	2,4,5-trihydroxyphenylalanine quinone
V _{max}	Maximum rate of enzyme reaction
WDW	Wet cell weight

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1. INTRODUCTION

1.1 General introduction

Sustainability has become the mantra of the 21st century and has been associated to all fields and facets of human existence, be it governance, engineering or lifestyle. Simply, sustainability is a call for change to better our collective present and future human existence. One of those changes is in the orientation of technological development (Brundtland Report, 1987, American Chemical Society, 2015). Although simple *N*-heterocycles occur in nature, they are currently produced through challenging chemical processes that use petroleum-derived feedstocks. This is unsustainable because of tense politics amongst petroleum producing countries, depleting petroleum reserves, fluctuation in price and supply as well as low eco-efficiency in its extraction due to intensive energy requirement and high emission of greenhouse gases (Xu et al., 2015c, Gober and Joullié, 2016, Lin and Tao, 2017). With the advances in metabolic engineering and synthetic biology, developing bioprocesses from bio-based sugar feedstocks offer an alternative and sustainable approach for the production of simple *N*-heterocycles (Kind and Wittmann, 2011, Lee et al., 2011, DeLoache et al., 2015).

Simple *N*-heterocycles such as pyrrole, pyridine and Δ^1 -piperidine are attractive building blocks (platform chemicals) in the production of natural and functional molecules or the development of new pharmaceuticals (synthetic drugs), agrochemicals, organic solvents and value products (Bhusal and Sperry, 2016). Δ^1 -Piperidine (2,3,4,5-tetrahydropyridine) is an unsubstituted six-membered *N*-heterocyclic ring involved in the natural biosynthesis of lysine-derived alkaloids e.g. piperidine, quinolizidine and lycopodium alkaloids (Shvekhgeimer, 1998, Gravel et al., 2006, Rouchaud and Braekman, 2009, Salame et al., 2010, Bunsupa et al., 2012a, Bunsupa et al., 2012b, Bunsupa et al., 2017). Δ^1 -Piperidine is an attractive platform compound because of its natural role as a precursor and has a reasonable carbon backbone for chemical derivatization into novel compounds of high therapeutic and industrial importance (Shvekhgeimer, 1998). Recently, Δ^1 -piperidine has

been reported as an intermediate for organic syntheses of valuable structurally diverse heterocycles (Unsworth et al., 2013, Unsworth et al., 2014) and therapeutic compounds including pelletierine, nitraramine and tetraponerines (Gravel et al., 2006, Rouchaud and Braekman, 2009, Monaco et al., 2011, Orejarena Pacheco et al., 2016).

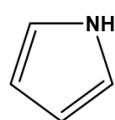
Therefore, this project explored the scope to develop a bioprocess for sustainable production of Δ^1 -piperidine from renewable (bio-based sugar) feedstocks. Previously reported tracer experiments with labelled precursors on plants involved in the synthesis of lysine-derived alkaloids showed that cadaverine, a product of lysine decarboxylation, is converted to Δ^1 -piperidine through a sequential catalytic deamination or transamination and spontaneous Schiff base cyclization reactions (Ma and Gang, 2004). In addition, cadaverine biosynthesis using microorganisms have been reported (Qian et al., 2010, Kind et al., 2011, Kind and Wittmann, 2011, Buschke et al., 2011, Buschke et al., 2013). If suitable enzymes can be identified, addition of only one final step to the reported cadaverine pathway(s) will represent a simple strategy for the direct microbial synthesis of Δ^1 -piperidine. In this study, identification of suitable enzyme for that step and the design of a bioprocess for Δ^1 -piperidine production from cadaverine and renewable feedstocks (bio-based sugar) were investigated.

2. LITERATURE REVIEW

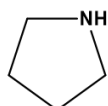
2.1 *N*-Heterocycles

N-Heterocycles are cyclic aromatic or aliphatic compounds with one or more nitrogen atoms (Fig. 2.1). They are widely found in nature as sub-structures of various biologically active compounds from secondary metabolism such as alkaloids, vitamins, DNA (purine and pyrimidine), pigments, hormones and antibiotics (Shi and Szostak, 2017).

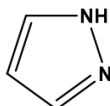
Simple *N*-heterocycles



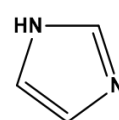
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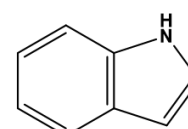
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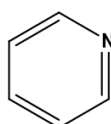
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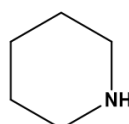
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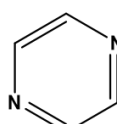
Indole



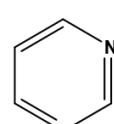
Pyridine



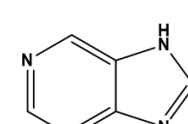
Piperidine



Pyrazine

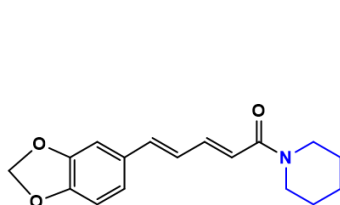


Pyrimidine

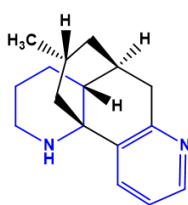


Purine

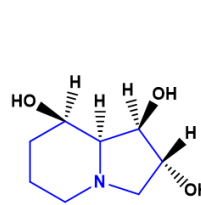
Complex *N*-heterocycles



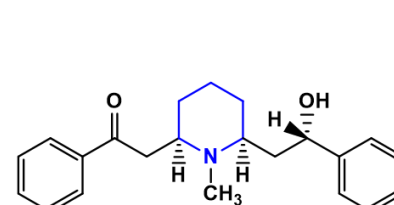
Piperine



(-)-Lycodine



(-)-Swainsonine



(-)-Lobeline

Fig. 2.1: Structures of selected *N*-heterocycles.

Rings in blue shows simple *N*-heterocycles as structural components of complex or functionalized *N*-heterocycles.

N-Heterocycles are attractive building blocks in the production of natural and functional molecules or development of new pharmaceuticals and agrochemicals (Vo et al., 2014, Vo and Bode, 2014, Xu et al., 2015c, Zhou et al., 2016, Shi and Szostak, 2017). Recent structural analysis of U.S. FDA approved drugs reveals that 59% of unique small-molecule drugs contain an *N*-heterocycle (Vitaku et al., 2014). The market size for *N*-heterocycles

was estimated to average more than one billion dollars annually (\$ 1000000000/year). About 10,000 tonnes of pyrrole and indole derivatives each are produced annually at an average unit cost of \$ 25000/tonne and \$ 20000/tonne respectively, while an average of 200,000 tonnes of pyridine derivatives are produced annually at an average unit cost of \$5000/tonne (Xu et al., 2015c).

Consequently, preparation of *N*-heterocycles has been at the core of chemical synthesis over decades and remarkable successes have been achieved (Vo and Bode, 2014, Steib et al., 2015b, Steib et al., 2015a, Zhou et al., 2016, Shi and Szostak, 2017). For instance, industrial production of pyrroles is carried out by reaction of petroleum-derived furan with ammonia in the presence of solid acid catalysts (Higasio and Shoji, 2001, Caiazza et al., 2004, Xu et al., 2015c, Bhusal and Sperry, 2016, Yao et al., 2017) (Fig. 2.2). Pyridines are prepared through condensation of aldehydes, ketones or α,β -unsaturated carbonyl compounds with ammonia or ammonia derivatives over zeolite catalysts (Shimizu et al., 1998, Shimizu et al., 2000, Reddy et al., 2012, Cespi et al., 2015), while indoles are synthesized from aniline and ethylene glycol (Campanati et al., 2005, Yao et al., 2016). Notably, all the raw materials for these compounds are sourced from petrochemical (crude oil) feedstocks involving energy intensive and multistep-chemical processes. Also, these chemical synthetic processes generate a range of unwanted waste products and consume valuable earth chemicals as catalyst.

Current methods for synthesis of *N*-heterocycles are unsustainable because of dwindling petroleum reserves, political instability amongst petroleum producing countries, price and supply fluctuation, and increased environmental deterioration associated with petrochemical exploration and current chemical synthesis (Kind and Wittmann, 2011, Lee et al., 2011). Considering the pharmaceutical and industrial importance of *N*-heterocycles, it is therefore, imperative to develop sustainable approaches to their production.

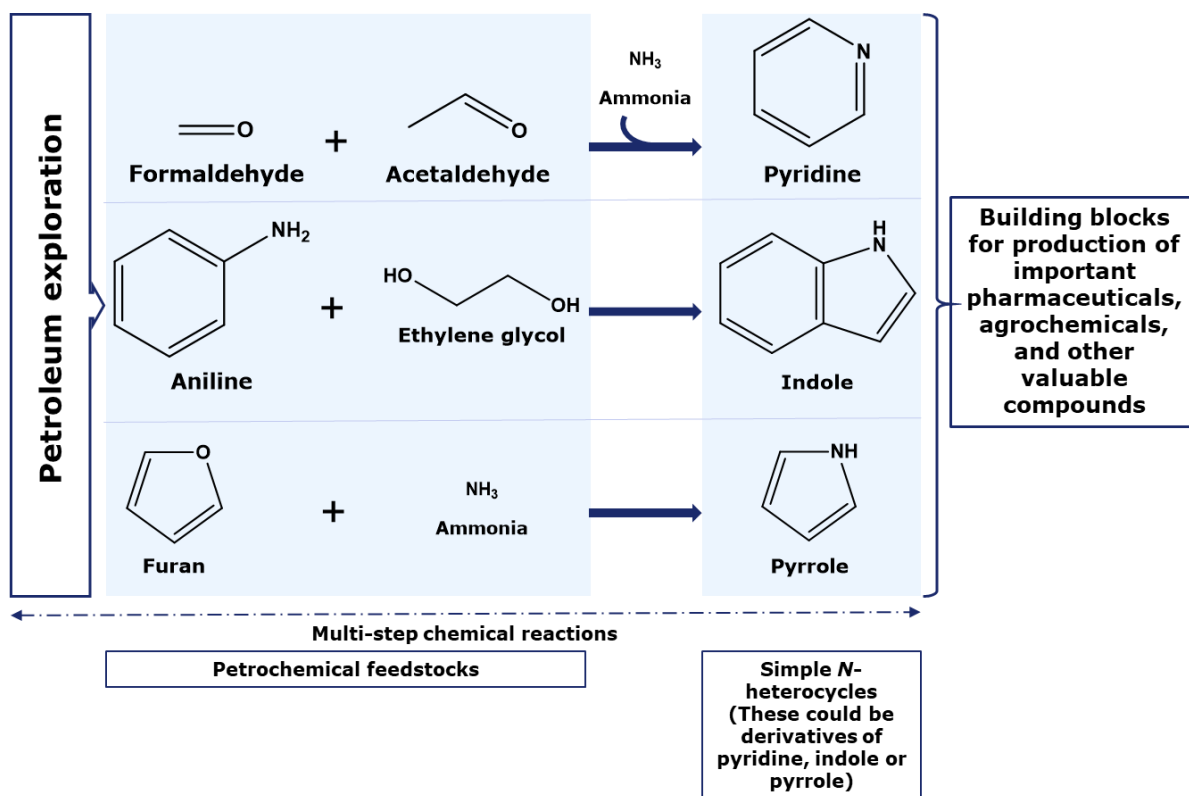


Fig. 2.2: Chemical production of simple *N*-heterocycles using petrochemical feedstocks.

2.1.1 Towards sustainable production of *N*-heterocycles

The increased adoption of green or sustainable production principles (see Appendix 11.1, Fig. 11.1) has led to the development of new chemical synthetic methods that reduce or eliminate the generation of environmentally hazardous substances and the use of toxic conventional solvents and high energy. Popular amongst these green methods are solvent-free or solvent toxic-free synthesis, photocatalytic synthesis, solid support (immobilized reactants) synthesis and microwave-assisted synthesis (Tucker, 2006, Anastas and Beach, 2007, Polshettiwar and Varma, 2008, Santagada et al., 2009, Kaur, 2014b, Kaur, 2014a, Vo and Bode, 2014, Steib et al., 2015b, Orejarena Pacheco et al., 2016, Zhou et al., 2016, Shi and Szostak, 2017). However, the sustainability of these methods is still in doubt because all of their feedstock are derived from crude oil while some still require high-energy input.

Thermo-catalytic (pyrolysis) conversion and ammonization of biomass (including cellulose, lignocellulose, hemicellulose, glycerol, furfural etc.) into *N*-heterocycles have been demonstrated as sustainable technologies (Xu et al., 2015c, Xu et al., 2015a, Xu et al., 2015b). Whereas this technology removes the use of crude oil derived feedstock, the energy intensiveness and use of catalysts limits its sustainability. The development of microbial bioprocesses (bioproduction) from renewable feedstock (biomass) could address most of the concerns raised earlier (Kind and Wittmann, 2011, Lee et al., 2011, Adkins et al., 2013, DeLoache et al., 2015).

Microbial bioprocess refers to the use of microorganisms (whole-cell) and/or their components (proteins and enzymes) to perform chemical transformation or conversion of compounds (renewable feedstock) of various types. Microbial bioprocess approaches are broadly divided into biotransformation (biocatalysis) and fermentation bioprocesses. Fermentation involves the synthesis of products from growth substrates (feedstock) through the host cells' native or engineered metabolism, while in biocatalysis, feedstocks are converted to the desired product(s) by non-growing (harvested) cells or purified enzymes, thereby separating the cell growth (enzyme production) and product formation stages. Amongst other advantages (Table 2.1), microbial bioprocesses are sustainable because they use cheap and abundant renewable raw materials and they are environmentally friendly due to low emission of greenhouse gases (Turner and Truppo, 2013, Kohls et al., 2014, Becker and Wittmann, 2015, Lin and Tao, 2017). In addition, advances in metabolic engineering, recombinant DNA technology and synthetic biology have accelerated the successful application of microbial bioproduction (Jones-Prather and Martin, 2008, Nielsen et al., 2014, Becker and Wittmann, 2015, Julleson et al., 2015).

Table 2.1: Advantages and disadvantages of microbial bioproduction in comparison with chemical catalysis (adapted from Lin and Tao, 2017).

Advantages	Disadvantages
Environmentally friendly – low emission of greenhouse gases and use of renewable materials most times treated as waste.	Catalyst stability: biocatalyst is susceptible to substrate or product inhibition/toxicity; inactivation may occur at high temperatures, at extremes of pH, or in organic solvents
High selectivity (which can be chiral, positional and functional group-specific) High catalytic efficiency	The cell membrane may act as a mass transport barrier
Multi-step reactions in the single strain with cofactor regeneration	Likely to have undesirable metabolic by-products, which may be toxic to the cells and difficult to separate
Milder operational conditions – low energy consumption	Contaminated water and biomass waste
Recycling microbial strain or enzyme is sometimes possible	
High catalytic efficiency	

Recently, microbial bioproduction using renewable feedstocks has been explored in developing bio-based building blocks such as butanol, citramalic acid, levulinic acid, 3-hydroxybutyrolactone, artemisinic acid, isoprene, acrylic acid, isobutanol, and styrene (Atsumi et al., 2008, Nikolau et al., 2008, McKenna and Nielsen, 2011, Zhao et al., 2011, Julleson et al., 2015, Gober and Joullié, 2016, Webb et al., 2018). Biocatalytic conversion of petrochemical derived feedstock into natural and non-natural *N*-heterocycles have been reported. Examples are the production of chiral amines using transaminases (TA), monoamine oxidases (MAO), imine reductases (IRED), amine dehydrogenases (ADH) and ammonia lyases (AL) (Koszelewski et al., 2010, Mitsukura et al., 2010, Huisman and Collier, 2013, Kohls et al., 2014, O'Reilly et al., 2014, Hussain et al., 2015, Both et al., 2016, Gomm et al., 2016, Lenz et al., 2017). Although reports for microbial bioproduction of complex *N*-heterocycles from renewable feedstock are available (Facchini et al., 2004, Nakagawa et al., 2011, DeLoache et al., 2015, Galanie et al., 2015, Winzer et al., 2015,

Nakagawa et al., 2016), such process for simple *N*-heterocycles have not yet been reported.

N-Heterocycles occur in nature as secondary metabolites from the products of primary metabolism, with amino acids serving as their main precursors. Most of the diverse group of natural *N*-heterocycles are alkaloids and are found in approximately 20% of plant species. Therefore, alkaloids are the representative candidates to understand the biosynthesis of both simple and complex *N*-heterocycles. Plants produce about 12,000 known alkaloids, many display potent medicinal activities and several are widely used as natural product based pharmaceuticals or as lead compounds for the development of new drugs (Facchini et al., 2004, Bunsupa et al., 2017).

However, unlike most other groups of natural products, different structural types of alkaloids have independent biosynthetic origins (Fig. 2.3) (Aniszewski, 2007). For example, isoquinoline alkaloids such as morphine and berberine are synthesized from tyrosine, indole alkaloids such as vinblastine are derived from tryptophan, and tropane alkaloids such as cocaine and scopolamine are produced from ornithine, while lycopodium, piperidine and quinolizidine alkaloids like hurperzine-A, piperine and sparteine respectively are derived from lysine (Ma and Gang, 2004, Bunsupa et al., 2017). Alkaloid biosynthetic pathways are complex and typically composed of multiple biocatalytic steps that not only form a basic structural nucleus (e.g. simple *N*-heterocycles) but also modify nascent alkaloid molecules through various carbon-ring modifications including hydroxylation, methylation, acetylation and glycosylation (Aniszewski, 2007, Bunsupa et al., 2017). Six-membered simple *N*-heterocycles are present in an extensive number of biologically active compounds.

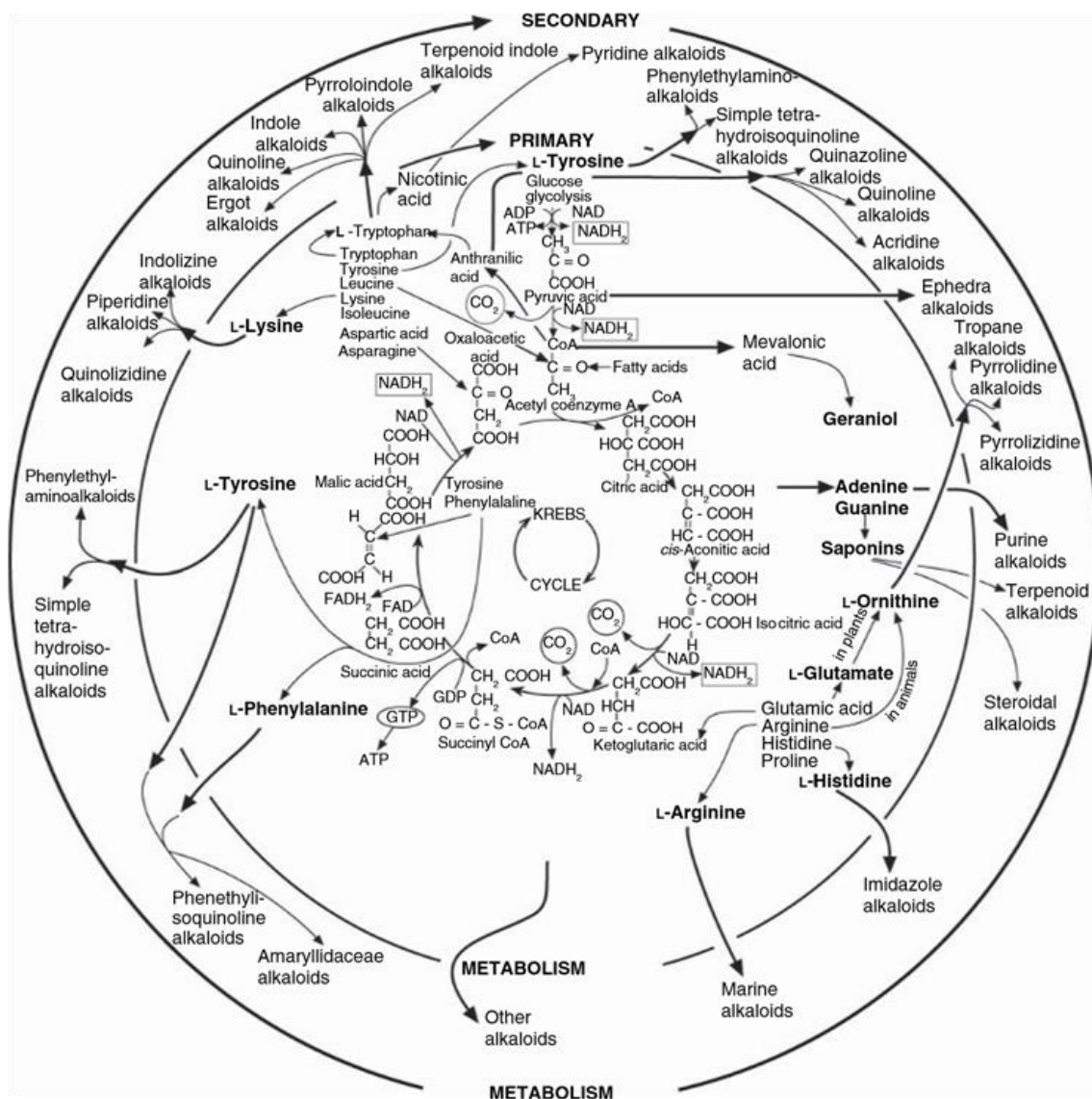


Fig. 2.3: Complex scheme of alkaloid synthetic pathways (reproduced from Aniszewski, 2007).

The lysine-derived alkaloids exhibit a wide range of pharmacological properties that are beneficial for humans. For instance, they have anticancer, anti-Alzheimer's disease, anti-inflammatory, hypocholesterolemic and antiarrhythmic effects. Lysine-derived alkaloids are widely distributed throughout the plant kingdom and are subdivided into piperidine, lycopodium, quinolizidine and indolizidine alkaloids (Fig. 2.4) (Leistner and Spenser, 1973, Bunsupa et al., 2012b, Bunsupa et al., 2017). Although biosynthetic pathways of lysine-derived alkaloids are not well documented, tracer experiments with labelled precursors have shown that they are formed from L-lysine *via* Δ^1 -piperidine and L-pipecolic acid Schiff bases (Fig. 2.5) (Leistner and Spenser, 1973, Gupta and Spenser, 1969, Golebiewski

and Spenser, 1988, Saito and Murakoshi, 1995, Dewick, 2002, Ma and Gang, 2004, Evans et al., 2009, Bunsupa et al., 2017).

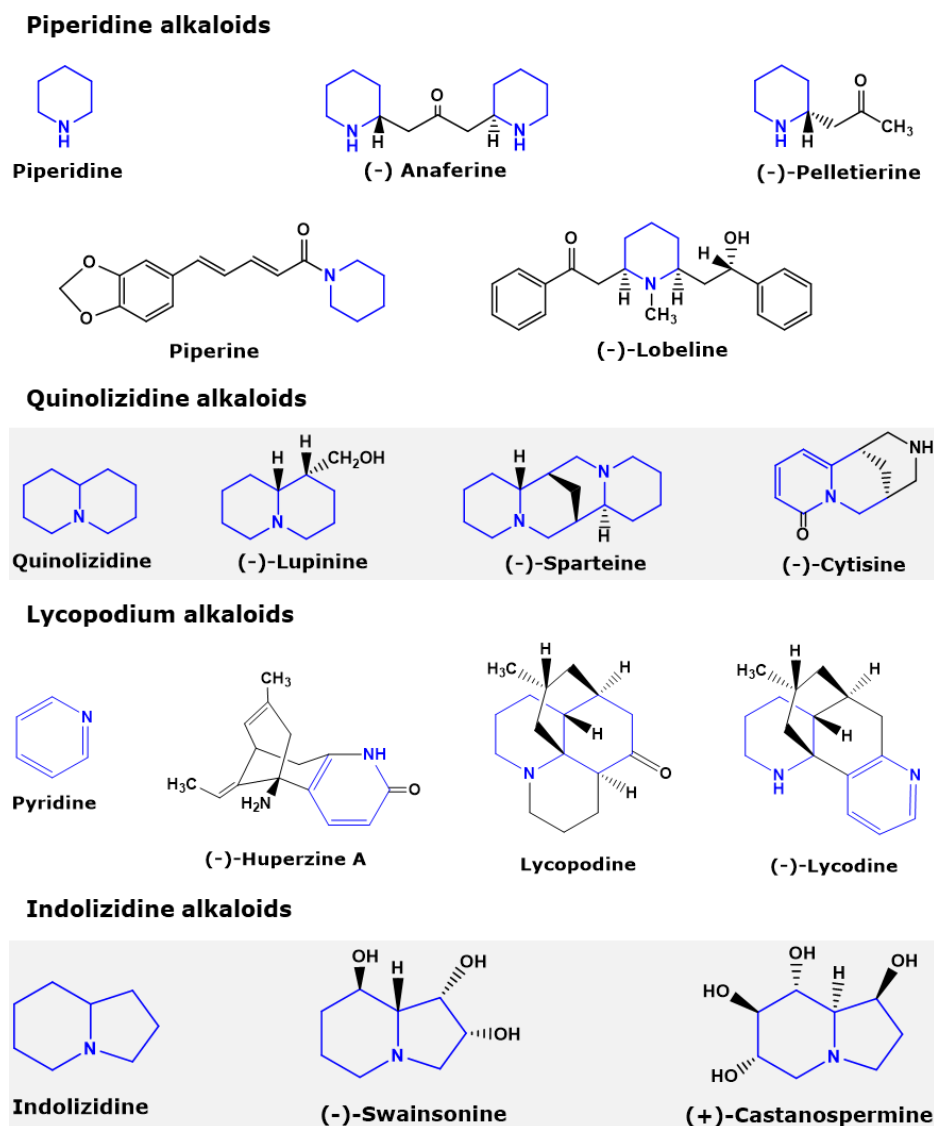


Fig. 2.4: Examples of lysine-derived alkaloids.

Rings in blue show the basic structural nucleus (simple *N*-heterocycles).

Δ^1 -Piperidine and L-pipecolic acid are both six-membered simple *N*-heterocycles, but Δ^1 -piperidine is an attractive platform compound because it has available carbon backbone for chemical derivatization into novel compounds of high therapeutic and industrial importance (Shvekhgeimer, 1998, Yokoshima et al., 2016). Δ^1 -Piperidine is a valuable simple *N*-heterocycle and its formation in nature is simple.

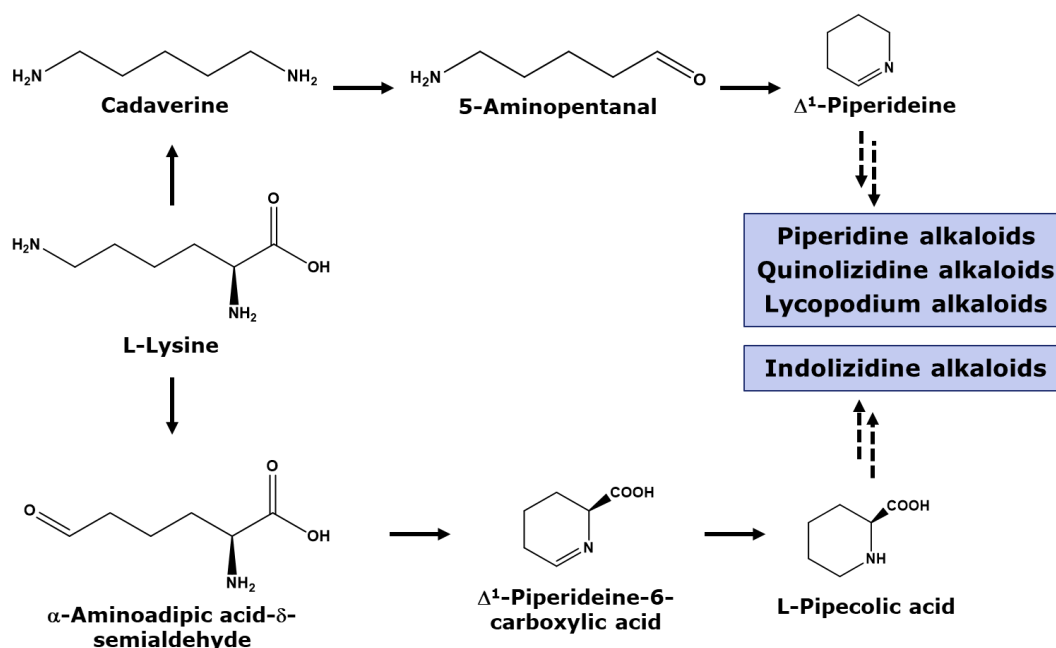


Fig. 2.5: Biosynthesis of universal intermediates for lysine derived alkaloids.

Dotted arrows indicate multiple reaction steps. Δ^1 -Piperideine and L-pipecolic acid are universal intermediates that can be modified by condensation, hydrolysis, or methylation to produce diverse Lys-derived alkaloids (Evans et al., 2009, Bunsupa et al., 2012a).

The goal of metabolic engineering and synthetic biology is to maximize yield of desired compound by redirecting cellular metabolic network (fluxes) (Nielsen et al., 2014, Julleson et al., 2015). They rely on optimization or reconstruction of existing natural pathways (Jones-Prather and Martin, 2008, Brockman and Prather, 2015) and involve iterative cycles of host genetic manipulation, an analysis of the consequences and a design of a further change to develop an optimized strain (Bailey, 1991, Nielsen, 2001). Genetic manipulation includes introduction of recombinant DNA cassettes encoding foreign enzymes, replacement of gene expression signals and /or gene deletion (Picataggio, 2009, Gibson et al., 2010, Annaluru et al., 2014, Julleson et al., 2015).

Different experimental strategies are employed to design and assemble biofactories (Lee et al., 2012). Metabolic routes are often changed to enhance the formation of target product by focusing on enhancing substrate uptake and utilization efficiency of different carbon sources. Naturally, microorganisms (*Saccharomyces cerevisiae* and *Escherichia coli*) preferentially consume six carbon sugars e.g. glucose. However, strains could be

designed to improve carbon efficiency and substrate uptake utilizing other readily available carbon sources e.g. pentoses (xylose) and lignocelluloses (Buschke et al., 2011, Lindner et al., 2011, Tai et al., 2016, Rossoni et al., 2018).

Similarly, when the originally designed pathway does not provide high yields, rerouting of metabolic pathway may be employed. This is often achieved by construction of new biosynthetic pathways with more efficient enzymes, *via* combinatorial heterologous expression of enzymes from various sources (Dellomonaco et al., 2011, Lee et al., 2012). Shen et al. (2011) increased the production of 1-butanol in an engineered *E. coli* strain by overexpressing *Treponema denticola* trans-2-enoyl CoA reductase (Ter) instead of butyryl-CoA dehydrogenase (Bcd-EtfAB) from *Clostridium acetobutylicum*.

Cofactors are required for normal functioning of many enzymes, and they are organic electron-mediators e.g. NADH and NADPH. The availability and the balance of necessary cofactors are important for the production of target product by increasing the growth of an organism. Therefore, to optimize cofactors, metabolic engineering approaches target at either increasing the production of the required cofactor or by changing cofactor preferences of relevant key enzymes (Shen et al., 2011). The former approach includes replacement of central metabolic enzymes that prefer other cofactors, redirection of metabolic flux through the pentose phosphate pathway and cross conversion of NADH and NADPH by co-expression of transhydrogenases (Chemler et al., 2010, Lee et al., 2011, Lee et al., 2012).

In addition, by-product elimination/reduction and precursor enrichment is a popularly practiced approach in metabolic engineering in which competing pathways for precursors or intermediates are removed through gene deletion, inactivation or attenuation. This pushes the flux towards the product of interest, thereby increasing its overall productivity (Qian et al., 2009, Qian et al., 2010, Lee et al., 2012). However, this approach may affect cell growth, thus it requires close monitoring.

Finally, transporter engineering has recently been employed. This involves the modification of transporters or engineering of strains, with efflux pumps to efficiently pump out the final product, thereby preventing its accumulation in the intracellular space. This serves to minimize the intracellular concentration of the target product, thus avoiding feedback inhibition and growth inhibition or toxicity (Yomantas et al., 2010, Dunlop et al., 2011). At the same time, import of excreted target products should be prevented while that of the carbon source is increased (Lee et al., 2007, Qian et al., 2010).

2.2 Δ^1 -Piperidine: a simple *N*-heterocycle

Δ^1 -Piperidine (2,3,4,5-tetrahydropyridine, C_5H_9N ; Fig. 2.6) is an unsubstituted six-membered *N*-heterocycle with distinct characteristics. Whereas all (2-) substituted derivatives of Δ^1 -piperidine are mostly monomers under ordinary conditions, unsubstituted Δ^1 -piperidine exists in solution as three isomeric trimers and a transient dimer at $pH \geq 7$ (Fig. 2.6; Shvekhgeimer, 1998, Monaco, 2011). This is because of its susceptibility to nucleophilic addition at the azomethine carbon (Joule and Mills, 2010). The structures, configurations and conformations of these compounds were determined by ^{13}C NMR spectrometry (Schopf et al., 1948, Kessler et al., 1977, Shvekhgeimer, 1998, Rouchaud and Braekman, 2009).

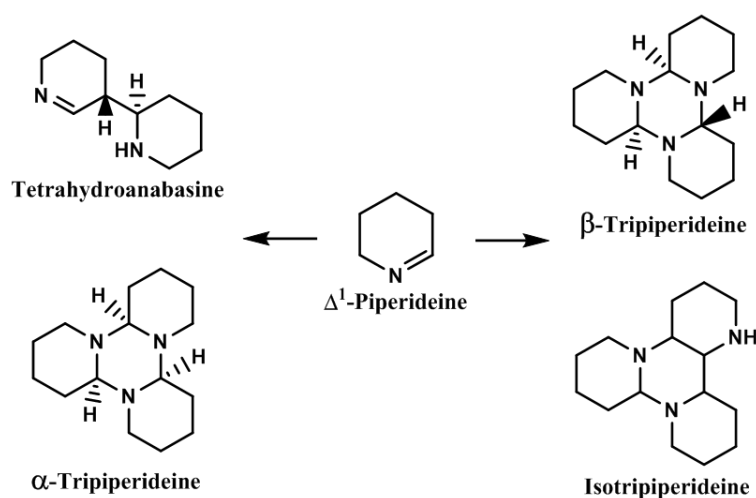


Fig. 2.6: Δ^1 -Piperidine (2,3,4,5-tetrahydropyridine) and its multimeric forms.

The evolution of monomeric Δ^1 -piperidine at different temperature and pH (Fig. 2.7) have been studied by NMR, UV and IR spectroscopy (Shvekhgeimer, 1998, Gravel et al., 2006, Rouchaud and Braekman, 2009, Salame et al., 2010). It was reported that at low temperatures, Δ^1 -piperidine occurs as the β -tripiperidine; while at room temperature, it existed as α -tripiperidine and isotripiperidine (Shvekhgeimer, 1998). At room temperature and pH 7 or slightly alkaline pH (≈ 8), as in nature, Δ^1 -piperidine occurs mainly in its trimeric forms. The α -tripiperidine is the major stable form at pH ≥ 12 , whereas at pH ≤ 2 (strongly acidic solutions), Δ^1 -piperidine exists only in its monomeric form. In addition, maximum formation of isotripiperidine was observed at pH between 9 and 10 (Gravel et al., 2006, Rouchaud and Braekman, 2009). The evolution and formation of the trimeric forms implies the occurrence of the dimer, tetrahydroanabasine, as a transient species and its rate of formation is maximum at pH 8 (Poupon et al., 2011, Salame et al., 2010). Notably, the tetrahydroanabasine can be isolated as a dihydrobromide crystalline salt (Schopf et al., 1948, Shvekhgeimer, 1998, Monaco et al., 2011, Poupon et al., 2011).

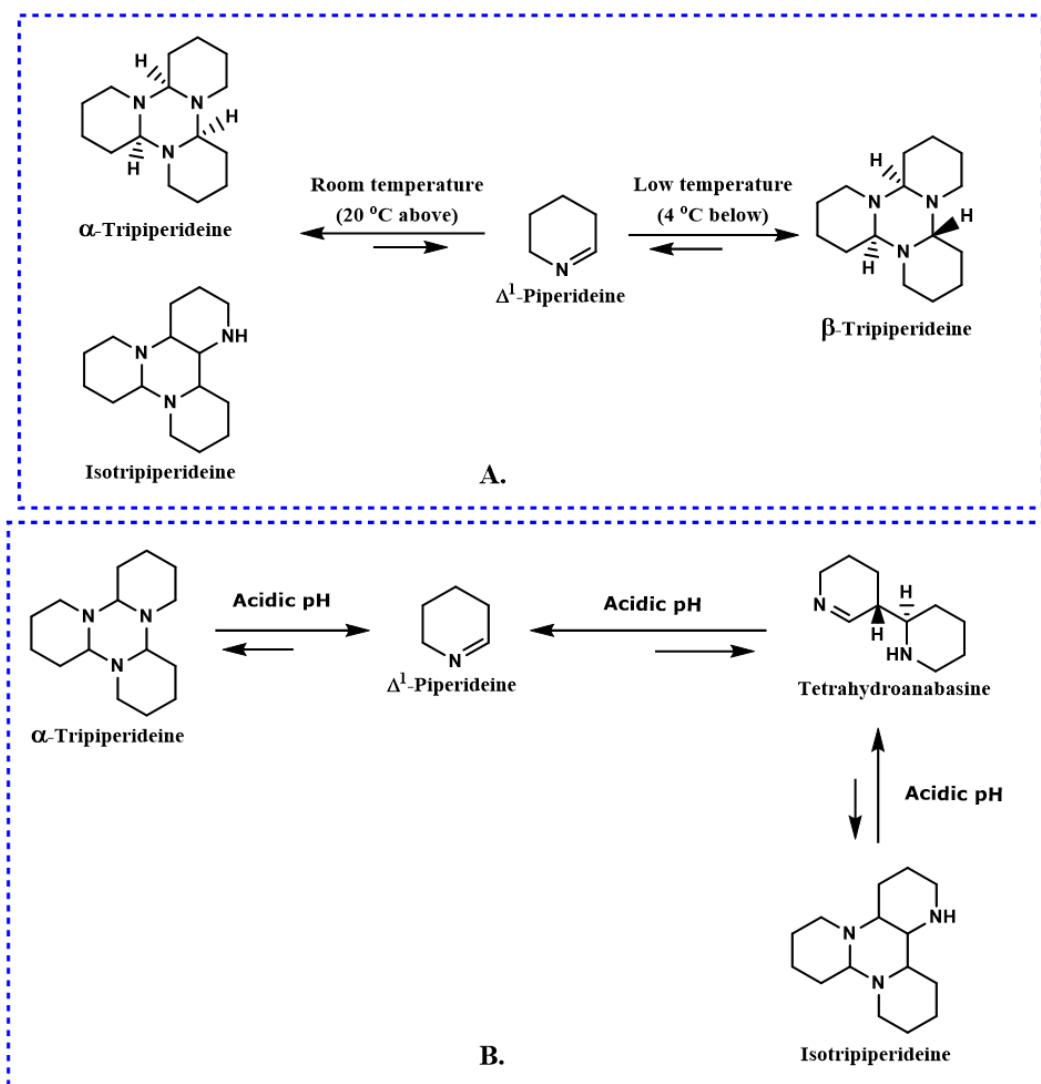


Fig. 2.7: Evolution of Δ^1 -piperidine: effect of Temperature (**A.**) and pH (**B.**).

In organic synthesis, Δ^1 -piperidine and its oxidized and reduced forms (piperidine and pyridine) are convenient starting compounds for the synthesis of lysine-derived alkaloids or development of novel molecules of industrial and pharmaceutical importance (Shvekhgeimer, 1998, Rouchaud and Braekman, 2009, Gravel et al., 2006, Salame et al., 2010, Bunsupa et al., 2012a). Chemical synthesis of unsubstituted Δ^1 -piperidine is commonly by a two-stage procedure, which includes treatment of piperidine with a chlorinating agent and reaction of the resulting chlorinated piperidine with a nucleophilic reagent (Hussain et al., 2015) (Fig. 2.8). The methods for synthesis of Δ^1 -piperidine and its substituted derivatives have been exhaustively reviewed (Shvekhgeimer, 1998).

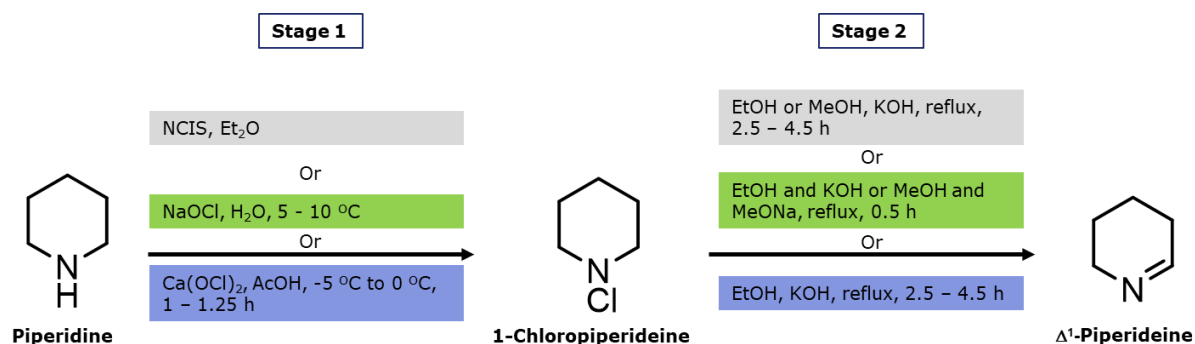


Fig. 2.8: Chemical synthesis of Δ^1 -piperidineine.

Reagents and conditions in the same colour represent a single synthetic batch. NCIS = *N*-Chlorosuccinimide, KOH = potassium hydroxide, EtOH = ethanol, MeOH = methanol, Et₂O = diethyl ether, NaOCl = sodium hypochlorite, MeONa = sodium methoxide, Ca(OCl)₂ = calcium hypochlorite, AcOH = acetic acid (Shvekhgeimer, 1998, Orejarena Pacheco et al., 2016).

Recently, Orejarena Pacheco et al. (2016) reported the total synthesis of tetraponerines T7 and T8 (a class of tricyclic alkaloids which cause paralysis and death presumably by inhibition of nicotinic acetylcholine receptors) using an efficient metal-free photocatalyst and Δ^1 -piperidineine as substrate (Fig. 2.9). Similarly, Rouchaud and Braekman (2009) used Δ^1 -piperidineine, as a substrate for organic syntheses of new analogues of the tetraponerines. The key step of this synthesis was a one-pot stereoselective process in which two molecules of Δ^1 -piperidineine or its dimer reacted with one molecule of diethyl malonate (Hussain et al., 2015) (Fig. 2.10). Also, pelletierine (piperidine alkaloid) and its analogues were produced in a biomimetic organocatalytic synthesis using Δ^1 -piperidineine as substrate (Fig. 2.11) (Monaco et al., 2011).

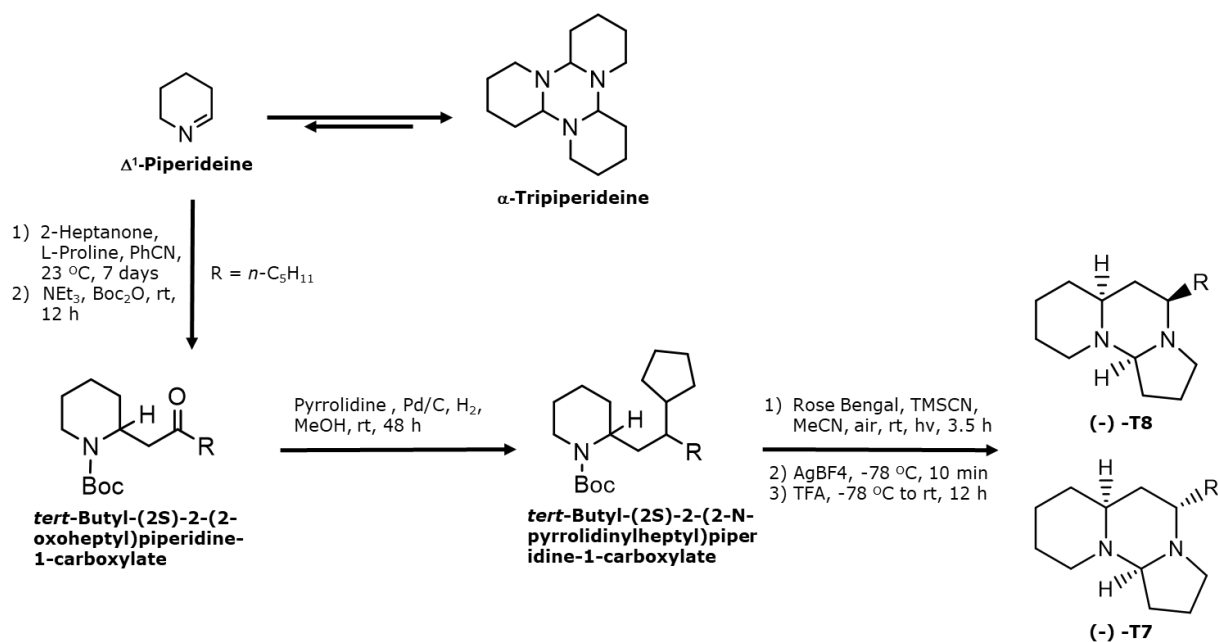


Fig. 2.9: Total synthesis of tetraoponerines T7 and T8.

Boc₂O = di-*tert*-butyl dicarbonate, rt = room temperature, hv = light, PhCN = benzonitrile, NEt₃ = trimethylamine, MeOH = methanol, Pd/C = palladium on carbon, TMSCN = trimethylsilyl cyanide, MeCN = acetonitrile, AgBF₄ = silver tetrafluoroborate, TFA = trifluoroacetic acid (Orejarena Pacheco et al., 2016).

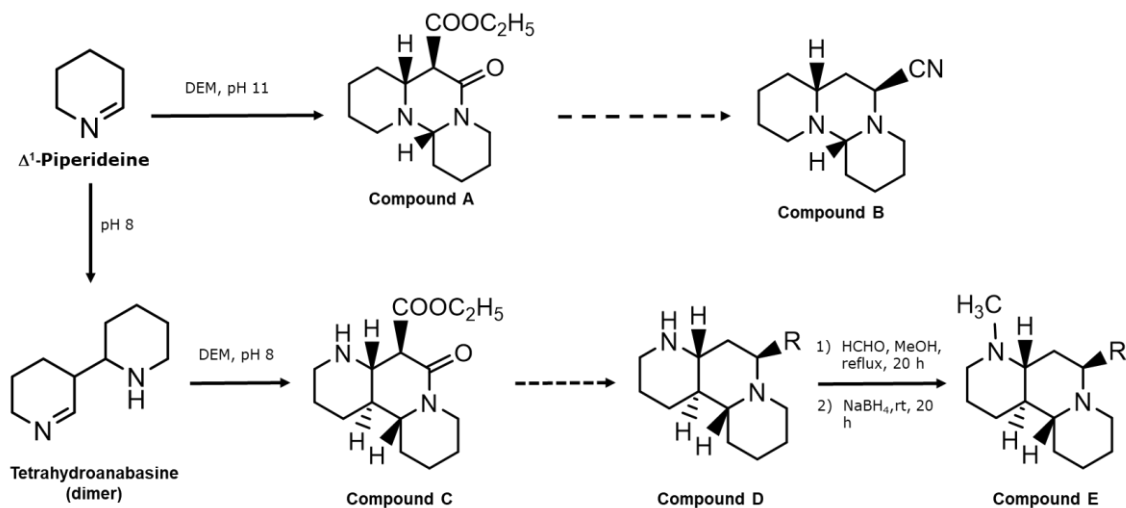


Fig. 2.10: Synthesis of key intermediates and new analogues of tetraoponerines.

Dotted arrows indicate multiple reaction steps. Compounds A, B and C = key intermediates, compounds D and E = new analogues of tetraoponerines, HCHO = formaldehyde, MeOH = methanol, DEM = diethyl malonate, NaBH₄ = sodium tetrahydroborate, R = C₃H₇, C₅H₁₁ and C₁₂H₂₅ (Rouchaud and Braekman, 2009).

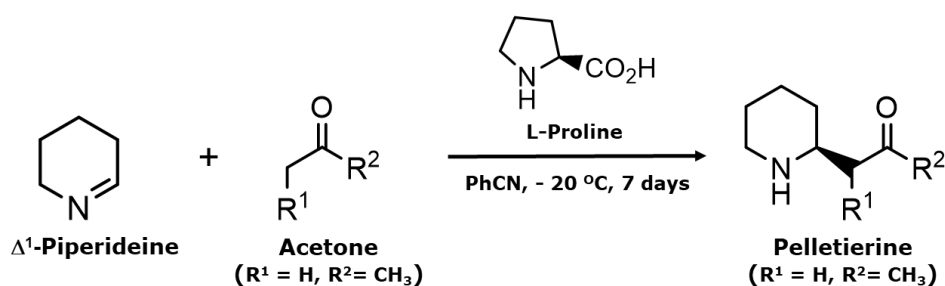


Fig. 2.11: Organocatalytic synthesis of pelletierine and its analogues. PhCN = benzonitrile (Monaco et al., 2011).

In the same vein, Δ^1 -piperideine have been used as a precursor for total synthesis of valuable structurally diverse heterocycles (Unsworth et al., 2013, Unsworth et al., 2014), while during investigation on one-step synthesis of complex polycyclic alkaloids, Gravel et al. (2006) reported the synthesis of nitramine by reacting chemically synthesised Δ^1 -piperideine and glutaraldehyde (Fig. 2.12). However, all of these organic syntheses used petrochemical feedstocks.

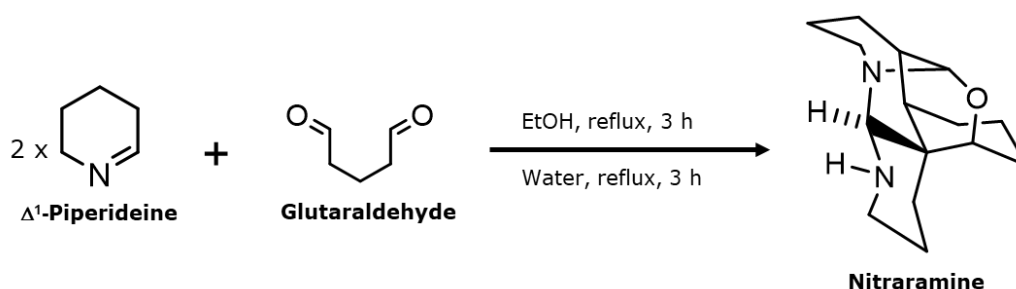


Fig. 2.12: Biomimetic synthesis of nitramine. EtOH = ethanol (Gravel et al., 2006).

In summary, Δ^1 -piperideine exists in solution as a complex diastereoisomeric mixture of its trimeric (major component) and monomeric form. Nonetheless, the use of Δ^1 -piperideine is not limited because when it is consumed in a chemical reaction, equilibrium adjustment causes the dissociation of the trimeric forms. Clearly, in a biosynthetic study that operates optimally in an aqueous environment, the speciation of Δ^1 -piperideine could present a challenge. However, it can be isolated in the trimeric forms (Gomm et al., 2016).

2.2.1 Δ^1 -Piperidine: Towards bioproduction

Δ^1 -Piperidine constitutes an important structural component of the lysine-derived alkaloids. Based on feeding experiments with labelled substrates using plants in their natural habitat (Ma and Gang, 2004), common characteristics in the biosynthesis of this alkaloid family, is that they are elaborated from the lysine derived Δ^1 -piperidine (see Fig. 2.5) (Szőke et al., 2013, Bunsupa et al., 2017). Although reports on organisms (plants, animals or microorganisms) accumulating or excreting Δ^1 -piperidine are not available, the wide biological occurrence of these lysine-derived alkaloids signify that the enzymatic toolkits for its bioproduction exists. The speciation of Δ^1 -piperidine does not prevent lysine-alkaloid synthesis due to plants having enzymes that will consume it down the pathway (see Fig 2.5) (Evans et al., 2009, Bunsupa et al., 2012a). The steps from lysine to Δ^1 -piperidine are proposed to be catalysed by decarboxylases and amine oxidases respectively.

Recently, in an attempt to identify smart amino donors for synthesis of optically pure chiral amines in a transaminase-mediated biotransformation, Gomm et al. (2016) reported the conversion of cadaverine to Δ^1 -piperidine and its trimers (α -tripiperidine and isotripiperidine) using a commercial ω -transaminase (ω -TA). To the best of our knowledge, microbial bioproduction of Δ^1 -piperidine employing the advances of metabolic engineering and synthetic biology is yet to be reported.

2.2.1.1 Candidate substrates for Δ^1 -Piperidine bioproduction

As established earlier, Δ^1 -piperidine is derived from lysine through decarboxylation and oxidative deamination (Bunsupa et al., 2017). The decarboxylation step yields cadaverine which further undergoes oxidative deamination into 5-aminopentaldehyde (5-aminopentanal). Because 5-aminopentaldehyde is chemically unstable, it undergoes spontaneous cyclization (Schiff base reaction) forming Δ^1 -piperidine (Fig. 2.13). The above Schiff base formation depends on water elimination, however, formation of peptide bonds are stable and does not dissociate. In addition, speciation of Δ^1 -piperidine favours

the forward reaction limiting the reversibility to the 5-aminopentaldehyde. In principle, the key is to form an aminoaldehyde, which can then be converted to a cyclic Schiff base, forming the target *N*-heterocycles. Therefore, cadaverine serves as a substrate and its biosynthesis could be explored for the design of Δ^1 -piperidine bioproduction in microorganisms (*Escherichia coli*).

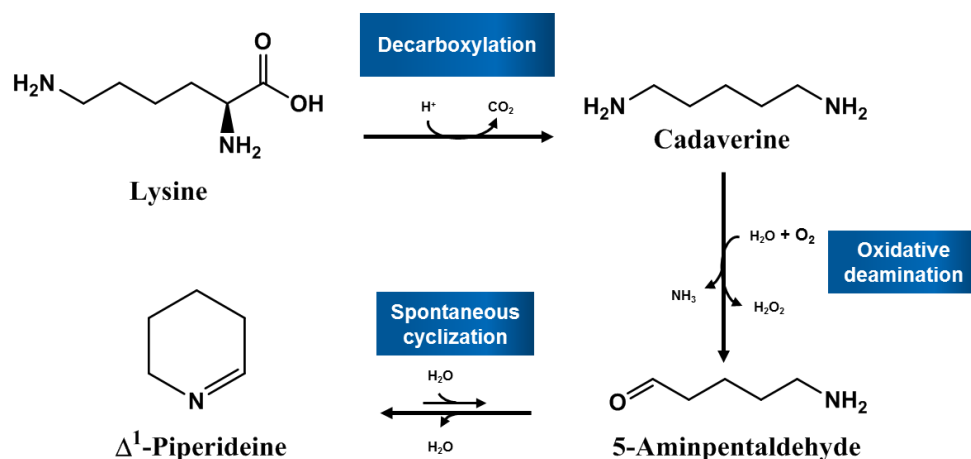


Fig. 2.13: Δ^1 -Piperidine bioproduction from lysine through decarboxylation and oxidative deamination.

Cadaverine (1, 5-diaminopentane) is a linear aliphatic diamine with five carbons. It was first isolated and described between the 17th and 20th centuries during systematic experiments on the putrefaction of human cadavers and this provided directly the basis for its trivial name cadaverine (Wallace et al., 2003, Kind and Wittmann, 2011). As a biogenic polyamine, cadaverine modulates cellular processes; making it essential for the growth and function of normal cells (Wallace et al., 2003, Cha et al., 2014, Slabu et al., 2016). It has been reported to protect microbial cells from oxidative and antibiotic stress (Kang et al., 2007, Tkachenko et al., 2009, Kanjee et al., 2011). Cadaverine has been used for production of bio-based polyamides (e.g. polyamide 54, made by polycondensation of cadaverine and succinic acid), polyurethane, chelating agents, and other additives (Mimitsuka et al., 2007, Kind et al., 2010, Kind and Wittmann, 2011).

In microorganisms, cadaverine is synthesized from lysine through decarboxylation by lysine decarboxylase (EC 4.1.1.18) (Kind et al., 2010, Mimitsuka et al., 2007, Mimizuka and Kazami, 2002). Overproduction of cadaverine using metabolically engineered *Corynebacterium glutamicum* (Mimitsuka et al., 2007, Tateno et al., 2009, Kind et al., 2011, Buschke et al., 2013) and *E. coli* (Qian et al., 2010) overexpressing lysine decarboxylase has been studied. However, yields remained relatively low [2.6g/l and 9.61g/l in *C. glutamicum* (Mimitsuka et al., 2007) and *E. coli* (Qian et al., 2010)].

2.2.1.2 Enzyme candidates for Δ^1 -piperidine bioproduction

Conversion of cadaverine to Δ^1 -piperidine through 5-aminopentanal intermediate, firstly, involves removing one of its terminal amine groups (-NH₂) and replacing it with an aldehyde group (-CHO). In principle, oxidative deamination or transamination can achieve this kind of reaction. In nature, enzymes in the class of amine oxidoreductases (EC. 1.4.3) and amine transferases (EC. 2.6.1) can catalyse this reaction.

2.2.1.2.1 Amine oxidases: Putrescine oxidase (PuO)

Amine oxidases (AO; EC. 1.4.3) are enzymes that catalyse the oxidative deamination of many biologically important alkylamines (aliphatic amines and psychotropic alkylamines) with the formation of the corresponding aldehyde, hydrogen peroxide and ammonia (Fig. 2.14) (Mondovì and Agrò, 1982). These enzymes are widely found in living organisms because of their roles in neurotransmission, cell growth and differentiation, neoplastic cell proliferation and in biocatalytic application (Pegg, 1988, Seiler et al., 1998, Abell and Kwan, 2001, Thomas and Thomas, 2001, Carr et al., 2005, Dunsmore et al., 2006). AOs are broadly classified into two subfamilies based on the use of copper and 2,4,5-trihydroxyphenylalanine quinone (Cu-TPQ; EC 1.4.3.6) or Flavin (FAD or FMN; EC 1.4.3.4) cofactors for catalysis (Kopacz et al., 2014, Bellelli et al., 2000).

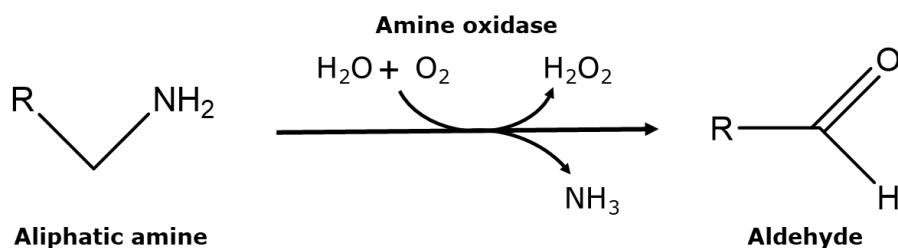


Fig. 2.14: Reaction of amine oxidase (AO) on aliphatic amines.

Copper amine oxidases (CuAO) function via a non-sequential (ping-pong, Fig. 2.15) mechanism and are homodimeric proteins consisting of 70-90KDa subunits, each containing a single copper ion (Cu^{2+}) and covalently bound redox 2,4,5-trihydroxyphenylalanine quinone (Cu-TPQ) cofactor. According to Sun et al. (2012) and Brazeau et al. (2004), the Cu-TPQ cofactor complex is formed by the post-translational modification of the conserved tyrosine side chain within the consensus active site sequence of Asn-Tyr-Asp/Glu. Plant CuAO are generally more catalytically active than those from other eukaryotes and prokaryotes (Bellelli et al., 2000, Brazeau et al., 2004). CuAO from *Huperzia serrata* suspected to be involved in the biosynthesis of huperzine-A (a lysine-derived alkaloid) has been cloned and characterized (Sun et al., 2012). *In vitro* analysis revealed the CuAO had the best substrate selectivity and activity on cadaverine with a K_m and K_{cat} (catalytic efficiency) of 300 μM and 1.0 s^{-1} respectively. However, the requirement of copper ion and the difficulty associated with post-translational modification of eukaryotic enzymes in bacteria (*E. coli*) limits the choice of this enzyme in a bioproduction design.

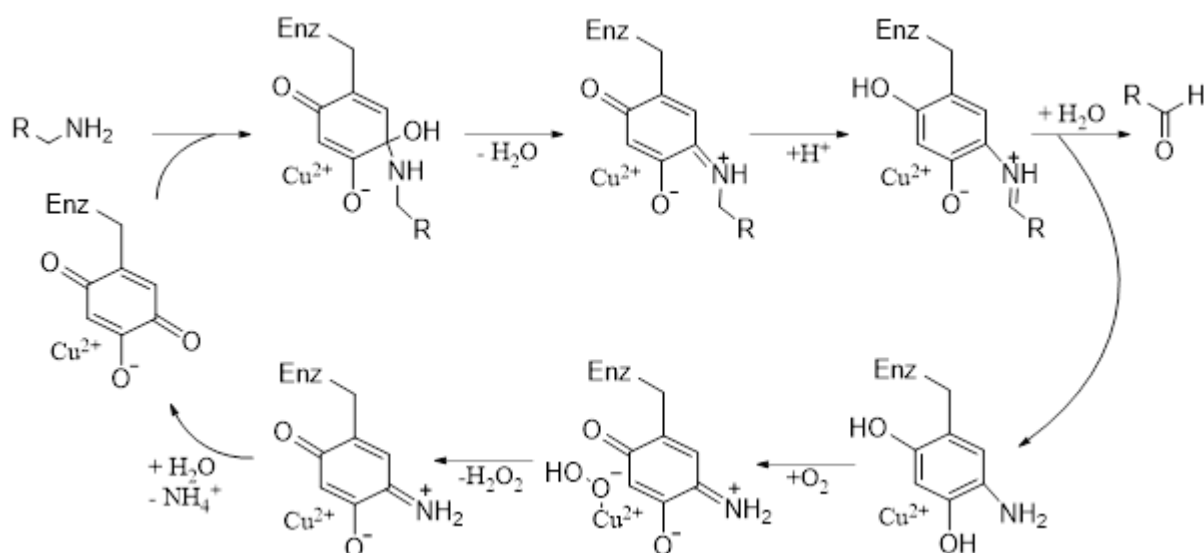


Fig. 2.15: Mechanism of copper amine oxidases.

The enzyme catalyse the oxidative deamination of primary amines ($R-C-NH_2$) to the corresponding aldehydes ($R-CHO$), with the concomitant reduction of dioxygen (O_2) to hydrogen peroxide (H_2O_2) via a non-sequential (ping-pong) mechanism involving a covalently bound redox cofactor, 2,4,5-trihydroxyphenylalanine quinone (TPQ), and a copper (Cu^{2+}) ion (Sun et al., 2012).

Flavin adenine dinucleotide (FAD; Fig. 2.16) proteins have several structural classes with majority containing a distinct domain that binds tightly but in a noncovalent manner with the adenosine dinucleotide phosphate (ADP) moiety of the FAD (Dym and Eisenberg, 2001). However, in some FAD proteins, the FAD cofactor is covalently bound. The most common covalent bond involves the C8 α -methyl moiety of the Flavin cofactor and a histidine or cysteine residue of the enzyme (Heuts et al., 2009). Noteworthy, the incorporation of FAD is autocatalytic (Kopacz et al., 2011) and the reactions catalysed by flavin-containing enzymes do not always share mechanistic pathways (Fig. 2.17) (Ghisla and Massey, 1989, Kopacz et al., 2014). FAD - amine oxidases are valuable biocatalysts for the oxidative deamination of biomolecules, as they selectively oxidize their substrate at a specific position, leaving other positions unaltered and their reactions are straightforward.

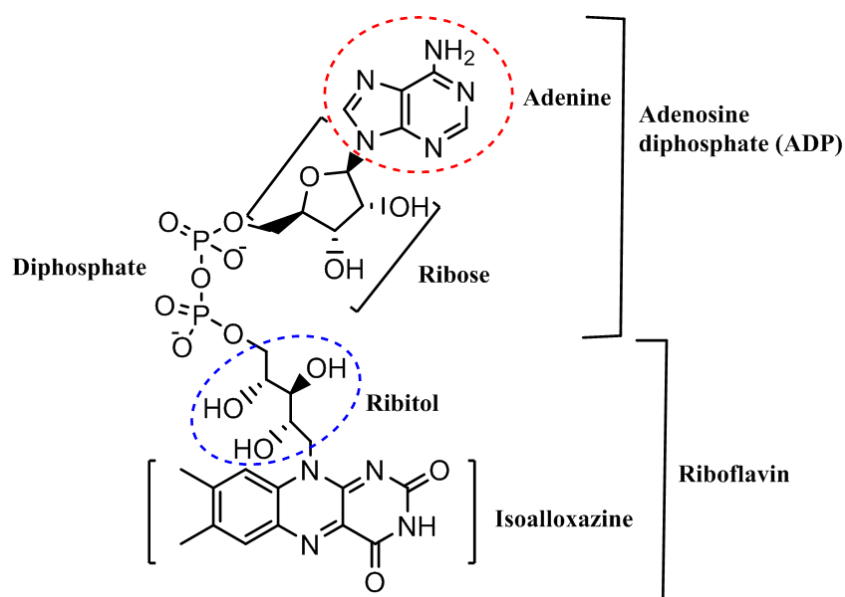


Fig. 2.16: Structure of Flavin adenine dinucleotide (FAD).

The redox chemistry of the FAD molecule is restricted to the isoalloxazine nucleus while the adenine moiety is not involved in catalysis but serves in anchoring the coenzyme at the active site of the protein (Ghisla and Massey, 1989).

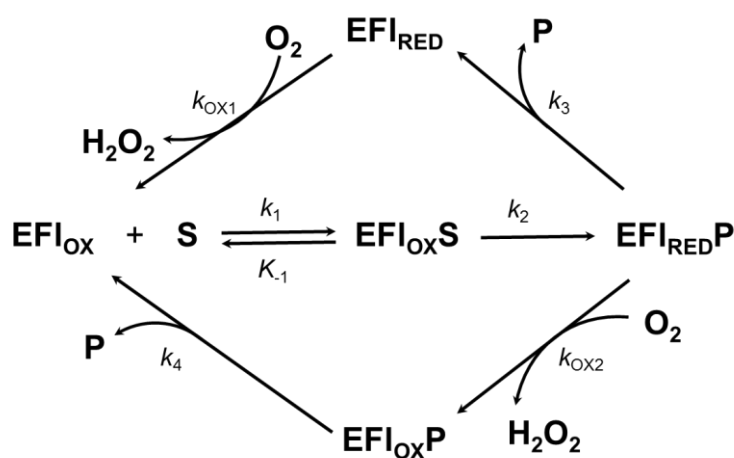


Fig. 2.17: Common kinetic mechanisms of FAD-amino oxidases.

The upper cycle represents a non-sequential (ping-pong) mechanism while the lower cycle an ordered sequential mechanism. **E_{ox}** represents oxidized form of enzyme; **E_{red}**, reduced form of enzyme; **S**, substrate; **P**, product (adapted from Kopacz et al., 2014).

Putrescine oxidase (PuO) is an FAD-dependent amine oxidase that catalyses the oxidative deamination of putrescine and some other aliphatic amines like cadaverine. PuO has been successfully cloned and characterized from *Micrococcus rubens* (Adachi et al., 1966, Yamada, 1971, DeSa and Brantner, 1972) and more recently from *Rhodococcus*

erythropolis (van Hellemond et al., 2008b). Putrescine oxidase from *R. erythropolis* (PuO_{Rh}) is a non-covalently bound FAD amine oxidase having a high degree of sequence homology with human and fungal monoamine oxidases (MAOs) (Kopacz et al., 2011). PuO_{Rh} contains one FAD molecule per protein dimer and mass spectrometry analysis revealed that this relatively low FAD content is the result of competitive noncovalent binding of ADP in the FAD binding site (van Hellemond et al., 2008a, Kopacz et al., 2011). According to van Hellemond and co-workers, it is a soluble dimeric flavoprotein consisting of 50 KDa subunits and reasonably thermostable with a half-life of 2 h at 50 °C, K_m and k_{cat} values of 18 ± 3 μM and 3.9 ± 0.2 s⁻¹ respectively for cadaverine.

Conclusively, AO are inexpensive in usage compared to other redox enzymes because of their ability to use molecular oxygen as electron acceptor instead of expensive coenzymes like NAD(P)H. The hydrogen peroxide (H₂O₂) and ammonia (NH₃) by-products can respectively be degraded by catalases to prevent oxidative stress and consumed as a source of nitrogen. PuO_{Rh} has a higher affinity and catalytic efficiency for cadaverine than CuAO and offers the further advantage that it does not require a Cu²⁺ cofactor.

2.2.1.2.2 Aminotransferase: Putrescine aminotransferase (PAT)

Aminotransferases are pyridoxal-5'-phosphate (PLP)-dependent enzymes that have shown remarkable versatility in the asymmetric synthesis of chiral amino building blocks (Slabu et al., 2016). Similar to many other PLP-dependent enzymes, aminotransferases are expected to undergo two half reactions (Fig. 2.18). The PLP cofactor of these enzymes bind covalently to the amino group of the substrate and act as an electrophile to stabilize different carbanionic intermediates (Eliot and Kirsch, 2004, Schiroli and Peracchi, 2015).

Putrescine aminotransferase (PAT; EC. 2.6.1.82) catalyses the transfer of an amino group from terminal diamine donor molecules to keto-acid acceptors by using pyridoxal-5'-phosphate as a cofactor (Cha et al., 2014). Based on its amino acid sequence alignment, it belongs to the class III pyridoxal-5'-phosphate (PLP)-dependent aminotransferase family, which include ornithine aminotransferase (OAT) and γ-aminobutyric acid

aminotransferase (GABA-AT) (Samsonova et al., 2003, Punta et al., 2012). In the first half-reaction, putrescine is converted to γ -aminobutyraldehyde, and the putrescine amino group is transferred to the PLP cofactor to yield pyridoxamine phosphate (PMP); this γ -aminobutyraldehyde is spontaneously cyclized to Δ^1 -pyrroline (Kurihara et al., 2008, Cha et al., 2014). In the second half-reaction, the PMP amino group is transferred to the α -ketoglutarate to yield L-glutamate and regenerate PLP (Fig. 2.18). Previous biochemical data show that the enzyme also can transaminate cadaverine and, in lower extent, spermidine, but not ornithine (Cha et al., 2014, Slabu et al., 2016). Alpha-ketobutyrate and pyruvate can also act as amino acceptors, although much less efficiently. The genes (yggG) from *Escherichia coli* K12, *Bacillus megaterium*, *Bacillus mycoides* and *Pseudomonas* sp. were successfully cloned and expressed in *E. coli* (Samsonova et al., 2003, Cha et al., 2014, Slabu et al., 2016, Wilding et al., 2017).

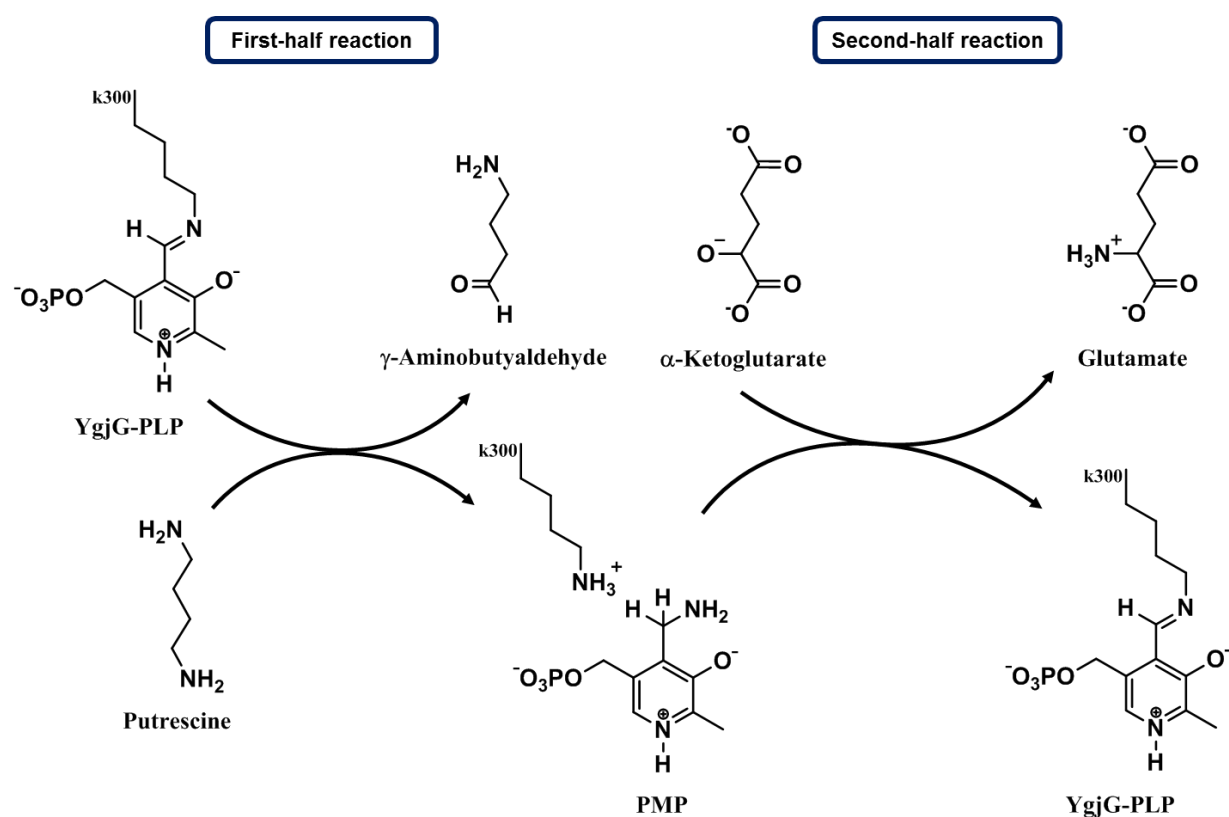


Fig. 2.18: Mechanism of PLP – aminotransferase showing the two half reactions catalysed by putrescine aminotransferase (YggG-PLP) using putrescine as substrate (Koszelewski et al., 2010, Cha et al., 2014, Schirotti and Peracchi, 2015).

In conclusion, putrescine aminotransferase and ω -transaminase have been used in whole-cell and cell-free biocatalysis for synthesis of optically pure chiral amines (O'Reilly et al., 2014, Gomm et al., 2016, Both et al., 2016). However, in a bioproduction design from renewable feedstock, PAT and ω -TA are not ideal because their requirement of co-substrate will reduce carbon efficiency as well as increase the energy demand and metabolic pressure on the cell factory.

2.2.1.3 Choice of microbial host

Δ^1 -Piperideine is not known to be accumulated in nature by microorganisms. Therefore, it is our opinion that Δ^1 -piperideine could be overproduced using recombinant bacteria because they lack necessary machinery for its secondary metabolism. Potentially, this is a signpost for good product yield and easy down-stream processing. Interestingly, amine oxidases (EC. 1.4.3) and amine transferases or transaminases (EC. 2.6.1) discussed earlier are found in microorganisms (O'Reilly et al., 2014, Hussain et al., 2015, Bezborodov and Zagustina, 2016, Gomm et al., 2016).

Therefore, *Escherichia coli* was chosen for this work because it is a well-reported, robust and reliable bio-factory that is easy to propagate and grows quickly, and it is generally recognized as safe (GRAS) for bioproduction. Most recombinant proteins can be cloned and expressed in *E. coli*, and *E. coli* is well documented for its advantages including low cost, easy transformation and fermentation, and high protein yields (Theisen and Liao, 2016).

3. AIMS AND OBJECTIVES

3.1 Aims and objectives

The aim of this study was to bioproduce Δ^1 -piperidine (a six-membered simple *N*-heterocycle) using metabolically engineered *Escherichia coli*, firstly, through biocatalysis (cadaverine to Δ^1 -piperidine) using purified enzyme and whole cells. Secondly, by fermentation of renewable feedstocks (xylose or glucose) to Δ^1 -piperidine using active whole cells (Fig. 3.1).

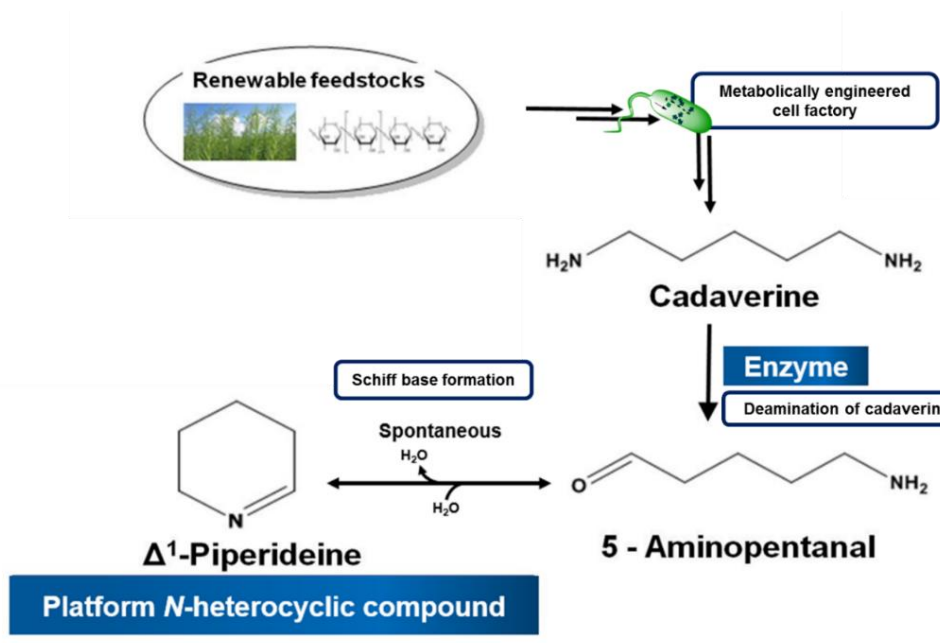


Fig. 3.1: Research strategy.

The objectives were:

1. Identify candidate six-membered simple *N*-heterocycle of economic importance and design its bioproduction using metabolically engineered microorganism.

*This study centred on sustainable production of *N*-heterocycles. *N*-heterocycles includes a broad range of simple and complex compounds, thus, the objective was to narrow down by identifying a natural candidate six-membered simple *N*-heterocycle of value that could be produced from renewable feedstocks using microorganisms and employ the gains of metabolic engineering to optimize its production. In this context, Δ^1 -piperidine has been identified.*

2. Identify candidate enzymes that could selectively catalyse conversion of cadaverine to Δ^1 -piperideine. Clone, express and characterize (if necessary) best candidate enzyme in *Escherichia coli*.

Having identified Δ^1 -piperideine as a candidate six-membered simple N-heterocycle of value that could be produced from renewable feedstocks using microorganisms, reviewed its biosynthesis in nature and retro-synthetically identified cadaverine as a terminal substrate, the objective was to identify candidate enzyme with potential to catalyse its formation in a microbial bioproduction set-up. This was done within the class of amine oxidases (EC. 1.4.3) and amine transferases or transaminases (EC. 2.6.1), since these are the enzymes that catalysed similar reaction in nature. The objective also, was to use the knowledge of metabolic engineering and synthetic biology to design an E. coli strain that could overexpress soluble, stable and active candidate enzyme of interest. Finally, the objective included characterisation of the overexpressed candidate enzyme of interest.

E. coli was chosen for this work because it is a well-reported, robust and reliable bio-factory that is easy to propagate and grows quickly, easy to manipulate genetically with the tools of metabolic engineering and synthetic biology, and it is generally recognized as safe (GRAS) for bioproduction. In addition, most recombinant proteins can be cloned and expressed in E. coli. Finally, the use of E. coli for protein expression is well documented for its advantages of low cost, easy transformation and fermentation, and high protein yields (Theisen and Liao, 2016).

3. Test purified enzyme in a cell-free reaction system and enzyme induced non-growing whole cells for activity on cadaverine to evaluate its ability to catalyse the target reaction.

The objective was to test the enzyme for bioconversion of cadaverine to Δ^1 -piperideine, firstly using purified enzyme and secondly using enzyme induced non-growing whole cells. Therefore, tests were done in crude extracts and the reaction conditions employed were based on literature. In order to be sure that the enzymes were expressed in active form, they were always tested on their natural substrates before being tested on the substrates of interest.

4. Design a metabolic pathway towards developing a metabolically engineered microbial strain that overproduces Δ^1 -piperideine from cadaverine and renewable feedstock (glucose and xylose).

Overproduction of cadaverine using metabolically engineered Corynebacterium glutamicum (Mimitsuka et al., 2007, Mimizuka and Kazami, 2002, Tateno et al., 2009, Kind et al., 2011, Buschke et al., 2013) and E. coli (Qian et al., 2010) has been studied. However, yields remained relatively low. Therefore, this study will build on these reports, and advances in metabolic engineering and synthetic biology to design a pathway for bioproduction of Δ^1 -piperideine.

5. To identify optimal analytical method(s) for Δ^1 -piperideine.

Δ^1 -Piperideine exists in solution as a complex diastereoisomeric mixture of its trimeric (major component) and monomeric form. Clearly, in a biosynthetic study that operates optimally in an aqueous environment, the speciation of Δ^1 -piperideine represents a challenge. However, it can be isolated in the trimeric forms (Gomm et al., 2016), therefore, effect of pH adjustment and extraction into organic solvents was explored. Also, the objective was to quantify each of the product species using quantitative structural analytical methods e.g. Nuclear Magnetic Resonance (NMR).

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

The following chemicals were purchased from Sigma-Aldrich: glucose, agar, carbenicillin, ethanol (EtOH), isopropanol, glycerol, imidazole, isopropyl β -D-1-thiogalactopyranoside (IPTG), β -mercaptoethanol, sodium dodecyl sulphate (SDS), dimethyl sulfoxide (DMSO), ethylenediaminetetracetic acid (EDTA), magnesium sulphate (MgSO_4), 99.9% deuterium oxide (D_2O), deuterated chloroform (CDCl_3), chloroform (CHCl_3), sodium acetate, hydrochloric acid (American Chemical Society), sodium chloride (NaCl), potassium chloride (KCl), sodium hydroxide (NaOH), sodium carbonate (NaHCO_3), sodium sulphate (Na_2SO_4), maleic acid, *o*-aminobenzaldehyde, and 3,4,5-trichloropyridine. Ethidium bromide (EtBr_2) and agarose were purchased from Fisher. Purity of all chemicals were within analytical standards.

4.1.2 Reagents

Tris/glycine/SDS Premixed Electrophoresis Buffer, EZBlue Stain solution, TAE buffer and Laemmli Sample Buffer were purchased from Bio-Rad. DNA Loading Dye, Gene Ruler™ 1 Kb Plus DNA Ladder and Spectra™ Multicolour Broad Range Protein ladder were purchased from Fermentas. EB buffer and TE buffer were purchased from Qiagen. BugBuster Reagent™ and nucleotides were purchased from Merck-Millipore. SOC medium and cOmplete EDTA-Free Protease Inhibitor were purchased from Novagen and Roche, respectively.

4.1.3 Enzymes

T4 DNA Ligase and Antarctic phosphatase were purchased from New England Biolabs (NEB Inc.). Restriction enzymes (FastDigest®) were purchased from Thermo Scientific. Benzonase® nuclease was purchased from Merck-Millipore.

4.1.4 Kits

QIAQuick Gel Extraction Kit, QIAprep MiniPrep and MidiPrep kit were purchased from Qiagen. TransformAid™ Bacterial Transformation Kit was purchased from Fermentas and DC™ Protein Assay Kit from Bio-Rad.

4.1.5 Plasmids

pET-20b(+) was purchased from Novagen. pBMH vector was obtained from Biomatik as a carrier of the synthesised gene. Plasmids used in this chapter are listed in Table 4.1.

Table 4.1: Plasmids used

Strain or plasmid	Features	Reference
pBMH_ <i>puo_{Rh}</i>	Carrier plasmid, with the <i>puo_{Rh}</i> gene codon optimized for expression in <i>E. coli</i> and the inclusion of <i>XhoI</i> and <i>NdeI</i> restriction sites	Synthesized by Biomatik
pET-20b(+)	Expression vector, 3716bp, pBR322_ori; Amp ^R , T7 promoter, 6XHis(C terminal)	Novagen
pET-20b_ <i>puo_{Rh}</i>	pET20b with the <i>puo_{Rh}</i> gene inserted between <i>NdeI</i> and <i>XhoI</i> sites	This study

Plasmids and their sources are shown. Ori: origin of replication, amp^R: ampicillin resistance marker, 6XHis: hexa-histidine tag.

4.1.6 Strains

Escherichia coli JM107 was purchased from Fermentas-Thermo Scientific (UK) and competent *E. coli* BL21(DE3) from New England Biolabs (NEB Inc.). Bacterial strains used in this chapter are listed in Table 4.2.

Table 4.2: Bacterial strains used

Strain or plasmid	Features	Reference
<i>Escherichia coli</i> strains		
JM 107	<i>endA1 glnV44 thi-1 relA1 gyrA96 Δ(lac-proAB) [F' traD36 proAB⁺ lacI^q lacZΔM15] hsdR17(R_K⁻ m_K⁺) λ⁻</i>	(Yanisch-Perron et al., 1985)
BL21(DE3)	<i>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int:::(lacI::PlacUV5::T7 gene1) i21 Δnin5</i>	NEB Inc. & Novagen
JM 107_pET20b_ <i>puo_{Rh}</i>	JM107 complemented with pET20b harbouring <i>puo_{Rh}</i> gene	This study
BL21(DE3)_pET20b	BL21(DE3) complemented with pET20b, used as a control recombinant strain	This study
BL21(DE3)_pET20b_ <i>puo_{Rh}</i>	BL21(DE3) complemented with pET20b harbouring <i>puo_{Rh}</i> gene	This study

4.2 Methods

4.2.1 *In silico* analysis

4.2.1.1 Search for candidate enzymes

Databases such as CAS Scifinder (www.cas.org) and NCBI Pubmed (www.ncbi.nlm.nih.gov) were used to obtain articles, patents, and other scientific publications. Protein sequences, substrate range information and other significant functional data, were collected with the help of four main on-line enzyme and genome databases: The Kyoto Encyclopaedia of Genes and Genomes (www.genome.jp/kegg), the Uniprot database (www.uniprot.org), the Comprehensive Enzyme Information System - BRENDA (www.brenda-enzymes.org) and the RCSB Protein Data Bank (www.rcsb.org).

4.2.1.2 Gene/Protein sequence alignments

Gene and protein sequence alignments and blasting during plasmid design and gene cloning using UniProt and NCIB online services (Chen et al., 2017). The organism from which the gene (amino acid) sequences were used and the respective GenBank (Benson

et al., 2013) sequence accession number are as follows: *Rhodococcus erythropolis* NCIMB 11540 (EU240877).

4.2.2 Microbiological methods

4.2.2.1 General handling and preparation of media

Except otherwise stated, *E. coli* JM107 and *E. coli* BL21(DE3), and their recombinant strains were grown on Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride) supplemented with 50 µg/mL carbenicillin (C) and 1% glucose (G) for selection and screening purposes. SOC medium was used as outgrowth media following transformation by heat shock and contained: tryptone (20 g/L), yeast extract (5 g/L), glucose (3.6 g/L), MgCl₂ (0.95 g/L), NaCl (0.5 g/L) and KCl (0.186 g/L).

Media were dissolved in distilled water (dH₂O) and then autoclaved. For the preparation of agar plates, agar (15 g/L) was also added before autoclaving. Agar media were then cooled to ≈50 °C and poured manually in Petri dishes. Carbenicillin and glucose were added separately as required from filter sterilised 50 mg/mL stock solution prepared in dH₂O and 200 g/L stock solution prepared in dH₂O, respectively.

All media used were autoclaved (121 °C and 15 psi for 15 min or where stated in the text) whereas antibiotics and glucose solutions were filter (0.2 µm filter) sterilized. In addition, sterility tests were conducted by incubating broth and agar plates overnight at 37 °C or room temperature and then inspect visually for turbidity or colonies respectively before use.

4.2.2.2 Strains and growth conditions

All broth cultivations were performed in Falcon tubes or Erlenmeyer flasks, with nominal volumes five times the culture volume unless otherwise stated.

For long-term and routine storage of all organisms, glycerol stocks (8%) of transformants and their controls, in LB broth (supplemented with the appropriate antibiotic) were prepared. To prepare glycerol stocks, fresh LB medium (5 mL, supplemented with the

appropriate antibiotic) was inoculated with a single colony of the strain and incubated (37 °C, 250 rpm) until the exponential growth phase was reached. Samples (900 µL) were transferred into cryo-vials together with sterile 80% glycerol (100 µL) and stored at -80 °C.

For protein expression or whole-cell biotransformation assays, experimental cultures were derived from glycerol stocks of recombinant and control (*E. coli* transformed with empty plasmid) strains grown overnight (16-18 h) on LB agar supplemented with the appropriate antibiotic at 37 °C. Single colonies from the plates were used to inoculate precultures. Except otherwise stated, precultures were grown overnight on 10 or 50 mL LB broth (supplemented with the appropriate antibiotic) in sterile 50 mL Falcon tubes or 250 mL Erlenmeyer flasks respectively and incubated at 37 °C and 250 rpm, until an optical density at 600nm (OD₆₀₀) of approximately 1 was reached. Thereafter, the cells were collected by centrifugation (6000 rpm, 4 °C, 15 min). The cell pellets were resuspended in 1 mL LB broth, the OD₆₀₀ was measured and the aliquot used for inoculation of main expression experiments to an initial OD₆₀₀ of 0.1, calculated using Eq. 1.0. All expression experiments were conducted in 250 mL or 500 mL Erlenmeyer flasks containing 50 ml or 100 mL medium as appropriate respectively, except otherwise stated. Protein expression was induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG, 0.4 mM) at an OD₆₀₀ of approximately 0.6. Induced cultures were incubated and harvested by centrifugation (6000 rpm, 10 min, 4 °C) 5 h post induction unless otherwise stated.

$$\text{Initial OD}_{600} \text{ (e.g. 0.1)} = \frac{X}{(Y+X)} * Z \quad \text{----- Eq. 1.0}$$

Where X = Volume of preculture to be used for inoculation of fermentation flasks

Y = Volume of appropriate medium in fermentation flask

Z = OD₆₀₀ of centrifuged and resuspended precultures

4.2.2.3 Enzyme expression

For optimisation of enzyme expression and for the preparation of cultures for enzymatic assays, *E. coli* BL21(DE3) transformed with pET-20b(+) containing the gene of interest (20 mL) were incubated at 37 °C with 250 rpm shaking. Each time, a control culture of *E. coli* transformed with empty plasmid was also prepared. When the OD₆₀₀ reached approximately 1, the cultures were collected by centrifugation (6000 rpm, 4 °C, 15 min), resuspended in fresh LB medium (5 mL), and used as the inoculum for a bigger culture (100 mL when optimizing enzyme expression and 400 mL when preparing cultures for enzymatic assays). When OD₆₀₀ reached approximately 0.6, expression of the enzymes was induced by addition of filter sterilized IPTG to a final concentration of 0.4 mM. Temperatures and incubation times are described in the text.

4.2.3 Molecular biology methods

4.2.3.1 Oligonucleotides (Primer design and synthesis)

The oligonucleotides used for gene sequencing, were synthesised by Integrated DNA Technology (US).

4.2.3.2 DNA extractions

Plasmid DNA was extracted and purified using the QIAprep Spin MiniPrep Kit (Qiagen) for small amounts of plasmid or QIAprep Spin MidiPrep Kit (Qiagen) for larger amounts of plasmid when required, such as for ligation reactions. LB medium (5 mL for minipreps, 100 mL for midipreps) supplemented with the appropriate antibiotic was inoculated with a single colony of the *E. coli* strains harbouring the target plasmids (sub-cultured in LB agar with the appropriate antibiotic). The cultures were incubated overnight (250 rpm, 37°C) and harvested by centrifugation (5000 rpm, 10 min, 4 °C). Cell pellets were resuspended in buffer P1 (250 µL for minipreps, 4 mL for midipreps). Lysis buffer P2 (250 µL for minipreps, 4 mL for midipreps) was added to the suspension and mixed by inverting 4-6 times. The lysis reaction was incubated (5 min, room temperature) and stopped by the addition of chilled buffer N3 (350 µL miniprep, 4 mL midiprep). The suspension was mixed

by inverting 4-6 times and cell debris removed by centrifugation (13,400 rpm, 10 min miniprep, 13,400 rpm, 30 min midiprep).

For minipreps, the supernatant was transferred to a QIAprep spin column in a collection tube. Spin columns were centrifuged (13,400 rpm, 1 min) and the flowthrough discarded. Trace nuclease activity was removed by washing the column with buffer PB (500 µL) and the column centrifuged (13,400 rpm, 1 min). The flow-through was discarded and the columns washed with buffer PE (750 µL). The column was centrifuged (13,400 rpm, 1 min) and the flow through discarded. To ensure that any residual buffer was removed, the centrifuge step was repeated. Spin columns were transferred to a fresh microfuge tube and the DNA eluted by adding buffer EB (30 µL). The column was allowed to stand for 5 min before centrifuging (13,400 rpm, 1 min).

For midipreps, the prepared supernatant was transferred to a fresh polypropylene tube (15 mL) and the centrifugation step repeated. The supernatant was loaded onto a QIAGEN-tip 100 column pre-equilibrated with buffer QBT (4 mL). Columns were allowed to drain by gravity flow and washed twice with buffer QC (10 mL). Columns were transferred to a fresh polypropylene tube (15 mL) and plasmid DNA eluted by adding buffer QF (5 mL). Plasmid DNA was precipitated by adding isopropanol (3.5 mL) and centrifuging (13,400 rpm, 30 min, 4 °C). The supernatant was discarded and the pellet washed with ethanol (70 % v/v, 2 mL). The suspension was centrifuged (13,400 rpm, 10 min, 4 °C) and the supernatant discarded. Pellets were allowed to air dry and resuspended in buffer EB (500 µL). Finally, plasmids were stored at -20 °C until required.

4.2.3.3 Gene synthesis

Selected genes of interest were synthesised by BioMatik, optimised for overexpression in *E. coli* and with the addition of *NdeI* and *XhoI* restriction sites. The genes were delivered in lyophilised form as inserts in the pBMH plasmid. Plasmids were reconstituted in EB buffer and used for further cloning procedures.

4.2.3.4 DNA cloning

The putrescine oxidase (*puORh*) gene synthesised in pBMH vector was obtained by digesting the plasmid using *Nde*I and *Xho*I restriction enzymes. Also, the cloning vector pET-20b(+) was linearized using the same restriction enzymes. Digestion reactions (50 µL) contained: plasmid DNA (200 ng, 30 µL), FastDigest® buffer (5 µL), *Nde*I (0.5 µL), *Xho*I (0.5 µL) (1 µL total volume of restriction enzymes) and nuclease free water. Reactions were incubated in a water bath at 37 °C for 3 h. All fragments were mixed with 6 x blue loading dye, analysed by agarose gel electrophoresis and purified using the QIAQuick Gel Extraction Kit (Qiagen).

For gel extraction and purification, target DNA was excised from agarose gels using a clean scalpel and transferred to a pre-weighed microfuge tube. The tube was reweighed and the weight of the gel slice calculated. About 1-2 volumes of diffusion buffer was added to 1 volume/weight of gel slice and the suspension was incubated (50 °C) until the gel slice was completely dissolved. The solution was centrifuged for 1 min and the supernatant carefully removed using Pasteur pipette. Three (3) volumes of buffer QG was added to each volume of the supernatant and mixed, and the mixture was observed for yellow colouration. Subsequently, the solution was transferred to a QIAquick spin column in a 2 mL collection tube. DNA was bound to the column by centrifugation (13,400 rpm, 1 min) and the flow-through discarded. The column was washed with buffer PE (750 µL) and centrifuged (13,400 rpm, 1 min). The wash step was repeated. QIAquick spin columns were transferred to a fresh microcentrifuge tube and the DNA eluted by adding buffer EB or DNase-free dH₂O (50 µL). The column was allowed to stand for 5 min before centrifuging (13,400 rpm, 1 min). DNA was stored at -20 °C until required.

After restriction enzyme digestion, agarose gel extraction and purification of DNA fragments, the 5'-ends of DNA was dephosphorylated. Dephosphorylation reactions (30 µL) contained: DNA fragment (75 ng, 20 µL), 10x Antarctic phosphatase reaction buffer (3 µL), Antarctic phosphatase (1 µL) and nuclease-free water. Reactions were incubated

in a water bath at 37 °C for 30 - 60 min. Subsequently, the reaction was stopped by heat-inactivation at 80 °C for 2 min.

4.2.3.4.1 Gene (DNA) assembly

Restriction enzyme cloning (ligation) was used to insert *puo_{Rh}* into linearized pET-20b(+) plasmid. Noteworthy, both cloning vector and gene insert were digested with the same restriction enzymes as reported in earlier. Gene insert was ligated into the prepared plasmid DNA using a 3:1 molar ratio of gene insert to plasmid. The ligation mixture (30 µL) was composed of linearized plasmid DNA (50 ng), gene insert (3:1 molar ratio with plasmids and varied depending on size), T4 ligase (5 U), 10 x T4 DNA ligase buffer (3 µL) and PCR grade water. The reaction was incubated (room temperature, 1 h) and the enzymes inactivated by heating (70 °C, 10 min). Chemically competent cells were transformed with ligation products by standard heat shock method.

4.2.3.4.2 Bacterial transformation

Transformation of *E. coli* JM107 was performed using the TransformAid™ Bacterial Transformation Kit. The protocol was conducted following the manufacturers instruction was adopted. Transformation of *E. coli* BL21(DE3) with plasmids extracted and purified from recombinant *E. coli* JM107 strain was performed by standard heat shock. The purchased competent cells were conserved in aliquots (20 µL) at -80 °C. On the day of transformation, the required amount of aliquots was thawed on ice for 2-5 min and the plasmids (10 ng) was directly added into the tubes. After gentle mixing by flicking the bottom of the tube with a finger a few times, the mixtures were incubated on ice for 5 min, heat shocked for exactly 30 s in a 42 °C water bath using a floating rack, and immediately placed back on ice for 2 min. Room temperature SOC medium (80 µL) was then added and the cells were incubated at 37 °C for 60 min with 250 rpm shaking. Transformants were selected by plating the culture (10 - 50 µL) on pre-warmed LB agar plates containing appropriate antibiotic and incubated overnight at 37 °C in an incubator.

4.2.3.4.3 DNA analysis

Restriction enzyme digests were performed to confirm plasmids had been correctly assembled by extracting the DNA using a MiniPrep or MidiPrep kit (Qiagen) from the cloning and expression hosts. Subsequently the digested plasmids were analysed by agarose gel electrophoresis (4.2.7.1) for characteristic fragment length polymorphism. Also, DNA sequencing was used to verify the correct DNA sequence and check for mutations in the plasmid construct.

4.2.4 Isolation and purification of enzymes

4.2.4.1 Preparation of soluble and insoluble fractions for SDS-PAGE analysis

E. coli BL21(DE3) cells expressing the enzymes of interest were sampled at intervals. Samples (1.5 mL) were centrifuged (Eppendorf centrifuge 5810R) at 8000 rpm and 4 °C for 10 min, and pellets were resuspended in the 100 mM potassium phosphate buffer (pH 8), BugBuster Reagent™, cOmplete 47 EDTA-Free Protease inhibitor (1 tablet every 50 mL) and Benzonase® (2.5 U/mL). The amount of buffer used for each sample was calculated depending on the OD₆₀₀ which the original sample was taken at, using the formula: Solution amount = (OD₆₀₀/(0.2) × 45 µL) / 2. This normalised the total protein concentration among the samples. Resuspended cells were incubated (37 °C, 250 rpm for 20 min) and an amount of liquid equal to the lowest quantity of BugBuster Reagent™ used for cell resuspension was transferred into a fresh tube. Tubes were centrifuged at 14000 rpm for 20 min at 4°C and the supernatant (20 µL) was transferred into another fresh tube. This was used as the soluble fraction sample. The remaining pellet was resuspended into 100 mM potassium phosphate buffer (pH 8), using the same buffer volume as the volume of liquid transferred before centrifugation. A sample (20 µL) was then transferred into a fresh centrifuge tube and used as insoluble fraction sample. The fractions were then analysed by Polyacrylamide Gel Electrophoresis (PAGE, 4.2.7.4).

4.2.4.2 Preparation of crude extract

Cell free extracts of *E. coli* cultures (50 mL) expressing the enzymes to be assayed were prepared by harvesting the cells by centrifugation (6000 rpm, 15 min, 4 °C). Thereafter, the cell pellets were resuspended and centrifuged three times in 100 mM sodium-potassium phosphate buffer pH 7.5. The cell pellets were stored at -80°C until required. Cells were resuspended in buffer containing 0.1 µL of benzonase® nuclease and half tablet of protease inhibitor cocktail. The cell resuspension (5 mL except otherwise stated) was then lysed by two passages in the Constant Systems cell disruption system at a pressure of 20,000 – 25,000 psi. Prior to the use of this system, all detachable components were placed on ice for 10-15 min. Cell debris was removed by centrifugation (18000 rpm, 4 °C, 15 min). Crude extract (clear supernatant) transferred into fresh tubes and stored on ice to be used the same day. In all cases, the buffer used was the same as that used for the enzymatic assays, unless differently indicated.

4.2.4.3 Enzyme purification

For enzyme purification, cells were grown at optimized conditions for soluble protein expression and were harvested by centrifugation at 6000 rpm and 4 °C for 15 min. Subsequently, crude extracts (4.2.4.2) were prepared after resuspended in buffer A (20 mM sodium phosphate buffer, 500 mM sodium chloride - NaCl, 30 mM imidazole pH 7.4). The enzyme crude extract was kept on ice and used for subsequent purification *via* immobilized metal (Nickel) ion affinity chromatography (IMAC) of His-tagged enzymes. PuO_{Rh} was purified at 4°C using an Akta protein purification system. Crude extracts (5-10 mL) were applied to a HisTrap FF crude column (1 mL) using buffer A, and proteins were eluted on a linear gradient with buffer B (20 mM sodium phosphate buffer, 500 mM sodium chloride, 500 mM imidazole pH 7.4) at a flow rate of 1 mL/min. Elution was monitored by absorbance at 280 nm. Fractions (2 mL) were analysed by SDS-PAGE (4.2.7.4), and those containing the protein of interest were combined. Purified protein was desalted by exchanging Buffer B with 100 mM sodium-potassium phosphate buffer pH 7.5 by several

concentration/dilution cycles using a Vivaspin sample concentrator. Enzyme activity unit was defined as the amount of enzyme that causes formation of 1.0 μM of Δ^1 -piperideine per hour.

4.2.5 *In-vitro* enzyme assays and analysis: Putrescine oxidase (PuO_{Rh})

o-Aminobenzaldehyde – Δ^1 -piperideine coupled assay: Δ^1 -piperideine reacts with *o*-ABA to give an orange coloured dihydroquinazolinium compound (Molar extinction coefficient 2800 $\text{mol}^{-1}\text{cm}^{-1}$), detectable at an absorption wavelength of 465 nm. The reaction mixture was prepared in 1 cm-light path Quartz cuvettes and contained 100 mM sodium-potassium phosphate buffer pH 7.5, 0.1 – 1.0 mM substrate (Cadaverine or putrescine), 1 - 10 mM *o*-ABA solution and 0.1 mL purified PuO_{Rh} was used to start the reaction, except otherwise stated. The final mixture was 1mL and enzyme activity was determined by changing the concentrations of substrate (cadaverine or putrescine - control). Continuous bubbling of filtered compressed air (0.24 mM O₂) through the phosphate buffer prior use equilibrated the mixture. The absorbance was measured at 435 and 465 nm for putrescine and cadaverine respectively at 30 °C or otherwise started. The orange adduct for cadaverine has an extinction coefficient of 2800 $\text{L mol}^{-1} \text{cm}^{-1}$ at 465 nm while the yellow adduct for putrescine is 1860 $\text{L mol}^{-1} \text{cm}^{-1}$ at 435 nm (Bachrach, 1962, Fothergill and Guest, 1977).

Putrescine oxidase activity was assayed by monitoring the rate of oxygen (O₂) consumption using a Hansatech Instruments Oxygen Electrode Measurement Systems with an S1 Clark type O₂ electrode disc (Oxygraph, Hansatech Instruments Ltd, Norfolk, UK). The cylindrical reaction vessel was fitted with a small magnetic stirrer and coupled to a heated circulating water-bath (Grant TC120) for regulation of the reaction temperature. The assay mixture contained 100 mM sodium-potassium phosphate buffer pH 7.5, 0.1 – 1.0 mM substrate (Cadaverine or putrescine), and 0.1 mL purified PuO_{Rh} was used to start the reaction, except otherwise stated. The rate of O₂ utilization was determined by measuring the slope of a tangent drawn to the O₂ concentration versus time curve.

Maximum velocities ($V_{\max}$) and Michaelis constants (K_m) were estimated using the Michaelis-Menten model of GraphPad Prism version 6.04.

4.2.6 Bioproduction of Δ^1 -piperideine from cadaverine

4.2.6.1 Bioconversion using purified enzyme (PuO_{Rh})

The activity of the PuO_{Rh} enzyme on cadaverine was assayed in small and scaled reactions. Reaction mixtures contained 30 mM cadaverine and 1.0 mg/mL of the purified PuO_{Rh} in 100 mM sodium-potassium phosphate buffer, pH 7.5, except otherwise stated. The mixture was equilibrated by continuous bubbling of filtered compressed air (0.24 mM O₂) through the phosphate buffer prior use. Incubations were performed at 30 °C and 150 rpm for 15 min unless otherwise stated. Reaction mixtures were filtered using a Vivaspin sample concentrator to remove enzyme and quench the reaction before direct LC-ESI-MS analysis. Samples were also analysed directly in aqueous phase using ¹H NMR spectroscopy.

4.2.6.2 Whole cell biotransformation using harvested cells overexpressing PuO_{Rh}

Harvested whole cells expressing PuO_{Rh} were used for biotransformation of cadaverine. *E. coli* strains were grown and induced in LB medium as described earlier (see section 4.2.2). Precultures were incubated at 37 °C and 250 rpm while the fermentation (expression) batches were incubated under optimized conditions (25 °C and 250 rpm) for soluble protein expression. After 3 - 5 h induction, cells were collected by centrifugation at 6000 rpm and 4 °C for 10 min. Thereafter, cells were resuspended in 100 mM sodium-potassium phosphate buffer pH 7.5 and centrifuged as stated above. This was repeated three times before final resuspension for further analysis. Reaction mixtures (10 mL) containing harvested cells (20 g/L) and cadaverine (1 - 5 mM) in 100 mM Na-K phosphate buffer pH 7.5 were incubated at 25 °C and 250 rpm in 50 ml Erlenmeyer flask except otherwise stated. Samples were collected aseptically at intervals in 1.5 mL Eppendorf tubes, and these were centrifuged at 6000 rpm, 4 °C for 10 min. Subsequently, 2 mL syringe and needle were used to draw up the supernatant which was filtered (using a 0.22 µm syringe

filter) into another labelled Eppendorf tube. Thereafter, samples examined under the microscope and were analysed immediately or stored at -20 °C for later analysis using HPLC and ¹H NMR.

4.2.7 Analytical methods

4.2.7.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to purify digested DNA fragments, and to estimate DNA concentration, using a Green Range™ Horizontal Gel Electrophoresis Unit (Scie-Plas). The agarose gel (1 % w/v) was prepared with Tris-acetate EDTA (TAE) buffer (Bio-Rad) containing agarose. TAE buffer was prepared from a 50x concentrate by diluting in dH₂O. After dissolving the agarose by microwaving (800 W, 2.5 min) the solution, ethidium bromide (EtBr₂) (1% w/v) was added to the cooled agar, mixed by swirling and the solution poured into the pre-assembled electrophoresis gel mould. The comb was inserted immediately. When solidification was completed, the comb was removed and the DNA samples were loaded in the wells. Before loading, DNA samples were pre-mixed with 6X blue gel loading dye (New England Biolabs). GeneRuler™ 1 Kb Plus DNA Ladder was also added as standard for each analysis. The agarose gel electrophoresis analyses were performed at 50-80 mV for 40-60 min using TAE buffer. Pictures of the gels were obtained an uGenius³ Gel Documentation System (Syngene).

4.2.7.2 Estimation / dilution of DNA concentration

Plasmid concentration was determined using a NanoDrop ND1000 or BioDrop Spectrophotometer. Restricted DNA fragment concentration was determined using agarose gel electrophoresis and by visually comparing bands with standards at known concentrations, and using a BioDrop Spectrophotometer.

4.2.7.3 Gene sequencing

The pET-20b(+) plasmid containing the gene of interest (*puo_{Rh}*, 20 µL), extracted and purified from *E. coli* cells, were diluted to a concentration of 75 ng/µL and shipped to

Eurofins MWG Operon (Germany). The T7 promoter (5'-TAATACGACTCACTATAGGG-3') and T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3') primers were used for sequencing the pET-20b(+) plasmid design.

4.2.7.4 Sodium dodecyl - Polyacrylamide gel electrophoresis (SDS - PAGE)

Protein expression was checked by SDS-PAGE. SDS-PAGE was conducted out with a Mini-Protean electrophoresis system using TGX precast gels. Samples (cells) were harvested and extracts prepared as reported previously (see 4.2.4.1). Soluble protein was analysed in comparison to total protein. Samples from protein purification process were directly used. All protein samples (20 µL) were mixed with 20 µL Laemmli sample buffer (contained 950 µL Laemmli buffer and 50 µL β-mercaptoethanol). Thereafter, the mixture was boiled at 105 °C for 5 min, and stored on ice until required. Subsequently, 10 µL of the above prepared samples were loaded in each well and the electrophoresis was run in 1x TGS (Tris-Glycine-SDS) running buffer at 150 V for 45-60 min. Thereafter, the gel was washed with distilled water three times and stained overnight with EZBlue Stain (Bio-Rad) or QC colloidal Coomassie protein stain. The solubility of the enzyme was estimated according to the fraction (soluble or insoluble) in which the protein band corresponding to the size of the enzyme was thickest by visual inspection. Pictures of the gels were obtained an uGenius³ Gel Documentation System (Syngene).

4.2.7.5 Quantification of enzyme (crude and purified) – DC assay

The protein concentrations in crude and purified enzyme samples were measured using the DCTM Protein Assay Kit (Bio-Rad). Samples were diluted 10 and 50 times, and then transferred (20 µL) into a microcentrifuge tube. Working Reagent A' (100 µL), composed of Reagent S (20 µL) and Reagent A (2 mL), was added and the sample mixed. Reagent B (800 µL) was added into each tube and mixed immediately. After incubation (15 min) at room temperature, absorbance at 750 nm was analysed using an Agilent 8453 UV-Vis spectrophotometer. Protein concentration was calculated based on freshly prepared

calibration curves of bovine serum albumin (BSA) protein standard in a range of 0.2 - 1.5 mg/mL.

4.2.7.6 Spectrophotometric analysis

Spectrophotometric measurements were made with a Shimadzu UV-2600 UV-Vis spectrophotometer, Shimadzu UV mini-1240 UV-Vis spectrophotometer or an Agilent Technologies Cary 8454 UV-Vis spectrophotometer coupled to heated circulating water-bath (Grant TC120). A 1.0 cm light path was used for all measurements.

Growth of recombinant cells were determined by measuring the optical density (absorbance) at 600 nm (OD_{600}). Samples (1 mL) collected aseptically at hourly or stated intervals were transferred to cuvettes and the optical density measured. Where the reading was outside of a range from 0.0 - 0.8, samples were diluted 1 in 10 in LB medium until they were within the specified range.

4.2.7.7 Dry and wet cell weight determination

Cell weight measurements were made in pre-weighed microfuge tubes that had been dried by incubating in the presence of dry silica in a sealed container. Samples (1 mL) were harvested by centrifugation (14000 rpm, 2 min) and the supernatant discarded. The centrifugation step was repeated and any residual medium removed using a pipette. For wet cell weights, the microcentrifuge tubes were reweighed, and for dry cell weights, the cell pellets were incubated at 70 °C to constant weight and reweighed. The difference between the empty microcentrifuge tube and the cell pellet was used to calculate the cell weight per litre of culture.

4.2.7.8 Dansyl chloride (DnsCl) derivatisation of cadaverine

Dansyl chloride is one of the most used derivatisation agents which reacts with either primary and secondary amino groups, and even tertiary amines (Fig. 4.1) in extreme reaction conditions, providing very stable derivatives and combining the unique feature of being both fluorescence and detectable in the UV region. Earlier reported methods were

modified (Smith and Davies, 1985, Marce et al., 1995, Vale and Gloria, 1997, Molins-Legua et al., 1999, Smela et al., 2003, Gosetti et al., 2007). One (1) mL of the sample (extract or standard) was mixed with 0.5 mL of saturated solution of Na_2CO_3 , 1 mL of DnsCl (5 mg DnsCl of per 1 mL acetone) was added and the mixture was vortexed for 1 min. Derivatisation proceeded for 1 h in dark at 40 °C. After derivatisation, 250 μL of ammonia was added, vortexed for 1 min and incubated in the dark at room temperature for 15 min to remove the excess of DnsCl. The separation after dansyl chloride derivatisation was carried out by HPLC (Agilent Technologies 1200 series).

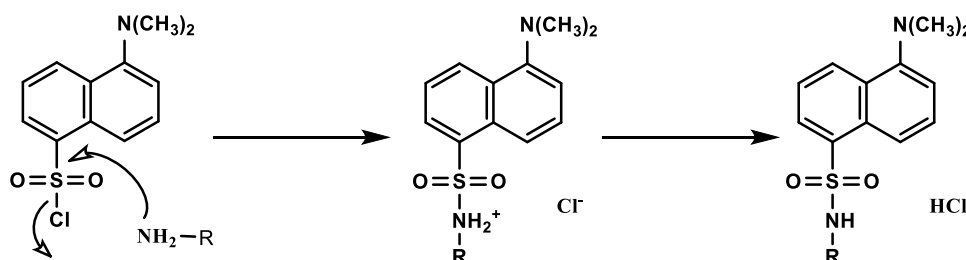


Fig. 4.1: Mechanism of biogenic amines derivatisation reaction with dansyl chloride.

4.2.7.9 High performance liquid chromatography with UV-detector

The separation of dansyl chloride derivatised samples were filtered and analysed by HPLC (Agilent Technologies 1200 series) on a Zorbax Eclipse XDB-C18 column (4.6 mm $\times$ 250 mm, particle size 5 μm). The injection volume was 10 μL and sample components were detected using a photometric UV/VIS detector at 254 nm. A water (H_2O)/acetonitrile (ACN) mixture in gradient elution mode was used as the eluent at a flow rate of 0.8 mL/min. The gradient used was 65% of acetonitrile at zero time, 65% at 10 min, 80% at 15 min and 100% at 20 min. After 20 min the percentage of acetonitrile was kept constant.

4.2.7.10 Liquid chromatography – electrospray ionisation – mass spectrometry (LC-ESI-MS)

LC-ESIMS spectra of samples were measured with an Agilent Technologies 1260 series HPLC coupled to an Agilent 6120 Quadrupole mass spectrometer fitted with an ESI probe

and a DAD UV detector. The instrument was controlled using LC-MSD Chemstation Rev. C.01.05 [39]. The reaction sample (products) were separated by reverse-phase HPLC on a Waters X-Bridge C18 column (3.5 μm , 2.1 mm x 30.0 mm). The injection volume was 2.0 μL at a flow rate of 0.8 mL/min. Gradient elution was performed with 0.1% ammonia in water (**A**) and acetonitrile (**B**): 0- 3.10 min, linear gradient from 5 to 100% **B**; 3.10 – 3.50 min, 100% **B**; 3.50 – 3.51 min, linear gradient from 100 to 5% **B**, and 3.51 – 4.50 min, 5% **B**. Elution was monitored by UV absorption at 254 nm. The ESI probe drying gas flow rate, nebuliser pressure, drying gas temperature and mass range were 13.0 L/min, 50 psig, 350 $^{\circ}\text{C}$ and 100 – 1000 amu respectively. All spectra were obtained in the positive ion mode, and at a rate of one scan every 0.2 s.

4.2.7.11 Preparation of samples for NMR

Samples of purified enzyme and harvested whole cell reactions were prepared as previously reported (see 4.2.6). Aqueous deuterated samples ($\text{H}_2\text{O}/\text{D}_2\text{O}$ ratio of 90/10%) were prepared by transferring 477 μL reaction sample into 50 mm NMR tubes, thereafter, 53 μL D_2O (99.9 %) was added, vortexed and allowed to stand for 30 – 60 min before been analysed. For quantitative NMR of aqueous samples, Maleic acid (Standard for qNMR TraceCERT[®]) was used as external standard in a stem co-axial insert containing 60 μL of 3.64 mM Maleic acid. Samples were extracted with deuterated chloroform (CDCl_3) and were analysed directly after transferring 530 μL into 50 mm NMR tubes. For quantitative NMR of chloroform samples, tubes contained 540 μL sample and 60 μL of 5 mM 3,4,5-Trichloropyridine 98% (Standard for qNMR TraceCERT[®]) was used as internal standard.

Also, samples for NMR were analysed at different pH directly in deuterated aqueous phase and after extraction in CDCl_3 . It should be noted that the enzyme reactions were carried out at pH 7.5, thereafter, reaction mixtures were adjusted to pH 1 or 12 using concentrated hydrochloric acid - HCl or 6 M potassium hydroxide - KOH respectively, before solvent extractions were carried out. For pH adjustments, a pH meter with microprobe that fits

into 1.5 – 2.0 mL microcentrifuge tubes to a volume displacement of about 20% was used. The pH probe was not washed until each adjustment batch was completed.

4.2.7.12 Proton nuclear magnetic resonance (^1H NMR)

NMR spectra of substrate and products in samples (purified PuO_{Rh} reaction mixture) were obtained at ambient temperature using a 400-MHz Bruker AV3400 spectrometer. Aqueous samples were diluted with 99.9% Deuterium oxide (H_2O :90% / D_2O :10%) and run using automated presaturation solvent suppression to reduce the intensity of the water signal. Data were processed and analysed using MestReNova-10.0.2-15465. Chemical shifts are quoted in parts per million (ppm), and coupling constants, J , are quoted in Hz. Multiplicity of each signal is designated by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; app, apparent; br, broad or combinations thereof.

Quantitative NMR (qNMR) analysis and subsequent calculations were carried out as previously recommended and reported by (Henderson, 2002, Rundlöf et al., 2010, Bharti and Roy, 2012, Rundlöf et al., 2014). Aqueous deuterated samples were analysed using Maleic acid (Standard for qNMR TraceCERT[®]) as external standard in a stem co-axial insert while 3,4,5-Trichloropyridine 98% (Standard for qNMR TraceCERT[®]) was used as internal standard for CDCl_3 samples.

4.2.7.13 Liquid – liquid (Solvent) extraction

The activity of the purified PuO_{Rh} enzyme whole cell bioconversion of cadaverine were assayed as previously reported in foam-plugged conical flasks. Reaction mixtures contained 30 mM cadaverine and 0.4 mg/mL of the purified PuO_{Rh} in 100 mM sodium-potassium phosphate buffer, pH 7.5. Incubations were performed at 25 °C and 150 rpm for 43 h, except otherwise stated. Continuous bubbling of compressed air (0.24 mM O_2) through the phosphate buffer prior use equilibrated the mixture. Five batches were prepared, a batch was evaporated to dryness without extraction while remaining four batches were extracted with different solvents (chloroform, diethyl ether, ethyl acetate and hexane) respectively. All experiments were carried out in triplicates.

After incubation, 500 μL of sample was deuterated using D_2O (90% sample + 10% D_2O) for ^1H qNMR analysis while the remaining sample was extracted using a solvent. Solvent extraction was carried out by mixing equal volumes (4.5 mL) of the sample with the solvent in the conical flask used for biotransformation reaction and was vigorously mixed at 10 s interval for 1 min before allowing to stand for 5 min. The solvent layer was transferred into a freshly labelled glass vial while the solvent extraction step above was repeated three times using the aqueous sample phase. Subsequently, the pooled solvent layer was dried using sodium sulphate (Na_2SO_4) powder for about 5 – 10 min. The solvent was transferred into a round-bottom flask and evaporated completely using a rotary evaporator set at appropriate pressure and 42 $^\circ\text{C}$. Deuterated chloroform (CDCl_3 , 4.5mL) was used to dissolve and wash the flask. Thereafter, about 560 μL of sample was used for ^1H qNMR analysis.

4.2.7.14 Microscopy

For microscopic examination of cells in a whole cell bioconversion of cadaverine, 10 μL of samples collected at intervals were dropped on a grease-free glass slide and covered with a glass slip. The slides were observed under the high power (x100) resolution of an Olympus confocal microscope using immersion oil and microscopic images were taken using a mobile camera attached to the microscope.

5. Results and Discussion: Expression and characterization of putrescine oxidase from *Rhodococcus erythropolis* (PuO_{Rh})

5.1 Introduction

Putrescine oxidase was identified as a suitable enzyme for the synthesis of Δ^1 -piperidine from cadaverine. Therefore, putrescine oxidase from *Rhodococcus erythropolis* (PuO_{Rh}) was cloned, expressed and characterized using a recombinant *Escherichia coli* strain.

Specifically, the objectives were to design and construct an *E. coli* strain that could overexpress soluble, stable and active PuO_{Rh}, to determine PuO_{Rh} activity in the cell free extract, and to purify the overexpressed soluble PuO_{Rh}. In addition, the activity of the purified enzyme was determined and finally, the purified PuO_{Rh} was biochemically characterised.

5.2 Cloning of PuO_{Rh}, transformation and expression in the expression host

5.2.1 Cloning of PuO_{Rh} and transformation in *E. coli* BL21(DE3)

The *R. erythropolis* NCIMB 11540 gene (*puo_{Rh}*; Appendix 11.5) encoding putrescine oxidase (PuO_{Rh}) [GenBank accession no. EU240877 (Benson et al., 2013)] with the 5' *Xho*I and the 3' *Nde*I restriction sites, was codon optimized for expression in *E. coli* and synthesized by Biomatik, within a carrier plasmid, named pBMH_*puo_{Rh}* (see Appendix 11.3). The gene was cloned into the pET-20b(+) expression system (see Appendix 11.3), which allows recombinant gene expression in cells containing the T7 RNA polymerase gene, under the control of the *lac*UV5 promoter. Expression of the T7 RNA polymerase can be induced by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) as the inducer of the *lac* promoter. Also, the pET-20b(+) vector contains an ampicillin resistance cassette that encodes for beta-lactamase, which is secreted by *E. coli* and degrades ampicillin, thus, conferring resistance to ampicillin and its derivatives. Carbenicillin is an ampicillin derivative that is more resistant to degradation by beta-lactamase; using this more stable antibiotic provides for a stronger selection pressure (Sanders and Sanders, 1983),

therefore, transformants in subsequent cloning reactions were selected by plating on media supplemented with carbenicillin.

To construct the recombinant pET-20b(+) plasmid, the *PuORh* gene was first extracted from pBMH_*puoRh* by double restriction digestion with *Xho*I and *Nde*I restriction enzymes, and purified by agarose gel electrophoresis (see Appendix 11.2), followed by gel extraction. The *puoRh* gene insert (1366 bp) was cloned using T4 DNA ligase, into the linearized pET-20b(+) vector (3586 bp), double digested with *Xho*I and *Nde*I (Fig. 5.1), resulting in the recombinant pET-20b(+)_*puoRh* plasmid (Fig. 5.2). The pET-20b(+) vector allowed the addition of a hexa-histidine ((His)₆) *N*-terminus tag and a stop codon to the *PuORh*.

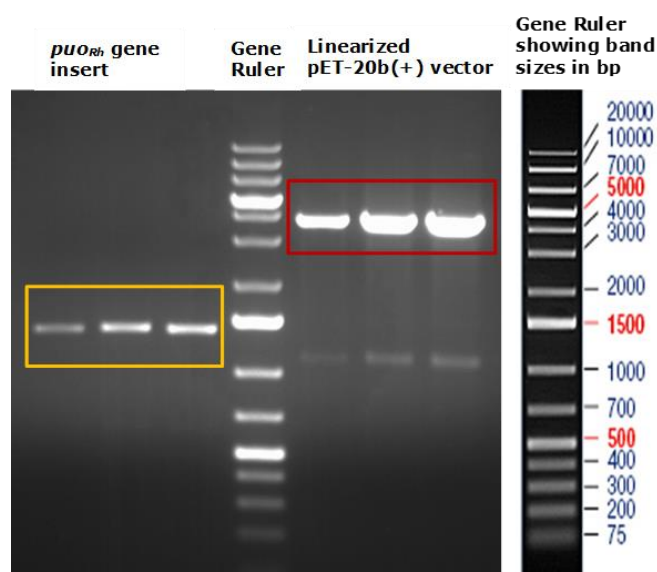


Fig. 5.1: Agarose gel electrophoresis of the extracted *puoRh* gene insert and linearized pET-20b(+) vector plasmid.

The *puoRh* gene insert and the linearized pET-20b(+) vector were earlier prepared by double digestion with *Xho*I and *Nde*I. **The yellow rectangle indicates the band corresponding to the *puoRh* gene insert (1366 bp) while the red rectangle indicates the band corresponding to the linearized pET-20b(+) vector plasmid (3586 bp).** Extra bands shown could be a result of star activity by the restriction enzymes.



Fig. 5.2: Plasmid map of pET-20b(+)_*puo_{Rh}*

The *puo_{Rh}* gene encoding PuO_{Rh} (codon optimized for *E. coli* and synthesized by Biomatik) was cloned into the pET20b plasmid using *Xho*I and *Nde*I restriction sites. The sites of restriction for cloning of the insert gene allowed the addition of a hexa-histidine ((His)₆) *N*-terminus tag to the PuO_{Rh}.

The recombinant pET-20b(+)_*puo_{Rh}* plasmid was first transformed into *E. coli* JM107 cloning host, using the heat shock technique. The resulting strain *E. coli* JM107_pET-20b(+)_*puo_{Rh}*, was selected on LB (Luria-Bertani) medium supplemented with 1% glucose and carbenicillin (50 µg/mL). The recombinant plasmid was amplified, isolated and subsequently retransformed into the *E. coli* BL21(DE3) expression host. The *E. coli* BL21(DE3)_pET-20b(+)_*puo_{Rh}* strain was also selected using carbenicillin, and was used in subsequent studies.

To verify the DNA cloning, the recombinant pET-20b(+)_*puo_{Rh}* was isolated from both transformed *E. coli* JM107 and *E. coli* BL21(DE3), and was digested using *Xho*I and *Nde*I. In both cases, two sharp bands were observed by agarose gel electrophoresis, representing the insert gene and the linearized pET-20b(+) (Fig. 5.3), confirming successful plasmid construction and transformation into *E. coli*. The *puo_{Rh}* gene was

sequenced, and the sequence data was aligned with the previously published gene sequence, confirming 100% identity. This indicates that no mutations were introduced during the cloning process.

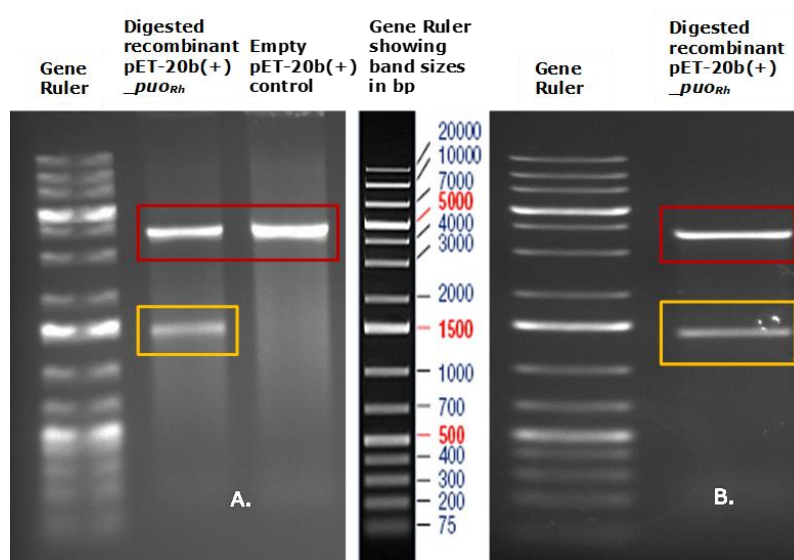


Fig. 5.3: Agarose gel electrophoresis of the recombinant pET-20b(+)_{*puoRh*} plasmid.

A. Digested recombinant pET-20b(+)_{*puoRh*} plasmid isolated from recombinant *E. coli* JM 107_ pET-20b(+)_{*puoRh*} and an empty pET-20b(+) plasmid as control. **B.** Digested recombinant pET20b(+)_{*puoRh*} plasmid isolated from recombinant *E. coli* BL21(DE3)_pET-20b(+)_{*puoRh*} (**the yellow rectangle indicates the band corresponding to the *puoRh* insert while red rectangle indicates the band corresponding to the linearized pET20b(+) vector**).

5.2.2 Recombinant PuO_{Rh} expression

The expression of soluble PuO_{Rh} was optimized by growing *E. coli* BL21(DE3)_pET-20b(+)_{*puoRh*} at different temperature and agitation speeds, and extracts were analysed by SDS-PAGE.

In all the growth conditions, the cultures grew exponentially (Fig. 5.4), but after induction of the enzyme using IPTG, the specific growth rate of the induced cultures (batches) were 12 – 30% lower than the growth rate of the uninduced cultures (Table 5.1). Overall, the observed differences in growth rate after induction was probably a result of the cells utilizing resources for expression of the recombinant protein. The recombinant strain had a higher growth rate at 37 °C and this was expected since the reported optimal temperature of *E. coli* is 37 °C (Theisen and Liao, 2016). Similarly, the optimal agitation

speed was 250 rpm and this is accurate because the higher presence of oxygen allows *E. coli* to complete oxidation of growth substrates and therefore is the most productive and the preferred metabolic mode (Guest et al., 1996, Partridge et al., 2006).

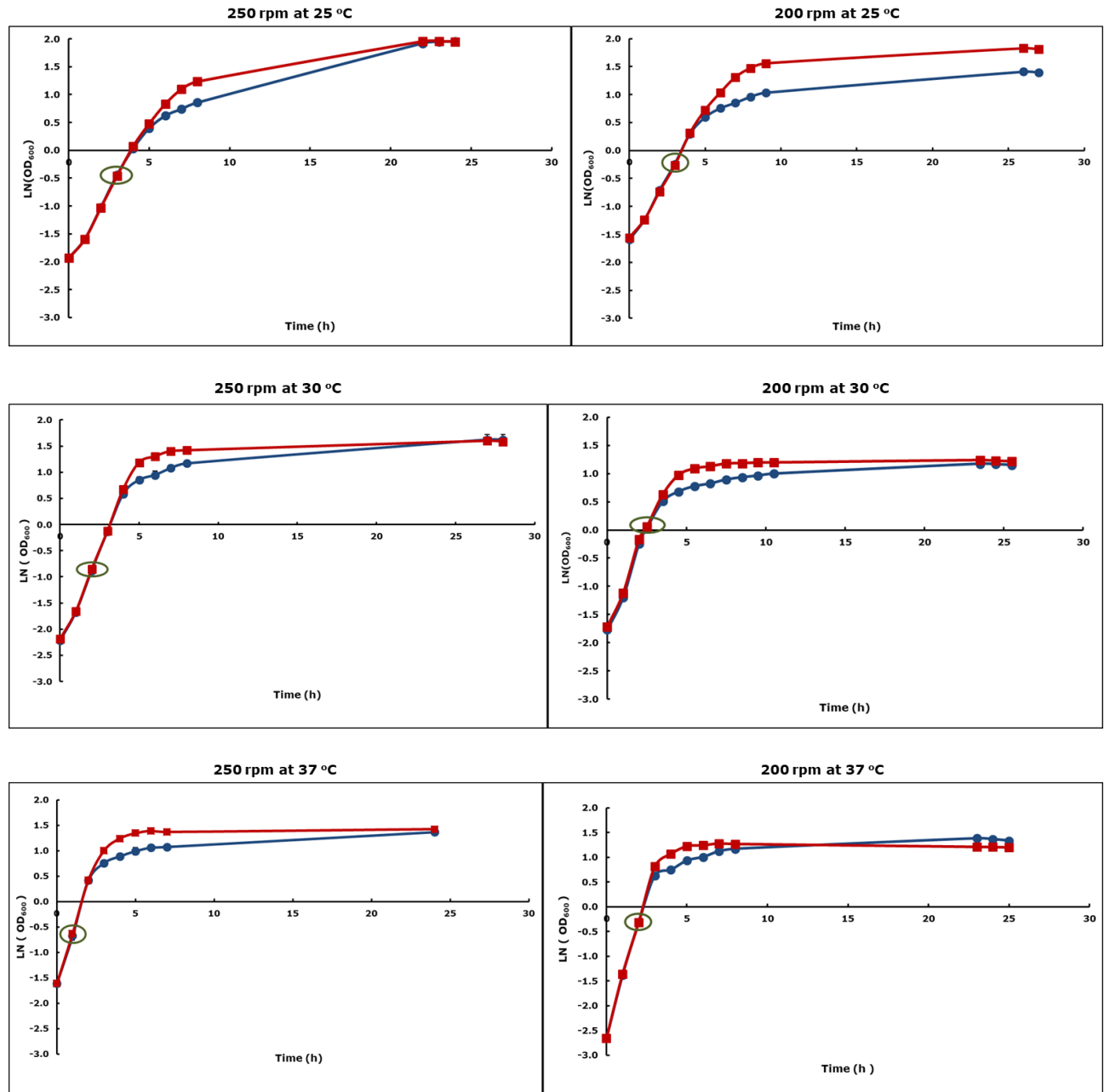


Fig. 5.4: Growth curve of recombinant *E. coli* BL21(DE3)_pET-20b(+)_puoRh

To optimize the overexpression of PuORh, *E. coli* BL21(DE3)_pET-20b(+)_puoRh was grown in 100 mL LB medium supplemented with 1% glucose and carbenicillin using 500 mL Erlenmeyer flasks. OD₆₀₀ was monitored at intervals up to 20 – 26 h and samples collected aseptically were diluted prior measurement. Blue line and circle markers represent batch induced with 0.4 mM IPTG while red line with square marker represent uninduced control batch. Green circle indicates point/time of induction. Values represent mean of triplicate experiments $\pm$ standard deviation.

Table 5.1: Growth rate of *E. coli* BL21(DE3)_pET-20b(+)_puORh

Specific growth rate, $\mu_{\max}$ (h ⁻¹) : post - induction						
	25 °C		30 °C		37 °C	
	250 rpm	200 rpm	250 rpm	200 rpm	250 rpm	200 rpm
Induced	0.35±0.00	0.33±0.01	0.59±0.03	0.32±0.01	0.72±0.03	0.53±0.02
Uninduced	0.43±0.00	0.43±0.01	0.69±0.00	0.46±0.01	0.82±0.00	0.69±0.00

The cells were grown different temperatures and agitation speeds in LB medium supplemented with 1% glucose and 50 µg/mL carbenicillin (van Hellemond *et al.*, 2008b). PuORh expression was induced with 0.4 mM IPTG when the cultures reached an optical density (OD₆₀₀) of 0.6. Thereafter, cell growth was monitored by measuring the OD₆₀₀ of samples collected at intervals. Growth rates were calculated from plots of natural logarithm of OD₆₀₀ against time. Values represent mean of triplicate experiments ± standard deviation.

Expression of PuORh was observed at different incubation conditions and the SDS-PAGE analysis of the soluble and insoluble protein content is shown in Fig. 5.4. In all cases, an increased temperature resulted in a decreased expression of soluble protein. This could indicate the formation of inclusion body (Villaverde and Mar Carrió, 2003, Sørensen and Mortensen, 2005). Most soluble protein expression was observed at 25 °C, however, when agitation was decreased, the protein became almost insoluble after 24 h (Fig. 5.5). The same was observed at 30 °C and 37 °C. This indicates that aeration could increase protein folding. Overall, expression of soluble protein by the recombinant *E. coli* decreased with increase in temperature, while increase in agitation speed resulted in an increase in expression of soluble protein. This is in agreement with previous reports on the expression of PuORh (van Hellemond *et al.*, 2008b).

Finally, the best result for soluble protein expression was obtained at 25 °C and 250 rpm (Fig. 5.5E). Although the expression of soluble protein increased over time, small fraction of insoluble protein was observed.

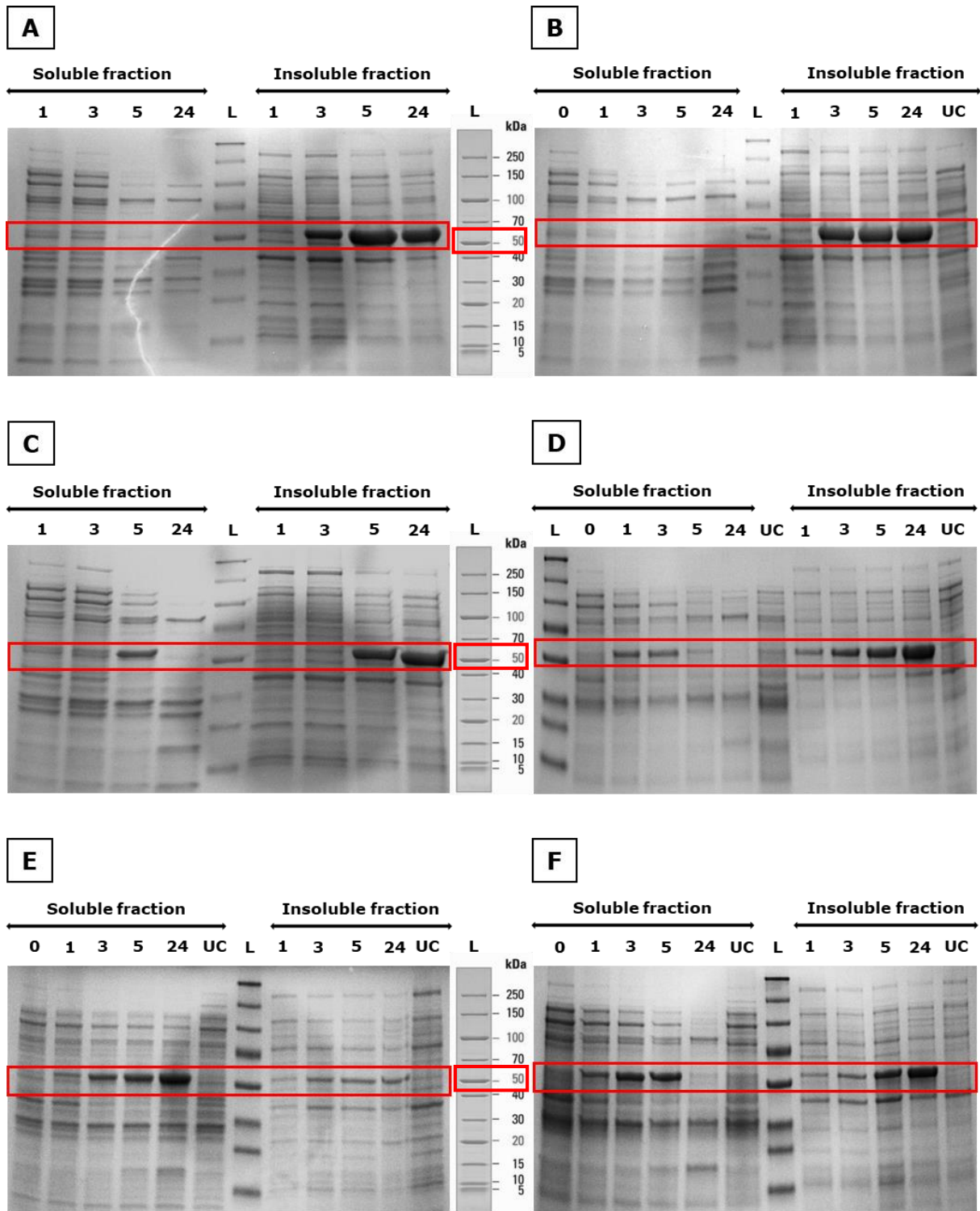


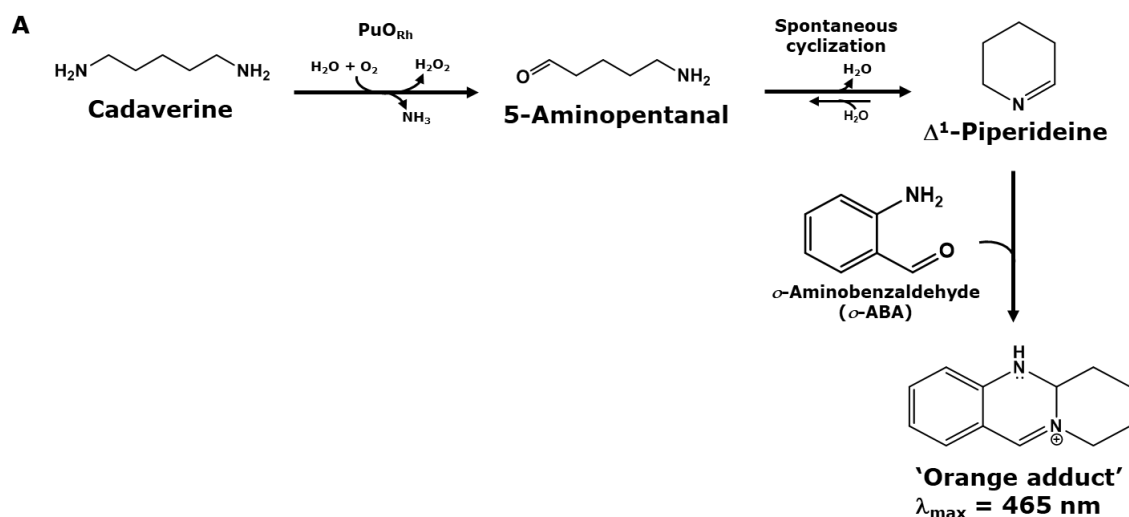
Fig. 5.5: SDS-PAGE of PuOR_h overexpression

PuOR_h overexpression by *E. coli* BL21(DE3)_{pET-20b₊-puOR_h during growth at **A.** 37 °C and 250 rpm, **B.** 37 °C and 200 rpm, **C.** 30 °C and 250 rpm, **D.** 30 °C and 200 rpm, **E.** 25 °C and 250 rpm, and **F.** 25 °C and 200 rpm. Recombinant strain was grown in LB medium supplemented with 1% glucose and carbenicillin. PuOR_h expression was induced with sterile IPTG (0.4 mM) at a culture OD₆₀₀ of 0.6. Samples were collected aseptically post-induction at intervals, lysed using BugBuster™ lysis reagent, and the protein content analysed by SDS-PAGE. **0,1,3,5&24:** sample collection time (h), **UC:** uninduced control, **L:** Spectra™ Multicolor Broad Range Protein Ladder. **Red rectangle highlights the band of PuOR_h and standard corresponding to the appropriate weight of ≈50 KDa.**}

5.2.3 Enzyme activity in cell free extract (crude enzyme)

The activity of the PuO_{Rh} in the cell-free extract was determined. The *o*-aminobenzaldehyde (*o*-ABA) coupled assay (Bachrach, 1962, Fothergill and Guest, 1977, Mitsakos et al., 2011) was used, because it not only shows the activity of the PuO_{Rh} enzyme, but also gives a preliminary confirmation of the product formed. In principle, the oxidation of cadaverine using putrescine oxidase yields 5-aminopentanal, which cyclises into the imine Δ^1 -piperideine. The cyclic imine reacts with *o*-ABA to form a coloured product (Fig. 5.6). Therefore, the PuO_{Rh} - *o*-ABA assay was performed and the formation of the orange adduct was monitored by UV-Vis spectrophotometry at 465 nm. The oxidation of putrescine was also assayed, with formation of yellow adduct monitored by UV-Vis spectrometry at 435 nm (Fig. 5.7).

Cells were harvested 5 h after induction at 25 °C and 250 rpm, lysed and cell debris were removed by centrifugation. The total protein concentration was determined by DC protein assay, and the enzymatic activity was determined using the *o*-ABA coupled assay.



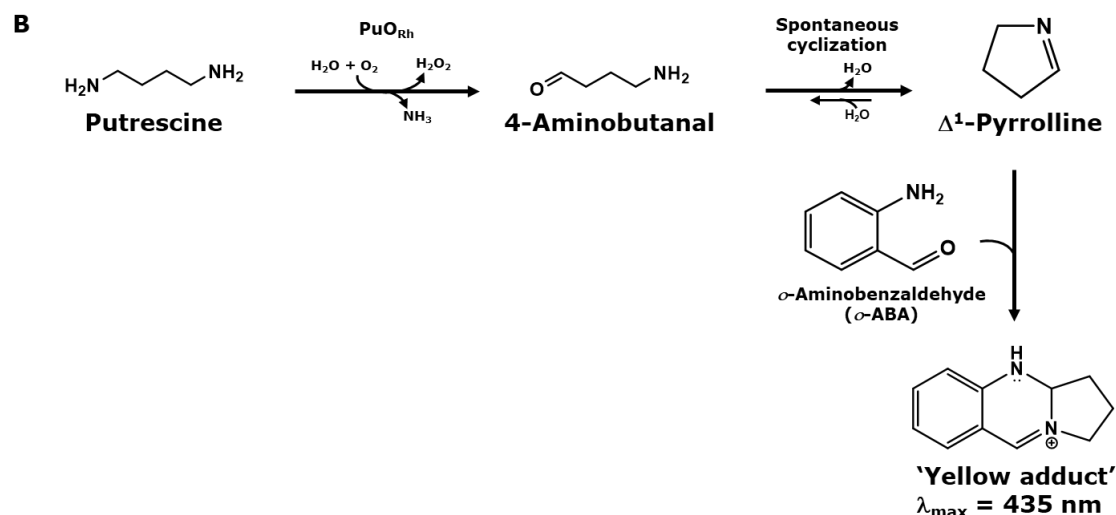


Fig. 5.6: PuOR_h - *o*-ABA coupled assay using cadaverine (**A**) and putrescine (**B**).

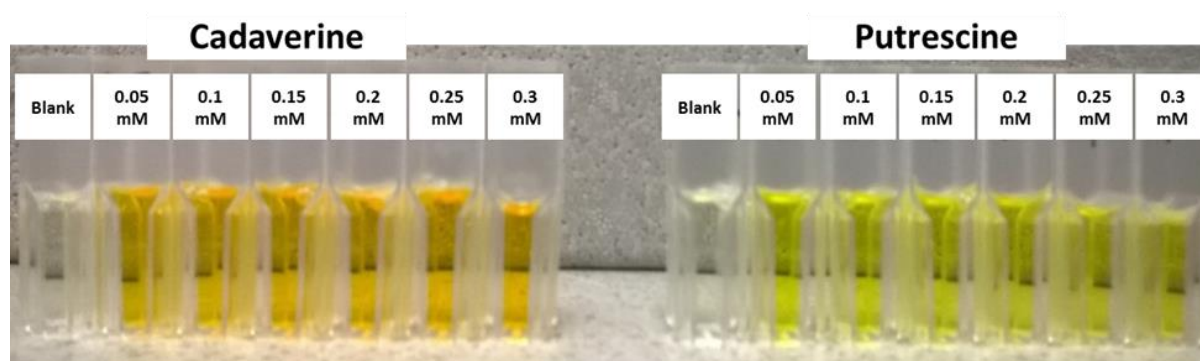


Fig. 5.7: Picture of PuOR_h - *o*-ABA coupled assay using cadaverine and putrescine reaction.

Orange and yellow dihydroquinazolinium adducts due to the reaction of *o*-ABA with the Δ^1 -piperidine and Δ^1 -pyrroline produced by oxidation of cadaverine and putrescine respectively catalysis by PuOR_h. The reaction mixture contained 100 mM Na - K phosphate buffer pH 7.5, 0.05 – 0.3 mM substrate (Cadaverine / Putrescine), 10 mM *o*-aminobenzaldehyde and 0.1 mg/mL purified PuOR_h. The mixture was incubated at 30 °C for 300 s.

The PuOR_h crude extract exhibited amino oxidase activity with both cadaverine and putrescine, with a higher substrate activity for putrescine (Fig. 5.8). No enzyme activity was detected when boiled PuOR_h crude extracts and crude extracts from recombinant *E. coli* strain with empty pET-20b(+) were used as negative controls. Control enzyme activity using crude extracts from PuOR_h uninduced recombinant *E. coli* did not show oxidase activity on cadaverine but showed activity on putrescine. This indicates basal (leaky) expression of active PuOR_h from the uninduced recombinant *E. coli*. However, basal protein

expression from pET (plasmid) constructs in expression hosts have been previously reported (Kelley et al., 1995, Grossman et al., 1998, Terpe, 2006). Finally, because no activity was found in the uninduced and empty plasmid controls, the results suggest that the observed oxidase activity is not as a result of the presence of other active enzymes (e.g. putrescine aminotransferase) which can utilize cadaverine or putrescine in the cell (Samsonova et al., 2003). This rather suggests that the activity was enzymatic due to the presence of active PuO_{Rh}.

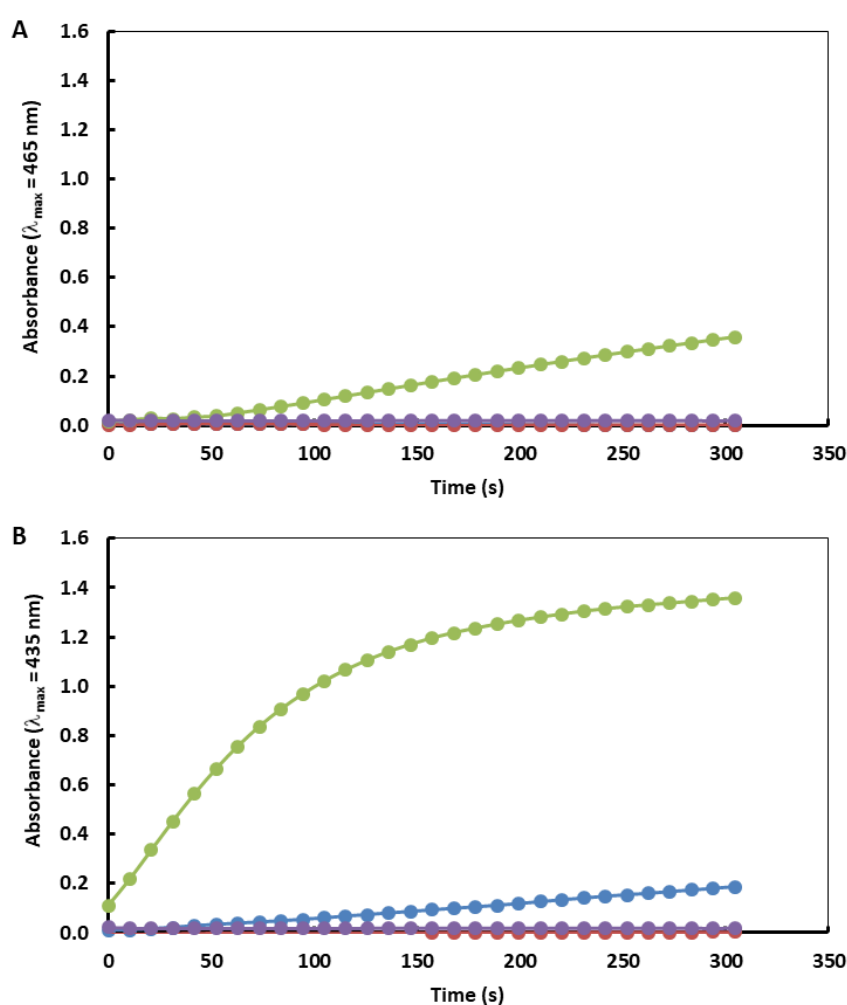


Fig. 5.8: PuO_{Rh} activity in cell free extracts (crude enzyme).

PuO_{Rh} oxidation of cadaverine (**A.**) and putrescine (**B.**) were assayed at 30°C. The reaction mixture contained 100 mM Na - K phosphate buffer pH 7.5, 1.0 mM Cadaverine or Putrescine, 0.01% *o*-aminobenzaldehyde and 1.0 mg/mL PuO_{Rh} crude extract. The absorbance was measured using Shimadzu UV-2600 spectrophotometer. The activity was measured using PuO_{Rh} crude extract [—], crude extract from uninduced recombinant *E. coli* [—], boiled (85 °C for 5 min) PuO_{Rh} crude extract [—] and crude extracts from recombinant *E. coli* strain with empty pET-20b(+)[—].

5.3 PuO_{Rh} purification and purified PuO_{Rh} activity

5.3.1 Purification by affinity chromatography

After confirming that the expressed PuO_{Rh} was active in cell-free extracts, PuO_{Rh} was isolated and purified. The hexa-histidine tag on the *N*-terminus of the expressed PuO_{Rh} enabled easy purification using immobilized metal (nickel) affinity chromatography (IMAC), from the soluble cell-free extracts. The purification process was monitored by UV absorbance at 280 nm and the chromatogram (Fig. 5.9) with a sharp absorbance peak indicated that the hexa-histidine tagged PuO_{Rh} was eluted on a linear gradient of 50 mM to 500 mM imidazole in sodium phosphate buffer.

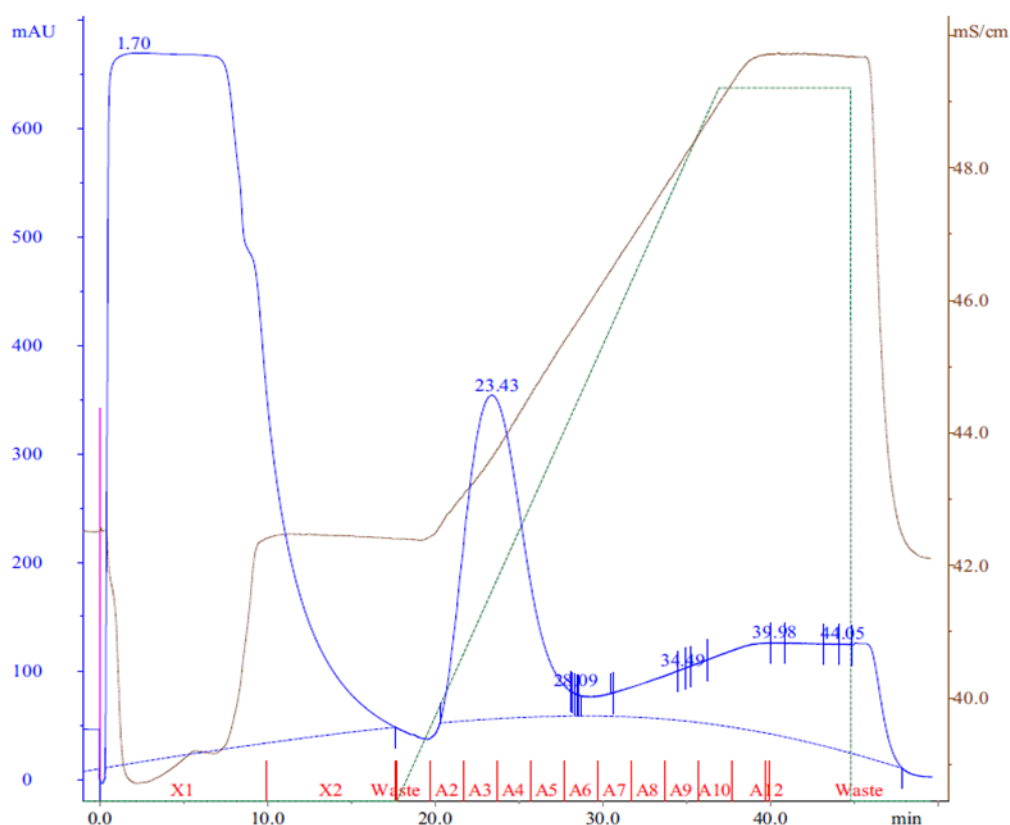


Fig. 5.9: Chromatogram of PuO_{Rh} purification.

Crude extract used was prepared from cells collected after 5 h PuO_{Rh} induction using 0.4 mM IPTG at 25 °C and 250 rpm. PuO_{Rh} was purified via immobilized metal (nickel) ion affinity chromatography (IMAC) of His-tagged enzymes at 4 °C using an Akta protein purification system. PuO_{Rh} was eluted on a linear gradient with buffer B (20 mM sodium phosphate buffer, 500 mM sodium chloride, 500 mM imidazole pH 7.4) at a flow rate of 1 mL/min. Elution was monitored by UV absorbance (280 nm). **Blue line represents UV absorbance during elution, brown line represent conductivity while red labels represent flow-through fractions.**

The fractions collected during the observed absorbance peak were separately analysed by SDS-PAGE. Thereafter, the fractions were pooled together, concentrated and desalted using centrifugal membrane filtration (Vivaspin), and analysed by SDS-PAGE. SDS-PAGE analysis showed that PuO_{Rh} was successfully purified (Fig. 5.10). Thereafter, the purified PuO_{Rh} was used for enzyme assays.

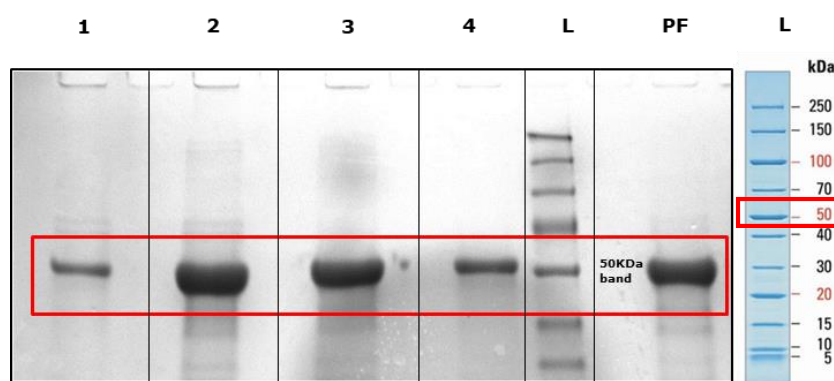


Fig. 5.10: SDS-PAGE analysis of fractions of purified PuO_{Rh}.

PuO_{Rh} was overexpressed by growing *E. coli* BL21(DE3)_pET-20b_puo_{Rh} at 25 °C and 250 rpm in LB medium supplemented with 1% glucose and carbenicillin. PuO_{Rh} expression was induced with sterile IPTG (0.4 Mm) at a culture OD₆₀₀ of 0.6, cells were harvested and cell free extracts prepared. PuO_{Rh} was purified by immobilized metal affinity chromatography using a HisTrap FF crude column and was monitored by UV absorption at 240 nm. Fractions were collected when a sharp absorbance peak was observed, and were analysed by SDS-PAGE separately and after pooling. **1,2,3&4:** fractions collected, **PF:** purified fraction pool, **L:** Spectra™ Multicolor Broad Range Protein Ladder. **Red rectangle highlights the band of PuO_{Rh} and standard corresponding to the appropriate weight of ≈50 KDa.**

The total protein content and specific activity of the cell free extract and the purified PuO_{Rh} were calculated (Table 5.2). After purification of the cell free extract, the total concentration of protein in the sample was reduced by three (3) fold. Similarly, total activity reduced by two fold, indicating that half of the PuO_{Rh} activity was preserved after the purification process. This could be as a result of suboptimal binding to the column or loss of protein during desalting. Other factors could be overloading of the column, incomplete elution or protein denaturation by the high buffer salt (imidazole) concentration. However, the specific activity of the enzyme increased after purification, indicating that the process was effective. Although the flow-through fraction during the

binding phase of the purification was not sampled or analysed in this study, it is highly recommended because it would have given clear answers to the cause of reduction observed in the total activity after purification.

Table 5.2: Activity of PuO_{Rh} before (cell free extract) and after purification

Purification Stage	Total Protein (mg/mL)	Total Activity (μmol/min)	Specific Activity (μmol/min/mg protein)	Yield (%)	Purification factor
Before (cell free extract)	26.50 ± 0.05	0.32 ± 0.01	12.08 ± 0.01	100	1
After (purified PuO _{Rh})	8.95 ± 0.15	0.16 ± 0.00	17.88 ± 0.00	50	1.48

PuO_{Rh} was expressed in *E. coli* BL21 (DE3)_pET-20b(+)_puo_{Rh} at a culture OD_{600nm} of 0.6 by the addition of IPTG (0.4 mM). Cells were harvested by centrifugation and lysed using a Constant Systems cell disrupter. Cell free extracts (supernatant) were collected after centrifugation (12000 rpm, 4°C, 10 min). PuO_{Rh} was purified by immobilized metal affinity chromatography using a HisTrap FF crude column. Cell free extract and purified PuO_{Rh} activity were determine using the *o*-ABA coupled colorimetric assay with putrescine as substrate at 30°C. The 1 mL reaction mixture contained 100 mM Na - K phosphate buffer pH 7.5, 1.0 mM Putrescine, 0.01% *o*-aminobenzaldehyde and 1 μL cell free extract or purified PuO_{Rh}. Values represent mean of triplicate experiments ± standard deviation.

5.3.2 Colorimetric assay of purified PuO_{Rh}

The kinetic parameters of PuO_{Rh} were previously reported by van Hellemond *et al.* (2008), using an indirect peroxidase coupled assay with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as the chromogenic substrate. The H₂O₂ formed by PuO_{Rh} was coupled to the Horseradish-peroxidase-mediated oxidation of ABTS resulting in the formation of a green product that can be measured at 420 nm. Similarly, such a coupled colorimetric assay based on the formation of Δ¹-piperidine was desirable for this research. Therefore, attempts were made to determine the steady state kinetic parameters of the purified PuO_{Rh} using the *o*-ABA coupled colorimetric assay with cadaverine and putrescine as substrates (see Fig. 5.6). PuO_{Rh} oxidation of putrescine was assayed and the absorbance at 435 nm monitored. Result indicated that putrescine was selectively oxidised because the absorbance increased over time. Similarly, PuO_{Rh} oxidation of cadaverine was conducted, however, an initial lag phase was observed which was not observed with putrescine (Fig. 5.11).

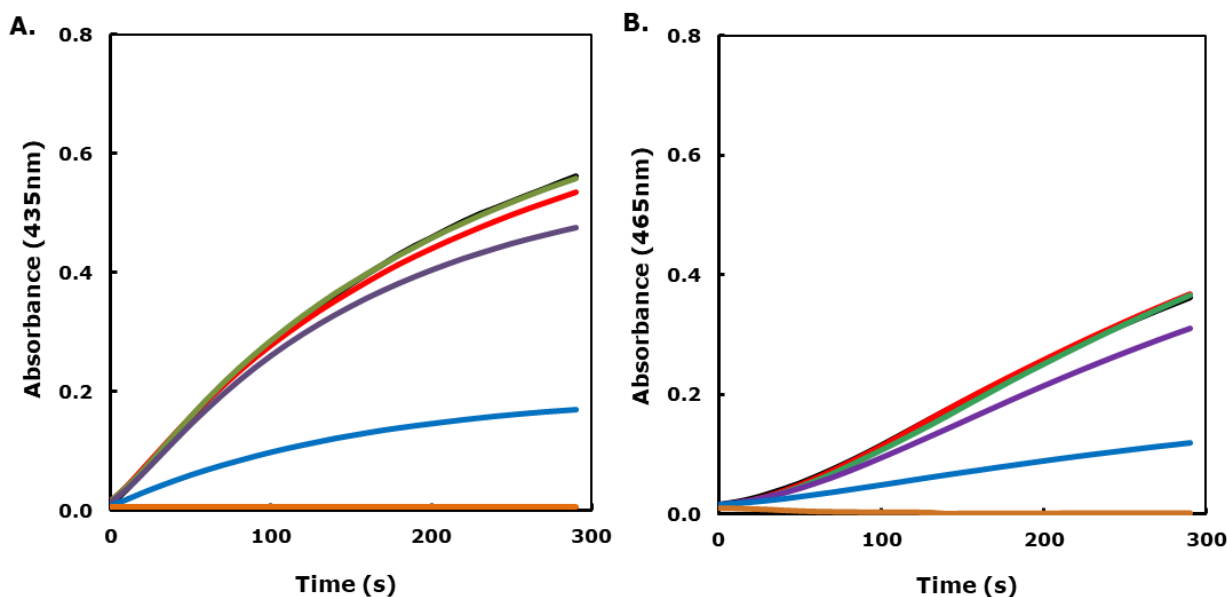


Fig. 5.11: PuOR_h - *o*-ABA assay on putrescine and cadaverine.

PuOR_h activity was determined with putrescine (**A.**) and cadaverine (**B.**) at 30 °C. The reaction mixture contained 100 mM sodium-potassium phosphate buffer pH 7.5, 0.1 – 1.0 mM substrate (Cadaverine / Putrescine), 1.0 mM *o*-aminobenzaldehyde and 0.1 mg/mL purified PuOR_h. The assay was started by the addition of PuOR_h and the reaction was monitored by UV-Vis spectrophotometry. The absorbance was measured at 465 and 435 nm for cadaverine and putrescine, respectively using Shimadzu UV-2600 spectrophotometer. The activity was measured using different concentrations of putrescine and cadaverine: 1.0 mM (—), 0.8 mM (—), 0.5 mM (—), 0.3 mM (—), and 0.1 mM (—), while set-up without enzyme was control (—).

In a similar reaction involving dihydrodipicolinate synthase (which catalyses the condensation of aspartate semi-aldehyde and pyruvate to give 4-hydroxy-2,3,4,5-tetrahydrodipicolinate – Fig. 5.12), Yugari and Gilvarg (1965) reported that the reaction of 4-hydroxy-2,3,4,5-tetrahydrodipicolinate with *o*-ABA, showed a lag phase and the chromophore formed slowly.

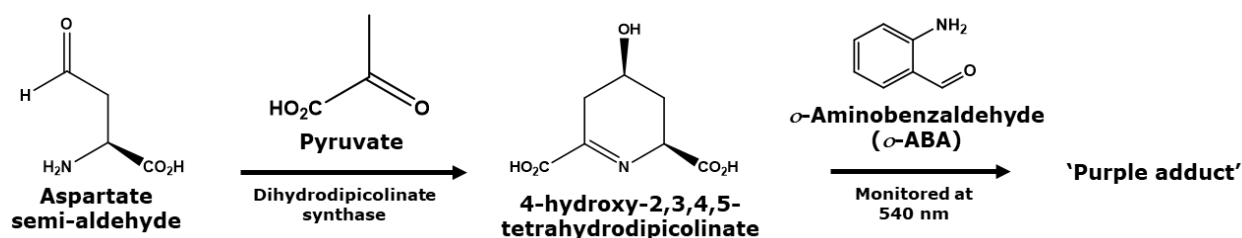


Fig. 5.12: Dihydrodipicolinate synthase - *o*-aminobenzaldehyde (*o*-ABA) assay

The initial rate of reaction is required to determine the kinetic parameters of an enzyme, hence the observed lag caused interference using cadaverine. Since the assay principle involves *o*-ABA reaction with Δ^1 -piperidine (see Fig. 5.6A), two reasons were envisaged for the observed lag: either the cyclization of 5-aminopentanal or the reaction between Δ^1 -piperidine and *o*-ABA. Given that cyclization is reported to be spontaneous, though not instantaneous (Monaco et al., 2011) and Δ^1 -piperidine is expected to reach a minimum concentration threshold in order to drive the adduct formation, it was reasoned that the slow initial formation of the adduct would explain the lag phase. Ideally, increasing the temperature of the reaction and the enzyme concentration, should favour the rate of 5-aminopentanal and Δ^1 -piperidine formation (Peterson et al., 2007). Therefore, the reaction was optimized by altering the temperature and the concentration of PuOR_h used in the assay. Also, the effect of *o*-ABA concentration was investigated.

When the enzyme concentration was increased from 0.1 mg/mL to 0.3 mg/mL, a negligible lag reduction was visually observed from the absorbance versus time plot (Fig. 5.13). Subsequently, the temperature of the assay was increased from 30 °C to 37 °C, using 0.3 mg/mL enzyme concentration. Visual comparison of the absorbance versus time plot, also, showed no significant reduction in the lag (Fig. 5.14). On the other hand, when the *o*-ABA concentration was increased up to 10 mM together with the increased enzyme concentration (0.3 mg/mL), a significant lag reduction was visualized (Fig. 5.15) in comparison with an assay using 1 mM *o*-ABA.

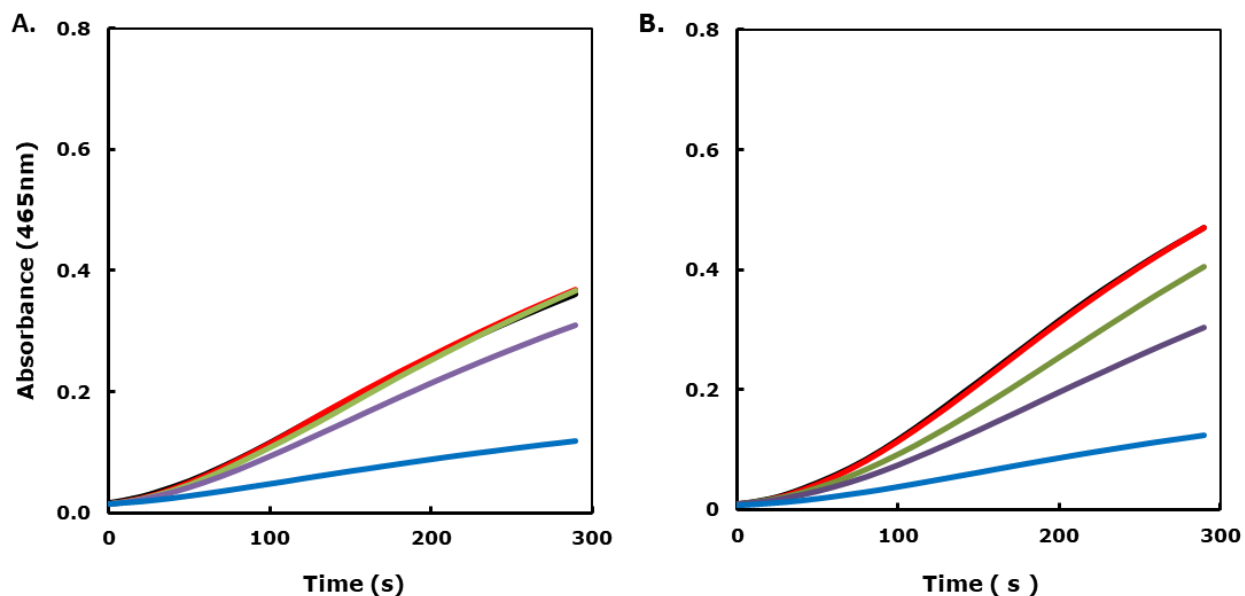


Fig. 5.13: Optimized PuOR_h - *o*-ABA assay with cadaverine: Effect of PuOR_h concentrations.

Catalysed oxidation of cadaverine using (A) 0.1 mg/mL and (B) 0.3 mg/mL PuOR_h at 30 °C. The reaction mixture contained 100 mM sodium-potassium phosphate buffer pH 7.5, 0.1 – 1.0 mM Cadaverine, 1 mM *o*-aminobenzaldehyde and purified PuOR_h. The absorbance was measured at 465 nm using Shimadzu UV-2600 spectrophotometer. The activity was assayed using different concentrations of cadaverine: 1.0 mM (—), 0.8 mM (—), 0.5 mM (—), 0.3 mM (—) and 0.1 mM (—).

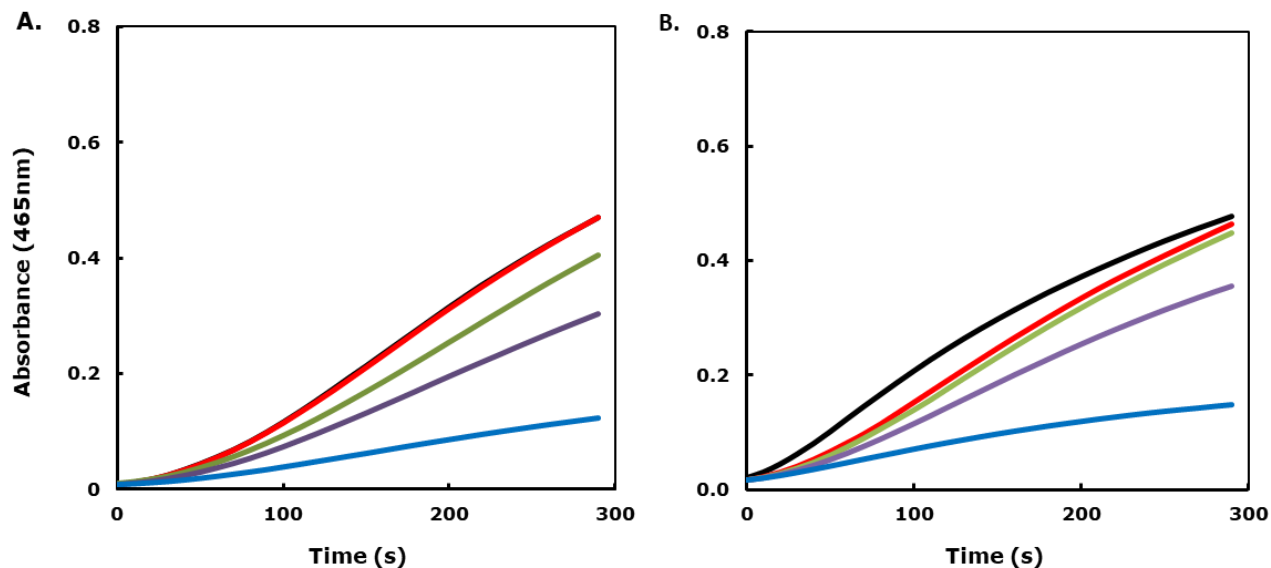


Fig. 5.14: Optimized PuOR_h - *o*-ABA assay with cadaverine: Effect of temperature.

PuOR_h catalysed oxidation of cadaverine at 30 °C (A) and 37 °C (B). The reaction mixture contained 100 mM sodium-potassium phosphate buffer pH 7.5, 0.1 – 1.0 mM Cadaverine, 1 mM *o*-aminobenzaldehyde and 0.3 mg/mL purified PuOR_h. The absorbance was measured at 465 nm using Shimadzu UV-2600 spectrophotometer. The activity was assayed using different concentrations of cadaverine: 1.0 mM (—), 0.8 mM (—), 0.5 mM (—), 0.3 mM (—) and 0.1 mM (—).

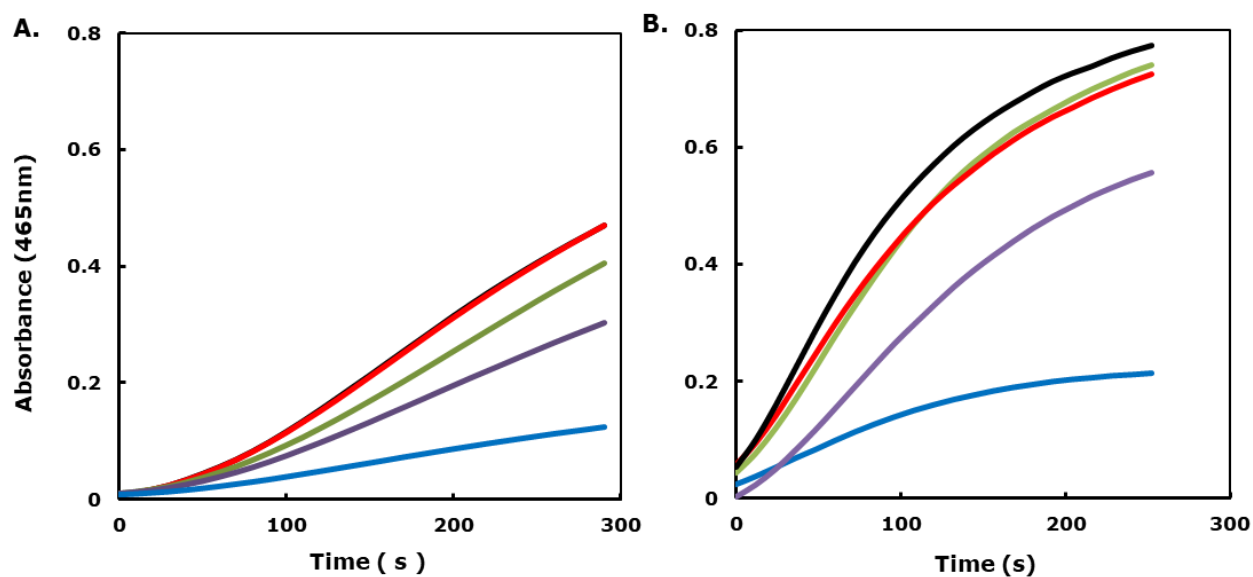


Fig. 5.15: Optimized PuOR_h - *o*-ABA assay with cadaverine: Effect of *o*-aminobenzaldehyde concentration.

PuOR_h catalysed oxidation of cadaverine using 1 mM (A) and 10 mM (B) *o*-aminobenzaldehyde at 30 °C. The reaction mixture contained 100 mM sodium-potassium phosphate buffer pH 7.5, 0.1 – 1.0 mM Cadaverine, 1 – 10 mM *o*-aminobenzaldehyde and 0.3 mg/mL purified PuOR_h. The absorbance was measured at 465 nm using Shimadzu UV-2600 spectrophotometer. The activity was assayed using different concentrations of cadaverine: 1.0 mM (—), 0.8 mM (—), 0.5 mM (—), 0.3 mM (—) and 0.1 mM (—).

In order to assess whether this was due to the combined increase of both *o*-ABA and PuOR_h concentrations, the experiment was repeated using lower enzyme concentration (0.1 mg/mL) at 30 °C and 37 °C respectively (Figure 5.16). Visual observation of the absorbance versus time plots show that while the lag was reduced at both temperatures, better reduction was obtained at 37 °C (Figure 5.16B). Interestingly, increasing the concentration of *o*-ABA increased the rate of reaction (by visual observation of the absorbance-time plot) even at lower enzyme concentration. Noteworthy, the *o*-ABA stock solution was prepared in water and in an effort to prepare stocks to give a higher concentration above 10 mM, it showed a partial solubility leaving precipitates in the *o*-ABA stock solution. Attempts to solve this challenge by dissolving *o*-ABA in 5% dimethyl sulphoxide (DMSO) gave a similar result. Nonetheless, the lag was reduced with an increased *o*-ABA concentration, signifying that the lag is dependent on the Δ^1 -piperidine - *o*-ABA reaction rather than an enzyme (PuOR_h) inhibition. This result supports the

hypothesis that a shift of the reaction towards formation of Δ^1 -piperidine or the orange adduct could eliminate the observed lag.

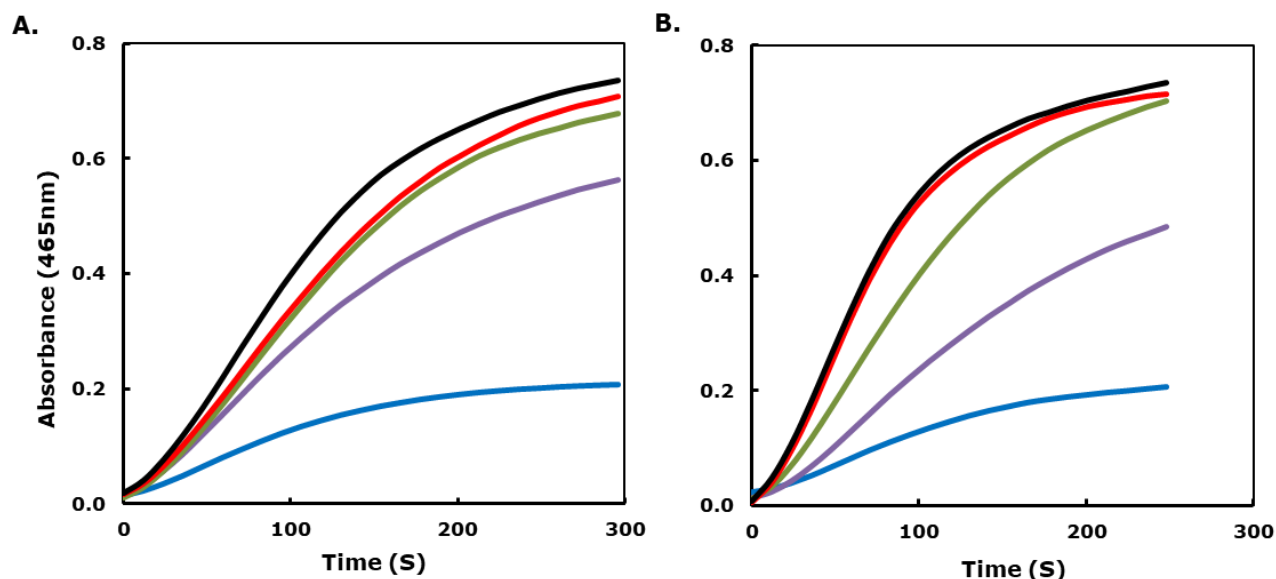


Fig. 5.16: Optimized PuOR_h - *o*-ABA assay on cadaverine: Effect of increased *o*-ABA concentration and reduced PuOR_h concentration at different temperatures.

PuOR_h catalysed oxidation of cadaverine at 30 °C (**A**) and 37 °C (**B**) using 10 mM *o*-aminobenzaldehyde. The reaction mix contained 100 mM sodium-potassium phosphate buffer pH 7.5, 0.1 – 1.0 mM Cadaverine, 10 mM *o*-aminobenzaldehyde and 0.1 mg/mL purified PuOR_h. The absorbance was measured at 465 nm for cadaverine using Shimadzu UV-2600 spectrophotometer. The reaction mixture contained 100 mM sodium-potassium phosphate buffer pH 7.5, 0.1 – 1.0 mM Cadaverine, 10 mM *o*-aminobenzaldehyde and 0.1 mg/mL purified PuOR_h. The absorbance was measured at 465 nm using Shimadzu UV-2600 spectrophotometer. The activity was assayed using different concentrations of cadaverine: 1.0 mM (—), 0.8 mM (—), 0.5 mM (—), 0.3 mM (—) and 0.1 mM (—).

In conclusion, to reliably determine the kinetic parameters of the enzyme, it is ideal to completely remove the observed lag. Therefore, the PuOR_h - *o*-ABA assay on cadaverine could not be used for PuOR_h characterization.

5.3.3 Oxygen consumption assay of purified PuO_{Rh}

To avoid the problems above, a more direct method was needed to measure PuO_{Rh} activity. Since PuO_{Rh} is an oxidase, oxygen consumption can be used to measure PuO_{Rh} activity on cadaverine and putrescine. This is a direct analysis of the activity of PuO_{Rh}, so it should represent an accurate and unbiased result. A linear decrease of oxygen concentration was observed initially with both substrates (Fig. 5.17), indicating that this assay can be used to determine activity of PuO_{Rh}. In comparison to the α -ABA coupled assay, the oxygen consumption assay showed no lag phase when cadaverine was used as substrate. Therefore, the kinetic parameters of PuO_{Rh} were determined by measuring the oxygen consumption in a catalysed oxidation of cadaverine and putrescine.

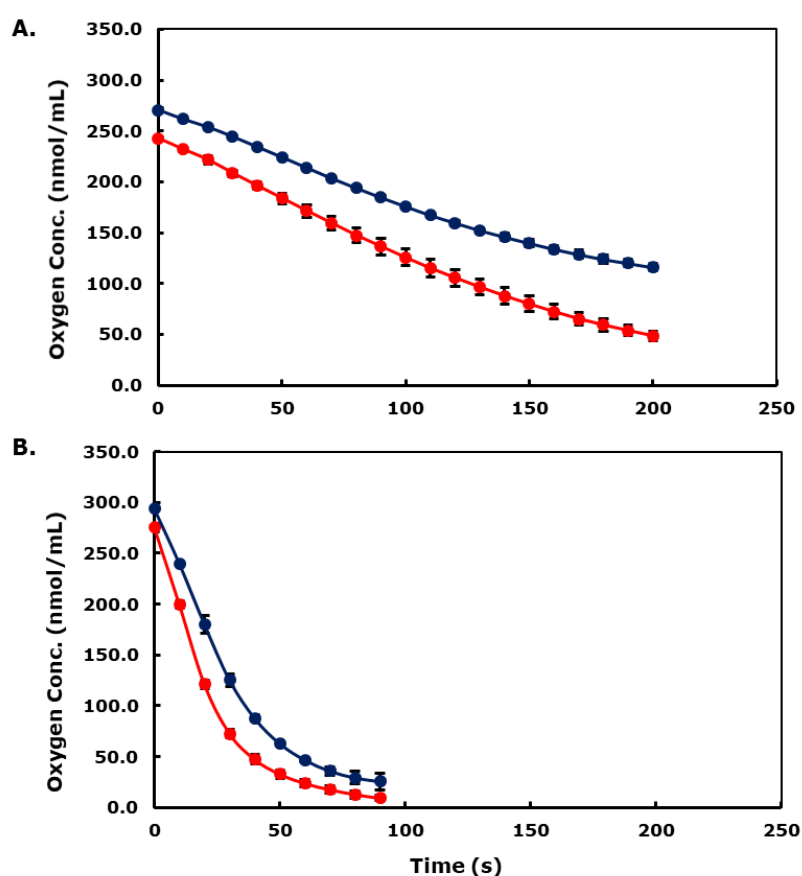


Fig. 5.17: Oxygen consumption assay of purified PuO_{Rh}

PuO_{Rh} (0.1 mg/mL) catalysed oxidation of cadaverine (**A**) and putrescine (**B**) was measured in 100 mM Na-K phosphate buffer pH 7.5 by monitoring the rate of oxygen consumption using a Clark-type Oxygen (O₂) electrode (Oxygraph, Hansatech Instruments Ltd, Norfolk, UK) maintained at 30 °C using a heated circulating water-bath (Grant TC120). Activity of the enzyme was assayed with different substrate concentrations: 1.0 mM [—] and 0.3 mM [—]. Values represent mean of triplicate experiments ± standard deviation.

5.4 Biochemical characterization of PuO_{Rh}

5.4.1 Steady-state kinetic parameters (Michaelis - Menten parameters)

The steady state kinetics of PuO_{Rh} has only been reported using ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] - horseradish peroxidase coupled assay, which depends on the hydrogen peroxide (H₂O₂), formed during the oxidation reaction (van Hellemond et al., 2008b). Indirect assays could be inaccurate because of the differences between the characteristics of an enzyme being analysed and the characteristics of the coupled assay system used (McClure, 1969, Storer and Cornish-Bowden, 1974, Uhr, 1990). For more accurate steady state kinetics of any enzyme, a direct assay is more appropriate. Therefore, the steady-state kinetics of PuO_{Rh} were determined by monitoring oxygen consumption; the K_M and k_{cat} values were 0.24 ± 0.05 mM and 26.6 ± 0.08 s⁻¹ for cadaverine, and 0.17 ± 0.03 mM and 147.4 ± 0.4 s⁻¹ for putrescine, respectively (Table 5.3 and Fig. 5.18). Whereas the k_{cat}/K_M values are lower than those reported using peroxidase-coupled assays, this result should represent the true kinetics of PuO_{Rh}. This result reveals that PuO_{Rh} has a better catalytic efficiency for putrescine than cadaverine, and this is in agreement with the report of van Hellemond et al. (2008b). To the best of my knowledge, this is the first report of PuO_{Rh} kinetic parameters using a direct assay.

Comparatively, the calculated parameters are lower in sharp contrast with the previous report by van Hellemond et al. (2008) and this could be because of the differences in the reaction conditions and methodology used. The results reported here are at pH 7.5 while van Hellemond et al. (2008) reported results at pH 8.5 (Table 5.3). On the other hand, comparison of this result to that of a recombinant copper (Cu²⁺) amine oxidase from *Huperzia serrata* with a K_m and k_{cat} of 0.3 mM and 1.0 s⁻¹ respectively for cadaverine (Sun et al., 2012), shows that PuO_{Rh} has better catalytic efficiency and substrate affinity for cadaverine.

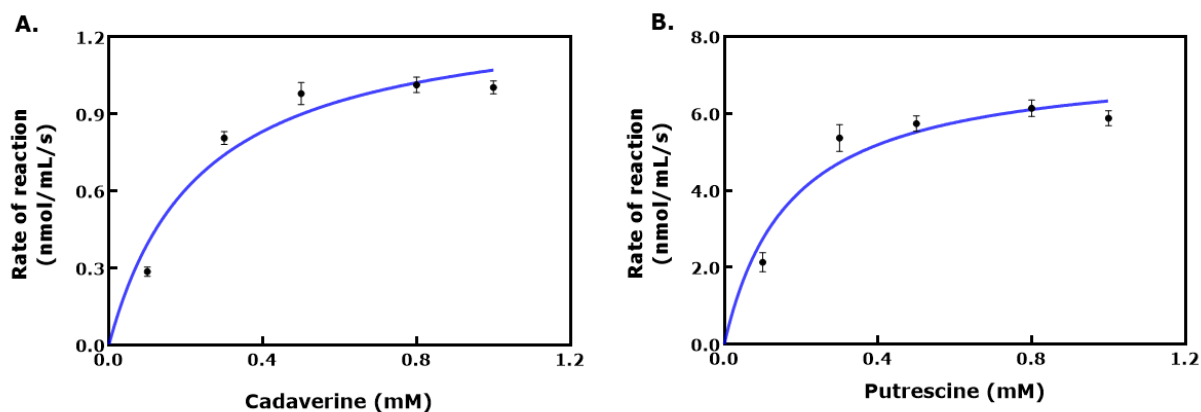
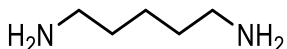
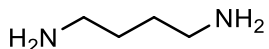


Fig. 5.18: Michaelis – Menten plot of PuO_{Rh} reaction using Cadaverine (**A.**) and Putrescine (**B.**)

PuO_{Rh} catalysed oxidation of cadaverine or putrescine (0.1 – 1.0 mM) was measured as reported in Fig. 5.17. The rate of O₂ utilization was determined by measuring the slope of a tangent drawn to the O₂ concentration versus time curve. Rate of reaction were used to fit a nonlinear Michaelis – Menten model/plot of enzyme activity against substrate concentration using GraphPad Prism version 6.04. Values represent mean of triplicate experiments $\pm$ standard deviation.

Table 5.3: Steady-state kinetic parameters of purified PuOR_h

Substrate	K _m (mM)	k _{cat} (s ⁻¹) [V _{max}]	k _{cat} / K _m (s ⁻¹ mM ⁻¹)	Methodology and assay conditions	Source & type of Oxidase	Reference
Cadaverine 	0.24±0.05	26.6±0.08	110.8	O ₂ consumption, Phosphate buffer pH 7.5, 30 °C	<i>R. erythropolis</i> & FAD oxidase	This study
Putrescine 	0.17±0.03	147.4±0.4	867.1	O ₂ consumption, Phosphate buffer pH 7.5, 30 °C	<i>R. erythropolis</i> & FAD oxidase	This study
Putrescine	0.23±0.07	106±0.05	463.5	o-Aminobenzaldehyde assay, Phosphate buffer pH 7.5, 30 °C	<i>R. erythropolis</i> & FAD oxidase	This study
Putrescine	0.0082	26.4±0.5	3200	HRP-ABTS enzyme assay, Tris-HCl buffer pH 8.0, 25 °C	<i>R. erythropolis</i> & FAD oxidase	van Hellemond et al. (2008b)
Cadaverine	0.018	3.9 ± 0.2	220	HRP-ABTS enzyme assay, Tris-HCl buffer pH 8.0, 25 °C	<i>R. erythropolis</i> & FAD oxidase	van Hellemond et al. (2008b)
Putrescine	0.038	[100 Ms ⁻¹]	ND	O ₂ consumption Tris-HCl buffer pH 8.6, 20 °C	<i>Micrococcus rubens</i> & FAD oxidase	DeSa and Brantner (1972)
Cadaverine	0.3	1.0	3.3	H ₂ O ₂ assay kit, Tris-HCl buffer pH 8.0, 25 °C	<i>Huperzia serrata</i> & copper amine oxidase	Sun et al. (2012)
Putrescine	1.2	1.9	1.6	H ₂ O ₂ assay kit, Tris-HCl buffer pH 8.0, 25 °C	<i>Huperzia serrata</i> & copper amine oxidase	Sun et al. (2012)

Kinetics parameters were estimated from the Michaelis-Menten model/plot (Fig. 5.18) using GraphPad Prism version 6.04. Values represent mean of triplicate experiments ± standard deviation. **K_m** = Michaelis constant (experimentally defined as the concentration at which the rate of the enzyme reaction is half **V_{max}**), **k_{cat}** = turnover number (denotes the maximum number of enzymatic reactions catalysed per second), while **k_{cat} / K_m** = catalytic efficiency of the enzyme reaction.

5.4.2 PuO_{Rh} spectral properties

Purified PuO_{Rh} was analysed by UV-Vis spectrophotometry. PuO_{Rh} exhibited the characteristic UV-visible spectrum of oxidized FAD (see Fig. 2.16 and 2.17) with three absorbance peaks at 274, 365 and 457 nm (Fig. 5.19) (Liu et al., 2010, Müller et al., 2018). Addition of a saturating amount of cadaverine converted the spectrum to that of a fully reduced form as indicated by the decrease in absorbance bands in the visible range (365 and 457 nm) (Kopacz et al., 2014, Müller et al., 2018). This suggests that the FAD molecules in the purified PuO_{Rh} were catalytically active in the presence of cadaverine. This is in agreement with previous report (Tavladoraki et al., 2006, van Hellemond et al., 2008b, Kopacz et al., 2014), who used a saturating amount of putrescine as the reducing substrate in Tris-buffer pH 8.0 to assess FAD activity in PuO_{Rh}.

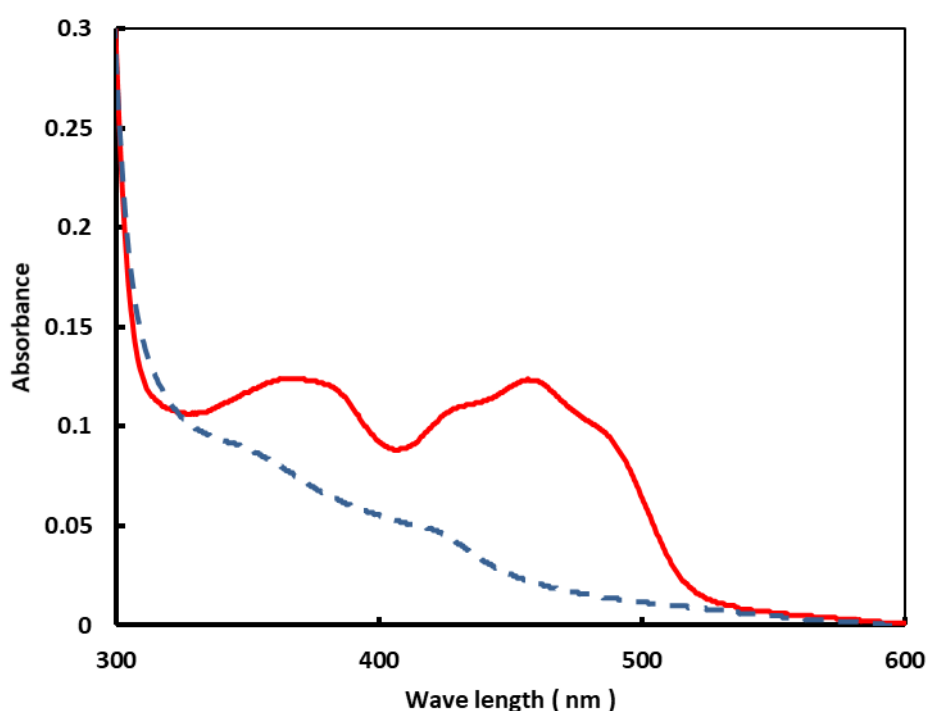


Fig. 5.19: PuO_{Rh} absorbance spectra.

PuO_{Rh} (1.0 mg/mL) absorbance spectra were recorded in 100 mM Na-K Phosphate buffer pH 7.5 at 25 °C. After adding 30 mM cadaverine, stirred and allowed to stand for 5 min, another spectrum was recorded for the reduced enzyme. Solid red line (—) represents oxidized PuO_{Rh} while dotted blue line (---) represents reduced PuO_{Rh}.

5.4.3 Effect of temperature on PuO_{Rh} activity

The activity of PuO_{Rh} was studied at different temperatures, constant pH and enzyme concentration to identify its optimum temperature. PuO_{Rh} activity increased directly with increase in temperature up to 55 °C and continued to be active until 75 °C (Fig. 5.20). The optimum temperature was 55 °C. These results are in agreement with earlier report (van Hellemond et al., 2008b); however, these authors did not present any data/result to support their report. Therefore, this report represent the first data supported findings using the direct enzyme assay.

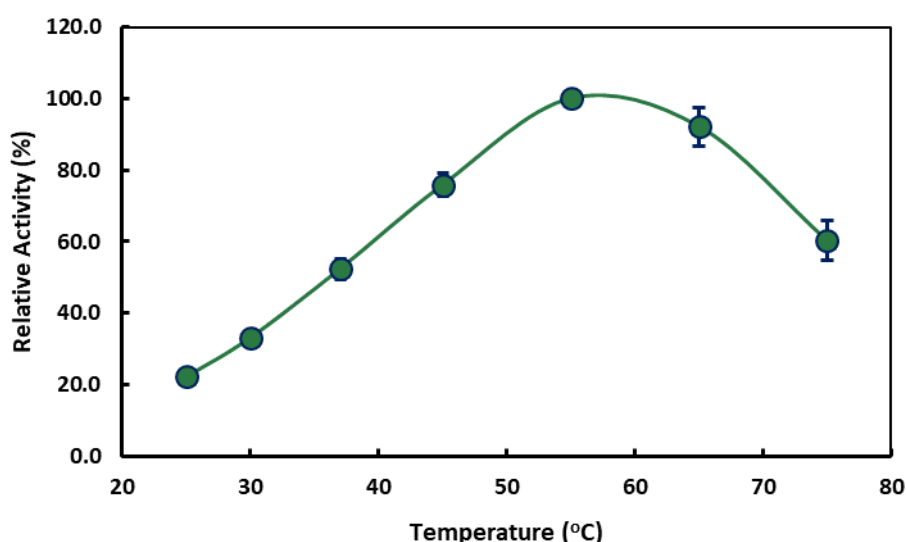


Fig. 5.20: Effect of temperature on PuO_{Rh} activity.

PuO_{Rh} (0.1 mg/mL) catalysed oxidation of cadaverine (0.8 mM) was measured in 100 mM Na-K phosphate buffer pH 7.5 by monitoring the rate of oxygen consumption using a Clark-type Oxygen (O₂) electrode (Oxygraph, Hansatech Instruments Ltd, Norfolk, UK) maintained at different temperature using a heated circulating water-bath (Grant TC120). Data points represent mean $\pm$ standard deviation from three independent experiments.

5.4.4 Effect of pH on PuO_{Rh} activity

The effect of pH on PuO_{Rh} activity was studied at 25, 30 and 37 °C, due to this temperature range being ideal for both microbial growth and active enzyme expression. Although 55 °C was earlier established as the optimum temperature, the aim was to check the effect of pH on PuO_{Rh} activity at a temperature range that best supports microbial growth and bioproduction. Results revealed that at all the temperatures examined, enzyme activity

decreased with increased acidity (pH 6.5) while the optimum pH was around 8.5 (Fig. 5.21). These reports also are in agreement with the report of van Hellemond et al. (2008b).

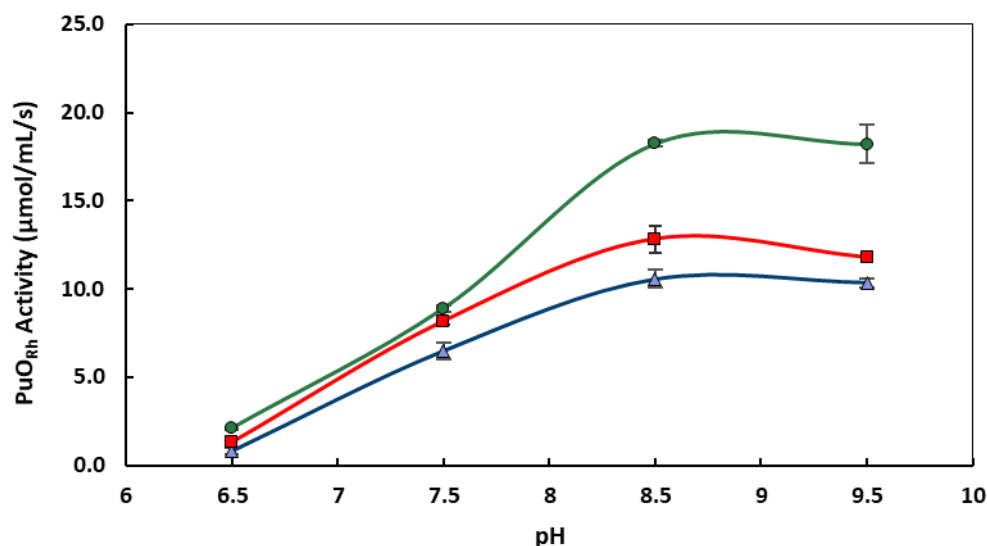


Fig. 5.21: Effect of pH on PuOR_h activity.

PuOR_h activity (0.1 mg/mL) on cadaverine was assayed using an Oxygen Electrode Measurement System as reported in Fig. 5.17. Assays for enzyme activity were in 100 mM sodium-potassium phosphate buffer and 0.8 mM cadaverine at different temperatures and pH. Data points represent mean of triplicate experiments $\pm$ standard deviation. The effect of pH was assayed at different temperatures; 37 °C —●—, 30 °C —■— and 25 °C —▲—.

5.4.5 Effect of temperature and pH on PuOR_h stability

To evaluate the effect of temperature and pH on the stability of PuOR_h, duplicate enzyme samples in buffer at pH 7.5 and 9.5 were independently incubated in a water bath at 25 °C and 55 °C. The incubated enzyme was sampled at intervals and its activity with cadaverine was assessed. PuOR_h showed high stability during incubation at 25 °C (Fig. 5.22) where enzyme activity was unaffected up to 65 h incubation. However, when incubated at its optimal temperature (55 °C), a sharp decrease of enzyme activity was observed both at pH 7.5 and 9.5. Almost 75% of activity was lost after 1 h incubation at pH 7.5. Thereafter, it remained stable and activity was only reduced slightly after 5 h. At pH 9.5, about 95% enzyme activity was lost after 1 h and complete inactivation was

observed after 2.5 h. This result indicated that optimal conditions for PuOR_{Rh} activity differs from conditions for its optimal stability. In conclusion, it is ideal to operate PuOR_{Rh} activity at 25 °C, however, operating temperature depends on the set up of the reaction.

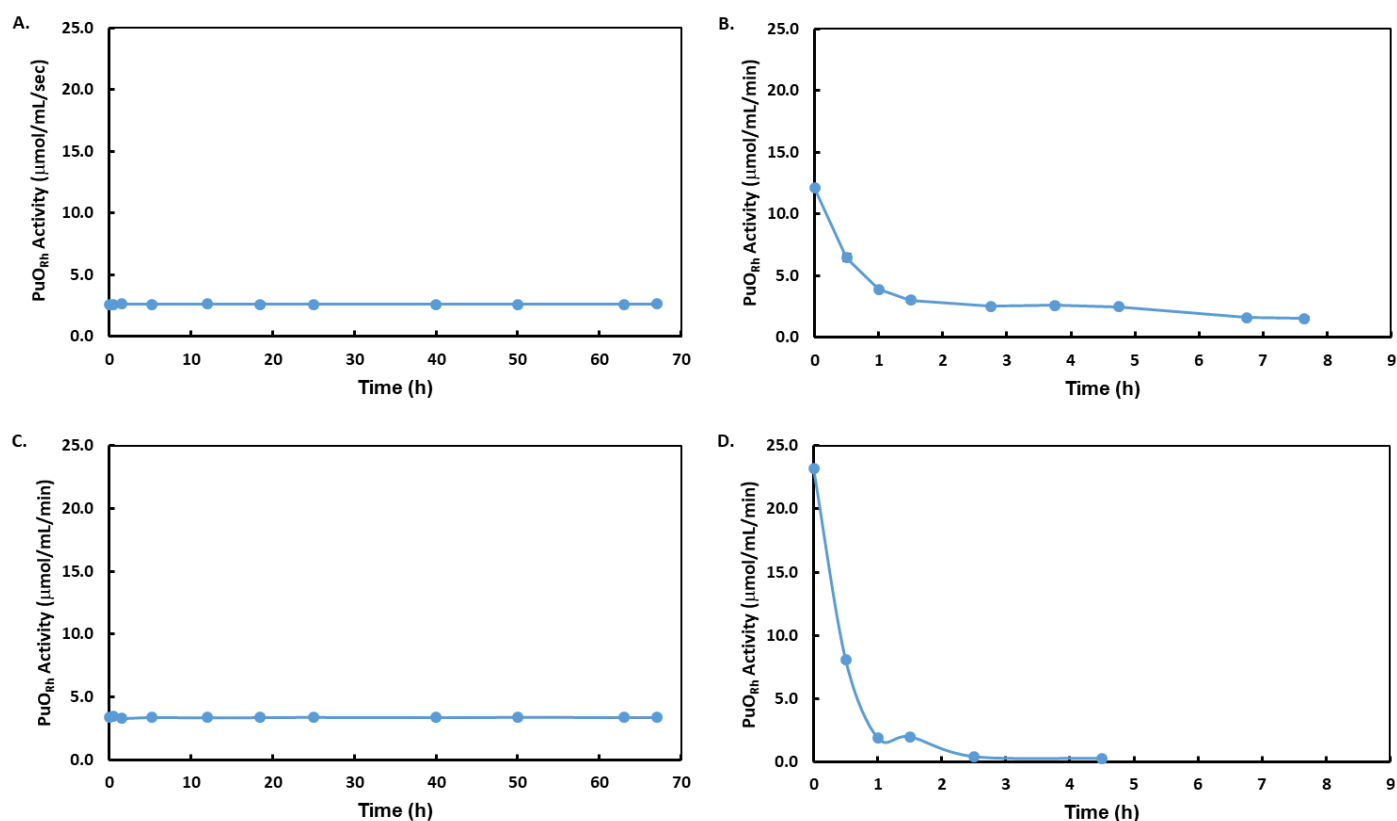


Fig. 5.22: Effect of pH and temperature on the stability of PuOR_{Rh}.

PuOR_{Rh} activity (0.1 mg/mL) on cadaverine was assayed using an Oxygen Electrode Measurement System as reported in Fig. 5.17. A single enzyme batch was divided into two equal parts in order to create identical starting activity of PuOR_{Rh}. Duplicate batches of PuOR_{Rh} in buffer adjusted to required pH 7.5 or 9.5 were independently incubated at 25 °C and 55 °C, and sampled for assays at intervals. Assays for enzyme activity were in 100 mM sodium-potassium phosphate buffer and 0.8 mM cadaverine at incubated temperature and pH: pH 7.5 and 25 °C (**A.**), pH 7.5 and 55 °C (**B.**), pH 9.5 and 25 °C (**C.**), and pH 9.5 and 55 °C (**D.**). Data points represent mean of triplicate experiments ± standard deviation.

5.4.6 Activation energy (E_a or ΔG^*) of PuO_{Rh} catalysed reaction

A catalyst provides an alternative route for the reaction with a lower activation energy. The rate of a reaction depends on the temperature at which it is run. In principle therefore, the rate of a reaction is inversely proportional to the activation energy and Arrhenius equation (Eq. 5.1) can be used to determine the activation energy for a reaction. A plot of the natural log of the rate constant for the reaction at different temperatures versus the inverse of the temperature in Kelvin is a straight line with a slope equal to $-E_a/R$. Therefore, the activation energy of an enzyme-catalysed reaction can be calculated (Berg et al., 2002).

$$\ln(K) = \ln(Z) - \frac{E_a}{R} \left(\frac{1}{T}\right) \quad \text{Eq. 5.1}$$

Where, **K** = Rate of reaction (reaction rate constant), **Z** = pre-exponential factor or collision frequency, **R** = Gas constant (8.31446 J K⁻¹ mol⁻¹), **T** = Absolute temperature (i.e. in kelvin), **E_a** = activation energy

To evaluate the activation energy of the PuO_{Rh} catalysed reaction, the rates of reactions at different temperatures were measured while the concentration of the enzyme remained constant. This was repeated or replicated at different pH to observe the effect of pH. The lowest activation energy was observed at pH 8.5 while the highest E_a was at pH 6.5 (Fig. 5.23). This result is consistent with the earlier results on the effect of pH and temperature on PuO_{Rh} activity (see section 5.4.3 & 5.4.4). Finally, this is the first report of the activation energy of PuO_{Rh} catalysed reaction and it is valuable for protein engineering, computational analysis of proteins and process optimization of bioproduction.

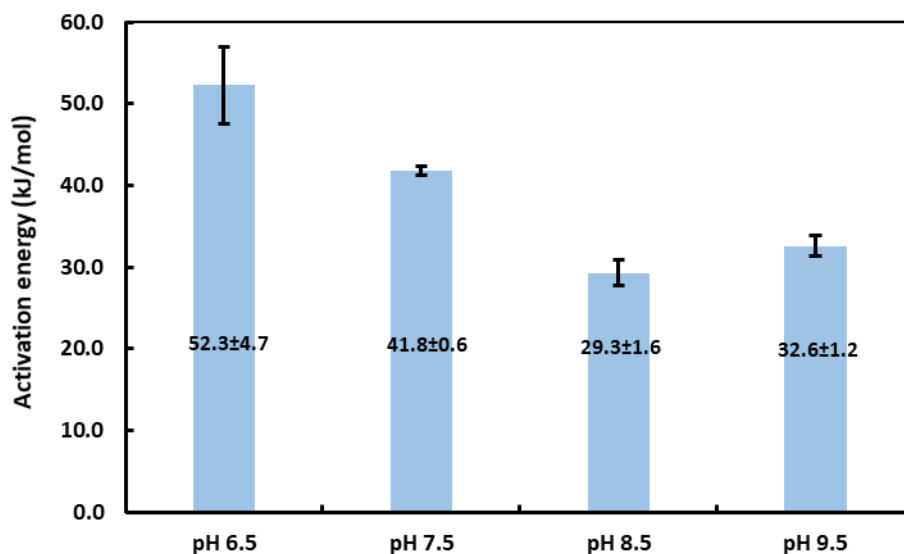


Fig. 5.23: Activation energy of PuO_{Rh} catalysed reaction at different pH.

PuO_{Rh} activity (0.1 mg/mL) on cadaverine was carried out using an Oxygen Electrode Measurement System as reported in Fig. 5.17. Assays for enzyme activity were in 100 mM sodium-potassium phosphate buffer and different concentrations of (0.1,0.3,0.5,0.8,1.0 mM) cadaverine at different temperatures and pH. Activation energy was determined using Arrhenius plot and equation. Data points represent mean of triplicate experiments ± standard deviation.

5.4.7 PuO_{Rh} substrate inhibition analysis

To assess the effect of substrate concentration on the activity of PuO_{Rh}, substrate inhibition analysis was conducted. Experimental set-ups monitored oxygen consumption using different and increasing concentrations of cadaverine. Reaction rates were calculated and fitted into Michaelis – Menten plots using GraphPad Prism. In an enzyme reaction, rate of reaction increases with increase in substrate concentration until the maximum rate of reaction is reached. At this point, rate of reaction is constant and increase in the substrate concentration will not result in an increased reaction rate. However, if the substrate inhibits the enzyme, once maximum reaction rate is achieved, further increase in the substrate concentration will result in decrease in the rate of reaction. Therefore, PuO_{Rh} activity is not inhibited by cadaverine up to 100 mM concentration (Fig. 5.24).

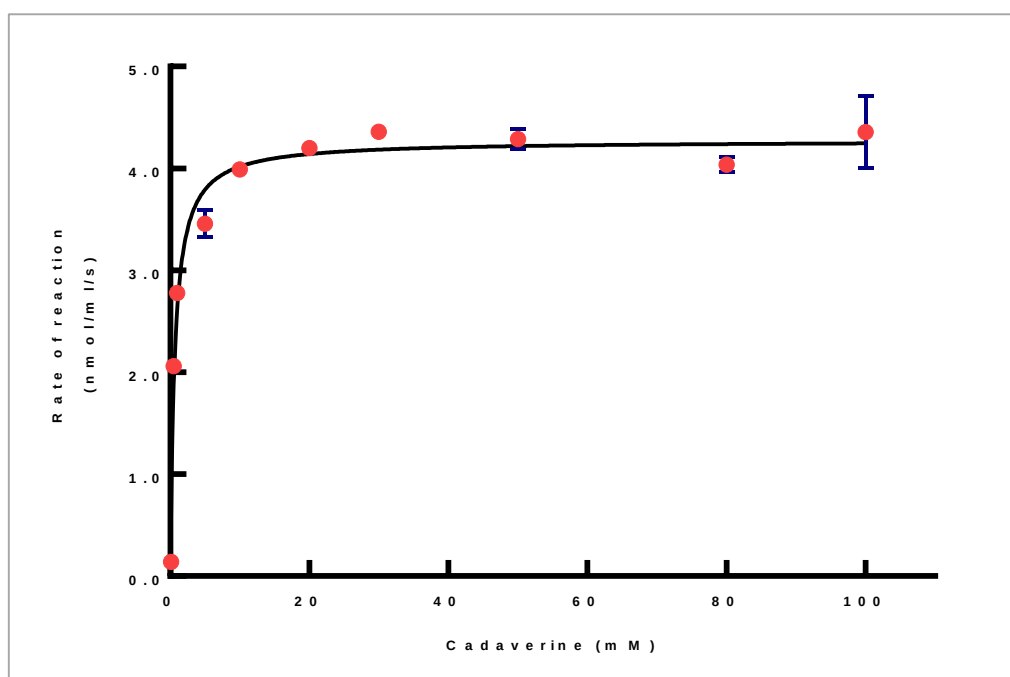


Fig. 5.24: Analysis of substrate inhibition on PuO_{Rh} activity.

PuO_{Rh} activity (0.1 mg/mL) on cadaverine was carried out using an Oxygen Electrode Measurement System as reported in Fig. 5.17. Assays for enzyme activity were in 100 mM sodium-potassium phosphate buffer and concentrations of (1.0 – 100.0 mM) cadaverine at 25 °C temperatures and pH 7.5. Reaction rates were determined and fitted into Michaelis – Menten plots using GraphPad Prism version 6.04. Data points represent mean of triplicate experiments $\pm$ standard deviation.

5.5 Discussion

Optimal soluble expression of PuO_{Rh} was observed at low temperature and high agitation. This could be because of increased oxygen concentration at the above conditions. Agitation of fermentation systems is known to increase the flow of oxygen, similarly, oxygen concentration increases with decrease in temperature (Guest et al., 1996). In addition, increased oxygen concentration has been reported to enable aerobic organisms to effectively oxidize growth substrates, therefore, optimizing their general metabolic activity (Partridge et al., 2006). Previously, van Hellemond et al. (2008b) reported that PuO_{Rh} was mainly present in the soluble fractions at 17 and 30°C, while at 37°C the insoluble fractions contained most of the overexpressed PuO_{Rh}. This is strongly in agreement with our observed results in this study.

The *o*-ABA coupled assay revealed an initial lag phase in the activity of PuOR_h on cadaverine. Although optimization of the reaction conditions reduced the lag phase, it was not eliminated. Therefore, further optimization studies are recommended. Nonetheless, the lag phase observed could be due to ring-closure, as formation of penta-cyclic ring (Δ^1 -pyrroline) from putrescine would be expected to proceed faster than the hexa-cyclic ring (Δ^1 -piperideine) from cadaverine. Again, the size of the cyclic rings could imply a preferential reaction favouring penta-cyclic ring (putrescine) and *o*-ABA compared to hexa-cyclic ring (cadaverine) and *o*-ABA (see Fig. 5.6). This result and explanation are supported by previously published report (Yugari and Gilvarg, 1965; see Fig. 5.12).

In both the *o*-ABA coupled and oxygen consumption assays, the activity of PuOR_h on putrescine was more than 5-folds higher compared to its activity on cadaverine. This is expected because putrescine is the primary substrate of PuOR_h, indicating that the active site of the enzyme is better adapted for diamines with four-carbon chain. This result is consistent with the reports of van Hellemond et al. (2008b). In addition, it presents a focus for protein engineering to improve the bonding affinity and activity of cadaverine and the active site of PuOR_h. However, this aspect of the work was not conducted.

Finally, PuOR_h showed no substrate inhibition with up to 100 mM cadaverine. This information is the first report of cadaverine inhibition on PuOR_h, and it is valuable for protein engineering and setting up an optimal bioproduction system using PuOR_h. In addition, Orejarena Pacheco, et al. (2016) reported total synthesis of tetraponerines, T7 and T8, using 34.3 mM Δ^1 -piperideine as starting material. This indicates that if PuOR_h efficiently converts 50% of 100 mM cadaverine to Δ^1 -piperideine, it can produce enough starting material for the synthesis of tetraponerines as reported, therefore, making it more sustainable. However, further concentrations of cadaverine (200 – 1000 mM) should be used in order to establish the minimum inhibitory concentration on the purified PuOR_h.

5.6 Conclusions

Putrescine oxidase from *Rhodococcus erythropolis* (PuO_{Rh}) was cloned and expressed in *E. coli* BL21(DE3) using the pET-20b(+) vector. Growth conditions were optimised and optimum expression of soluble enzyme was determined to be at 25 °C and 250 rpm. Crude enzyme assay using *o*-aminobenzaldehyde (*o*-ABA) coupled colorimetric analysis indicated that the overexpressed PuO_{Rh} was active on cadaverine and putrescine. *o*-ABA coupled assay was used for this work because it is quick, gives preliminary confirmation of the reaction product and has been previously reported (Bachrach, 1962, Fothergill and Guest, 1977, Mitsakos et al., 2011). Because our interest molecule was Δ^1 -piperidine, the assay indicated Δ^1 -piperidine was produced during catalysed oxidation of cadaverine, thus, cadaverine was the substrate of interest in this study. Subsequently, the hexa-histidine tagged PuO_{Rh} was successfully purified by immobilized nickel affinity chromatography (IMAC).

Attempts were made to characterize PuO_{Rh} using the *o*-ABA coupled assay. This was because PuO_{Rh} had only been previously characterized using ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] – horseradish peroxidase coupled assay (van Hellemond et al., 2008b). Thus, the purified PuO_{Rh} was used to catalyse the oxidation of putrescine and monitored using the *o*-ABA coupled assay. Based on the absorbance of the reaction, it progressed linearly and this means that the initial rate of the reaction could be calculated accurately. Contrarily, the *o*-ABA coupled assay revealed an initial lag phase in the activity of PuO_{Rh} on cadaverine.

Therefore, PuO_{Rh} was characterized using substrate consumption analysis. The steady state kinetics were determined by monitoring oxygen consumption; the K_M and k_{cat} values were 0.24 ± 0.05 mM and 26.6 ± 0.08 s⁻¹ for cadaverine, and 0.17 ± 0.03 mM and 147.4 ± 0.4 s⁻¹ for putrescine, respectively. Optimal temperature and pH for PuO_{Rh} catalytic oxidation of cadaverine were 55 °C and 8.5 respectively. Also, at pH 7.5 and 9.5, PuO_{Rh} was stable and active at 25 °C up to 65 h while it showed a 75 - 95% loss of activity at 55

°C in 1 h. Activation energy of PuO_{Rh} catalysed oxidation of cadaverine was lowest at pH 8.5 while substrate inhibition analysis revealed that cadaverine does not inhibit PuO_{Rh} activities. Spectral analysis of purified PuO_{Rh} indicated that it was bound to oxidized FAD. The spectral characteristics of the purified PuO_{Rh} is insightful because the enzyme's activity could be further studied using the dissociation of the enzyme complex (Liu et al., 2010, Müller et al., 2018).

Finally, this chapter focused mainly on cloning, expression and characterization of PuO_{Rh}. In the next chapters, the suitability of PuO_{Rh} for cell-free biocatalysis and whole-cell bioconversion of cadaverine to Δ^1 -piperidine were investigated.

6. Results and Discussion: Bioconversion of cadaverine using purified PuOR_h

6.1 Introduction

Δ^1 -Piperideine was chosen as the target molecule for this work because the metabolic engineering design for its production is simple. One-step enzyme catalysed oxidation of cadaverine yields 5-aminopentanal, which spontaneously cyclises into Δ^1 -Piperideine. Putrescine oxidase from *Rhodococcus erythropolis* (PuOR_h) was identified as a candidate enzyme to catalyse the one-step conversion of cadaverine. PuOR_h was cloned, and enzyme assays (*o*-ABA assay and oxygen consumption) indicated that the PuOR_h over-expressed was active. Therefore, the suitability of PuOR_h for cell-free bioconversion of cadaverine to Δ^1 -piperideine was explored.

However, the enzymatic assay methods are not suitable for the detection of the desired products. This is because Δ^1 -piperideine reacts spontaneously in aqueous solution to form multimeric products (see Fig. 2.6; tetrahydroanabasine, α -tripiperideine, β -tripiperideine and isotripiperideine) and the ratio of these products depends on pH (Rouchaud and Braekman, 2009, Monaco et al., 2011., Schopf, 1948). To characterize the reaction outcome, analytical methods that rely more on structural identification of compounds, mass spectrometry (MS) and nuclear magnetic resonance (NMR) were used. In addition, the effectiveness of liquid-liquid extraction for selective recovery of Δ^1 -piperideine and its multimeric species from aqueous medium was investigated.

6.2 Product detection and identification

6.2.1 Liquid chromatography mass spectrometric (LC-MS) analysis

The *o*-aminobenzaldehyde coupled assay results (see section 5.3.2) indicated that Δ^1 -piperideine was formed during PuOR_h catalysed oxidation of cadaverine. To validate the Δ^1 -piperideine product structure, the reaction mixture of cadaverine oxidation in the presence of purified enzyme (PuOR_h) was analysed by mass spectrometry. In the absence of

commercially available Δ^1 -piperidine standard, this analytical method was useful to identify the reaction products.

Reaction samples were directly analysed for Δ^1 -piperidine and its multimers by liquid chromatography mass spectrometry (LC – MS; Fig. 6.1). Peaks were observed, of molecular weights 167.2 and 250.2 (mass-to-charge ratio, m/z) equivalent to the charged dimer (tetrahydroanabasine) and trimers (isotripiperidine, α -tripiperidine and β -tripiperidine) respectively. Also, unlabelled peaks in the chromatogram (Fig. 6.1A) gave mass spectrum outside the expected molecular weights of the products, and this could be due to electron spray ionization. The three trimeric forms of Δ^1 -piperidine have the same molecular weight of 249.2. However, the trimer β -tripiperidine was previously reported not to occur at 30 °C (Shvehgheimer, 1998, Rouchaud and Braekman, 2009), therefore, the observed peak with molecular weight 250.2 could be either the charged isotripiperidine or α -tripiperidine. By contrast, the Δ^1 -piperidine forming activity was undetectable in control reactions without the purified PuO_{Rh} or cadaverine respectively. Whereas peaks with molecular weights equivalent to Δ^1 -piperidine were not detected, the presence of tetrahydroanabasine, isotripiperidine and α -tripiperidine indicated that Δ^1 -piperidine was produced in the enzyme assay containing cadaverine. However, these results are only indicative and the solvents in LC analysis could influence the speciation and equilibrium between Δ^1 -piperidine and its multimeric species. Therefore, to acquire structural information and to confirm the reaction products *in situ*, the products of enzyme reaction were analysed by ^1H NMR.

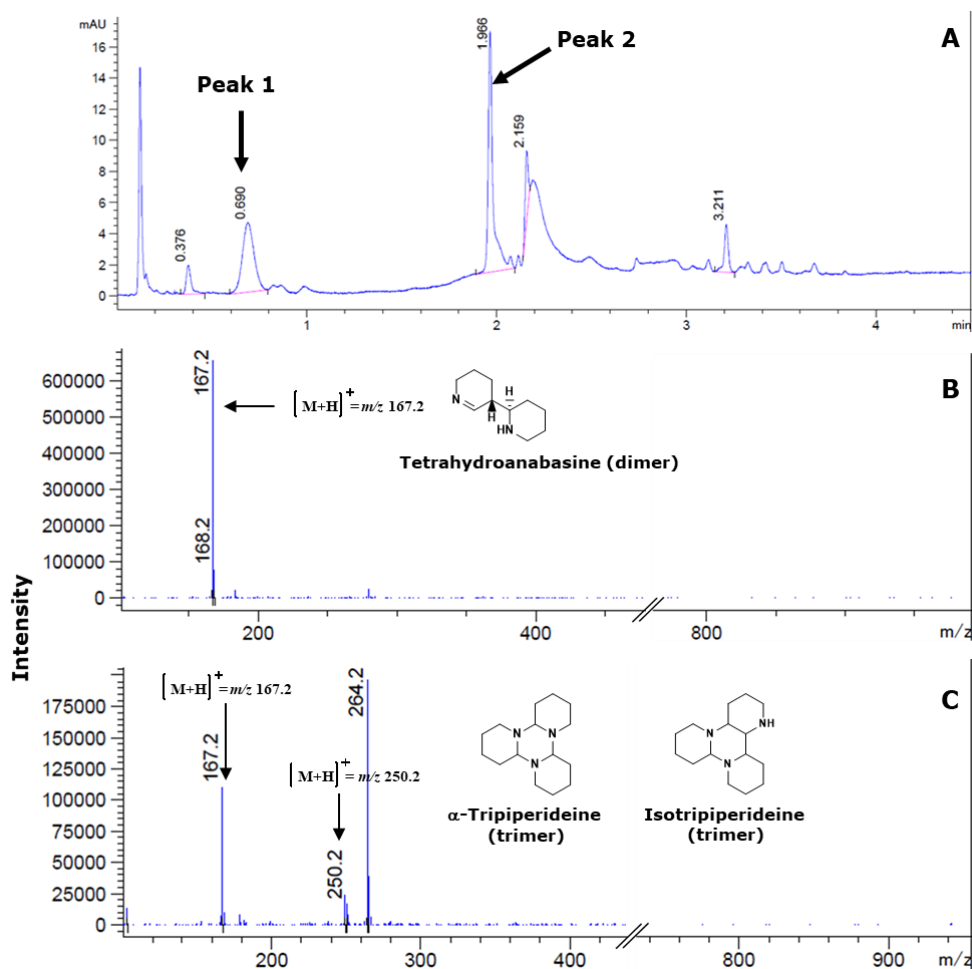


Fig. 6.1: LC - ESI - MS analysis of PuO_{Rh} catalysed oxidation of cadaverine.

E. coli BL21(DE3)_pET-20b(+)_puORh was grown on LB medium supplemented with glucose and carbenicillin. PuO_{Rh} expression was induced at a culture OD_{600nm} of 0.6 by the addition of sterile IPTG (0.4 mM). Cells were harvested by centrifugation 5 h post induction and PuO_{Rh} purified by IMAC. The enzyme reaction mixture (100 mM Na-K phosphate buffer pH 7.5, 30 mM cadaverine and 0.1 mg/mL purified PuO_{Rh}) was incubated (30 °C, 150 rpm, 15 min) and analysed for Δ^1 -piperideine and its multimers by LC - ESI - MS (Agilent Technologies 1260 series HPLC coupled to an Agilent 6120 Quadrupole mass spectrometer fitted with an ESI probe and a DAD UV detector) in positive mode. **A.** = liquid chromatogram, **B.** = mass spectrum of peak 1 and **C.** = mass spectrum of peak 2.

6.2.2 Proton nuclear magnetic resonance (¹H NMR) analysis

Enzymatic reactions at pH 7.5 were analysed by ¹H NMR in a 9 to 1 ratio with deuterium oxide (aqueous sample/D₂O ratio of 9/1). Controls without the enzyme were run in parallel to ensure that observed activity occurred because of the PuO_{Rh}. The spectra were analysed by comparison with literature reports (Santamaria et al., 1977, Barker et al., 2011, Monaco et al., 2011, Li et al., 2013, Unsworth et al., 2014, Unsworth et al., 2013, Salame et al., 2010, Gomm et al., 2016, Orejarena Pacheco et al., 2016). Noteworthy, Δ^1 -

piperidine and tetrahydroanabasine have characteristic chemical shifts between 7.5 – 8.0 ppm due to the presence of an imine proton (hydrogen on the carbon that has double bond with nitrogen). Because tetrahydroanabasine is a transient intermediate between Δ^1 -piperidine and its trimeric forms, the observed chemical shift between 7.5 – 8.0 ppm was attributed to Δ^1 -piperidine (Salame et al., 2010, Monaco et al., 2011).

The NMR analysis (Fig. 6.2) showed that cadaverine was almost entirely unreacted after 15 min, as suggested by the presence of its distinctive three peaks at 2.84 - 2.95 ppm, 1.60 – 1.62 ppm, and 1.35 – 1.37 ppm. Characteristic chemical shifts for 5-aminopentanal (≥ 8.5 ppm), the direct product of PuO_{Rh} activity on cadaverine (see Fig. 5.6A), and for Δ^1 -piperidine were not detected in the reaction mixture. Instead, characteristic chemical shifts for isotripiperidine at 3.55 – 3.77 ppm and α -tripiperidine at 1.72 – 2.1 ppm were observed in minor amounts. In addition, overlapping chemical shifts between 1.3 – 1.84 ppm, characteristic of Δ^1 -piperidine, isotripiperidine, and α -tripiperidine were observed. In comparison, only chemical shifts for cadaverine were observed in controls set-ups without PuO_{Rh} .

The fact that 5-aminopentanal was not observed, was not surprising. Firstly, this is because of its spontaneous cyclization (Schiff base reaction) to Δ^1 -piperidine, and secondly because Δ^1 -piperidine does not easily undergo hydrogenation of the carbon – nitrogen double (peptide, $\text{C}=\text{N}$) bond (Joule and Mills, 2010). Instead, Δ^1 -piperidine forms isotripiperidine and α -tripiperidine by the nucleophilic addition of nitrogen of one molecule to the azomethine carbon of a second molecule (Joule and Miller, 2010), thereby preventing the reversibility of the cyclization reaction. Also, double deamination of cadaverine leading to formation of glutaraldehyde (Salame et al., 2010), was not observed and this is supported by van Hellemond et al., 2008 who suggested that a minimum of two amino groups are required to be a substrate for PuO_{Rh} .

Published NMR spectra of Δ^1 -piperidine and its speciation products were in chloroform (CDCl_3) (Salame et al., 2010, Unsworth et al., 2014, Gomm et al., 2016, Orejarena-

Pacheco et al., 2016). To compare with the reported CDCl_3 spectra of the reaction products, reaction mixtures at pH 7.5 were extracted and analysed in CDCl_3 . The NMR analysis (Fig. 6.3) showed the presence of isotripiperideine and α -tripiperideine in the extracted sample. Δ^1 -piperideine was also detected, as suggested by the presence of the imine proton between 7.8 -7.9 ppm. Chemical shifts for cadaverine were not observed, indicating cadaverine was not extracted from the aqueous enzyme reaction mixture. Cadaverine did not extract into chloroform because at pH 7.5, it exists as a hydrophilic ion (salt), thus partitioned into the aqueous phase (Cai et al., 2007).

Finally, these results showed that the enzyme reaction samples contained a mixture of Δ^1 -piperideine and the trimeric species at pH 7.5 and this is in agreement with literature (Gravel et al., 2006, Rouchaud and Braekman, 2009, Poupon et al., 2011). To the best of our knowledge, this is the first report on PuO_{Rh} catalysed oxidation of cadaverine to Δ^1 -piperideine. Therefore, PuO_{Rh} could be used for bioproduction of Δ^1 -piperideine from cadaverine.

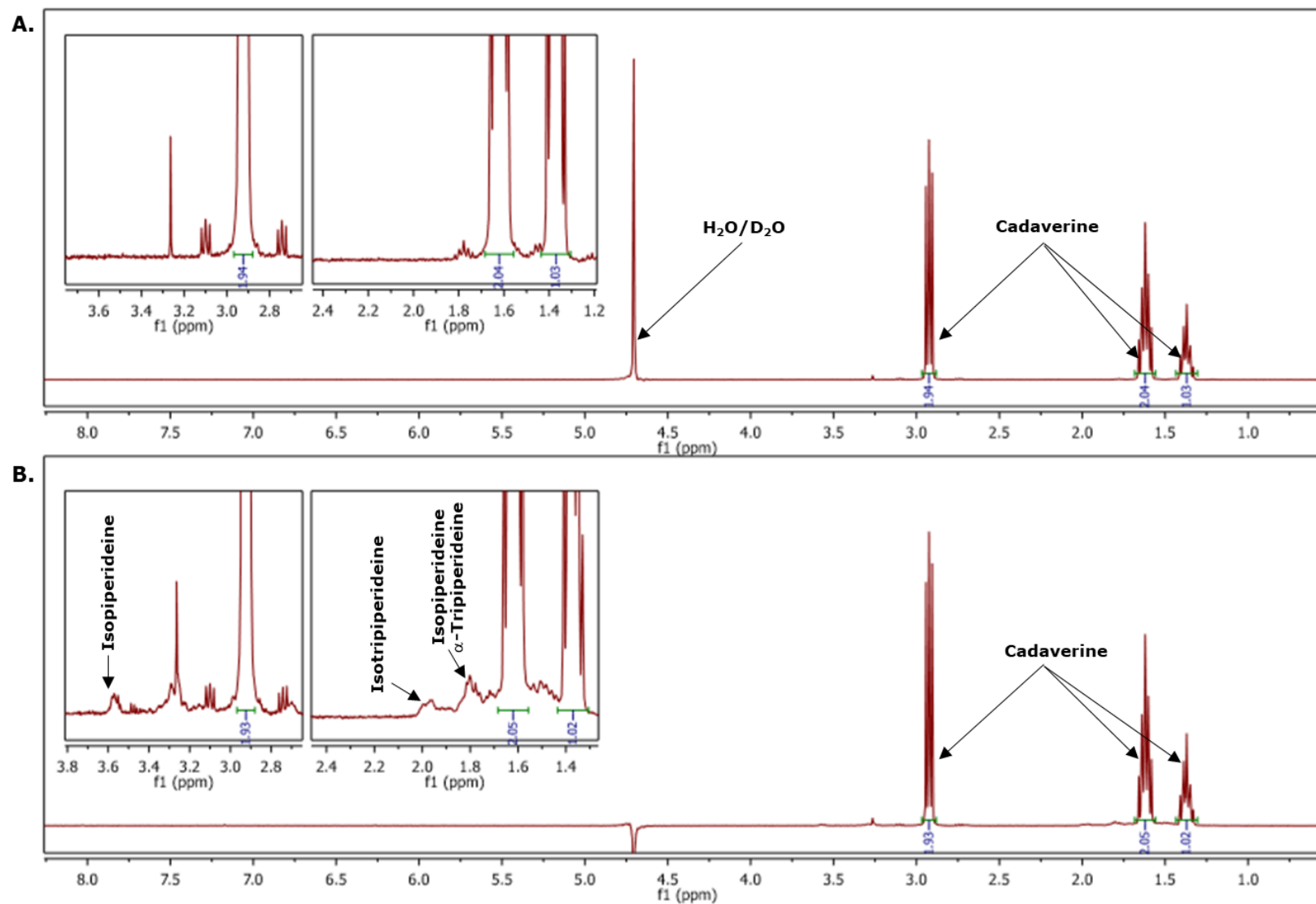


Fig. 6.2: ^1H NMR spectrum (D_2O) of (A.) Control set-up without enzyme and (B.) PuORh catalysed oxidation of cadaverine at pH 7.5.

PuORh catalysed oxidation of cadaverine was assayed. The reaction mixture contained O_2 equilibrated 100 mM sodium – potassium (Na-K) phosphate buffer pH 7.5, 30 mM cadaverine and 0.1 mg/mL purified PuORh . After incubation (30 $^\circ\text{C}$, 150 rpm, 15 min). Samples were deuterated with 99.9% Deuterium oxide (H_2O :90% / D_2O :10%) and analysed by ^1H NMR at ambient temperature using a 400-MHz Bruker AV3400 spectrometer, and run using automated presaturation solvent suppression to reduce the intensity of the water signal. Inserts are zoomed sections according to the scales.

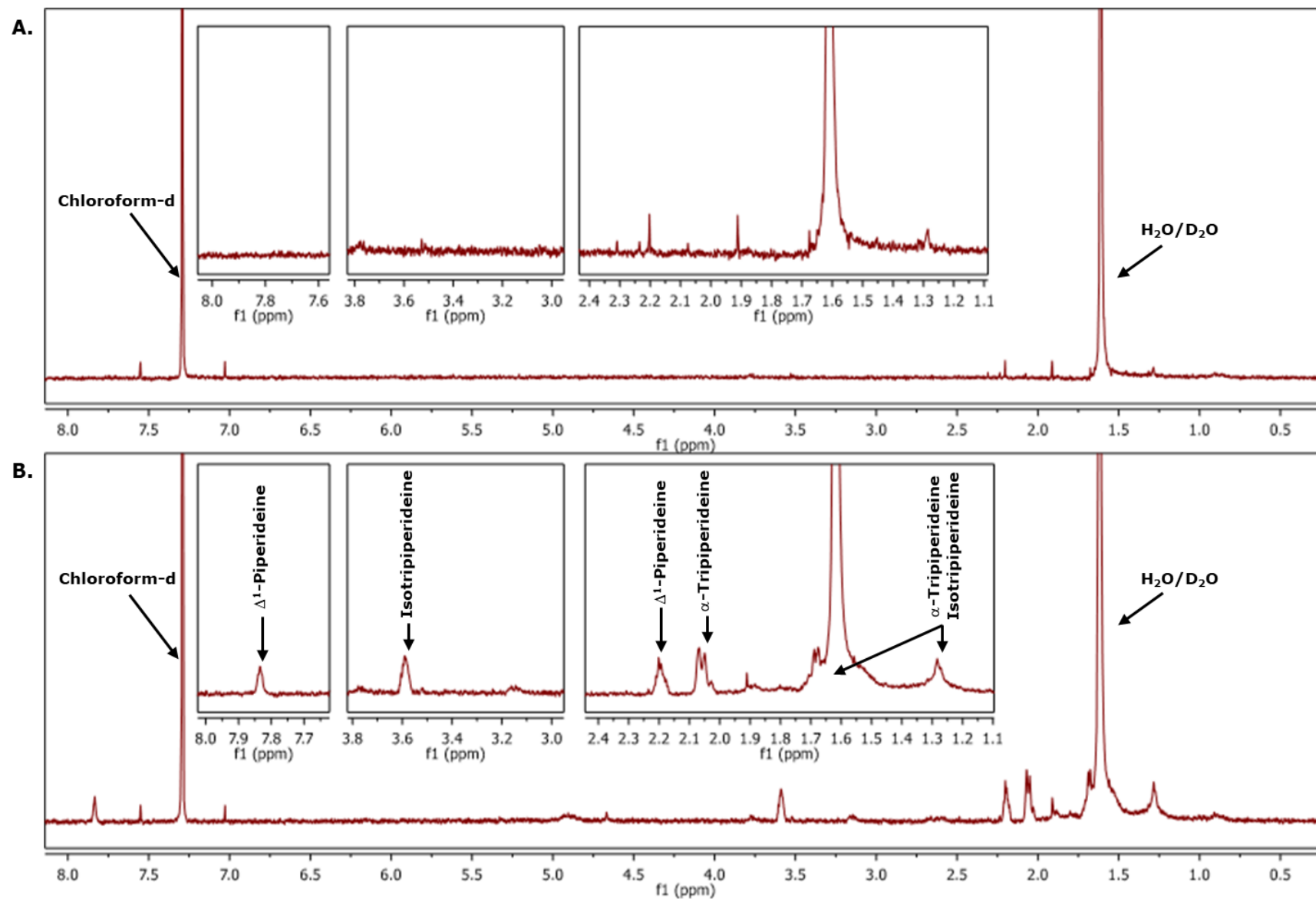


Fig. 6.3: ^1H NMR spectrum (CDCl_3) of (A.) Control set-up without enzyme and (B.) PuORh catalysed oxidation of cadaverine at pH 7.5 extracted with chloroform-d.

PuORh reaction mix was prepared as reported in Fig. 6.2 except that reaction mixtures at pH 7.5 were extracted with deuterated chloroform (CDCl_3) and dried with Na_2SO_4 and analysed in CDCl_3 by ^1H NMR at ambient temperature using a 400-MHz Bruker AV3400 spectrometer. Inserts are zoomed sections according to the scales.

To acquire quantitative information about the product formation and species distribution, the enzyme reaction was analysed using quantitative NMR (qNMR) after 40 h to enable maximum conversion. Maleic acid was used as external standard (see set-up and calibration; Methods and Appendix 11.6.3) for the deuterated aqueous samples because it has a single non-interfering chemical shift (6.2 – 6.4 ppm) with those of the products (Bharti and Roy, 2012, Rundlöf et al., 2014). The enzyme reaction was assayed at pH 7.5 using purified PuO_{Rh}, and incubated at 25 °C because of the recorded longer shelf life of the enzyme (see section 5.4.5).

As before, Δ^1 -piperidine, α -tripiperidine and isotripiperidine were present in all the samples (Fig. 6.4). Although the maximum total product yield of 43 % was observed after 24 h, it decreased to 35 % after 40 h. The concentration of Δ^1 -piperidine and isotripiperidine increased consistently over time. In contrast, the concentration of α -tripiperidine remained low for 18 h, and then increased to a maximum concentration after 24 h, followed by a sharp decrease after 40 h. At pH 7.5, Δ^1 -piperidine and α -tripiperidine are expected to be the most predominant species, but the result indicates Δ^1 -piperidine and isotripiperidine to be the most predominant species and this is in contrast with earlier reports (Rauchaud and Braekman, 2009 and Poupon et al., 2011). The observed decrease in concentration of α -tripiperidine after 40 h against an increase in the concentration of isotripiperidine suggests the instability of the α -tripiperidine while indicating its isomerization into isotripiperidine (Rauchaud and Braekman, 2009).

Also, since Δ^1 -piperidine is in principle the only carbon compound resulting from the PuO_{Rh} bioconversion of cadaverine, this result therefore indicates that substantial amount of carbon (about 58 % carbon substrate) was lost after 40 h. At the moment, this cannot be explained.

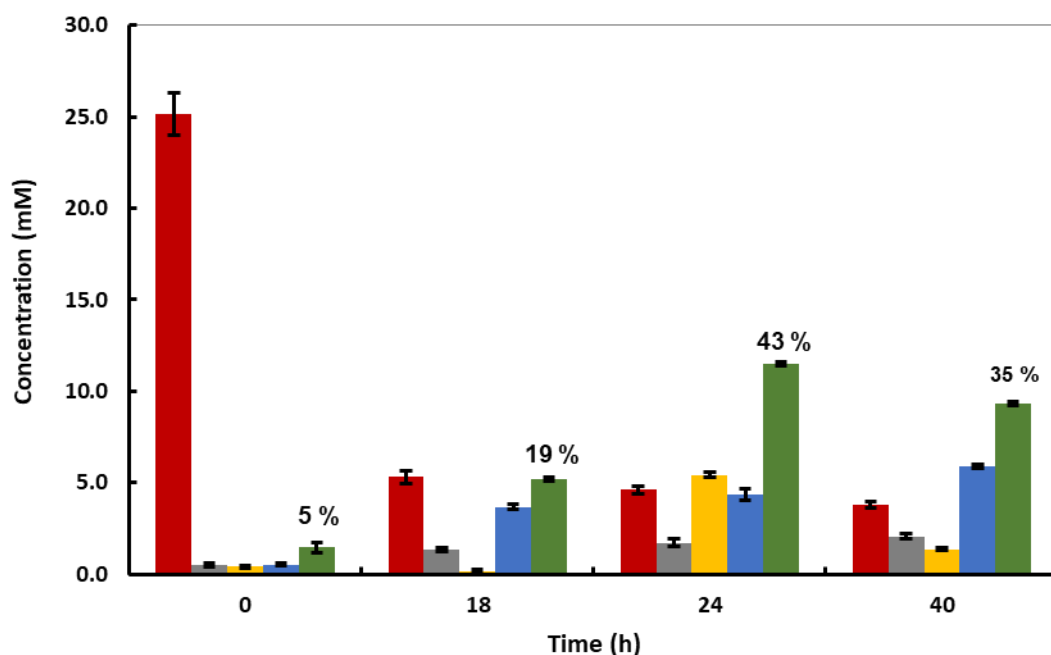


Fig. 6.4: Quantitative analysis of PuOR_h catalysed oxidation of cadaverine.

Cadaverine (27 mM) oxidation was studied using purified PuOR_h (1.0 mg/mL) in reaction mixtures containing Na-K phosphate buffer pH 7.5 (100 mM) and incubated (25 °C, 150 rpm). Samples were collected at intervals and were analysed by qNMR. Deuterated samples (H₂O/D₂O ratio of 90/10%) were prepared, and maleic acid was used as the external standard for qNMR in a stem co-axial insert (OD 2 mm). Spectra were obtained at ambient temperature using a 400-MHz Bruker AV3400 spectrometer, and run using automated presaturation solvent suppression to reduce the intensity of the water signal. Data represent mean of triplicate experiments $\pm$ standard deviation: ■ Residual cadaverine, ■ Δ^1 -Piperidine, ■ α -Tripiperidine, ■ Isotripiperidine and ■ Total Product (sum of the concentrations of Δ^1 -piperidine, α -tripiperidine and isotripiperidine calculated). Shown values represent total product yield (calculated as a percentage of initial cadaverine concentration used).

6.3 pH-dependent Speciation of Δ^1 -piperidine

The speciation of Δ^1 -piperidine has been reported to be pH-dependent (Schopf et al., 1948, Monaco et al., 2011). Therefore, to investigate the effect of pH in aqueous samples, experiments were repeated at pH 7.5, however, higher concentration of cadaverine was used and experiments were incubated longer. Thereafter, the reaction mixture was divided into three equal portions. One portion was analysed by qNMR at pH 7.5, while the other portions were first adjusted to pH 1 and 12 respectively before analysis.

Δ^1 -Piperidine, α -tripiperidine and isotripiperidine were present at all the pH measured (Fig. 6.5). The concentration of the α -tripiperidine measured in all the samples were lowest and not significantly different. The concentration of isotripiperidine slightly decreased when the pH was adjusted from pH 7.5 to pH 12. Adjusting the pH to 1 resulted

in a 77% increase in the concentration of Δ^1 -piperidine, with a concomitant increase in total yield of products measured.

It is possible that these changes were due to solubilisation of products as the pH was changed. The concentration of α -tripiperidine was expected to be highest at pH 12 since it has been reported to be predominant at pH 12 and above (Rauchaud and Braekman, 2009), but these results indicated the contrary. This result supports previous reports in the literature that Δ^1 -piperidine formation is favoured in acidic medium (Monaco et al., 2011). Similar to the earlier result, the concentration of the residual cadaverine when added to the concentration of the products does not account for the material balance (concentration of initial cadaverine), suggesting that the products might have precipitated. However, precipitates were not observed visually during the experiments.

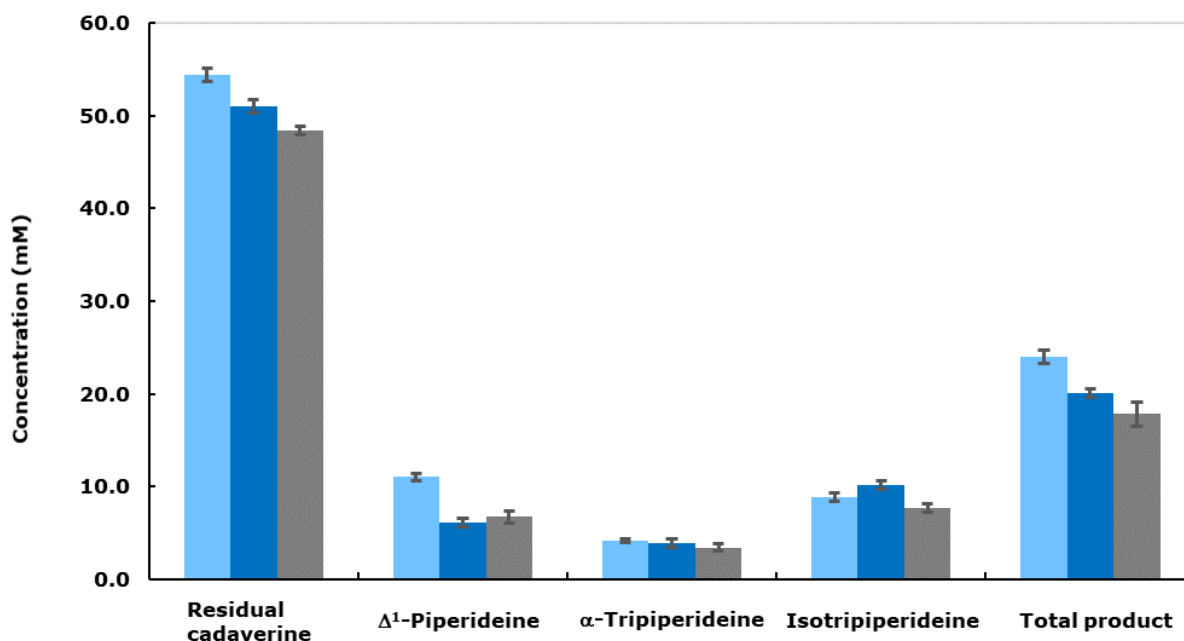


Fig. 6.5: Effect of pH on the speciation of Δ^1 -piperidine (product of PuO_{Rh} oxidation of cadaverine).

Cadaverine (91 mM) oxidation by purified PuO_{Rh} (1.0 mg/mL) was carried out in Na-K phosphate buffer (100 mM; pH 7.5) and reaction mix was incubated (25 °C, 150 rpm, and 92 h). Thereafter, the reaction mixture was divided into three equal portions. One portion was analysed by qNMR at pH 7.5, while the other portions were first adjusted to pH 1 and 12 using concentrated hydrochloric acid and 6 M potassium hydroxide respectively before analysis. The qNMR analysis was as reported in Fig. 6.4. The product concentrations are shown, together with the total of products (sum of the concentrations of Δ^1 -piperidine, α -tripiperidine and isotripiperidine calculated). ■ pH 1, ■ pH 7.5, and ■ pH 12. Values represent mean of triplicate experiments $\pm$ standard deviation.

6.4 Recovery of product by solvent extraction

Preliminary analysis of samples extracted with chloroform indicated that the reaction products (Δ^1 -piperidine and its multimers) were recovered in chloroform. Therefore, the effectiveness of liquid-liquid extraction for the selective recovery of the products from the aqueous reaction mixture with different organic solvents was studied using qNMR.

Product recovery from PuO_{Rh} bioconversion of cadaverine was analysed after extraction with CDCl_3 from the reaction mixture. Analysis of the deuterated aqueous reaction mixture gave a total product yield of 14% while the chloroform extract of the reaction mixture analysed in deuterated chloroform (CDCl_3) resulted to a fourfold (58%) total product recovery (Fig. 6.6). This indicated products are soluble in chloroform and were possibly forming microcrystals in the aqueous medium. Supportively, chloroform has been reported for extraction of naturally occurring complex *N*-heterocycles (e.g. alkaloids) (El Jaber-Vazdekis et al., 2006).

Further extraction or recovery of the reaction products was attempted using diethyl ether. Δ^1 -Piperidine and its multimers could not be detected from the diethyl ether extract (layer) analysed. Extraction with ethyl acetate yielded Δ^1 -piperidine, α -tripiperidine and isotripiperidine up to a total product yield of 51 % (Fig. 6.6). Again, this indicates approximately threefold increase in the total product recovered when compared to the yield observed in aqueous sample analysed in deuterium oxide (D_2O). *n*-Hexane is a non-polar solvent that is widely used for oil extraction because of its low boiling point (63 – 69 °C) and excellent solubilizing ability (Kumar et al., 2017). Extraction of the reaction products using *n*-hexane gave a total product recovery of 12%, which is lower compared to the 14% total product yield from aqueous samples (Fig. 6.6). Hexane, therefore, showed poor solubilisation of Δ^1 -piperidine.

Overall, Δ^1 -piperidine, α -tripiperidine and isotripiperidine were extracted in chloroform, and ethyl acetate, with α -tripiperidine having the highest recovery in all the extractants reported here (Fig. 6.6). Comparatively, the best total product recovery was recorded with

chloroform. However, when the aqueous sample was dried under rotary evaporator and the products resuspended and analysed in deuterated chloroform, a total product recovery of 67% was observed, which was 9% higher than the recovery in CDCl_3 (Fig. 6.6). This could indicate that the products were precipitating out of the aqueous reaction mixture and were very soluble in chloroform. Considering the energy intensiveness and time required to evaporate water from aqueous reaction mixture, and the possibility of thermal decomposition of the products, chloroform extraction seem the best practice for the recovery of Δ^1 -piperidine, α -tripiperidine and isotripiperidine from the reaction mixture, and presents the feasibility of *in situ* extraction. However, the economic worth of the extraction processes can only be truly established after a techno-economic analysis (TEA) and life-cycle analysis (LCA), which are outside the scope of this work.

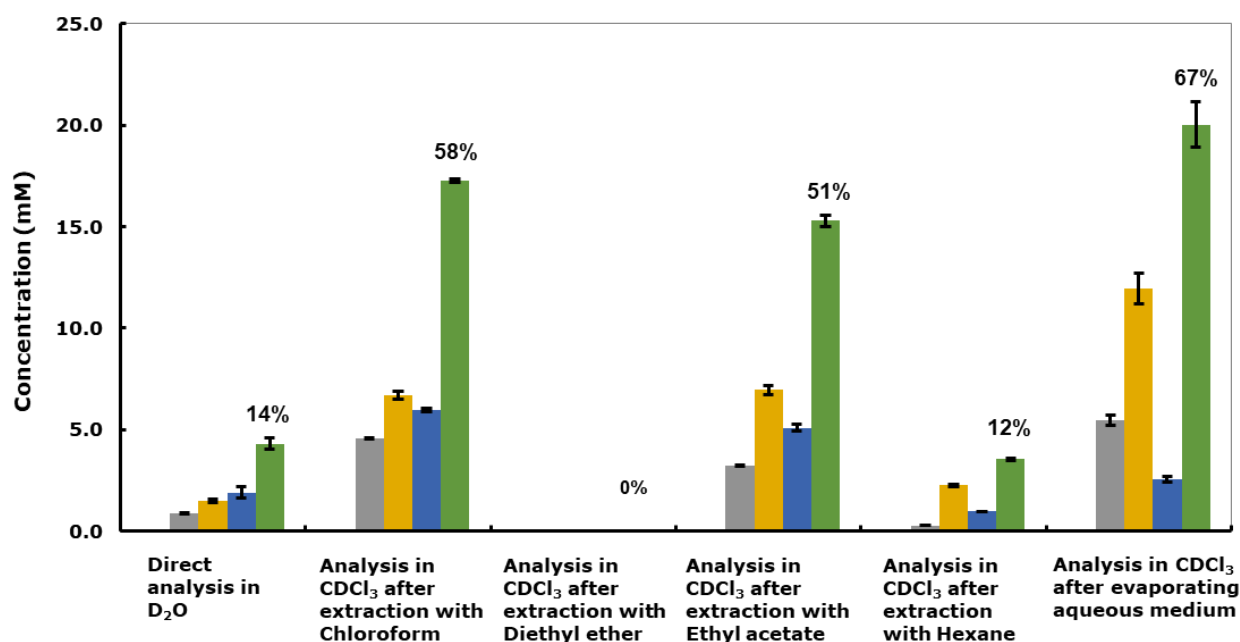


Fig. 6.6: PuO_{Rh} catalysed oxidation of cadaverine before and after solvent extraction.

Reaction mixture containing 30 mM cadaverine and 0.4 mg/mL purified PuO_{Rh} in 100 mM Na-K phosphate buffer pH 7.5 were prepared and incubated (25 °C, 43 h, 150 rpm). About 4.5 mL was extracted with chloroform (repeated three times and the chloroform pooled). The chloroform was dried using Na_2SO_4 before complete evaporation in a rotary evaporator. Products were resuspended in 4.5 mL deuterated chloroform and subsequently analysed by qNMR using 3,4,5-trichloropyridine as internal standard. Deuterated samples were prepared and analysed as earlier reported. $\square$ Δ^1 -piperidine, $\square$ α -tripiperidine, $\square$ isotripiperidine and $\square$ total product (sum of the concentrations of Δ^1 -piperidine, α -tripiperidine and isotripiperidine calculated). Values represent total product yield (calculated as a percentage of initial cadaverine concentration used). Data points represent mean of triplicate experiments $\pm$ standard deviation.

6.5 Conclusions and Discussion

The suitability of PuO_{Rh} for cell-free bioconversion of cadaverine to Δ^1 -piperidine was studied. Purified PuO_{Rh} reactions mix was analysed qualitatively by ESI – MS, LC-MS and ¹H NMR, while quantitative ¹H NMR was used to quantify the reaction products. The formation of Δ^1 -piperidine was demonstrated directly for the first time. However, Δ^1 -piperidine reacts spontaneously in aqueous solution to form the multimeric products (see Figure 2.6). α -Tripiperidine and isotripiperidine were found in all the samples. Product concentrations increased over 24 h with maximum total product yield of 43% (Fig. 6.4). Only 60% and 49% of starting material after 24 h and 40 h respectively could be accounted for. Therefore, PuO_{Rh} was confirmed suitable for biotransformation of cadaverine to Δ^1 -piperidine.

Also, studies on the effect of pH on the speciation of Δ^1 -piperidine showed that it is stable in the monomeric form (Δ^1 -piperidine) at acidic pH, while the trimeric form, isotripiperidine is favoured at neutral to slightly alkaline pH. In terms of the dominant species at different pH, these results are in agreement with previous reports (Rouchaud and Braekman, 2009, Poupon et al., 2011). In contrast to these published reports, all the species (Δ^1 -piperidine and its trimeric form) were found at all the pH analysed. However, a limitation of the reported results in this study was the short incubation time between pH adjustment and sample analysis. Although a time course study on the effect of pH adjustment would have given more insights, these experiments were not conducted due to time pressure.

Generally, for a bioproduction setup to be economically attractive, it is imperative to develop efficient downstream process for the desired product/molecule (Sheldon and Woodley, 2018). Liquid-liquid extraction using organic solvents was identified as a potential method for the recovery of Δ^1 -piperidine and its multimeric species from aqueous solution (Rosinha Grundtvig et al., 2018). Experiments to confirm the efficiency of extraction using chloroform, diethyl ether, ethyl acetate and hexane were conducted.

The extraction experiments revealed that Δ^1 -piperidine and its multimeric species were lost in the aqueous solution because total product recovery after extraction in chloroform (58%) was fourfold higher than the yield when directly analysed in aqueous solution (14%). This could be as a result of product precipitation or products were lost during extraction processes. Similar loss of products in liquid-liquid extraction have been previously reported (Cai et al., 2007, Rosinha Grundtvig et al., 2018). However, further scaled experiments and process optimization are needed to properly elucidate how the products are lost.

Again, Δ^1 -piperidine and its multimeric species could not be extracted in diethyl ether while hexane extracted a total product below the directly analysed aqueous samples. Ethyl acetate recorded a reasonable yield of 51%. The result of the extraction using diethyl ether is in contrast to the report of Orejarena Pacheco et al. (2016), though their report was in a chemical synthesis of Δ^1 -piperidine. Although complete evaporation of aqueous samples and subsequent analysis after resuspension in deuterated chloroform gave 67% recovery, it is also not recommendable because of high-energy requirement and possibility of thermal decomposition of the products. Nonetheless, the reported temperature does not affect the equilibrium between Δ^1 -piperidine and its multimeric species (Shvekhgeimer, 1998), and the thermal stability or shelf life of Δ^1 -piperidine is yet to be reported. Therefore, chloroform was identified as suitable extractant. Hence, recovery of Δ^1 -piperidine and its multimeric species from aqueous medium can be efficiently conducted through liquid-liquid extraction using chloroform. However, in terms of cost and sustainability, recommendations cannot be made as a lifecycle analysis (da Silva et al., 2012) and techno-economic analysis (Barker et al., 2011) are required.

Finally, based on published literatures, this is the first report on bioconversion of Δ^1 -piperidine from cadaverine using PuO_{Rh} and its recovery from aqueous medium using liquid-liquid extraction. Further experiments therefore will focus on bioproduction of Δ^1 -piperidine using PuO_{Rh} induced whole cells.

7. Results and Discussion: Bioconversion of cadaverine using whole cell containing PuO_{Rh}

7.1 Introduction

Whole cell bioconversion has been widely reported for the sustainable production of valuable compounds with a wide range of application in pharmaceutical and chemical industries (Lin and Tao, 2017, Heyland et al., 2010, Ke et al., 2016, Dong et al., 2017). Amongst others, whole cell bioconversion is cost effective because it bypasses the need for cell lysis and enzyme purification, and the concerns of substrate/product toxicity (de Carvalho, 2017, Lin and Tao, 2017).

Therefore, this result section focused on the design of an effective whole cell biocatalyst with putrescine oxidase (PuO_{Rh}) for bioconversion cadaverine to Δ^1 -piperidine. Also, attempts were made to optimise the downstream process through liquid-liquid extraction.

7.2 Development of the whole-cell cadaverine oxidation

7.2.1 Cadaverine consumption and product detection in a whole cell biotransformation

To investigate the activity of PuO_{Rh} *in vivo*, harvested whole cells expressing PuO_{Rh} were used for biotransformation of cadaverine. Cadaverine consumption was monitored by HPLC analysis and results with PuO_{Rh} induced resting cells showed active and complete consumption of cadaverine after 19 h of incubation (Fig. 7.1). Control set-up with PuO_{Rh} uninduced cells showed slight biotransformation of cadaverine while control experiments with cells containing empty plasmids showed neither cadaverine uptake nor biotransformation. Similarly, cadaverine was not detected in control set-ups without cadaverine.

This result indicates active uptake of cadaverine by resting cells, suggesting an efficient transport system for cadaverine in *E. coli*. Interestingly, a putative cadaverine transport protein CadB that had both cadaverine uptake activity at neutral pH (dependent on proton

motive force) and cadaverine excretion activity (acting as a cadaverine-lysine antiporter) has been reported in *E. coli* (Soksawatmaekhin et al., 2004). Control experiments indicated leaky expression of PuO_{Rh} in the uninduced set-up and this is supported because the control with cells containing empty plasmids (lacking *puORh* gene) did not consume cadaverine throughout the observed duration. Also, this indicates that the observed cadaverine consumption with cells expressing PuO_{Rh} is not because of the presence of other active enzymes (e.g. putrescine aminotransferase) which can utilize cadaverine in the cell (Samsonova et al., 2003). Control results indicate that cadaverine is not constitutively produced by *E. coli* and the presence of PuO_{Rh} does not induce its production.

However, this brings to question the functionality of the reported CadB transport protein in *E. coli*. It could be suggested that the transport protein is activated by the presence of a cadaverine-degrading enzyme or there is an unreported cadaverine uptake system in *E. coli* since the control results showed that it cannot be attributed to diffusion. Observed anomaly in the concentration of cadaverine, can be attributed to pipetting error due to heterogeneous mixing of the substrate or volume of sample used, and cell surface adsorption in the system. In conclusion, PuO_{Rh} can consume cadaverine *in vivo*, however, to produce Δ^1 -piperidine from sugar-based feedstock, an upstream pathway regulation to overproduce cadaverine is required.

Furthermore, samples collected from harvested whole cell biotransformation of cadaverine after 24 h, were analysed at pH 7.5 in deuterium oxide (D₂O) by ¹H NMR. Result from experimental set-up containing PuO_{Rh} induced resting cells and cadaverine, indicated complete consumption of cadaverine after 24 h. In addition, the spectra showed chemical shifts corresponding to Δ^1 -piperidine, α -tripiperidine and isotripiperidine (Fig. 7.2).

Control experiments containing only PuO_{Rh} induced cells or cells with empty plasmid (without *puORh* gene) confirmed that the organism does not synthesize cadaverine while control set-up containing cadaverine and cells with empty plasmid confirmed that the cells were not expressing other enzymes that could metabolise cadaverine and Δ^1 -piperidine.

This is in agreement with previously published reports (Hafner et al., 1979, Qian et al., 2010). Thus, these validate the result of the positive experiment as a function of the PuO_{Rh} enzyme. It also confirmed that the presence of PuO_{Rh} does not activate cadaverine synthesis in *E. coli*; therefore, a functional metabolic pathway needs to be engineered into *E. coli* in order to synthesize Δ^1 -piperidine from renewable feedstock. Although enzymes (e.g. lysine decarboxylase and putrescine aminotransferase) which metabolise cadaverine have been reported in *E. coli*, their induction systems are not well documented (Zabriskie and Jackson, 2000, Tomar et al., 2013, Kurihara et al., 2008).

According to Lin and Tao (2017), one of the advantages of whole cell biocatalysis is that it is efficient and provides fewer environmental problems by minimizing side reactions which generate unwanted by-products and reduces cost of downstream processing. The result reported here, showed that low amount of by-products was generated and this indicates an increased ease for product separation with minimal waste or contaminant generation.

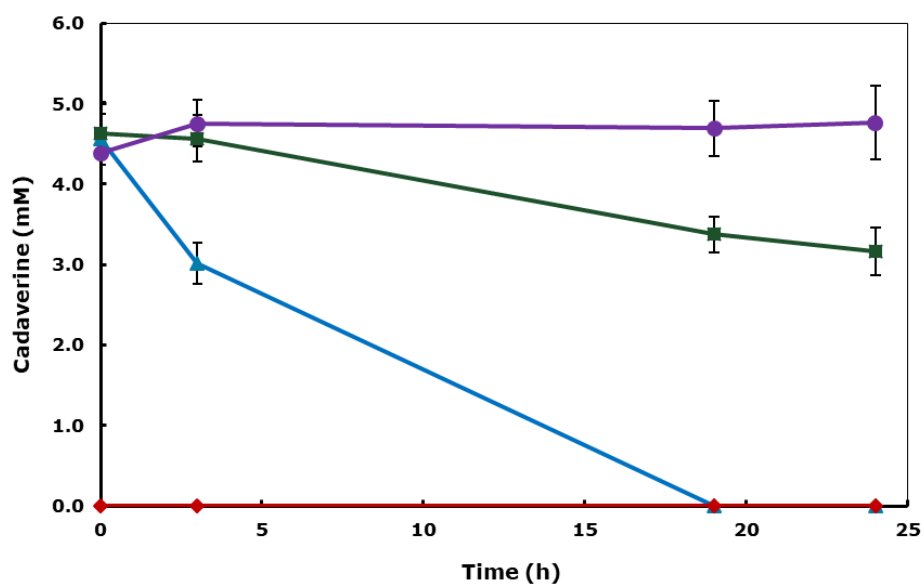


Fig. 7.1: Cadaverine consumption in a whole cell biotransformation

E. coli BL21(DE3) pET20b-*puo_{Rh}* and *E. coli* BL21(DE3) pET20b (control with empty plasmid) were grown on LB containing carbenicillin at 25 °C and 250 rpm. IPTG (0.4 mM) was added when the OD₆₀₀ reached 0.6 and cells were harvested by centrifugation (6000 rpm, 10 min, 4 °C). The cell pellets were repeatedly washed three times by resuspension in Na-K phosphate buffer (100 mM; pH 7.5) and centrifuged as above. Reaction mixtures containing washed harvested cells (DCW = 1.0±0.1 mg/mL, WCW = 6.8±0.7 mg/mL) and cadaverine (5 mM) in Na-K phosphate buffer were incubated as above. Samples were collected at intervals and centrifuged. The supernatants were filtered (0.22 µm filter) and derivatized using dansyl chloride, and analysed by HPLC. Cadaverine concentration was extrapolated from calibration curve with known concentrations. Different set-ups were used: ▲ induced cells + 5 mM cadaverine, ■ uninduced cells + 5 mM cadaverine, ● cells with empty pET20b + 5 mM cadaverine, and ◆ induced cells without cadaverine. Data points represent mean of triplicate experiments ± standard deviation.

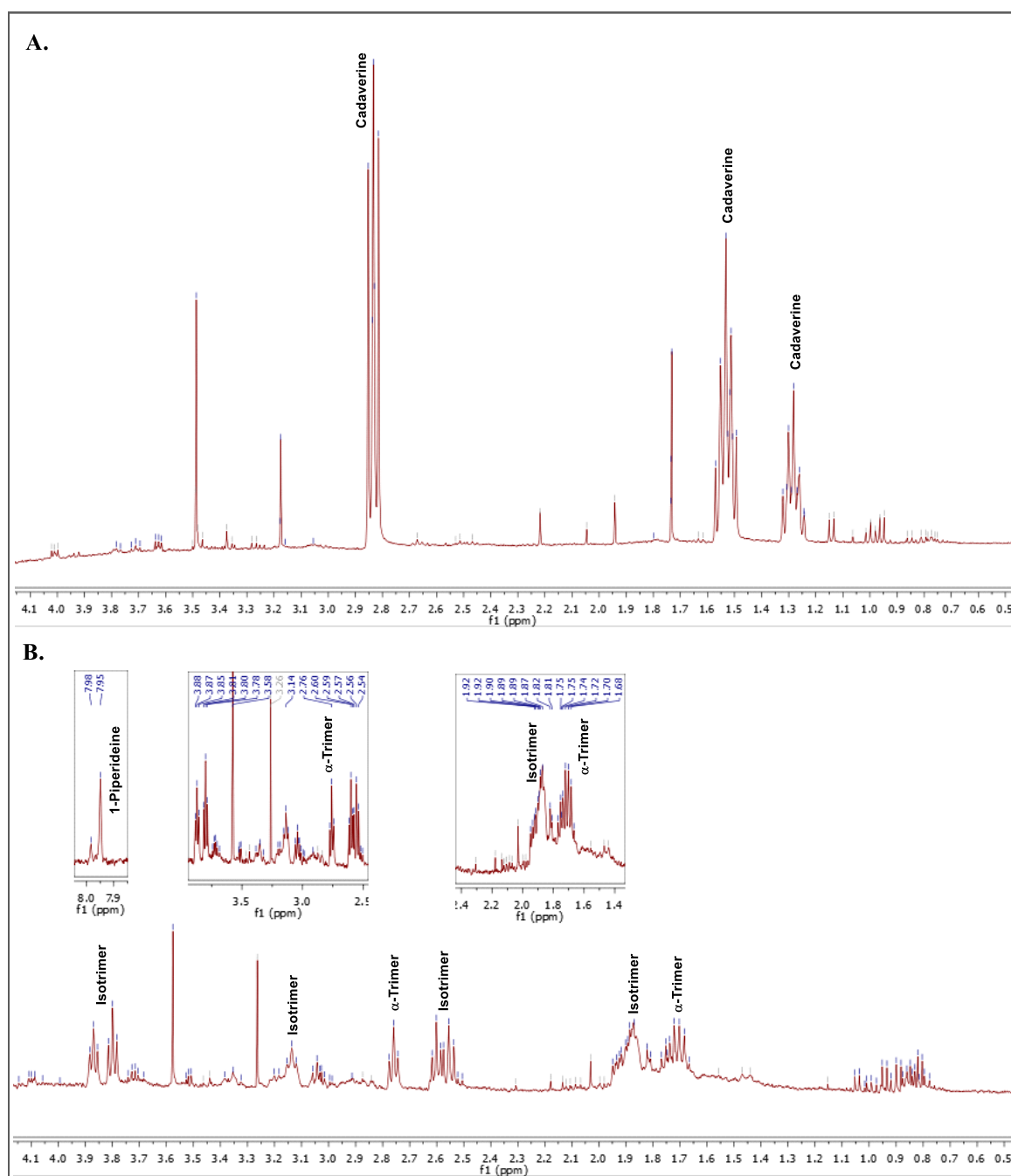


Fig. 7.2: ^1H NMR (400 MHz) δ (D_2O) of PuORh induced resting whole cell biotransformation of cadaverine after 0 h (**A**) and 24 h (**B**).

Whole cell biotransformation of cadaverine using harvested PuORh induced cells and sample preparation are as reported in Fig. 7.1 without derivatization. Thereafter, aqueous samples at pH 7.5 were diluted with 99.9% Deuterium oxide (H_2O :90% / D_2O :10%). 500 μL was transferred into NMR tubes and sent for ^1H NMR analysis at ambient temperature using a 400-MHz Bruker AV3400 spectrometer, and run using automated presaturation solvent suppression to reduce the intensity of the water signal.

7.2.2 Time-course analysis on whole cell biotransformation of Cadaverine

Previously, the consumption of cadaverine in a PuO_{Rh} induced whole cell biotransformation showed complete consumption after 19 h and ¹H NMR analysis revealed the presence of Δ^1 -piperidine and its trimers in the reaction samples. Therefore, qNMR analyses of samples collected at intervals (up to 32 h) from a PuO_{Rh} induced whole cell biotransformation (pH 7.5) were conducted. This is important to determine the yield of the desired products (Δ^1 -piperidine and its multimeric forms), understand whether the products are further consumed and the effect of cellular (membrane) barriers on product transport into the reaction medium.

Results validated complete consumption of cadaverine between 14 – 24 h and, Δ^1 -piperidine, α -tripiperidine and isotripiperidine were observed in good yields in all the samples. The concentration of isotripiperidine increased consistently over time while that of Δ^1 -piperidine remained almost constant all through the duration of analysis. Total product yield of 63 % after 24 h incubation was recorded with the isotripiperidine giving the highest concentration (Fig. 7.3). Although after 32 h, 30 % of the carbon substrate could not be accounted for, a total product yield/recovery of 70 % shows successful biotransformation using harvested whole cells. It could be possible that the products partitioned into the cell cytoplasm or were trapped in the cell debris during centrifugation. Therefore, extraction of the cells is recommended in further experiments.

Since these analyses were conducted using reaction medium and without lysing the cells, it suggests that Δ^1 -piperidine and its trimeric forms were exported from the *E. coli* strain. However, the transport mechanism or associated proteins are not currently known. Also, the carbon (substrate) loss could be attributed to product partitioning into cell cytoplasm. Observed cadaverine transformation recorded at 0 h was due to delay in processing and analysis of samples. This result represent a higher product yield and recovery efficiency compared to that observed in the purified PuO_{Rh} catalysis (see Fig. 6.4). Whereas a higher concentration of cadaverine was used in the purified PuO_{Rh} catalysis, the whole cell

biotransformation has shown to be efficient. This is highly desirable as a sustainable approach to bio-produce Δ^1 -piperidine, because the cost and time for purification of enzyme are conserved.

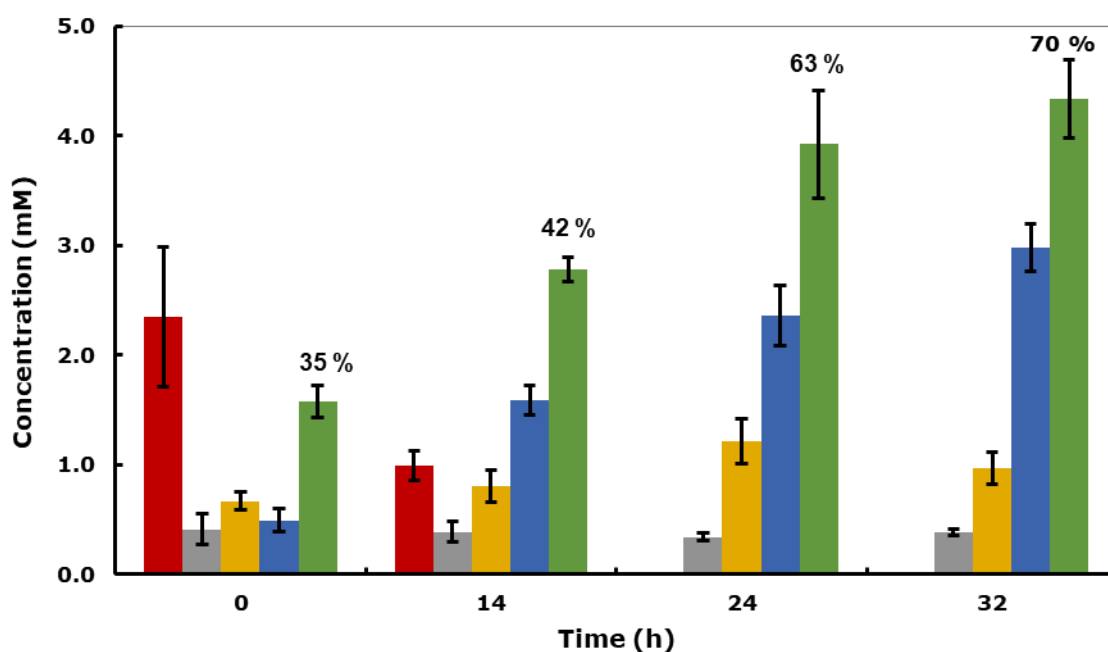


Fig. 7.3: Whole cell biotransformation of cadaverine and product analysis

E. coli BL21(DE3) pET20b-*puo_{Rh}* and *E. coli* BL21(DE3) pET20b (cell with empty plasmid) were grown and samples prepared as reported in Fig. 7.1, but using 6.19 mM cadaverine. Samples were further prepared for qNMR analysis as reported in Fig. 6.4. Thereafter, aqueous deuterated samples (H_2O/D_2O ratio of 90/10%) at pH 7.5 were prepared using Maleic acid (Standard for qNMR TraceCERT®) as external standard in a stem co-axial insert. Spectra of deuterated samples were obtained at ambient temperature using a 400-MHz Bruker AV3400 spectrometer, and run using automated presaturation solvent suppression to reduce the intensity of the water signal. Data represent mean of triplicate experiments $\pm$ standard deviation: ■ Residual cadaverine, ■ Δ^1 -Piperidine, ■ α -Tripiperidine, ■ Isotripiperidine and ■ Total Product (sum of the concentrations of Δ^1 -piperidine, α -tripiperidine and isotripiperidine calculated). Shown values represent total product yield (calculated as a percentage of initial cadaverine concentration used).

The cadaverine consumption studies indicated complete cadaverine assimilation. As earlier suggested, it indicated the presence of a transport mechanism for cadaverine uptake and product release into the medium. However, it is possible the cells lysed during incubation, thereby released the enzyme to oxidize cadaverine in the reaction mixture. Therefore, to gain an insight to how the substrate and products were transported across the cell, samples collected at interval were observed under the microscope. Pictures showed normal rod-shaped *E. coli* cells with a good cell density visually observed at the start of the

experiment (Fig. 7.4). The density of the cells decreased over time and after 32 h, aggregated lysed cells were observed.

These results indicate that the enzyme could have been released into the reaction medium, thus, contradicts earlier suggestion of substrate uptake and product export mechanism. However, this is unclear because products were observed at the beginning of the experiments. Also, the cell lysis could not be attributed to cadaverine toxicity because the concentration (6.19 mM) used in this experiment, was far lower than the reported tolerance limit of up to 200 mM using *E. coli* K12 strain (Qian et al., 2010). It is possible cell lysis was due to starvation. Further study is required to understand the exact cause of cell lysis, and the mechanism of substrate uptake and product export.

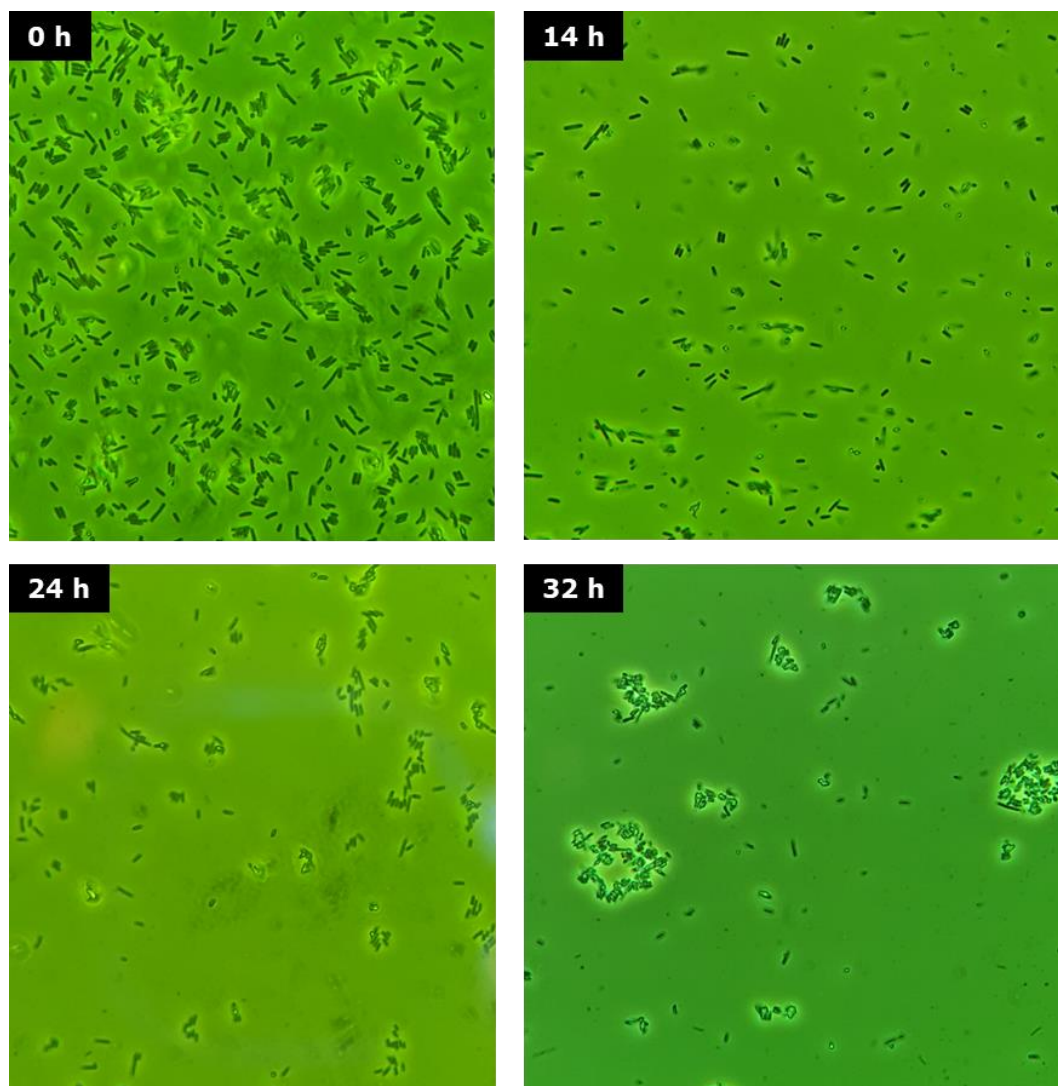


Fig. 7.4: Microscopic image of harvested whole cells during biotransformation of cadaverine

Whole cell biotransformation using harvested *E. coli* BL21(DE3) pET20b-*puORh* was as reported in Fig. 7.3. Samples were collected at intervals and 10 μ L was mounted on a grease-free glass slide, covered with a glass slip and observed under a microscope (Olympus CX41).

7.3 Recovery of product by solvent extraction: chloroform

Previously, product recovery experiments by solvent extraction from purified enzyme bioconversion of cadaverine, identified chloroform as the best solvent amongst others used. In addition, pH-dependent speciation of Δ^1 -piperidine was demonstrated. Therefore, to determine the effect of pH on the recovery of products by solvent extraction, harvested whole cells were used for bioconversion of cadaverine. Subsequently, the pH of the reaction mixtures was adjusted, and after product extraction using chloroform, samples were analysed in CDCl_3 by qNMR.

Results (Fig. 7.5) indicates Δ^1 -piperidine, α -tripiperidine and isotripiperidine were presents at all the pH analysed. Total product recovery at pH 7.5 increased approximately two-fold compared to the amount detected in the aqueous sample. At pH 7.5, extraction in chloroform significantly increased the recovery of Δ^1 -piperidine and α -tripiperidine. Adjustment of pH to 1 and 12 dramatically increased the amount of total product recovered by 47% and 46% respectively. The amounts of α -tripiperidine and isotripiperidine recovered at pH 12 were slightly higher compared to recovery at pH 1. On the contrary, Δ^1 -piperidine recovery was favoured at pH 1.

Results on the recovery of Δ^1 -piperidine at pH 1 and those of α -tripiperidine at pH 12 are in agreement with previous reports (Monaco et al., 2011, Rouchaud and Braekman, 2009), however, it was expected that the recovery of isotripiperidine would have been lower compared to α -tripiperidine at pH 12. In addition, it is not clear why the recovered concentrations of α -tripiperidine and isotripiperidine at pH 1 were higher than the concentration of Δ^1 -piperidine. This contradicts published previous reports (Monaco et al., 2011), as is expected to be the dominant species at pH 1. Again, the recovery yields indicate that the products are probably lost in aqueous solution. However, as in previous experiments, visible precipitates were not observed. Further scaled studies are needed to confirm if the products precipitated out in aqueous solution.

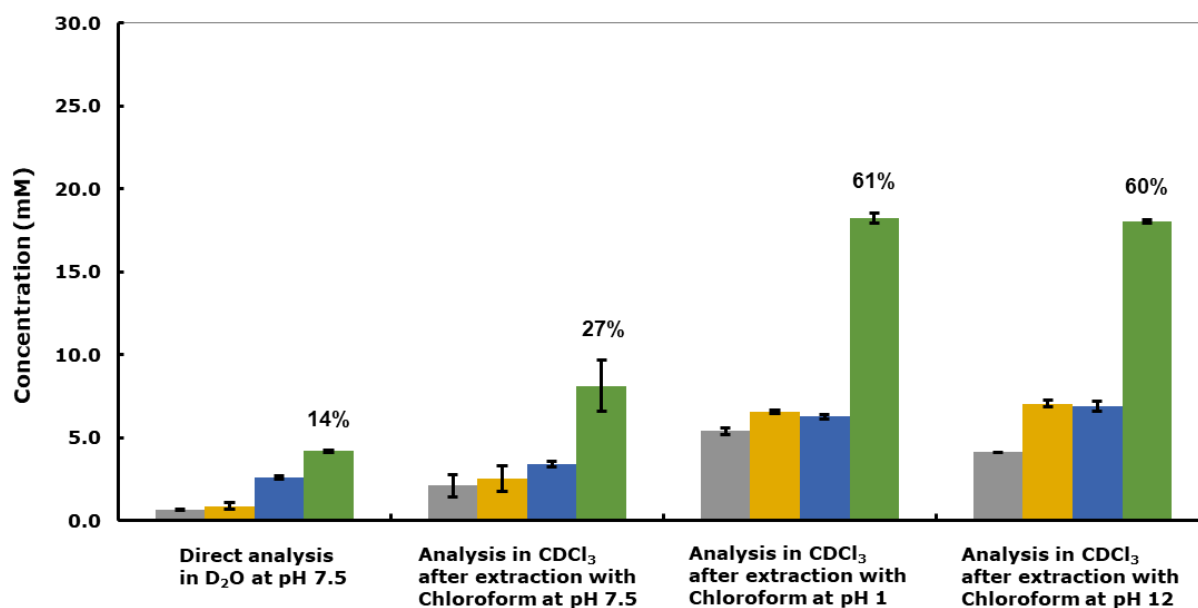


Fig. 7.5: Whole cell biotransformation of cadaverine: effect of pH on the recovery of products by chloroform extraction.

E. coli BL21(DE3) pET20b-*puORh* culture was grown on LB containing carbenicillin at 25 °C and 250 rpm. IPTG (0.4 mM) was added when the OD₆₀₀ reached 0.6 and cells were harvested by centrifugation (6000 rpm, 10 min, 4 °C). The cell pellets were repeatedly washed three times by resuspension in Na-K phosphate buffer (100 mM; pH 7.5) and centrifuged as above. Reaction mixtures containing washed harvested cells (DCW = 1.0±0.2 mg/mL, WCW = 7.0±0.4 mg/mL) and cadaverine (30 mM) in Na-K phosphate buffer were incubated (250 rpm, 46 h, 25 °C). Subsequently, reaction mixtures collected and centrifuged. pH of Supernatants were adjusted to pH 1 and 12 using concentrated hydrochloric acid and 6 M potassium hydroxide respectively, before extraction with equal volume of chloroform (repeated three times and the chloroform pooled). The chloroform was dried using Na₂SO₄ before complete evaporation in a rotary evaporator. Also, reaction mixture at pH 7.5 were extracted as above. Products were resuspended in deuterated chloroform and subsequently analysed by qNMR using 3,4,5-trichloropyridine as internal standard. Deuterated samples at pH 7.5 were prepared and analysed as earlier reported. Δ^1 -piperidine, α -tripiperidine, isotripiperidine and total product (sum of the concentrations of Δ^1 -piperidine, α -tripiperidine and isotripiperidine calculated). Values represent total product yield (calculated as a percentage of initial cadaverine concentration used). Data points represent mean of triplicate experiments ± standard deviation.

7.4 Conclusions and Discussion

Putrescine oxidase from *R. erythropolis* (PuOR_h) has been demonstrated to oxidize cadaverine to Δ^1 -piperidine *in vivo* and *in vitro*. In addition, the reaction products were released into the reaction medium and that makes product recovery an easy task. Comparatively, a higher total product yield was observed in the whole cell bioconversion than the purified PuOR_h bioconversion. In terms of product speciation in both whole cell and purified PuOR_h bioconversion, the concentration of isotripiperidine consistently increased all through the duration of analysis, α -tripiperidine increased with a maximum concentration recorded at 24 h but it decreased thereafter between 32 – 40 h. These

results are consistent in purified PuO_{Rh} and whole cell systems, and since the isotripiperideine is the reported predominant species at pH 7.5, the results are in agreement with published literatures (Gravel et al., 2006, Poupon et al., 2011).

Different concentrations of cadaverine up to 30 mM were used in the *in vivo* studies, but this does not limit the reported results because the results are comparative when scaled or normalised, and these concentrations are not inhibitory to the activity of PuO_{Rh}. Nevertheless, further experiments using a consistent higher concentration (up to 100 mM) are recommended.

Although microscopic examination of cultures indicated cells were lysed over time, whole cell biotransformation of cadaverine presents a cheaper approach to bio-based production of Δ^1 -piperidene, because the cost and time for purification of enzyme are eliminated. This result indicated that the toxicity of Δ^1 -piperideine needs to be determined, again, this experiment was not conducted because Δ^1 -piperideine was commercially unavailable at the time of this study. On the other hand, the purified enzyme system generates less waste and presents the opportunity of enzyme recycling when immobilized, and integration of *in situ* product extraction system.

According to Rosinha Grundtvig et al. (2018), the choice of a solvent for a bioprocess should comprise a balance between the effects on the environment, effects on human health, safety hazards, biocatalyst/microorganism activity, solubility and selectivity of substrates and/or products and recovery. This balance is important because it is not always possible to find a solvent that fully covers all these criteria (Jessop, 2011, Rosinha Grundtvig et al., 2018). Although, the solvents reported in this study have been similarly used in bioprocesses (Doukyu and Ogino, 2010, Devi et al., 2017), they do not represent the best sustainable (green) choices (Prat et al., 2016). Therefore, greener solvents for bioprocesses including ionic liquids, deep eutectic solvents and supercritical carbon dioxide (Lou et al., 2005, Wimmer and Zarevúcka, 2010, Jessop, 2011) could be explored to further this study.

Finally, these results indicate that metabolic engineering and synthetic biology approaches using PuO_{Rh} could be rationally applied to design and construct a biosynthetic pathway for production of Δ^1 -piperidiene from renewable feedstocks.

8. OVERALL DISCUSSION

The suitability of putrescine oxidase from *Rhodococcus erythropolis* (PuO_{Rh}) for cell-free and whole-cell bioproduction of Δ^1 -piperidine were studied. PuO_{Rh} was characterised for the first time by monitoring oxygen concentration in a direct substrate consumption assay. These results indicated that PuO_{Rh} was not inhibited by cadaverine and are valuable in further studies.

Unsuccessful attempts were made to characterize PuO_{Rh} using *o*-aminobenzaldehyde (*o*-ABA) coupled colorimetric assay (Fig. 5.6, page 73). Although the assay was sensitive to detect enzyme activity and a preliminary qualitative indication of the product of the reaction, it was not possible to calculate the initial rate of the reaction. This was because the reaction showed an initial lag phase even after the reaction conditions were optimized. Further optimization studies are suggested to eliminate the initial lag phase. On an application point of view, this assay can be adapted as a quick qualitative assay in environmental pollution control and medical diagnostics.

Bioconversion of cadaverine using purified PuO_{Rh} and whole cells expressing PuO_{Rh} were also demonstrated qualitatively and quantitatively. The results represent a yield of the Δ^1 -piperidine and its trimeric species (α -tripiperidine and isotripiperidine), and the preliminary effect of pH on Δ^1 -piperidine speciation in aqueous medium have been reported. This study indicates that PuO_{Rh} is suitable for bioproduction of Δ^1 -piperidine. Whereas, all the species of Δ^1 -piperidine were present at all the pH analysed, pH adjustments could still be used to shift the product mixture to the specie of interest. Although the yield of the product is far below competition with chemical process or industrial demands, it represents an excellent proof of concept and can be optimized through protein engineering and process optimization or combination of both approaches. In addition, statistical analysis like design of experiments can be employed to optimize the reaction conditions (Villegas-Torres et al., 2018).

Amine oxidases have been well reported for their use in the synthesis of chiral amines (O'Reilly et al., 2014, Villegas-Torres et al., 2015). This is mostly in a one-pot or cascade reaction with imine reductases. PuO_{Rh} have not been reported in such a reaction. Thus, it will be ideal to explore the potentials of this enzyme for the purpose of imine synthesis. In addition, this will give the opportunity to explore the substrate range of PuO_{Rh} to make other heterocycles e.g. 2-methylpiperidine. Because of the multimeric products formed by Δ^1 -piperideine in solution (unstable), another aspect that could be explored is to further design reaction steps to enzymatically oxidize or reduce it to pyridine or piperidine respectively (Fig. 8.1). Although these compounds are not synthesized naturally and enzymes that catalyse their formation are not known, it is possible to identify enzymes that could accept Δ^1 -piperideine as a substrate for such reactions.

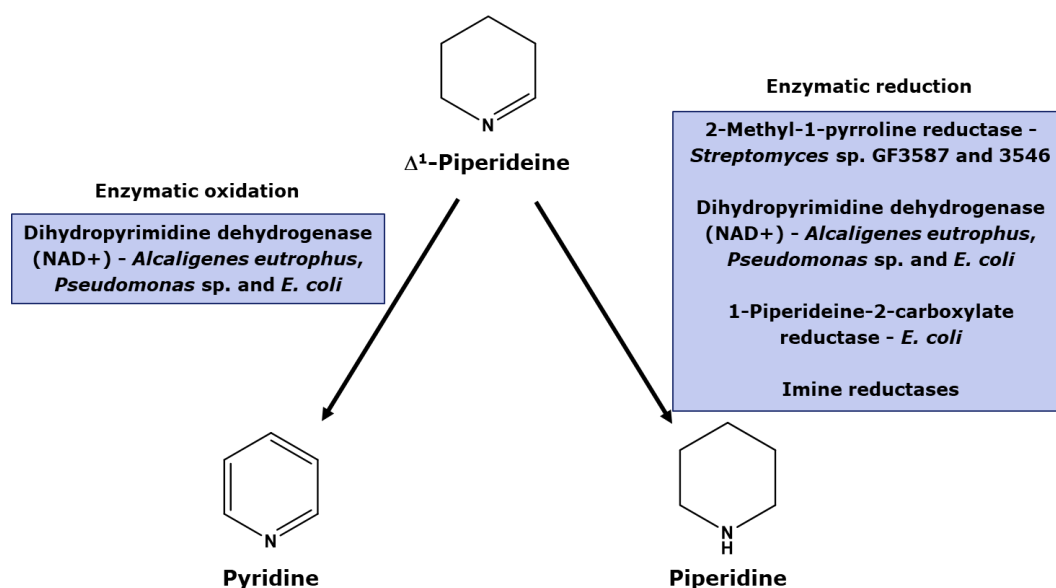


Fig. 8.1: Enzymatic oxidation and reduction of Δ^1 -piperideine

Possible enzymes that could catalyse the reactions are shown in blue boxes.

Reaction products were recovered by solvent (liquid – liquid) extraction and chloroform was identified as the best extractant. This result indicates that the reported biosynthetic approach can be integrated to a chemical/organic synthetic process. Also, depending on which species is required in the chemical synthesis, pH adjustment can be used to drive equilibrium to its formation. In addition, harvested whole cell biotransformation of

cadaverine resulted to the yield of Δ^1 -piperidine, α -tripiperidine and isotripiperidine, which were successfully extracted with chloroform at different pH range. Again, these results suggest that bioconversion of cadaverine in a biphasic system could be more appropriate, however, the inhibition and toxicity of the solvents on the enzyme and whole cells respectively needs to be established. To our knowledge at the time of this report, this work represents the first report on the bioproduction of Δ^1 -piperidine, a simple *N*-heterocycle using engineered *E. coli* strains overexpressing putrescine oxidase.

Due to global environmental deterioration and widely accepted campaign for sustainable strategies, production of chemicals, fuels and materials biologically using renewable feedstocks as a replacement to the conventional chemical synthesis using petrochemical-based feedstocks have grown. Currently, researches integrating bioprocesses and chemical synthesis have been on the rise (Zhao et al., 2018). Δ^1 -Piperidine is naturally an intermediate for synthesis of biologically active compounds and has the potential of been a substrate for the synthesis of novel functional compounds. Fortunately, these products are well extracted in organic solvents, thus, integrating our cell-free or whole cell biotransformation experiments will represent a sustainable approach to boycott the use of petrochemical feedstocks for organic synthesis. This research therefore, represent a step towards achieving a sustainable production strategy for Δ^1 -piperidine and its species.

Interestingly, recent researches aimed at synthesizing piperidine-type alkaloids and their analogues, tetraopenerines and its analogues, and complex polycyclic alkaloids have used Δ^1 -piperidine and its species as substrates, though synthesized chemically (Gravel et al., 2006, Rouchaud and Braekman, 2009, Monaco et al., 2011, Barker et al., 2011, Li et al., 2013, Unsworth et al., 2014, Unsworth et al., 2013, Salame et al., 2010, Orejarena Pacheco et al., 2016). Replacement of this phase of synthesis of these medically and pharmaceutically important compounds with our reported results/experiments represents a huge step towards sustainable production.

In conclusion, Δ^1 -piperidine exists in solution as a complex diastereoisomeric mixture of its trimeric (major component) and monomeric form. Nonetheless, the use of Δ^1 -piperidine is not limited because when it is consumed in a chemical reaction, equilibrium adjustment causes the dissociation of the trimeric forms (Rouchaud and Braekman, 2009, Orejarena Pacheco et al., 2016). The production of Δ^1 -piperidine using a bio-based process is not currently available. Therefore, results presented in this thesis gives a proof of concept for the sustainable production of Δ^1 -piperidine.

9. CONCLUSIONS AND FUTURE PERSPECTIVES

The primary aim of this study was to develop a sustainable bioprocess for simple *N*-heterocycles because they are valuable in the agrochemical and pharmaceutical industries. Therefore, early focus was to identify valuable simple *N*-heterocycles that could be produced easily by employing the advances of metabolic engineering as a proof of concept. These simple compounds occur naturally as subunits of plant and animal metabolites, predominantly alkaloids. Δ^1 -Piperideine, a six membered *N*-heterocycle derived from cadaverine was subsequently selected after literature analysis on the biosynthesis of lysine-derived alkaloids. From a chemistry perspective, it is a simple reaction involving deamination or transamination and a spontaneous Schiff base cyclization, while biologically; enzymes that could catalyse such reactions exist in nature (Fig. 2.13, page 33).

Amine oxidases (putrescine oxidase) and transaminases (putrescine aminotransferase) were considered as potential bacterial enzymes for conversion of cadaverine to Δ^1 -piperideine. However, because of cofactor requirement and higher carbon efficiency, putrescine oxidase was the enzyme of choice. Consequently, this study has successfully engineered an *E. coli* strain overexpressing putrescine oxidase from *R. erythropolis* (PuO_{Rh}) and characterized the enzyme by monitoring its oxygen consumption during cadaverine catalysis. The reported kinetic parameters are valuable in the design and optimization of biocatalytic processes using PuO_{Rh}. Protein engineering and computational modelling of enzymes have been used previously to optimize the catalytic efficiency of enzymes (Sheldon and Woodley, 2017). Thus, these kinetic data are useful for computational analyses to improve the activity of PuO_{Rh} on cadaverine. Because of the huge resource requirement, this aspect of the work was not pursued.

Bioconversion of cadaverine using purified PuO_{Rh} has also been carried out and the results represent a yield of the Δ^1 -piperideine and its trimeric forms (α -tripiperideine and isotripiperideine), and the preliminary effect of pH on Δ^1 -piperideine speciation in aqueous

medium have been reported. Reaction products were recovered by solvent (liquid – liquid) extraction and chloroform was identified as the best extractant. In addition, harvested whole cell biotransformation of cadaverine resulted to the yield of Δ^1 -piperidine, α -tripiperidine and isotripiperidine, which were successfully extracted with chloroform at different pH range. To our knowledge at the time of this report, this work represents the first report on the bioproduction of Δ^1 -piperidine, a simple *N*-heterocycle using engineered *E. coli* strains overexpressing putrescine oxidase.

A further research objective will focus on the design of a Δ^1 -piperidine pathway in *E. coli* for efficient conversion of cadaverine to this platform compound. Therefore, the potential of Δ^1 -piperidine bioproduction directly from a renewable feedstock, in particular xylose and glucose can be developed *via* the lysine/cadaverine pathway. Therefore, a new route leading to the overproduction of Δ^1 -piperidine *via* the cadaverine biosynthetic pathway was proposed (Fig. 9.1). Although this pathway was not demonstrated experimentally due to time pressure, its feasibility is almost 100%. This route and the enzymes involved have been studied, characterized and their genes sequenced. To achieve Δ^1 -piperidine overproduction, the pathway will be optimized, firstly, by overexpressing some key enzymes (see Appendix 11.4 & 11.5) and where necessary, by deletion or attenuation of reactions opposing flux to Δ^1 -piperidine. Interestingly, stoichiometric balance analysis of the proposed pathway showed a well-distributed mass balance with better cofactor regeneration and high carbon efficiency (see Appendix 11.8). Nonetheless, metabolic engineering tools are quite robust and its iterative cycle could be applied to increase the yield and productivity of the product of interest. Finally, metabolic modelling can be applied to this pathway at this stage to optimize the metabolic flux to Δ^1 -piperidine.

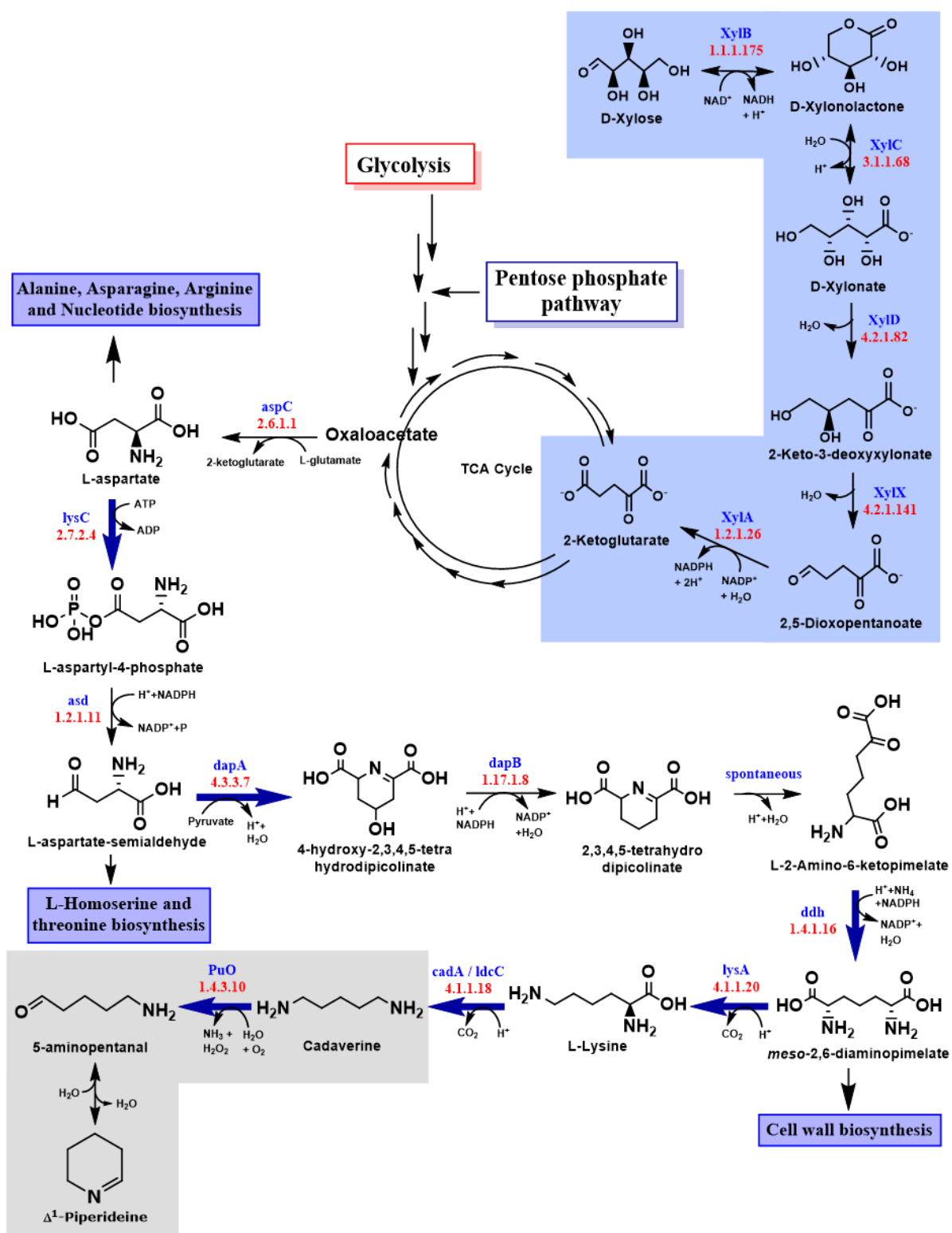


Fig. 9.1: Proposed pathway for xylose conversion to Δ^1 -piperidine.

Enzymes involved are as follows: 1.1.1.175 - xylose dehydrogenase, 3.1.1.68 - D-xylonolactonase, 4.2.1.82 - D-xylonate dehydrogenase, 4.2.1.141 - 2-keto-3-deoxyxylonate dehydratase, 1.2.1.26 - 2-ketoglutarate semialdehyde dehydrogenase, 2.6.1.1 - Aspartate transaminase, 2.7.2.4 - aspartate kinase, 1.2.1.11 - aspartate semialdehyde dehydrogenase, 4.3.3.7 - 4-hydroxy-tetrahydrodipicolinate synthase, 1.17.1.8 - 4-hydroxy-tetrahydrodipicolinate reductase, 1.4.1.16 - meso-diaminopimelate dehydrogenase, 4.1.1.20 - meso-diaminopimelate decarboxylase, 4.1.1.18 - lysine decarboxylase, 1.4.3.10 - Putrescine oxidase. **Bold blue arrows represent proposed steps for upregulation by enzyme overexpression.**

10. REFERENCES

- ABELL, C. W. & KWAN, S.-W. 2001. Molecular characterization of monoamine oxidases A and B. *Prog. Nucleic Acid Res. Mol. Biol.*, 65, 129-156.
- ADACHI, O., YAMADA, H. & OGATA, K. 1966. Purification and properties of putrescine oxidase of *Micrococcus rubens*. *Agric. Biol. Chem.*, 30, 1202-10.
- ADKINS, J., JORDAN, J. & NIELSEN, D. R. 2013. Engineering *Escherichia coli* for renewable production of the 5-carbon polyamide building-blocks 5-aminovalerate and glutarate. *Biotechnol. Bioeng.*, 110, 1726-1734.
- AMERICAN CHEMICAL SOCIETY, N. 2015. *Rachel Carson's Silent Spring* [Online]. Available: <http://www.acs.org/content/acs/en/education/whatischemistry/landmarks/rachel-carson-silent-spring.html> [Accessed 30th May 2015].
- ANASTAS, P. T. & BEACH, E. S. 2007. Green chemistry: the emergence of a transformative framework. *Green Chemistry Letters and Reviews*, 1, 9-24.
- ANISZEWSKI, T. 2007. *Alkaloids - Secrets of Life:: Alkaloid Chemistry, Biological Significance, Applications and Ecological Role*, Elsevier Science.
- ANNALURU, N., MULLER, H., MITCHELL, L. A., RAMALINGAM, S., STRACQUADANIO, G., RICHARDSON, S. M., DYMOND, J. S., KUANG, Z., SCHEIFELE, L. Z., COOPER, E. M., CAI, Y., ZELLER, K., AGMON, N., HAN, J. S., HADJITHOMAS, M., TULLMAN, J., CARAVELLI, K., CIRELLI, K., GUO, Z., LONDON, V., YELURU, A., MURUGAN, S., KANDAVELOU, K., AGIER, N., FISCHER, G., YANG, K., MARTIN, J. A., BILGEL, M., BOHUTSKI, P., BOULIER, K. M., CAPALDO, B. J., CHANG, J., CHAROEN, K., CHOI, W. J., DENG, P., DI CARLO, J. E., DOONG, J., DUNN, J., FEINBERG, J. I., FERNANDEZ, C., FLORIA, C. E., GLADOWSKI, D., HADIDI, P., ISHIZUKA, I., JABBARI, J., LAU, C. Y. L., LEE, P. A., LI, S., LIN, D., LINDER, M. E., LING, J., LIU, J., LIU, J., LONDON, M., MA, H., MAO, J., MCDADE, J. E., MCMILLAN, A., MOORE, A. M., OH, W. C., OUYANG, Y., PATEL, R., PAUL, M., PAULSEN, L. C., QIU, J., RHEE, A., RUBASHKIN, M. G., SOH, I. Y., SOTUYO, N. E., SRINIVAS, V., SUAREZ, A., WONG, A., WONG, R., XIE, W. R., XU, Y., YU, A. T., KOSZUL, R., BADER, J. S., BOEKE, J. D. & CHANDRASEGARAN, S. 2014. Total synthesis of a functional designer eukaryotic chromosome. *Science (Washington, DC, U. S.)*, 344, 55-58.

- ATSUMI, S., HANAI, T. & LIAO, J. C. 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature (London, U. K.)*, 451, 86-89.
- BACHRACH, U. 1962. Spermidine Oxidase from *Serratia marcescens*. *Journal of Biological Chemistry*, 237, 3443-3448.
- BAILEY, J. E. 1991. Toward a science of metabolic engineering. *Science*, 252, 1668-75.
- BARKER, G., MCGRATH, J. L., KLAPARS, A., STEAD, D., ZHOU, G., CAMPOS, K. R. & O'BRIEN, P. 2011. Enantioselective, Palladium-Catalyzed α -Arylation of N-Boc Pyrrolidine: In Situ React IR Spectroscopic Monitoring, Scope, and Synthetic Applications. *The Journal of Organic Chemistry*, 76, 5936-5953.
- BECKER, J. & WITTMANN, C. 2015. Advanced Biotechnology: Metabolically Engineered Cells for the Bio-Based Production of Chemicals and Fuels, Materials, and Health-Care Products. *Angewandte Chemie International Edition*, 54, 3328-3350.
- BELLELLI, A., MORPURGO, L., MONDOVI, B. & AGOSTINELLI, E. 2000. The oxidation and reduction reactions of bovine serum amine oxidase. A kinetic study. *Eur J Biochem*, 267, 3264-9.
- BENSON, D. A., CAVANAUGH, M., CLARK, K., KARSCH-MIZRACHI, I., LIPMAN, D. J., OSTELL, J. & SAYERS, E. W. 2013. GenBank. *Nucleic Acids Res*, 41, D36-42.
- BERG, J. M., TYMOCZKO, J. L. & STRYER, L. 2002. Free Energy Is a Useful Thermodynamic Function for Understanding Enzymes. In: BERG J.M., TYMOCZKO J.L. & L., S. (eds.) *Biochemistry*. 5th ed.: New York: W H Freeman.
- BEZBORODOV, A. M. & ZAGUSTINA, N. A. 2016. Enzymatic biocatalysis in chemical synthesis of pharmaceuticals (Review). *Applied Biochemistry and Microbiology*, 52, 237-249.
- BHARTI, S. K. & ROY, R. 2012. Quantitative ^1H NMR spectroscopy. *TrAC Trends in Analytical Chemistry*, 35, 5-26.
- BHUSAL, R. P. & SPERRY, J. 2016. Flexible synthesis of diverse N-heterocycles from substrates attainable from biomass. *Green Chemistry*, 18, 2453-2459.

- BOLOGNA, F. P., ANDREO, C. S. & DRINCOVICH, M. F. 2007. Escherichia coli Malic Enzymes: Two Isoforms with Substantial Differences in Kinetic Properties, Metabolic Regulation, and Structure. *Journal of Bacteriology*, 189, 5937-5946.
- BOTH, P., BUSCH, H., KELLY, P. P., MUTTI, F. G., TURNER, N. J. & FLITSCH, S. L. 2016. Whole-Cell Biocatalysts for Stereoselective C–H Amination Reactions. *Angewandte Chemie International Edition*, 55, 1511-1513.
- BRAZEAU, B. J., JOHNSON, B. J. & WILMOT, C. M. 2004. Copper-containing amine oxidases. Biogenesis and catalysis; a structural perspective. *Archives of Biochemistry and Biophysics*, 428, 22-31.
- BROCKMAN, I. M. & PRATHER, K. L. 2015. Dynamic metabolic engineering: New strategies for developing responsive cell factories. *Biotechnol J*, 10, 1360-9.
- BRUNDTLAND REPORT. 1987. 1987 | *BRUNDTLAND REPORT: OUR COMMON FUTURE* [Online]. Available: <http://www.dac.dk/en/dac-cities/sustainable-cities/historic-milestones/1987--brundtland-report-our-common-future/> [Accessed 30th May 2015].
- BUNSUPA, S., KATAYAMA, K., IKEURA, E., OIKAWA, A., TOYOOKA, K., SAITO, K. & YAMAZAKI, M. 2012a. Lysine decarboxylase catalyzes the first step of quinolizidine alkaloid biosynthesis and coevolved with alkaloid production in Leguminosae. *Plant Cell*, 24, 1202-1216.
- BUNSUPA, S., YAMAZAKI, M. & SAITO, K. 2012b. Quinolizidine alkaloid biosynthesis: recent advances and future prospects. *Frontiers in Plant Science*, 3, 239.
- BUNSUPA, S., YAMAZAKI, M. & SAITO, K. 2017. Lysine-derived Alkaloids: Overview and Update on Biosynthesis and Medicinal Applications with Emphasis on Quinolizidine Alkaloids. *Mini-Reviews in Medicinal Chemistry*, 17, 1002-1012.
- BUSCHKE, N., BECKER, J., SCHÄFER, R., KIEFER, P., BIEDENDIECK, R. & WITTMANN, C. 2013. Systems metabolic engineering of xylose-utilizing *Corynebacterium glutamicum* for production of 1,5-diaminopentane. *Biotechnology Journal*, 8, 557-570.

- BUSCHKE, N., SCHRÖDER, H. & WITTMANN, C. 2011. Metabolic engineering of *Corynebacterium glutamicum* for production of 1,5-diaminopentane from hemicellulose. *Biotechnology Journal*, 6, 306-317.
- CAI, Y., CAI, Y. E., SHI, Y., LIU, J., MOU, S. & LU, Y. 2007. A liquid-liquid extraction technique for phthalate esters with water-soluble organic solvents by adding inorganic salts. *Microchimica Acta*, 157, 73-79.
- CAIAZZO, A., DALILI, S., PICARD, C., SASAKI, M., SIU, T. & YUDIN, A. K. 2004. New methods for the synthesis of heterocyclic compounds. *Pure and Applied Chemistry*, 76, 603-613.
- CAMPANATI, M., FRANCESCHINI, S., PICCOLO, O. & VACCARI, A. 2005. Reaction pathway in the vapour-phase synthesis of indole and alkylindoles. *Journal of Catalysis*, 232, 1-9.
- CARR, R., ALEXEEVA, M., DAWSON, M. J., GOTOR-FERNANDEZ, V., HUMPHREY, C. E. & TURNER, N. J. 2005. Directed evolution of an amine oxidase for the preparative deracemisation of cyclic secondary amines. *ChemBioChem*, 6, 637-639.
- CESPI, D., BEACH, E. S., SWARR, T. E., PASSARINI, F., VASSURA, I., DUNN, P. J. & ANASTAS, P. T. 2015. Life cycle inventory improvement in the pharmaceutical sector: assessment of the sustainability combining PMI and LCA tools. *Green Chemistry*, 17, 3390-3400.
- CHA, H. J., JEONG, J. H., ROJVIRIYA, C. & KIM, Y. G. 2014. Structure of putrescine aminotransferase from *Escherichia coli* provides insights into the substrate specificity among class III aminotransferases. *PLoS One*, 9, e113212.
- CHEMLER, J. A., FOWLER, Z. L., MCHUGH, K. P. & KOFFAS, M. A. G. 2010. Improving NADPH availability for natural product biosynthesis in *Escherichia coli* by metabolic engineering. *Metab. Eng.*, 12, 96-104.
- CHEN, C., HUANG, H. & WU, C. H. 2017. Protein Bioinformatics Databases and Resources. *Methods Mol Biol*, 1558, 3-39.
- DA SILVA, L. C. A., HONORATO, T. L., CAVALCANTE, R. S., FRANCO, T. T. & RODRIGUES, S. 2012. Effect of pH and Temperature on Enzyme Activity of Chitosanase Produced

- Under Solid Stated Fermentation by *Trichoderma* spp. *Indian Journal of Microbiology*, 52, 60-65.
- DE CARVALHO, C. C. C. R. 2017. Whole cell biocatalysts: essential workers from Nature to the industry. *Microbial Biotechnology*, 10, 250-263.
- DELLOMONACO, C., CLOMBURG, J. M., MILLER, E. N. & GONZALEZ, R. 2011. Engineered reversal of the β -oxidation cycle for the synthesis of fuels and chemicals. *Nature (London, U. K.)*, 476, 355-359.
- DELOACHE, W. C., RUSS, Z. N., NARCROSS, L., GONZALES, A. M., MARTIN, V. J. J. & DUEBER, J. E. 2015. An enzyme-coupled biosensor enables (S)-reticuline production in yeast from glucose. *Nature Chemical Biology*, 11, 465.
- DESA, R. J. & BRANTNER, R. 1972. Putrescine oxidase from *Micrococcus rubens*. Purification and properties of the enzyme. *J. Biol. Chem.*, 247, 5527-34.
- DEVI, N. A., RADHIKA, G. B. & BHARGAVI, R. J. 2017. Lipase catalyzed transesterification of ethyl butyrate synthesis in n-hexane— a kinetic study. *Journal of Food Science and Technology*, 54, 2871-2877.
- DEWICK, P. M. 2002. *Medicinal Natural Products: A Biosynthetic Approach*, Wiley.
- DONG, H., SECUNDO, F., XUE, C. & MAO, X. 2017. Whole-Cell Biocatalytic Synthesis of Cinnamyl Acetate with a Novel Esterase from the DNA Library of *Acinetobacter hemolyticus*. *J Agric Food Chem*, 65, 2120-2128.
- DOUKYU, N. & OGINO, H. 2010. Organic solvent-tolerant enzymes. *Biochemical Engineering Journal*, 48, 270-282.
- DUNLOP, M. J., DOSSANI, Z. Y., SZMIDT, H. L., CHU, H. C., LEE, T. S., KEASLING, J. D., HADI, M. Z. & MUKHOPADHYAY, A. 2011. Engineering microbial biofuel tolerance and export using efflux pumps. *Molecular Systems Biology*, 7, 1-7.
- DUNSMORE, C. J., CARR, R., FLEMING, T. & TURNER, N. J. 2006. A Chemo-Enzymatic Route to Enantiomerically Pure Cyclic Tertiary Amines. *J. Am. Chem. Soc.*, 128, 2224-2225.

- DYM, O. & EISENBERG, D. 2001. Sequence-structure analysis of FAD-containing proteins. *Protein Sci.*, 10, 1712-1728.
- EL JABER-VAZDEKIS, N., GUTIERREZ-NICOLAS, F., RAVELO, A. G. & ZARATE, R. 2006. Studies on tropane alkaloid extraction by volatile organic solvents: dichloromethane vs. chloroform. *Phytochem Anal*, 17, 107-13.
- ELIOT, A. C. & KIRSCH, J. F. 2004. Pyridoxal phosphate enzymes: mechanistic, structural, and evolutionary considerations. *Annu Rev Biochem*, 73, 383-415.
- EVANS, W. C., EVANS, D. & TREASE, G. E. 2009. *Trease and Evans Pharmacognosy*, Saunders/Elsevier.
- FACCHINI, P. J., BIRD, D. A. & ST-PIERRE, B. 2004. Can Arabidopsis make complex alkaloids? *Trends in Plant Science*, 9, 116-122.
- FALCO, S. C., GUIDA, T., LOCKE, M., MAUVAIS, J., SANDERS, C., WARD, R. T. & WEBBER, P. 1995. Transgenic Canola and Soybean Seeds with Increased Lysine. *Nat Biotech*, 13, 577-582.
- FOTHERGILL, J. C. & GUEST, J. R. 1977. Catabolism of L-lysine by *Pseudomonas aeruginosa*. *J Gen Microbiol*, 99, 139-55.
- GALANIE, S., THODEY, K., TRENCHARD, I. J., FILSINGER INTERRANTE, M. & SMOLKE, C. D. 2015. Complete biosynthesis of opioids in yeast. *Science*, 349, 1095-1100.
- GHISLA, S. & MASSEY, V. 1989. Mechanisms of flavoprotein-catalyzed reactions. *European Journal of Biochemistry*, 181, 1-17.
- GIBSON, D. G., GLASS, J. I., LARTIGUE, C., NOSKOV, V. N., CHUANG, R.-Y., ALGIRE, M. A., BENDERS, G. A., MONTAGUE, M. G., MA, L., MOODIE, M. M., MERRYMAN, C., VASHEE, S., KRISHNAKUMAR, R., ASSAD-GARCIA, N., ANDREWS-PFANNKUCH, C., DENISOVA, E. A., YOUNG, L., QI, Z.-Q., SEGALL-SHAPIRO, T. H., CALVEY, C. H., PARMAR, P. P., HUTCHISON, C. A., III, SMITH, H. O. & VENTER, J. C. 2010. Creation of a bacterial cell controlled by a chemically synthesized genome. *Science (Washington, DC, U. S.)*, 329, 52-56.

- GOBER, C. M. & JOULLIÉ, M. M. 2016. Joining Forces: Fermentation and Organic Synthesis for the Production of Complex Heterocycles. *The Journal of Organic Chemistry*, 81, 10136-10144.
- GOLEBIEWSKI, W. M. & SPENSER, I. D. 1988. Biosynthesis of the lupine alkaloids. II. Sparteine and lupanine. *Canadian Journal of Chemistry*, 66, 1734-1748.
- GOMM, A., LEWIS, W., GREEN, A. P. & O'REILLY, E. 2016. A New Generation of Smart Amine Donors for Transaminase-Mediated Biotransformations. *Chemistry – A European Journal*, 22, 12692-12695.
- GOSETTI, F., MAZZUCCO, E., GIANOTTI, V., POLATI, S. & GENNARO, M. C. 2007. High performance liquid chromatography/tandem mass spectrometry determination of biogenic amines in typical Piedmont cheeses. *Journal of Chromatography A*, 1149, 151-157.
- GRAVEL, E., POUPON, E. & HOCQUEMILLER, R. 2006. Biomimetic investigations from reactive lysine-derived C5 units: one step synthesis of complex polycyclic alkaloids from the Nitraria genus. *Tetrahedron*, 62, 5248-5253.
- GROSSMAN, T. H., KAWASAKI, E. S., PUNREDDY, S. R. & OSBURNE, M. S. 1998. Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability. *Gene*, 209, 95-103.
- GUEST, J. R., GREEN, J., IRVINE, A. S. & SPIRO, S. 1996. The FNR Modulon and FNR-Regulated Gene Expression. In: LIN, E. C. C., AND LYNCH, A. S., (ed.) *Regulation of Gene Expression in Escherichia coli*. Boston, MA: Springer US.
- GUPTA, R. N. & SPENSER, I. D. 1969. Biosynthesis of the Piperidine Nucleus: THE MODE OF INCORPORATION OF LYSINE INTO PIPECOLIC ACID AND INTO PIPERIDINE ALKALOIDS. *Journal of Biological Chemistry*, 244, 88-94.
- HAFNER, E. W., TABOR, C. W. & TABOR, H. 1979. Mutants of Escherichia coli that do not contain 1,4-diaminobutane (putrescine) or spermidine. *Journal of Biological Chemistry*, 254, 12419-26.

- HENDERSON, T. J. 2002. Quantitative NMR Spectroscopy Using Coaxial Inserts Containing a Reference Standard: Purity Determinations for Military Nerve Agents. *Anal. Chem.*, 74, 191-198.
- HEUTS, D. P. H. M., SCRUTTON, N. S., MCINTIRE, W. S. & FRAAIJE, M. W. 2009. What's in a covalent bond? *The FEBS Journal*, 276, 3405-3427.
- HEYLAND, J., ANTWEILER, N., LUTZ, J., HECK, T., GEUEKE, B., KOHLER, H. P., BLANK, L. M. & SCHMID, A. 2010. Simple enzymatic procedure for L-carnosine synthesis: whole-cell biocatalysis and efficient biocatalyst recycling. *Microb Biotechnol*, 3, 74-83.
- HIGASIO, Y. S. & SHOJI, T. 2001. Heterocyclic compounds such as pyrroles, pyridines, pyrrolidins, piperdines, indoles, imidazol and pyrazins. *Applied Catalysis A: General*, 221, 197-207.
- HUISMAN, G. W. & COLLIER, S. J. 2013. On the development of new biocatalytic processes for practical pharmaceutical synthesis. *Curr Opin Chem Biol*, 17, 284-92.
- HUSSAIN, S., LEIPOLD, F., MAN, H., WELLS, E., FRANCE, S. P., MULHOLLAND, K. R., GROGAN, G. & TURNER, N. J. 2015. An (R)-Imine Reductase Biocatalyst for the Asymmetric Reduction of Cyclic Imines. *ChemCatChem*, 7, 579-583.
- JESSOP, P. G. 2011. Searching for green solvents. *Green Chemistry*, 13, 1391-1398.
- JONES-PRATHER, K. L. & MARTIN, C. H. 2008. De novo biosynthetic pathways: rational design of microbial chemical factories. *Curr. Opin. Biotechnol.*, 19, 468-474.
- JOULE, J. A. & MILLS, K. 2010. *Heterocyclic chemistry / John A. Joule, Keith Mills*, Oxford, Wiley-Blackwell.
- JULLESSON, D., DAVID, F., PFLEGER, B. & NIELSEN, J. 2015. Impact of synthetic biology and metabolic engineering on industrial production of fine chemicals. *Biotechnology Advances*, 33(7):1395-402.
- KANG, I.-H., KIM, J.-S., KIM, E.-J. & LEE, J. K. 2007. Cadaverine protects *Vibrio vulnificus* from superoxide stress. *J. Microbiol. Biotechnol.*, 17, 176-179.

- KANJEE, U., GUTSCHE, I., ALEXOPOULOS, E., ZHAO, B., EL BAKKOURI, M., THIBAUT, G., LIU, K., RAMACHANDRAN, S., SNIDER, J., PAI, E. F. & HOURY, W. A. 2011. Linkage between the bacterial acid stress and stringent responses: the structure of the inducible lysine decarboxylase. *EMBO J.*, 30, 931-944.
- KAUR, N. 2014a. Microwave-Assisted Synthesis of Fused Polycyclic Six-Membered N-Heterocycles. *Synthetic Communications*, 45, 273-299.
- KAUR, N. 2014b. Synthesis of Fused Five-Membered N,N-Heterocycles Using Microwave Irradiation. *Synthetic Communications*, 1-32.
- KE, C., YANG, X., RAO, H., ZENG, W., HU, M., TAO, Y. & HUANG, J. 2016. Whole-cell conversion of L-glutamic acid into gamma-aminobutyric acid by metabolically engineered *Escherichia coli*. *Springerplus*, 5, 591.
- KELLEY, K. C., HUESTIS, K. J., AUSTEN, D. A., SANDERSON, C. T., DONOGHUE, M. A., STICKEL, S. K., KAWASAKI, E. S. & OSBURN, M. S. 1995. Regulation of sCD4-183 gene expression from phage-T7-based vectors in *Escherichia coli*. *Gene*, 156, 33-36.
- KESSLER, H., MOEHRLE, H. & ZIMMERMANN, G. 1977. Determination of the configuration and conformation of .alpha.-, .beta.-, and isotripiperidine carbon-13 nuclear magnetic resonance spectroscopy. *The Journal of Organic Chemistry*, 42, 66-72.
- KIND, S., JEONG, W. K., SCHRÖDER, H. & WITTMANN, C. 2010. Systems-wide metabolic pathway engineering in *Corynebacterium glutamicum* for bio-based production of diaminopentane. *Metabolic Engineering*, 12, 341-351.
- KIND, S., KREYE, S. & WITTMANN, C. 2011. Metabolic engineering of cellular transport for overproduction of the platform chemical 1,5-diaminopentane in *Corynebacterium glutamicum*. *Metab. Eng.*, 13, 617-627.
- KIND, S. & WITTMANN, C. 2011. Bio-based production of the platform chemical 1,5-diaminopentane. *Appl. Microbiol. Biotechnol.*, 91, 1287-1296.
- KOHL, H., STEFFEN-MUNSBURG, F. & HÖHNE, M. 2014. Recent achievements in developing the biocatalytic toolbox for chiral amine synthesis. *Current Opinion in Chemical Biology*, 19, 180-192.

- KOPACZ, M. M., HEUTS, D. P. H. M. & FRAAIJE, M. W. 2014. Kinetic mechanism of putrescine oxidase from *Rhodococcus erythropolis*. *FEBS J.*, 281, 4384-4393.
- KOPACZ, M. M., ROVIDA, S., VAN DUIJN, E., FRAAIJE, M. W. & MATTEVI, A. 2011. Structure-Based Redesign of Cofactor Binding in Putrescine Oxidase. *Biochemistry*, 50, 4209-4217.
- KOSZELEWSKI, D., TAUBER, K., FABER, K. & KROUTIL, W. 2010. w-Transaminases for the synthesis of non-racemic α -chiral primary amines. *Trends in Biotechnology*, 28, 324-332.
- KUMAR, S. P. J., PRASAD, S. R., BANERJEE, R., AGARWAL, D. K., KULKARNI, K. S. & RAMESH, K. V. 2017. Green solvents and technologies for oil extraction from oilseeds. *Chemistry Central Journal*, 11, 9.
- KURIHARA, S., ODA, S., TSUBOI, Y., KIM, H. G., OSHIDA, M., KUMAGAI, H. & SUZUKI, H. 2008. γ -Glutamylputrescine Synthetase in the Putrescine Utilization Pathway of *Escherichia coli* K-12. *Journal of Biological Chemistry*, 283, 19981-19990.
- KWON, Y. D., KWON, O. H., LEE, H. S. & KIM, P. 2007. The effect of NADP-dependent malic enzyme expression and anaerobic C4 metabolism in *Escherichia coli* compared with other anaplerotic enzymes. *Journal of Applied Microbiology*, 103, 2340-2345.
- LEE, J. W., KIM, T. Y., JANG, Y. S., CHOI, S. & LEE, S. Y. 2011. Systems metabolic engineering for chemicals and materials. *Trends Biotechnol*, 29, 370-8.
- LEE, J. W., NA, D., PARK, J. M., LEE, J., CHOI, S. & LEE, S. Y. 2012. Systems metabolic engineering of microorganisms for natural and non-natural chemicals. *Nat. Chem. Biol.*, 8, 536-546.
- LEE, K. H., PARK, J. H., KIM, T. Y., KIM, H. U. & LEE, S. Y. 2007. Systems metabolic engineering of *Escherichia coli* for L-threonine production. *Mol Syst Biol*, 3, 149.
- LEISTNER, E. & SPENSER, I. D. 1973. Biosynthesis of the piperidine nucleus. Incorporation of chirally labeled (1-3H)cadaverine. *J Am Chem Soc*, 95, 4715-25.

- LENZ, M., BORLINGHAUS, N., WEINMANN, L. & NESTL, B. M. 2017. Recent advances in imine reductase-catalyzed reactions. *World Journal of Microbiology and Biotechnology*, 33, 199.
- LI, G., QIAN, S., WANG, C. & YOU, J. 2013. Palladium(II)-Catalyzed Dehydrogenative Cross-Coupling between Two C sp³-H Bonds: Unexpected C-C Bond Formation. *Angewandte Chemie International Edition*, 52, 7837-7840.
- LIN, B. & TAO, Y. 2017. Whole-cell biocatalysts by design. *Microb Cell Fact*, 16, 106.
- LINDNER, S. N., SEIBOLD, G. M., HENRICH, A., KRÄMER, R. & WENDISCH, V. F. 2011. Phosphotransferase System-Independent Glucose Utilization in *Corynebacterium glutamicum* by Inositol Permeases and Glucokinases. *Applied and Environmental Microbiology*, 77, 3571-3581.
- LIU, B., LIU, H., ZHONG, D. & LIN, C. 2010. Searching for a photocycle of the cryptochrome photoreceptors. *Current Opinion in Plant Biology*, 13, 578-586.
- LOU, W.-Y., XU, R. & ZONG, M.-H. 2005. Hydroxynitrile Lyase Catalysis in Ionic Liquid-containing Systems. *Biotechnology Letters*, 27, 1387-1390.
- MA, X. & GANG, D. R. 2004. The Lycopodium alkaloids. *Natural Product Reports*, 21, 752-772.
- MAJUMDER, A., GUPTA, R. & JAIN, A. 2013. Microwave-assisted synthesis of nitrogen-containing heterocycles. *Green Chemistry Letters and Reviews*, 6, 151-182.
- MARCE, M., BROWN, D. S., CAPELL, T., FIGUERAS, X. & TIBURCIO, A. F. 1995. Rapid high-performance liquid chromatographic method for the quantitation of polyamines as their dansyl derivatives: application to plant and animal tissues. *J. Chromatogr. B: Biomed. Sci. Appl.*, 666, 329-35.
- MCCLURE, W. R. 1969. Kinetic analysis of coupled enzyme assays. *Biochemistry*, 8, 2782-2786.
- MCKENNA, R. & NIELSEN, D. R. 2011. Styrene biosynthesis from glucose by engineered *E. coli*. *Metab. Eng.*, 13, 544-554.

- MIMITSUKA, T., SAWAI, H., HATSU, M. & YAMADA, K. 2007. Metabolic engineering of *Corynebacterium glutamicum* for cadaverine fermentation. *Biosci., Biotechnol., Biochem.*, 71, 2130-2135.
- MIMIZUKA, T. & KAZAMI, J. 2002. *Overexpression of E. coli lysine decarboxylase and lysine/cadaverine antiporter gene for cadaverine biosynthesis*. Host and method for producing cadaverine, JP Patent 2002223770.
- MITSAKOS, V., DEVENISH, S. R. A., O'DONNELL, P. A., GERRARD, J. A. & HUTTON, C. A. 2011. LC-MS and NMR characterization of the purple chromophore formed in the o-aminobenzaldehyde assay of dihydrodipicolinate synthase. *Bioorganic & Medicinal Chemistry*, 19, 1535-1540.
- MITSUKURA, K., SUZUKI, M., TADA, K., YOSHIDA, T. & NAGASAWA, T. 2010. Asymmetric synthesis of chiral cyclic amine from cyclic imine by bacterial whole-cell catalyst of enantioselective imine reductase. *Organic & Biomolecular Chemistry*, 8, 4533-4535.
- MOLINS-LEGUA, C., CAMPINS-FALCO, P., SEVILLANO-CABEZA, A. & PEDRON-PONS, M. 1999. Urine polyamines determination using dansyl chloride derivatization in solid-phase extraction cartridges and HPLC. *Analyst*, 124, 477-482.
- MONACO, M. R., RENZI, P., SCARPINO SCHIETROMA, D. M. & BELLA, M. 2011. Biomimetic Organocatalytic Asymmetric Synthesis of 2-Substituted Piperidine-Type Alkaloids and Their Analogues. *Organic Letters*, 13, 4546-4549.
- MONDOVÌ, B. & AGRÒ, A. F. 1982. Structure and Function of Amine Oxidases. In: BOSSA, F., CHIANCONE, E., FINAZZI-AGRÒ, A. & STROM, R. (eds.) *Structure and Function Relationships in Biochemical Systems*. Boston, MA: Springer US.
- MÜLLER, P., IGNATZ, E., KIONTKE, S., BRETTEL, K. & ESSEN, L.-O. 2018. Sub-nanosecond tryptophan radical deprotonation mediated by a protein-bound water cluster in class II DNA photolyases. *Chemical Science*, 9, 1200-1212.
- NAKAGAWA, A., MATSUMURA, E., KOYANAGI, T., KATAYAMA, T., KAWANO, N., YOSHIMATSU, K., YAMAMOTO, K., KUMAGAI, H., SATO, F. & MINAMI, H. 2016. Total biosynthesis of opiates by stepwise fermentation using engineered *Escherichia coli*. *Nat Commun*, 7, 10390.

- NAKAGAWA, A., MINAMI, H., KIM, J.-S., KOYANAGI, T., KATAYAMA, T., SATO, F. & KUMAGAI, H. 2011. A bacterial platform for fermentative production of plant alkaloids. *Nature Communications*, 2, 326.
- NIELSEN, J. 2001. Metabolic engineering. *Applied Microbiology and Biotechnology*, 55, 263-283.
- NIELSEN, J., FUSSENEGGER, M., KEASLING, J., LEE, S. Y., LIAO, J. C., PRATHER, K. & PALSSON, B. 2014. Engineering synergy in biotechnology. *Nat. Chem. Biol.*, 10, 319-322.
- NIKOLAU, B. J., PERERA, M. A. D. N., BRACHOVA, L. & SHANKS, B. 2008. Platform biochemicals for a biorenewable chemical industry. *Plant J.*, 54, 536-545.
- O'REILLY, E., IGLESIAS, C., GHISLIERI, D., HOPWOOD, J., GALMAN, J. L., LLOYD, R. C. & TURNER, N. J. 2014. A Regio- and Stereoselective ω -Transaminase/Monoamine Oxidase Cascade for the Synthesis of Chiral 2,5-Disubstituted Pyrrolidines. *Angewandte Chemie International Edition*, 53, 2447-2450.
- OGAWA-MIYATA, Y., KOJIMA, H. & SANO, K. 2001. Mutation Analysis of the Feedback Inhibition Site of Aspartokinase III of Escherichia coli K-12 and its Use in L-Threonine Production. *Bioscience, Biotechnology, and Biochemistry*, 65, 1149-1154.
- OREJARENA PACHECO, J. C., LIPP, A., NAUTH, A. M., ACKE, F., DIETZ, J.-P. & OPATZ, T. 2016. A Highly Active System for the Metal-Free Aerobic Photocyanation of Tertiary Amines with Visible Light: Application to the Synthesis of Tetraoponerines and Crispine A. *Chemistry – A European Journal*, 22, 5409-5415.
- PARTRIDGE, J. D., SCOTT, C., TANG, Y., POOLE, R. K. & GREEN, J. 2006. Escherichia coli Transcriptome Dynamics during the Transition from Anaerobic to Aerobic Conditions. *Journal of Biological Chemistry*, 281, 27806-27815.
- PEGG, A. E. 1988. Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. *Cancer Res.*, 48, 759-74.

- PETERSON, MICHELLE E., DANIEL, ROY M., DANSON, MICHAEL J. & EISENTHAL, R. 2007. The dependence of enzyme activity on temperature: determination and validation of parameters. *Biochemical Journal*, 402, 331.
- PICATAGGIO, S. 2009. Potential impact of synthetic biology on the development of microbial systems for the production of renewable fuels and chemicals. *Curr Opin Biotechnol*, 20, 325-9.
- POLSHETTIWAR, V. & VARMA, R. S. 2008. Microwave-assisted organic synthesis and transformations using benign reaction media. *Acc Chem Res*, 41, 629-39.
- POUPON, E., SALAME, R. & YAN, L.-H. 2011. Biomimetic Synthesis of Ornithine/Arginine and Lysine-Derived Alkaloids: Selected Examples. *Biomimetic Organic Synthesis*. Wiley-VCH Verlag GmbH & Co. KGaA.
- PRAT, D., WELLS, A., HAYLER, J., SNEDDON, H., MCELROY, C. R., ABOU-SHEHADA, S. & DUNN, P. J. 2016. CHEM21 selection guide of classical- and less classical-solvents. *Green Chemistry*, 18, 288-296.
- PRESTON, T. R. 1995. Tropical animal feeding. A manual for research workers.: FAO Animal Production and Health
- PUNTA, M., COGGILL, P. C., EBERHARDT, R. Y., MISTRY, J., TATE, J., BOURSNEILL, C., PANG, N., FORSLUND, K., CERIC, G., CLEMENTS, J., HEGER, A., HOLM, L., SONNHAMMER, E. L. L., EDDY, S. R., BATEMAN, A. & FINN, R. D. 2012. The Pfam protein families database. *Nucleic Acids Research*, 40, D290-D301.
- QIAN, Z.-G., XIA, X.-X. & LEE, S. Y. 2009. Metabolic engineering of *Escherichia coli* for the production of putrescine: A four carbon diamine. *Biotechnol. Bioeng.*, 104, 651-662.
- QIAN, Z.-G., XIA, X.-X. & LEE, S. Y. 2010. Metabolic engineering of *Escherichia coli* for the production of cadaverine: A five carbon diamine. *Biotechnol. Bioeng.*, 108, 93-103.
- REDDY, J. K., MOTOKURA, K., KOYAMA, T.-R., MIYAJI, A. & BABA, T. 2012. Effect of morphology and particle size of ZSM-5 on catalytic performance for ethylene conversion and heptane cracking. *Journal of Catalysis*, 289, 53-61.

- ROSINHA GRUNDTVIG, I. P., HEINTZ, S., KRUHNE, U., GERNAEY, K. V., ADLERCREUTZ, P., HAYLER, J. D., WELLS, A. S. & WOODLEY, J. M. 2018. Screening of organic solvents for bioprocesses using aqueous-organic two-phase systems. *Biotechnol Adv*, 36, 1801-1814.
- ROSSONI, L., CARR, R., BAXTER, S., CORTIS, R., THORPE, T., EASTHAM, G. & STEPHENS, G. 2018. Engineering *Escherichia coli* to grow constitutively on D-xylose using the carbon-efficient Weimberg pathway. *Microbiology*, 164, 287-298.
- ROUCHAUD, A. & BRAEKMAN, J.-C. 2009. Synthesis of New Analogues of the Tetraonerines. *European Journal of Organic Chemistry*, 2009, 2666-2674.
- RUNDLÖF, T., MATHIASSEN, M., BEKIROGLU, S., HAKKARAINEN, B., BOWDEN, T. & ARVIDSSON, T. 2010. Survey and qualification of internal standards for quantification by ¹H NMR spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis*, 52, 645-651.
- RUNDLÖF, T., MCEWEN, I., JOHANSSON, M. & ARVIDSSON, T. 2014. Use and qualification of primary and secondary standards employed in quantitative ¹H NMR spectroscopy of pharmaceuticals. *Journal of Pharmaceutical and Biomedical Analysis*, 93, 111-117.
- SAITO, K. & MURAKOSHI, I. 1995. Chemistry, biochemistry and chemotaxonomy of lupine alkaloids in the leguminosae. In: ATTA UR, R. (ed.) *Studies in Natural Products Chemistry*. Elsevier.
- SALAME, R., GRAVEL, E., RETAILLEAU, P. & POUPON, E. 2010. Biomimetically relevant self-condensations of C5 units derived from lysine. *Organic & Biomolecular Chemistry*, 8, 2522-2528.
- SAMSONOVA, N. N., SERGEY V SMIRNOV, IRINA B ALTMAN & PTITSYN, L. R. 2003. Molecular cloning and characterization of *Escherichia coli* K12 *ygjG* gene. *BMC Microbiology*, 3, 1-10.
- SANDERS, C. C. & SANDERS, W. E., JR. 1983. Emergence of resistance during therapy with the newer beta-lactam antibiotics: role of inducible beta-lactamases and implications for the future. *Rev Infect Dis*, 5, 639-48.

- SANTAGADA, V., FRECENTESE, F., PERISSUTTI, E., FIORINO, F., SEVERINO, B. & CALIENDO, G. 2009. Microwave assisted synthesis: a new technology in drug discovery. *Mini Rev Med Chem*, 9, 340-58.
- SANTAMARIA, J., HERLEM, D. & KHUONG-HUU, F. 1977. Oxydation photochimique D'amines tertiaires et D'alcaloïdes—VIII11R. Beugelmans, D. Herlem, H.-P. Husson, F. Khuong-Huu et M.-T. Le Goff, Oxydation photochimique d'amines tertiaires et d'alcaloïdes—VII. *Tetrahedron Letters* 435 (1976). *Tetrahedron*, 33, 2389-2392.
- SCHIROLI, D. & PERACCHI, A. 2015. A subfamily of PLP-dependent enzymes specialized in handling terminal amines. *Biochim Biophys Acta*, 1854, 1200-11.
- SCHOPF, C., KOMZAK, A., BRAUN, F., JACOBI, E., BORMUTH, M. L., BULLNHEIMER, M. & HAGEL, I. 1948. Polymers of Δ^1 -piperidine. *Ann.*, 559, 1-42.
- SEILER, N., ATANASSOV, C. L. & RAUL, F. 1998. Polyamine metabolism as target for cancer chemoprevention (review). *Int. J. Oncol.*, 13, 993-1006.
- SHELDON, R. A. & WOODLEY, J. M. 2018. Role of Biocatalysis in Sustainable Chemistry. *Chemical Reviews*, 118, 801-838.
- SHEN, C. R., LAN, E. I., DEKISHIMA, Y., BAEZ, A., CHO, K. M. & LIAO, J. C. 2011. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. *Appl. Environ. Microbiol.*, 77, 2905-2915.
- SHI, S. & SZOSTAK, M. 2017. Synthesis of Nitrogen Heterocycles Using Samarium(II) Iodide. *Molecules*, 22, 2018.
- SHIMIZU, S., ABE, N., IGUCHI, A. & SATO, H. 1998. Synthesis of pyridine bases: general methods and recent advances in gas phase synthesis over ZSM-5 zeolite. *Catalysis Surveys from Asia*, 2, 71-76.
- SHIMIZU, S., WATANABE, N., KATAOKA, T., SHOJI, T., ABE, N., MORISHITA, S. & ICHIMURA, H. 2000. Pyridine and Pyridine Derivatives. *Ullmann's Encyclopedia of Industrial Chemistry*. Wiley-VCH Verlag GmbH & Co. KGaA.

- SHVEKHGEIMER, A. M.-G. 1998. 2,3,4,5-Tetrahydropyridine (Δ^1 -piperidine) and its derivatives. Synthesis and chemical properties. *Russian Chemical Reviews*, 67, 1031-1060.
- SLABU, I., GALMAN, J. L., WEISE, N. J., LLOYD, R. C. & TURNER, N. J. 2016. Putrescine Transaminases for the Synthesis of Saturated Nitrogen Heterocycles from Polyamines. *ChemCatChem*, 8, 1038-1042.
- SMELA, D., PECHOVA, P., KOMPRDA, T., KLEJDUS, B. & KUBAN, V. 2003. Liquid chromatographic determination of biogenic amines in a meat product during fermentation and long-term storage. *Czech J. Food Sci.*, 21, 167-175.
- SMITH, M. A. & DAVIES, P. J. 1985. Separation and Quantitation of Polyamines in Plant Tissue by High Performance Liquid Chromatography of Their Dansyl Derivatives. *Plant Physiology*, 78, 89-91.
- SOKSAWATMAEKHIN, W., KURAISHI, A., SAKATA, K., KASHIWAGI, K. & IGARASHI, K. 2004. Excretion and uptake of cadaverine by CadB and its physiological functions in *Escherichia coli*. *Mol Microbiol*, 51, 1401-12.
- SØRENSEN, H. P. & MORTENSEN, K. K. 2005. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *Journal of Biotechnology*, 115, 113-128.
- STEIB, A. K., KUZMINA, O. M., FERNANDEZ, S., MALHOTRA, S. & KNOCHEL, P. 2015a. ChemInform Abstract: Chemoselective Chromium(II)-Catalyzed Cross-Coupling Reactions of Dichlorinated Heteroaromatics with Functionalized Aryl Grignard Reagents. *ChemInform*, 46.
- STEIB, A. K., KUZMINA, O. M., FERNANDEZ, S., MALHOTRA, S. & KNOCHEL, P. 2015b. Chemoselective Chromium(II)-Catalyzed Cross-Coupling Reactions of Dichlorinated Heteroaromatics with Functionalized Aryl Grignard Reagents. *Chemistry – A European Journal*, 21, 1961-1965.
- STORER, A. C. & CORNISH-BOWDEN, A. 1974. The kinetics of coupled enzyme reactions. Applications to the assay of glucokinase, with glucose 6-phosphate dehydrogenase as coupling enzyme. *Biochemical Journal*, 141, 205-209.

- SUN, J., MORITA, H., CHEN, G., NOGUCHI, H. & ABE, I. 2012. Molecular cloning and characterization of copper amine oxidase from *Huperzia serrata*. *Bioorganic & Medicinal Chemistry Letters*, 22, 5784-5790.
- SZŐKE, É., LEMBERKOVICS, É. & KURSINSZKI, L. 2013. Alkaloids Derived from Lysine: Piperidine Alkaloids. In: RAMAWAT, K. G. & MÉRILLON, J.-M. (eds.) *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*. Berlin, Heidelberg: Springer Berlin Heidelberg.
- TAI, Y.-S., XIONG, M., JAMBUNATHAN, P., WANG, J., WANG, J., STAPLETON, C. & ZHANG, K. 2016. Engineering nonphosphorylative metabolism to generate lignocellulose-derived products. *Nat Chem Biol*, 12, 247-253.
- TATENO, T., OKADA, Y., TSUCHIDATE, T., TANAKA, T., FUKUDA, H. & KONDO, A. 2009. Direct production of cadaverine from soluble starch using *Corynebacterium glutamicum* coexpressing α -amylase and lysine decarboxylase. *Appl. Microbiol. Biotechnol.*, 82, 115-121.
- TAVLADORAKI, P., ROSSI, M. N., SACCUTI, G., PEREZ-AMADOR, M. A., POLTICELLI, F., ANGELINI, R. & FEDERICO, R. 2006. Heterologous Expression and Biochemical Characterization of a Polyamine Oxidase from *Arabidopsis* Involved in Polyamine Back Conversion. *Plant Physiology*, 141, 1519-1532.
- TERPE, K. 2006. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology*, 72, 211.
- THEISEN, M. & LIAO, J. C. 2016. Industrial Biotechnology: *Escherichia coli* as a Host. In: WITTMANN, C. & LIAO, J. C. (eds.) *Industrial Biotechnology*. Wiley-VCH Verlag GmbH & Co.
- THOMAS, T. & THOMAS, T. J. 2001. Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell. Mol. Life Sci.*, 58, 244-258.
- TKACHENKO, A. G., SHUMKOV, M. S. & AKHOVA, A. V. 2009. Adaptive functions of *Escherichia coli* polyamines in response to sublethal concentrations of antibiotics. *Mikrobiologiya*, 78, 32-41.

- TOMAR, P. C., LAKRA, N. & MISHRA, S. N. 2013. Cadaverine. A lysine catabolite involved in plant growth and development. *Plant Signaling Behav.*, 8, E25850/1-E25850/15.
- TUCKER, J. L. 2006. Green Chemistry, a Pharmaceutical Perspective. *Organic Process Research & Development*, 10, 315-319.
- TURNER, N. J. & TRUPPO, M. D. 2013. Biocatalysis enters a new era. *Curr Opin Chem Biol*, 17, 212-4.
- UHR, M. L. 1990. Coupled Enzyme Systems: Exploring Coupled Assays with Students. *Biochemical Education*, 18, 48 - 50.
- UNSWORTH, W. P., COULTHARD, G., KITSIOU, C. & TAYLOR, R. J. K. 2014. Direct Imine Acylation for Molecular Diversity in Heterocyclic Synthesis. *The Journal of Organic Chemistry*, 79, 1368-1376.
- UNSWORTH, W. P., KITSIOU, C. & TAYLOR, R. J. K. 2013. Direct Imine Acylation: Rapid Access to Diverse Heterocyclic Scaffolds. *Organic Letters*, 15, 258-261.
- VALE, S. R. & GLORIA, M. B. A. 1997. Determination of biogenic amines in cheese. *J. AOAC Int.*, 80, 1006-1012.
- VAN HELLEMOND, E. W., MAZON, H., HECK, A. J., VAN DEN HEUVEL, R. H. H., HEUTS, D. P. H. M., JANSSEN, D. B. & FRAAIJE, M. W. 2008a. ADP Competes with FAD Binding in Putrescine Oxidase. *J. Biol. Chem.*, 283, 28259-28264.
- VAN HELLEMOND, E. W., VAN DIJK, M., HEUTS, D. P., JANSSEN, D. B. & FRAAIJE, M. W. 2008b. Discovery and characterization of a putrescine oxidase from *Rhodococcus erythropolis* NCIMB 11540. *Appl Microbiol Biotechnol*, 78, 455-63.
- VILLAVERDE, A. & MAR CARRIÓ, M. 2003. Protein aggregation in recombinant bacteria: biological role of inclusion bodies. *Biotechnology Letters*, 25, 1385-1395.
- VILLEGAS-TORRES, M. F., WARD, J. M. & BAGANZ, F. 2018. Optimisation of enzyme cascades for chiral amino alcohol synthesis in aid of host cell integration using a statistical experimental design approach. *J Biotechnol*, 281, 150-160.

- VITAKU, E., SMITH, D. T. & NJARDARSON, J. T. 2014. Analysis of the Structural Diversity, Substitution Patterns, and Frequency of Nitrogen Heterocycles among U.S. FDA Approved Pharmaceuticals. *Journal of Medicinal Chemistry*, 57, 10257-10274.
- VO, C.-V. T., LUESCHER, M. U. & BODE, J. W. 2014. SnAP reagents for the one-step synthesis of medium-ring saturated N-heterocycles from aldehydes. *Nature Chemistry*, 6, 310.
- VO, C. V. & BODE, J. W. 2014. Synthesis of saturated N-heterocycles. *J Org Chem*, 79, 2809-15.
- WALLACE, H. M., FRASER, A. V. & HUGHES, A. 2003. A perspective of polyamine metabolism. *Biochem. J.*, 376, 1-14.
- WANG, B., WANG, P., ZHENG, E., CHEN, X., ZHAO, H., SONG, P., SU, R., LI, X. & ZHU, G. 2011. Biochemical properties and physiological roles of NADP-dependent malic enzyme in *Escherichia coli*. *The Journal of Microbiology*, 49, 797-802.
- WEBB, J. P., ARNOLD, S. A., BAXTER, S., HALL, S. J., EASTHAM, G. & STEPHENS, G. 2018. Efficient bio-production of citramalate using an engineered *Escherichia coli* strain. *Microbiology*, 164, 133-141.
- WILDING, M., SCOTT, C., NEWMAN, J. & PEAT, T. S. 2017. Crystal structure of a putrescine aminotransferase from *Pseudomonas* sp. strain AAC. *Acta Crystallogr F Struct Biol Commun*, 73, 29-35.
- WIMMER, Z. & ZAREVÚCKA, M. 2010. A Review on the Effects of Supercritical Carbon Dioxide on Enzyme Activity. *International Journal of Molecular Sciences*, 11, 233.
- WINZER, T., KERN, M., KING, A. J., LARSON, T. R., TEODOR, R. I., DONNINGER, S. L., LI, Y., DOWLE, A. A., CARTWRIGHT, J., BATES, R., ASHFORD, D., THOMAS, J., WALKER, C., BOWSER, T. A. & GRAHAM, I. A. 2015. Morphinan biosynthesis in opium poppy requires a P450-oxidoreductase fusion protein. *Science*, 349, 309.
- XU, L., HAN, Z., YAO, Q., DENG, J., ZHANG, Y., FU, Y. & GUO, Q. 2015a. Towards the sustainable production of pyridines via thermo-catalytic conversion of glycerol with ammonia over zeolite catalysts. *Green Chemistry*, 17, 2426-2435.

- XU, L., JIANG, Y., YAO, Q., HAN, Z., ZHANG, Y., FU, Y., GUO, Q. & HUBER, G. W. 2015b. Direct production of indoles via thermo-catalytic conversion of bio-derived furans with ammonia over zeolites. *Green Chemistry*, 17, 1281-1290.
- XU, L., YAO, Q., DENG, J., HAN, Z., ZHANG, Y., FU, Y., HUBER, G. W. & GUO, Q. 2015c. Renewable N-Heterocycles Production by Thermocatalytic Conversion and Ammonization of Biomass over ZSM-5. *ACS Sustainable Chemistry & Engineering*, 3, 2890-2899.
- YAMADA, H. 1971. Putrescine oxidase (*Micrococcus rubens*). *Methods Enzymol.*, 17, 726-730.
- YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, 33, 103-19.
- YAO, Q., XU, L., GUO, C., YUAN, Z., ZHANG, Y. & FU, Y. 2017. Selective production of pyrroles via catalytic fast pyrolysis of cellulose under ammonia atmosphere at low temperature. *Journal of Analytical and Applied Pyrolysis*, 124, 409-414.
- YAO, Q., XU, L., ZHANG, Y. & FU, Y. 2016. Enhancement of indoles production and catalyst stability in thermo-catalytic conversion and ammonization of furfural with NH₃ and N₂ environments. *Journal of Analytical and Applied Pyrolysis*, 121, 258-266.
- YOKOSHIMA, S., ISHIKAWA, M., BENIYAMA, Y. & FUKUYAMA, T. 2016. Chemical Transformation of an Intermediate in the Synthesis of Huperzine A, Leading to a Diverse Array of Molecules. *Chemical and Pharmaceutical Bulletin*, 64, 1528-1531.
- YOMANTAS, Y. A. V., TOKMAKOVA, I. L., GORSHKOVA, N. V., ABALAKINA, E. G., KAZAKOVA, S. M., GAK, E. R. & MASHKO, S. V. 2010. Aromatic Amino Acid Auxotrophs Constructed by Recombinant Marker Exchange in *Methylophilus methylotrophus* AS1 Cells Expressing the *aroP*-Encoded Transporter of *Escherichia coli*. *Applied and Environmental Microbiology*, 76, 75-83.
- YUGARI, Y. & GILVARG, C. 1965. The condensation step in diaminopimelate synthesis. *J. Biol. Chem.*, 240, 4710-16.

- ZABRISKIE, T. M. & JACKSON, M. D. 2000. Lysine biosynthesis and metabolism in fungi. *Nat. Prod. Rep.*, 17, 85-97.
- ZELDER, O., JEONG, W. K., KLOPPROGGE, C., HEROLD, A. & SCHRÖDER, H. 2009. *Process For The Production Of Cadaverine*. US Patent WO2007113127A1.
- ZHAO, J., LICHMAN, B. R., WARD, J. M. & HAILES, H. C. 2018. One-pot chemoenzymatic synthesis of trolline and tetrahydroisoquinoline analogues. *Chem Commun (Camb)*, 54, 1323-1326.
- ZHAO, Y., YANG, J., QIN, B., LI, Y., SUN, Y., SU, S. & XIAN, M. 2011. Biosynthesis of isoprene in *Escherichia coli* via methylerythritol phosphate (MEP) pathway. *Appl. Microbiol. Biotechnol.*, 90, 1915-1922.
- ZHOU, L., LOKMAN HOSSAIN, M. & XIAO, T. 2016. Synthesis of N-Containing Heterocyclic Compounds Using Visible-light Photoredox Catalysis. *The Chemical Record*, 16, 319-334.

11. APPENDICES

11.1 Green or sustainable production (chemistry) principles

The World Commission on Environment and Development defined sustainable production as "ensuring that production meets the needs of the present without compromising the ability of future generations to meet their own needs" (Brundtland Report, 1987, Preston, 1995). To this can be added the need to respond to the pressures increasingly coming to bear in both industrialized and developing countries to safeguard natural resources. Worldwide campaign to follow up on this and protect the environment, led to the advent of green or sustainable production (Fig. 11.1) in the early 1990s; a widely adopted strategy to meet the fundamental scientific challenges to protect the human health and the ecosystem while simultaneously achieving commercial viability (Tucker, 2006, Majumder et al., 2013).

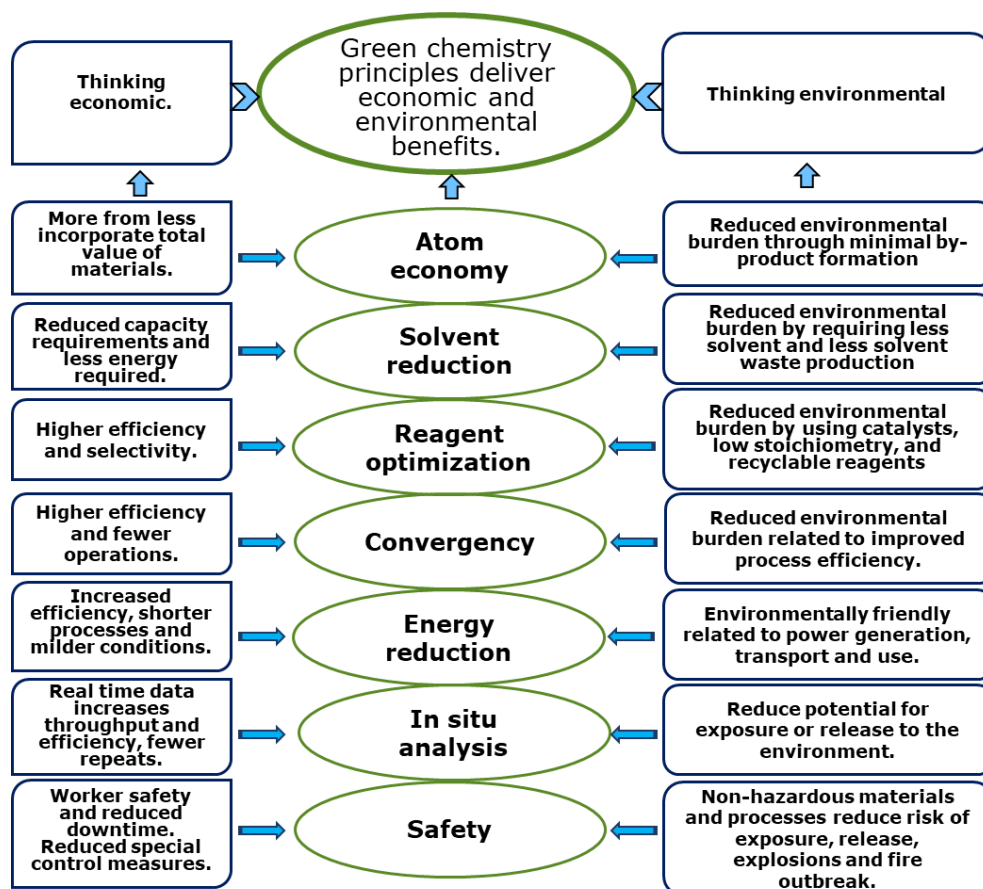


Fig. 11.1: Benefits of Green Chemistry Principles.

Source: Tucker (2006) with modifications.

11.2 Plasmid construction

The carrier vector pBMH_*puORh* gave two bands (Fig. 11.2a), with the PuO_{Rh} having a band weight of 1366bp as shown in yellow rectangle while the pET-20b(+) vector (Fig. 11.2b) after linearization gave a single band of 3586bp (red rectangle). These results are accurate because there is only one restriction site for *Xho*I and *Nde*I respectively in the plasmids. The bands corresponding to the *puORh* gene insert and linearized pET-20b(+) vector were excised, extracted and purified using gel purification kit and analysed by SDS-PAGE.

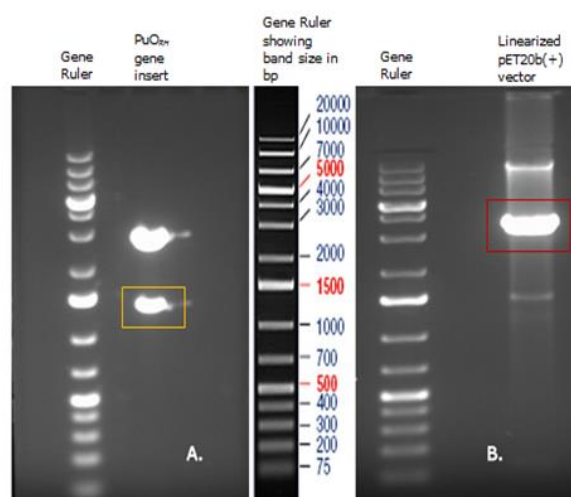


Fig. 11.2: Agarose gel electrophoresis of *Xho*I and *Nde*I digested pBMH_*puORh* and pET-20b(+) plasmids.

A. Digested pBMH_PuORH carrier plasmid (yellow rectangle indicate band corresponding to *puORh* insert). **B.** Digested pET-20b(+) vector plasmid (red rectangle indicates band corresponding to linearized pET-20b(+) vector). Plasmids were digested in 1.5 mL Eppendorf tubes using *Xho*I and *Nde*I in fast digest green buffer according to manufacturer's instruction. Digest mix was incubated for 3 h in a water bath at 37 °C and enzymes were inactivated at 80°C. Subsequently, 20 µL of the digest mixture were analysed by SDS-PAGE at 60 V for 60 min.

11.3 Plasmid systems for overexpression of target enzymes

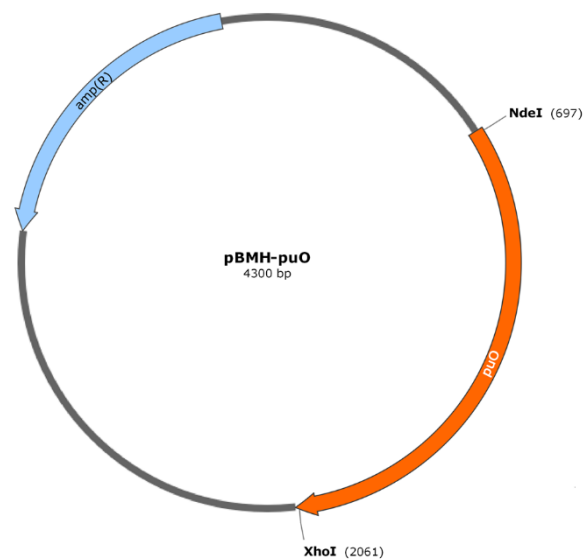


Fig. 11.3: Plasmid map of pBMH_*puo*_{Rh}

Carrier plasmid containing *puo*_{Rh} codon optimized for *E. coli* and synthesized by Biomatik.

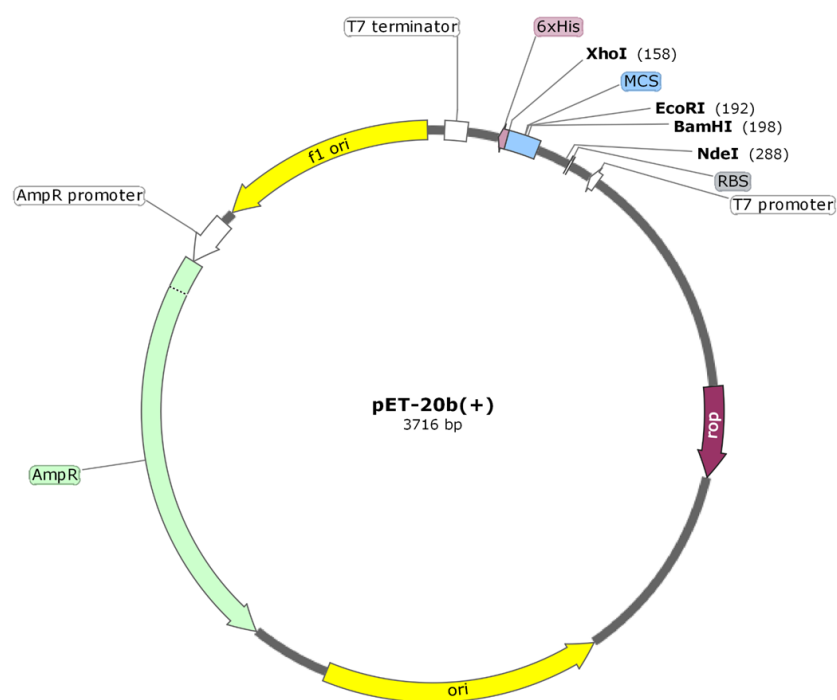


Fig. 11.4: Plasmid map of pET-20b(+)

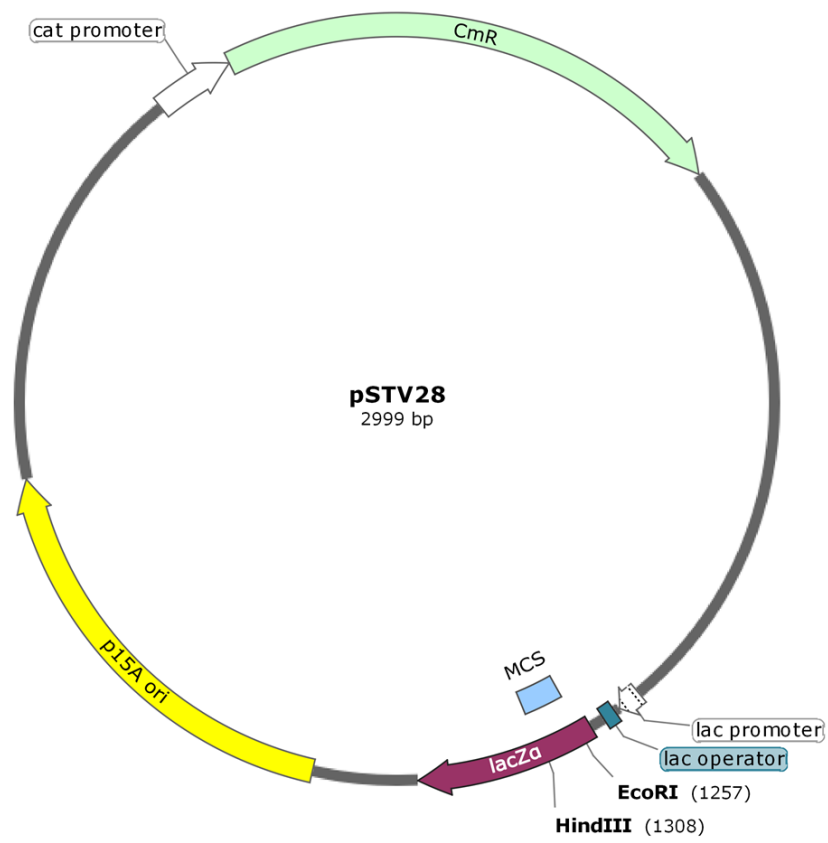


Fig. 11.5: Plasmid map of pSTV28

11.4 Theoretical pathway design

Pentose (C5) sugars can be readily obtained from hemicellulose, a major waste material derived from processing crops, forestry, and food (Buschke et al., 2011, Buschke et al., 2013, Tai et al., 2016). A route from waste C5 sugars to chemicals and fuels is extremely attractive, since this avoids any competition with the C6 sugars required for human food (Tai et al., 2016, Buschke et al., 2011). Recently, an efficient chassis organism to convert C5 sugars directly to TCA (Tricarboxylic acid) cycle intermediate was developed (Rossoni et al., 2018). The developed route converts C5 sugar (xylose) to 2-ketoglutarate *via* a five step reaction (Fig. 9.1 - blue shade). The metabolic route is efficient for utilizing pentose sugars because it can provide up to 100% atom efficiency from sugar to chemical products (Tai et al., 2016, Rossoni et al., 2018).

Therefore, it is desirable to use this published pathway and chassis organism for production of useful chemicals such as platform *N*-heterocycle, Δ^1 -piperidine. A metabolic route to produce Δ^1 -piperidine will be designed and cloned into *E. coli* strain and subsequently used for Δ^1 -piperidine synthesis from xylose (see Fig. 9.1). To exploit the proposed pathway, selected gene/enzymes (Table 11.1) catalysing important steps will be upregulated or overexpressed using a plasmid vector. However, this will be guided by previous reports on lysine and cadaverine biosynthesis using *E. coli* and *C. glutamicum* (Table 11.2). Some of the genes will be sourced from *C. glutamicum*, therefore will be codon optimized for *E. coli*. After overproduction of cadaverine is demonstrated, gene deletion or attenuation will be considered for further optimization of the pathway.

Table 11.1: List of selected enzymes for upregulation

Enzyme	Organism	Gene name
Aspartate kinase (2.7.2.4)	<i>E. coli</i> str. K12 substr. MG1655	lysC
4-Hydroxy-2,3,4,5-tetrahydrodipicolinate synthase (4.3.3.7)	<i>C. glutamicum</i> ATCC 13032	dapA
meso-2,6-Diaminopimelate dehydrogenase (1.4.1.16)	<i>C. glutamicum</i> ATCC 13032	ddh
meso-2,6-Diaminopimelate decarboxylase (4.1.1.20)	<i>E. coli</i> W3110	lysA
Lysine decarboxylase (4.1.1.18)	<i>E. coli</i> W3110	cadA
Putrescine oxidase (1.4.3.10)	<i>R. erythropolis</i> NCIMB 11540	puo

Table 11.2: Summary of previous reports on lysine and cadaverine biosynthesis using *E. coli* and *C. glutamicum*.

Reference	Upregulated/overexpressed genes and approach	Deregulated genes and approach	Result
Qian et al. (2010)	<p>i. CadA (lysine decarboxylase) in a plasmid.</p> <p>ii. DapA (4-Hydroxy-2,3,4,5-tetrahydrodipicolinate synthase), dapB (4-Hydroxy-2,3,4,5-tetrahydrodipicolinate reductase) and lysA (meso-2,6-Diaminopimelate decarboxylase). Through replacement of their native promoter with the strong <i>trc</i> promoter in the genome.</p> <p>iii. Integrating ddh (from <i>C. glutamicum</i>) with its own promoter and transcription terminator into <i>E. coli</i> genome.</p>	Deactivation of cadaverine utilization and degradation pathways – speE (putrescine/cadaverine aminopropyltransferase), speG (spermidine acetyltransferase), puuA (glutamate-putrescine/glutamate-cadaverine Ligase), puuP (putrescine importer), and ygjG (putrescine/cadaverine aminotransferase).	Developed an engineered <i>E. coli</i> strain that constitutively produced 9.61 g/l of cadaverine (231 mmol/mol glucose yield of cadaverine) by fed-batch cultivation from glucose.
Adkins et al. (2013)	Lysine feedback resistant dapA (from <i>C. glutamicum</i>) and lysC (with T352I mutation) in a plasmid.	LdcC and cadA (lysine decarboxylases genes in <i>E. coli</i>).	Engineered <i>E. coli</i> strain producing 5-aminovalerate and glutarate by optimizing the strain to over produce lysine up to 2.25 g/l from glucose (138mmol/mol glucose yield of lysine).
Kind et al. (2010)	<p>i. LysC (with T311I point mutation), dapB, ddh, lysA and ldcC in a plasmid.</p> <p>ii. Increased precursor supply towards oxaloacetate formation by amplification of pycA (pyruvate carboxylase) with P458 point mutation.</p>	Attenuation of flux into the competing threonine pathway (hom – homoserine dehydrogenase) and deletion of pepck (phosphoenolpyruvate carboxylase).	Employed systems-wide metabolic pathway engineering to develop a <i>C. glutamicum</i> strain resulting to a high yield of 200 mmol/mol cadaverine from glucose.

11.4.1 Exploiting lysine pathways in *E. coli* and *C. glutamicum*

Although cadaverine (1,5-diaminopentane) is directly synthesised in *Escherichia coli* by the decarboxylation of lysine, cadaverine is not normally accumulated (Qian et al., 2010, Kang et al., 2007, Tkachenko et al., 2009, Kanjee et al., 2011). Consequently, the first objective of the pathway design was to overproduce cadaverine using *E. coli* by increasing metabolic flux towards lysine production and upregulating lysine decarboxylation. The lysine biosynthetic pathway (Fig. 11.6) already exists in *E. coli* for the production of *s*-diaminopimelate (DAP, a building block for cell wall synthesis) and lysine (an amino acid for protein synthesis). However, this pathway does not normally lead to overproduction of lysine. In *Corynebacterium glutamicum*, which have been successfully engineered to over produce more than a thousand metric tons of lysine per year (Kind et al., 2010), lysine biosynthesis involves a similar pathway but with fewer reaction steps (Fig. 11.7). Engineered *E. coli* strain with lysine production titre over 10 g/L has been reported and this was achieved by overexpressing enzymes from *C. glutamicum* (Qian et al., 2010).

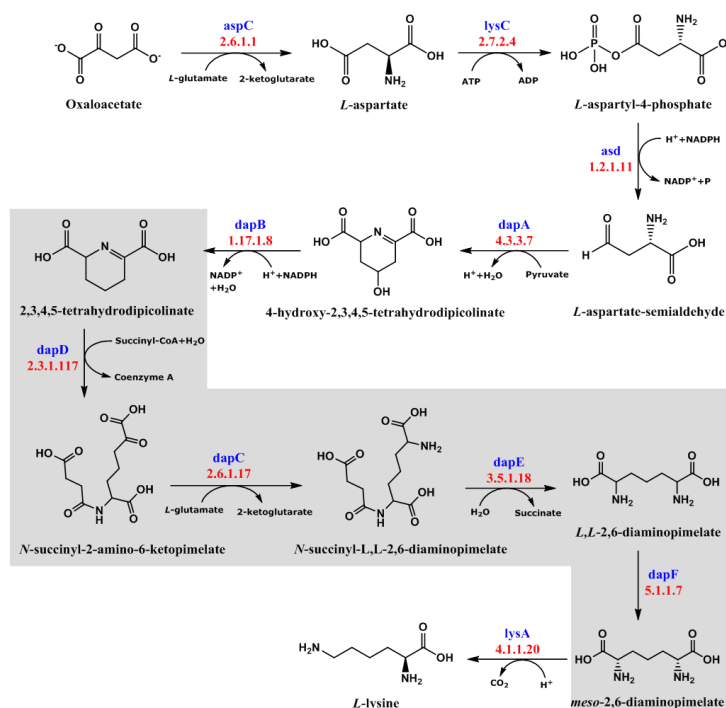


Fig. 11.6: Lysine biosynthesis via oxaloacetate/*s*-aspartate in *E. coli*

Enzymes involved are as follows: **2.6.1.1** - Aspartate transaminase, **2.7.2.4** - aspartate kinase, **1.2.1.11** - aspartate semialdehyde dehydrogenase, **4.3.3.7** - 4-hydroxy-tetrahydrodipicolinate synthase, **1.17.1.8** - 4-hydroxy-tetrahydrodipicolinate reductase, **2.3.1.117** - tetrahydrodipicolinate succinylase, **2.6.1.17** - *N*-succinyldiaminopimelate aminotransferase, **3.5.1.18** - *N*-succinyl-L-diaminopimelate desuccinylase, **5.1.1.7** - diaminopimelate epimerase, **4.1.1.20** - diaminopimelate decarboxylase.

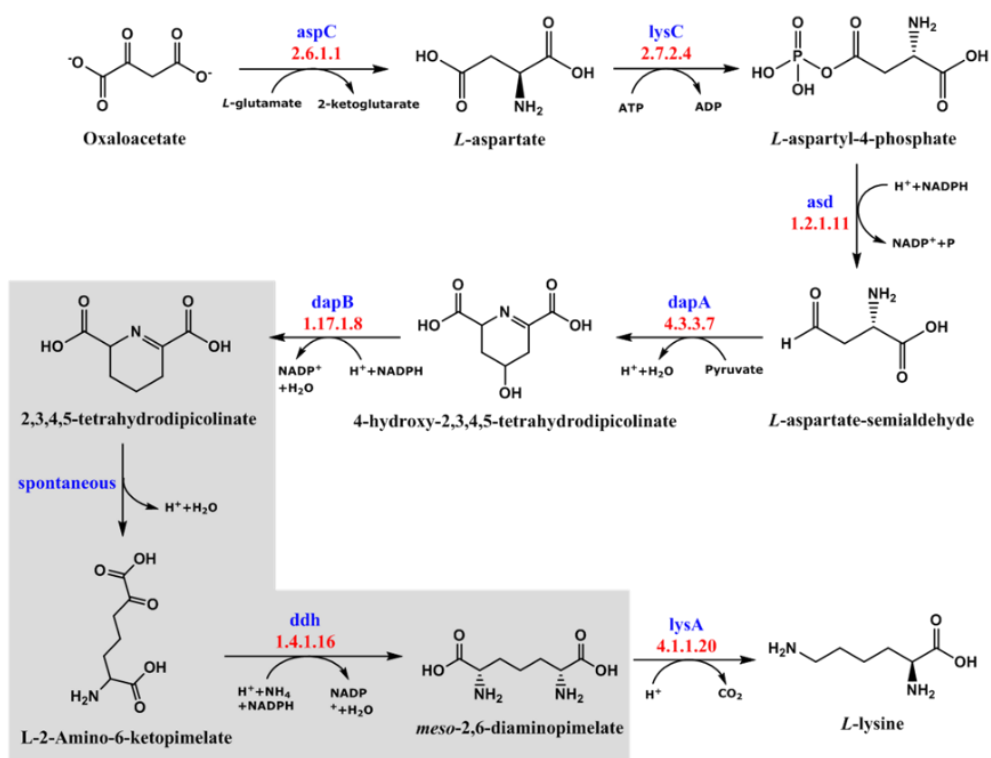


Fig. 11.7: Lysine biosynthesis via oxaloacetate/s-aspartate in *Corynebacterium glutamicum*.

Enzymes involved are as follows: 2.6.1.1 - Aspartate transaminase, 2.7.2.4 - aspartate kinase, 1.2.1.11 - aspartate semialdehyde dehydrogenase, 4.3.3.7 - 4-hydroxy-tetrahydrodipicolinate synthase, 1.17.1.8 - 4-hydroxy-tetrahydrodipicolinate reductase, 1.4.1.16 - meso-diaminopimelate dehydrogenase, and 4.1.1.20 - diaminopimelate decarboxylase.

11.4.2 Plasmid library design

The chassis organism published earlier by Rossoni et al. (2018) serves as a template organism to further engineer the proposed pathway in this study. The designed plasmid in the chassis organism is a ColE1 plasmid; therefore, the plasmid designs proposed in this study will use a ColE1 compatible plasmid vector such as pACYC184 or pSTV28.

Plasmid library 1 – A plasmid with an operon containing *puo_{Rh}*, *cadA*, *lysA*, *ddh*, *dapA*, *lysC* genes.

Justification:

- Adkins et al. (2013) produced 2.25 g/l lysine from glucose in *E. coli* by only overexpressing feedback resistant *dapA* (*C. glutamicum*) and *lysC* in a plasmid.

- ii. Qian et al. (2010) produced 9.61 g/l lysine from glucose (fed-batch cultivation) in *E. coli* by upregulating *dapA*, *dapB* and *lysA* through replacement of their native promoter with a strong *trc* promoter, cloning *ddh* from *C. glutamicum* with its own promoter and terminator, and overexpressed *cadA* using a plasmid.
- iii. Ddh from *C. glutamicum* removes two steps from the native lysine biosynthesis pathway in *E. coli*, therefore improves its efficiency.

Plasmid library 1 is the target design; however, other options that could be designed are listed below.

Plasmid library 2 - A plasmid design with an operon containing *puo*, *cadA*, *lysA*, *ddh* genes.

Justification:

- i. Adkins et al. (2013) produced 2.25 g/l lysine from glucose in *E. coli* by only overexpressing feedback resistant *dapA* (*C. glutamicum*) and *lysC* in a plasmid.

Plasmid library 3 - A plasmid with an operon overexpressing *puo*, *cadA*, *ddh*, *dapA*, *lysC* genes.

Justification:

- i. Adkins et al. (2013) produced 2.25 g/l lysine from glucose in *E. coli* by only overexpressing feedback resistant *dapA* (*C. glutamicum*) and *lysC* in a plasmid.
- ii. Ddh from *C. glutamicum* removes two steps from the native lysine biosynthesis pathway in *E. coli*, therefore improves its efficiency.

After the design of the pathway and plasmids, experimental efforts were made. Taking a retrosynthetic approach, a plasmid containing lysine decarboxylase (CadA) and putrescine oxidase (PuOR_h) was designed (Fig. 11.8). The aim of this design was to develop a bacterial strain that could convert lysine into Δ^1 -piperidine. Unfortunately, further work was not conducted using this designed plasmid due to time pressure.

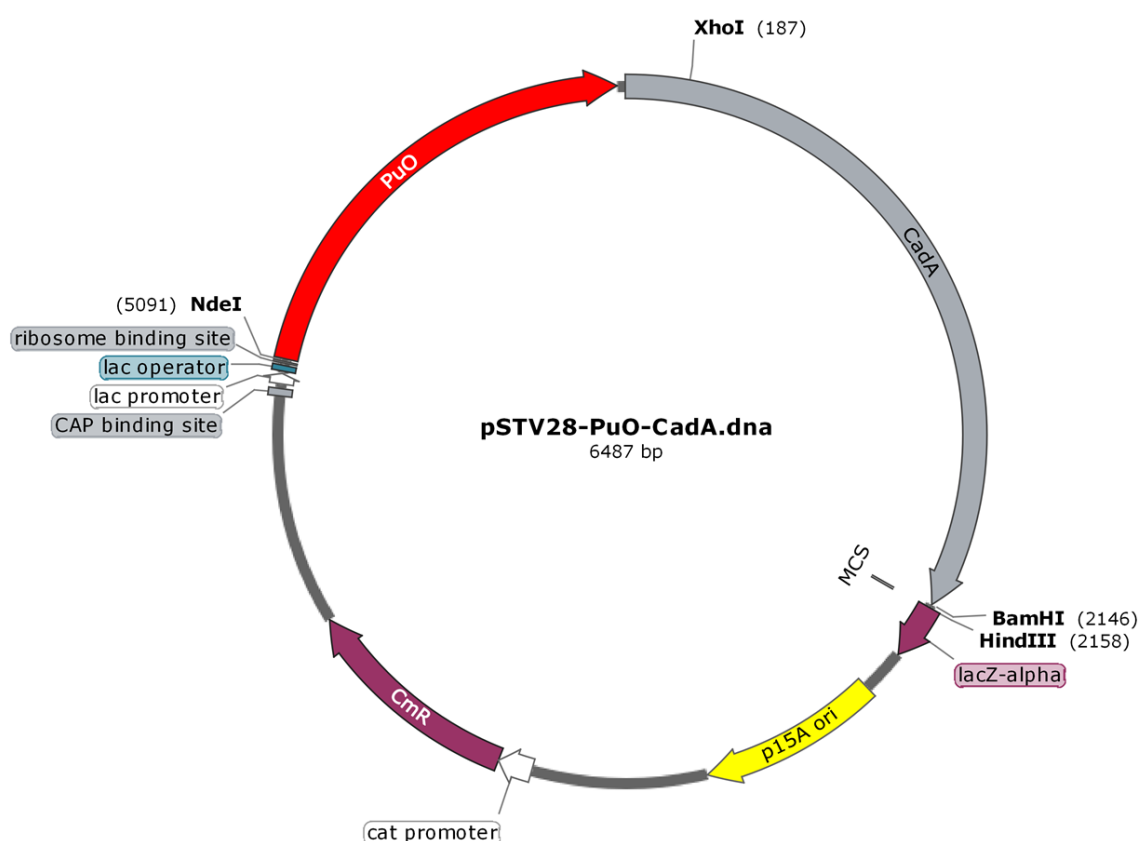


Fig. 11.8: Plasmid map of pSTV28_*puo_{Rh}*_cadA

The *puo_{Rh}* gene encoding PuO_{Rh} (previously codon optimized for *E. coli* and synthesized by Biomatik) and *cadA* gene encoding CadA were amplified by PCR from pET-20b(+)_*puo_{Rh}* and *E. coli* MG1655 genome, and cloned/assembled into the pSTV28 plasmid by NEBHIFI Builder.

The genes selected in this pathway have been cloned, expressed and sequenced elsewhere. These sequences are readily available in Genbank and the techniques to do the practical experiments are well documented. However, as this study progressed, it was difficult to carry out the experiments because of time pressure. Finally, the above-proposed pathway (Fig. 9.1) represents a sustainable and simple proof of concept for bioproduction of Δ^1 -piperidine and its multimeric species from renewable feedstocks.

11.5 Gene and amino acid sequences

Putrescine oxidase - *Rhodococcus erythropolis* NCIMB 11540 (PuO_{Rh})

Cadaverine to 5-Aminopentanal: Putrescine oxidase (PuO):

Putrescine oxidase catalyses the oxidative deamination of cadaverine into 5-aminopentanal. 5-aminopentanal subsequently undergoes spontaneous (non-enzyme catalysed) Schiff-base formation to Δ^1 -piperidine. Although, enzymes [speE (putrescine/cadaverine aminopropyltransferase), speG (spermidine acetyltransferase) and puuA (glutamate-putrescine/glutamate-cadaverine Ligase)] catalysing consumption of cadaverine have been reported in *E. coli* (Zelder et al., 2009, Qian et al., 2010), overexpression of PuO will increase carbon flow towards formation of Δ^1 -piperidine. Putrescine oxidase from *Rhodococcus erythropolis* (PuO_{Rh}) has been previously overexpressed in *E. coli* and characterized (van Hellemond et al., 2008b, Kopacz et al., 2014), therefore it will be used further for this work.

Gene sequence (1362 bp, GenBank Accession Number: EU240877)

```
GTGCCTACTCTCCAGAGAGATGTTGCAATCGTCGGCGCCGGCCCCCTCTGGCCTGGCAGCGGCAACCGCGC
TGCGCAAGGCCGGCTTGTCCTGTCGCCGTGATCGAAGCACGCGATCGTGTCTGGCGGCCGCACGTGGACCGA
CACCATCGACGGCGCAGTCTCTGGAGATCGGGCGCCAGTGGGTCTCCCCGACCAGACTGCTCTGATCTCC
TTGCTCGACGAACTCGGCCTGAAGACTTTTCGAGCGCTACCGCGAGGGCGAGTCCGTCTACATCTCGTCAG
CAGGCGAGCGGACTCGATACACGGGCGATTCTTCCCCACGAACGAGACCACCAAGAGGAGATGGACCG
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AAGCCGAGTGATCTGGCCGTACCAACCAACCTCTACTCCCGGATCTCCTACGATCCCCGCTGCCGCGT
CGTCAGCACCAGATGCATCAGCATCAGTCTCTCGGCCTCGTCATCAAGGTGCACGCCGTGTACGAGACGC
CTTTCTGGCGCGAAGACGGCCTCTCCGGCACCGGCTTCGGCGCGTCCGAGGTAGTGCAGGAGGTGTACGA
CAACACCAACCACGAGGACGATCGCGGCACCTGGTTCGCTTTTGTCTCCGACGAGAAGGCCGACGCGATG
TTCGAGCTTTCCGCCGAGGAGCGTAAGGCCACGATTCTGGCCTCACTCGCCCGCTACCTGGGCCCCGAAGG
CCGAAGAGCCGGTTGTGTACTACGAATCCGACTGGGGCTCGGAGGAATGGACCCGCGGTGCGTACGCGGC
GAGCTTCGATCTCGGCGGCCTGCACCGCTACGGCGCGGATTCCCGCACGCCCGTCGGACCGATCCACTTC
TCGTGCTCCGACATCGCAGCCGAGGGGTACCAGCACGTGACGGTGCCGTTCGGATGGGTGACGCGACCG
CCGCCGACATCATCGCCCGCAGCAAGGCCTGA
```

Codon optimized gene sequence (The gene sequence used was codon optimized for expression in *E. coli* by the synthesizing company, Biomatik. Added start codon is indicate in green)

```
atggttccgaccctgcagcgtgatgtggctatcgtgggtgcgggtccgtctggcctggctgctgctactgctctgcgcaaagcg
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aattggcgccagtggttagcccgaccagactgcgctgatctctgctggtgatgaactgggtctgaaaaccttcgaacgct
accgtgaaggtgaatctgtttacatcagctccgctggtgaacgcactcgctataccggtgactctttcccgaccaacgagacca
```

ctaagaaagagatggaccgcctgatcgatgaaatggatgacctggcagctcagattggcgctgaagaaccgtggcgccacc
 cgctggctcgtgatctggacaccgtttcttttaaacagtggtgatcaaccagctgacgacgcagaggcgctgataacattg
 gtctgttcacgctggcggtatgctgaccaagccggcacacagcttttctgactgcaggcagttctgatggcagcatctcgg
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 gcggacattatcgcgctctaaagcgctc

Amino acid sequence (453 aa, NCBI accession number: ABY74497)

"VPTLQRDVAIVGAGPSGLAAATALRKAGLSVAVIEARDRVGGRTWTDITDIDGAVLEIGGQWVSPDQ
 TALISLLDELGLKTFERYREGESVYISSAGERTRYTGDSFPTNETTKKEMDRLIDEMDDLAAQIGAE
 PWAHPLARLDLTVSFKQWLINQSDDAEARDNIGLFIAGGMLTKPAHSFSAALQAVLMAASAGSFS
 SHL VDEDFILDKRVIGGMQQVSIRMAEALGDDVFLNAPVRTVKWNESGATVLADGDIRVEASRVILAVP
 PNLYSRISYDPPLRRQHQMHHQSLGLVIKVHAVYETPFWREDGLSGTGFGASEVVQEVYDNTNH
 EDDRGTLVAFVSDEKADAMFELSAEERKATILASLARYLGPKAEPPVYYESDWGSEEWTRGAYAA
 SFDLGGLHRYGADSRTPVGPIHFSCSDIAAEGYQHVDGAVRMGQRTAADIIARSKA"

11.5.1 Theoretical pathway design: Gene sequence of enzymes for upregulation

Aspartate kinase (LysC, EC 2.7.2.4): *Escherichia coli* str. K-12 substr. MG1655

Aspartate to L-aspartyl-4-phosphate:

About 27% of *E. coli*'s nitrogen flows through aspartate because it serves as a substrate for synthesis of other amino acids (Alanine, asparagine, and arginine) and nucleotides (uridine triphosphate and cytidine triphosphate). Therefore, the phosphorylation of aspartate to L-aspartyl-4-phosphate catalysed by aspartate kinase (LysC, EC 2.7.2.4, and encoded by *lysC* gene) will be up regulated in order to increase carbon flow towards lysine biosynthesis. Also, it has been reported that the missense mutation T352I on *E. coli*'s aspartate kinase, completely eliminates lysine allosteric inhibition (Falco et al., 1995, Ogawa-Miyata et al., 2001, Adkins et al., 2013). This will be explored to optimize the pathway flux to our product of interest.

Protein translation:

"MSEIVVSKFGGTSVADFDAMNRSADIVLSDANVRLVVLASAGITNLLVALAEGLEPGERFEKLDAI
RNIQFAILERLRYPNVIREEIERLLENITVLAEEAALATSPALTDELVSHGELMSTLLFVEILRERDVQA
QWFDVRKVMRTNDRFGRAEPDIAALAEALQLLPRLNEGLVITQGFIGSENKGRITTLGRGGSDYT
AALLAEALHASRVDIWTDPGIYTTDPRVVSAKRIDEIAFAEAAEMATFGAKVLHPATLLPAVRSDI
PVFVGSSKDPAGGTLVCNKTENPPLFRALALRRNQTLTLHSLNMLHSRGFLAEVFGILARHNISVD
LITTSEVSVALTLDTTGSTSTGDTLLTQSLLMELSALCRVEVEEGLALVALIGNDLSKACGVGKEVFG
VLEPFNIRMICYGASSHNLCFLVPGEDAEQVVQKLHSLNLF"

RBS: TTAAGTATAAGAAGGAGATATACA

Gene sequence:

ATGAACCGCAGCGCTGATATTGTGCTTTCTGATGCCAACGTGCGTTTAGTTGTCCTCTCGGCTTC
TGCTGGTATCACTAATCTGCTGGTCGCTTTAGCTGAAGGACTGGAACCTGGCGAGCGATTCTGAA
AACTCGACGCTATCCGCAACATCCAGTTTGCCATTCTGGAACGTCTGCGTTACCCGAACGTTAT
CCGTGAAGAGATTGAACGTCTGCTGGAGAACATTACTGTTCTGGCAGAAGCGGCGGCGCTGGCA
ACGTCTCCGGCGCTGACAGATGAGCTGGTCAGCCACGGCGAGCTGATGTCGACCCTGCTGTTTG
TTGAGATCCTGCGCGAACGCGATGTTTCAGGCACAGTGGTTTGATGTACGTAAAGTGATGCGTAC
CAACGACCGATTTGGTCGTGCAGAGCCAGATATAGCCGCGCTGGCGGAACTGGCCGCGCTGCA
GCTGCTCCACGTCTCAATGAAGGCTTAGTGATCACCCAGGGATTTATCGGTAGCGAAAATAAAG
GTCGTACAACGACGCTTGCCGTGGAGGCAGCGATTATACGGCAGCCTTGCTGGCGGAGGCTT
TACACGCATCTCGTGTTGATATCTGGACCGACGTCCCGGGCATCTACACCACCGATCCACGCGTA
GTTTCCGCAGCAAAACGCATTGATGAAATCGCGTTTGCCGAAGCGGCAGAGATGGCAACTTTTG
GTGCAAAAGTACTGCATCCGGCAACGTTGCTACCCGCAGTACGCAGCGATATCCCGGTCTTTGT
CGGCTCCAGCAAAAGACCCACGCGCAGGTGGTACGCTGGTGTGCAATAAACTGAAAATCCGCCG
CTGTTCCGCGCTCTGGCGCTTCGTCGCAATCAGACTCTGCTCACTTTGCACAGCCTGAATATGCT
GCATTCTCGCGGTTTCCTCGCGGAAGTTTTCGGCATCCTCGCGCGGCATAATATTTTCGGTAGACT
TAATCACACGTGAGAAGTGAGCGTGGCATTAAACCCTTGATACCACCGGTTCAACCTCCACTGGC
GATACGTTGCTGACGCAATCTCTGCTGATGGAGCTTTCCGCACTGTGTCGGGTGGAGGTGGAAG
AAGGTCTGGCGCTGGTCGCGTTGATTGGCAATGACCTGTCAAAAGCCTGCGGCGTTGGCAAAGA
GGTATTCGGCGTACTGGAACCGTTCAACATTCGCATGATTTGTTATGGCGCATCCAGCCATAACC
TGTGCTTCCTGGTGCCCGGCGAAGATGCCGAGCAGGTGGTGCAAAAAGTGCATAGTAATTTGTT
TGAGTAA

4-hydroxy-2,3,4,5-tetrahydrodipicolinate synthase (dapA, EC 4.3.3.7):

Corynebacterium glutamicum ATCC 13032

L-Aspartate-semialdehyde to 4-hydroxy-2,3,4,5-tetrahydrodipicolinate:

The first step in the synthesis of lysine is the condensation of pyruvate and aspartate semialdehyde to form 4-hydroxy-2,3,4,5-tetrahydrodipicolinate. It is catalysed by 4-hydroxy-2,3,4,5-tetrahydrodipicolinate synthase (DapA, EC 4.3.3.7). The enzyme is the product of the *dapA* gene, and is a homotetramer with subunits having a molecular weight of 31KD. Lysine has been shown to allosterically inhibit DapA in Gram-negative bacteria e.g. *E. coli*, therefore it is considered as a rate-limiting enzyme of the pathway. However, *dapA* from Gram-positive bacteria such as *Corynebacterium glutamicum* shows little or no

lysine inhibition, and it has been successfully overexpressed in *E. coli* (Qian et al., 2010, Adkins et al., 2013). Also, L-aspartate-semialdehyde serves as the intermediate for L-homoserine biosynthesis which is required for cellular metabolism, therefore, *dapA* from *C. glutamicum* will be heterogeneously overexpressed in order to drive carbon flow towards lysine synthesis and prevent lysine feedback inhibition. The supply of pyruvate also affects this reaction step. Since pyruvate is not synthesized from the designed xylose metabolic pathway, it is expected that it will be supplied by the oxidative decarboxylation of malate by malic enzyme. Malic enzyme from *E. coli* have been isolated and characterised, and its gene has been sequenced and annotated (Wang et al., 2011, Bologna et al., 2007, Kwon et al., 2007). Therefore, upregulation of this enzyme may be considered at the later stage of this study for pathway optimization.

Protein translation:

"MSTGLTAKTGVEHFGTVGVAMVTPFTESGDIDIAAGREVAAYLVDKGLDSLVLAGTTGESPTTTAA
EKLELLKAVREEVGDRAKLIAGVGTNNRTSVELAEAAASAGADGLLVTPYYSKPSQEGLLAHFGAI
AAATEVPICLYDIPGRSGIPIESDTMRRLSELPTILAVKDAKGDLVAATSLIKETGLAWYSGDDPLNLV
WLALGSGFISVIGHAAPTALRELYTSFEEDLVRAREINAKLSPLVAAQGRLLGGVSLAKAALRLQGI
NVGDPRLPIMAPNEQEALREDMKKAGVL"

RBS 1: TCGGCACCGTTGGAGTAGCA

RBS 2: ACAGGTTTAACAGCTAAGACCGGAGTAGAGCACTTCGGCACCGTTGGAGTAGCA

Gene sequence:

ATGGTTACTCCATTACGGAATCCGGAGACATCGATATCGCTGCTGGCCGCGAAGTCGCGGCTT
ATTTGGTTGATAAGGGCTTGGATTCTTTGGTTCTCGCGGGCACCCTGGTGAATCCCCAACGACA
ACCGCCGCTGAAAACTAGAACTGCTCAAGGCCGTTCTGTAGGAAGTTGGGGATCGGGCGAAG
CTCATCGCCGGTGTCTGGAACCAACAACACGCGGACATCTGTGGAAGTTGCGGAAGCTGCTGCTT
CTGCTGGCGCAGACGGCCTTTTAGTTGTAACCTCTTATTACTCCAAGCCGAGCCAAGAGGGATTG
CTGGCGCACTTCGGTGCAATTGCTGCAGCAACAGAGGTTCCAATTTGTCTCTATGACATTCCTGG
TCGGTCAGGTATTCCAATTGAGTCTGATACCATGAGACGCCTGAGTGAATTACCTACGATTTTGG
CGGTCAAGGACGCCAAGGGTGACCTCGTTGCAGCCACGTCATTGATCAAAGAAACGGGACTTGC
CTGGTATTCAGGCGATGACCCACTAAACCTTGTGTTGGCTTGCTTTGGGCGGATCAGGTTTCATTT
CCGTAATTGGACATGCAGCCCCACAGCATTACGTGAGTTGTACACAAGCTTCGAGGAAGGCGA
CCTCGTCCGTGCGCGGGAAATCAACGCCAACTATCACCGCTGGTAGCTGCCCAAGGTCGCTTG
GGTGGAGTCAGCTTGGCAAAAGCTGCTCTGCGTCTGCAGGGCATCAACGTAGGAGATCCTCGAC
TTCCAATTATGGCTCCAAATGAGCAGGAAGTTGAGGCTCTCCGAGAAGACATGAAAAAGCTGTA
A

meso-2,6-Diaminopimelate dehydrogenase (ddh, EC 1.4.1.16):
Corynebacterium glutamicum ATCC 13032

L-2-Amino-6-ketopimelate to meso-2,6-diaminopimelate:

Diaminopimelate dehydrogenase (Ddh, EC 1.4.1.16) is a NADPH-dependent enzyme that catalyses the reductive amination of *L*-2-amino-6-ketopimelate (the acyclic form of *L*-2,3,4,5-tetrahydrodipicolinate) to *meso*-Diaminopimelate. *C. glutamicum* is among the small group of Gram-positive bacteria that possess Ddh activity, as a result, can synthesise lysine by both the dehydrogenase or succinylase pathway. Ddh is coded by the *ddh* gene in *C. glutamicum* and it has been sequenced and studied in *E. coli* (Falco et al., 1995, Kind et al., 2010, Qian et al., 2010). Therefore, *ddh* gene will be cloned and overexpressed in *E. coli* to further increase flux to the target product.

Protein translation:

"MTNIRVAIVGYGNLGRSVEKLIQKPDMDLVGIFSRRLDTKTPVFDVADVDKHADDVDVFLCM
GSATDIPEQAPKFAQFACTVDYDNHRDIPRHRQVMNEAATAAGNVALVSTGWDPGMFSINRVYA
AAVLAEHQQHTFWGPGLSQGHSDALRRIPGVQKAVQYTLPSDALEKARRGEAGDLTGKQTHKRQ
CFVVADAADHERIENDIRTMPDYFVGVEVEVNFIDEATFDSEHTGMPHGHHVITTDGTTGGFNHTVE
YILKLDNRNPFTASSQIAFGRAAHMKQQGQSGAFTVLEVAPYLLSPENLDDLIARDV"

RBS: AATTTTGGAGGATTACAAGAAC

Gene sequence:

ATGACCAACATCCGCGTAGCTATCGTGGGCTACGGAAACCTGGGACGCAGCGTCGAAAAGCTTA
TTGCCAAGCAGCCCGACATGGACCTTGTAGGAATCTTCTCGCGCCGGGCCACCCTCGACACAAA
GACGCCAGTCTTTGATGTCGCCGACGTGGACAAGCACGCCGACGACGTGGACGTGCTGTTCTCTG
TGCATGGGCTCCGCCACCGACATCCCTGAGCAGGCACCAAAGTTCGCGCAGTTCGCCTGCACCG
TAGACACCTACGACAACCACCGCGACATCCCACGCCACCGCCAGGTCATGAACGAAGCCGCCAC
CGCAGCCGGCAACGTTGCACTGGTCTCTACCGGCTGGGATCCAGGAATGTTCTCCATCAACCGC
GTCTACGCAGCGGCAGTCTTAGCCGAGCACCAGCAGCACACCTTCTGGGGCCAGGTTTGTAC
AGGGCCACTCCGATGCTTTGCGACGCATCCCTGGCGTTCAAAAGGCAGTCCAGTACACCCTCCC
ATCCGAAGACGCCCTGGAAAAGGCCCGCCGCGCGCAAGCCGGCGACCTTACCGGAAAGCAAAC
CCACAAGCGCCAATGCTTCGTGGTTGCCGACGCGGCCGATCACGAGCGCATCGAAAACGACATC
CGCACCATGCCTGATTACTTCGTTGGCTACGAAGTCGAAGTCAACTTCATCGACGAAGCAACCTT
CGACTCCGAGCACACCGGCATGCCACACGGTGGCCACGTGATTACCACCGGCGACACCGGTGG
CTTCAACCACACCGTGGAATACATCCTCAAGCTGGACCGAAACCCAGATTTACCGCTTCCTCAC
AGATCGCTTTTCGGTCGCGCAGCTACCGCATGAAGCAGCAGGGCCAAAGCGGAGCTTTACCGT
CCTCGAAGTTGCTCCATACCTGCTCTCCCCAGAGAACTTGGACGATCTGATCGCACGCGACGTCT
AA

meso-2,6-Diaminopimelate decarboxylase (LysA, EC 4. 1. 1. 20): *Escherichia coli* str. K12 substr. MG1655

meso-2,6-Diaminopimelate to Lysine:

Diaminopimelate decarboxylase (LysA, EC 4. 1. 1. 20), the product of *lysA* gene is a PLP-dependent enzyme that is responsible for catalysing the final reaction of the lysine biosynthesis pathway. In this non-reversible reaction, LysA converts the substrate meso-DAP to lysine and carbon dioxide. In this work, the *lysA* gene will be upregulated to effectively increase flux towards product of interest.

Protein translation:

"MPHSLFSTDTDLTAENLLRLPAEFGCPVWVYDAQIIRRQIAALKQFDVVRFAQKACSNIHILRLMRE QGVKVDVSVSLGEIERALAAAGYNPQTHPDDIVFTADVIDQATLERVSELQIPVNAGSVDMLDQLGQV SPGHRVWLRVNPFGFHGHSQKTNTGGENSKHGIWYTDLPALDVIQRHHLQLVGIHMHIGSGVDY AHLEQVCGAMVRQVIEFGQDLQAISAGGGLSVPYQQGEEAVDTEHYGLWNAAREQIARHLGHPV KLEIEPGRFLVAQSGVLITQVRSVKQMGSRHFLVDAGFNDLMPAMYGSYHHISALAADGRSLEH APTVETVVAGPLCESGDVFTQQEGGNVETRALPEVKAGDYLVLHDTGAYGASMSSNYSRPLLPEVL FDNGQARLIRRRQTIEELLALELL"

RBS: GCACTTATCTGGAGTTTGT

Gene sequence:

ATGCCACATTCACCTGTTTCAGCACCGATACCGATCTCACCGCCGAAAATCTGCTGCGTTTGCCCGC TGAATTTGGCTGCCCCGGTGTGGGTCTACGATGCGCAAATTATTCGTCGGCAGATTGCAGCGCTG AACAGTTTTGATGTGGTTCGCTTTGCACAGAAAGCCTGTTCCAATATTCATATTTTTCGCTTAATG CGTGAGCAGGGCGTGAAAGTGATTCCGTCTCGTTAGGCGAAATAGAGCGTGCGTTGGCGGCG GGTACAATCCGCAAACGCACCCCGATGATATTGTTTTACGGCAGATGTTATCGATCAGGCGAC GCTTGAACGCGTCAGTGAATTGCAAATCCGGTGAATGCGGGTTCTGTTGATATGCTCGACCAAC TGGGCCAGTTTTCGCCAGGGCATCGGGTATGGCTGCGCGTTAATCCGGGGTTTGGTCACGGAC ATAGCCAAAAAACCAATACCGGTGGCGAAAACAGCAAGCACGGTATCTGGTACACCGATCTGCC CGCCGCACTGGACGTGATAACGTCATCATCTGCAGCTGGTCGGCATTACATGCACATTGGT CTGGCGTTGATTATGCCCATCTGGAACAGGTGTGTGGTGCTATGGTGCGTCAGGTCATCGAATT CGGTCAGGATTTACAGGCTATTTCTGCGGGCGGTGGGCTTTCTGTTCCCTTATCAACAGGGTGAA GAGGCGGTTGATACCGAACATTATTATGGTCTGTGGAATGCCGCGCGTGAGCAAATCGCCCGCC ATTTGGGCCACCCTGTGAAACTGGAAATTGAACCGGGTCGCTTCCTGGTAGCGCAGTCTGGCGT ATTAATTACTCAGGTGCGGAGCGTCAAACAAATGGGGAGCCGCCACTTTGTGCTGGTTGATGCC GGGTTCAACGATCTGATGCGCCCGGCAATGTACGGTAGTTACCACCATATCAGTGCCCTGGCAG CTGATGGTCGTTCTCTGGAACACGCGCCAACGGTGGAACCGTCGTCGCCGGACCGTTATGTGA ATCGGGCGATGTCTTTACCCAGCAGGAAGGGGGAAATGTTGAAACCCGCGCCTTGCCGGAAGT GAAGGCAGGTGATTATCTGGTACTGCATGATACAGGGGCATATGGCGCATCAATGTCATCCAAC TACAATAGCCGTCCGCTGTTACCAGAAGTTCTGTTTGATAATGGTCAGGCGCGGTTGATTCGCCG TCGCCAGACCATCGAAGAATTACTGGCGCTGGAATTGCTTTAA

Lysine decarboxylase (CadA, EC 4. 1. 1. 18): *Escherichia coli* str. K12 substr. MG1655

Lysine to cadaverine: Lysine decarboxylase (LdcC and CadA):

Lysine decarboxylase catalyses the decarboxylation of lysine to cadaverine. There are two lysine decarboxylases in *E. coli*, encoded by the *cadA* and *ldcC* genes. The *cadA* gene product, lysine decarboxylase 1 (CadA), is a more active and inducible enzyme with a pH optimum of 5.5. The constitutively expressed *ldcC* gene product, lysine decarboxylase 2 (LdcC), differs from the *cadA*-encoded enzyme in that it is weakly expressed, more thermosensitive and has activity over a broad range of pH with a pH optimum of 7.6. This pH favours optimal growth of *E. coli* and functioning of other enzymes especially putrescine oxidase (PuO) which catalyses the final step to Δ^1 -piperidine. However, CadA has been overexpressed for cadaverine production in *E. coli* and a good yield (231 mmol/mol glucose yield of cadaverine) was obtained (Qian et al., 2010). Therefore, overexpression of the *cadA* encoded lysine decarboxylase will be carried out.

Protein translation:

"MNVIAILNHMGVYFKEEPIRELRALERLNFQIVYPNDRDDLKLIENNARLCGVIFDWDKYNLELCE
EISKMNENLPLYAFANTYSTLDVSLNDRLLQISFFEYALGAAEDIANIKQTTDEYINTILPPLTKALFK
YVREGKYTFCTPGHMGGTAFQKSPVGSIFYDFGPNTMKSDISISVSELGSLLDHSGPHKEAEQYIA
RVFNADRSYMTNGTSTANKIVGMYAPAGSTILIDRNCHKSLTHLMMMSDVTPIYFRPTRNAYGIL
GGIPQSEFQHATIAKRVKETPNATWVPVHAVITNSTYDGLLYNTDFIKKTLDVKSIHFDSA WVPYTNF
SPIYEGKCGMSGGRVEGKVIYETQSTHKLLAAFSQASMIHVKGDVNEETFNEAYMMHTTTSPHYGI
VASTETAAAMMKGNAGKRLINGSIERAIKFRKEIKRLRTESDGWFFDVWQPDHIDTTECWPLRSDS
TWHGFKNIDNEHMYLDPIKVTLLTPGMEKDGTM SDFGIPASIVAKYLDEHGIVVEKTGPYNLLFLFSI
GIDKTKALSLLRALTDFKRAFDLNLRVKNMLPSLYREDPEFYENMRIQELAQNIHKLIVHHNLPDLMY
RAFEVLPTMVMTPYAAFQKELHGMTEEVYLDENVGRINANMILPYPPGVPLVMPGEMITEESRPVLEF
LQMLCEIGAHPGFETDIHGAYRQADGRYTVKVLKEESKK"

RBS: TTACCTGGAGATATGACT

Gene sequence:

ATGAACGTTATTGCAATATTGAATCACATGGGGGTTTATTTTAAAGAAGAACCCATCCGTGAACCT
CATCGCGCGCTTGAACGTCTGAACCTCCAGATTGTTTACCCGAACGACCGTGACGACTTATTTAA
ACTGATCGAAAACAATGCGCGTCTGTGCGGCGTTATTTTGAAGTGGGATAAATATAATCTCGAGC
TGTGCGAAGAAATTAGCAAAATGAACGAGAACCTGCCGTTGTACGCGTTCGCTAATACGTATTCC
ACTCTCGATGTAAGCCTGAATGACCTGCGTTTACAGATTAGCTTCTTTGAATATGCGCTGGGTGC
TGCTGAAGATATTGCTAATAAGATCAAGCAGACCACTGACGAATATATCAACACTATTCTGCCTCC
GCTGACTAAAGCACTGTTTAAATATGTTTCGTGAAGGTAAATATACTTTCTGTACTCCTGGTCACAT
GGGCGGTACTGCATTCCAGAAAAGCCCGGTAGGTAGCCTGTTCTATGATTCTTTGGTCCGAATA
CCATGAAATCTGATATTTCCATTTAGTATCTGAACTGGGTTCTCTGCTGGATCACAGTGGTCCAC
ACAAAGAAGCAGAACAGTATATCGCTCGCGTCTTTAACGCAGACCGCAGCTACATGGTGACCAA

CGGTACTTCCACTGCGAACAAAATTGTTGGTATGTACTCTGCTCCAGCAGGCAGCACCATTTCTGA
TTGACCGTAACTGCCACAAATCGCTGACCCACCTGATGATGATGAGCGATGTTACGCCAATCTAT
TTCCGCCCCGACCCGTAACGCTTACGGTATTCTTGGTGGTATCCCACAGAGTGAATTCAGCACGC
TACCATTGCTAAGCGCGTGAAAGAAACACCAAACGCAACCTGGCCGGTACATGCTGTAATTACCA
ACTCTACCTATGATGGTCTGCTGTACAACACCGACTTCATCAAGAAAACACTGGATGTGAAATCC
ATCCACTTTGACTCCGCGTGGGTGCCTTACACCAACTTCTCACCGATTTACGAAGGTAAATGCGG
TATGAGCGGTGGCCGTGTAGAAGGGAAAGTGATTTACGAAACCCAGTCCACTCACAAACTGCTG
GCGGCGTTCTCTCAGGCTTCCATGATCCACGTTAAAGGTGACGTAAACGAAGAAACCTTTAACGA
AGCCTACATGATGCACACCACCACTTCTCCGCACTACGGTATCGTGGCGTCCACTGAAACCGCTG
CGGCGATGATGAAAGGCAATGCAGGTAAGCGTCTGATCAACGGTTCTATTGAACGTGCGATCAA
ATTCCGTAAAGAGATCAAACGTCTGAGAACGGAATCTGATGGCTGGTTCTTTGATGTATGGCAGC
CGGATCATATCGATACGACTGAATGCTGGCCGCTGCGTTCTGACAGCACCTGGCACGGCTTCAA
AAACATCGATAACGAGCACATGTATCTTGACCCGATCAAAGTCACCCTGCTGACTCCGGGGATG
GAAAAAGACGGCACCATGAGCGACTTTGGTATTCCGGCCAGCATCGTGGCGAAATACCTCGACG
AACATGGCATCGTTGTTGAGAAAACCGGTCCGTATAACCTGCTGTTCTGTTGAGCATCGGTATC
GATAAGACCAAAGCACTGAGCCTGCTGCGTGCTCTGACTGACTTTAAACGTGCGTTTCGACCTGA
ACCTGCGTGTGAAAAACATGCTGCCGTCTCTGTATCGTGAAGATCCTGAATTCTATGAAAAATG
CGTATTCAGGAAGTGGCTCAGAATATCCACAACTGATTGTTACCCACAATCTGCCGGATCTGAT
GTATCGCGCATTTGAAGTGCTGCCGACGATGGTAATGACTCCGTATGCTGCATTCCAGAAAGAG
CTGCACGGTATGACCGAAGAAGTTTACCTCGACGAAATGGTAGGTTCGTATTAACGCCAATATGAT
CCTTCCGTACCCGCCGGGAGTTCCTCTGGTAATGCCGGGTGAAATGATCACCGAAGAAAGCCGT
CCGGTTCTGGAGTTCCTGCAGATGCTGTGTGAAATCGGCGCTCACTATCCGGGGCTTTGAAACCG
ATATTCACGGTGCATACCGTCAGGCTGATGGCCGCTATACCGTTAAGGTATTGAAAGAAGAAAG
CAAAAAATAA

11.6 Calibration curves

11.6.1 Protein concentration determination

Total protein concentrations were measured using a DC Protein Assay. Bovine serum albumin (BSA) was used as the protein reference standard. BSA (5 mg) was dissolved in the same buffer as the reaction was to be performed in (1 mL) and a series of dilutions prepared (0.2 to 1.5 mg/mL). Each sample (20 μ L) was transferred to a microcentrifuge tube and mixed with reagent A (100 μ L) and reagent B (800 μ L). The reaction was incubated at room temperature (15 - 25 min) and the absorbance at 750 nm measured. The buffers for each enzyme assay was the same and the calibration curve was freshly prepared for each experiment, along with the unquantified protein samples. Some examples of a BSA calibration curve are shown (Fig. 11.9). The calibration curve was then used to extrapolate protein concentration of samples.

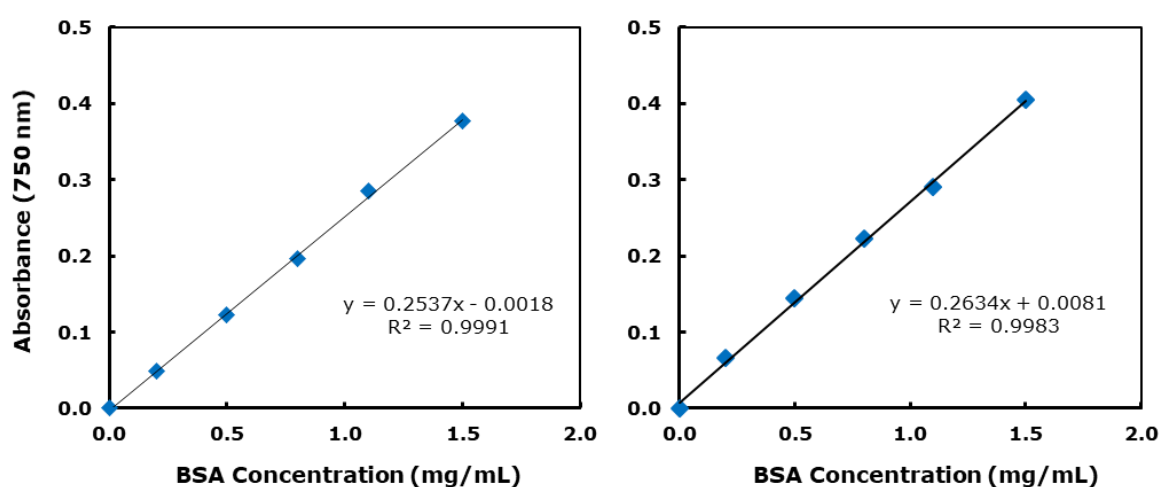


Fig. 11.9: BSA (DC assay) calibration curve

Values represent mean of triplicate experiments $\pm$ standard deviation.

11.6.2 HPLC analysis

To observe the consumption of cadaverine in a PuO_{Rh} catalysed reaction, HPLC standard curves were generated using known concentrations of cadaverine. Cadaverine was first derivatised using dansyl chloride (DNS-Cl). The peak area was measured and standard curve was plotted against cadaverine concentration. The amount of cadaverine used to generate the standard curve was dependent on the expected titre of cadaverine used in the enzyme assays, so that the amount consumed would fall within the range of the calibration curve. Sample chromatograms of derivatized cadaverine are shown in Fig. 11.10, while the sample calibration curves used for cadaverine consumption studies are shown in Fig. 11.11.

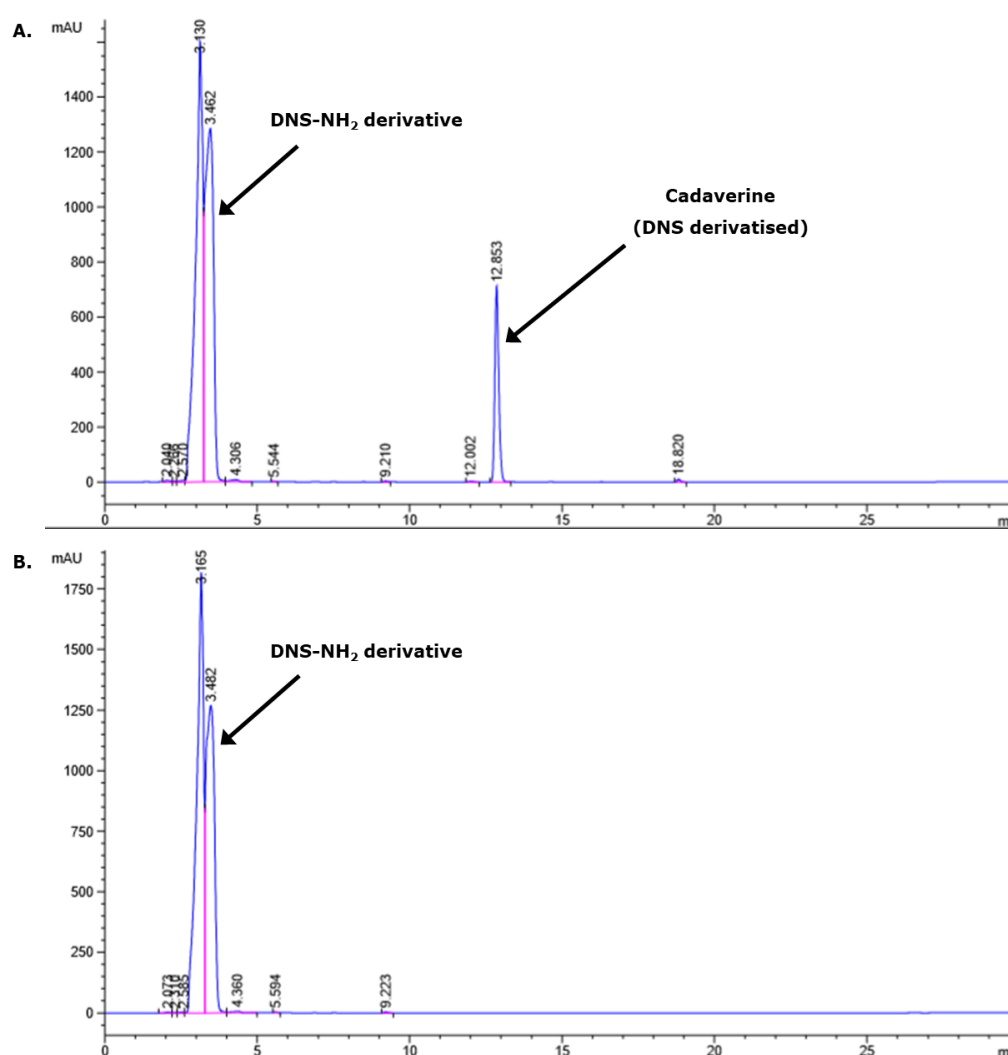


Fig. 11.10: HPLC analysis of cadaverine after DNS-Cl derivatisation.

A. Sample containing cadaverine, and **B.** Sample without cadaverine

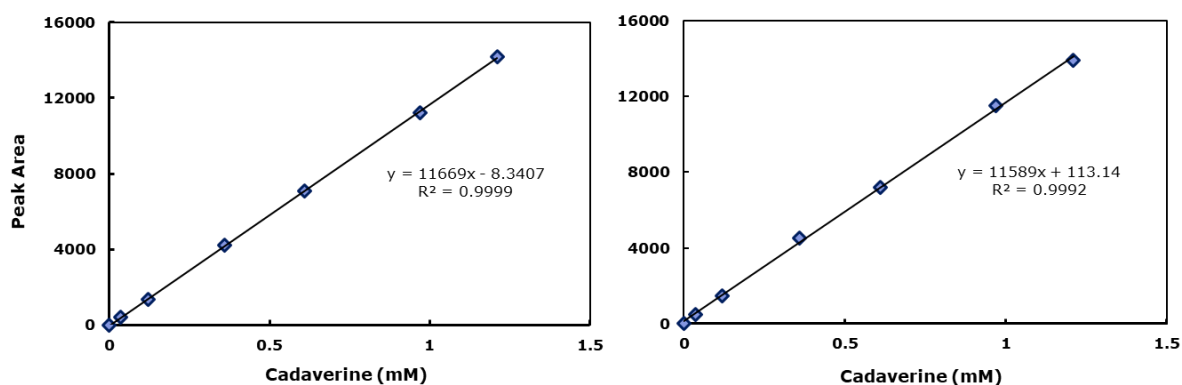


Fig. 11.11: Standard curves for dansyl-derivatized cadaverine

Values represent mean of triplicate experiments $\pm$ standard deviation.

11.6.3 Quantitative ^1H NMR analysis: Calibration of external standard

To calculate the concentration of Δ^1 -piperidine, α -tripiperidine and isotripiperidine produced in the PuO_{Rh} catalysed oxidation of cadaverine, maleic acid (MA) was used as an external standard for qNMR analysis of aqueous samples in deuterium oxide (D_2O). To achieve this, maleic acid was filled in a co-axial stem insert (Fig. 11.12) and its concentration was firstly calibrated using known and validated concentrations of cadaverine. After the NMR analysis, the calibrated concentration of the maleic acid in the co-axial stem insert was calculated using Eq. 11.1. Thereafter, the same insert was used in all the experimental sample qNMR analysis and concentrations of the substrate and products calculated using above equation and calibrated maleic acid concentration (internal standard). Sample NMR spectrum of the maleic acid calibration set-up is shown (Fig. 11.13).

In addition, to validate the concentrations of cadaverine used, standard curves were generated from known concentrations of cadaverine. This is to confirm that the integral area of the peaks increases with increase in cadaverine concentration. Characteristically, cadaverine shows three different peaks in D_2O . The peak areas for each peak were integrated and used to plot standard curves of the integral area against cadaverine concentration. The calibration curves used are shown in Fig. 11.14.

$$\text{Conc. (M)std} = \frac{P_x * I_{\text{std}} * N_x * \text{Conc. (M)}_x}{I_x * N_{\text{std}} * P_{\text{std}}} \quad \text{Eq. 11.1}$$

Where, I, N, Conc. (M) and P are integral area, number of nuclei, molar concentration and purity of analyte (x) and standard (std), respectively.

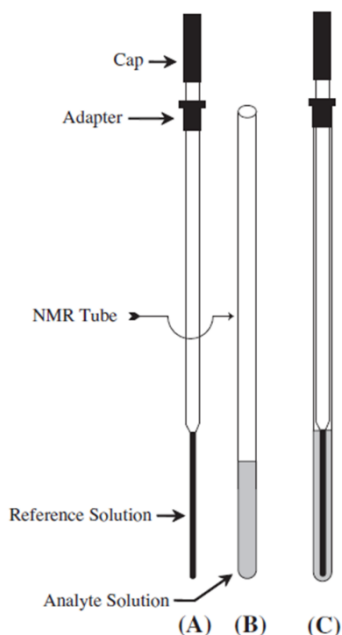


Fig. 11.12: A stem co-axial insert with its plastic cap and NMR sample-tube adapter

The co-axial insert (A) and NMR sample tube with sample (B) assembled for analysis (C). Reproduced from Bharti and Roy (2012).

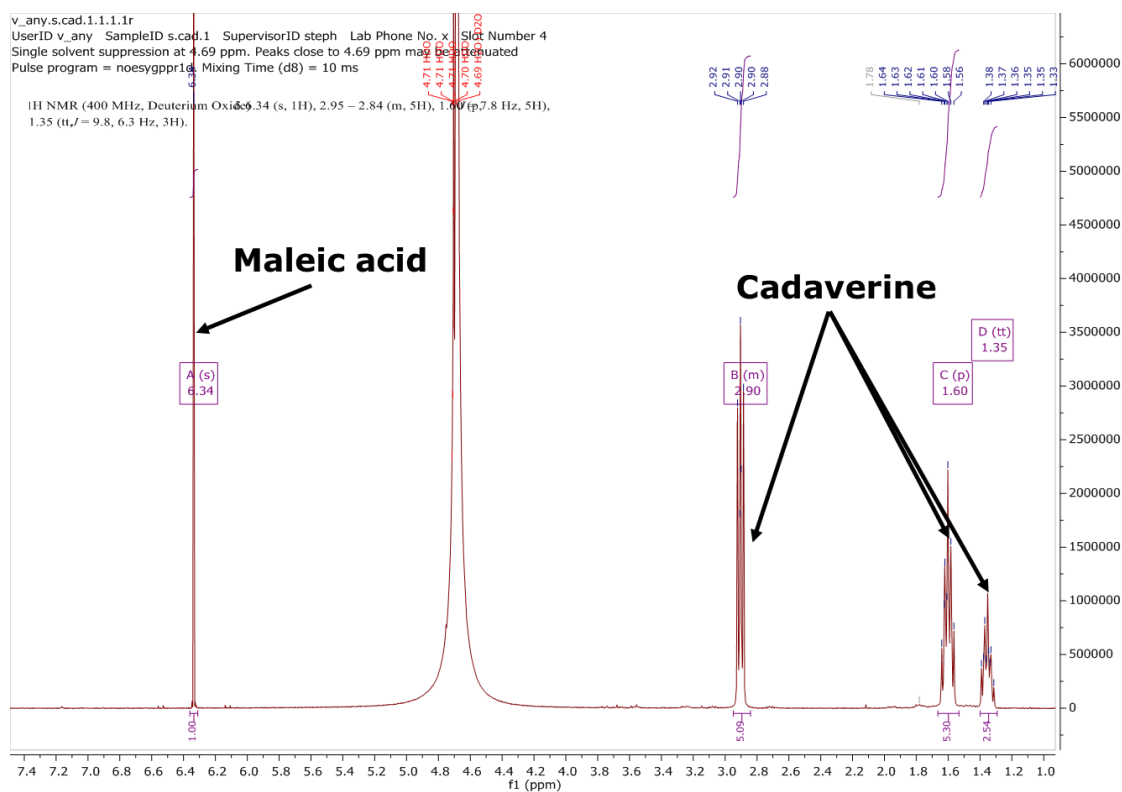


Fig. 11.13: NMR spectrum of the maleic acid calibration set-up

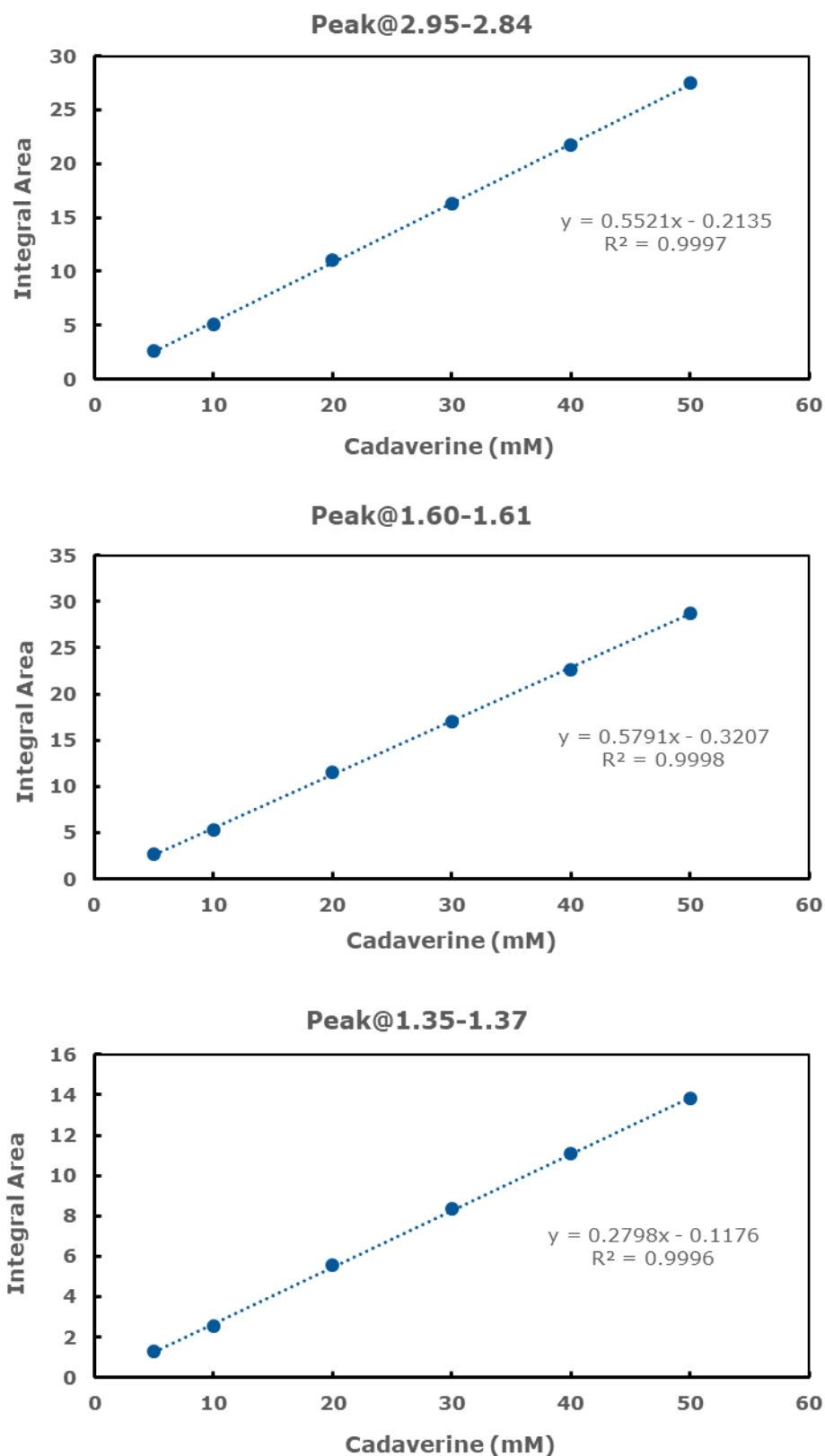


Fig. 11.14: Cadaverine qNMR calibration curve.

Cadaverine (5 - 50 mM) was prepared in D₂O. Deuterated samples (H₂O/D₂O ratio of 90/10%) were prepared, and maleic acid was used as the external standard for qNMR in a stem co-axial insert (OD 2 mm). Spectra were obtained at ambient temperature using a 400-MHz Bruker AV3400 spectrometer, and run using automated presaturation solvent suppression to reduce the intensity of the water signal. Values represent mean of triplicate experiments $\pm$ standard deviation.

11.7 Raw spectra for qNMR

Sample spectra for purified PuOR_h bioconversion of cadaverine (see Fig. 6.4)

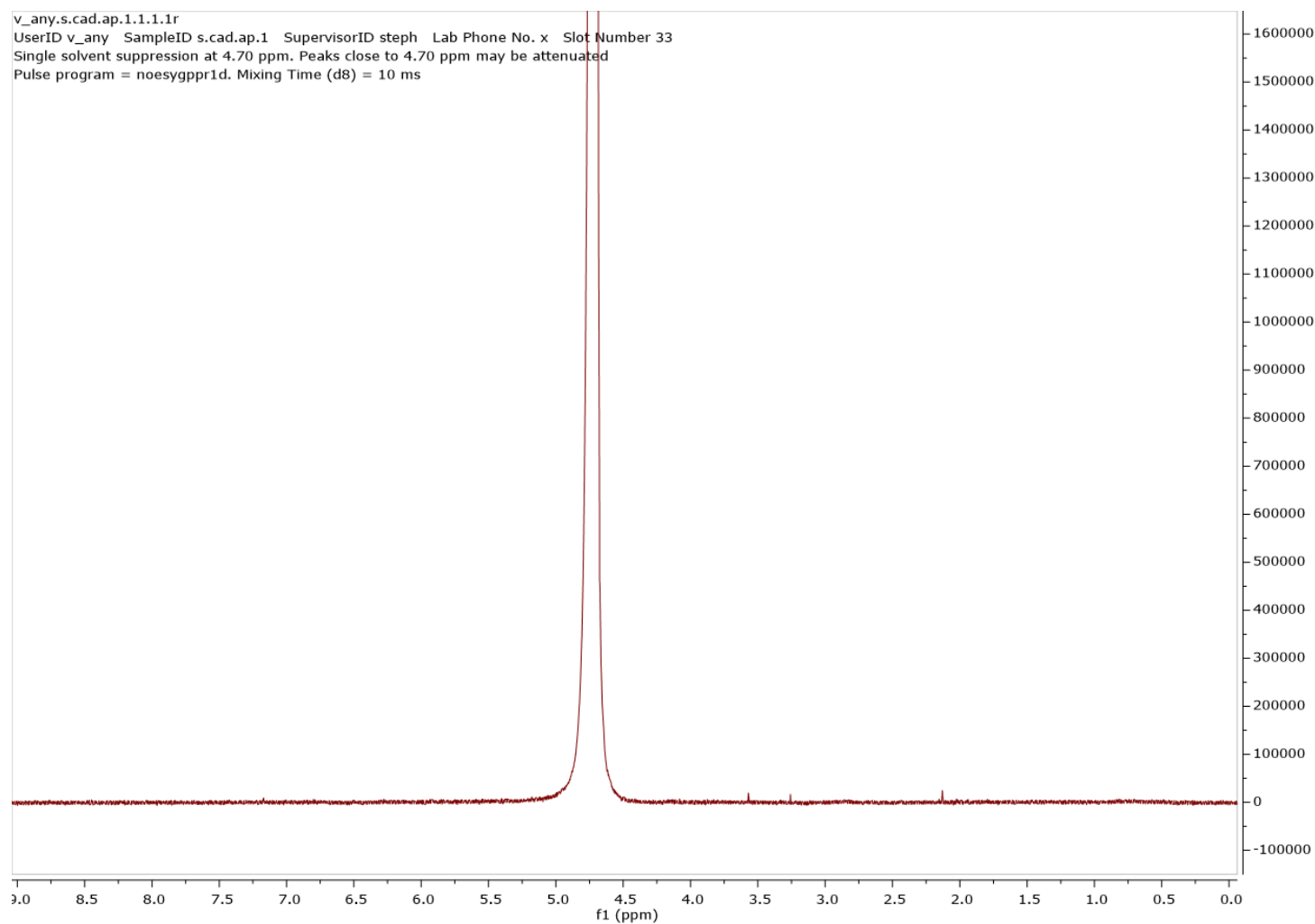


Fig. 11.15: Control analysis containing only buffer and purified enzyme.

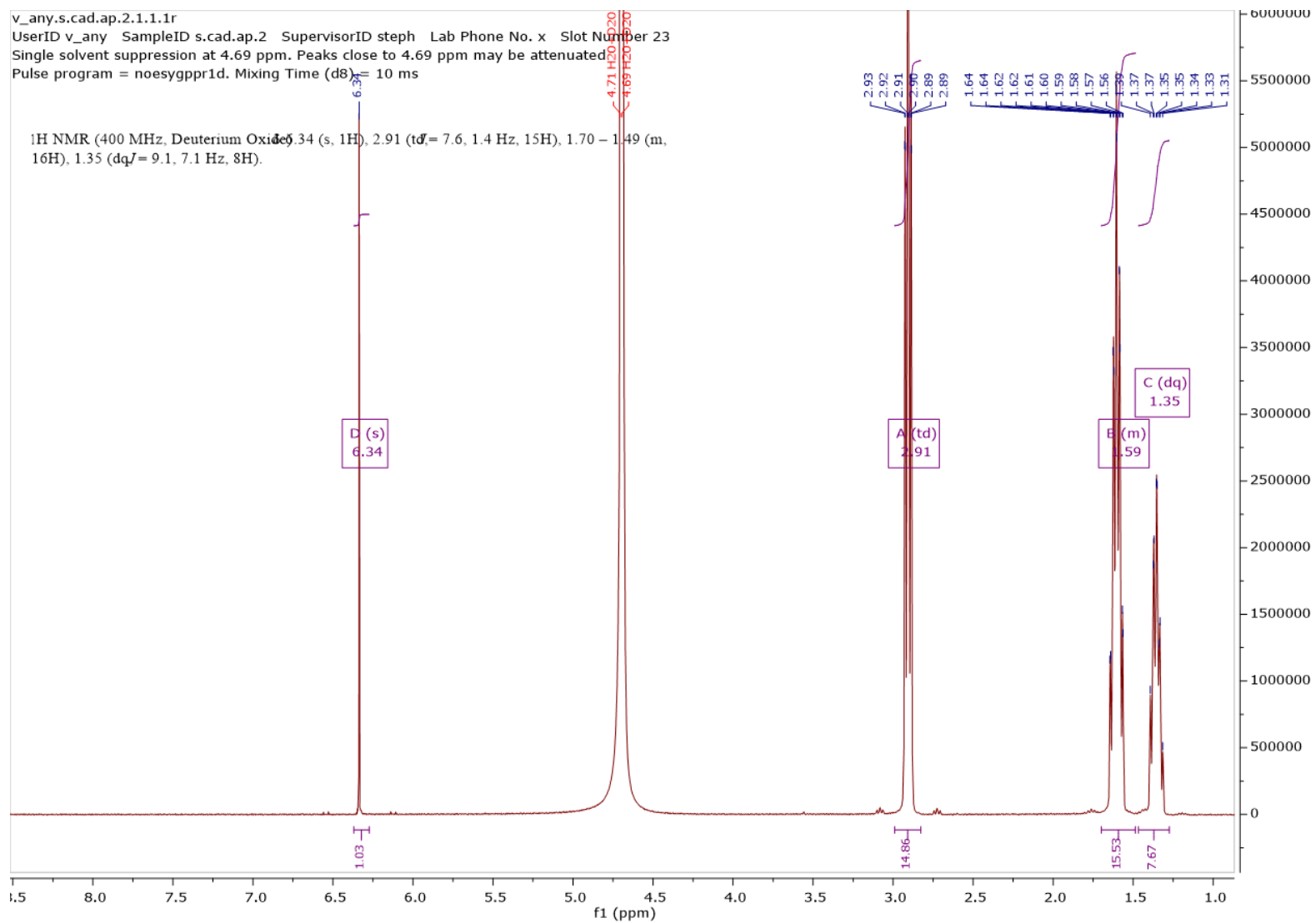


Fig. 11.16: Control analysis containing only buffer and cadaverine without purified enzyme.

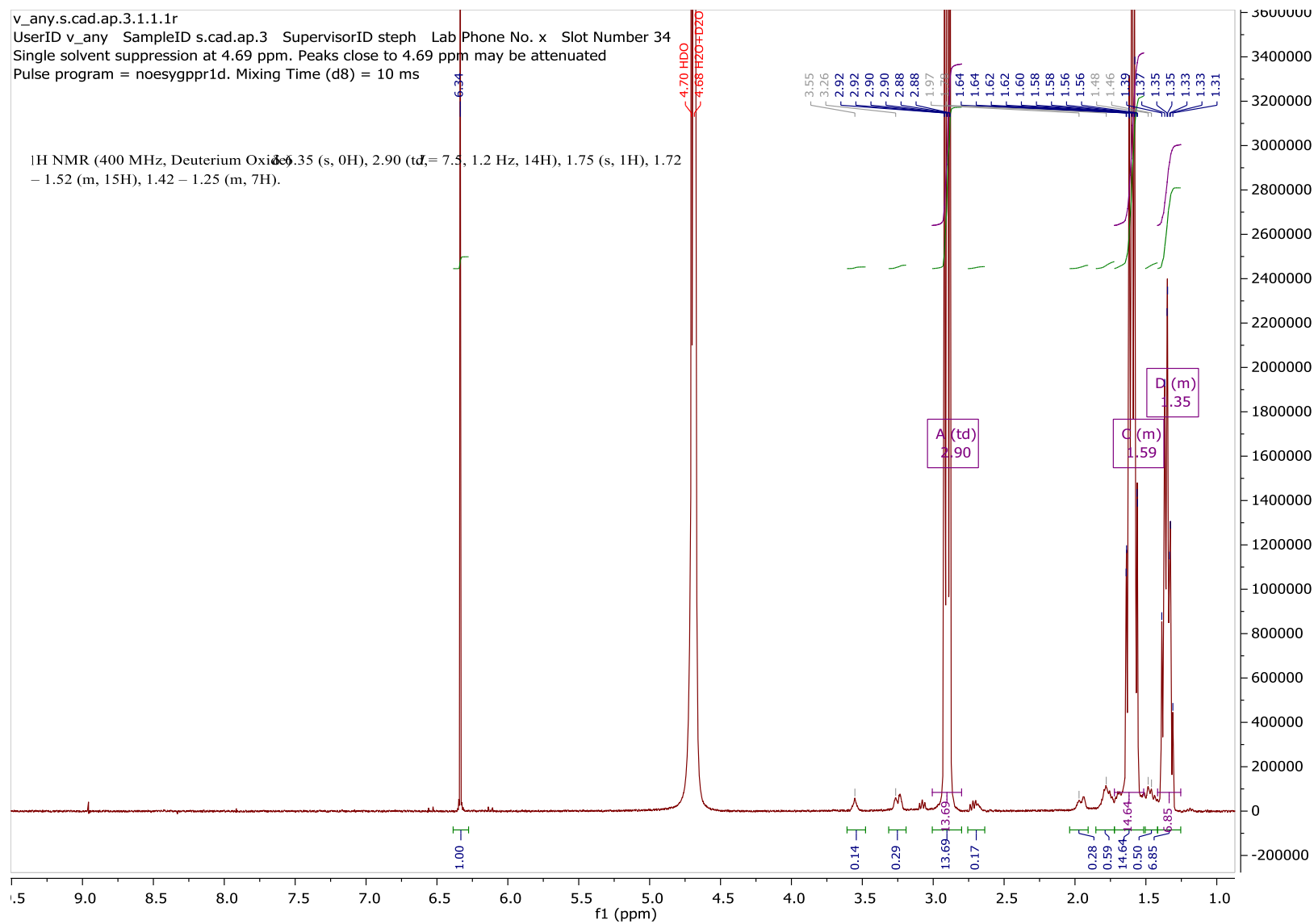


Fig. 11.17: Purified PuO_{Rh} bioconversion of cadaverine at 0 h

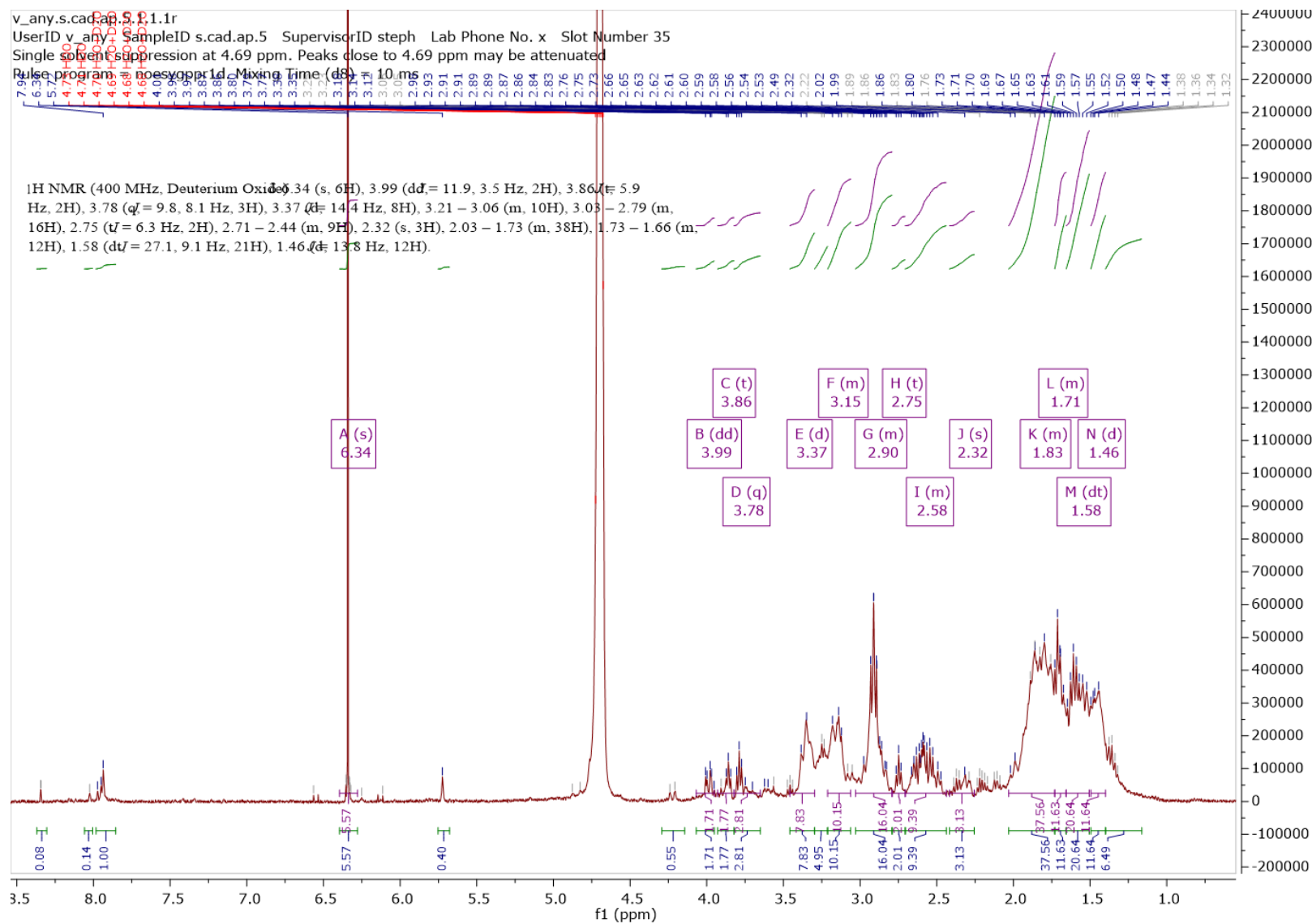


Fig. 11.18: Purified PuORh bioconversion of cadaverine at 18 h.

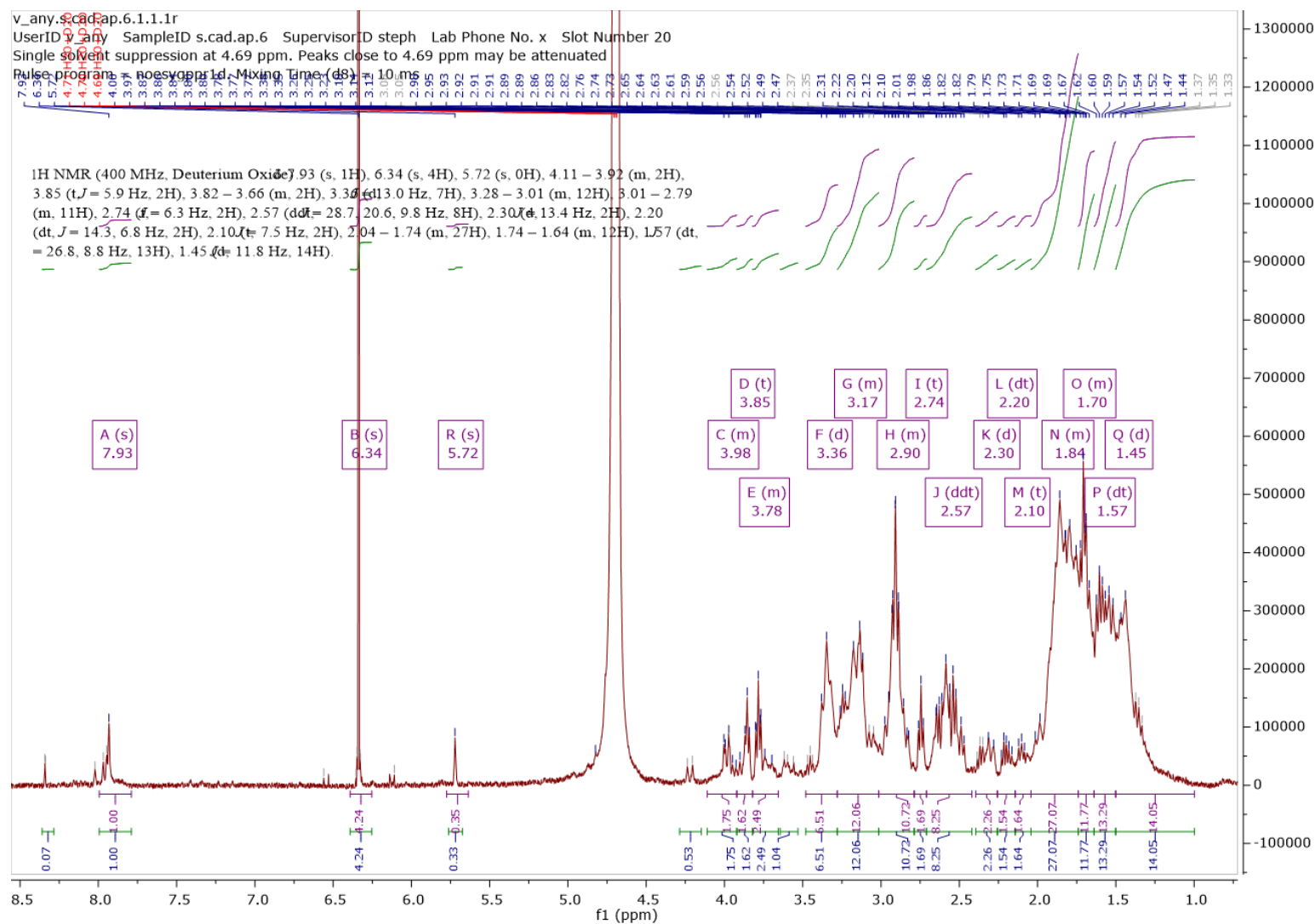


Fig. 11.19: Purified PuORh bioconversion of cadaverine at 24 h.

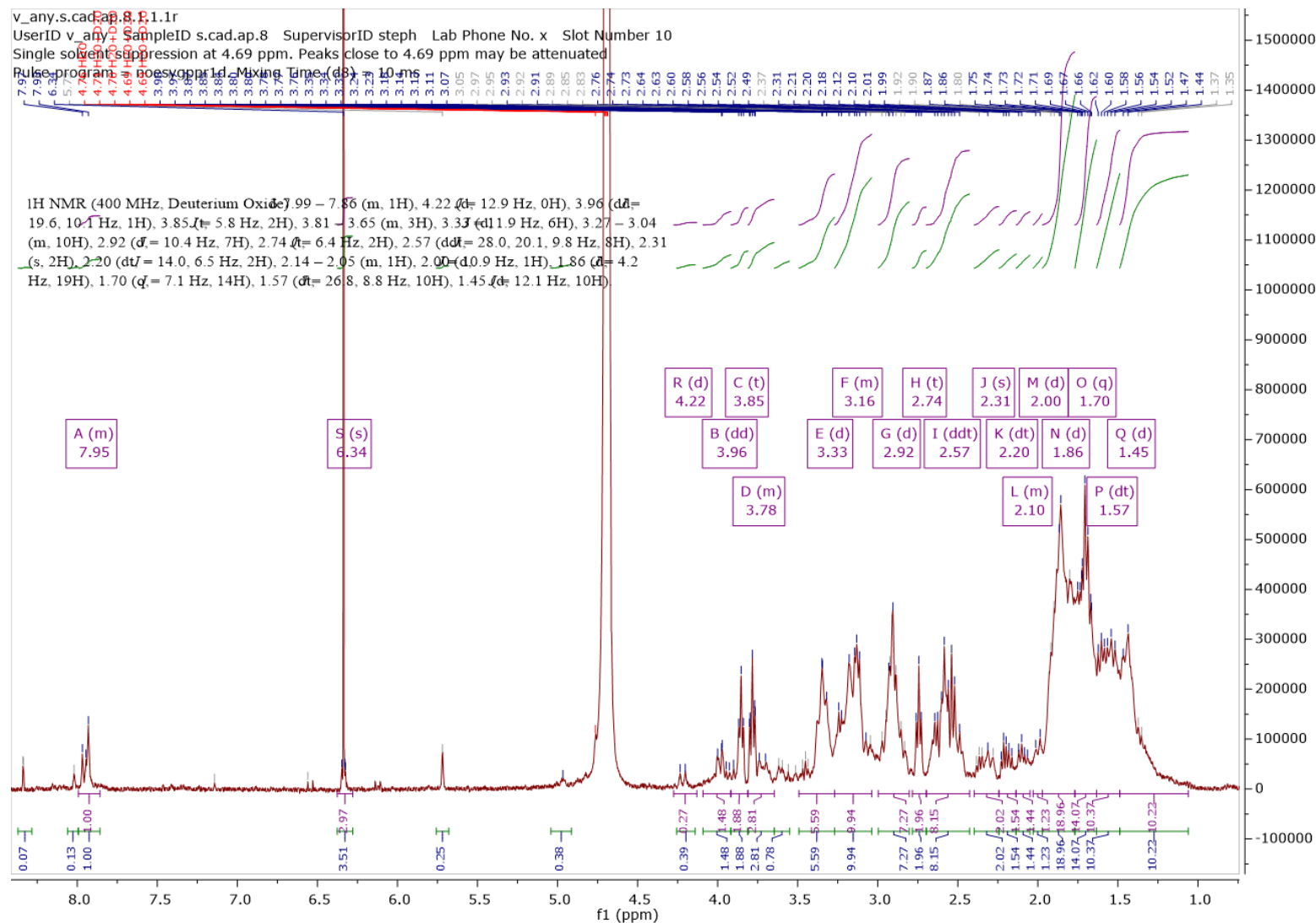


Fig. 11.20: Purified PuO_{Rh} bioconversion of cadaverine at 40 h.

11.8 Theoretical pathway design: stoichiometric matrix

Lysine pathway proposed																			
Reaction steps	Oxaloacetate	NADP ⁺	NADPH	Glutamate	2-Ketoglutarate	ATP	ADP	Pyruvate	CO ₂	H ₂ O ₂	NH ₃	H ⁺	P _i	H ₂ O	O ₂	5-Aminopentanal			
1		-1			-1	1													
2							-1	1											
3			1	-1									-1	1					
4									-1				1		1				
5			1	-1									-1		1				
6													1		1				
7			1	-1								-1	-1		1				
8									1				-1						
9									1				-1						
10											1	1			-1	-1			
Total		-1	3	-3	-1	1	-1	1	-1	2	1	0	-3	1	3	-1	1		
Oxaloacetate + 3NADPH + 2H ₂ O → 5-Aminopentanal + 4H ⁺ + 3NADP ⁺ + 2H ₂ O + CO ₂																			
Oxaloacetate + 3NADPH + Glutamate + ATP + Pyruvate + 3H ⁺ + O ₂ → 3NADP ⁺ + 2-ketoglutarate + ADP + 2CO ₂ + H ₂ O ₂ + PO ₄ ³⁻ + 3H ₂ O + 5-aminopentanal																			
Xylose pathway																			
Reaction steps	Xylose	NAD ⁺	NADH	NADP ⁺	NADPH	H ⁺	H ₂ O	2-Ketoglutarate											
1	-1	-1		1			1												
2							1	-1											
3								1											
4								1											
5					-1	1	2	-1											
Total	-1	-1		1	-1	1	4	0											
Xylose + NAD ⁺ + NADP ⁺ + 2H ₂ O → 2-Ketoglutarate + 4H ⁺ + NADPH + NADH + 2H ₂ O																			
Xylose + NAD ⁺ + NADP ⁺ → 2-Ketoglutarate + 4H ⁺ + NADPH + NADH																			
Lysine pathway - natural occurring																			
Reaction steps	Oxaloacetate	NADP ⁺	NADPH	Glutamate	2-Ketoglutarate	ATP	ADP	Pyruvate	Succinyl-CoA	Succinate	CO ₂	H ₂ O ₂	NH ₃	H ⁺	P _i	H ₂ O	O ₂	CoA	5-Aminopentanal
1	-1				-1	1													
2							-1	1											
3			1	-1											-1	1			
4											-1				1			1	
5			1	-1											-1			1	
6										-1							-1		1
7					-1	1													
8											1						-1		
9 NR																			
10												1			-1				
11												1			-1				
12													1	1			-1	-1	1
Total	-1	2	-2	-2	-2	2	-1	1	-1	-1	1	2	1	1	-3	1	-1	-1	1
Oxaloacetate + 2NADPH + 2H ₂ O → 5-Aminopentanal + 4H ⁺ + 2NADP ⁺ + 2H ₂ O																			
Oxaloacetate + 2NADPH + 2Glutamate + ATP + Pyruvate + Succinyl-CoA + 3H ⁺ + H ₂ O + O ₂ → 5-Aminopentanal + 2NADP ⁺ + 2 2-Ketoglutarate + ADP + Succinate + 2CO ₂ + H ₂ O ₂ + NH ₃ + PO ₄ ³⁻ + CoA																			

Fig. 11.21: Theoretical pathway design: stoichiometric matrix

Stoichiometric balance of proposed pathway

Oxaloacetate + 3NADPH + Glutamate + ATP + Pyruvate + 3H⁺ + O₂ → 3NADP⁺ + 2-ketoglutarate + ADP + 2CO₂ + H₂O₂ + PO₄³⁻ + 3H₂O + 5-aminopentanal

Stoichiometric balance of published xylose pathway

Xylose + NAD⁺ + NADP⁺ → 2-Ketoglutarate + 4H⁺ + NADPH + NADH

Stoichiometric balance of native pathway (*E. coli* lysine pathway plus Δ¹-piperidine pathway)

Oxaloacetate + 2NADPH + 2Glutamate + ATP + Pyruvate + Succinyl-CoA + 3H⁺ + H₂O + O₂ → 5-Aminopentanal + 2NADP⁺ + 2 2-Ketoglutarate + ADP + Succinate + 2CO₂ + H₂O₂ + NH₃ + PO₄³⁻ + CoA