Enzymatic production of Volatile Organosulphur Compounds for the Flavour and Fragrance Industry

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Abstract

Volatile organosulphur compounds (VOSC) are a diverse group of chemical entities, which are among the highest impact aroma chemicals (HIACs) in the flavour and fragrance (F&F) industry. These compounds may be synthesized by both chemical processes, or through the use of biological pathways referred to 'natural production' methods.

The overall aim of this investigation was to establish methods for the enzymatic production of the VOSCs methanethiol, hydrogen sulphide, thiomenthone and other valuable F&F compounds from natural precursors. The first part of this project was focussed on identifying novel bacterial and plant C-S lyase enzymes, which can cleave the bond between sulphur and carbon in methionine, cysteine and related conjugates synthesised through Michael addition reaction. Methods for the optimal production of recombinant C-S lyases were developed using *E. coli* as a bacterial host. The collection of C-S lyases selected were cloned and then used for *in vitro* natural production studies. A comprehensive understanding of the factors controlling their effective use in biotransformation studies included the dependence and optimisation of cofactor, temperature and pH in order to choose the most appropriate enzymes for the production of each VOSC. In addition, methods for the enzymatic methylation of VOSC and other free thiols were developed using an *S*-methyl transferase derived from *Arabidopsis thaliana*. Finally, building on these single-step reactions, the concept of linked enzymatic reactions *in vivo* and *in vitro* to generate *S*-methylated VOSCs directly from amino acid derivatives was investigated.

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Abbreviations

[^{3}H]-SAM: *S*-adenosyl-L-[^{3}H-methyl]-L-methionine

At: *Arabidopsis thaliana*

bp: base pair of DNA

BBT: 4,4'-thio*bis*-benzenethiol

CAM: Chloramphenicol

CBL: Cystathionine beta lyase (from *E.coli*)

CDNB: 1-chloro-2,4-dinitrobenzene

cDNA: Complementary deoxyribonucleic acid

Ci: Curies

C-S lyase: Carbon - sulphur bond lyase

CMTP: Methylated fenclorim thiol (4-chloro-6-(methylthio)-2-phenylpyrimidine)

CPM: Counts per minute

Cys: Cysteine

Da: Dalton

dATP: 2-'Deoxyadenosine-5'-Triphospate

DCM; Dichloromethane

DNA: Deoxyribonucleic acid

DNPH: 2,4-Dinitrophenylhydrazine

DPM: Disintegrations per minute

DTB: Desthiobiotin

DTNB: 5'5-dithio-*bis*-(2 nitrobenzoic acid)

DTT: 1,4-dithiothreitol

EC: Enzyme Commission

F: Fenclorim

F&F: Flavour and Fragrance

FAC: *S*-(fenclorim)-*N*-acetylcysteine

FγEC, *S*-(4-chloro-2-phenyl-pyrimidin-6-yl)-γ-glutamylcysteine

FACC: *S*-(4-*N*-acetylcysteine-2-phenylpyrimidin-6-yl)-cysteine

FACG: *S*-(4-*N*-acetylcysteine-2-phenylpyrimidin-6-yl)-glutathione

FAD: Flavin adenine dinucleotide

FC: Fenclorim cysteine (*S*-(4-chloro-2-phenylpyrimidin-6-yl)-cysteine)

FG: *S*-(4-chloro-2-phenylpyrimidin-6-yl)-glutathione

FMC: Fenclorim malonyl cysteine (*S*-(4-chloro-2-phenylpyrimidin-6-yl)-*N*-malonylcysteine)

FT: 4-chloro-6-(thio)-2-phenylpyrimidine

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GC: Gas chromatography

GCMS: Gas chromatography mass spectrometry

GM: Genetically modified

GSH: Glutathione

GST: Glutathione-*S*-transferase

HABA: 2-(4-hydroxyphenylazo)-benzoic acid

HCl: Hydrochloride

HIACs: High Impact Aroma Chemicals

HPLC: High-performance liquid chromatography

IPTG: Isopropylthiogalactoside

ITC: Isothermal Titration Calorimetry

KAN: Kanamycin

Kod: DNA polymerase from the thermophile *Thermococcus kodakaraensis*

kDa: Kilodalton

K_M: Michaelis constant

MalY: Cystathionine β-lyase from (*E.coli)*

MBq*:* Megabecquerel

MCS: Multiple cloning site

Met: Methionine

MetC: Cystathionine β-lyase from (*E.coli)*

MGL: Methionine gamma lyase from (*P. putida)*

MPS: Methyl phenyl sulphide

MPMC: *S*-(4-(methylthio)-2-phenylpyrimidin-6-yl)-*N*-malonylcysteine

MS: Mass spectrometry

MS-MS: Tandem mass spectrometry

MTL: Methanethiol

MVK: Methyl vinyl ketone

NADH: Nicotinamide adenine dinucleotide

NADPH: Reduced adenine dinucleotide phosphate

LB: Luria broth

LCMS: liquid chromatography mass spectrometry

LDH: Lactate dehydrogenase

OD: Optical density

OM: Optimised medium

OMT: *O*-methyltransferase

PAPS, 3'-phosphoadenosine-5'-phosphosulfate

PCNB: Pentachloronitrobenzene

PCR: Polymerase chain reaction

PCTA: pentachlorothioanisole

Pfu: Phusion High-Fidelity Polymerase

PMP: Pyridoxyamine 5’-phosphate

PLP: Pyridoxal- 5’- phosphate

ppb: Parts per billion

PG: Propylene glycol

PVPP: Polyvinylpolypyrrolidone

Rpm: Revolution per minute

RT: Retention time

SAIL: Syngenta Arabidopsis insertion library

SAH: *S*-adenosyl-homocysteine

SAM: *S*-adenosyl-L-methionine

SDS: Sodium dodecyl sulfate

SDS-PAGE: polyacrylamide gel electrophoresis

SMT: *S*-methyl transferase

SPC: *S*-phenyl-L-cysteine

SPME: Solid-phase microextraction

Taq: DNA polymerase (from *Thermus aquaticus*)

TCEP: Tris (2-carboxyethyl)phosphine hydrochloride

TEMED:Tetramethylethylenediamine

TFA: Trifluoroacetic acid

TMT1: Thiol methyl transferase *(*from *Arabidopsis thaliana*)

Tris: Trihydroxymethylaminomethane

TRP: Tryptophanase (from *E.coli*)

UBC: Ubiquitin C gene

X-GAL: (5-bromo-4-chloro-3-indolyl-β-d-galactopyranose)

V/V: Volume/ Volume

V/W: Volume/ Weight

V_{max}: Maximum enzymatic activity

VOSC: Volatile organosulphur compounds

Declaration

No material presented in this thesis has previously been submitted as part of any other degree. Except where acknowledged through referencing, all material is the work of the author.

Statement of copyright

Publications arising from work described in this thesis

"Halomethane biosynthesis: Structure of a SAM-dependent halide methyltransferase from *Arabidopsis thaliana"* Jason W. Schmidberger, Agata B. James, Robert Edwards, James H. Naismith, David O'Hagan Angewande Chemie International Edition English. 2010 May 10, vol 49, issue 21, pages: 3646-3648.

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1. Introduction

This introductory chapter will discuss the background and work already documented on the subject of enzymatic production of Flavour and Fragrance (F&F) compounds with the main focus on those, containing an organosulphur moiety. Initially, the chapter will define general flavour groups of F&F compounds. Some industry aspects will be discussed and an overview of current chemical applications given. The biochemical literature covering sulphur pathways in natural product metabolism will be also reviewed, including a section on sulphur amino acid metabolism in plants and microorganisms. Furthermore, several carbon – sulphur lyases from various organisms, which have previously been identified, will be reviewed. This will include aspects of their activity and their potential application as specific biocatalysts for the biosynthesis of highly valuable natural products. Finally, the direct methylation of free thiols by *S*-methyltransferase will be discussed.

1.1 Flavour and Fragrance chemistry

Much of the research into F&F chemistry has been driven by commercial interest with the aroma constituents being isolated from different natural sources. Many F&F compounds have been known as flavourings for centuries, but have only been chemically identified over the past 50 years due to sensitive detection methods employing GC or LC-MS. A brief list of some of the achievements in F & F chemical industry is presented in Table 1.1-1.

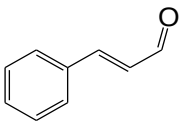
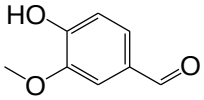
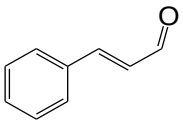
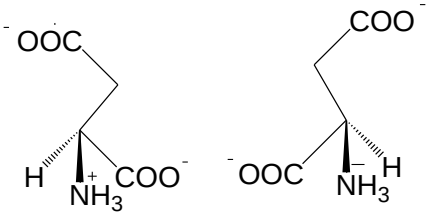
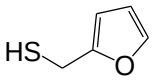
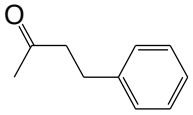
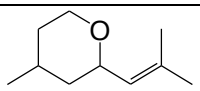
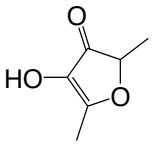
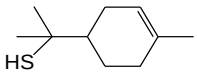
Date	Achievements in F&F research	Structure
1834	Identification of cinnamaldehyde - the first flavour compound	
1871	Identification of vanillin	
1884	Synthesis of cinnamaldehyde	
1887	Enantiomers of aspartate found to have different tastes: *S*-aspartate (bitter), *R* –aspartate (sweet)	
1926	Identification of furfuryl mercaptan in coffee	
1937	Identification of raspberry ketone	
1952	Development of Gas Chromatography (James & Martin)	
1955	Perkin-Elmer released first commercial GC	
1959	Discovery of Rose oxide	
1964	Identification of furaneol strawberries-like	
1982	Identification of powerful VOSC grapefruit mercaptan	
1989	Solid Phase Micro Extraction (SPME)	

Table 1.1-1. Important milestones in development of flavourings chemistry and the identification of important aroma chemicals (adapted from Rowe, D.J. 2005).

1.1.1 Flavour and Fragrance industry

The F&F products are desired in many applications in developed countries. The production and use of flavours and fragrances on an industrial scale started in the nineteenth century with the isolation of single chemicals responsible for the characteristic aroma of natural products (*e.g.*, cinnamaldehyde isolated from cinnamon oil, or benzaldehyde from bitter almond oil Fisher and Scott 2001). The synthesis of aroma chemicals that reproduced the characteristic odour and taste of natural products then became commonplace and gave rise to a new branch of chemical specialties. The rapid expansion of the flavour and fragrance (F&F) industry worldwide, however dates only to the last 100 years, and has been driven by the demand for a broader range of consumer products with unique properties.

Flavour and fragrance ingredients are highly valuable additives widely used in the food and personal care industries. Using natural sources, the isolation of single flavouring chemicals from a mixture requires costly methods such as fractional distillation and is often not cost effective when an active ingredient is found at trace levels. The use of plant and microbial enzymes for bioproduction is now seen as the viable alternative to produce F&F compounds on a large scale. Hence, processes have been developed using whole-cell systems (microbial or plant), immobilized enzymes, or genetically modified bacteria and yeast for enhancing total flavour of materials or for the formation of single aroma chemicals. Today the industry is worth over 20 billion dollars and is dominated by 10 major companies: Givaudan (Swiss, 19.1 % market share), Firmenich (Swiss, 13.9 %), International Flavors and Fragrances (USA, 11.6 %), Symrise (German, 9.8 %) and Takasago (Japan, 6.1 %), Sensient

Technologies (USA, 2.7 %), Mane SA (USA, 2.7 %), T. Tasegawa (Japan, 2.3 %) Robertet (France 2.2 %) and Frutarom (Israel 2.1%) (www.leffingwell.com 2010). The flavourings company Frutarom Limited specialise in the manufacture of high impact sulphur aroma chemicals and plays a key role in the F&F market.

1.1.2 Legislation

The flavour and fragrance industry is strictly regulated as described in Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 covering flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC). Under current legislation "natural flavouring substance" means a flavouring substance obtained by appropriate physical, enzymatic or microbiological processes from material of vegetable, animal or microbiological origin either in the raw state or after processing for human consumption by one or more of the traditional food preparation processes listed in Annex II of the EC regulation. European legislation restricts the methods of preparation to either physical extraction from natural material or *via* enzymatic and microbiological natural catalysis. Natural flavouring substances have to correspond to substances that are characterised and chemically identified in Nature. However, no definition of enzymatic process, or limitation of the source or type of enzyme used in manufacture is given in the 88/388/EEC document. Furthermore, all starting materials must be natural, which is a significant change to previously granted legislation and will affect future methods used for the production of flavours and fragrances.

1.1.3 Natural flavour compounds

Customers have become more sensitive about the methods of production of food additives and there is now a higher demand to develop environmentally acceptable systems for chemical manufacture. Natural aromatic chemicals can be extracted directly from plant or animal sources, or generated by human activity in different ways. First, by thermal reactions where flavours are generated due to high temperatures and pressures in cooked food. This process is known as the Maillard reaction (Cerny 2007). Second, by fermentation, by yeast and other microorganisms. Replicating these natural flavour and fragrance enzymatic processes can be performed by isolated enzymes, which lead to the generation of specific chemicals of interests (Dia *et al*. 2010). The formation of volatile organosulphur compounds (VOSCs) in fermented food is a subject of interest for the F&F industry. Such compounds are essential for the aroma of many food products like cheeses or fermented beverages, in which they can play an attractive or a repulsive role, depending on their identity and their concentration (Vermeulen, *et al*. 2005). VOSCs are produced essentially from common sulphur-bearing precursors like methionine (Landaud *et al*. 2008). Because of their low odour threshold and flavour notes, these compounds are frequently responsible for essential sensorial properties of the final product. Sulphur compounds are very powerful and important family of aroma chemicals. Humans and animals have evidently evolved to specifically recognise such chemicals, perhaps as a means of avoiding unpleasant or toxic foods, or conversely as attractants in some exotic fruits (Schwab *et al*. 2008). Sulphur volatiles are extremely potent and detectable at low concentrations. Since early times, many *Allium* species, such as garlic (*A. sativum* L.), onion

(*Allium cepa* L.) chives (*A. schoenoprasum* L.), leeks (*A. porrum* L.) and shallots (*A. ascalonicum*) have been used as foods and spices (Jabrane *et al*., 2011), (Iciek *et al*., 2009). Similarly, the characteristic fresh, savoury flavours and odours of a number of important vegetables are the result of the enzymatic degradation of secondary sulphur-containing amino acids, usually alkyl cysteines and/or their sulfoxides (Ho and Mazelis, 1993). VOSCs being high impact aromas are subsequently found at only trace levels in fruit tissue. For example the major character impact volatile in grapefruit, mercaptohexanol (grapefruit mercaptan). Most vegetable aromas are released during cellular disruption, which releases enzymes that act upon non-volatile precursors. Before the cell walls of the vegetable are broken, the aroma chemicals are bonded as non-volatile precursor (Halkier and Gershenzon, 2006).

1.1.4 Enzymatic and microbiological catalysis

High consumer demand for natural flavours has stimulated a number of research projects aimed at developing naturally derived products using microbes or isolated enzymes (Serra et al., 2005). The use of natural enzymes as catalysts for biotransformations in organic chemistry has recently expanded and become an important part of innovative biochemistry (Jackel and Hilvert, 2010). There are many advantages of using isolated enzymes as natural biocatalysts with reactions processing under mild optimum conditions e.g; pH 5-8 and 20-40°C (Rich *et al.,* 2002). As such, less energy is required for such processes and the environmental impact and energy costs are reduced. Enzymes are totally degradable, non-toxic and catalytically highly efficient (Dia et al., 2010). However, enzymes do have their disadvantages with high

substrate and product concentrations inhibiting many enzymes and hence reducing productivity. Furthermore, some enzymes require cofactors which can be expensive and difficult to recycle (Bugg, 2004). Nevertheless, the use of biocatalysts to derive important, highly valuable compounds offers many advantages and serves as a novel route for large scale F&F production (Serra, et al., 2005)

1.1.5 Chirality of flavour compounds

Many flavour compounds exist in different chiral conformations. There are many significant differences between stereoisomers of the same odorant compound, both in the type and strength of the F&F chemical, (Brenna *et al*., 2003). As an example in (figure 1.1-1) demonstrates the variable odours associated with one of the F&F compounds - thiomenthone (8-mercapto-*p*-menthan-3-one) is presented, while all four isomers can be synthesized *via* Michel-type additions with hydrogen sulphide acting on pulegone only the (1*S*, 4*S*) and (1*S*, 4*R*) diastereoisomers are found in nature oil derived from buchu leaf. This is most likely due to the action of enzymes, which are enantiomer specific.

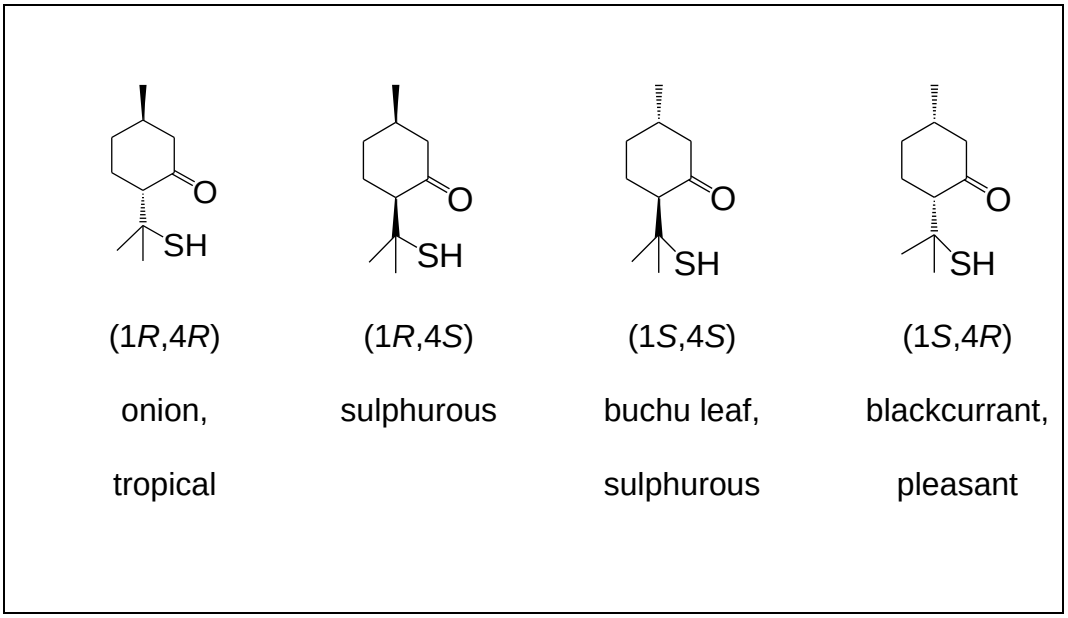


Figure 1.1-1. Flavour characterization of different forms of thiomenthone.

1.2 Chemistry of sulphur and VOSCs

1.2.1 Sulphur characterisation

The element sulphur is essential to living organisms. In order to understand the properties of sulphur metabolites, an understanding of the element itself and its chemistry is first required. Sulphur belongs to group 16, period 3 of the periodic table of elements. Sulphur is a group six p-block element situated below oxygen and above selenium. The sulphur atom possesses six valence electrons and has four high energy 3p orbitals. Sulphur is electronically very active and frequently has catalytic or electrochemical functions in biomolecules. Organic sulphur variants include mercaptides, mercaptans and thioethers (sulphides) and disulphides (figure 1.2-1.).

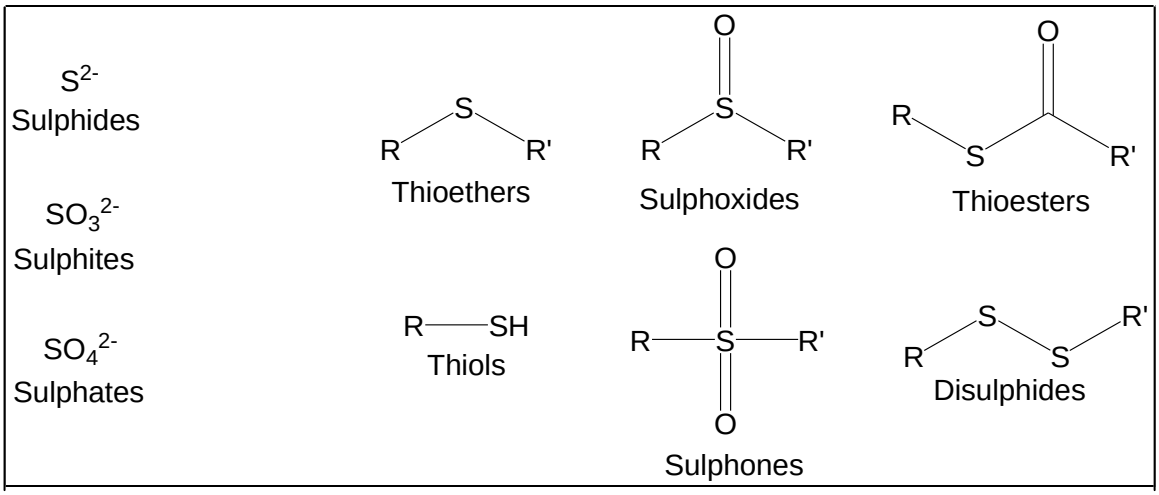


Figure 1.2-1. The main chemical groups containing sulphur.
Inorganic: sulphides, sulphites, sulphates and organic: thiols (mercaptans) and thioethers (sulfides) disulfides R' stands for functional group.

Sulphur volatiles are extremely potent and easily detectable at very low concentrations, therefore they are referred to as high -impact aroma chemicals (HIACs) and this particular group of natural products have an important role in the flavour and fragrance industries. Free thiol groups are over 500 times more pungent than the corresponding oxygen analogue (Wakabayashi, 2004) as presented in table 1.2-1. Sulphur has classically been used as a soft nucleophile, which is able to attack carbon-carbon double bonds in preference to carbon-oxygen double bonds (Soda, 1987). These reactions lend to the production of sulphur containing aldehydes and ketones.

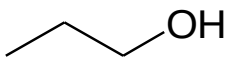
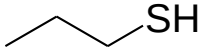
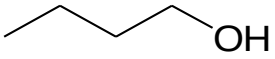
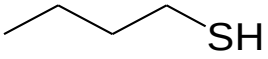
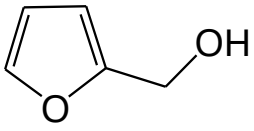
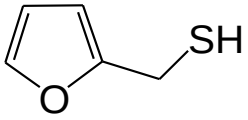
propan-1-ol 	pungent 9000 ppb	propane-1 thiol 	cabbage-like 3 ppb
butan-1-ol 	fruity 500 ppb	butane-1-thiol 	onion-like 6 ppb
(furan-2-yl)methanol 	nearly odourless	(furan-2-yl)methanethiol 	roasted coffee 0.005 ppb

Table 1.2-1 Characterisation of some odour properties of sulphur-containing compounds vs corresponding oxygen analogues adapted from (Wakabayashi, 2004).

1.2.2 Sulphur metabolism in plants

Plants carry out the important role of fixing inorganic sulphate (SO_4^{2-}) from the soil and reducing it to sulphide (S^{2-}) prior to incorporation into organic compounds. Sulphate is taken up by the roots before being incorporated into essential amino acids and secondary plant metabolites. Once inside the leaf, the sulphate can be stored in vacuoles or metabolised in plastids through reduction and assimilation. The pathways of sulphur uptake and assimilation have been well characterised in the model plant *Arabidopsis thaliana* where regulation of the pathways and adaptation to low sulphur environments have been studied as reviewed in figure 1.2-2 (Leustek, *et al.,* 2000; Hell,1977). Sulphur is essential for the production of group-transfer coenzyme A, vitamin B1), *S*-adenosyl methionine and is a critical component of proteins and phytochelatins (Hawkesford and De Kok, 2006). Uptake and assimilation of

sulphur into Cysteine and methionine is regulated by post-transcriptional and posttranscriptional mechanisms (Giovanelli 1987).

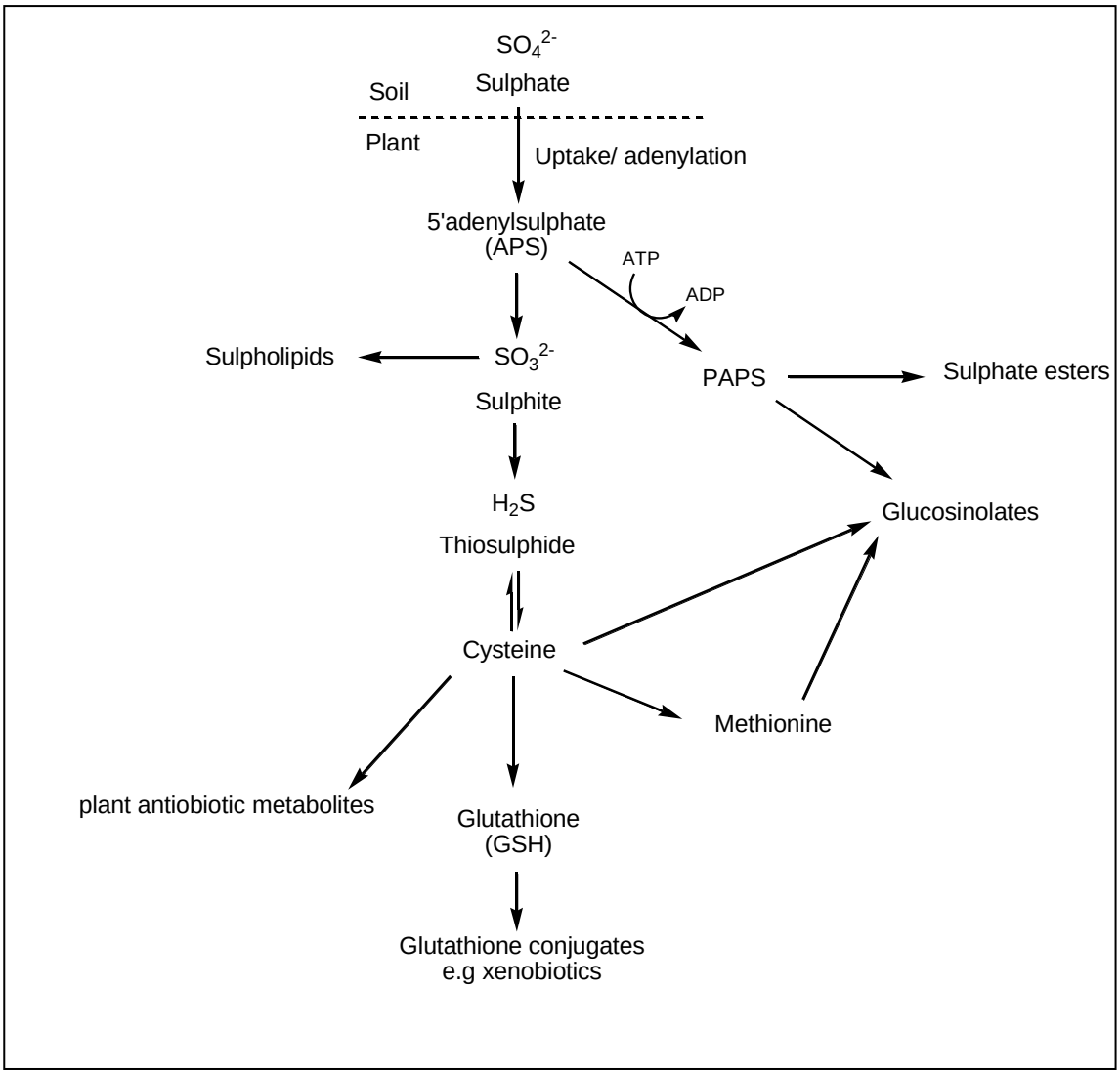


Figure 1.2-2. Sulphur metabolism in plant adapted from (Rausch and Wachter 2005). PAPS, 3'-phosphoadenosine-5'-phosphosulfate

1.2.3 Sulphur metabolism in microorganisms

The formation of HIACs by microorganisms plays an important role in the production of food and beverages. Depending on the type of fermentation and concentration of released flavour and fragrance constituents, the taste and

odour can be very aromatic and attractive, or repulsive and unpleasant. VOSCs found in wine, beer and cheese mainly arise from the sulphur-bearing precursor amino acid- methionine (Kadota and Ishida, 1972). Bacterial C-S lyase enzymes are well known to generate characteristic, sulphur derived VOSCs in resembling ripe cheese (Fernandez *et al*., 2000). Furthermore, compounds like methanethiol, dimethyl disulphide dimethyltrisulphide, released by enzymatic metabolism, play an important role in Cheddar cheese flavour (Starkenmann *et al*., 2008), (Landaud *et al*., 2008) In bacteria the synthesis of homocysteine, the direct precursor of methionine, proceeds *via* a transsulphuration reaction involving two pyridoxal 5'-phosphate (PLP)-dependent enzymes.

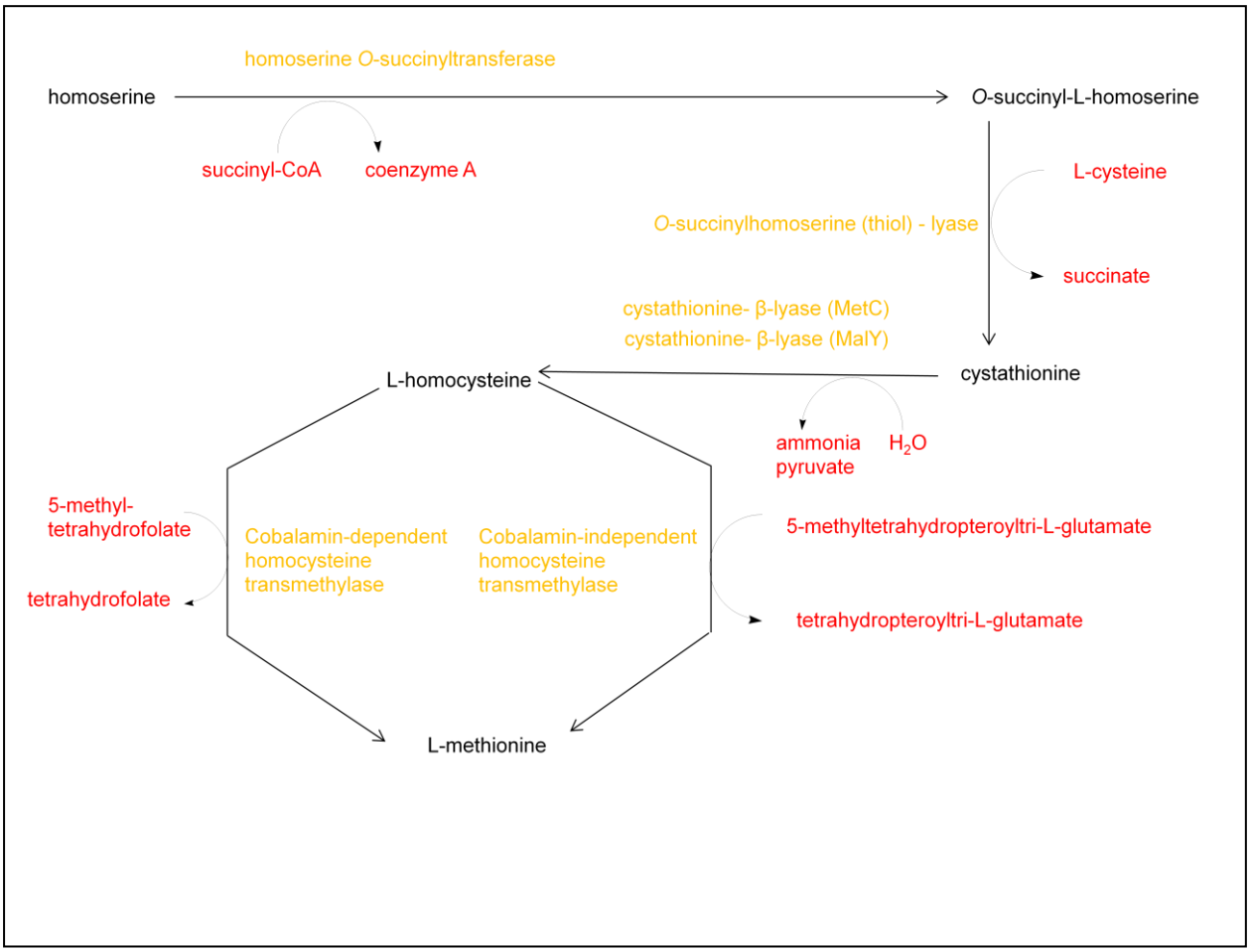


Figure 1.2-3. Metabolism of sulphur-containing amino acids adapted from biocyc.org

Transsulphuration results in the formation of cystathionine, which is an intermediate in both degradative and synthetic pathways (Soda, 1987). Cystathionine is then cleaved by cystathionine beta lyase to produce homocysteine, pyruvate, and ammonia (Aitken and Kirsch 2005; Awano *et al*., 2005; Vermeij and Kertesz 1999).

1.3 Characterisation of Pyridoxal phosphate dependent enzymes

Pyridoxal phosphate (PLP) dependent enzymes can be found in all living organisms, where they catalyse a number of reactions with a range of compounds including amino acids. The PLP- dependent proteins are divided into five main groups based on their functionality, namely: oxidoreductases, transferases, hydrolases, isomerases and lyases (Percudani and Peracchi, 2003). The lyase group is the second largest family of enzymes with a subfamily group (C-S lyase) shown to catalyse the cleavage of the carbon-sulphur bonds in various amino acids. These enzymes can release thiol compounds from cysteine or methionine conjugates with each enzyme operating on a limited number of substrates of similar structure to generate products under specific conditions (Eliot and Kirsch 2004). The PLP dependent enzymes presented in figure 1.3-1 catalyse different types of reactions. These enzymes contains a conserved residue of Tyrosine 114 which is present in the active site and proven crucial for enzymatic activity (Inoue 2000). Pyridoxal 5'-phosphate shown in figure 1.3-2 is an essential cofactor derived from a member of pyridoxine vitamins (Bugg 2004).

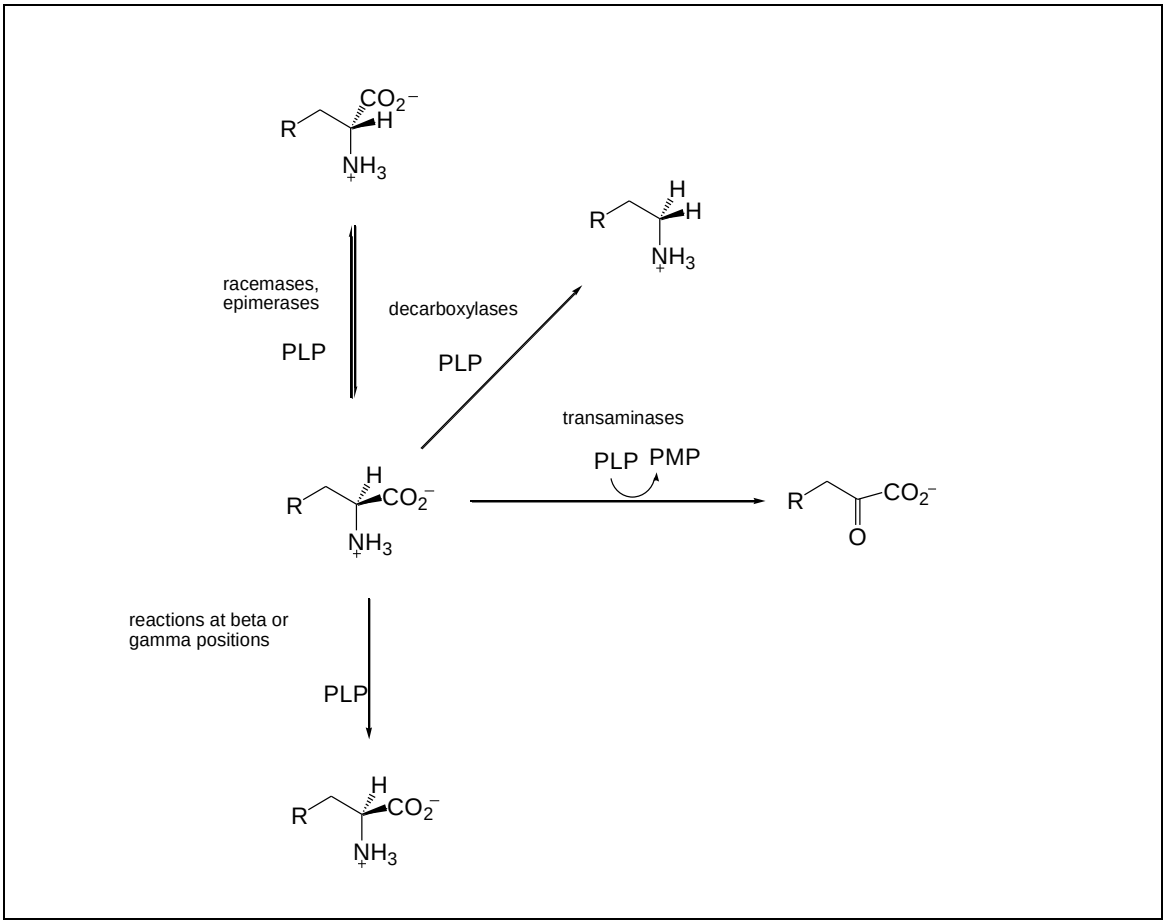


Figure 1.3-1. Enzymatic transformations of α-amino acids.

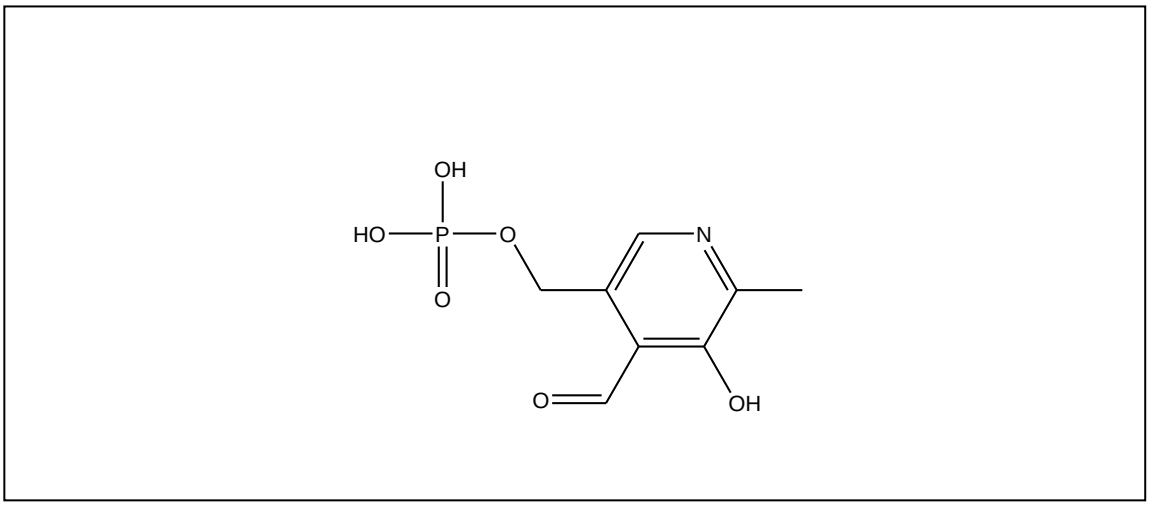


Figure 1.3-2. The structure of pyridoxal 5'-phosphate (PLP).

The PLP dependent enzymes bind their cofactor through an imine linkage between the aldehyde group of PLP and the ε-amino group of a conserved lysine residue (Bugg, 2004; Percudani and Peracchi 2009).

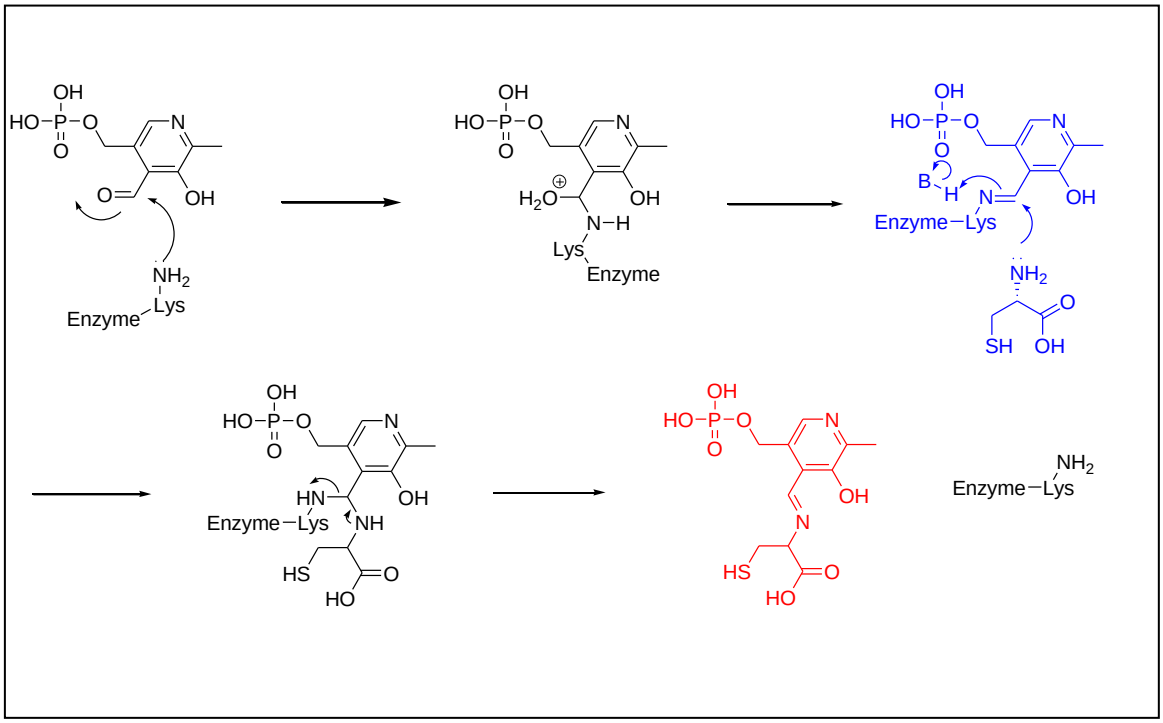


Figure 1.3-3. Formation of the Schiff base *in vivo* via the enzyme-pyridoxal phosphate internal Schiff base (blue) to the substrate-pyridoxal phosphate external Schiff base (red). Adapted from (Wakabayashi 2004).

As an example of the action of catalysis of these enzymes the conversion of cystathionine to cysteine and ketobutyrate occurs in a multistep reaction and is catalysed by cystathionine γ- lyase The reaction begins with the formation of an imine, which is deprotonated at the α carbon to give isomeric imine, then the deprotonation of the imine result in an enamine production (Breitinger *et al*., 2001; Clausen *et al*., 1996).

1.3.1 Characterisation of C-S lyase enzymes

Carbon – sulphur lyases form a novel source of natural catalytic agents for use in the F&F industry. The search for the most active and selective C-S lyases would ideally give better results for the production of some highly desired chemicals. Some of C-S lyases with great potential from an F&F perspective are described below.

1.3.1.1 Methionine γ -lyase (EC 4.4.1.11) from *Pseudomonas putida*

L- Methionine γ-lyase (MGL) from *Pseudomonas putida* is a PLP-dependent multifunctional enzyme (Alexander *et al*., 1994) and has a classification number EC 4.4.1.11. MGL is a tetrameric protein composed of identical subunits (Motoshima *et al*., 2000). The molecular weight of one subunit is 42,626 Da being composed of 398 amino acid residues. Each subunit binds one molecule of the cofactor-PLP. MGL is a member of *γ*-family and possess a conserved tyrosine residue (Tyr114) which is crucial for activity (Inoue *et al*., 2000). The formyl group of pyridoxal 5'-phosphate is bound in an aldimine linkage to the t-amino group of lysine residues of the protein (Tanaka *et al*., 1977). Methionine γ-lyase also catalyzes α, β-elimination and β-replacement of various *S*-substituted derivatives of homocysteine, cysteine and methionine (Inoue *et al*., 1995). However, the enzyme cannot catalyze α, β-elimination reactions with cystine, alanine, serine D-cysteine or D-methionine.

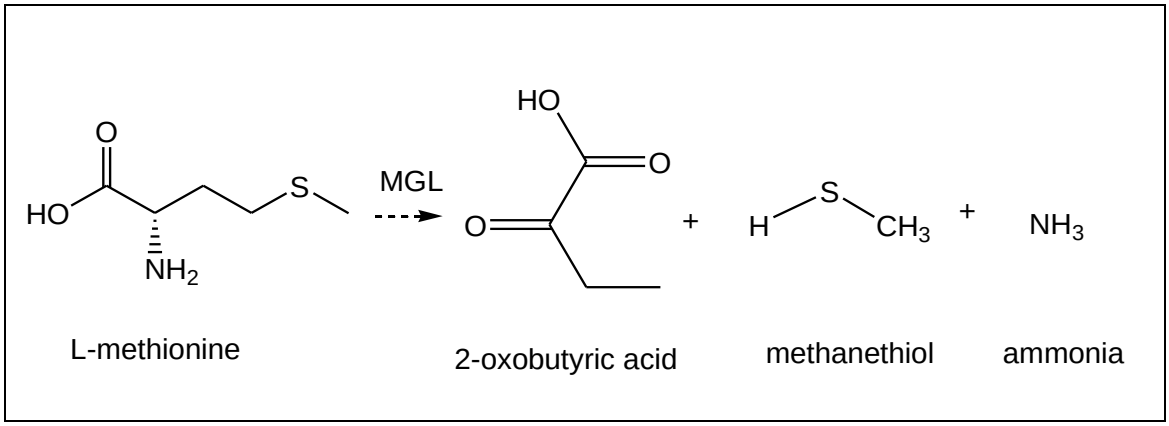


Figure 1.3-4. Conversion of L-methionine into 2-oxobutyric acid, ammonia and methanethiol.

1.3.1.2 Tryptophanase from *E. coli*

Tryptophanase (EC 4.1.99.1) (TRP) known also as tryptophan indole-lyase from *Escherichia coli* is a PLP-dependent enzyme and a tetramer of four (52.8 kDa) subunits. Each monomer binds one molecule of PLP to Lys270 by forming an aldimine bond (Kogan *et al*., 2004). TRP mainly catalyses the synthesis and degradation of the natural substrate tryptophan *via* β-elimination (Newton *et al*., 1965). The enzyme also acts on other L-amino acids and their analogues (reactions 2-5). In each case, the enzyme catalyzed β- replacement reactions (Newton and Snell 1964). The enzyme is inactive in the absence of PLP (Tsesin *et al*., 2007). Instead, in the apo-form, two sulphate ions occupy the phosphoryl-binding site of the cofactor binding site of enzyme (Ku *et al*., 2006). It has been proven that Lys269 is crucial in the formation of a PLP-quinonoid intermediate with Arg419 is necessary for the β-elimination reaction and His463 and Tyr74 determining substrate preferences (Ku *et al*., 2006).

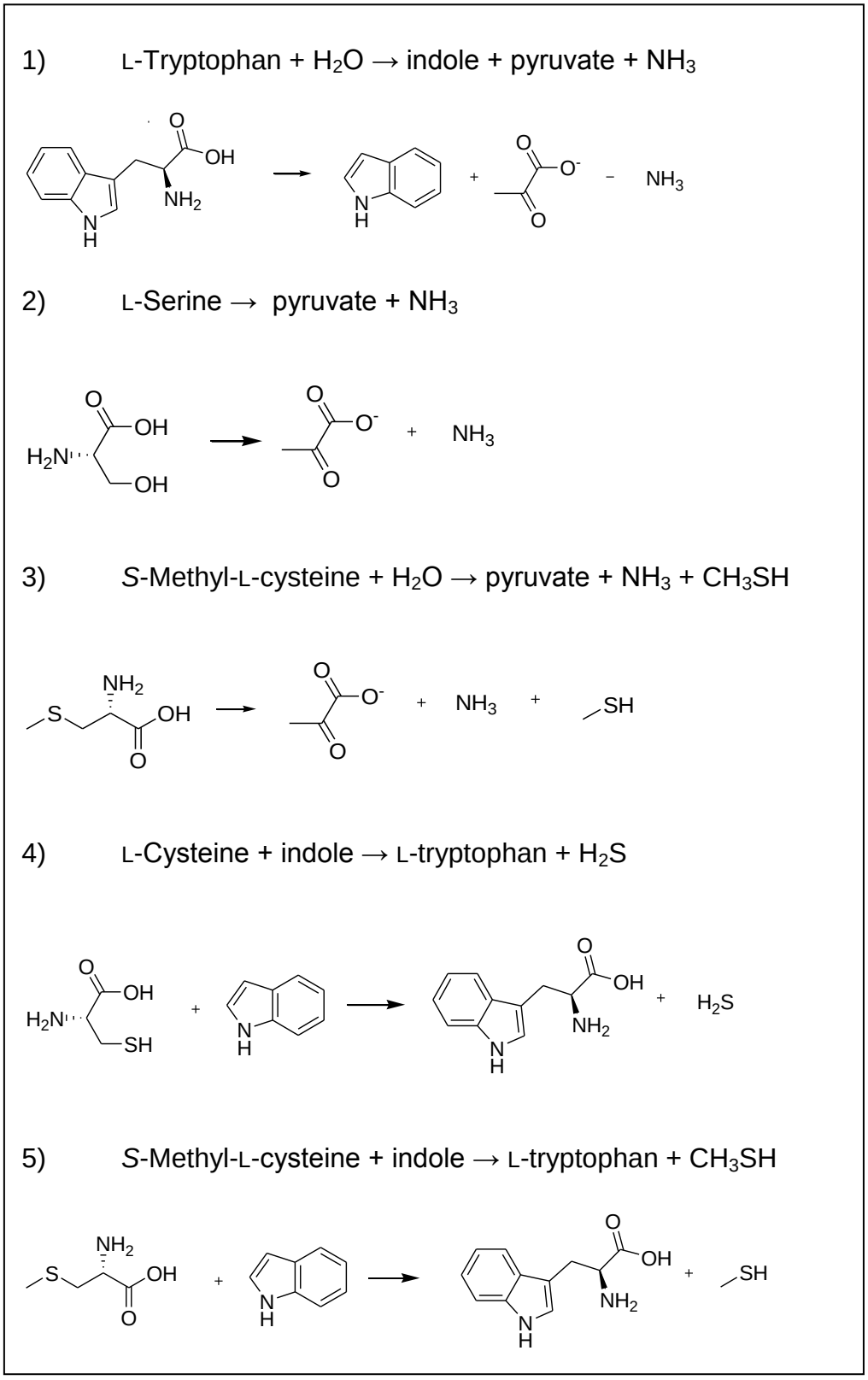


Figure 1.3-5. The list example reactions catalysed by tryptophanase from *E. coli*.

It was reported that cations such as NH_4^+ and K^+ are essential for enzyme activation and turnover (Newton *et al*., 1965; Ku *et al*., 2006; Tsesin *et al*.,

2007) with the optimum temperature for enzyme activity being 37°C and with maximal activity between pH 7.2 - 8.

1.3.1.3 MalY and MetC- Cystathionine β-lyase from *E. coli*

Two pyridoxal 5'-phosphate cystathionine β lyases from *Escherichia coli,* which are encoded by two genes Maly and MetC degrade cystathionine to pyruvate, ammonia and homocysteine (Uren, 1987). MetC is a tetrameric protein with the molecular weight of each subunit being approximately 43,000 Da (Clausen *et al*., 1996; Dwivedi *et al*., 1982). Each monomer consists of 395 amino acids and contains one covalently-bound molecule of PLP linked to the amino group of Lys 210 (Clausen *et al*., 1996). Recent investigations have provided new insights into the connection between the structure and the catalytic mechanism of the enzyme. It was suggested that Tyr111 is responsible for active site shielding with Ser339, Arg59 and Arg372 forming the characteristic docking site for the substrate (Clausen *et al*., 2000). Unfortunately, there is not much information in the literature about the relative importance of MetC in *E. coli.* It is unclear if MetC or MalY are responsible for cystathionine conversion in methionine synthesis (Faleev *et al*., 2009). In the pathway cystathionine is cleaved to homocysteine (figure1.3-6) and methylated to methionine (Zdych *et al*., 1995) (figure 1.2-3).

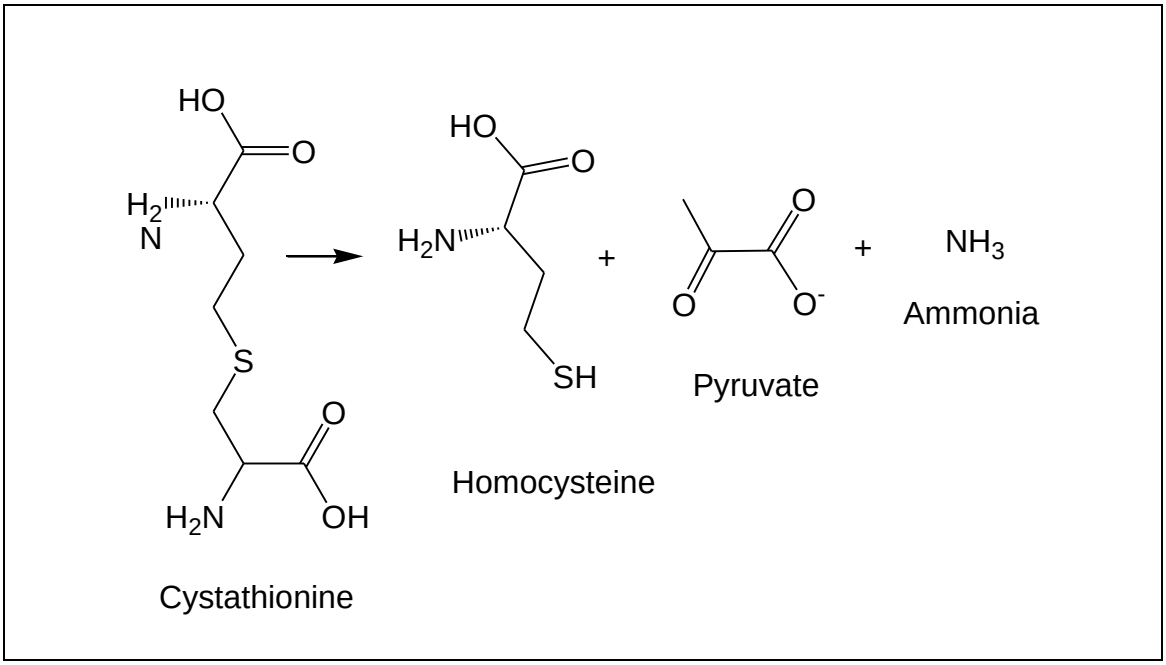


Figure 1.3-6. The general scheme of conversion of L-cystathionine into homocysteine, pyruvate and ammonia.

The optimum pH of reactions for both enzymes is pH 8.2 with maximal activity determined at 37^{o}C (Zdych *et al*., 1995). The differential expression of MalY and MetC has been the subject of some study with the MalY gene, known as a repressor of the maltose system (Bertoldi *et al*., 2005). It was proven that the MalY enzyme can completely replace MetC in the *E. coli* biochemical pathway but MetC was not able to regulate the maltose system and had no effect on the other maltose transport genes (Zdych *et al*., 1995; Boos *et al*., 1998). It has been reported that the active site of MalY protein is solvent-exposed and does not bind the PLP molecule very strongly. In addition, the binding pocket of MalY is larger than MetC and can show activity toward amino acid-sugar conjugates with sulphur atoms in the β position (Clausen *et al*., 2000). Cystathionine β-lyase can convert cystathionine to homocysteine but is also able to catalyze an α,γ- elimination of cysteine and its derivative (Clausen *et al*., 2000). MetC was

also shown to exhibit α,γ-elimination activity toward methionine (Fernandez *et al*., 2000). MalY is a homodimeric protein with each subunit consisting of 399 amino acids with a conserved amino acid residue – lysine 233 at the pyridoxal phosphate binding site (Zdych *et al*., 1995). Each subunit of MalY has a relative molecular mass of 43500 Da and an unusual mechanism of PLP binding compared to the other C-S lyases. The active holoenzyme exists as a dimer containing two molecules of PLP, whereas the inactive apoenzyme dissociates into monomers. In the presence of the cofactor the enzyme can refold and can become an active dimer again with a 85% of its original activity (Bertoldi *et al*., 2005).

1.3.1.4 Cystathionine β,γ-lyases from *Saccharomyces cerevisiae*

Studies by Yamagata and co-workers (Yamagata *et al*., 1993) have shown that cystathionine γ-lyase from yeast catalyses the γ-elimination of cystathionine to L-cysteine, 2-oxobutanoate and ammonia. Cystathionine β-lyase is also responsible for converting cystathionine to L-homocysteine, pyruvate and ammonia (figure 1.3-7). The enzyme exhibits both activities *in vivo* depending on the availability of the substrates. For example, high concentration of L-cysteine inhibits the activity of cystathionine β-lyase (Yamagata, *et al.* 2003). It was found that the transsulphuration pathway in *S. cerevisiae* allows the interconversion of homocysteine and cysteine *via* the intermediary formation of cystathionine. Unfortunately, the enzyme mechanism of cystathionine β-lyase is not well understood are and there is no structural information available for the enzyme. Cystathionine γ-lyase (EC 4.4.1.1) is encoded by the CYS3 gene and has a tetrameric structure (Thomas *et al*., 1997). Each subunit consists of 393

amino acids residues, each with a molecular weight of approximately 42,000Da. Cystathionine β-lyase binds two molecules of PLP per subunit.

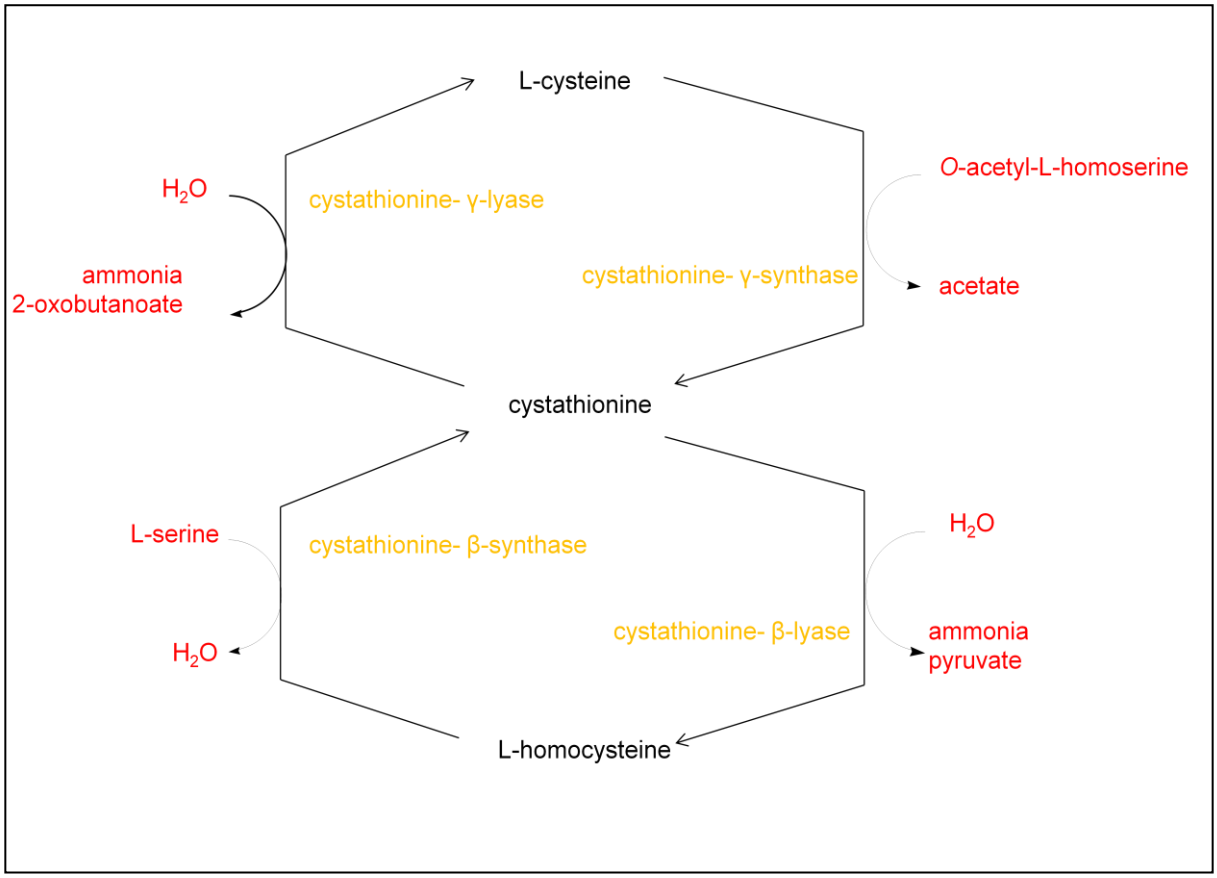


Figure 1.3-7. Pathway of sulphur amino acid biosynthesis in *S. cerevisiae*. Two target enzymes are marked by blue boxes. Adopted from biocyc.org

1.3.1.5 Cystathionine β-lyases from *Arabidopsis thaliana*

Cystathionine β-lyase from *Arabidopsis thaliana* (CBL) is a tetramer composed of four identical subunits of 46 kD, each being associated with one molecule of pyridoxal 5'-phosphate (Ravanel *et al*., 1996; Breitinger, *et al.* 2001). The enzyme is essential for the *de novo* biosynthesis of methionine in plants and cleavage of cystathionine to produce homocysteine (Breitinger *et al*., 2001). Cystathionine β-lyase is synthesized as a 50.4 kDa precursor protein, that is processed on transport into the chloroplast to yield the mature enzyme cystathionine β-lyase with the exact location of the maturation site is not known. (Ravanel et al., 1996) A Schiff base is formed between the cofactor and the holoenzyme at the amino group of Lys-278 (Ravanel et al., 1996).

1.4 Characterisation of glucosinolates in plants

Glucosinolates are amino acid-derived natural plant products, (Kyung and Lee 2001); which upon tissue disruption are hydrolysed by the endogenous enzyme myronidase, which is usually stored in distinct subcellular compartment to potentially toxic isothiocyanates, thiocyanate or nitriles (Rask *et al*., 2000). Glucosinolates are widely found in members of *Brassicaceae, Capparidaceae, Resedaceae,* and *Caricaceae* families. Every glucosinolate contains a central carbon with structural diversity due to different side groups as shown in figure 1.4-1 (Vergara *et al*., 2008).

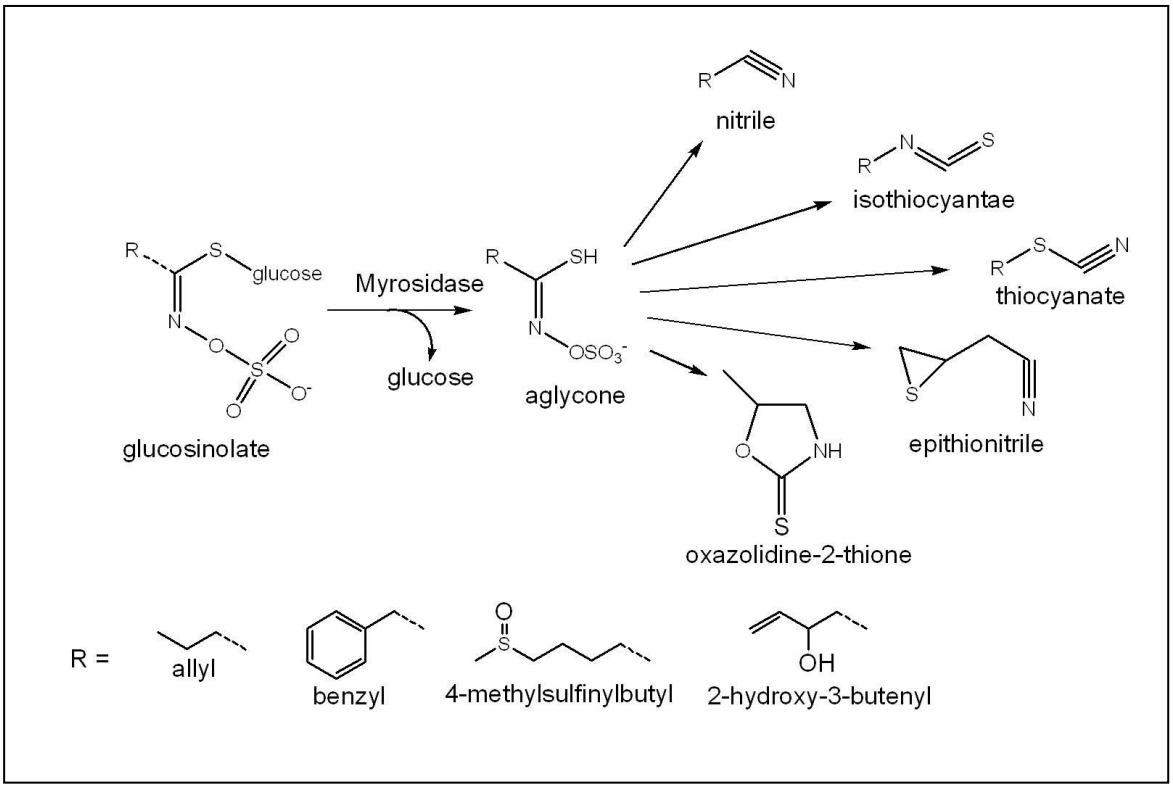


Figure 1.4-1. The general structure of glucosinolates and their enzymatic degradation products adapted from Rask *et al*. 2000.

the products released on myronidase action have important roles as repellents against herbivorous insects and microorganisms, but also as volatile attractants of specialized insects (Wittstock and Halkier, 2002; Hopkins *et al*., 2009). For humans, these compounds serve as flavour compounds (Tripathi and Mishra, 2007). More recently, it has been determined that these compounds also serve as cancer-preventative agents with protective activities associated with sulphoraphane and phenylisothiocyanate derived from glucoraphanin and gluconasturtiin respectively (Toribio *et al.,* 2007; Lee 2009). Some active degradation products of glucosinolate are also known as flavour compounds. For example, sinigrin found in mustard seeds, provides the plant with a defence against herbivores by releasing reactive products like allyl isothiocyanate as presented on figure 1.4-2 (Al-Gendy *et al*., 2010)

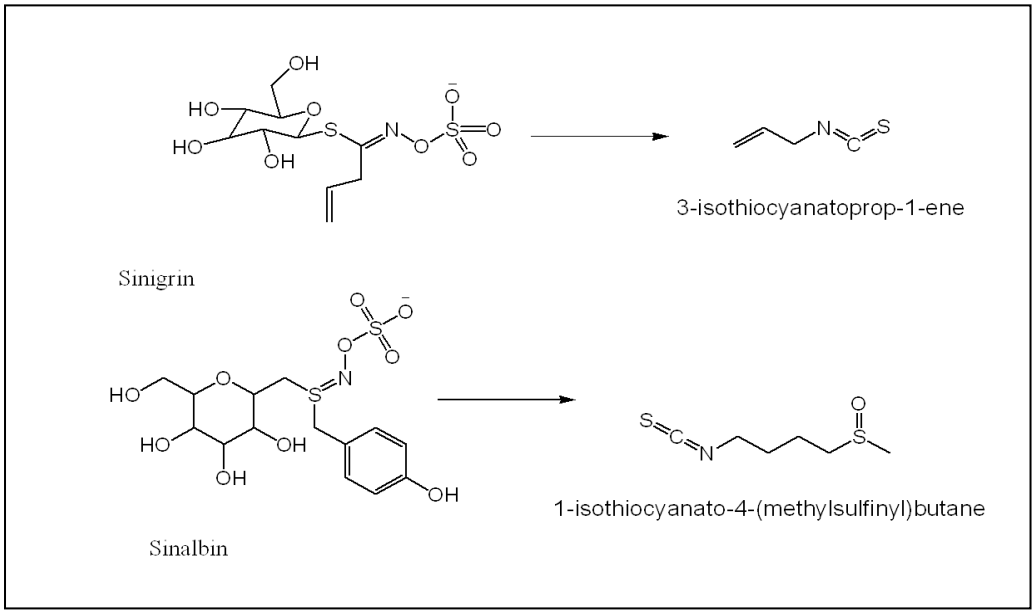


Figure 1.4-2. Example of flavour glucosinolates (adapted from Al-Gendy *et al.*, 2010).

There is some evidence that food processing can lead to glucosinolate decomposition resulting in changes in flavour composition (Bones and Rossiter, 2006; Halkier and Gershenzon, 2006).

1.5 Methylation of free thiols

Little is known concerning the enzymes involved in direct methylation of free thiols *in vivo*. For example, plant *S*-methyltransferases also active towards halide/thiocyanate nucleophiles (HTMT), have been previously isolated and studied from several plants: *Batis maritima* (Ni and Hager 1999), Wollastonia-Biflora Leaves (James *et al.,* 1995; Trossat *et al.,* 1996), *Brassica* (Attieh *et al.,* 2000) and *A. thaliana* (Attieh *et al.,* 2002; Nagatoshi and Nakamura, 2007). In brief, *S*-adenosyl-L-methionine (SAM) dependent methyltransferases act on halide ions using SAM in a nucleophilic substitution reaction, to generate halomethane derivatives (Attieh *et al.,* 2000). As an enzymatic group, these

methyltransferases additionally show activity towards thiol substrates such as bisulfide or thiocyanate (Itoh *et al.,* 2009). It has been suggested that, members of the methyltransferase family may be involved in the detoxification of sulphur compounds produced by the degradation of glucosinolates to release them as highly volatile compounds (methanethiol and methyl thiocyanate), which are believed to possess anti-pathogenic properties (Itoh et al., 2009). *S*-adenosyl methionine (SAM), the methyl donor for all methyltransferases, is synthesized by *S*-adenosyl methionine synthetase from methionine and ATP (figure 1.5.1). In the presence of the methyltransferase enzyme, the methyl group is being attached to positively charged sulphur atom and the co-product of the reaction *S*-adenosyl homocysteine (SAH) is released. Methylation can also occur by transfer of the methyl group from tetrahydrofolate, which is synthesised from folic acid though this actually is not known to be associated with *S*-methyltransferase (Bugg, 2004). Three distinct *S*-methyltransferases have been found in the model organism *Arabidopsis thaliana* with these enzymes primarily responsible for the production of methylated chloride, bromide and iodide ions. A phylogenetic analysis using the *A. thaliana* structural gene (AtHOL1) suggests a wide distribution of these enzymes throughout the plant kingdom, (Nagatoshi, 2007).

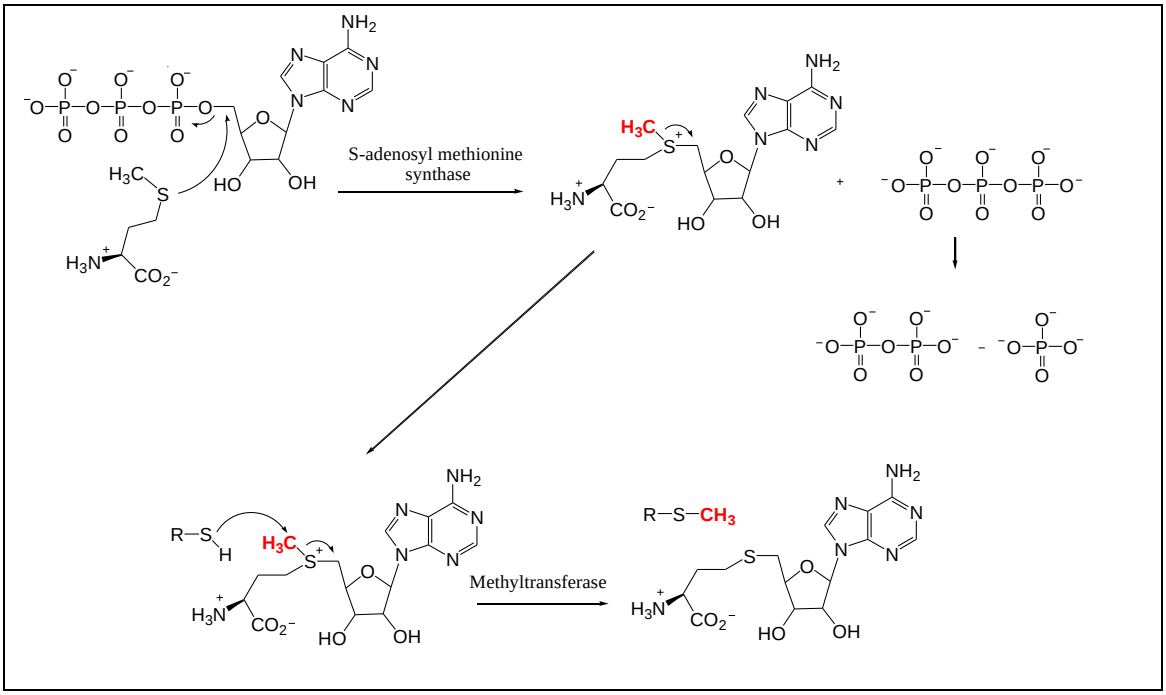


Figure 1.5-1. Biosynthesis of *S*-adenosyl methionine and methyl group transfer adapted from (Bugg, 2004.

A product of the AtHOL1 gene descended as methyl transferase TMT1 has been shown to possess high activity towards methylthiocyanate and based on this fact, is believed to metabolize glucosinolate breakdown products (Rhew *et al.,* 2003). A series of sulphydryl compounds including F&F precursors has been found to act as methyl accepting substrates in the presence of *S*-methyltransferase from *Catharanthus roseus* (Madagascar periwinkle) (Coiner *et al.,* 2006). For example, the enzyme named CrSMT1 originally identified as an *O*-methyl transferase also showed useful activities toward *S*-acceptors including flavour compounds like furfuryl thiol, mercapto-hexanol and pyridinethiol.

1.6 The role of methylation in xenobiotic metabolism in plants

It has been previously reported that methylation also plays an important role in the detoxification of xenobiotic compounds in *Euglena gracilis,* onion and *A. thaliana* (Drotar and Fall 1985). More recently the importance of methylation in xenobiotic has been reported in metabolism of herbicide safener fenclorim (Brazier-Hicks *et al.,* 2008). Safeners are usually co–applied with herbicide to protect crops as they act by transcriptionally activating the expression of genes encoding several classes of detoxifying enzymes, notably the glutathione transferases (GSTs) (Wu *et al.,* 1999).This well established system represents an example of an inducible multiple step metabolic pathway, where detailed information on the content of intermediates is available (Liu et al., 2009). As presented in figure 1.6-1 the metabolism of fenclorim leads to the formation of 4-chloro-6-(methylthio)-2-phenylpyrimidyne. The methyltransferase activity associated with the formation of this intermediate was determined, though the enzymes responsible were not identified (figure 1.6-2) Methylation of foreign thiols has been also studied in *E. gracilis* where the cellular SMT shows activity toward foreign compounds like PCNB, benzenethiol, chlorophenol and thiosalicylic acid (Drotar and Fall, 1985).

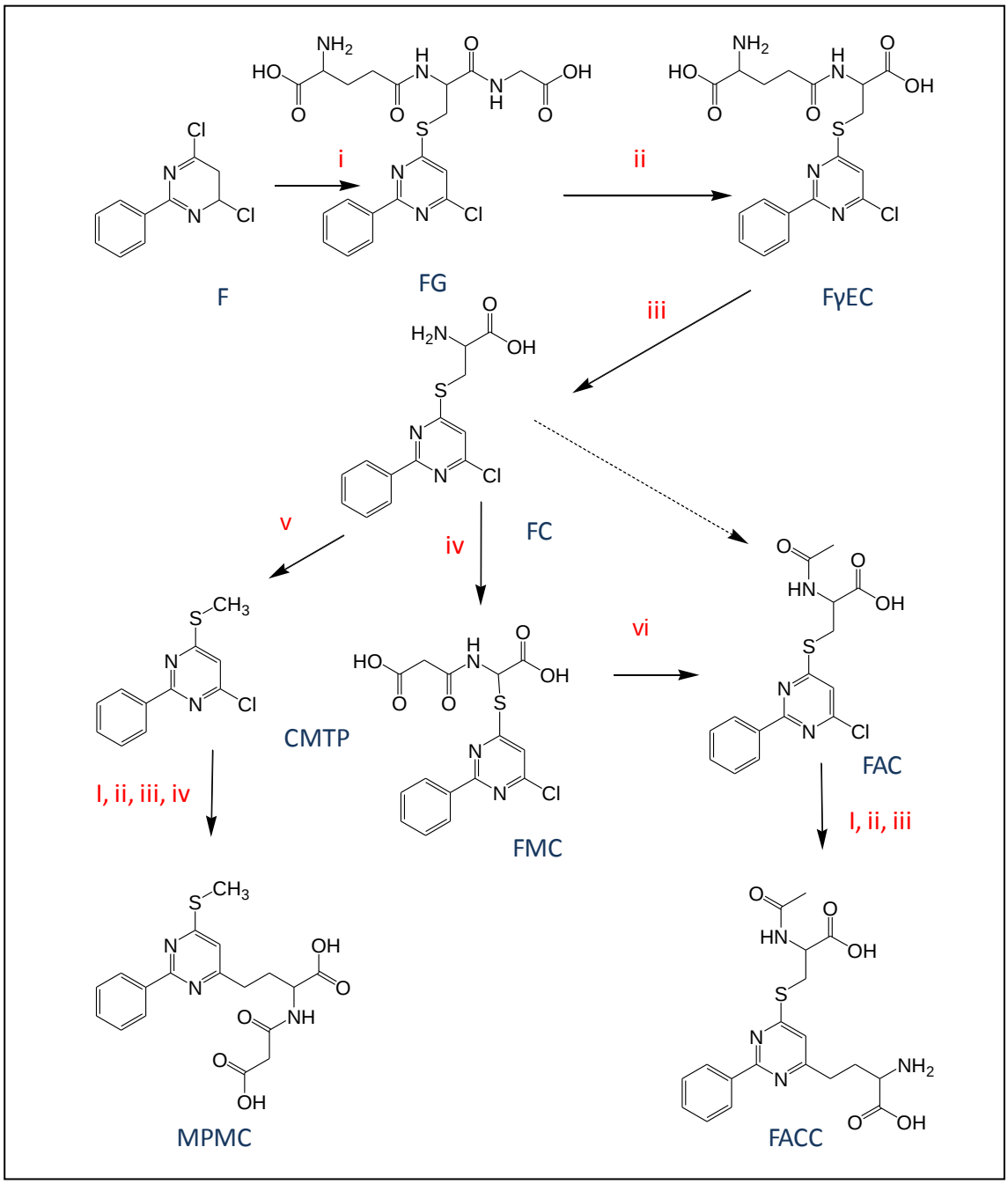


Figure 1.6-1. The proposed scheme of fenclorim metabolism in *A. thaliana* adapted from (Brazier-Hicks *et al.*, 2008).
The *numbered arrows* refer to the following reactions: glutathionylation (*i*), removal of a glycine moiety (*ii*), removal of a γ-glutamyl moiety (*iii*), *N*-malonylation (*iv*), C-S lyase mediated cleavage of the cysteine moiety and subsequent *S*-methylation of the exposed thiol (*v*), and decarboxylation (*vi*).

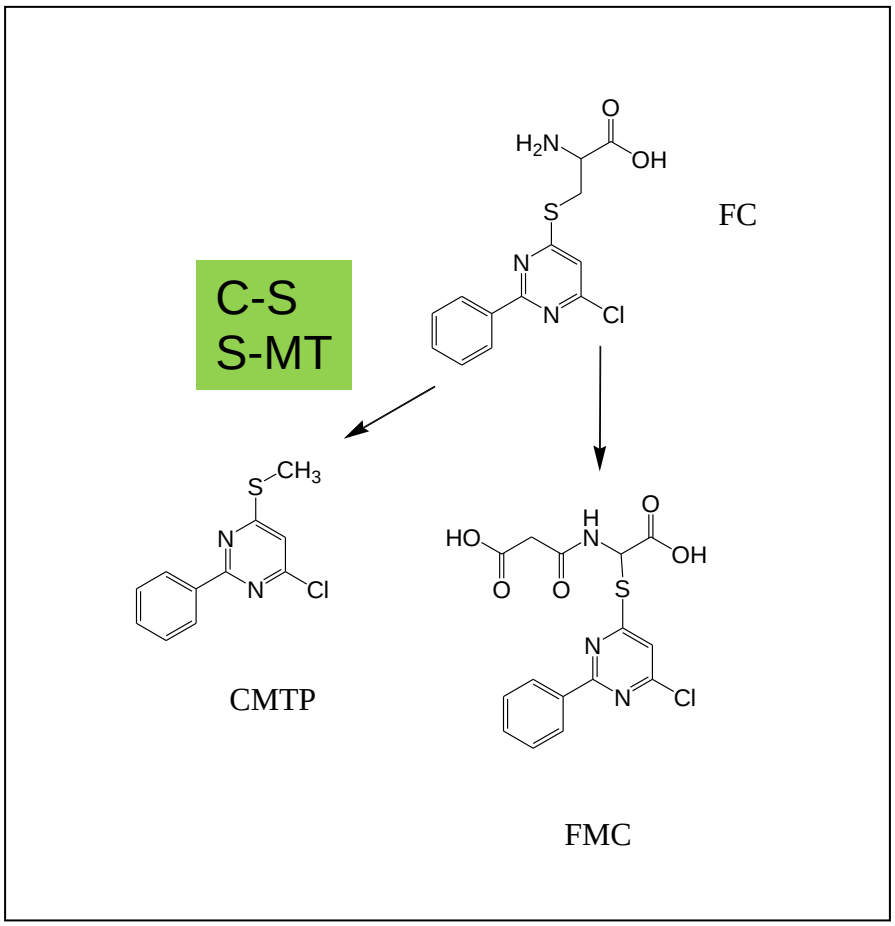


Figure 1.6-2. The putative reaction scheme for coupled enzymatic reaction using a beta- lyase and *S*-methyltransferase in fenclorim metabolism in *A. thaliana* (Brazier-Hicks *et al.*, 2008).
Green box –proposed complex of two enzymes: carbon-sulphur lyase (C-S) and sulphur-methyltransferase (S-MT); FC: (Fenclorim cysteine, S-(4-chloro-2-phenylpyrimidin-6-yl)-cysteine); CMTP: Methylated fenclorim thiol (4-chloro-6-(methylthio)-2-phenylpyrimidine); FMC: Fenclorim malonyl cysteine (S-(4-chloro-2-phenylpyrimidin-6-yl)-N-malonylcysteine).

Other studies on the metabolism of the fungicide pentachloronitrobenzene (PCNB) in *A.cepa* have also suggested that *S*-methylation is involved in the detoxification pathway (Lamoureux and Rusness, 1980). These findings suggest that the enzymatic methylation of foreign compounds is a widespread phylogenetically environmental biotransformation.

1.7 Biosynthesis pathways for the natural production of volatile organosulphur compounds

In this project, studies on the enzymatic production of VOSCs have been undertaken with the aim of developing methods for the direct or indirect generation of compounds of interest for flavour and fragrance applications. In order to obtain the desired sulphur contained F&F compound the pathway for generation of free thiols (BioPath VOSC) was proposed and will be further investigated in detail. A general overview of designed pathway is presented in figure 1.7-1.

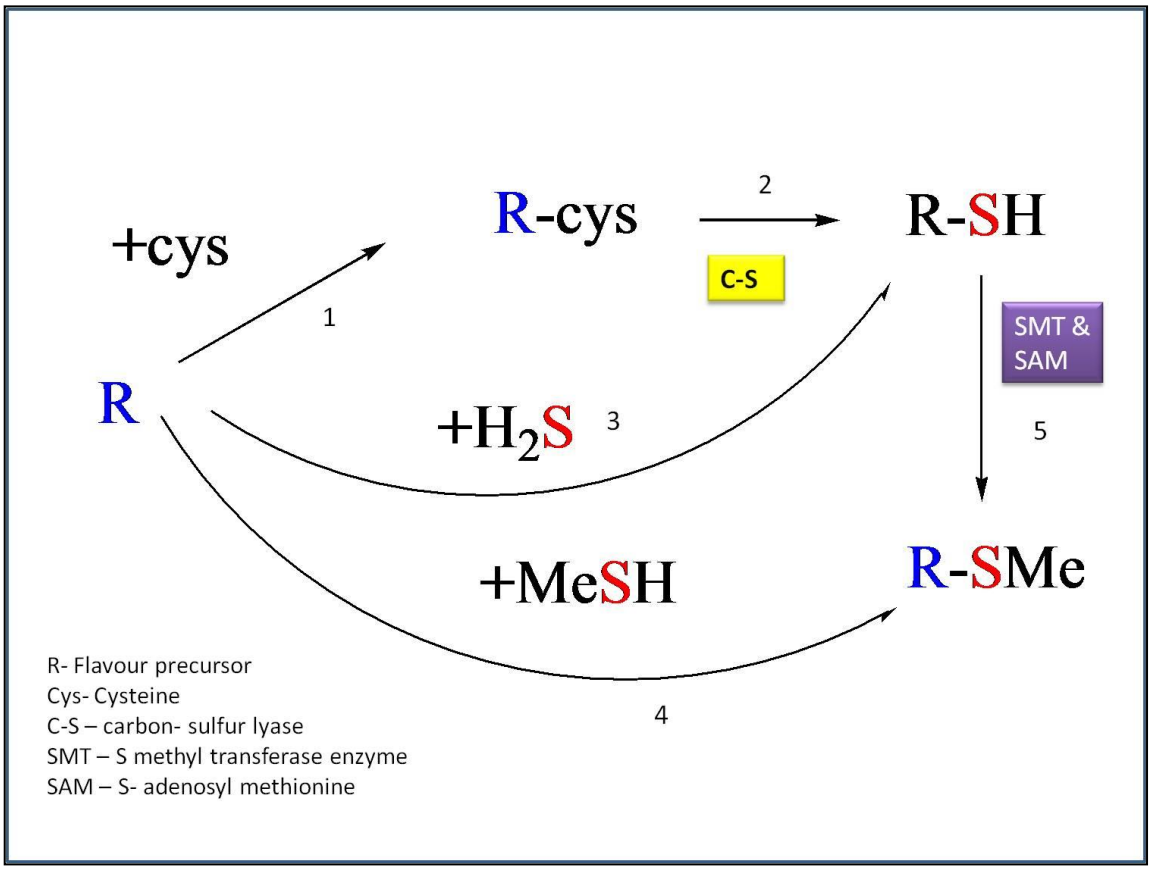


Figure 1.7-1. Biosynthesis pathway for the natural production of organosulphur compounds for the F&F industry (BioPath VOSCs).

In the first reaction, Michael-type addition of flavour precursor (α, β-unsaturated carbonyls) to L-cysteine leads to the formation of L-cysteine conjugates. In the next step C–S lyase enzymes cleave the carbon sulphur bond in cysteine resulting in incorporating a sulphur group into the flavour compound.

In reaction number 3, the desired free thiol compounds can be obtained by direct reaction of flavour precursors and naturally generated hydrogen sulphide. As shown in reaction 5 free thiol obtained in reaction two and three can then be subsequently methylated by sulphur methyl transferase in the presence of *S*-adenosyl methionine (SAM). Alternatively, another route for the production of the same type of methylated compounds involving one-step mechanism has been proposed in reaction 4 as a direct addition of methanethiol naturally produced by MGL to flavour precursor. The proposed pathway will be investigated in further detail in the following chapters of this thesis.

1.8 Aims and Objectives

The aim of this work was to establish several routes for the enzymatic production of High-Impact volatile organosulphur containing compounds. Emphasis was placed on identifying the most active and highly expressed enzymes, which could be used as industrial biocatalysts as an alternative to the chemical synthesis of Flavour and Fragrance compounds.

The project was broken down into the following objectives:

- Source, clone and express a collection of highly active carbon-sulphur lyase enzymes from different sources (microbial, plant).
- Determine and compare their activity toward sulphur-containing amino acids and other thiol compounds of interest.
- Develop methods to measure enzymatic activity and products formation.
- Source, clone and express new thiol methyl transferase from *Arabidopsis thaliana* and test the enzyme activity towards the range of free thiols.
- Investigate the possibility of producing methylated forms of free thiol compounds obtained from industry partner Frutarom Ltd Hartlepool.
- Optimise the production of derived compounds so as to be suitable to take to commercial scale.
- Characterise the concept of construction of the C-S lyase/ *S*-methyltransferase pathway in bacteria, in order to investigate the coupling of microbial/plant enzymes activities to directly produce methylated thiols of interest *in vivo*.

2 Materials and methods

2.1 Materials

All chemicals and materials were purchased from Sigma – Aldrich and of analytical grade unless stated. The *S*-adenosyl-L-[^{3}H-methyl]-L-methionine (MBa/mmol) was obtained from GE Healthcare. Cysteine conjugates were kindly donated by Frutarom Limited (Hartlepool, UK) unless stated otherwise. DNA primers were synthesised by MWG - (Biotech, Ebersberg, Germany). Enzymes for molecular biology procedures were obtained from New England Biolabs. KOD HiFi DNA Polymerase and the pET24a vector system were obtained from Novagen. The Wizard® plus SV miniprep DNA purification system and pGEM T Easy vector system were purchased from Promega. Agarose, peptone and agar were purchased from Melford. The coding sequence of (CrSMT1) from *Catharanthus roseus* was synthesized by GeneScript USA Inc.

All buffers were prepared in de-ionised water followed by filtration and de-gassing. *Arabidopsis thaliana* seeds were supplied from the Nottingham Arabidopsis Stock centre.

2.1.1 Bacterial medium

2.1.1.1 Luria-Bertani medium (LB)

Medium for bacterial growth was prepared as the following formulation; peptone (10 g/l), NaCl (10 g/l), yeast extract (5 g/l). LB-agar was prepared as above with the addition of agar (15 g/l). Both LB and LB-agar were adjusted to pH 7.0 with NaOH followed by autoclaving to sterilise.

2.1.1.2 Optimised medium (OM)

Medium composition: yeast extract (30 g/l), NaCl (1 g/l), $(NH_4)H_2PO_4$ (1.88 g/l), K_2HPO_4 (13.1 g/l) and glycerol (25 g/l) and pH was adjusted to pH 7.0, followed by autoclaving to sterilise.

2.1.2 Synthesis of fenclorim-*S*-conjugates

The synthesis was performed according to the method described previously (Brazier-Hicks, *et al*., 2008). To a 4 mL solution of fenclorim (0.1 mmol) in ethanol /acetonitrile (1:1) (v/v)) cysteine was added (0.2 mmol) in distilled water with the addition of TCEP reagent (Tris (2-carboxyethyl)phosphine hydrochloride). After adjusting to pH 9 with triethylamine, the mixture was diluted with distilled water to a final volume of 6 ml and then incubated overnight at 25°C. The fenclorim glutathione conjugate was purified on an X Bridge PrepC18 5µm column connected to mass-directed HPLC (Waters). Mobile phase – Solvent A: water with 0.1 % formic acid; solvent B: Methanol with 0.1 % formic acid. Gradient conditions: 90 % A to 5 in 14 min: 10 % to 95 % flow rate at 17 ml/min. The purified fractions of interest were dried under vacuum and purity was confirmed by LCMS.

2.1.3 Purification of cysteine conjugates

After dissolving in methanol/water (1:1 *v*/*v*), the cysteine conjugates were purified on an X Bridge Prep C18 5 µm column connected to HPLC (Waters) according to method described in section 2.1.2. The purified fractions of interest were dried under vacuum. Purity was determined by LCMS.

2.1.4 HPLC separation of cysteine conjugate isomers

500μl samples were injected onto a C18 (15 x 1 cm, 5 μm) column and analysed using a Dionex UltiMate 3000 HPLC (Solvent A: 0.5 % formic acid in water, Solvent B: 0.5 % formic acid in acetonitrile) at a flow rate of 0.8 ml/min starting at 10 % B to 100 % B over 12 ml.

2.1.5 Reduction of organic disulphide bonds

Disulphide bond reductions were carried out by mixing appropriate volumes of tris (2-carboxyethyl) phosphine TCEP HCl defined earlier in (0.1 mM in Tris-HCl pH 7.5) and substrate solution (0.1 mM in Tris-HCl (pH 7.8) buffer) in 1:1 molar ratio, accordingly to the manufacturer's protocol, followed by incubation at room temperature for 1 h.

2.2 Methods

2.2.1 Gene Cloning

2.2.1.1 Polymerase chain reaction (PCR)

Standard PCR reactions were performed in a total volume of 50 µl using a 1:11 dilution of Taq PCR buffer (449 mM Tris, pH 8.8, 112 mM $(NH_4)_2SO_4$, 23 mM $MgCl_2$, 3 µM mercaptoethanol, 44 µM EDTA, MgCl2, each of dATP, dCTP, dTTP and dGTP 2mM or 10x KOD HiFi polymerase buffer (20mM Tris-HCl,pH 7.5, 8mM $MgCl_2$, 7.5 mM DTT, 50 µg/ml BSA,150 µm each of dATP, dCTP, dTTP and dGTP. To initiate the reaction, 0.5 µl Taq polymerase or KOD polymerase was added respectively during step 1 as detailed in Table 2.2-1., 2.2-2. The mixture was then subjected to the program detailed in Table 2.2-3 in an Eppendorf Mastercycler Gradient PCR machine. After denaturation at 94°C for 2 min, the samples were subjected to 20-28 cycles at 94°C for 30 s, 50-65°C for 45 s and 72°C for 45 s to 1 min 30 s. After the last cycle, samples were incubated at 72°C for 5 min to fill-in the protruding ends of newly synthesized PCR products.

PCR products were analysed on a 0.8 % agarose gel, and if required, purified from the gel using a Promega Wizard® SV Gel and PCR Clean-Up kit (Promega UK, Southampton, UK).

Reagents	Amount [µl]
Template-DNA	2
KOD HiFi DNA Polymerase-buffer (10x)	5
dNTP (2 mM)	5
Forward primer (+) [100 ng/µl]	2.5
Reverse primer (-) [100 ng/µl]	2.5
KOD-Polymerase [2500 U/ml]	0.5
$MgCl_2$ 23 mM	2
H_2O	30.5

Table 2.2-1. The list of reagents used in PCR reaction with Kod HiFi DNA Polymerase.

Reagents	Amount [µl]
Template-DNA	1
Taq-Polymerase-buffer (11x)	4.5
Forward primer (+) [100 ng/µl]	2.5
Reverse primer (-) [100 ng/µl]	2.5
Taq Polymerase [2500 U/ml]	1
H_2O	38.5

Table 2.2-2. The list of reagents used in PCR reaction with Taq DNA Polymerase.

Step	Cycle	Temperature [°C]	Time (min : sec)
Denaturation	1	94	2 min
Denaturation Annealing Amplification	25	94 55 72	15 s 1:30 s 1:30 s
Amplification	1	72	5 min
Cooled	1	4	5 min

Table 2.2-3. The standard PCR Programme used for the amplification of DNA.

2.2.1.2 DNA agarose gel electrophoresis

Agarose gels were prepared by microwaving 1 x TAE (Tris-acetate-EDTA) (4.84 % w/v Tris base, 1.14 v/v glacial acetic acid) and 0.8 % (w/v) molecular biology agarose until the agarose had melted. The mixture was cooled to 55^{o}C and supplied ethidium bromide was added (10 µl/ l). The gel was poured into a gel tray of a horizontal gel electrophoresis BIO-RAD apparatus. DNA samples were prepared by diluting with 6x loading buffer (0.25% v/v xylene, 0.25 % w/v bromophenol blue, 15 % w/v Ficoll) and loaded into the gel wells. Gels were loaded with a lane containing a 1 kb DNA marker GibcoBRL® prior to electrophoresis 1x TAE buffer at 120 V for approximately 20 minutes.

2.2.1.3 Purification of DNA

The DNA fragments of interest were excised from a 0.8 % agarose gel after visualizing under UV light and placed in 1.5 ml tubes containing 500 µl of binding buffer (6M Sodium Perchlorate, 50mM Tris-HCl, 10mM EDTA, pH 8.0). Agarose was dissolved by incubation in a water bath for approximately 10 minutes at 55^{o}C and 10 µl of silica fines suspension (166 mg/ml in water, World Mineral Ltd, Hessle, UK) was added and incubated for 5 min at room temperature. The tube was spun at 1000 *g* for 1 min using a microcentrifuge and the supernatant was discarded. The pellet was resuspended in 125 µl of wash buffer 0.4M NaCl, 2mM EDTA, 2mM Tris-HCl pH 7.5), centrifuged at 1000 g for 1 min and the supernatant discarded; the pellet was washed again in 125 µl of 80 % ethanol and the pellet then dried at 37^{o}C for 10 min and resuspended in 15 µl of sterile water and incubated for 5 min at 50^{o}C. The sample was

microfuged for 1 min at 1000 *g* and the supernatant containing DNA collected and stored at -20 ºC.

2.2.1.4 Restriction enzyme digests

PCR products and plasmid DNA were digested with various restriction enzymes obtained from Promega and New England Biolabs. 2-10 of enzyme units were added to the sample (0.5-2 μg DNA), with the appropriate buffer to a final volume of 20 μl volume and incubated at 37°C for 1-3 h. The digests were analysed by agarose gel electrophoresis and the desired products excised.

2.2.1.5 Partial digestion of DNA with *Nde*I

Partial cleavage was accomplished by using a reduced concentration of restriction enzyme *Nde*I (New England Biolabs) and *Xho*I with appropriate buffer in a final volume of 100 µL at 37°C for 15 min.

A set of different dilutions (1- 20 fold) of enzyme was used to produce the digest products of interest. The reactions were terminated by placing samples in a heat block at 70°C for 10 min and examined by agarose gel 0.8 % (as described in section 2.2.1.2). The DNA fragment of interest was then purified (2.2.1.3) and used for further ligation.

2.2.1.6 Tailing PCR products using dATP

PCR products obtained with *Pfu* polymerase did not contain an adenosine nucleotide at their 3`- terminus and therefore were not compatible for direct ligation into pGEM®-T easy vector (Promega). Therefore the A-tailing reaction for blunt-ended PCR fragments was performed, 1-7 µl of PCR reaction was

mixed with 10x Buffer pGEM®-T, dATP 0.2 mM, 5 units of *Taq* DNA Polymerase and incubated in a thermal block for 30 min at 70ºC.

2.2.1.7 Ligation

For direct cloning, ligations were carried out following the manufacturer's instructions (Promega pGEM-T Easy vector kit). For cloning into the pET24a vector, 1 µl (10×) ligation buffer buffer (as supplied with the enzyme) and 1 µl T4 ligase were incubated with the gene insert ligation prepared in a insert: vector 3:1 molar ratio (total reaction volume 10 µl) at 15ºC overnight.

2.2.1.8 Transformation

The ligated pGEM-T plasmids were transformed into Top 10 competent *E. coli* cells (Invitrogen) and plated onto LB-Amp plates (50 µg/ml) spread with X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranose) and incubated overnight at 37°C. Transformation of competent cells was carried out following the manufacturer's instructions. In brief, 20 µl of competent cells were incubated on ice with 1 µl plasmid for 5 minutes. The cells were then incubated in a water bath at 42ºC for 30 seconds before being returned to ice for a further minute (Sambrook and Russel 2001). 80 µl LB medium was added and the cells incubated at 37ºC for one hour. The cells were then plated on to LB agar with appropriate antibiotic selection (100 µg/ml Amp).

2.2.1.9 Plasmid Preparation

Plasmid DNA was prepared from 10ml of liquid bacterial culture using The Wizard® plus SV minipreps DNA purification system (Promega) according to the manufacturer's instructions. Plasmids were eluted in 100 μl of water.

2.2.1.10 Cloning of *Crstm1* into pET-STRP3 vector

The pET-STRP3 vector was already available in-house (courtesy of Dr. D. P. Dixon). The plasmid was purified as described in section 2.2.1.9 and the plasmid (15 µL) was digested with the restriction enzymes *Nde*I (1 µL) and SalI (1 µL) in Buffer 3 (2 µL) (New England Biolabs, MA, USA) and H_2O (1 µL) at 37°C. Vector (1 µL) was ligated with Crstm1 (3 µL) using T4 DNA ligase (1 µL) (Fermentas Life Sciences, York, UK) in ligation buffer (1 µL) (Fermentas Life Sciences, York, UK). The ligation mixture was transformed into XL-10 gold genotype ultracompetent cells (TetrΔ (mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac The [F' proAB laclqZΔM15 Tn10 (Tetr) Amy Camr] Agilent Technologies Ltd, Cheshire, UK) (as described in section 2.2.1.7). The construct was then purified (as described in section 2.2.1.9) and its sequence confirmed by DNA sequencing.

2.2.1.11 DNA sequencing and analysis

Double stranded cDNAs were sequenced using an Applied Biosystems 3730 DNA Analyser by the University of Durham sequencing service. DNA sequences were edited, translated and restriction sites determined using the DNA sequence editing and analysis program DNA for Windows 2.4.0 (software written by Dr D.P. Dixon, Centre for Bioactive Chemistry, University of Durham,

UK). DNA and protein sequences were aligned using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and sequence similarities were determined using BLAST (http://blast.ncbi.nlm.nih.gov/).

2.2.2 Recombinant protein expression in bacteria

2.2.2.1 Preparation of competent cells

Competent cells Rosetta-gami^{TM} 2 *E. coli* were prepared as described by Sambrook. (Sambrook and Russel 2001). A single bacterial colony was used to inoculate a 100 ml LB starter culture in 1 l flask, containing 35 µg/ml chloramphenicol. The culture was incubated for 3 h at 37ºC with shaking at 200 rpm. When at A 600 nm of ~ 0.4 was reached, bacterial cells were transferred into ice-cold 50 ml polypropylene tubes, cooled on ice for 10 min and then pelleted for 10 min at 2700 g at 4ºC. Medium was decanted and the remaining pellet was resuspended in 30 ml of an ice-cold, sterile solution of $MgCl_2$/$CaCl_2$ (80 mM $MgCl_2$ and 20mM $CaCl_2$ the solution was filter sterilized (0.45 µm) and stored on ice for 20 min). Centrifugation was performed again (10 min at 2700 g at 4ºC), the supernatant was discarded and the cells were resuspended in 2 ml of 0.1 M $CaCl_2$ with 10 % glycerol. The cells were distributed into several pre-chilled sterile microfuge tubes divided into 50ul aliquots then frozen immediately by immersion of the microfuge tubes in liquid nitrogen prior to storing at –80ºC.

2.2.2.2 Transformation of expression cells

For protein expression, Rosetta-gami™ 2 *E. coli* (genotype: Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F′[lac+ lacIq pro] gor522::Tn10 trxB pRARE2 (CamR, StrR, TetR) were used (unless stated otherwise). Transformation was carried out as for Top 10 cells, except kanamycin (50 μg/ml) and chloramphenicol (35 μg/ml) antibiotic selection was applied.

2.2.2.3 Bacterial starting cultures

A transformed single *E. coli* colony was used to inoculate a 10 ml LB starter culture, containing 100 μg/ml kanamycin and 100 μg/ml chloramphenicol (unless stated otherwise). The culture was incubated overnight at 37ºC with shaking at 200 rpm.

2.2.2.4 Ovexpression of proteins induced with IPTG or lactose

A 500 ml culture was inoculated with 10 ml of the starter culture, and the cells grown under the same conditions for approximately 4 hours until the culture reached mid-log phase of growth. At an optical density at 600 nm of 0.500, the cells were induced with 0.1 mM IPTG or 0.01mM lactose . The culture was then incubated overnight at 37ºC with shaking at 220 rpm. The bacteria were then harvested by centrifugation (10000 g, 10 min) and the pellets frozen at -80ºC prior to use.

2.2.3 Protein purification and analysis

2.2.3.1 Bacterial protein extraction with the B-Per® kit

For direct extraction from bacteria, *E. coli* cells were lysed using a B-Per® kit (Thermo Pierce Chemical, Rockford) following the manufacturer's instructions. Briefly, the cells were suspended in B-Per® supplemented with 5 mM $MgCl_2$, 5 µg/ml DNase I and 200 µg/ml lysozyme and gently shaken for 15 min at room temperature (RT). Debris and inclusion bodies were removed by centrifugation at 1000 g for 15 min at 4°C.

2.2.3.2 Sonication of cells

Bacterial cell pellets were resuspended in 5 ml of His loading buffer (20 mM imidazole, 20 mM Tris in HCL pH 7.8 containing 0.5 M NaCl) and sonicated for 30 seconds on a 33 % power settings using a Bandelin Sonopus UW 2070. The bacterial lysate was then centrifuged (14000 g, 15 min, 4°C).

2.2.3.3 His-Tagged protein purification

The soluble protein fraction was loaded on to a 5 ml Ni-NTA column (Qiagen) and washed with His loading buffer (20 mM imidazole, 20 mM Tris in HCl, 0.5 M NaCl pH 7.8) until detector baseline was achieved. The retained protein was recovered using His elution buffer (300 mM imidazole, 20 mM Tris in HCl, 0.5 M NaCl pH 7.8). Protein elution was monitored by determining UV absorbance at 280 nm.

2.2.3.4 Strep-Tagged protein purification

The frozen bacterial cell pellets were thawed at room temperature and re-suspended in 30 ml HEPES-buffered saline [20 mM HEPES free acid, 150 mM NaCl, 1mM EDTA final pH 7.5]. An approximately 5 ml aliquot of re-suspended *E. coli* cells was taken and the cells lysed via sonication. Avidin (2 µg/ml) and DNase I (5 µg/ml) were added and the lysate spun down (1800g, 15 min, 4°C). Purification was performed using a 1 ml *Strep*-Tactin Superflow High Capacity column (Stratech Scientific Ltd, Suffolk, UK). The column was firstly pre-equilibrated with HEPES buffer at 22°C and then loaded with cell lysate at a flow rate of 1 ml/min. Once all unbound protein had been removed from the column (as judged by the 280 nm measurement), recombinant protein was eluted with 5 ml desthiobiotin (DTB; 2.5 mM in HEPES buffer), with the recombinant protein eluting in a single 1ml elution fraction. Recombinant protein was stored at 4°C and the column regenerated with 10 ml 2-(4-hydroxyphenylazo) benzoic acid (HABA; 1 mM in HEPES buffer).

2.2.3.5 Mono Q protein purification

Proteins were applied to a mono Q 1 ml column in 50 mM Tris/HCl, pH 7.8. Following a wash with 5 ml of Tris/HCl pH 7.8 buffer, the bound proteins were eluted with a linear gradient of NaCl (0M - 1M) at 1ml/min and 1ml fractions were collected.

2.2.3.6 Hydrophobic interaction chromatography using octyl Sepharose

Ammonium sulphate was added to soluble fraction to a concentration of 1M prior to loading onto an octyl Sepharose column (47 ml) in 50 mM phosphate

buffer, pH 7.8 containing 1 M $(NH_4)_2SO_4$ at 5 ml/min. Proteins were eluted with a linearly decreasing concentration of $(NH_4)_2SO_4$ (1-0 M) over 200 ml. Following an 80ml wash with phosphate buffer, ethylene glycol (5 ml) was passed through the column to elute any remaining bound protein. Fractions (8 ml) were collected and 100 μl aliquots assayed for enzyme activity using the radiochemical assay described in section 2.2.5.7. Active fractions were pooled and applied to the next column, or precipitated with $(NH_4)_2SO_4$ and stored at -20°C.

2.2.3.7 Gel filtration chromatography using Superdex 200

In quantifying cofactor binding, a gel filtration column was used to determine the amount of pyridoxal phosphate covalently bound to the native protein. A purified fraction of enzyme derived from His-tagged affinity chromatography (2.5 ml) was applied to a superdex gel filtration column (7.8 ml) in 50 mM phosphate buffer, pH 7.8, 0.15 M NaCl at a flow rate of 0.5 ml/min and the absorbance monitored for pyridoxal phosphate at 280 nm.

2.2.3.8 Protein desalting method

After purification, over-expressed proteins were desalted by one of the methods, described below.

2.2.3.8.1 HI Trap ™ desalting column

Hi Trap ™ desalting columns (250x16mm no.54822 Supelco), were attached to FPLC system (ACTA GE Healthcare pH/C-900) and were used to remove salt and other low molecular weight components from protein samples. A sample (1 ml) was loaded onto a pre equilibrated column at flow rate at 1 ml/min. Desalted

protein was then collected. The column was then re-equilibrated, following the manufacturer's instructions with approximately 10 ml buffer before applying the next sample.

2.2.3.8.2 Zebra desalting column

Protein elutant was load onto disposable Zebra 2 ml column (Thermo scientific) packed with Sephadex G25. Columns were prewashed with 5 ml of the appropriate buffer, then protein samples (0.5 ml) were loaded and columns spun at 200*g* for 1 min. Eluted proteins were used directly for assays.

2.2.3.8.3 Dialysis tube

In this method, desalting was accomplished using cellulose dialysis tubing (D9777-100FT Sigma) after washing the tubing in running water for few minutes. The protein elutant was placed inside cellulose tubing and dialysed overnight at 4°C against 500 volume of the appropriate buffer.

2.2.3.8.4 PD10 column

PD10 sephadex columns (GE healthcare) were prewashed with 25 ml of appropriate buffer, then a 2.5 ml of sample was loaded onto the column. The protein was then eluted with 3.5 ml of buffer with the first 2.5 ml collected and used for direct assay. Any remaining salt was removed from column by washing it with approximately 10 ml of buffer prior to re-use.

2.2.3.9 Protein determination

The concentrations of the protein solutions were determined by measuring absorbance at 280 nm (Beckman coulter DU 530 UV/ Vis) with extinction coefficients calculated from the amino acid compositions using the Richard’s

protein calculator program (http://www.mrc-lmb.cam.ac.uk/ms/methods /proteincalculator.html). Total protein content was determined using the Bradford protein dye-binding reagent according to manufacturer's instructions (BIO-RAD) with γ-globulin used as the reference protein. Alternatively for comparative purposes, the assay based on a modified method devised by Bradford (Bradford 1976) was used.

2.2.3.10 Protein analysis by SDS-PAGE

SDS-PAGE gels were prepared using a kit from Biorad according to the method of Laemmli (1970). Resolving gels (10-12 %) were polymerised from acrylamide/ *bis*-acrylamide in 375 mM Tris/ HCl, pH 9.0 containing 0.1 % (v/v) TEMED (Tetramethylethylenediamine), 0.1% (w/v) SDS and 0.1 % (w/v) ammonium persulphate. The stacking gel was polymerised from 4 % acrylamide/ *bis*-acrylamide, 126 mM Tris/ HCl, pH 6.8, 0.1% (v/v) TEMED, 0.1 % (w/v) SDS and 0.05 % (w/v) ammonium persulphate. Protein samples were diluted with an equal volume of 2x loading buffer (0.1 M Tris-HCl, pH 6.8, 20 % (v/v) glycerol, 200mM DTT, 4% (w/v) SDS, 0.2 % (w/v) bromophenol blue) and incubated at 95°C for 10 min. The samples were, than loaded into wells and electrophoresed in SDS-PAGE running buffer (25 mM Tris, 192 mM glycine,0.1 % SDS, pH 8.3) at 200 V until the dye front reached the bottom of the gel (approximately 40 min). After carrying out electrophoresis, the gels were removed from the apparatus. Gels were washed twice thoroughly with 100 ml of distilled water to remove the SDS and stained with colloidal Coomassie blue reagent (0.01 % in ethanol:water 95:5 (v/v) after fixing in 10 % (v/v) phosphoric acid:water 85:15 (v/v). Destaining was carried out using distilled water.

2.2.3.11 LC-MS protein analysis

LCMS (liquid chromatography mass spectrometry) analysis of natural products was performed using a Waters Q TOF Premier mass spectrophotometer connected with HPLC C18 (100 × 2 mm) column. The eluents were: solvent A 0.1 % formic acid and solvent B, acetonitrile, 0.1 % formic acid. The program of elution was as follows:

Time (min)	Flow Rate (mL/min)	% A	% B
0	0.200	95.0	5.0
0.50	0.200	95.0	5.0
1.00	0.200	30.0	70.0
1.30	0.050	30.0	70.0
3.50	0.400	5.0	95.0
4.50	0.400	95.0	5.0
6.00	0.200	95.0	5.0

Table 2.2-4 LCMS program used for protein analysis.

A volume of 10 µl of sample was injected. The eluant was monitored for UV absorbing metabolites between 210 nm and 500 nm using a Waters 996 photodiode array detector. This was followed by ESI-TOF MS on a Micromass spectrometer operating in positive mode.

2.2.3.12 Manipulating protein concentration

In order to increase protein concentration, Vivaspin 2 disposable concentrators (Sartorius Stedim Biotech GMbH Germany) were used according to the

manufacturer's protocol and samples (1ml) spun down at 800 *g*. Once the desired concentration was achieved, concentrators were inverted and spun at 200 *g*. Proteins collected from concentrator were used for assay or stored at 4°C.

2.2.3.13 Preparation of apoenzyme

Cofactor-Free MetC and MalY lyases were prepared by dialysing against potassium phosphate buffer, pH 8.0, containing 1-10 μM hydroxylamine for up to 2 h at 4°C, followed by desalting on a disposable column as described in section 2.2.3.8.2 using 20 mM potassium phosphate buffer pH 8.0.

2.2.3.14 Preparation of holoenzyme

Previously prepared apoenzyme was incubated with 20 μM pyridoxal 5'-phosphate for 1 h at 37°C in potassium phosphate buffer, pH 8.0.

2.2.4 Plant studies

2.2.4.1 Sterilization of seeds

Arabidopsis thaliana seeds were soaked in 0.1 % Triton X-100 (Sigma Aldrich), vortexed and placed on a rotary shaker for 20 minutes. The first wash was replaced with 95 % ethanol and 5 % Triton X-100 and the seeds vortexed occasionally over 5 minutes and the supernatant was discarded. Seeds were then washed with 10 % solution of bleach in water containing 0.1 % Triton X-100 and finally, after 10 min, rinsed with sterile, deionised water in a sterile plant culture hood.

2.2.4.2 Plant materials and growth conditions

Arabidopsis thaliana seeds used for plant studies were sown on moist, potting soil (multipurpose compost and sand ratio 4:1) and germinated under controlled conditions for 3-4 weeks in plant growth rooms at the Biological Sciences Department (Durham, UK). Seeds were grown under a 16 hour photoperiod (24°C) with an 8 hour dark period (22°C). The light intensity was 80 µmol of µEinstein m^{-2} s^{-2} (in the photosynthetically active range). Tissue was frozen in liquid nitrogen prior to storage at -80°C.

2.2.4.3 Arabidopsis root culture

Arabidopsis root culture medium was composed of Sucrose (10 g/l) and Gamborg's B-5 (3.9 g/l) (Melford) with the pH adjusted to pH 5.8 with 1 M potassium hydroxide. Flasks containing 80 ml of medium were covered with cotton wool, foil and then autoclaved. Approximately 20-30 sterilized seeds were added to each flask. Cultures were grown in the dark at 25°C with constant shaking at 120 rpm. Material was harvested after 14 days, frozen in liquid nitrogen and stored at -80°C. Control treatments consisted of 0.1 % v/v acetone.

2.2.4.4 Cell culture treatment

Chemical treatments were diluted in acetone to the final concentration of 10 µM and were added separately to the cell cultures at a final concentration of 0.1 % *v*/*v* acetone and the cells grown on for 5 days. The cultures were harvested after 4, 8, 12 and 24 h of incubation. In each case, chemicals were diluted 1 in 1000 on addition to cultures, with additions of the respective solvents (0.1 %)

alone used as control treatments. On harvesting, cells were separated from the growth medium by filtration prior to weighing, freezing in liquid nitrogen and storage at –80°C. Media samples were analysed directly by LCMS.

2.2.4.5 Protein extraction

Tissue was homogenized in liquid nitrogen using a pestle and mortar. Protein was extracted with 3 v/w 0.2 M Tris-HCL buffer (pH 7.5) and 5 % polyvinylpolypyrrolidone (PVPP). The extract was filtered through miracloth and then centrifuged (10,000 g, 15 min, 4°C). Protein was precipitated from the supernatant fraction by the addition of ammonium sulphate up to 80 % saturation and then centrifuged (5000 g, 30 min, 4°C). Pellets were stored at -80°C and desalted before use on an FPLC desalting column (HiTrap desalting column, GE Healthcare).

2.2.4.6 Protein purification and analysis

All chemicals columns and FPLC systems were from GE Healthcare unless otherwise stated. Chromatography steps were either carried out at 4°C using a GradiFrac low pressure system, or at room temperature using the ACTA BASIC. Protein elution was measured as UV absorbance at 280 nm.

2.2.4.7 GST assay

CDNB (1-chloro-2,4-dinitrobenzene) assays were based on the protocol described by Habig *et al*. 1974. Assays (total volume 1 ml), were performed in 0.1 M potassium phosphate buffer (pH 6.5) at a temperature of 30°C. The reaction was performed as described: 875 µl of buffer was pre-warmed to 30°C

followed by the addition of glutathione (5 mM, pH 7), recombinant enzyme (50 µl of a 1 in 10 dilution of crude extract) and CDNB (1.25 mM, from a 40 mM stock in ethanol) with thorough mixing. The reaction was followed by monitoring the increase in absorbance at a wavelength of 340 nm for 30 s.

Absorbance readings were corrected for the non-enzymatic rate by the replacement of recombinant enzyme with the appropriate volume of buffer. Enzymatic rates were then expressed as nmol of the *S*-glutathionylated-DNB product formed per second per mg of recombinant protein using the molar extinction coefficient of the product (9.6 mM^{-1} cm^{-1}).

2.2.4.8 DNA extraction from plants

A single small *Arabidopsis thaliana* leaf was placed in an an Eppendorf tube with 400 µl of extraction buffer (200 mM Tris in HCl, pH 8, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS, and 1 % DTT) and incubated at 95°C for 5 min. The sample was centrifuged at 10,000 *g* for 5 min and 300 µl of supernatant placed in a new tube. An equal volume of isopropanol was added, incubated for 2 min at room temperature and centrifuged at 9352 *g* for 5 min. The supernatant was discarded and the pellet washed with 70 % ethanol.

2.2.4.9 Extraction of total RNA

Buffers and water used in the extraction of RNA were autoclaved at 150°C for 15 min to ensure they were RNA*ase* free. For the same reason, sterile plasticware was used and the pestle and mortar baked in an oven at 200°C overnight. Total RNA was extracted from *A. thaliana* tissue, using TRI Reagent™ (Sigma) as described in the accompanying protocol. Around 50 mg

of each tissue was processed with 2 ml of TRI Reagent and the resulting RNA pellet washed with 75 % ethanol before being stored in ethanol at -20°C, or dried and then dissolved in 25 μl of ultrapure sterile water for further processing. The concentration and quality of the RNA was determined by measuring the $OD_{260/280}$ ratio ($OD_{260/280}$ ratio > 1.7: pure RNA without DNA contamination, OD_{260} 1.0 = 40 μg /ml RNA).

2.2.4.10 Real time PCR analysis

Equal amounts of RNA previously isolated from *Arabidopsis thaliana* (section 2.2.3.9) were used to synthesize cDNA using Maloney Murine Leukemia Virus reverse transcriptase (Promega). Sets of specific primers were designed to each gene as listed in appendix 9.4 using Primer 3 (http://frodo.wi.mit.edu/primer3/input.html). Each set of primers had an optimum temperature of 60°C and gave a final product around 200 bp. The genes of *gapdh (At1g13440)*, gst*u19 (At1g78380)* and *ubc (At5g25760)* were used as a positive control. Real time PCR was performed in a Rotogene 3000 (Qiagen) using SYBR Green Jump Start Taq ready mix™ (Sigma). Analysis was carried out by using Rotogene 6.0 software in comparison with the expression of positive control genes. Primer sequences can be found in appendix 9.5.

2.2.4.11 Metabolite analysis by LC-MS

LCMS (liquid chromatography mass spectrometry) analysis of natural products was performed using Waters Q TOF Premier mass spectrophotometer after elution through a HPLC C18 (100 × 2 mm) column. The eluents were: solvent A 0.1 % formic acid and solvent B, acetonitrile, 0.1 % formic acid. The program of

elution was as follows: a linear increase of 5 % to 100 % B from 0 to 12 min. A volume of 10 µl of sample was injected with a flow rate of 0.2 ml min^{-1} and the eluant was monitored for UV absorbing metabolites between 210 nm and 500 nm using a Waters 996 photodiode array detector. This was followed by ESI-TOF MS operating in positive mode.

2.2.4.12 Profile of metabolites in *Arabidopsis thaliana*

Metabolites were extracted from root cultures by homogenizing in 3 v/w of methanol per 1 g of tissue. The initial analysis of the metabolites were carried at 287 nm on HPLC.

2.2.5 Assay methods

Enzyme assays were run in triplicate with the specific activities (nkat/mg protein) determined as the means of 3 replicates. The level of variability (error) was displayed as +/- one standard deviation from the mean. Heat-treated protein samples, denatured at 95°C for 5 min were run in parallel to determine the chemical rate, which was subtracted from the total rate (+ enzyme) to determine the true enzymatic rate. Prior to assay, enzymes were pre-incubated for 5 min in the presence of cofactor at the desired temperature (unless otherwise stated).

2.2.5.1 DNPH assay

The standard assay reaction (final volume 1 ml) consisted L-Methionine (0.01 - 100 m M) dissolved in 0.1 M potassium phosphate buffer pH 7.8, containing Methionine γ lyase, which had been enzyme pre-incubated with pyridoxal phosphate (10 nM) unless otherwise stated. Oxobutyrate formation was

monitored by mixing of 25 µl from the assay reaction mixture with 50 µl of 2, 4-dinitrophenylhydrazine (DNPH; 0.1 M in 1 M HCl) and 1 mM in 0.1 M potassium phosphate buffer pH 7.8. After incubation for 5 min at 37°C, 40 µl of deactivation solution (1M NaOH) was added in order to stop the reaction. The absorbance was then measured at 445 nm (Beckman Coulter DU 530UV/Vis spectrophotometer). The 2-oxobutyric acid formation was determined by constructing a standard curve with known amount of the product determined using 2, 4-dinitrophenylhydrazine (DNPH). The obtained extinction coefficient for the reaction product was (ε = 65.88 mM^{-1} cm^{-1}).

2.2.5.2 Continuous assay

The continuous assay concentration of fee thiols was measured by the coupled measurement of the absorption of NADH (Shimadzu UV-1800 UV spectrophotometer Shimadzu Corporation, Japan) according to the method previously described by Nishiya *et al*., (2005). The assay was performed using a Peltier temperature controlled cell (Shimadzu). The assay reagents were prepared by dissolving NADH (0.28 mM) and 60 U/ml of lactate dehydrogenase (Sigma) in 100 mM potassium phosphate buffer (pH 8). 750 µl of reaction reagent was mixed with 200 µl substrate solution (0.1 mM - 250 mM in 100 mM potassium phosphate buffer pH 8). The reaction was initiated with the addition of 50 µl enzyme previously incubated at 37°C with cofactor. Rates of reaction were monitored continuously in a final volume of 1 ml, with the reagent and substrate (without enzyme) used as a control.

2.2.5.3 Ellman's C-S lyase assay

Protein samples in 0.1 M potassium phosphate buffer (pH 7.8) were incubated with substrates (0.1-5 mM) at 37°C. C-S lyase activity was determined by adding an equal volume of 4 mg/ml Ellman's reagent (5'5-dithio-*bis*-(2 nitrobenzoic acid) dissolved in 0.1 M potassium phosphate buffer pH 7.8 to the assay at timed intervals. After 1 min incubation, the absorbance was determined at 412 nm (Shimadzu UV-1800 UV spectrophotometer Shimadzu Corporation, Japan). The reaction was then stopped by the addition of 100 µl of methanol and after centrifugation (1600x g 5 min), products were analysed by HPLC-MS. Extracts (25 µl) were injected onto UPLC^{TM} BEH C18 (1.7µM; 2.1 ×100mm) and eluted with a solvent gradient starting at 20 % B rising to 100 % B over 9 min. The eluate was analysed for UV absorbance at 264 nm with standards used to determine the retention time of products.

2.2.5.4 Cofactor binding studies by isothermal titration calorimetry

Purified MetC protein was dialyzed overnight against buffer (50 mM potassium phosphate pH 7.8). All calorimetric experiments were conducted using a Microcal (Northampton, MA) VP-ITC microcalorimeter. The calorimeter was calibrated as described in the MCS-ITC manual. During titration, the reference cell was filled with distilled water. Prior to the experiment, the protein analyte (2 ml) was degassed under vacuum. Cofactor was then injected in 5 µl pulses at 37°C into a cell containing a known amount of enzyme in buffer. During the titration the reaction mixture was continuously stirred at 307 rpm. On each injection the released heat was measured The raw experimental data were

presented as the amount of heat produced per second following each injection of ligand into the enzyme solution as a function of time.

2.2.5.5 HPLC assay

The enzyme activities were determined by incubating 50-150 µg of protein with various thiol substrates at concentration 0.01- 0.1 mM in Tris-HCl (pH 7.8). After incubating at 37°C for 5 minutes the reaction was initiated by adding SAM to 0.1 mM. The reaction was stopped after 30 min by addition of 100 µl of methanol. After centrifugation (1200x g 5 min) products were analysed by HPLC.

2.2.5.6 Gas chromatography product analysis

Enzyme activity and product formation were determined using gas chromatography (GC). Protein samples were dissolved in 490 µl of 0.1 M potassium phosphate buffer, pH 7.8 containing 10 µM - 1 mM substrate, in air-tight 1 ml glass vials which were sealed and incubated at 28-55°C for 5 min (unless stated otherwise). Products were partitioned into dichloromethane and dried over sodium sulphate prior to injection (5 µl) onto a capillary GC column; CP SIL 5B. A Thermo Focus GC system was used with a splitless injection (unless stated otherwise) under the following parameters; injector temperature: 200°C, initial oven temperature: 80°C, ramp rate 5°C /min to a maximum temperature: 200°C, carrier gas: Nitrogen, column pressure 30kPa. Products were identified with a flame ionisation (FID) detector with the identity of the sulphur volatiles confirmed and quantified using authentic standards.

2.2.5.7 Radiochemical assay

Desalted protein (20-100 µg) in 20 mM Tris-HCl buffer (pH 7.8) in a total volume 65 µl was mixed with 5 µl of substrate in methanol to give a final substrate concentration of 66 µM. The reaction was initiated by adding 5 µl of *S*-adenosyl-L-[^{3}H-methyl]-L-methionine (50000 dpm, specific activity 68 µCi /mmol and incubated at 27°C for 10 min. Controls, were performed using methanol in place of acceptor substrate. The reaction was stopped with the addition of 125 µl of 0.3 mM HCl and the reaction products partitioned with 200 µl of water-saturated ethyl acetate. A sample of the radioactive methylated product (100 µl) was taken from the upper organic phase and quantified in a liquid scintillation counter (The Tri-Carb Perkin Elmer).

2.2.5.8 Crude protein assays

Crude plant protein was extracted in (2 v/w of 100 mM Tris buffer pH 7.5, 1 mM DTT) and desalted prior to activity assays on Zeba desalting columns (Pierce). Crude total protein concentrations were measured by Bradford assay.

2.2.5.9 Quantitation of enzyme activity

The specific enzyme activity was determined using pure proteins as moles of substrate converted per unit of time (enzyme activity) per mass of enzyme. The specific activity was measured in nkat/mg.

2.2.6 Transformations

2.2.6.1 Medium scale biotransformation reaction conditions

Reactions were conducted on a 50 ml scale in round bottomed flasks (150 ml) at 37°C with stirring. 10 mg of enzyme were used in each reaction, unless specified otherwise.

2.2.6.2 Feeding of thiols to *E. coli* expressing enzymes

E. coli transformed with MetC or arabidopsis TMT1 or coupled construct: TMT1/CBL, TMT1/MetC or TMT1/TRP in Rosetta-gami^{TM} 2 were grown to OD 0.5 and IPTG added to a final concentration of (0.01 - 0.4 mM). Cultures were grown overnight in airtight vials with 50 µg of the appropriate antibiotic per ml, at 28 ºC or 37ºC with shaking at 200 rpm. Metabolites were extracted directly from the culture suspension into ethyl acetate and measured by GC-MS unless stated otherwise.

2.2.6.3 Analysis of reaction products using gas chromatography

100 µl of reaction products were portioned into an equal volume of organic solvent and analysed on Focus GC Thermo with capillary GC column (30m x 0.25 mm). Capillary GC was performed using systems with following parameters:

(I): Splitless injection, injector temperature: 200°C, initial oven temperature: 50°C, ramp rate 20°C /min to a maximum temperature: 200°C, carrier gas: Nitrogen, column pressure 30 kPa.

(II): Splitless injection, injector temperature: 200°C, Initial oven temperature: 50°C, ramp rate 30°C /min to a maximum temperature: 300°C, carrier gas: Nitrogen, column pressure 30kPa.

2.2.6.4 Analysis of reaction products using gas chromatography mass spectrometry (GCMS)

Aliquots of reaction products (500µl), were partitioned into an equal volume of dichloromethane and dried using sodium sulphate. 5µl samples were analysed using a Thermo-Finnigan Trace GCMS (Thermo) with a Phenonemex Zebron ZB-5 capillary GC column (30m x 0.25mm), using the Department of Chemistry Analytical Services (Durham, UK). The following parameters were used; splitless injection, injector temperature: 200°C, Initial oven temperature: 80°C, ramp rate 2°C /min to a maximum temperature: 200°C, running time 21.5 minutes, carrier gas: Nitrogen, column pressure 30kPa.

2.2.6.5 Solid phase micro-extraction using SPME

Volatile compounds were measured using solid phase micro extraction (SPME) using a Divinylbenzene/Carboxen/Polydimethylsiloxane fibre (Supelco, Bellefonte, PA) coupled to GC. All assays were performed with samples (200 µl) placed in vials sealed with metal crimp tops with silicone septa. Fibres were conditioned before use according to the manufacturer's instruction (50/30 µm DVB/CAR/PDMS for 1 h at 270°C). The SPME fibre was inserted into the headspace of the pre-warmed injector (10 min at 60°C). Each vial was sampled only once, although each experiment was performed in triplicate. Internal standards (Sigma) were used to confirm product identity. After extraction, fibres

were removed from vials and eluted by placing into the injector port of the gas chromatograph. Analysis was performed using GC system (II) described in section (2.2.5.6).

3 Cloning, expression and purification of C-S lyases

3.1 Introduction

This chapter describes the cloning, expression and biochemical characterisation of methionine γ-lyase (MGL) from *Pseudomonas putida*; tryptophanase (TRP), cystathionine β-lyase (MetC) and cystathionine β-lyase MalY from *Escherichia coli*; cystathionine γ (γ-yeast) and β (β-yeast)-lyase from *Saccharomyces cerevisiae* and cystathionine β-lyase (CBL) from *Arabidopsis thaliana.* This diverse library of C-S lyases should provide a rich source of diverse enzymes with interesting and important activities in generating natural free thiols. The main aim of this part of the project was to produce proteins in a soluble and active form for further functional studies. The desired PCR products encoding the enzymes of interest were gel purified by electrophoresis and the coding sequences inserted into pGEM-T vectors which were then used to transform Top 10 cells. Independent clones containing the insert of the correct size were isolated and the DNA sequence confirmed. Target cDNA clones were subcloned into pET24a and the respective recombinant proteins expressed in *E. coli* and then purified on a Ni-NTA column as described in the methods (2.2.3.3). The mass of each protein was confirmed by SDS-PAGE and by mass spectrometry. Several factors including: medium composition, cofactor, temperature, storage stability and buffer pH were investigated in detail in order to optimise the production of the most appropriate enzymes for future reactions to be carried out on a larger scale. Once generated, the industrial potential of selected enzymes and their activity towards natural substrates were tested.

3.2 Cloning of C-S lyases

The first objective of this project was to source the coding sequences of different carbon-sulphur lyases. Based on previous literature studies, cystathionine β-lyase (MetC), cystathionine β-lyase MalY from *E. coli*; cystathionine γ, β-lyase from *S. cerevisiae* and cystathionine β- lyase (CBL) from *A. thaliana* were chosen for further study. Two C-S lyase enzymes, MGL from *P. putida* and TRP from *E. coli* had been previously cloned in-house and were available for testing. Primers listed in annex 9.1 were designed to amplify target DNA from *E. coli*, *S. cerevisiae a*nd *A. thaliana* respectively. The *Nde*I and *Xho*I restriction sites were engineered at the 5' and 3' ends of the coding sequence respectively. The addition of the restriction enzyme sites permitted directional cloning in the previously chosen vector system. Each PCR reaction was performed according to methods described in section 2.2.1.1. All PCR products were amplified by KOD polymerase to ensure the highest fidelity, (unless stated otherwise). All PCR were carried out using an Eppendorf Mastercycler (Eppendorf AG, Germany). PCR products were analysed on a 0.8 % agarose gel (figure3.2-1), to confirm the expected size for each product. Two clones encoding MGL and TRP were obtained from Dr Mark Skipsey (Durham University). Prior to ligation, PCR products were extracted from the agarose gel and purified prior to removal of primers, nucleotides and buffer components (as described in section 2.2.1.3).

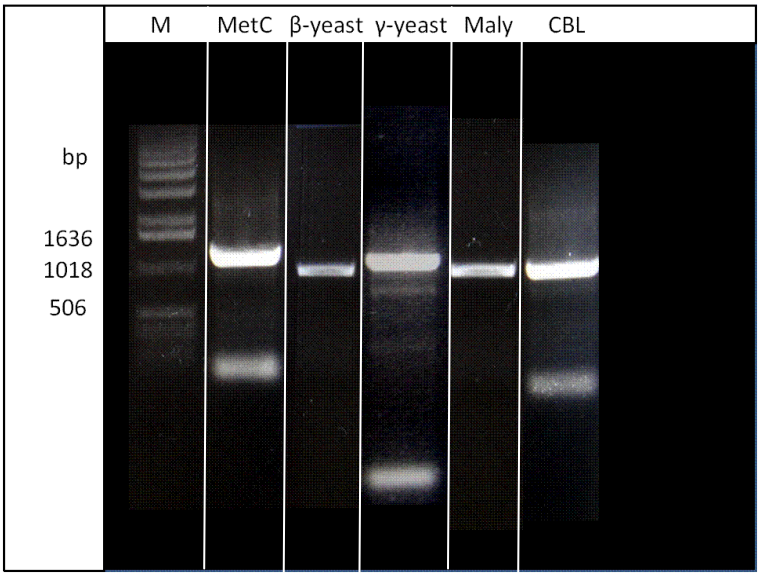


Figure 3.2-1. Verification of PCR products on agarose gels.
Lane MetC - 1181 bp; lane β-yeast 1021 bp; γ-yeast - 1181 bp; lane MalY- 1171bp; lane lane CBL 1177 bp; lane M. 1 Kbp DNA marker ladder (Bio-Rad).

The purification method is based on the fact that DNA can be bound to silica particles in the presence of salt and in the last step of purification, DNA is eluted by incubation in water at 50ºC. In the next step, the use of *Taq* DNA polymerase, which has terminal transferase activity, lead to the addition of dA (deoxyriboadenosine) at the 3' ends of the PCR amplified DNA. The PCR products were then inserted into the sequencing vector pGEM-T Easy using with 3'-dT ends. The pGEM-T Easy vector system allows the recloning of the fragment of DNA into another expression vector for further studies and is well known for its high efficiency for cloning PCR products. The ligation of pGEM-T and DNA fragment was performed using the protocol described in section (2.2.1.7). The plasmids containing the correct gene were transformed into Top10 expression cells and grown overnight at 37^{o}C as previously described in section (2.2.2.3). In order to perform mini-prep analysis, pGEM-T transformant colonies were grown on an agar plate containing ampicillin, IPTG, and X-Gal. The positive

clones containing the desired DNA fragments which appeared as white colonies, were selected and used to inoculate 10 ml LB starter cultures, as previously described (section 2.2.2.3). The authenticity of each insert was confirmed by restriction digest analysis of chosen colonies, performed following the Promega restriction enzyme protocol. The digested plasmid and insert were run on a 0.8 % agarose gel and subsequently purified. The MalY DNA fragment has an internal restriction site *Nde*I (figure3.2-2). Therefore, a partial restriction digestion was necessary to obtain the full-length DNA fragment. This experiment was performed according to the methods described in section (2.2.1.5). The resulting products were analysed on 0.8 % agarose gel and are presented in (figure 3.2-3). A product of an 1171 kb was isolated from the gel and ligated into the pET24a vector.

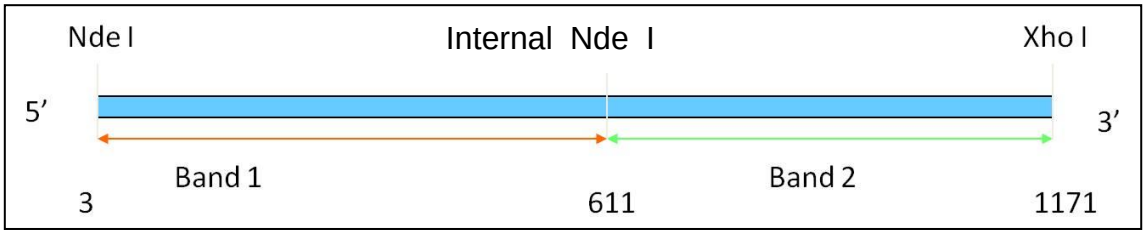


Figure 3.2-2. Schematic of the partial restriction digest of the MalY clone with *Nde*I.

DNA inserts were cut from the agarose gel, purified and cloned into pET24a, an expression plasmid with complementary restriction sites and a kanamycin selection marker with a T7 promoter (T7 RNA polymerase gene under *lacUV5* control). Expression was induced by the addition of IPTG (an analog of lactose, which inhibits the *lac* repressor). The pET24a vector was also digested with the restriction enzyme *Nde*I and *Xho*I to prevent self-ligation.

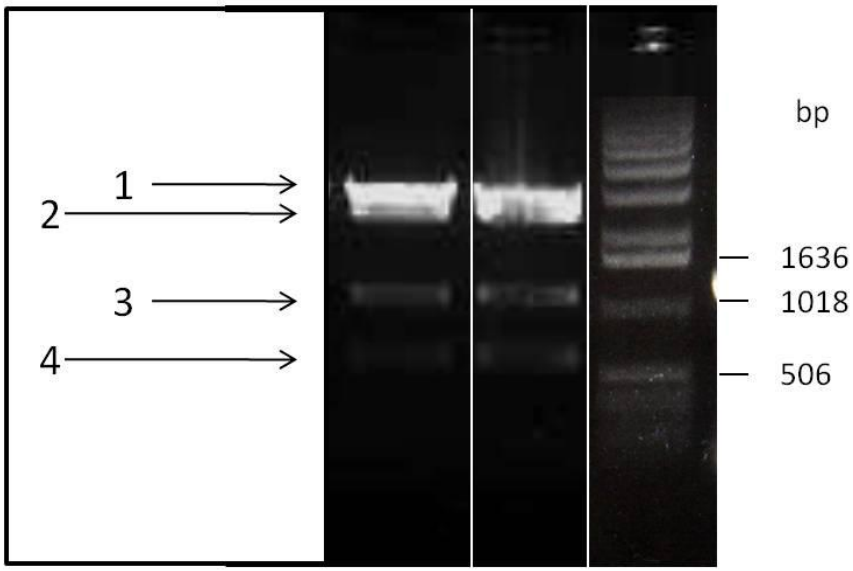


Figure 3.2-3. Verification of the partial restriction analysis of MalY on agarose gels.
Arrows indicate DNA fragments as follow: 1- undigested vector; 2- pGEM-T; 3- full length MalY; 4- incomplete 609bp fragment. The 1kb DNA marker ladder used was obtained from Bio-Rad.

All clones were subjected to DNA sequencing to ensure that the amplified and cloned DNA fragments were correct. Each ligation product was used to transform Rosetta-*gami*^{TM} 2 *E. coli* competent cells following heat-shock. These host strains alleviate codon bias and enhance disulfide bond formation of the resulting recombinant proteins in the cytoplasm. Rosetta-*gami*^{TM} 2 *E. coli* competent cells are also compatible with kanamycin-resistant vectors and carry the chloramphenicol-resistant pRARE*2* plasmid, which supplies seven rare tRNAs. The positive clones were analysed by direct PCR screening of the bacterial colonies. Because MalY and MetC are genes isolated from *E. coli*, the 5'-T7 promoter primer was used in conjunction with the gene-specific 3' primer to amplify these sequences, hence discriminating them from bacterial genomic sequences. All genes of interest were successfully cloned and the further expression purification and characterisation will be described in the next section.

3.3 Expression and purification of C-S lyases

The methods chosen for recombinant genes expression and protein purification depend on many variables. The combination of the appropriate vector, bacterial host and growing culture conditions is essential to obtain high yields of active enzymes (Weickert *et al*., 1996). The expression system based on the pET24 vector contains the T7-*lac* promoter and the kanamycin resistance marker and was expected to deliver very efficient regulation of expression. In the case of pET-based systems, induction of T7 RNA polymerase by IPTG, leads to the formation of recombinant proteins. In this study, all enzymes were designed to incorporate an additional C-terminal sequence of 8 amino acids (LEHHHHHH), which was added to facilitate His-tag purification. In order to purify fusion proteins from bacterial host cells, two different methods were tested. Extraction can affect the target proteins conformation and as a result, enzyme activity, therefore the main objective of this step of purification was to obtain soluble fraction of protein in active form (Murby *et al.,* 1996). The first method used sonication for mechanical cell disruption and was performed according to the method described in section 2.2.3.2. This disruption procedure proved relatively effective, although the high temperature generated during the process reduced the activity of all tested proteins. Therefore, sonication steps were performed for 30 sec at 33 % power with 2 min intervals. All protein samples were kept on ice at all times. In some cases this method was not fully effective due to the high concentration of bacterial DNA present and even after prolonged sonication, the lysate was not clarified. The second type of cell disruption performed used the Thermo Scientific B-PER Bacterial Protein Extraction Reagent kit, which is designed to extract soluble protein from bacterial cells. These easy-to-use cell lysis solutions use

mild non-ionic detergents to disrupt cells and solubilize proteins without denaturation. Enzyme supplements including DNase I and Lysozyme allowed for the improved recovery of high molecular weight proteins, which are otherwise difficult to purify. The procedure was performed as described in section 2.2.3.1. The recombinant expressed His-tagged proteins were then purified using a 5 ml nickel-chelate column according to the method described in section 2.2.3.3. Imidazole was used to elute the His-tagged proteins which were bound to nickel ions attached to the surface of beads in the chromatography column. An excess of imidazole was passed through the column, which displaced the His-tag from its nickel co-ordination, freeing the His-tagged proteins. The column was cleaned between each run to avoid crossed contamination. Imidazole was then removed from each fraction using a His Trap Desalting column, or dialysis, as described in section 2.2.3.8. The clarified fraction containing each protein was then subjected to buffer exchange to remove excess salt. The purified enzymes were analysed by SDS-PAGE and the molecular masses estimated (figure3.3-2). The purified proteins at this stage were relatively pure. Unfortunately, cystathionine β-lyase (β-yeast) from *S. cerevisiae* (55 kDa) was not soluble under these conditions (figure 3.3-1) and the expressed protein could only be observed in the insoluble crude bacterial lysate. As other enzymes were readily soluble, further work on this protein was not pursued. All purified proteins were analysed by time-of-flight mass spectrometry following electrospray ionisation as described in section (2.2.3.11).

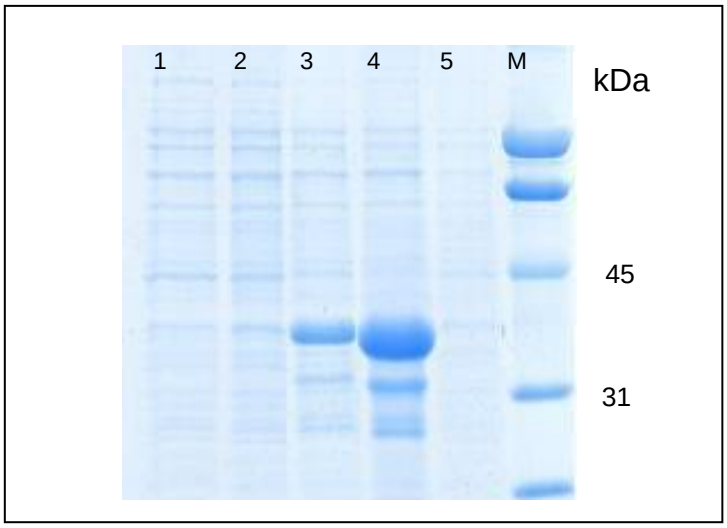


Figure 3.3-1. An SDS-PAGE gel stained with Coomassie Blue showing a β-lyase from yeast. (Molecular weight of 38036.5 Da). Lane 1 and 2 control; lane 3 crude total protein; lane 4 crude insoluble; lane 5 pure soluble protein fraction; lane M molecular-weight markers (kDa). Overexpressed protein was organised as insoluble particles, known as inclusion bodies.

The results presented in (figure 3.4-3 - 3.4-8) were compared to the mass calculated using the Prot Param calculator (http://expasy.org/cgi-bin/protparam). All lyase proteins were shown to have a significant of incorporation of the cofactor PLP (protein peak with 227 Da adduct) with the exception of TRP. The level of expression of each lyase was determined with each batch overexpressed in standard LB medium. In order to estimate the amount of purified, soluble enzyme different methods of estimation were used including: BIO-RAD, which has been performed according to manufacturer's instructions based on Bradford assay described in detail in section 2.2.3.9, or by measurement of the absorbance of the pure protein at 280nm using the calculated extinction coefficient.

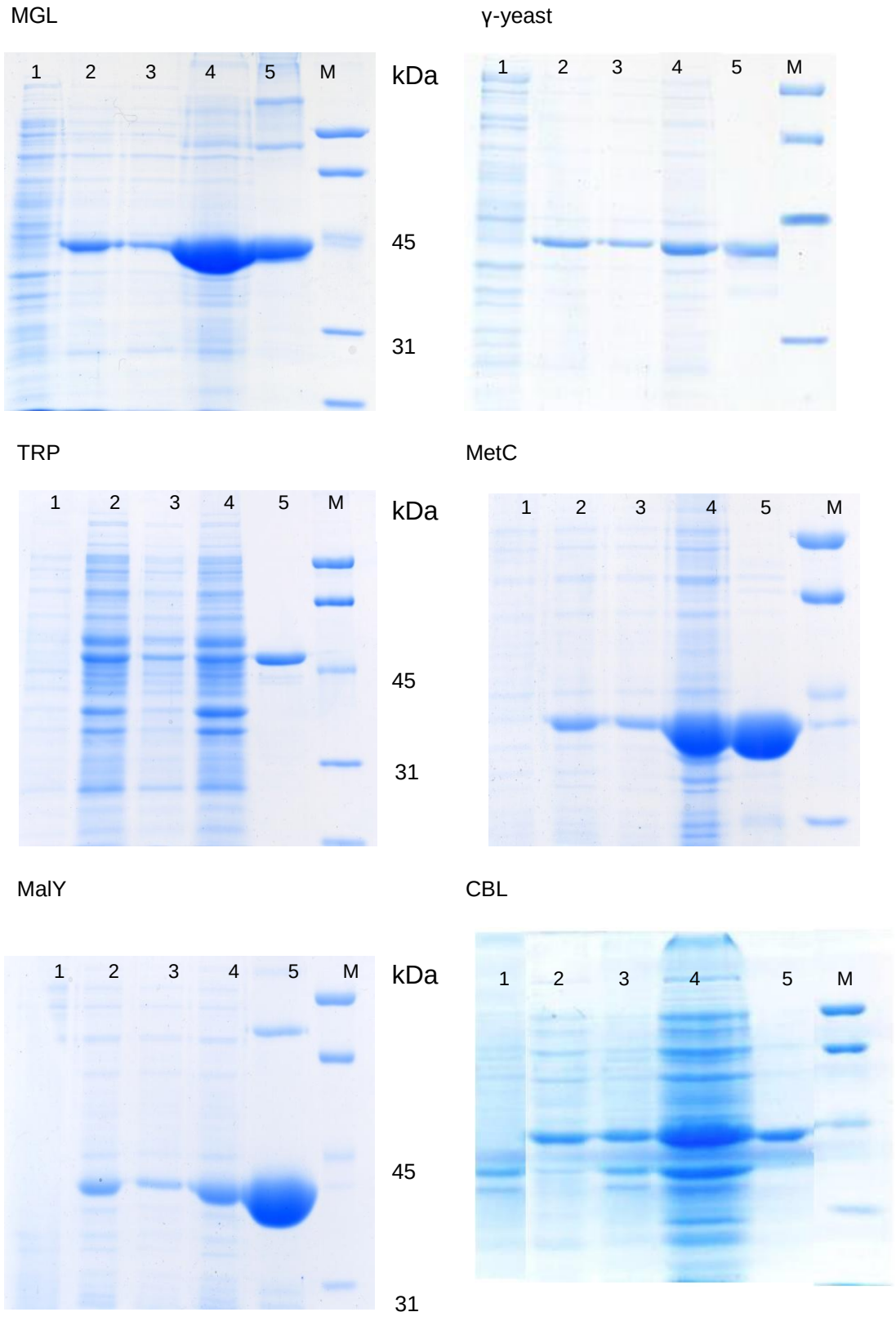


Figure 3.3-2. An SDS-PAGE gel stained with Coomassie Blue showing proteins recovered from Ni-NTA column from *E. coli*, *A. thaliana* and *S. cerevisiae*. Lane 1. control; lane 2,3 crude total protein; lane 4 crude insoluble; lane 5 pure soluble protein; lane M molecular-weight markers (kDa). MGL- methionine γ lyase from *P. putida*, γ-yeast from *S. cerevisiae*, (TRP - Tryptophanase beta lyase from *E.coli*, MetC - Cystathionine beta lyase from *E. coli*, CBL - Cystathionine beta lyase from *A. thaliana*).

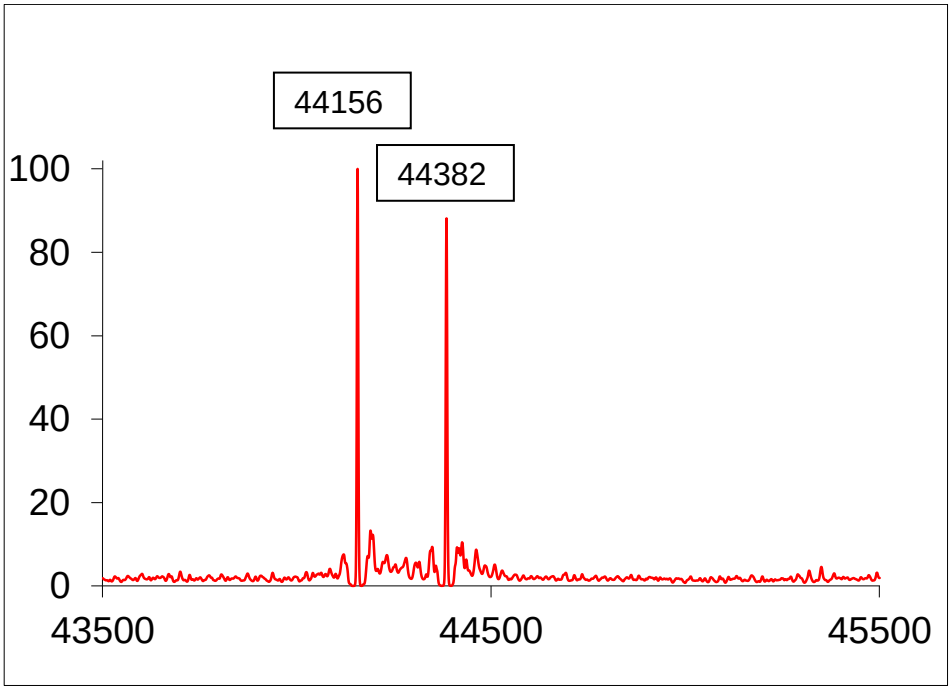


Figure 3.3-3. Positive ionisation ES-MS spectra of purified recombinant methionine γ lyase from *Pseudomonas putida.*
Spectrum range 43.5-45.5 kDa. The predicted mass of MGL was 44156; MGL with incorporation of PLP was 44382.

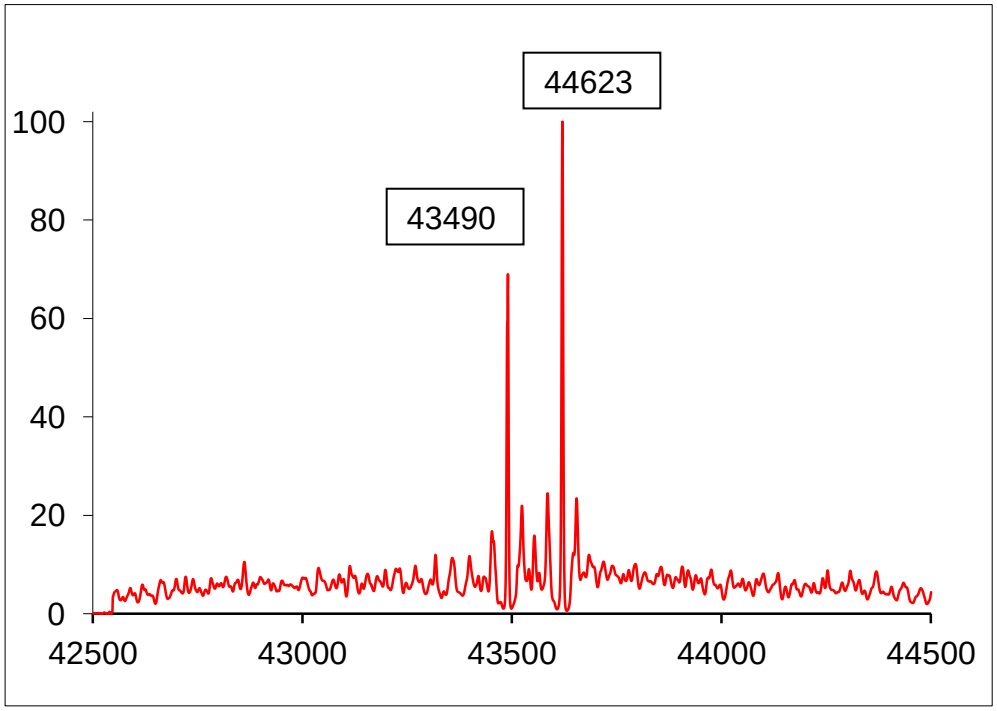


Figure 3.3-4. Positive ionisation ES-MS spectra of purified recombinant γ-yeast from S. cerevisiae
Spectrum range was 42.5-44.55 kDa. The predicted mass of γ-yeast was 43490 and γ-yeast with incorporation of PLP was 44623.

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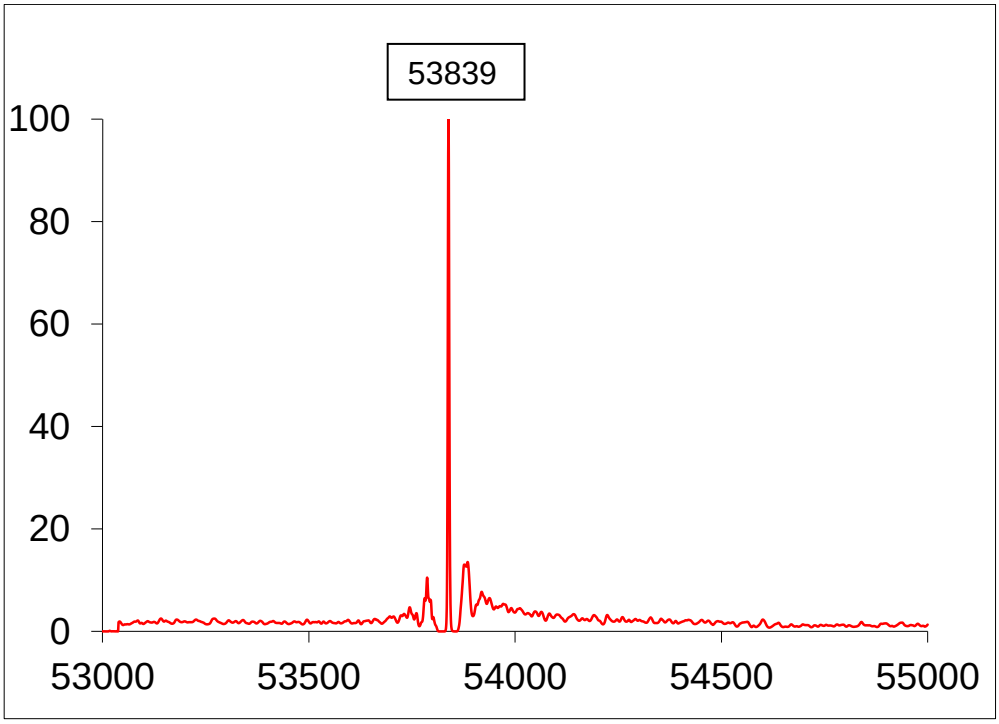


Figure 3.3-5. Positive ionisation ES-MS spectra of purified recombinant tryptophanase from *E. coli.*
Spectrum range was 53-55 kDa. The predicted mass of TRP was 53839.

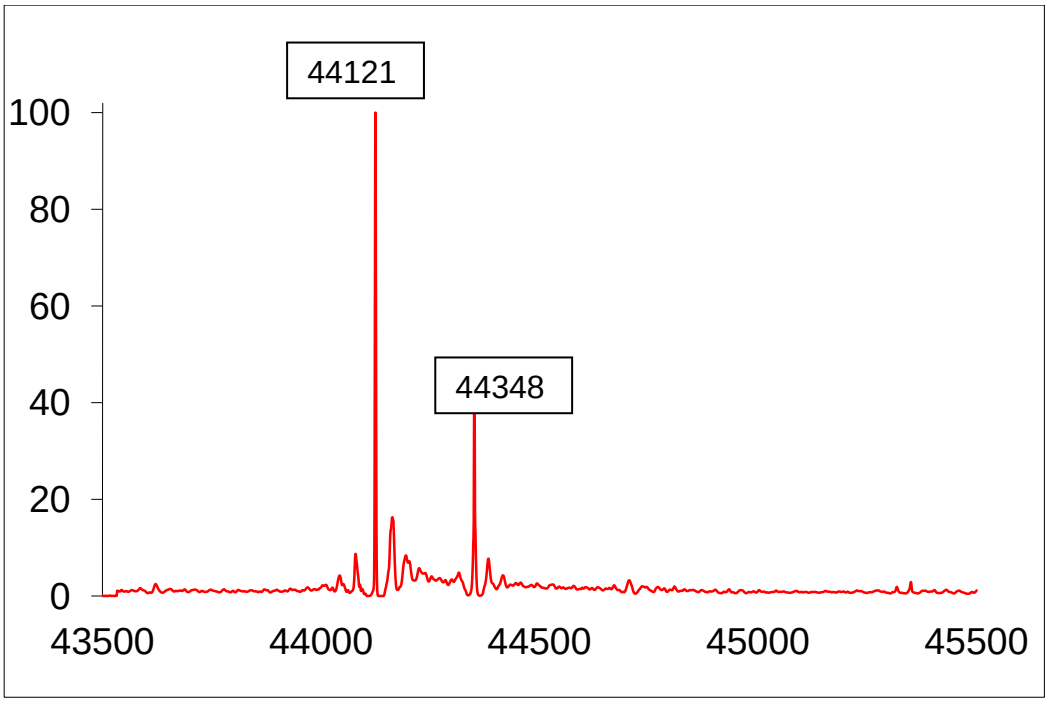


Figure 3.3-6. Positive ionisation ES-MS spectra of purified recombinant cystathionine β-lyase from *E. coli.*
Spectrum range was 43.5 – 45.5 kDa. The predicted mass of MetC was 44121, MetC with incorporation of PLP was 44348.

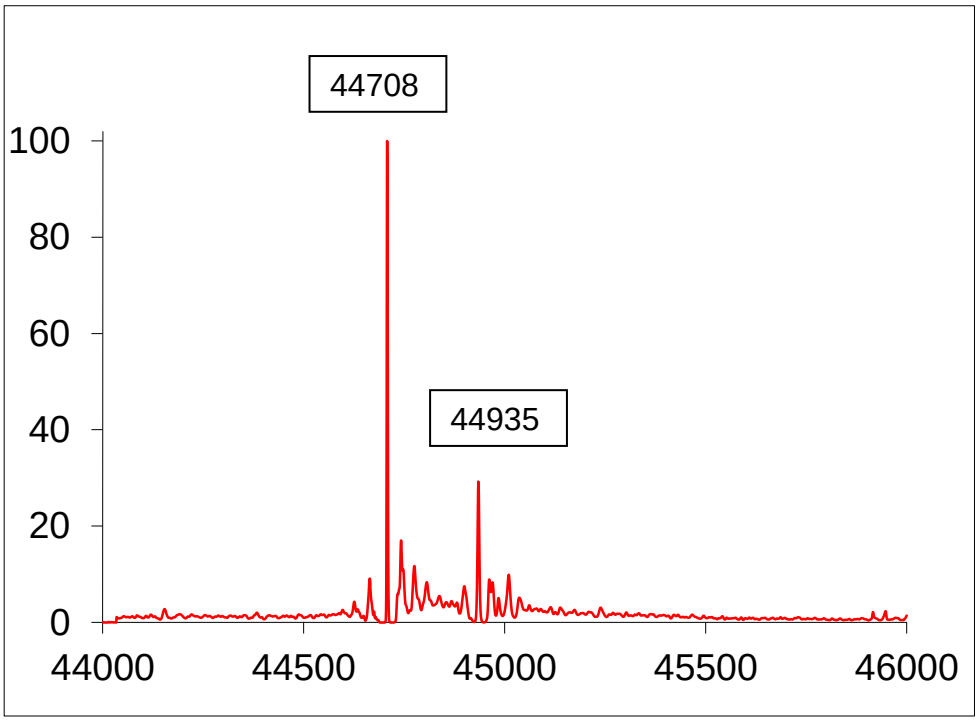


Figure 3.3-7. Positive ionisation ES-MS spectra of purified recombinant cystathionine β-lyase from *E. coli*.
Spectrum range was 44 - 46 kDa. The predicted mass of MalY was 44708 and MalY with incorporation of PLP was 44935.

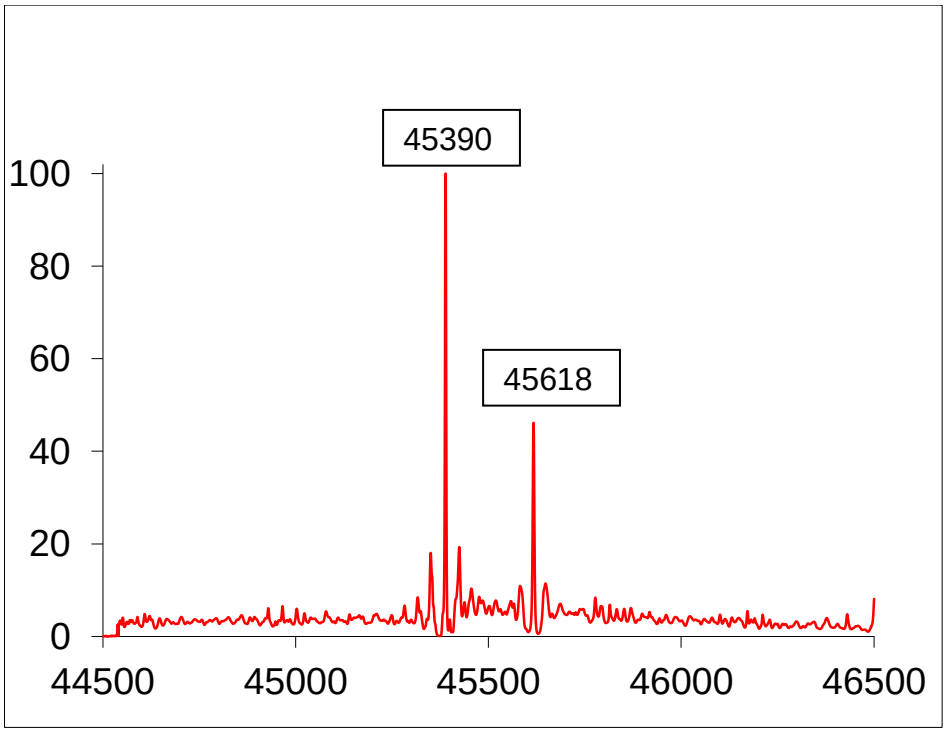


Figure 3.3-8. Positive ionisation ES-MS spectra of purified recombinant cystathionine β-lyase from *A. thaliana*.
Spectrum range 44.5 – 46.5 kDa. The predicted mass of CBL was 45390, CBL with incorporation of PLP was 45618.

The second method of protein quantification proved to be relatively accurate and was used in the majority of further experiments to determine the amount of purified proteins. In each case buffer of the same concentration and pH was used as a blank sample. A comparison of protein expression yields obtained in these studies is presented in table 3.3-1. Further growing condition optimization was to be investigated in the next section.

Name of the enzyme	**Total amount of soluble enzyme produced from 500ml of LB**
MGL	101± 3.9 mg
γ-yeast	98 ± 2.1 mg
TRP	81 ± 4.1 mg
MetC	118 ± 8.9 mg
MalY	101 ± 1.7 mg
CBL	99 ± 4.8 mg

Table 3.3-1. The comparison of yields of purified enzymes.

3.4 Preliminary enzymatic activity comparison.

In order to find out the most active natural catalyst, the collection of overexpressed enzymes were tested against natural substrates. The comparison of activity of gamma enzymes revealed, that MGL showed the higher activity towards methionine as compared with the γ-yeast protein as presented in figure 3.4.1.

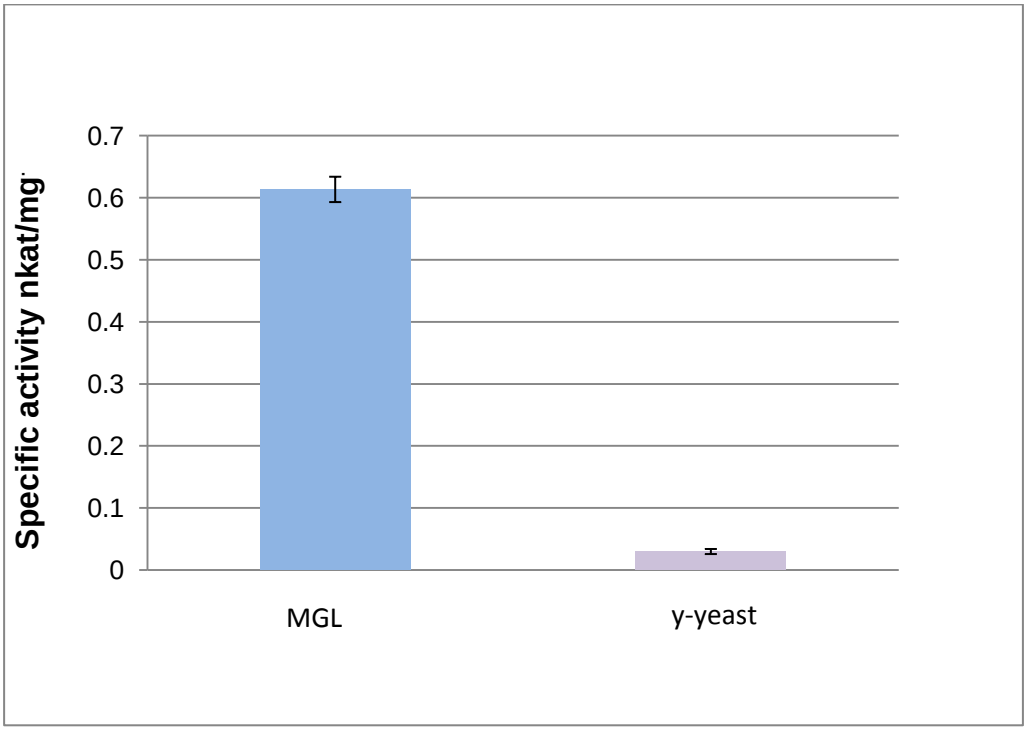


Figure 3.4-1. Comparison of activity of MGL and γ-yeast towards methionine.

Among the beta group of enzymes MetC, showed the highest rate of elimination reactions towards substrate cystathionine as compared with TRP, MalY or CBL as presented in figure 3.4.2. All reactions were performed at the same conditions (KP buffer pH 7.8 at 37°C) according to the method described in chapter 2.2.4.2. Further, more detailed activity analysis was then performed in order to identify and choose the most efficient enzymes for scaling up reactions.

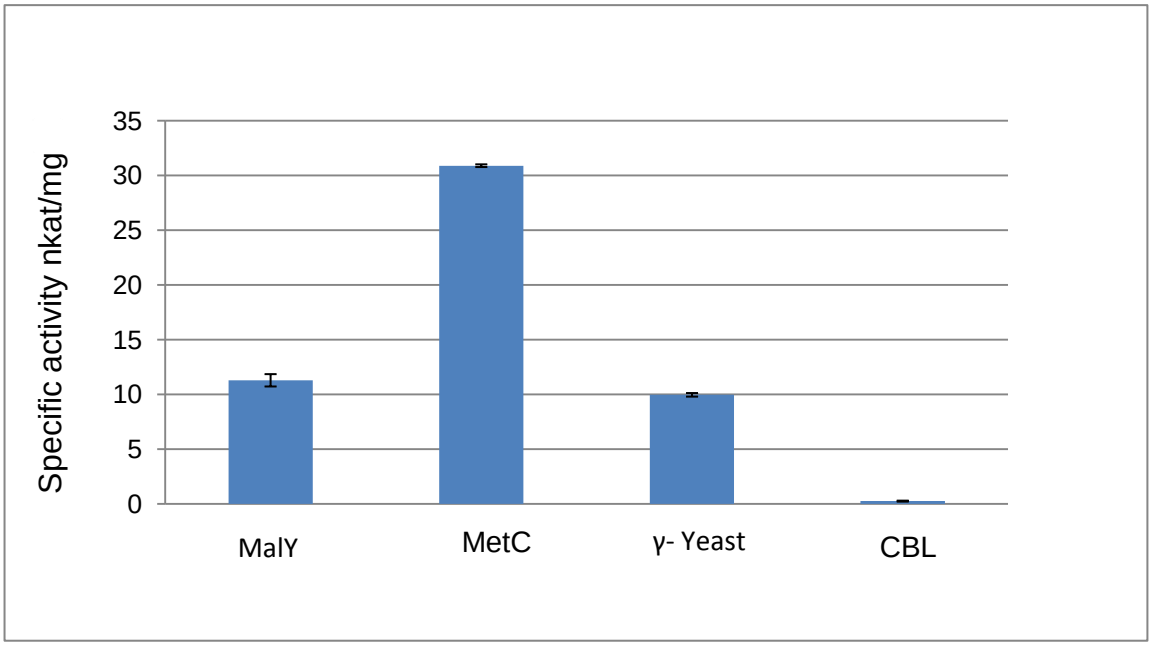


Figure 3.4-2. Comparison of beta lyase activity enzymes (MetC, MalY, γ-yeast, CBL) towards natural substrate L- cystathionine.

3.5 Storage and enzyme stability

In order to select the most useful enzymes, it was essential to test enzyme stability after purification. The series of tests conducted included the storage of proteins under various conditions. As bacterial cell pellets stored at -20^{o}C all enzymes were stable over 6 months without any significant loss of activity after purification (figure 3.5-2). Within the γ-group of lyases, Methionine gamma lyase from *P. putida* was found to be more stable then the γ- lyase from *S. cerevisiae*. It was found, that the methionine gamma lyase enzyme could be kept at room temperature for a few days without appreciable loss of activity and the pure extracted enzyme could be stored as a stock solution at -20^{o}C in glycerol buffer (1:1) with no loss in activity determined over 3 months. Within the beta lyases, MetC proved to be the most stable enzyme in comparison to MalY, CBL and TRP. MetC was stable at -20^{o}C in the presence of 50 % glycerol for 3 months

and could be stored at 4°C for up to 3 months with 80 % of its activity remaining. In order to determine the stability of the enzymes under assay conditions, enzymes were incubated at 37°C for 5 hours. MGL and MetC were the most stable enzymes and retained 75 % and 90 % of their activity over the test period respectively. Detailed comparison of the stability of the enzymes under different conditions is presented in figure 3.5-3. In all experiments, the presence of additional pyridoxal 5- phosphate increased the stability of the tested enzymes. This result suggested the important role of the cofactor structural conformation and the presence of PLP proved to be an important factor in the storage of the lyases.

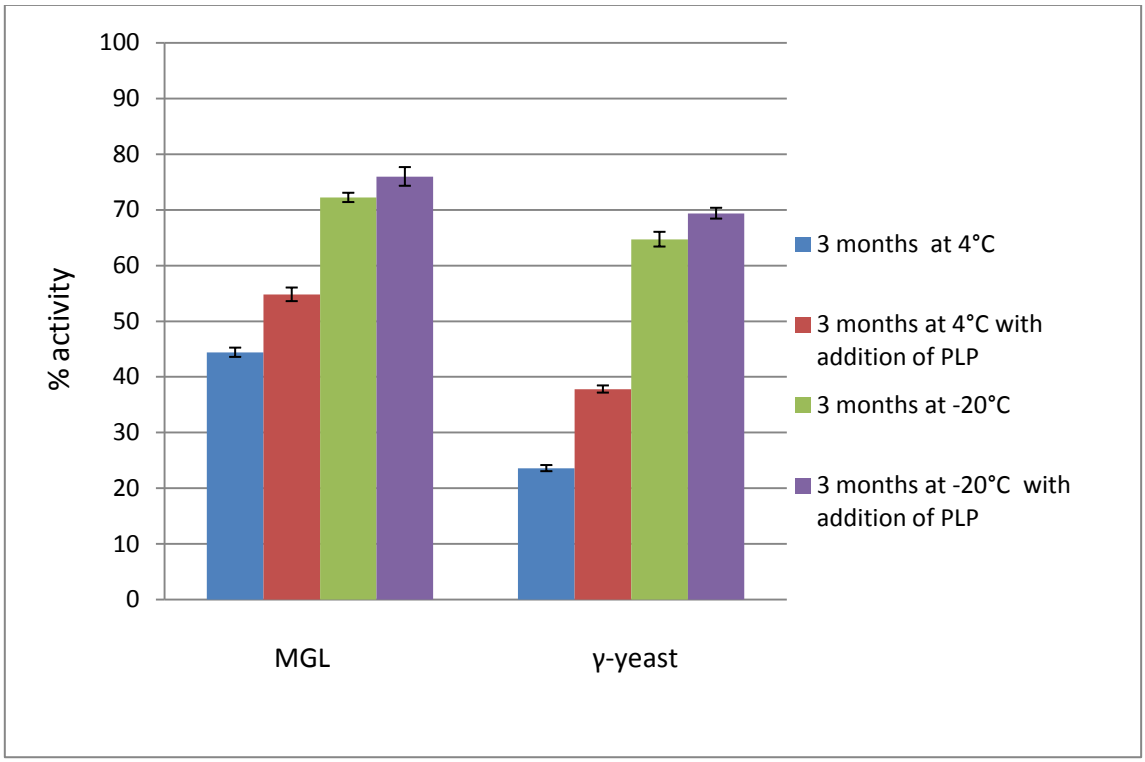


Figure 3.5-1. The effect of storage on the stability of γ-lyase enzymes.

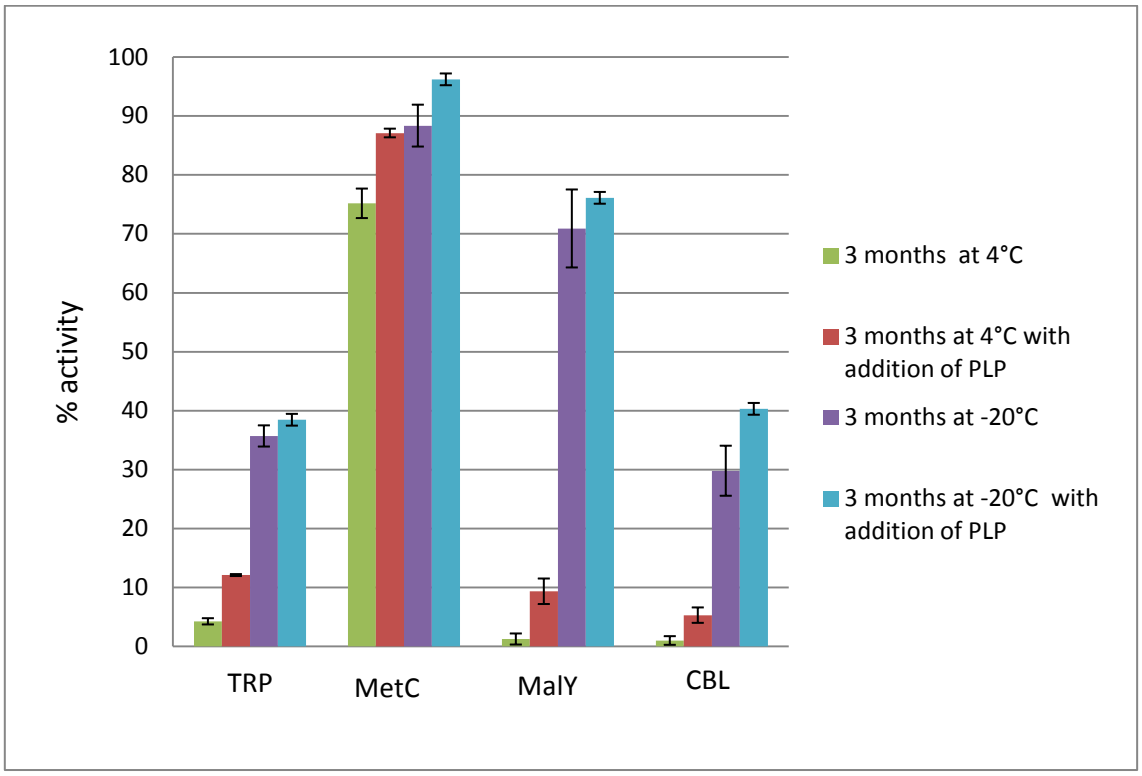


Figure 3.5-2. The effect of storage condition upon the stability of β-lyase enzymes.

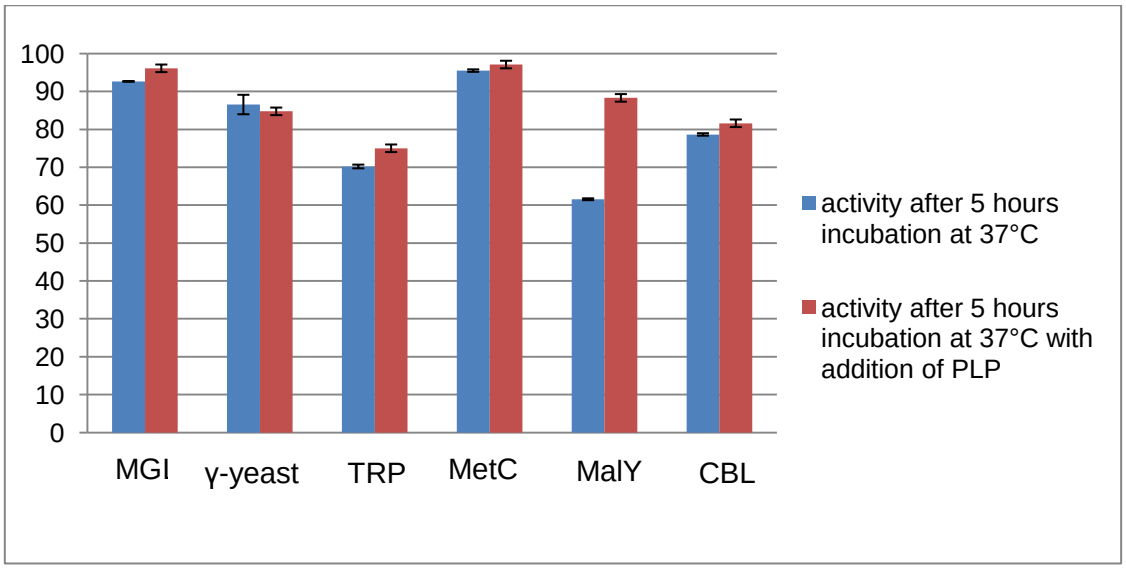


Figure 3.5-3. The effect of incubation at 37 °C upon the stability of C-S lyase enzyme activity.

3.6 Optimisation of growing conditions

The fast growth rate and ease of recombinant protein purification technology using *E. coli* based vectors make the system suitable for fast and reliable protein overproduction. However, the method needs to take into account many different factors such as media composition, temperature and optimal timing for the high yield of complex proteins as soluble, active enzymes. Therefore, the development of the optimal expression strategies is critical in obtaining high levels of the desired recombinant proteins. In order to optimize the production of recombinant protein in *E. coli* host, the effects of various parameters including incubation temperature and the amount of the overexpression inducer-IPTG were investigated. High-density *E. coli* cultures were utilised, employing a defined medium in order to limit production costs. Methods of increasing cofactor (pyridoxal phosphate) biosynthesis in the expression host were also investigated. The *in vitro* activity of all described C-S lyases is sensitive to the amount of PLP bound to the holoenzyme. In order to improve culture growing conditions two different media were investigated. Plasmid-carrying transformants were incubated overnight with 50 µg of the appropriate antibiotic per ml, at 28 ºC or 37ºC with shaking at 200 rpm. To produce large amounts of the C-S lyase proteins in *E. coli*, the bacterial growth media was optimized for high protein overexpression as well as high specific enzyme activity. According to Hill *et al*., (1996), *E. coli* can synthesise pyridoxal phosphate from many basic carbon sources, therefore C-S lyases proteins were overproduced in medium supplemented with additional source of carbon – glycerol (25 g ml/l) (Dempsey 1971). In addition, increased amounts of yeast extract (30 g/l), a decreased amount of NaCl (1 g/l) and an additional content of $NH_4H_2PO_4$ (1.88 g/l), K_2HPO_4

(13.1 g/l) were added as described in section (2.1.1.2). The results obtained after overnight incubation of MetC and MGL are shown in figure 3.6-1. In comparison to standard Luria broth, the optimised medium increased the expression of enzymes of interest, leading to a considerable improvement of recovered enzyme activity. Significantly, all enzymes over-expressed in these studies showed a yellow colourization in comparison to enzymes overexpressed in standard LB broth. Furthermore, LC-MS traces of all enzymes showed a higher binding and accumulation of the natural cofactor PLP (data not shown). Additionally the optimal amount of inducing agent IPTG was also tested and determined to be 0.1 mM. Using these optimised procedures overexpression of MGL and MetC resulted in great improvement in expression of 16.5 % and 18.5 % respectively. In both cases, the recombinant enzyme was isolated at higher yield in comparison to standard Luria Broth giving the yield of pure protein per 500ml of cell culture (MGL - 121 mg and MetC - 148 mg).

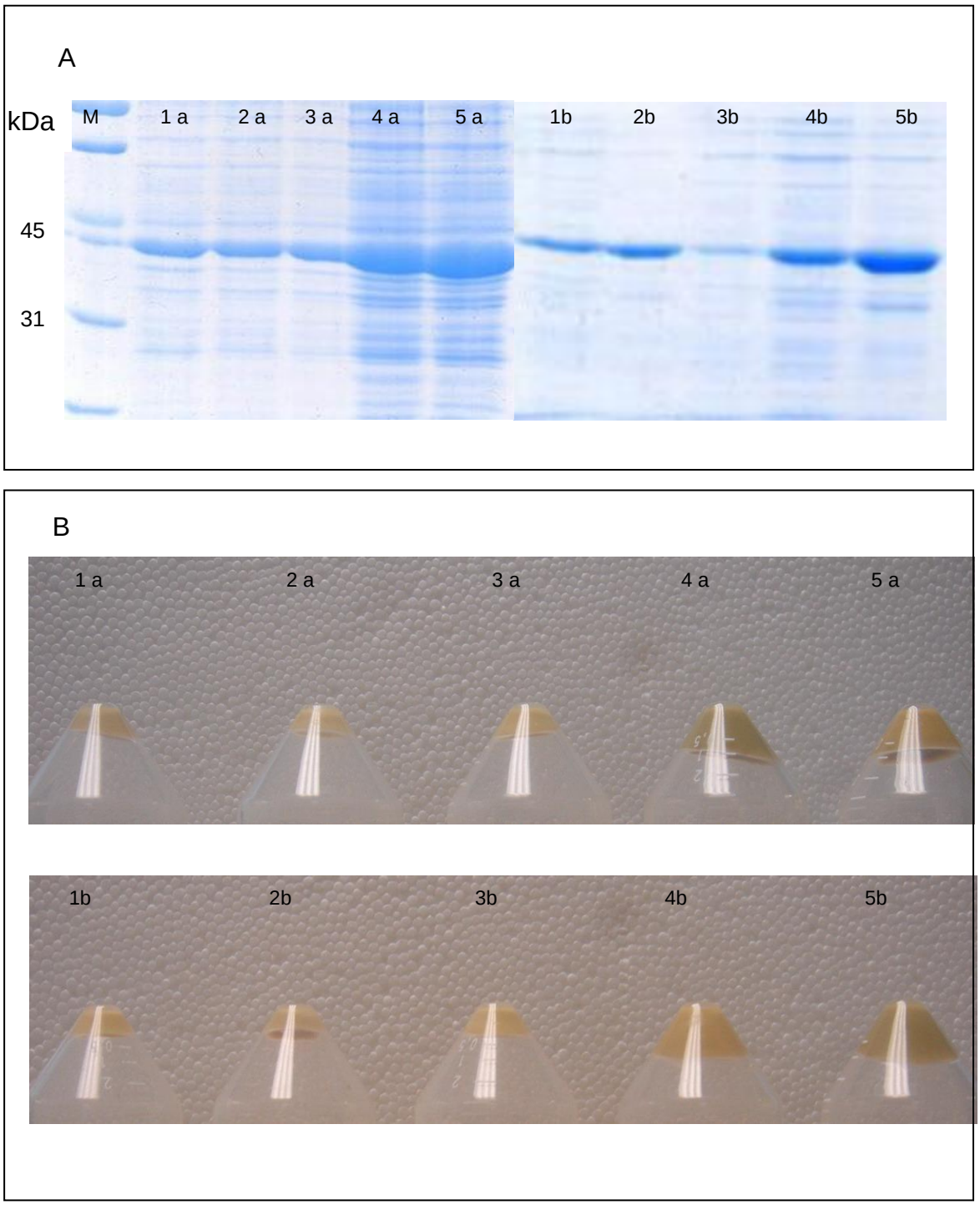


Figure 3.6-1. Effect of temperature and medium composition on the overexpression of MetC and MGL.

Different levels of expression determined by SDS-PAGE (picture A) and bacterial pellets (picture B). Abbreviations: Line M, Marker; MetC, a; MGL, b; 1, LB medium at 37°C; line 2, LB with addition of glycerol at 37°C; line 3, LB with addition of glycerol at 28°C; line 4, optimized medium at 37°C; line 5, optimized medium at 28°C.

3.7 Incorporation of pyridoxal phosphate into C-S lyases

As previously described in the chapter 1.3, pyridoxal phosphate (PLP) is a natural cofactor and has an enormous influence on C-S lyase activity and stability. In these C-S lyase proteins, the cofactor PLP is linked via a Schiff base to the side chain of a lysine. As shown in chapter 3.6 significant amount of PLP was covalently bound to purified proteins figure 3.7-1. In particular MetC protein was found to bind PLP with high affinity in comparison to the other tested proteins, MalY or TRP. Following purification of enzyme MetC, a different colourisation could be observed, therefore further determination of residual PLP was performed according to method 2.2.3.7. The results of this visual difference were verified by HPLC method according to method 2.2.4.5. as presented in figure 3.7-2. HPLC data confirmed the significant presence of PLP bound to native protein after purification of around 64 %. This data seems correlate with the activity studies where incubation with PLP led to increase of MetC activity by further 41 %.

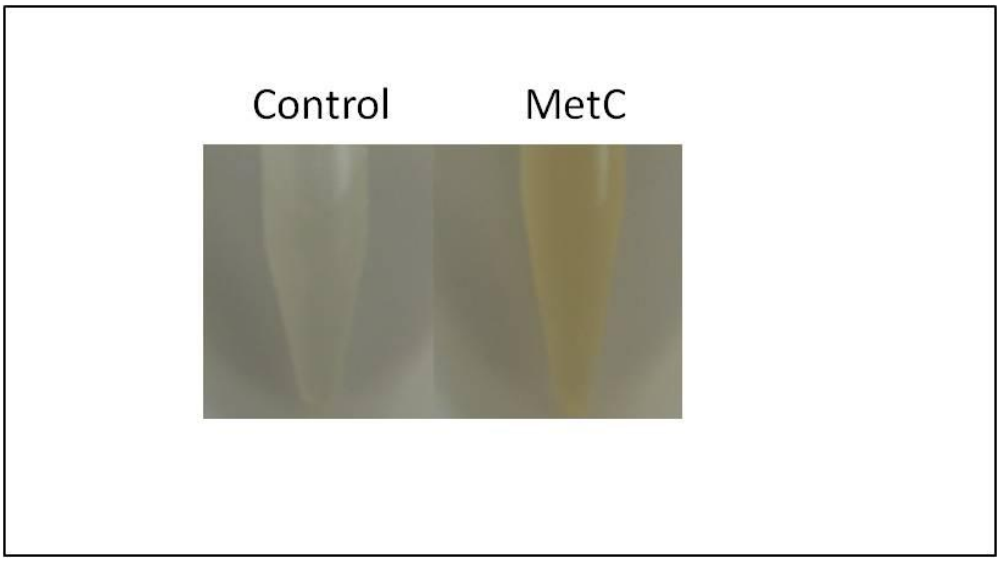


Figure 3.7-1. Accumulation of pyridoxal phosphate in purified MetC.
Yellow colourisation represent cofactor PLP covalently bounded to the purified protein.

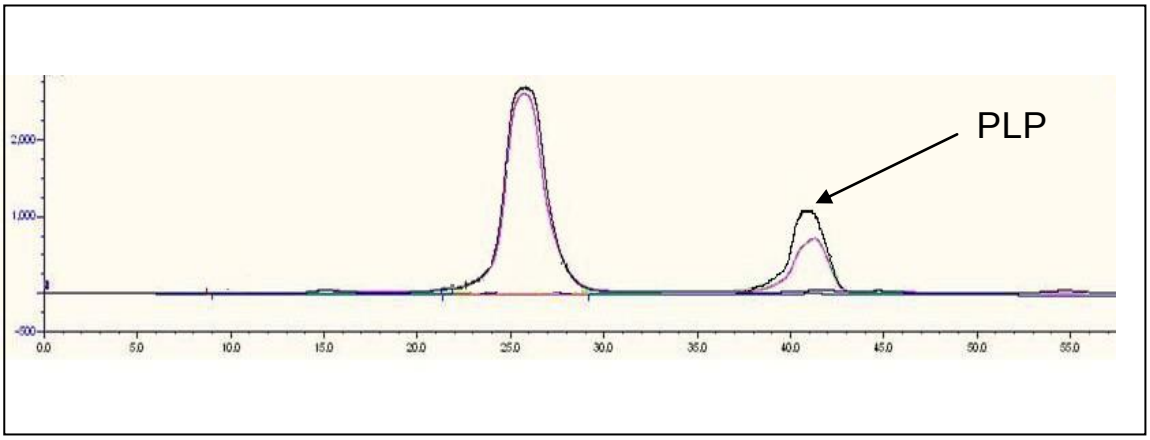


Figure 3.7-2. HPLC elution profile of MetC after purification (purple) and MetC incubated with additional PLP (black).

The interaction of MetC with PLP was studied by isothermal titration calorimetry (ITC) at 37°C in aqueous solution to determine the strength, stoichiometry, and quantity of PLP binding to each enzyme after purification. This simple rapid method for determination of the dissociation binding constants for MetC-PLP interaction was performed according to method 2.2.5.4 (Wiseman *et al*., 1989). The binding isotherm for MetC-PLP interaction was obtained by carrying out a titration calorimetric experiment using known concentrations of PLP. The obtained data proved further binding of PLP over 40 minutes of incubation.

Interestingly, it has been reported that the active site of MalY protein is solvent-exposed and does not bind PLP molecule very strongly (Clausen *et al*., 2000). This protein has an unusual mechanism of PLP binding compared to the other C-S lyases; as an active holoenzyme exist as a dimer containing two molecules of PLP and as the apoenzyme dissociate into two separate monomers. Hydroxylamine is a carbonyl-specific reagent that reversibly dissociates PLP from the holoenzyme. Holo-MetC and Holo-MalY was treated with 100 µM hydroxylamine as described in section 2.2.3.14. Treatment of MetC with

hydroxylamine, which reacts with the internal aldimine to form a PLP oxime, results in the loss of PLP.

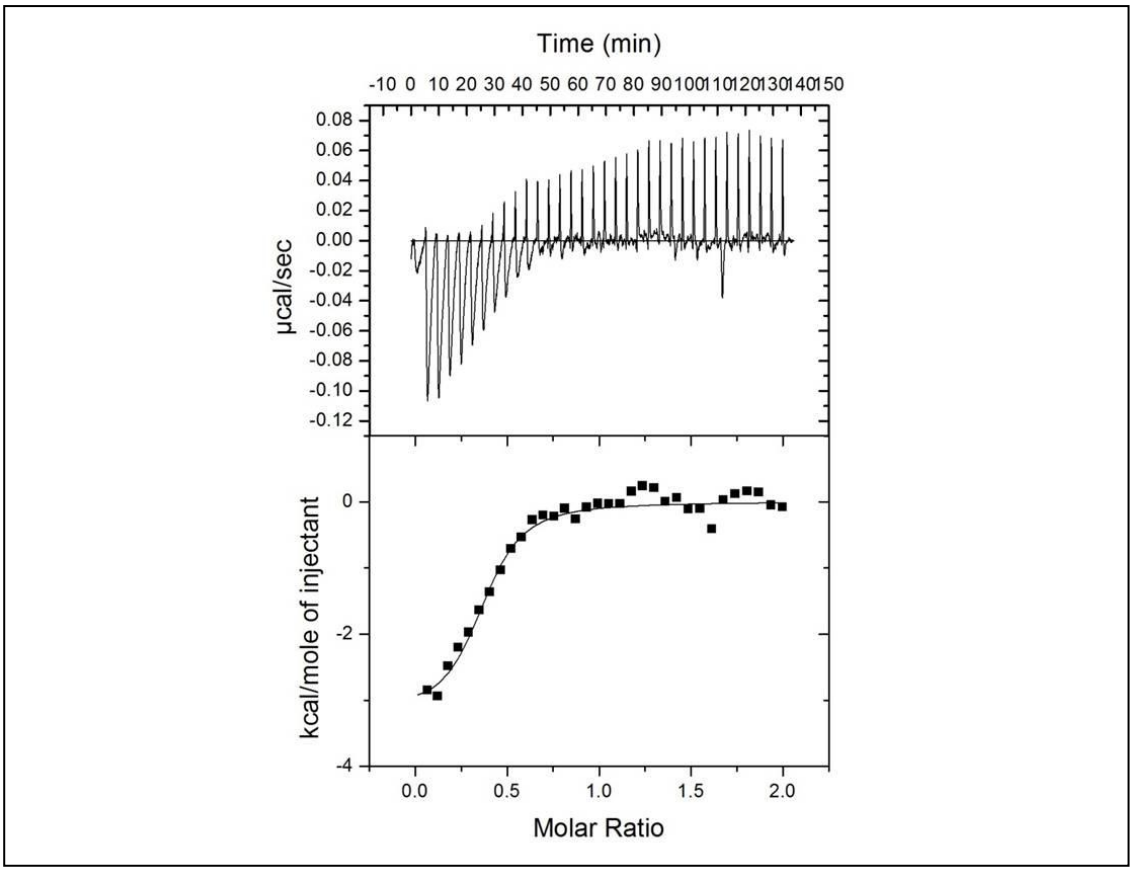


Figure 3.7-3. Isothermal titration calometric cofactor binding studies with MetC. Data shows linear binding of PLP to MetC enzyme over time until saturation point.

Neither enzyme showed any activity towards L-cystathionine. After incubation with cofactor as described in section 2.2.3.13 MetC and MalY showed only 34 % and 12 % recovery of its original activity respectively. This data confirms the crucial role of PLP enzymatic activity and may suggest that PLP plays an important role in maintaining structural stability of enzymes.

3.8 Cloning for industrial purposes

In order to be able to overexpress and purify enzymes on an industrial scale, it was required to use a non-licensed expression system, which falls outside the currently protected patents for the applied production of commercial enzymes. The pET vector system, which was use to overexpress proteins for detailed studies, was used under terms of a non-commercial closure and could not be used in any unlicensed industrial applications. Instead, the vector pGEX with a lactose induced *tac* promoter was designed by Dr Ian Cummins, to avoid licensing issues using a *tac* promoter vector modified by site-directed mutagenesis to allow tag-free expression of required enzymes. Two enzymes: MetC and MGL, which showed the highest beta and gamma activity respectively, were cloned into the *tac* vector for over-expression. In each case, the sequence was excised by the introduction of an *Nde*I site at the start codon, together with an *Xho*I site after the stop codon. This allowed the direct cloning of inserted native sequences without any purification tags. The respective coding DNA of MGL and MetC was obtained after PCR gene amplification using primers (MGL Forward, MGLstop Reverse) and (MetC Forward, MetCstop Reverse) respectively (appendix 9.1) according to the method 2.2.1.1. The MetC coding sequence was then cloned into *Nde*I/*Xho*I digested pTAC (pGEX derived vector (created by Ian Cummins) and utilised to create pTAC-MetC and pTAC-MGL. Further cloning was performed, using standard molecular biology methods outlined in section 2.2.1. Sequence analysis of the two plasmid constructs revealed no PCR errors introduced during cloning. Another important step to commercial expression was a choice of host for protein overexpression. Both pTAC-MetC and pTAC-MGL were transformed into the BL21 strain of *E. coli* as

previously used Rosetta- gami^{TM} 2 would require a licence from Brookhaven National Laboratories. Induction of expression was performed by the addition of 10 mM lactose. The efficiency of expression using this custom vector was analysed after the overnight production of the protein at 30ºC, according to the method described in section (2.2.2.4). As shown on figure 3.7.-1 the colour of the bacterial pellets obtained were initially different. MGL expression turned the bacterial pellet pink, while MetC expression resulted in a yellow pellet most likely, due to the high concentration of covalently bound cofactor - PLP.

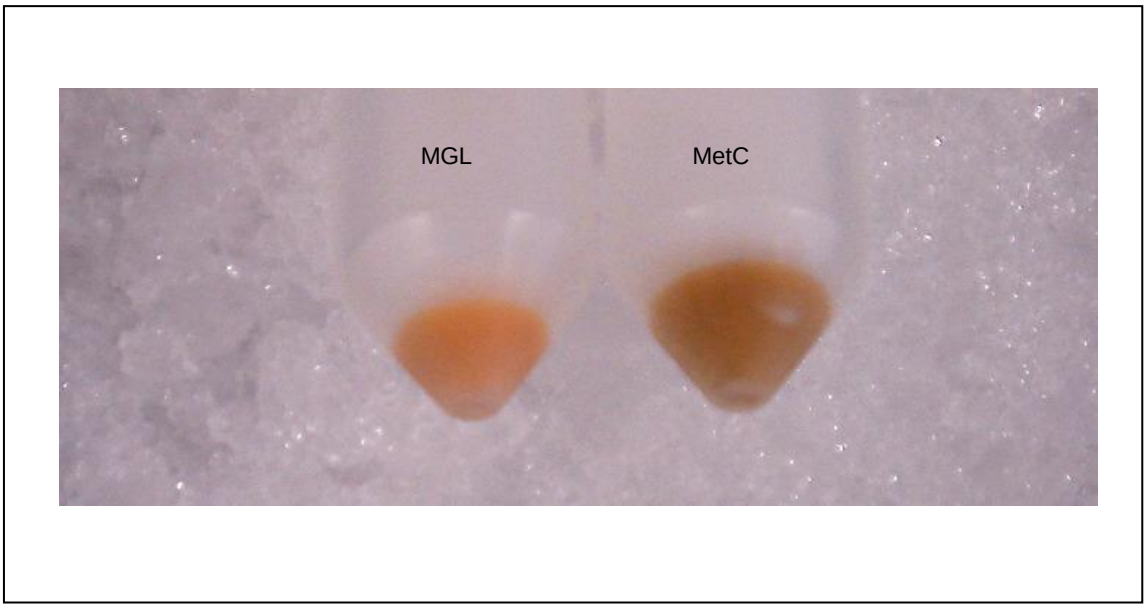


Figure 3.8-1. Picture showing different colourization of bacterial pellets of MGL (pink) and MetC (yellow).

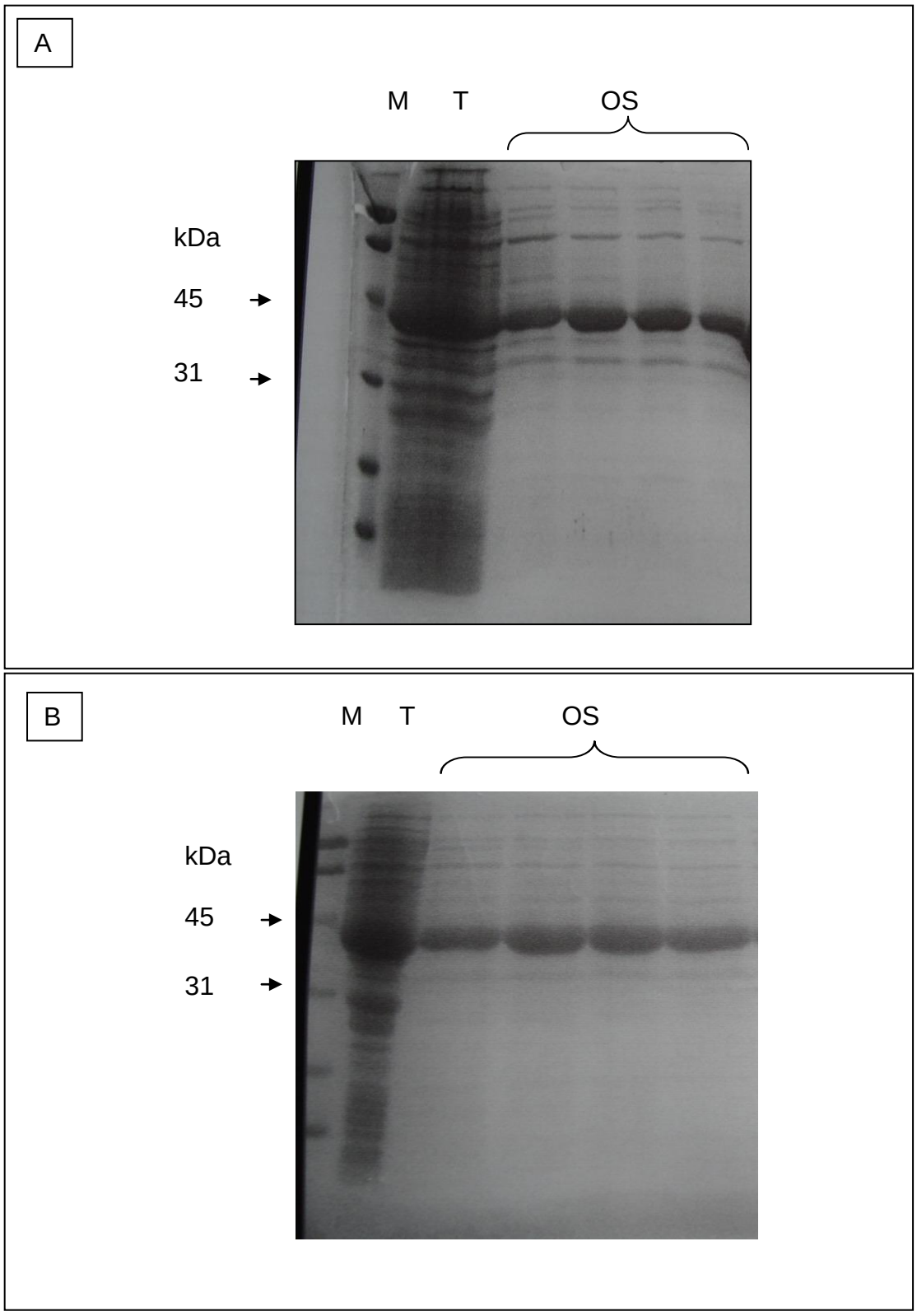


Figure 3.8-2. The SDS-PAGE gel showing MetC and MGL in pGex vector stained with Coomassie Blue.
(A) MetC in pGEX vector and (B) MGL in pGEX vector. Marker (M), total soluble protein (T), Octyl sepharose peaks (OS)

Pellets were subjected to further purification as described in section 2.2.3.6. After extraction, both proteins were analysed by SDS-PAGE figure 3.8.2. Recombinant expression of MetC in *E. coli* BL21 resulted in high level expression of the recombinant proteins. The 500 ml of MGL and MetC culture yielded approximately 67 mg and 82 mg protein respectively. The Octyl Sepharose purification method for recovering recombinant protein was less effective in comparison to the previously used His-tagged and also proved to result in some impurities. Analysis of MGL enzymes by LC-MS showed that, very little pyridoxal phosphate-containing protein was evident (figure 3.8-3). In contrast, MetC showed a greater association with the cofactor (figure 3.8-4).

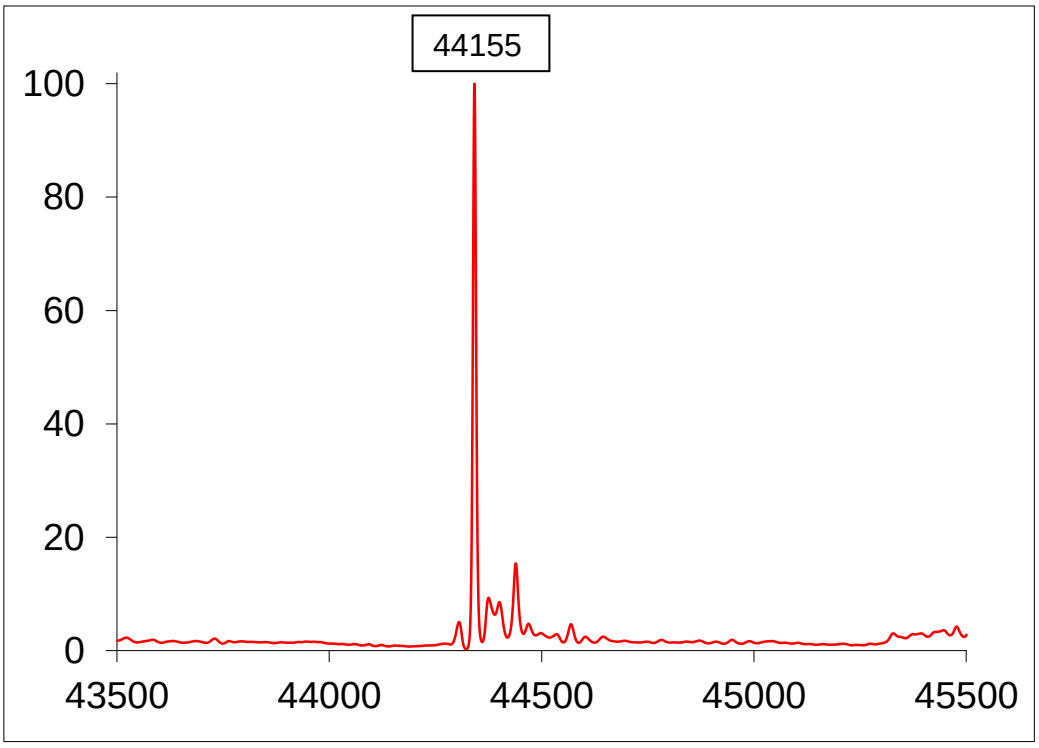


Figure 3.8-3. Positive ionisation ESMS spectra of purified recombinant Methinionine γ lyase *from P. putida*.
Range 43.5-45.5 kDa The predicted mass of MGL was 44155.

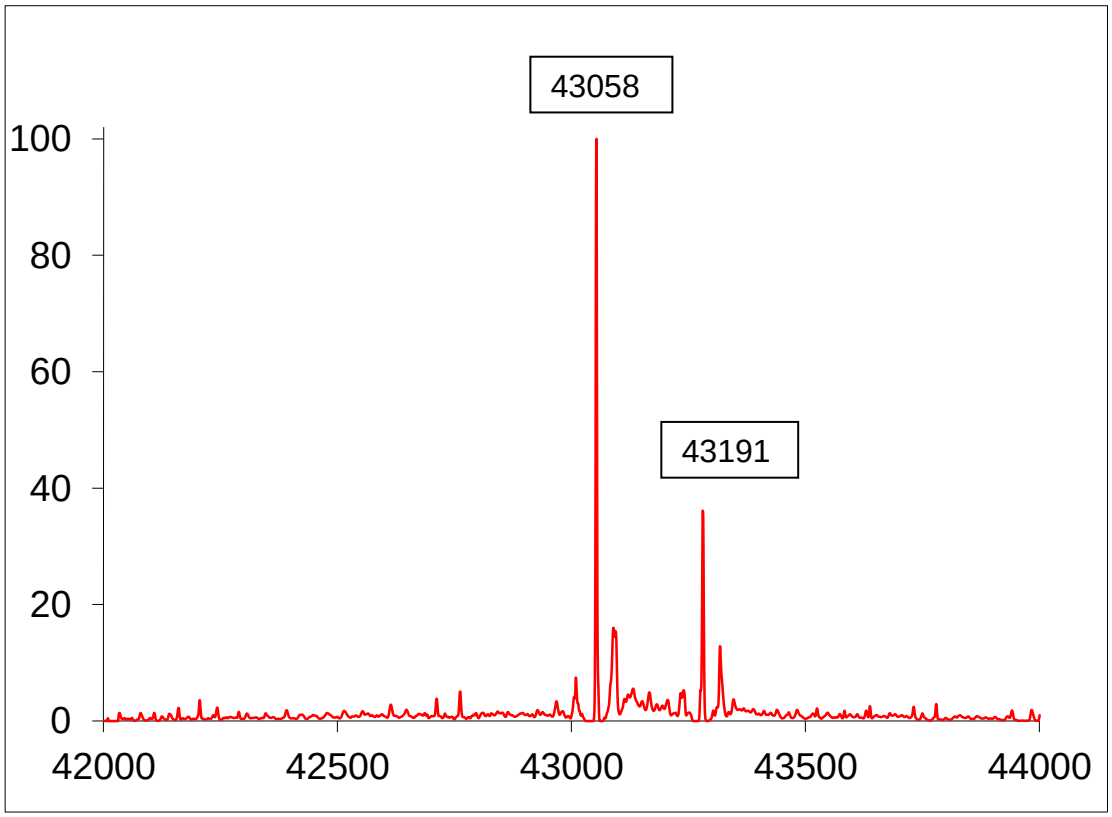


Figure 3.8-4. Positive ionisation ESMS spectra of purified recombinant Cystathionine β-lyase from *E. coli.*
Range 42-44 kDa. The predicted mass of MetC was 43058 MetC with incorporation of PLP 43191.

3.9 Summary and comparison of enzyme expression

The initial idea for the project was to source the most efficient enzymes in term of expression, solubility and activity for use in commercial biotransformation. As such a priority for the study was a rapid cloning and overexpression of lyase enzymes for industrial applications. An analysis of the factors affecting the recombinant enzyme produced by fermentation was conducted. All cloned enzymes were found to differ in terms of their enzymatic properties, such as thermal stability, catalytic efficiency and incorporation of the cofactor PLP. All target proteins shown to be highly expressed and relatively easy to purify. Although Cystathionine β- lyase from *S. cerevisiae* was expressed at a high level in *E.coli*, it formed only insoluble inclusion bodies, making it impossible to isolate in active form. Since other β-C-S lyases were readily soluble, work on this protein was not pursued further. The high activity of MGL towards methionine led to the enzyme being an attractive candidate for biotechnological applications requiring the cleavage of carbon–sulphur bond in the gamma position of amino acid to produce natural substrates like methanethiol. When compared with the enzyme from yeast, MGL showed better storage characteristics, more efficient overexpression and significantly higher specific activity. MetC, which cleaves the sulphur- carbon bond in the beta position of cystathionine, appeared to be the most appropriate enzyme to test on cysteine conjugates. Although MalY showed high levels of overexpression, the enzyme lost activity rapidly when stored at 4^{o}C. As a result, the best candidate for the cleavage of sulphur-carbon bond in the beta position was determined to be MetC from *E. coli* and in the gamma position the MGL from *P. putida.* Both proteins gave high yields when

overexpressed. They also showed high incorporation of the cofactor pyridoxal-5’ phosphate and relatively high activity towards their natural substrates after purification. The activity of both selected enzymes on sulphur-containing compounds of interest were therefore tested and evaluated in the following chapters.

4 Use of methionine gamma lyase for production of methanethiol

4.1 Introduction

The enzymatic conversion of methionine to volatile organosulphur compounds (VOSCs) is of great importance in flavour formation during many natural ly occurring processes such as cheese ripening (Hanniffy *et al*., 2009). Methionine is a crucial precursor of a variety of VOSC compounds such as methanethiol, dimethyl sulphide, dimethyl trisulphide or *S*-methylthioester (Weimer *et al.,* 1999). In this work, methanethiol is of potential utility in generating VOSCs in exit reactions from mainstream thiol production as well as being a value added natural product for the industrial partner - Frutarom Ltd.

The aim of this study was to investigate the ability of gamma lyase enzymes to generate methanethiol from methionine, optimize reaction conditions, scale up medium-sized production and finally develop the methods for trapping the volatile product.

4.2 Development of assay

In order to determine the rate of enzymatic reaction, it was important to develop a highly sensitive, fast and reliable method. There are many different thiol detection assays described in the literature (Jocelyn 1972, Takakura *et al*., 2004) based on spectrophotometric, fluorescence and GC-MS and LC-MS methods. Furthermore, due to the highly volatile nature of free thiols being released by enzymatic activity, the use of indirect measurement method was also investigated (Hansen *et al*., 1985). In order to establish the activity of two gamma

lyases enzymes, different techniques were tested according to the methods described in section 2.2.5.

4.2.1 DNPH Assay

The DNPH assay was adapted from the method described previously by (Anthon and Barrett 2003) and was used to measure pyruvic acid formation. This sensitive method is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form coloured 2,4-dinitrophenylhydrazone derivatives, which can then be detected and quantitated spectrophotometrically.

In this study, biosynthesis of methanethiol was monitored by using the (DNPH) reagent to monitor the 2-oxobutyric acid formation according to the method described in section 2.16. A standard curve and extinction coefficient (figure 4.2-1) were determined to quantify enzymatic activity based on the reaction scheme as presented in figure 4.2.2.

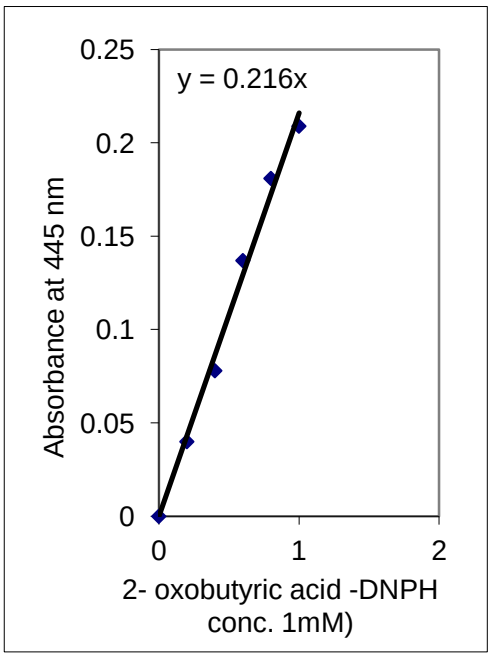


Figure 4.2-1. Calibration curve generated using 2-oxobutyric acid. The extinction coefficient was determined at ε = 65.88 mM^{-1} cm^{-1}.

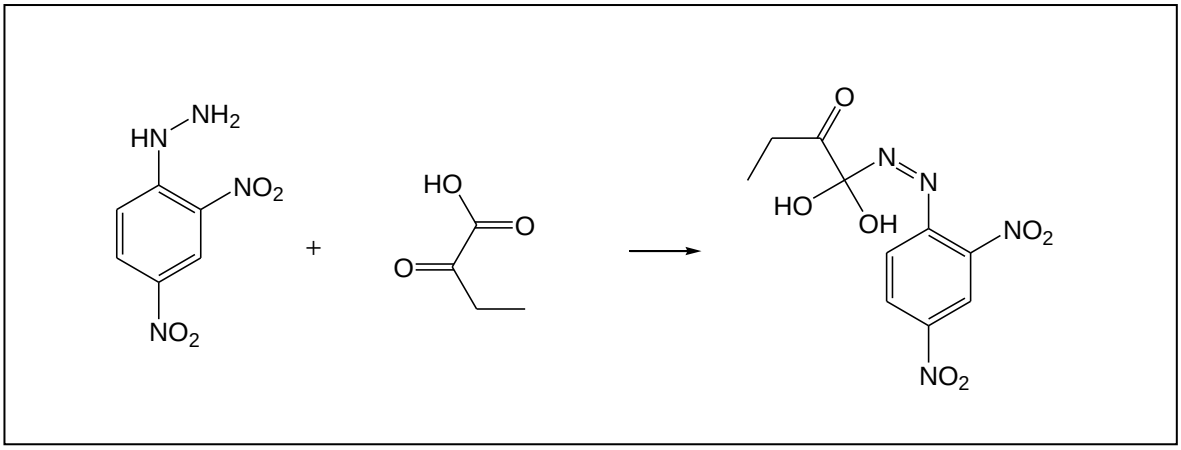


Figure 4.2-2. The scheme of 2-oxobutyric acid reaction with DNPH.

4.2.2 Lactate dehydrogenase continuous assay

The lactate dehydrogenase continuous assay is an indirect method of measuring reaction rates previously described by Nishiya *et al*., 2005.

In this study, the 2-oxobutyrate produced from methionine in the presence of gamma C-S lyase was used by lactate dehydrogenase as a substrate and the rate of reaction monitored spectrophotometrically by measuring the loss of NADH (ε = 6.22 mM^{-1} cm^{-1}). The continuous assay was performed according to the method described in section 2.2.5.2 and the general scheme is shown in figure 4.2-3. The optimum lactate dehydrogenase concentration was determined to ensure that the rate of change in absorbance was directly proportional to the methionine γ-lyase activity. In order to ensure that any changes in the assay conditions would not affect the activity of LDH enzyme each solution was preincubated prior to addition of gamma C-S lyase to obtain the non-specific rate of NADH conversion to NAD+.

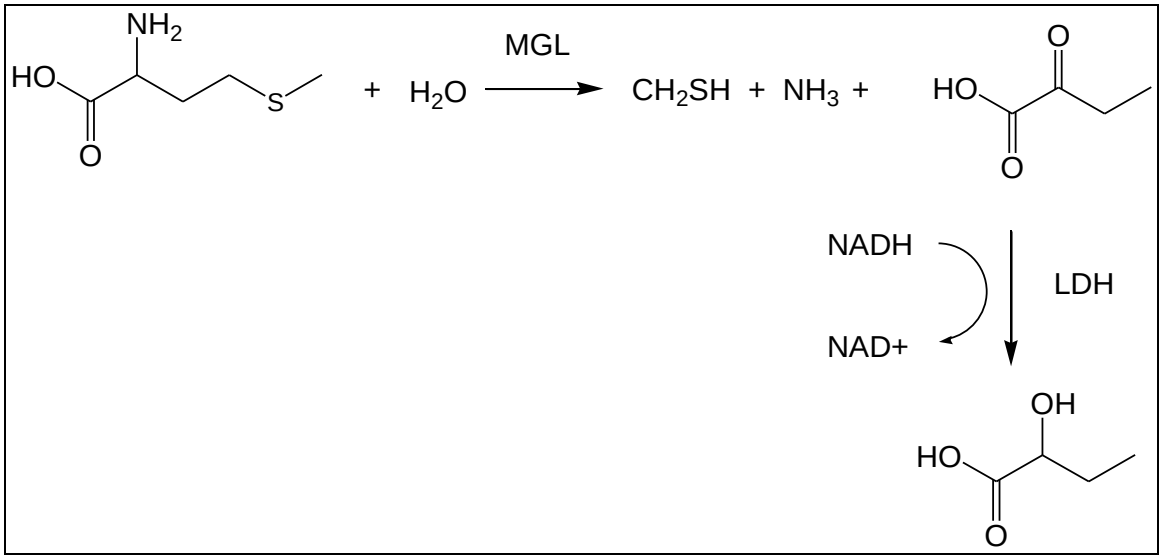


Figure 4.2-3. The principle of lactate dehydrogenase continuous assay.
Reaction catalysed by methionine gamma lyase (MGL), methionine being converted to oxobutyrate, along with methanethiol and ammonia. Oxobutyrate can then be used as substrate in coupled reaction by lactate dehydrogenase (LDH). The rate of both reactions can be monitored by measuring loss of NADH at 340nm.

4.2.3 Ellman's assay

The Ellman's assay described previously (Ellman, 1959) and developed by Dr E. Tapp (Tapp and Brassington 2007) in which 5'5-dithio-*bis*-(2 nitrobenzoic acid) (DTNB) is used to react with released SH groups to produce a yellow substance 5-thio-(2-nitrobenzoic acid with a maximum absorbance at 412 nm, which allows the quantification of free thiol groups in solution. In order to monitor enzymatic activity, a standard curve was prepared according to method 2.2.5.3 using reduced glutathione and the assay was found to be linear up to 100 nmol of thiol in 1 ml of reagent (figure 4.2-5.).

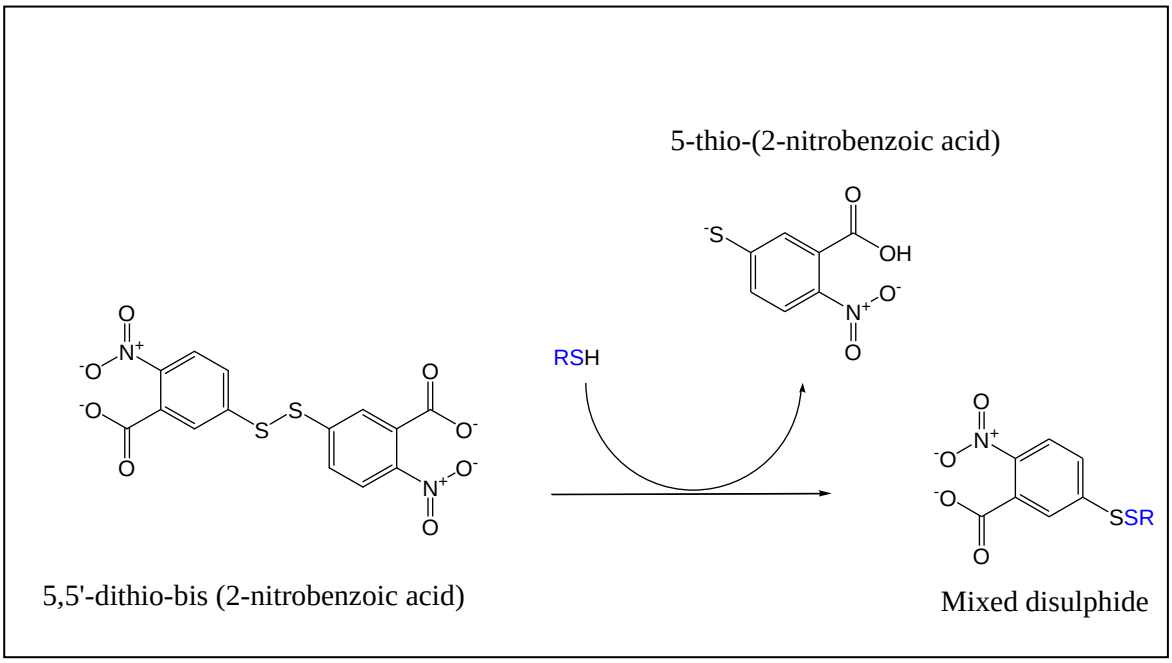


Figure 4.2-4. The principle of Ellman's assay.

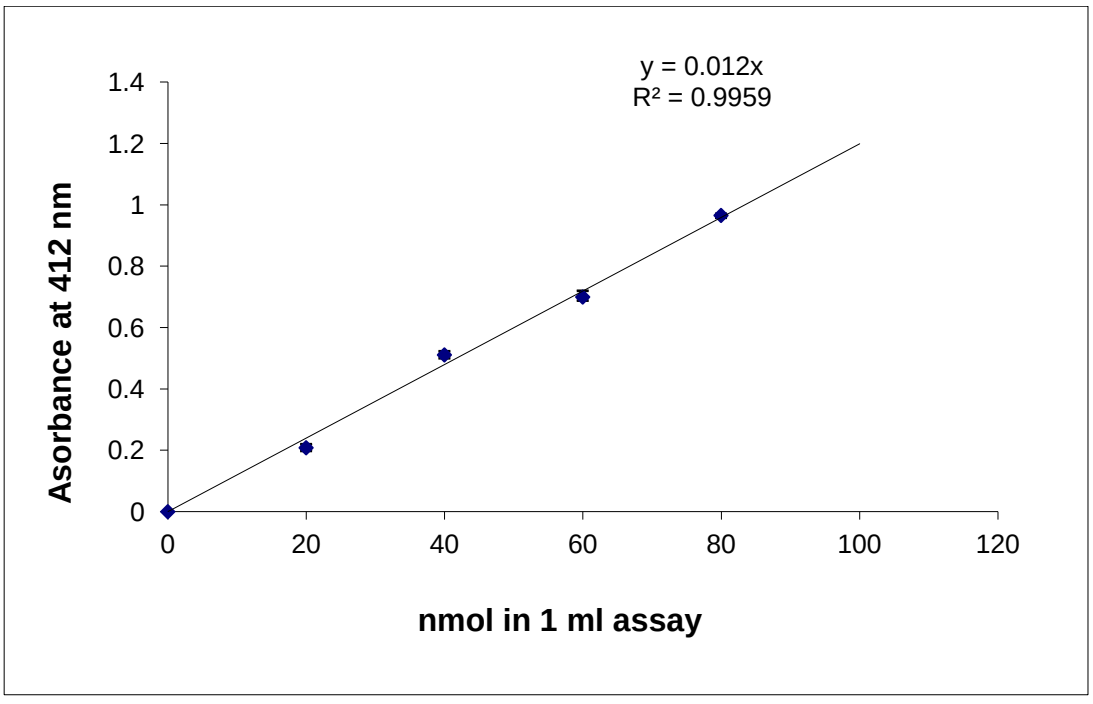


Figure 4.2-5. The standard curve illustrating the direct correlation between reduced glutathione concentration and absorbance (412 nm) using Ellman's reagent.

4.2.4 Assay method comparison

Evaluation of three different quantitative and semi-quantitative techniques for measuring enzymatic activity revealed that the lactate dehydrogenase continuous approach was the most reliable and sensitive assay, permitting the simple determination of reaction rates. The LDH continuous assay was chosen for the routine determination of enzymatic activity in this work. Ellman's assay was also relatively simple and a very sensitive assay, however it was not always accurate due to the highly volatile nature of some of the free thiol compounds products. For example, when analysing methanethiol production determined by the DNPH assay, consistency was not observed probably due to the loss of methanethiol from the assays. The comparison of enzymatic activity of methionine gamma lyase from *P. putida* and *S. cerevisiae* towards methionine measured by different method is presented in table 4.2.1.

Enzyme	Specific activity (nkat mg^{-1}) Calculated by DNPH assay	Specific activity (nkat mg^{-1}) Calculated by LDH assay	Specific activity (nkat mg^{-1}) Calculated by Ellman's assay
MGL *P. putida*	0.68 +/- 0.22	0.58 +/- 0.02	0.37 +/- 0.24
γ-yeast *S. cerevisiae*	0.021 +/- 0.35	0.021 +/- 0.05	0.001 +/- 0.025

Table 4.2-1. Comparison of enzymatic activity towards L-methionine measured by various methods.

4.3 Comparison of gamma lyases

The initial activity comparison of cystathionine gamma lyase from *S. cerevisiae* (γ-yeast) and *P. putida* (MGL) was presented in chapter 3.4. It was established that, MGL had higher catalytic activity toward L-methionine than γ-yeast being 0.6 and 0.02 nkat respectively. Activity of MGL and γ-yeast was improved by cultivating the host bacteria in optimized medium raising yields by 22 % and 27 % respectively. Activity of MGL and γ-yeast enzymes was significantly higher after 15 minute pre-incubation with natural cofactor pyridoxal phosphate (PLP) with the activity increased by 42 % and 45 % respectively. Further parameters such as pH, buffers, temperature, incubation time, and substrate concentration were also investigated. All reactions were routinely followed by measuring the quantity of oxobutyric formed. Maximal activity for both enzymes was observed at pH 8.0 in 0.1 M KP buffer. Similar activity curves were obtained in 0.1 M Tris buffer with the activity being relatively stable at pH values between pH 7.0 and pH 8. The effects of changes in temperature on the rate of enzyme catalysed reaction was then studied. Cystathionine γ-lyase from *S. cerevisiae* had an optimum temperature of 37^{o}C showing 0.02 nkat activity. Interestingly MGL enzyme activity increased with temperature until an optimum of 52°C was reached, above this point, activity decreased possibly as a result of protein denaturation. The activity of MGL at 52°C was 29 % higher than at 37°C. Enzyme assays were carried out at different time scales allowed comparison of activity against time.

4.4 Scaling up the production of methanethiol

The medium scale production of methanethiol was performed according to the method described in section 2.2.6.1. MGL gene in pGEX (35mg) was pre-

incubated in 10 mM pyridoxal phosphate for 30 min at ambient temperature and a desalted aliquot analysed by LC-MS, showing significant incorporation of cofactor (data not shown). The reaction vessel was placed on a stirrer at 37ºC and the enzyme added by syringe vessel sealed with new seals and flushed with nitrogen gas. The reaction was allowed to proceed overnight and an aliquot was then removed by syringe to assay for thiol and methionine content. The vessel was then connected to 3 propylene glycol traps in series in ice, each containing 30 ml propylene glycol PG solvent to capture volatiles.

4.5 Trapping and detection of methanethiol

A simple trapping system was manufactured by glassblowers at Durham chemistry department and was used in larger scale experiments to trap the volatile methanethiol in propylene glycol as presented in figure 4.5.1. Trapping vessels were placed on ice throughout the experiment. Ellman's assay as previously described in section 2.2.5.3 was used to quantify methanethiol levels trapped in propylene glycol and reaction solution. Methanethiol presence was also tested using GCMS figure 4.5-2 with a retention time of 2.31 recorded for authenticated standards obtained from Frutarom Ltd. The GC method encountered several difficulties due to the high viscosity of propylene glycol (PG). Several modifications of the GC conditions as time and oven temperature were tested (data not shown) but could not improve the method. Methanethiol could not be detected as a single peak. It was determine that this experiment showed poor efficiency in trapping methanethiol using a continuous nitrogen flush through propylene glycol, due to the loss of the volatiles through the traps. Instead, the reaction with methionine was carried out in a sealed vessel at pH 8.0 and the

yield of the performed reaction estimated by Frutarom Ltd. to be around 78 %. Unfortunately, using the propylene glycol traps the efficiency of the trapped methanethiol was only around 12 %.

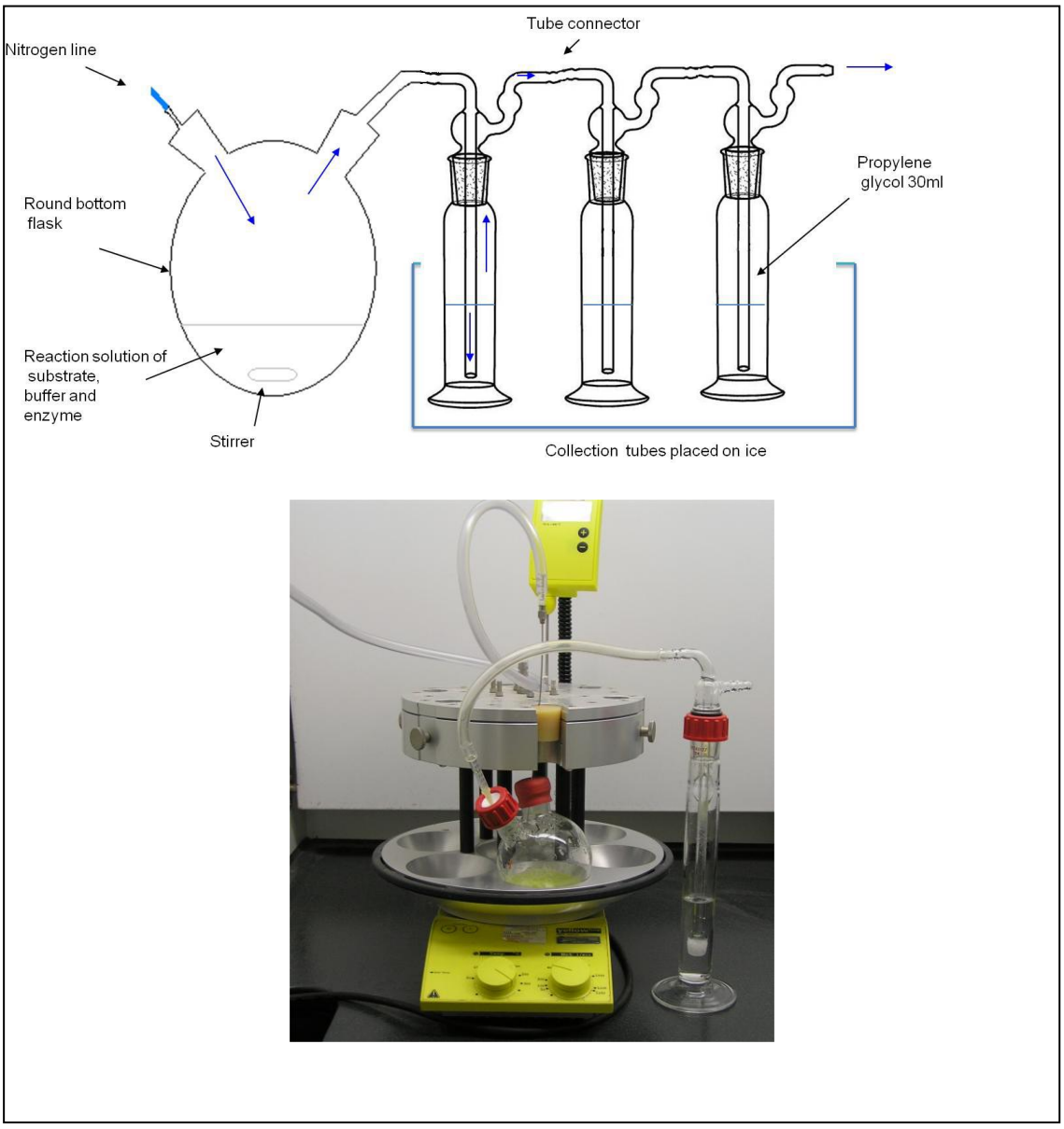


Figure 4.5-1. The scheme showing the equipment used for methanethiol trapping.

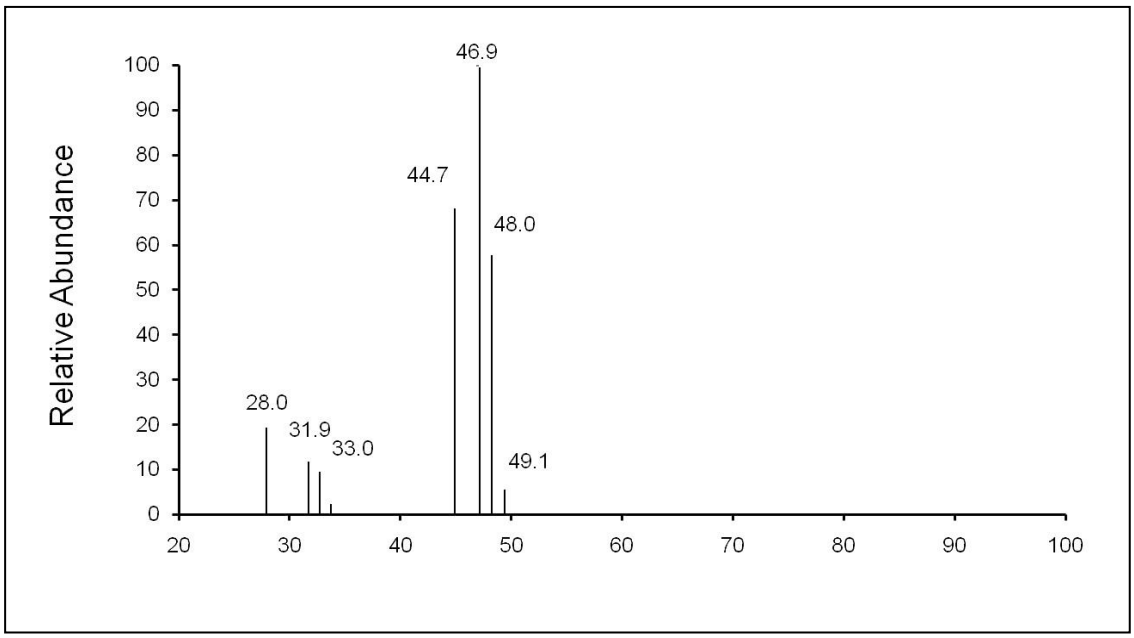


Figure 4.5-2. GCMS spectra of methanethiol (retention time 2.31 minute).

4.6 Summary

The conversion of methionine to volatile sulphur compounds (VOSCs) is of great importance in flavour formation and is the focus of biotechnological approaches toward food flavour improvement or enhancement (Landaud *et al*., 2008). In this work, significant progress has been made toward the natural production of methanethiol from methionine using MGL. As previously described in chapter 3, conditions for the optimal expression in pET and pGEX vectors of MGL were determined. Furthermore, specially optimised growing media have allowed for the purification of the active holoenzyme containing pyridoxal phosphate. However, additional work is still needed for the unequivocal identification of the factors, which may affect reaction on a large scale. Notably, more detailed work is needed in order to improve the trapping of highly volatile methanethiol, which is essential in development of cost effective production on a factory scale.

5 Use of beta C-S lyases to produce *S*-containing high impact aroma chemicals

5.1 Introduction

This chapter explores natural routes toward production of high-impact organosulphur compounds. Previously sourced and cloned groups of lyases, which cleave carbon – sulphur bonds in the beta position of cysteine conjugates were tested against cysteine conjugates in order to obtained desirable free thiol compounds. In particular, natural production of hydrogen sulphide and thiomenthone were investigated in detail.

5.2 Comparison of assay methods

In this study, various methods were compared to obtain a sensitive and simple assay for determining enzyme activity. Protein products from four different constructs of pET24a-gene with MalY, MetC and TRP were tested for enzyme activity. All samples were pre-incubated with pyridoxal phosphate in 0.1M phosphate buffer, pH 7.8 and then subsequently assayed for carbon-sulphur lyase activity toward the panel of substrates using Ellman's method (2.2.5.3.) and the lactate dehydrogenase continuous assay (2.2.5.2). The initial data generated from enzyme assays in the presence of cysteine conjugates shown that, the enzymatic activity measured by Ellman’s method was lower than the continuous assay on average by 7.3 (± 2.4) %. Under optimal conditions, continuous assay was proven to be an accurate indirect determination of free thiols production. In

order to confirm the production of desired compounds LC-MS and GC-MS methods were also investigated.

5.3 Assay optimisation

All tested enzymes (MalY, MetC and TRP) were incubated with pyridoxal phosphate prior to use and showed an optimum pH profile between 7.8 and 8.4 without any significant loss of activity. All of the enzymes tested showed a very similar performance in buffers tested at pH 7.8. The most effective buffer - 0.1 M potassium phosphate buffer pH 7.8 was selected for all assays. Results for MetC are presented in figure 5.3.-1. MetC from *E. coli* was found to be the most active enzyme with a broad activity and was therefore studied in more detail.

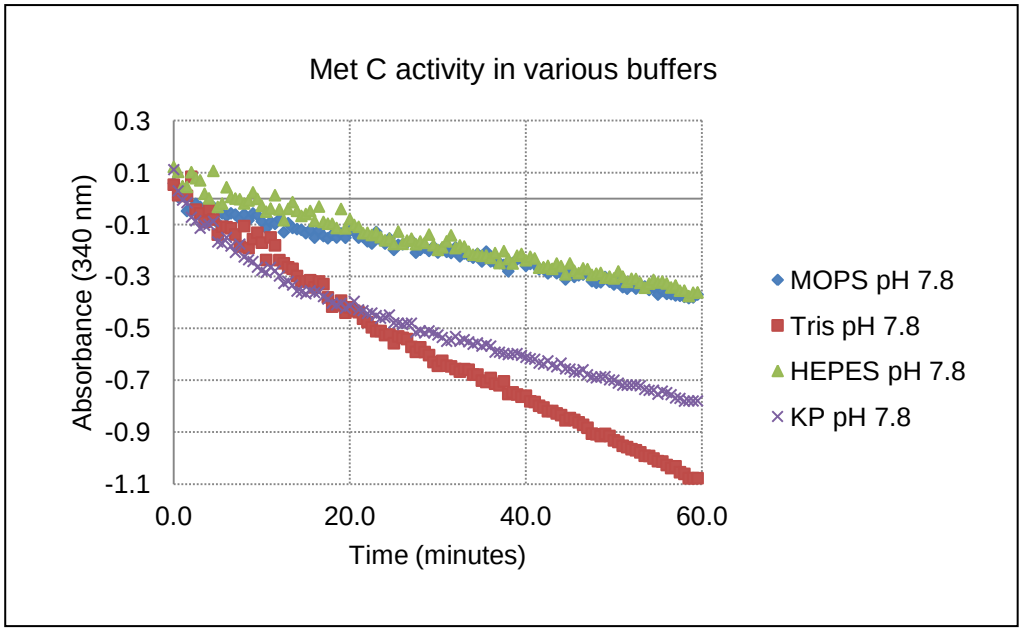


Figure 5.3-1. The comparison of MetC activity towards cystathionine in various buffers by LDH assay.

5.4 Comparison of beta C-S lyase enzyme activity

In order to compare enzymes activity, all enzymes were tested towards cysteine conjugates as presented in table 5.4-1. MetC had the greatest specific activity towards all suitable substrates, demonstrating relatively high levels of activity across the different chemicals. The highest activity was observed with 1-Penten-3-one and pulegone cysteine conjugate, 57 and 43.5 nkat mg^{-1}, using 1mM substrate respectively. MalY and TRP revealed much lower specific activity with all tested substrates. Pulegone cysteine substrate was relatively insoluble which most probably increased the consistency of assay.

Unfortunately, none of tested enzymes showed activity towards cysteine conjugate of furfural or cuminaldehyde. Furthermore as shown in chapter 3 in comparison to other tested beta enzymes, MetC showed very efficient storage properties and high expression, therefore is the most favourable enzyme in this project. Natural production of thiomenthone will be studied in more details in chapter 5.5.2. The range of possible substrates for MetC may be broader and further activity studies could reveal many interesting properties of MetC.

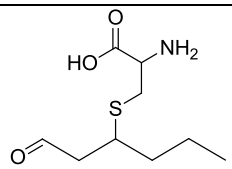
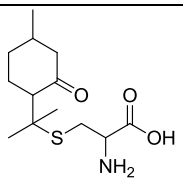
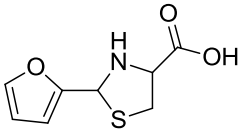
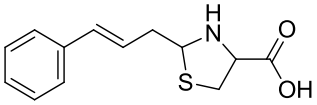
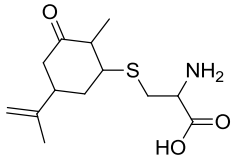
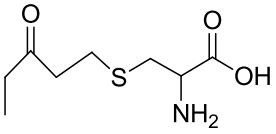
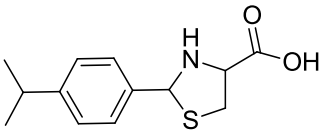
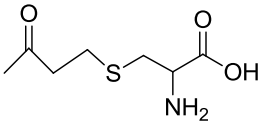
Name of F&F precursor	Structure of substrates	MetC	MalY	TRP
Trans-2-hexenal		14.1 +/- 3.6	2.3+/- 0.8	1.3+/- 0.6
Pulegone		43.5 +/- 6.8	6.7+/-2.9	2 +/- 0.8
Furfural		0	0	0
Cinnamaldehyde		6.5 +/- 0.3	0.23+/-0.04	0.3+/- 0.03
Carvone		12.4 +/- 4.5	0.2+/- 0.1	1.1 +/- 0.3
1-Penten-3-one		57 +/- 1.5	9.2 +/- 4.5	5.8 +/- 0.8
Cuminaldehyde		0	0	0
Methyl Vinyl Keton		17 +/- 0.09	1.3 +/- 0.7	0.2 +/- 0.09

Table 5.4-1. The comparison of activities of beta lyases against various F&F cysteine conjugates measured by Ellman's assay. Structures of the F&F precursors and specific activities obtained with C-S lyases in presence of cysteine conjugates of presented flavour precursors shown in second column (nkat mg $^{-1}$, using 1mM substrate).

5.5 The enzymatic production of High-Impact Aroma Chemicals

In this chapter, work continued on optimising the biogenesis of thiol compounds and in particular identifying the barriers to progressing this technology through medium-scale production. The most active and stable enzyme MetC was tested using L-cystine and pulegone cysteine in the course of the biofactory project. Furthermore, this amino acid is a direct source of hydrogen sulphide, which can then be used directly to react with certain aldehydes to produce free thiols as presented in section 1.7. Cysteine conjugates used in this work, have been previously identified, synthesised, isolated and characterised by commercial partner Frutarom Ltd. All C-S lyase substrates were resuspended in 50 % MeOH at 1 mg/ml and analysed by reversed-phase HPLC-MS in order to determine their final purity.

5.5.1 Natural production of hydrogen sulphide from cysteine and cystine.

The generation of hydrogen sulphide from L-cystine has been previously studied by Morra (Morra *et al.,* 1991*).* In this work, production of natural hydrogen sulphide was achieved by enzymatic processing of natural cystine to cysteine, which was then acted upon by a MetC to release hydrogen sulphide, which can be reacted directly with other F&F precursors as suggested in pathway in chapter 1.7. The substrate cystine was not highly soluble except at low pH therefore the reaction was carried out using suspension of the substrate. The optimised media previously employed for CS-lyase fermentation was utilised for these studies. The pGEX MetC in *E. coli* BL21 strains were grown overnight at 37°C in 200 ml LB medium (with appropriate antibiotic selection) at 200 rpm. The reaction was

performed at 50 ml scale with 10 mg/ml cystine, in the presence of 8 mg of MetC. The reaction was performed over 2h. The yield of reaction was very high over 93 %, however only 15 % of produced thiol was trapped in propylene glycol traps as previously described in chapter 4.5 as measured by Ellman's assay (2.2.4.3). The high levels of hydrogen sulphide were escaping the system during production and was detected by lead acetate paper. The MetC specific activity of 13 nkat/mg was measured by the LDH assay. Ultimately, the reaction was conducted with very slow stir to avoid breakage of the nitrogen bleed and loss of volatile product during bleed replacement. Production of hydrogen sulphide was confirmed by GC-MS using method 2.2.5.4.

Fractions of propylene glycol were collected and analysed directly by GC (data not shown). This study shows that hydrogen sulphide can be made in a natural way from both cysteine and cystine on a medium scale with relatively high yield of reaction. Unfortunately, using the propylene glycol traps the efficiency of the trapped product was only around 6 %. Therefore, a more efficient method of trapping the hydrogen sulphide needed to be developed.

5.5.2 Thiomenthone production

Thiomenthone is a cassis-like odourant naturally found in buchu leaf oil and can be biosynthesized via elimination of cysteine by carbon- sulphur lyase such a MetC. Thiomenthone is also, a very powerful material in exotic fruit formulation and black currant odorants. It is used widely in alcoholic beverages, apricot, herbal flavours, mango pineapple and tropical fruits. Thiomenthone has four isomers, of which two occur in nature (Wakabayashi *et al*., 2002).

For medium scale production the pulegone cysteine conjugate (0.1 M) was solubilised in 0.1 M phosphate buffer pH 8.0. The pulegone conjugate is poorly

soluble in water and can clearly be seen in each reaction as an insoluble white solid. Enzyme was pre–incubated at 37^{o}C with the pyridoxal phosphate for 30 minutes and the biosyntheses were initiated by addition of substrate. Assays of thiomenthone formation were monitored at 37^{o}C in 0.1 M potassium phosphate buffer pH 8.0 containing 1, 10, 100 mM pulegone cysteine, 4 mM pyridoxal phosphate and 10 mg of MetC. In brief, recombinant *E. coli* expressing MetC were cultured at a 50ml scale under conditions previously established for production of MGL chapter 4.4. Initially, the solution was translucent as the substrate became increasingly soluble at 37^{o}C, however after ~30 minutes the solution became an opaque and oily emulsion. Thiol (S–H) moieties were detected by addition of 10 µl of the assay to 1 ml of 0.1 M potassium phosphate buffer pH 8.0 containing 50 mg/ml Ellman's reagent (5,5'-Dithio-(bis-2-nitrobenzoic acid). Experiments were incubated at room temperature for 2h, with observation for loss of conjugate from the reaction. The pH was tested at the end of each reaction and was found to be stable at pH 8. Three different reactions were performed at different concentration of substrate: 1, 10 and 100 mM. The most efficient reaction occurred at 1 mM concentration of pulegone cysteine resulting in 92 % yield of reaction. The reaction performed at higher, 100mM concentration gave a lower yield of 24 %. The concentrations of thiomenthone achieved in these reactions appear to be especially high at 1mM concentration, giving 92 % molar yield. These data suggested potential product inhibition. This could be due to one or more of each of the three reaction products, ammonia, pyruvate or thiomenthone. It is known from previous studies that thiomenthone is very susceptible to dimerisation in the presence of oxygen (Wakabayashi *et al*., 2002) therefore the reaction was performed in a sealed vessel. Samples tested

after 1 week of storage show dimerisation of thiomenthone, therefore they were treated with a 3x molar excess of either dithiothreitol or mercaptoethanol to reduce the proposed dimer back to thiomenthone for quantification. The product of the reaction was confirmed by GC-MS as presented in figure 5.5.1.

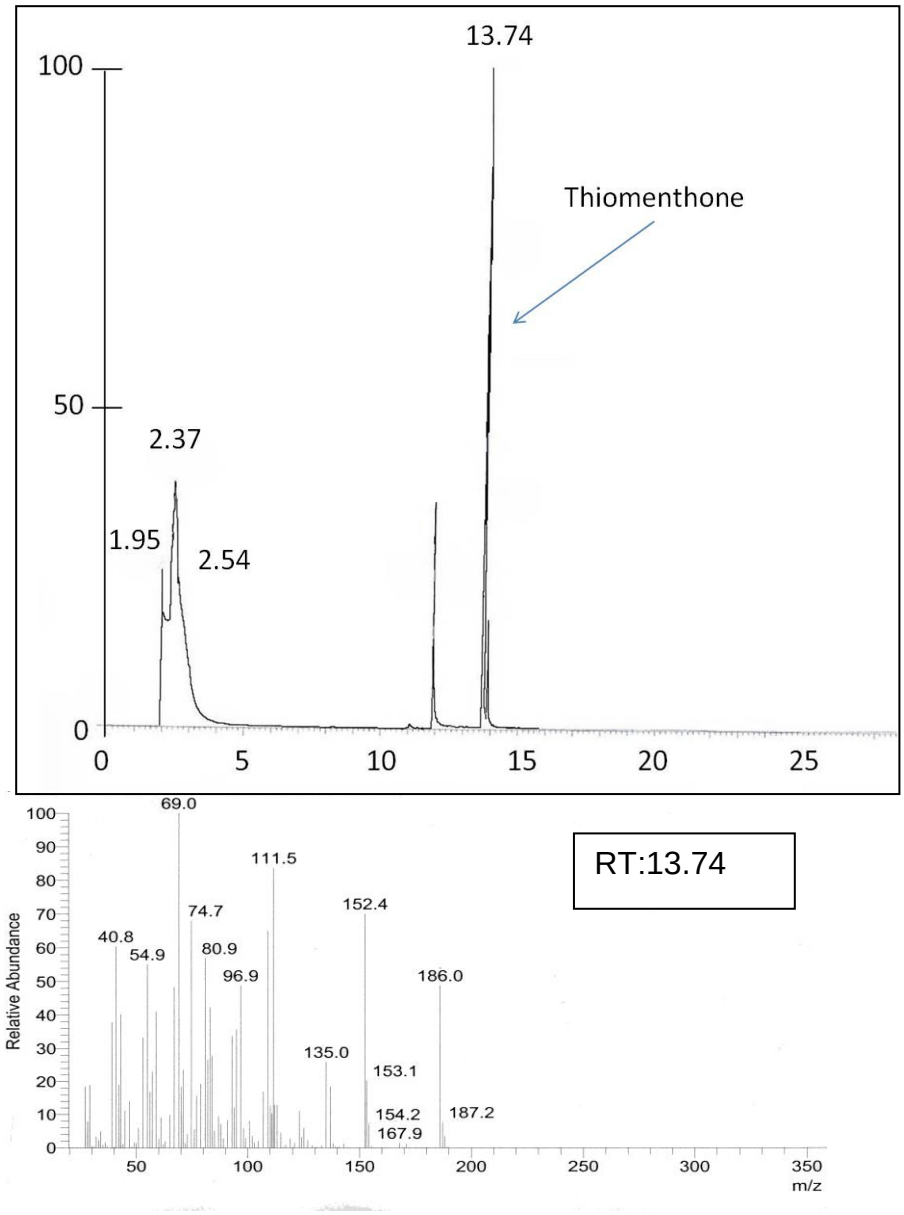


Figure 5.5-1. GCMS chromatogram showing thiomenthone.

The production of thiomenthone was also investigated *in vivo.* In brief, recombinant *E. coli* expressing MetC were cultured at a 50 ml according to method 2.2.6.2 in the presence of pulegone cysteine. Experiments were incubated at room temperature for approximately 2, 4 and 6 h, with observation over the first few hours for growth density.

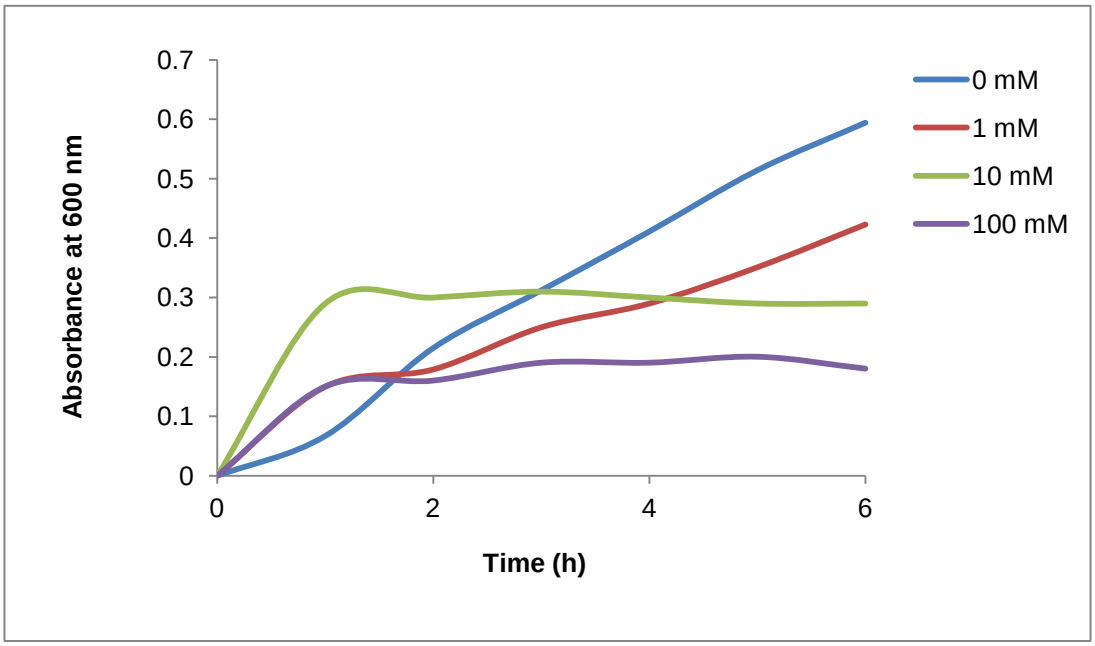


Figure 5.5-2. Growth studies of *E. coli* expressing MetC measured as changes in optical density over time in a presence of pulegone cysteine.
Blue line -control, showing growth of *E. coli* without pulegone cysteine; red line in presence of 1mM of pulegone cysteine: green line - pulegone (10 mM); purple line pulegone cysteine (100mM).

As presented in figure 5.5-2, high concentration of pulegone cysteine caused inhibition of growth. The concentration of pulegone cysteine at 1 mM was found to be the highest optimal concentration. Prior to feeding studies on medium scale, *E. coli* cultures expressing MetC were grown to OD 0.6 and supplemented with pulegone cysteine at final concentration of 1 mM as described in section

2.2.6.2. Thiomenthone could be detected only in small quantities, therefore it was not possible to estimate the yield of this reaction.

5.6 Conclusion

The selection of tested C-S lyases which, cleave carbon – sulphur bond in beta position of cysteine conjugates showed a broad spectrum of activities. The most active enzyme - MetC showed very high activity towards cysteine conjugates such as 1-penten-3 one, pulegone, methyl vinyl ketone and cuminaldehyde.

Initial laboratory medium scale production of thiomenthone and hydrogen sulphide was successful. Although successful large scale of biosynthesis of thiomenthone from pulegone cysteine conjugate was obtained; notably, more detailed work is needed in order to improve the trapping of highly volatile products, which is essential in the development of cost effective production on a factory scale. The other possibility is to directly react products like hydrogen sulphide with flavour precursors as suggested in chapter 1.7. Furthermore, enzymes, which can tolerate higher temperature could be used in reactions at increased temperature, that promotes higher reaction rates. Sourcing beta C-S lyases from alternative thermophilic microbes, which can tolerant highly temperature would be highly desirable.

6 Methylation of free thiols

6.1 Introduction

As detailed in chapter 1.5, direct enzymatic methylation of free thiol compounds can lead to useful precursors and become a possible route for production of desired F&F chemicals. In recent years, there has been increasing interest in identifying sulphur-methyl transferases (SMTs) in biological samples in order to explain their roles in various pathways. Unfortunately, there is limited information on SMT enzymes. The model plant *A. thaliana* contains a wide selection of methyl transferases utilizing *S*-adenosyl methionine as methyl donor enzymes including, *O*-methyltransferases (OMTs) and three *S*-methyltransferase (SMTs) dedicated to small sulphur molecule conjugation (Nagatoshi and Nakamura 2007). This new class of SMTs are responsible for methylation of the hydrolysis products of glucosinolates to volatile sulphur compounds (Nagatoshi and Nakamura 2007). In particular, the SMT named TMT1 encoded by the *ATHOL1* gene has been shown to possess relatively high activity towards free thiols. Furthermore, an enzyme previously determined to be an *O*-methyltransferases from *Catharanthus roseus* (CrSMT1) has recently been shown to function as a SMT with a potent ability to methylate free thiols (Coiner *et al.,* 2006). This chapter describes the cloning of two selected methylation enzymes (CrSMT1) from *C. roseus* and TMT1 from *A. thaliana*, the expression of the respective proteins initial enzyme activity studies, the properties of purified recombinant proteins and finally their potential for catalyzing the direct methylation of sulphur containing flavour and fragrance compounds. In addition, the potential of TMT1 to act on pesticide metabolites such as fenclorim was examined to test the possible role of TMT1 in plant xenobiotic metabolism. Although, fenclorim is not a

F&F related compound, it was of interest to the Edward's research group (Brazier-Hicks *et al*., 2008).

6.2 Cloning, expression and purification of *S*-methyl transferases

6.2.1 The sulphur methyl transferase (CrSMT1) from *Catharanthus roseus*

The coding sequence of the sulphur methyltransferases (CrSMT1) from *Catharanthus roseus* was obtained by total gene synthesis (GeneScript USA Inc.) and supplied as a DNA MiniPrep in the pUC57 vector. The CrSMT1 gene was subsequently excised from the vector using the restriction enzymes *Nde*I and *Xho*I and then ligated into *Nde*I and *Xho*I digested pET24a to make a C-terminus His-Tag fusion as described in chapter 2.2.1. The clone of CrSMT1 was subjected to DNA sequencing to confirm the sequence accuracy. Unfortunately the expression of the insert in pET24a C-terminal His Tag was unsuccessful (figure 6.2-2 lane 2), so two different expression vectors were used namely the pET28a (N-terminal HisTag) and pET-STRP3 cut with *Nde*I and *Sal*I. These vectors were already available in-house, courtesy of Dr Melissa Brazier-Hicks and Dr D.P. Dixon respectively. In the case of the strep vector CrSMT1 from *Catharanthus roseus* the open reading frame was cloned into the vector pET-STRP3, which is a modified version of pET28a (Novagen) allowing an N-terminal *Strep* II tag fusion. CrSMT1 was amplified using proof-reading DNA polymerase KOD (Novagen), the 5' primer CrSMT1*Nde*I and the 3' primer CrSMT1*Sal*I Stop (chapter 9.1) to introduce a *Sal*I site after the translation termination codon. The resulting PCR product was digested with *Nde*I and *Sal*I and ligated into *Nde*I and

*Xho*I digested pET-STRP3 to create pET-STRP3-CrSMT1 and into *Nde*I and *Xho*I digested pET-28a to obtain pET-28a-CrSMT1 respectively see (figure 6.2-1).

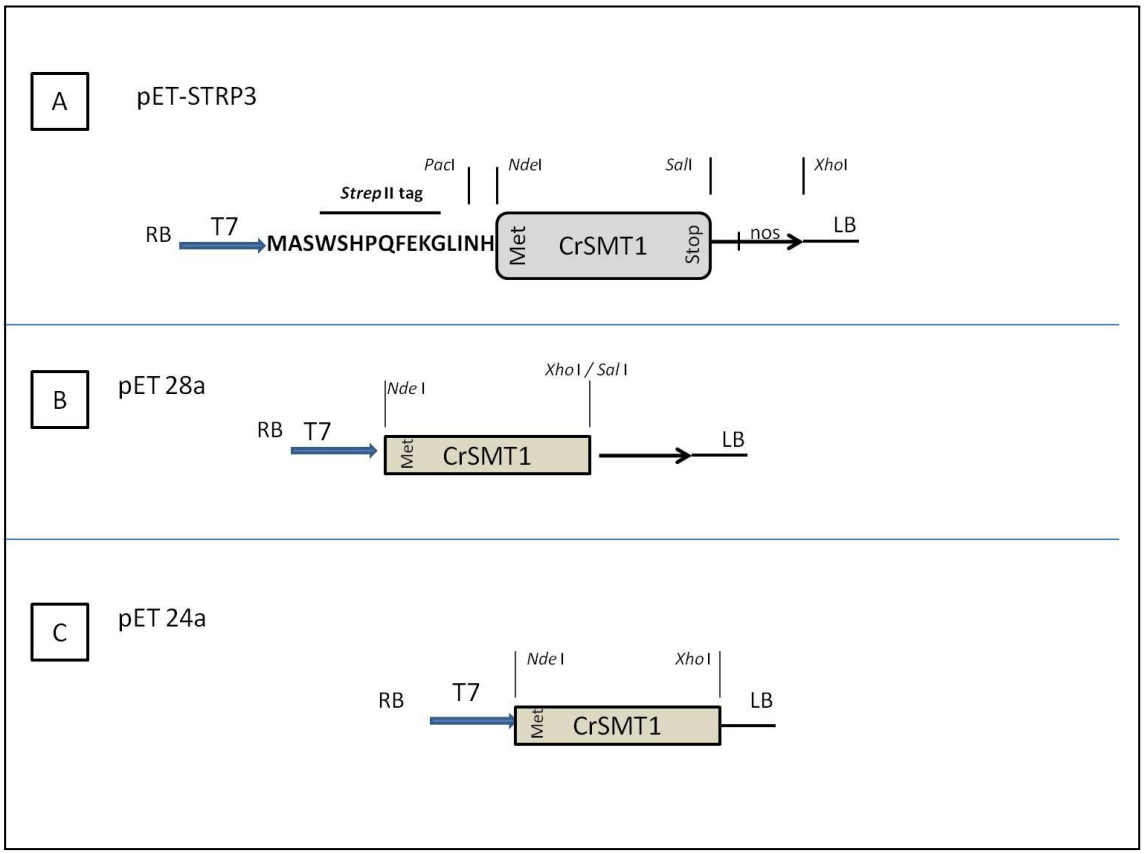


Figure 6.2-1. The scheme of the three pET vectors used in overexpression of TMT1.
Strep II tag is shown fused to the N-terminus of the respective gene along with restriction enzyme sites. RB – right border repeat; LB – left border repeat (Dixon *et al.*, 2009).

The recombinant enzymes were then expressed in *E. coli* and the results analysed by SDS-PAGE (figure 6.2-2). The SDS-PAGE analysis clearly demonstrates very poor level of expression of recombinant protein in the lysates from all three vectors grown in shake flask cultures, with the predicted protein band at 39.9 kDa not determined (figure 6.2-2).

Further attempts to express soluble recombinant CrSMT1 were unsuccessful and protein failed to express in the soluble form. Similarly, it was not possible to

confirm the presence of purified protein using mass spectrometry of the affinity purified fractions. Lysates were also analysed for SMT activity utilising the analysis of radiochemical assay described in 2.2.5.7. Unfortunately, no activity was detected.

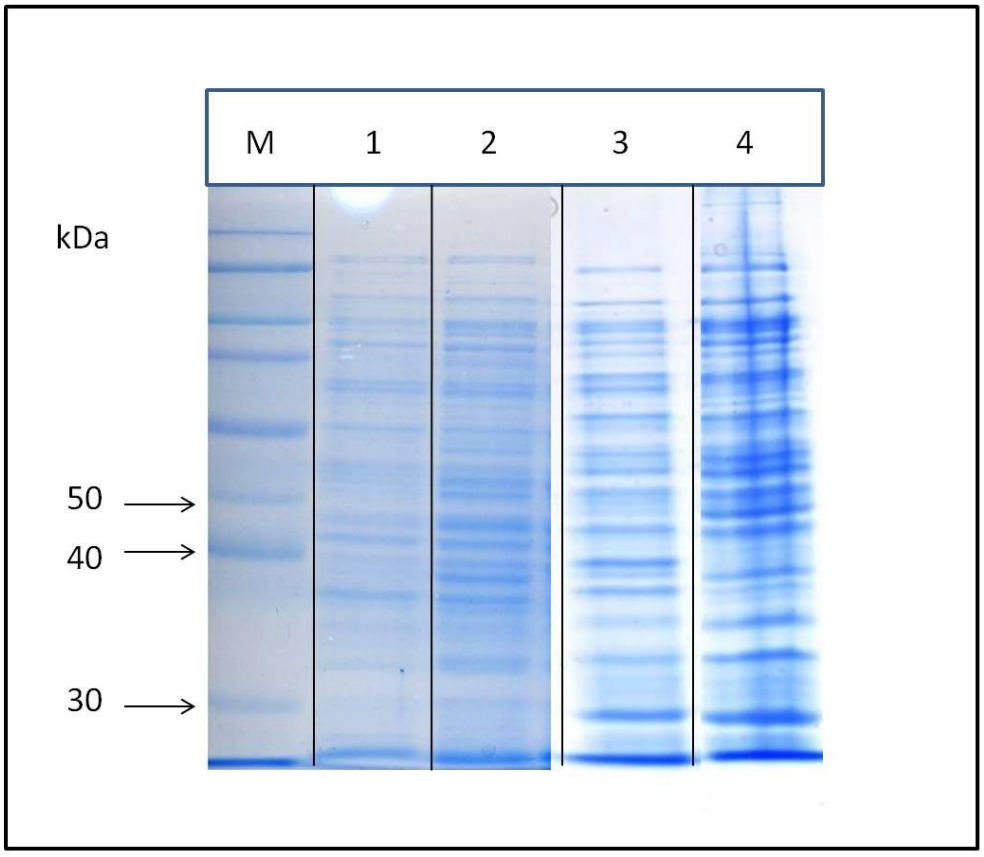


Figure 6.2-2. SDS-PAGE analysis of CrSMT1 protein expression in 250 ml shake flask cultures. M: protein marker, sizes are indicated in kilo Dalton at the side of gel; lane 1: control; lane 2: peT-24a-CrSMT1; lane 3: pET-28a-CrSMT1; lane 4: pET-STRP3-CrSMT1.

No further work on CrSMT1 was undertaken due to time constraints, though the use of an alternative expression host such as yeast may have been successful.

6.2.2 The *S*-methyl transferase from *Arabidopsis thaliana*

The coding sequence of *S*-methyl transferase (TMT1) was amplified from *Arabidopsis thaliana* cDNA using the PCR primers TMT1 listed in section 9.1. The amplified fragments were ligated into the sequencing vector pGEM-T easy and transformed into ultracompetent cells XL 10 – Gold (Stratagene). Prior to DNA sequencing. The desired vectors were cut with *Nde*I and *Xho*I restriction enzymes and the insert cloned into the same sites in the expression vectors pET24a (Novagen) allowing for the His –Tag purification of the respective proteins. The constructs were used to transform Rosetta-gami^{TM} 2, with TMT1 synthesis induced by the addition of 1 mM IPTG to the LB culture medium when the cells were in the mid-log phase of growth. TMT1 was purified using nickel-chelate chromatography and the recovered protein dialysed overnight against 10 mM Tris-HCl (pH 7.8) at 4°C and concentrated by using a centrifugal concentrator (Viva Spin, Sartorius) to improve recovery. The purified TMT1 protein was then analysed by time-of-flight mass spectrometry following electrospray ionisation (ESI-TOF-MS) as described in section 2.2.3.11 and a dominant mass ion of 26217 was determined (figure 6.2.4). The highest overexpression yield of total 98 ± 1.3 mg protein per 500ml of bacterial culture was achieved using overnight culture at 28°C.

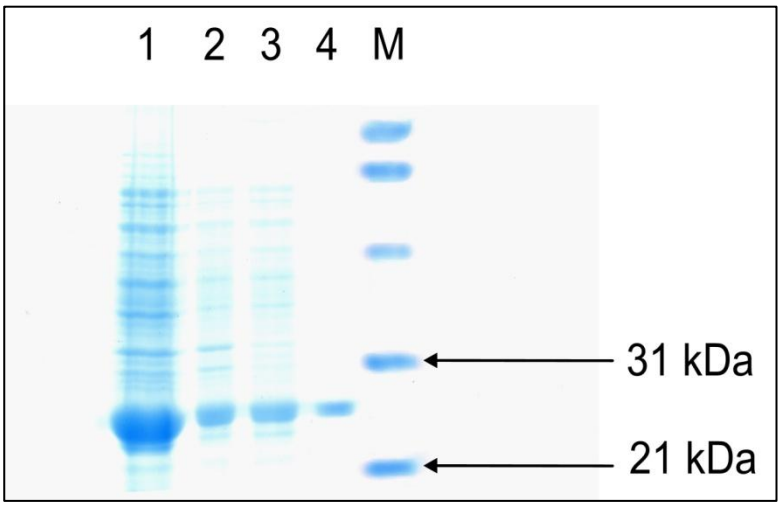


Figure 6.2-3. SDS -PAGE analysis of TMT1 expressed in *E. coli.*
Total IPTG-induced protein (lane 1), soluble protein fraction (lane 2) and insoluble protein fraction (lane 3). Lane 4 shows TMT1 following purification by Nickel affinity chromatography. Molecular weight markers (Lane M) are also shown with molecular masses as indicated.

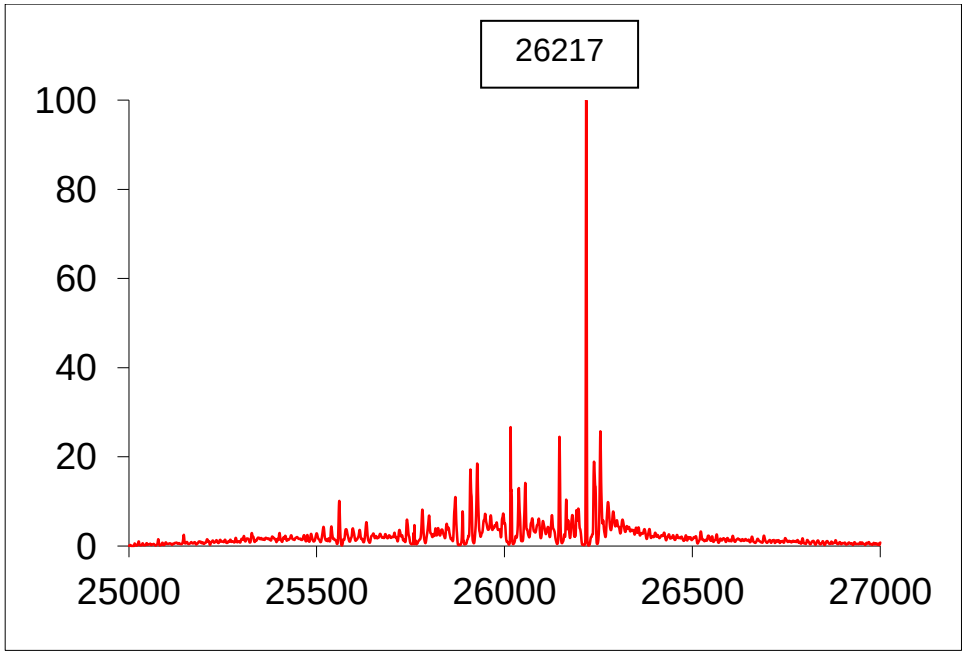


Figure 6.2-4. Deconvoluted mass spectrometry analysis of TMT1 protein after His-tagged purification.
Spectrum range 25-27 kDa. The predicted mass of TMT1 was 26217.

6.3 Activity of *S*-methyl transferase

The purified recombinant TMT1 protein was directly assayed for enzyme activity using both HPLC and radiochemical based assays according to the method described in section 2.2.4.5 and 2.2.4.7 respectively. The native TMT1 enzyme activity was tested toward two model substrates namely: 4,4-*bis*thiobenzenothiol (BBT) and thiophenol. Once both methods were tested and optimised, activity was then determined with a broader range of free thiol substrates.

6.3.1 Development of HPLC based enzyme assay

Enzyme assays were performed according to the methods described in section 2.2.5.5 with activity determined by HPLC (2.2.4.11). The HPLC assay consisted of the TMT1 enzyme (100 µg) incubated with acceptor substrate 0.2 - 1 mM and SAM 0.5 mM - 2 mM. Initial studies concentrated on the methylation of two substrates thiophenol (figure 6.3-1) and 4,4'-thiobisbenzenothiol (figure 6.3-2). However, as it was difficult to quantify product formation using diode array data, a radiochemical assay was also performed. To confirm the identity of the reaction products the GCMS method (2.2.6.4) was used to confirm the accurate molecular mass of the methylated product formation, as presented in figure 6.3-3.

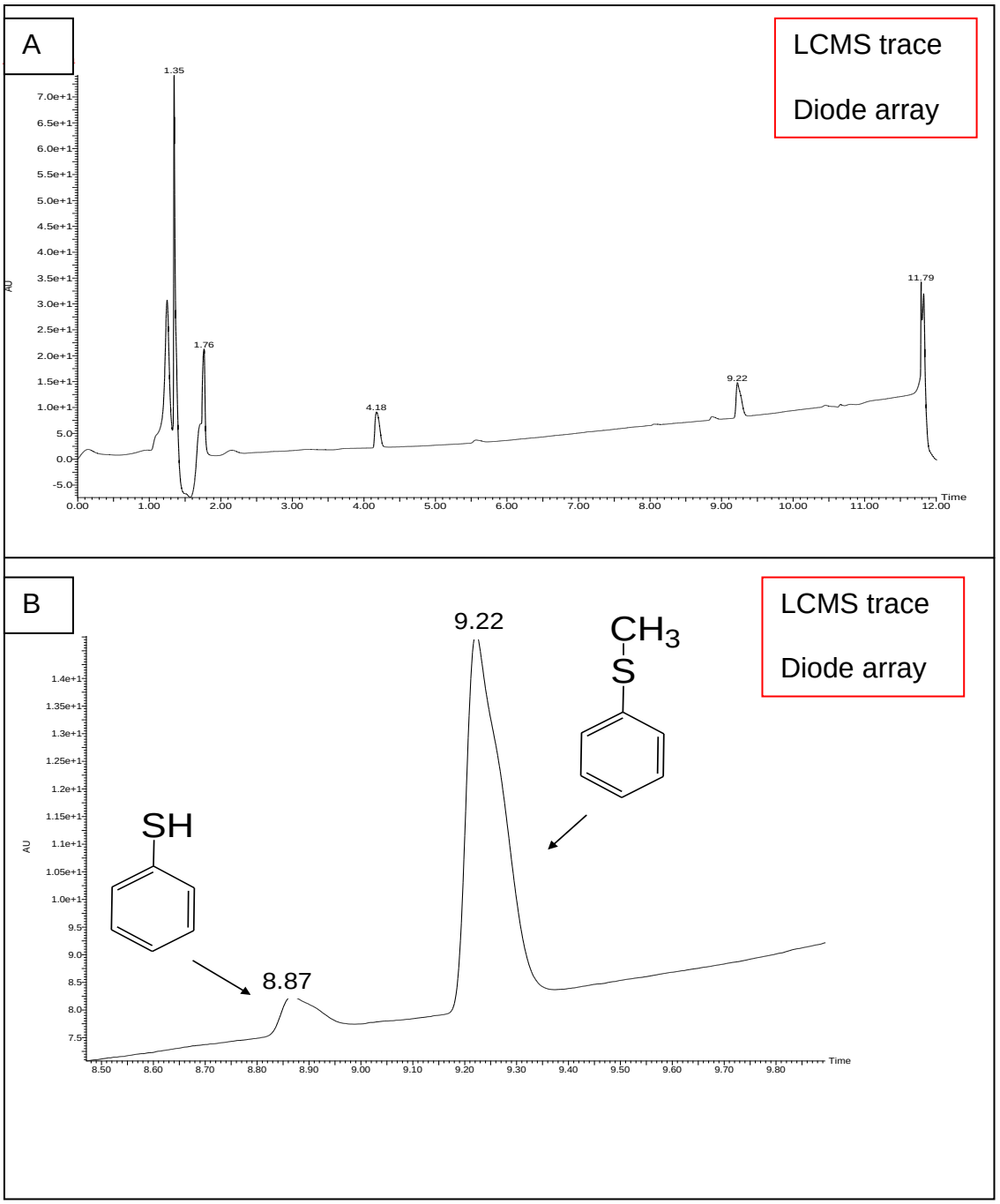


Figure 6.3-1. HPLC-MS of TMT1 assays performed with thiophenol, metabolites detected from UV absorbance at 280 nm.

A: Total LCMS chromatogram. B: LCMS trace shows detection of eluant at 280 nm thiophenol and methylthiophenol at retention time 8.87 and 9.22 respectively.

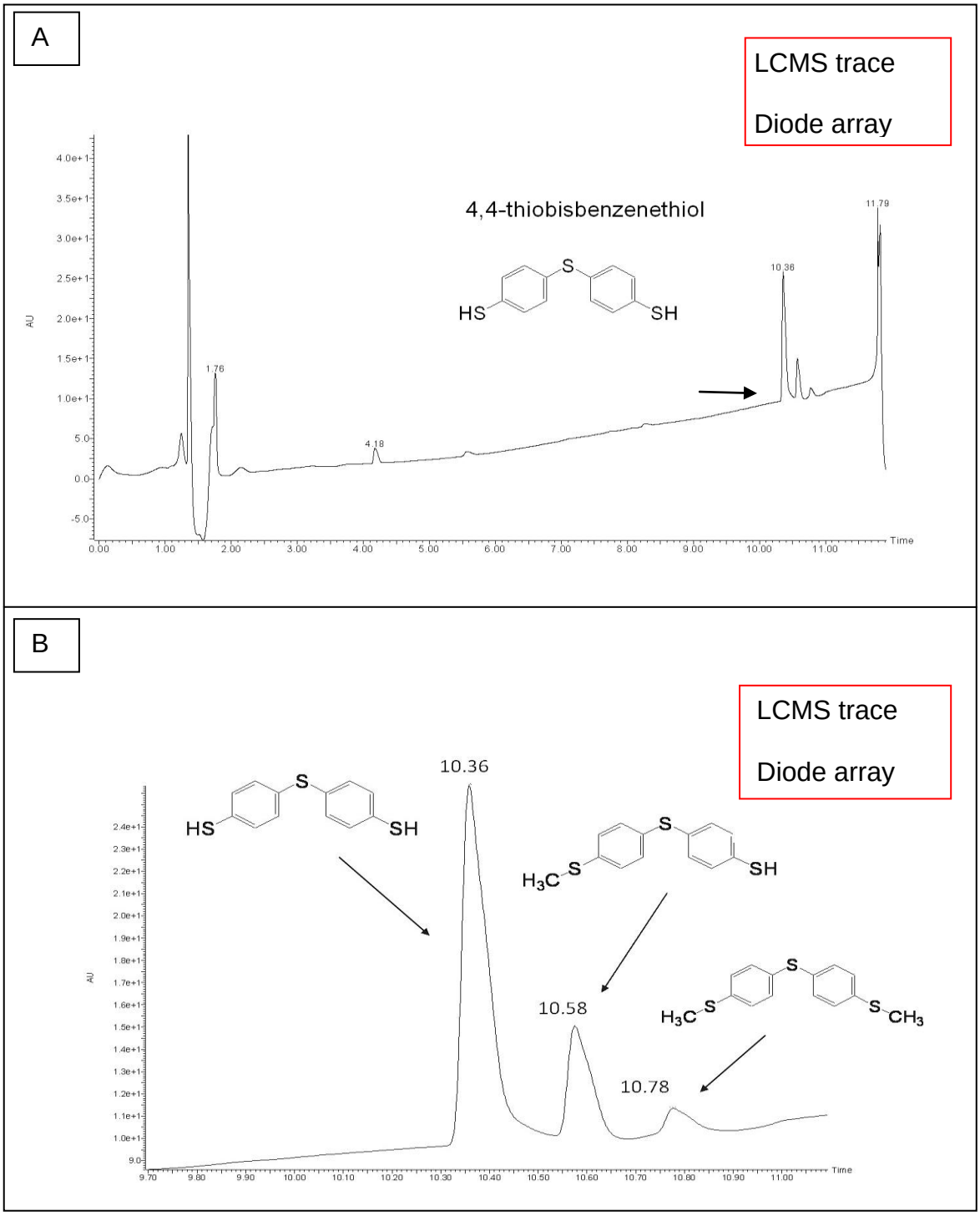


Figure 6.3-2. HPLC-MS of TMT1 assays performed with 4,4'-thio*bis*-benzenethiol with metabolites detected from UV absorbance at 280 nm.
A: Total LCMS chromatogram. B. The peak at 10.36 confirmed the substrate; the peaks at 10.58 and 10.78 showed single and double methylation respectively.

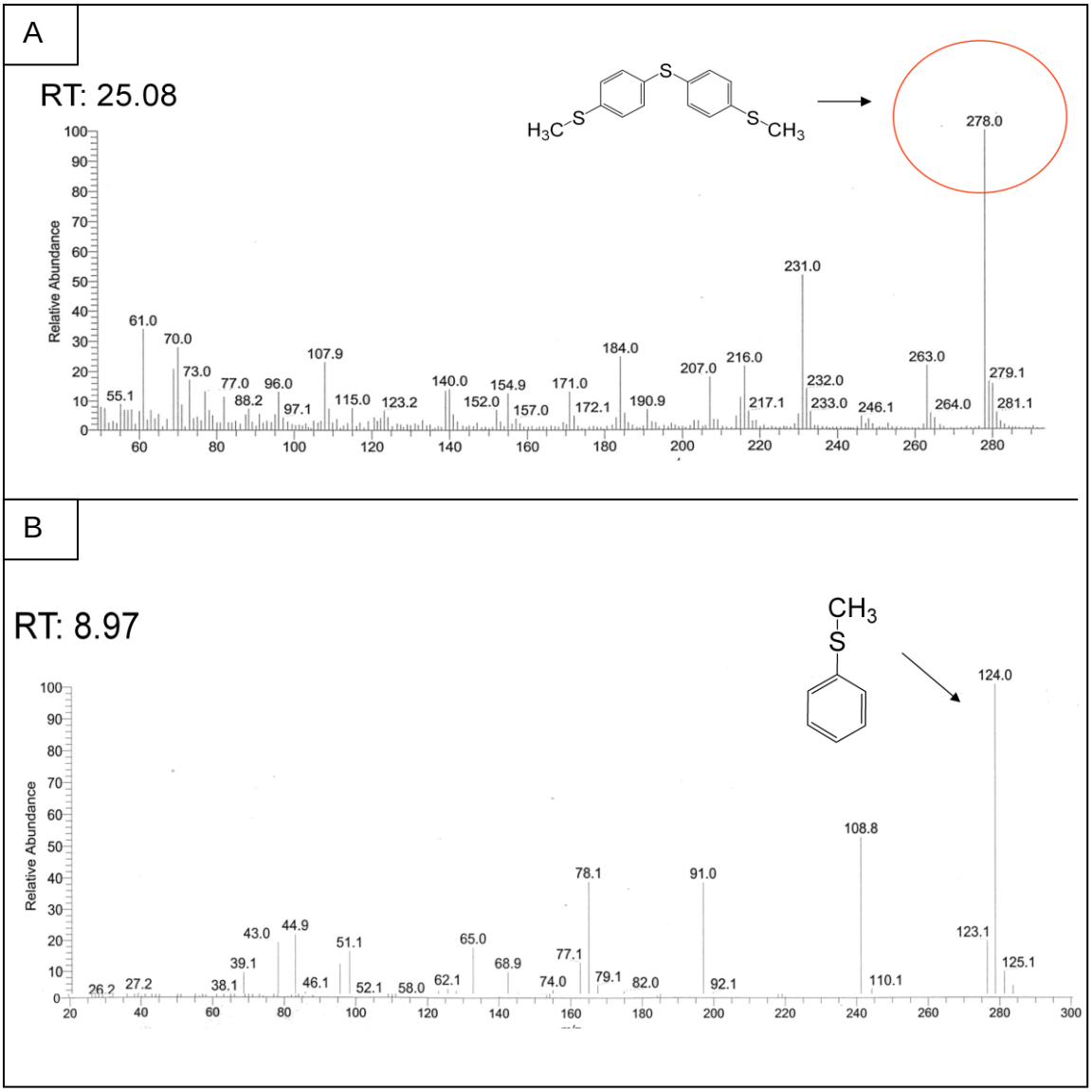


Figure 6.3-3. The GCMS confirmation of methylated products.
Picture A: GCMS trace showing methylated 4,4-*bis*thiobenzenothiol at retention time 25.08 (Predicted mass = 278). Picture B: GCMS trace showing methylthiophenol at retention time 8.79 (predicted mass = 124.

6.3.2 Development of radiochemical based assay

The radiochemical assay was used to better quantify enzyme activity. This method allowed the determination of methylation of thiol groups in small samples, at optimum pH and was successfully applied to the quantification of product formation. The method, adapted from a glucosyltransferase assay of

Brazier-Hicks (2003) was described in the chapter 2.2.5.7 and was based on the ability of the purified enzyme (TMT1) to transfer the ^{3}H-methyl group of *S*-adenosyl-L-[^{3}H-methyl]-L-methionine (SAM) to thiol acceptors. After partitioning reaction products from the upper (organic) phase were quantified by liquid scintillation counting (Tri-Carb 2900TR). In order to determine the kinetic values associated with *S*-methyl transferases from *A. thaliana* (TMT1), radiochemical assays were conducted using varying concentration of substrate adjusted with a mixture of labelled and unlabelled SAM and thiol acceptor using different buffers (pH 6.5 to 10). As previously described in chapter 6.3.1, TMT1 was shown to methylate thiophenol and 4,4'-thio*bis*-benzenethiol (BBT) therefore, these chemicals were used as model substrates to develop and optimize the radiochemical assay method. The reaction mixtures were incubated for 5-30 minutes to determine the optimum time. All experiments gave a linear rate of product formation with respect to all substrates tested over the 2-30 min incubation tested (data not shown). Assay mixtures with boiled enzyme were used as a control. All enzyme assays were performed in triplicate and then averaged after background and control correction. The pH dependence of the reactions was tested between pH 6.5 to 11 with the greatest product formation determined between pH 7.5 – 8.3. Thereafter assays were performed at pH 7.8. Assays were also performed in the range from 10°C to 36°C and showed a broad optimum centred around 28°C. The results of these experiments are presented in table 6.3-1.

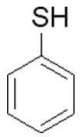
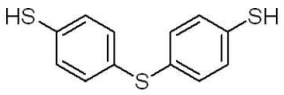
Name	Structure	Mass ion (M^{+1})	Specific activity pkat/mg
Thiophenol		**111**	**0.0775**
4,4'- thiobisbenzenethiol		**250**	**0.1467**

Table 6.3-1. Characteristics of thiophenol and 4,4'-thiobisbenzenethiol and activity determined as substrates of TMT1.

6.3.3 Activity of TMT1 towards a range of free thiols

In order to find out substrate preferences, the activity of TMT1 was determined with the range of free thiols presented in figure 6.3-5. Three of the substrates listed required chemical reduction prior to enzymatic reactions by using Tris (2-carboxyethyl)phosphine hydrochloride (TCEP). TCEP is an odourless, non-volatile agent, which reduces organic disulfides to thiols rapidly and quantitatively in water at room temperature. The compounds that required TCEP treatment are presented in table 6.3-2 with the associated reduction reaction shown in figure 6.3-4 (Ruegg and Rudinger, 1977). The radiochemical assay was then used for the determination of methylation of the respective thiol groups, with the results presented in figure 6.3-7. Data is presented as the relative activity determined in comparison to the 100 % relative activity obtained with thiophenol.

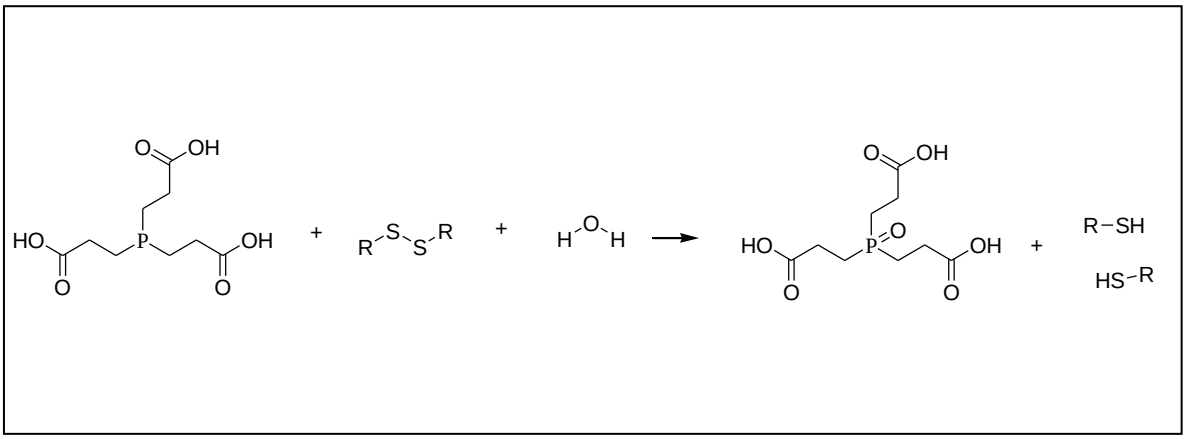


Figure 6.3-4. Reduction of organic disulphide bonds using TCEP.

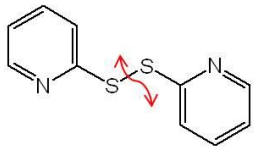
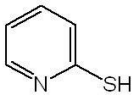
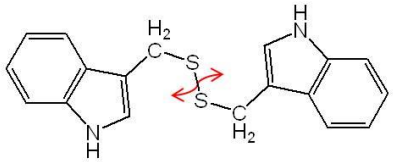
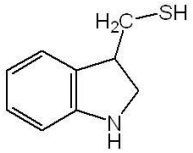
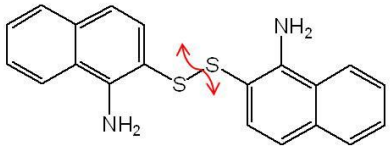
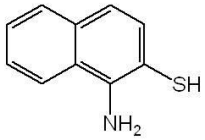
Starting compound	Substrate after TCEP reduction	Name of the substrate
		2,2-Dithiopyridine
		(1H-indol-3-yl)methanethiol
		2,2 Dithio -Di (1-napthyl amine)

Table 6.3-2. TCEP reduction of starting organic disulphide compounds.
Red arrow shows the reduction of disulphide bond in presence of the TCEP.

The activity of TMT1 was determined toward selected compounds in triplicate over a time course of 5, 10 and 15 min. It was found that a linear rate of product formation was achieved over this period.

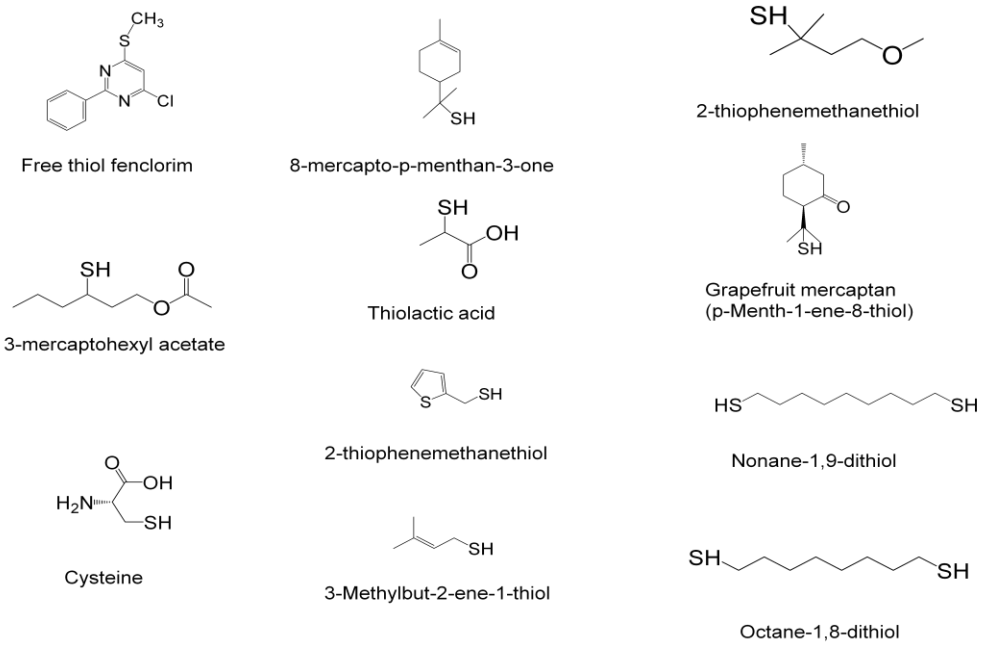


Figure 6.3-5. The list of free thiols tested by radiochemical assay.

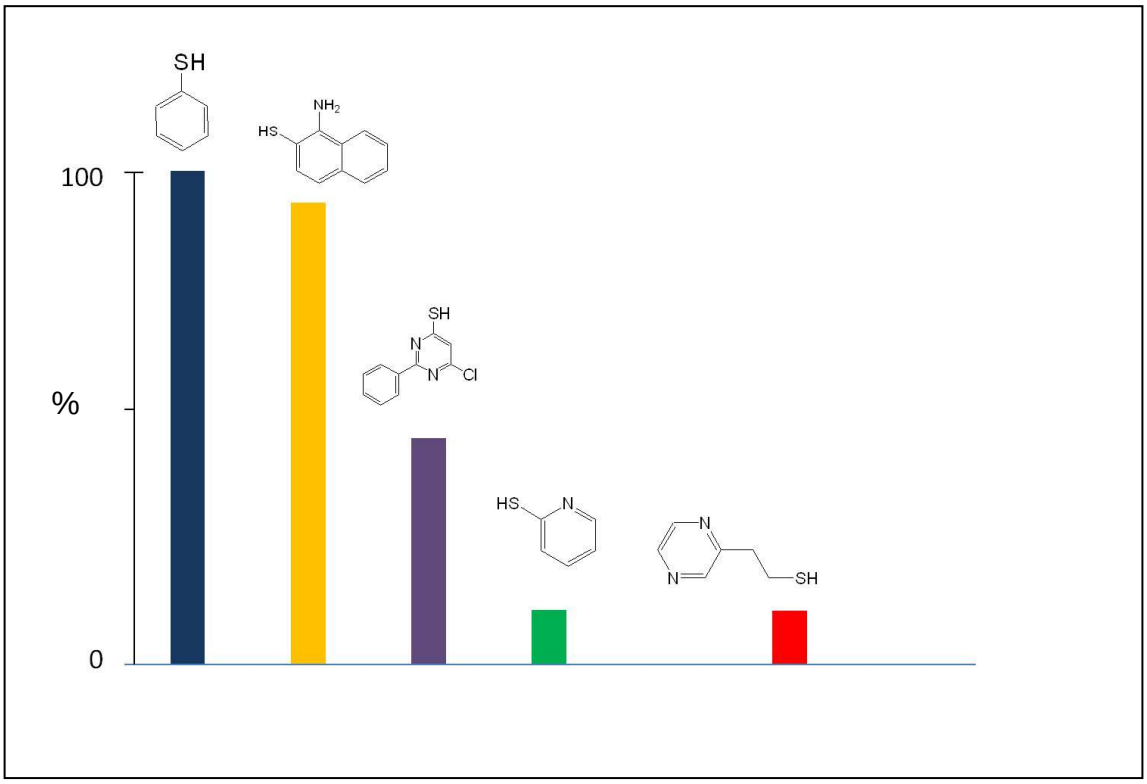


Figure 6.3-6. Activity of TMT1 towards selected compounds confirmed by radiochemical assay. 100 % = 0.079 pkats/mg pure recombinant protein. No activity was determined with the other thiols tested.

From the results, it can be seen that TMT1 showed a preference for aromatic, rather than aliphatic forms of free thiol compounds. Direct analysis of the enzymatic products was then attempted. Several difficulties were encountered, including the unstable nature of the free thiol substrates, disulphide formation, solubility issues and cost of SAM- the donor of methyl group, limiting the scale of reactions. Initial HPLC analysis of the free thiol profiles was carried out using UV absorbance (280nm) for detection, although this method was found to be unsatisfactory as only 3-mercapto-2-pentanone (figure 6.3-8) was easily detected. LCMS analysis of the products was found to be ineffective due to the lack of ionization of the majority of the methylated products. An alternative, GCMS method was also not successful. Low recoveries of products was thought to be a potential reason for the lack of detection. As a consequence only the methylation of furfuryl mercaptan could be confirmed, as presented in figure 6.3-9. The interest in the activity of TMT1 protein toward thiol acceptors was then extended to the intermediates formed in metabolism of compounds called herbicide safeners, which are agrochemicals which increase herbicide tolerance in plant (Brazier-Hicks *et al*., 2008). Activity of TMT1 toward the metabolite formed from the safener fenclorim 4-chloro-6-(thio)-2-phenylpyrimidine (FT) was tested by radiochemical assay using *S*-adenosyl-L-[^{3}H-methyl]-L-methionine using FT as substrate. Significant activity of 0.156 pkat/mg was determined. Product methylation was confirmed by LCMS with the presence of a m/z =237^{+1} mass ion, corresponding to the methylated FT-derivative, thiol 4-chloro-6-(methylthio)-2-phenylpyrimidine (CMTP) as shown in figure 6.3-7.

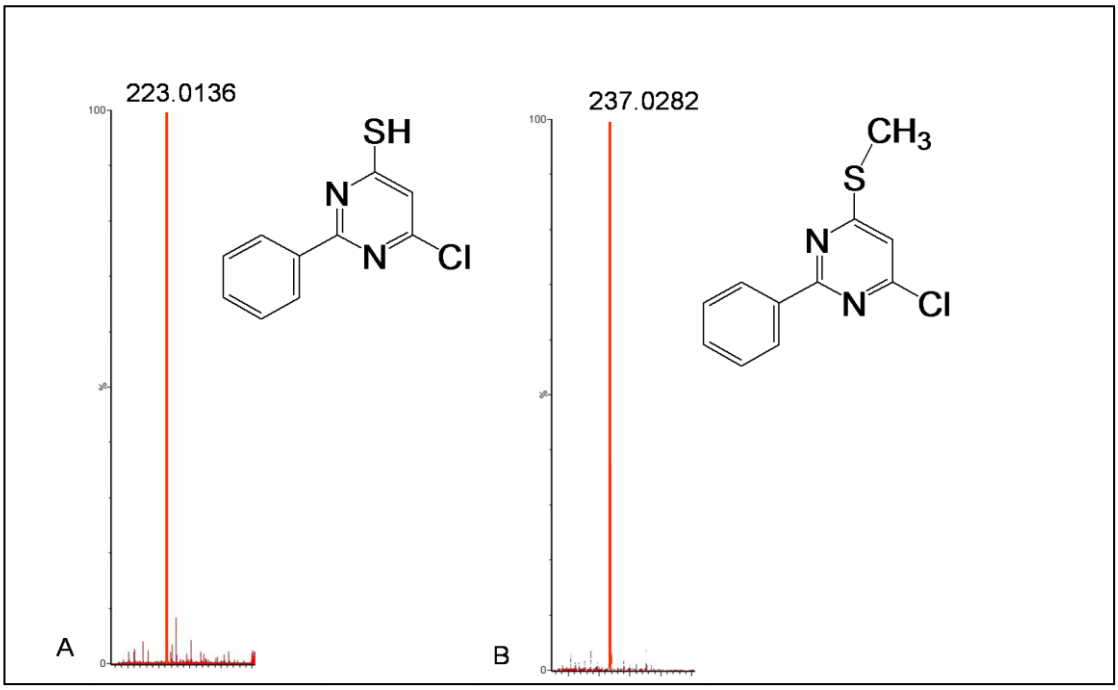


Figure 6.3-7. LCMS trace confirming methylation of the thiol derivative of fenclorim in the presence of TMT1 and *S*-adenosyl methionine as observed with ES$^+$ mass spectrometry.

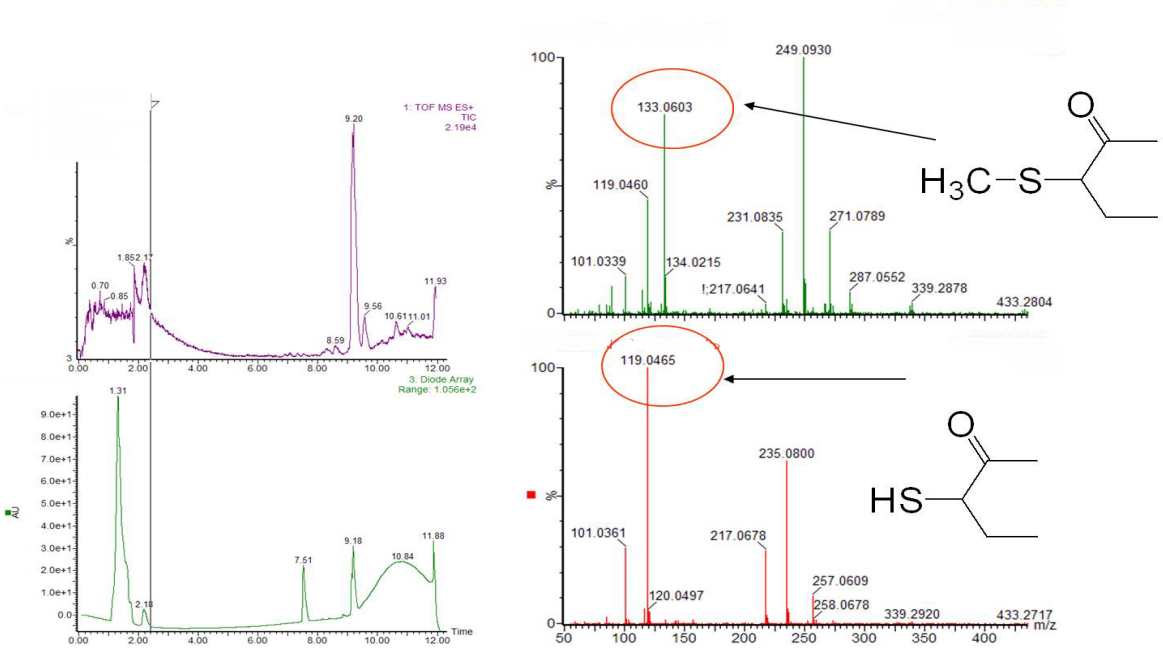


Figure 6.3-8. LCMS chromatogram showing substrate, 3-mercapto 2-pentanone and methylated product.

The implication of this latest finding could be that TMT1 might be involved in methylation of glutathionine conjugate intermediates as previously suggested in section 1.6. However, it is hard to predict the role of this enzyme in fenclorim metabolism based on *in vitro* activity alone.

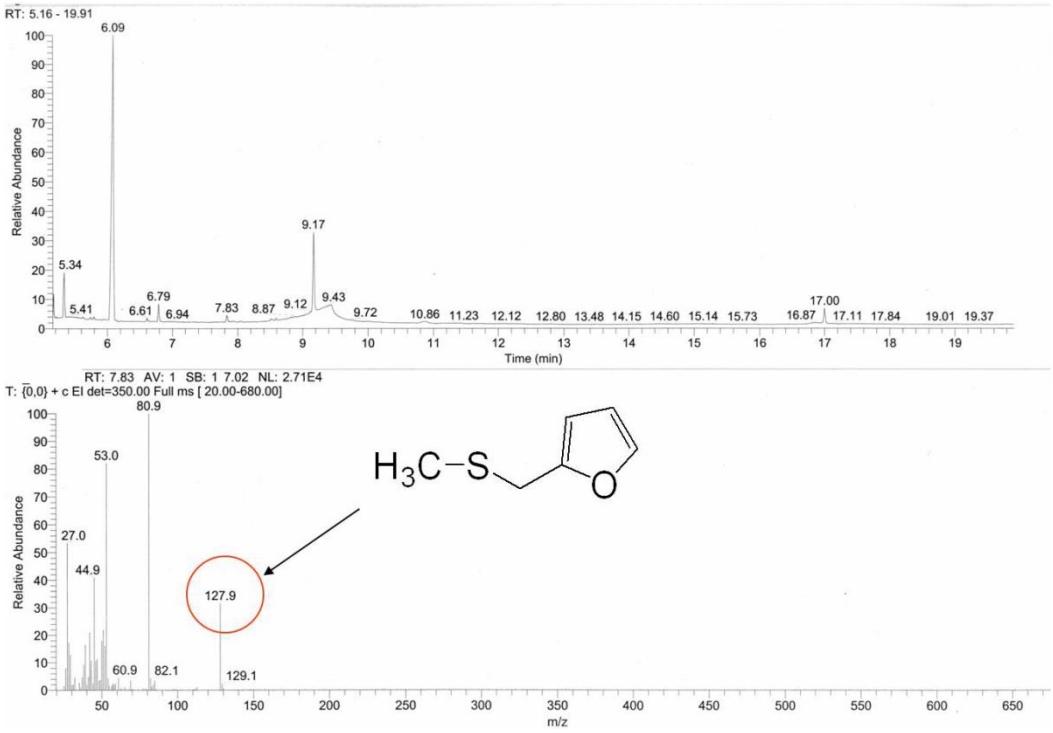


Figure 6.3-9. GCMS chromatogram showing substrate, furfuryl mercaptan and methylated product.

6.4 Partial purification of *S*-methyl transferase from *A. thaliana*

To further investigate the enzyme responsible for methylating FT the *S*-methyl transferase involved in the methylation of the fenclorim metabolite was carried out. In addition, the investigation also studied the relationship between methylating activities toward naturally occurring and synthetic phenols in this species. The aim was to test the activity of crude plant extract from *A. thaliana* towards FT substrate. The crude enzyme extract was isolated from *A. thaliana* cell cultures and subjected to ammonium sulphate precipitation as described in

section 2.2.4.5. Partial purification of fractions was then performed according to the method described in section 2.2.3.5 using a Mono Q column. Each fraction was tested for methyltransferase activity by radiochemical assay as described in section 2.2.5.7 and submitted to LCMS analysis. The formation of methylated product was successfully confirmed in 2 fractions as indicated in figure 6.4-1.

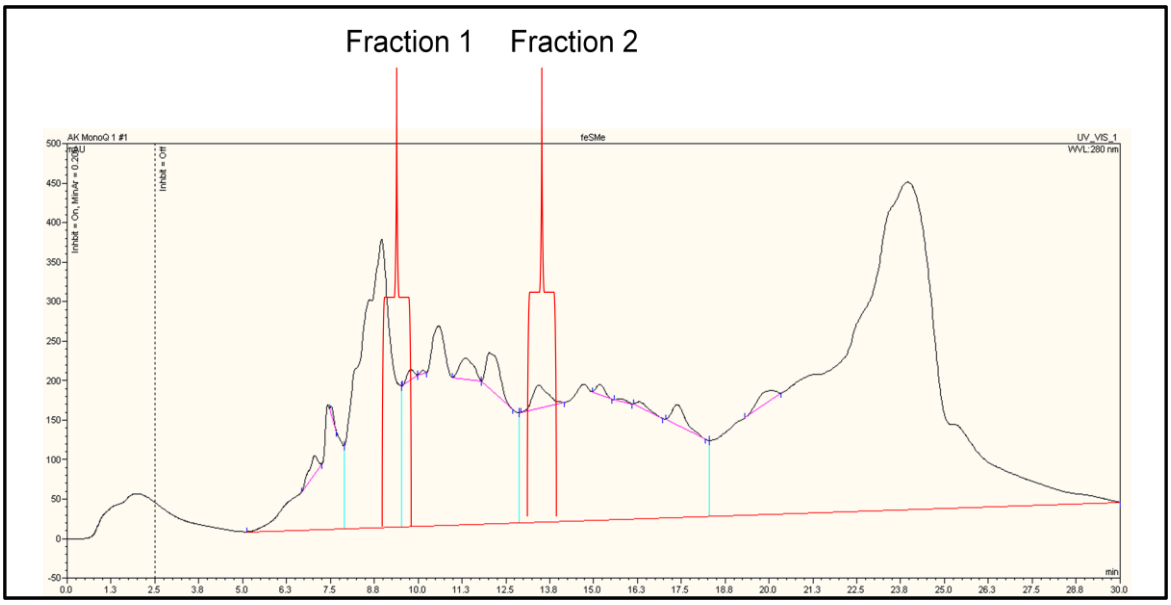


Figure 6.4-1. Partial purification of a protein fraction containing *S*-methyltransferase activity. Results are shown using a bar graph with product formation presented as radioactivity disintegrations per minute.

6.5 Methylation of VOSCs and fenclorim

In an attempt to identify the associated changes in gene expression and potential metabolic pathways underpinning the different responses in *A. thaliana* plants was tested by qPCR method according to protocol described in section 2.2.4.10. The studies with fenclorim had indicated that the SMT activity was up-regulated by the safener so it was therefore of interest to determine if TMT1 was similarly induced. The housekeeping genes of *gapdh (At1g13440), gstu19 (At1g78380)* and *ubc (At5g25760)* were used as a positive control and were successfully up –

regulated. The results obtained suggest that the genes encoding the three known SMTs: TMT1, TMT2,and TMT3 are most likely not directly involved in the xenobiotics pathway, though the enzyme activity showing methylation of fenclorim could be measured in crude extracts as presented in section 6.4. None of the tested genes showed induction after incubation with fenclorim or thiomenthone. All qPCR reaction products were verified on the agarose gel as presented in figure 6.5-2.

	At2g43910	At2g43920	At2g43940
Con 0hr	1.00	1.00	1.00
F1 0h	0.81	0.72	0.71
M1 0h	0.89	0.43	1.11
Con 8hr	0.89	0.14	0.73
F1 8h	1.21	0.97	1.35
M1 8h	1.16	0.70	0.80

Figure 6.5-1. The qPCR results of induction of transcripts by fenclorim (F1) and thiomenthone (M1) in A. thaliana crude extracts.

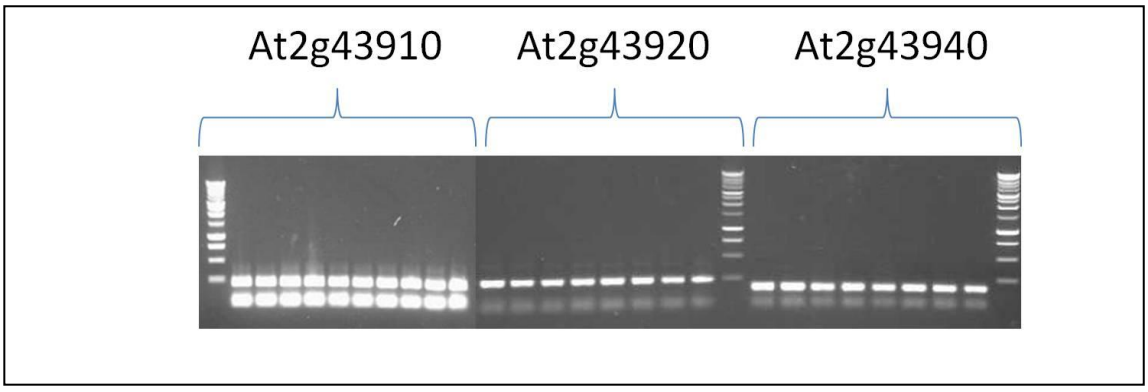


Figure 6.5-2. Verification of the qPCR product of on 1.2 % agarose gel. 1 Kbp DNA marker ladder (Bio-Rad was used.

6.6 Feeding studies

Development of a route for production of *S*-methylated compounds by reconstitution of the respective pathways in recombinant bacteria as an alternative production of methylated free thiols was investigated *in vivo* according to method described in section 2.2.6.1. The growth medium containing *E. coli* expressing plant TMT1 culture was supplemented with thiophenol (0.1 mM) to test the possible toxicity of compound. Interestingly, the *E. coli* host with overexpressed methylation enzyme TMT1 shown greater acceptance of toxic thiophenol as shown in figure 6.6.1, resulting in a final optical density of 0.45 in comparison to the *E. coli* control 0.32. The concentration of thiophenol at 0.1mM was found to be the highest optimal concentration between 10 mM-100 mM was lethal. In order to explore possibility of the natural methylation other potential substrate like: thiophenol, furfuryl mercaptan, 3-mercaptohexan-1-ol, 3-mercapto-2-pentanone, 2-methyl-3-furanthiol or thiomenthonewere tested. Prior to feeding studies on a medium scale, *E. coli* cultures expressing plant TMT1 were grown to OD 0.6 and supplemented with either thiophenol, furfuryl mercaptan, 3-mercaptohexan-1-ol, 3-mercapto-2-pentanone, 2-methyl-3-furanthiol or thiomenthone at final concentration of 0.1 mM as described in section 2.2.6.2. Further characterisation of the metabolites was carried out by GC-MS. Unfortunately, none of the other tested methylated compounds could be detected. Only methyl thiophenol was identified by GC-MS as described in section 2.2.6.4. The methylated forms of tested Frutarom compound were not found in the cells or media after extraction from bacterial culture (data not shown) possibly due to low insolubility of Frutarom compounds. Furthermore, it was found that, methylation *in vivo* in the presence of the methyl donor SAM from

bacterial hosts could not lead to successful, clearly detectable methylation of the compounds tested. It was not clear if this was due to problems with extraction of these highly volatile compounds.

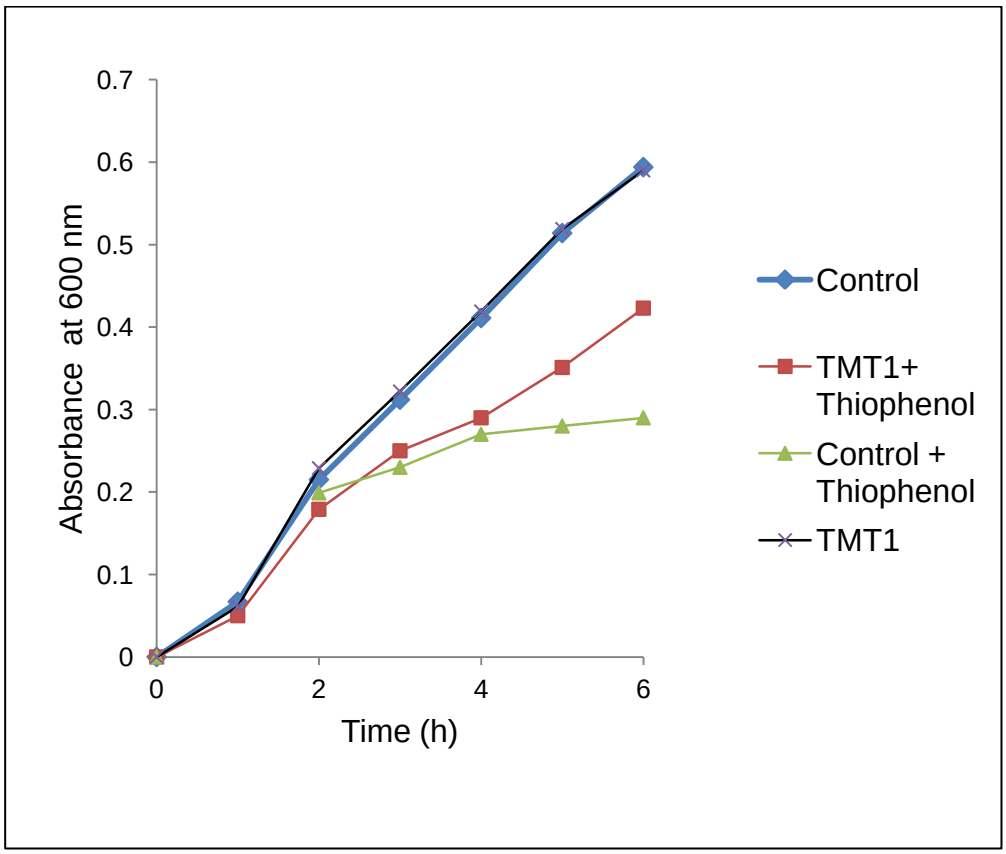


Figure 6.6-1. Growth studies of growth of *E. coli* measured as changes in optical density over time. Blue line -control showing growth of *E. coli*; green line - *E. coli* with thiophenol (0.1mM); black line - growth of *E. coli* expressing *S*-methyl transferase TMT1 from *A. thaliana*; red line -growth of *E. coli* with thiophenol (0.1M).

Each culture was then analyzed for the presence of the respective methylated products. Only thiophenol was shown to be converted to its thiomethyl derivative as presented in figure 6.6-2.

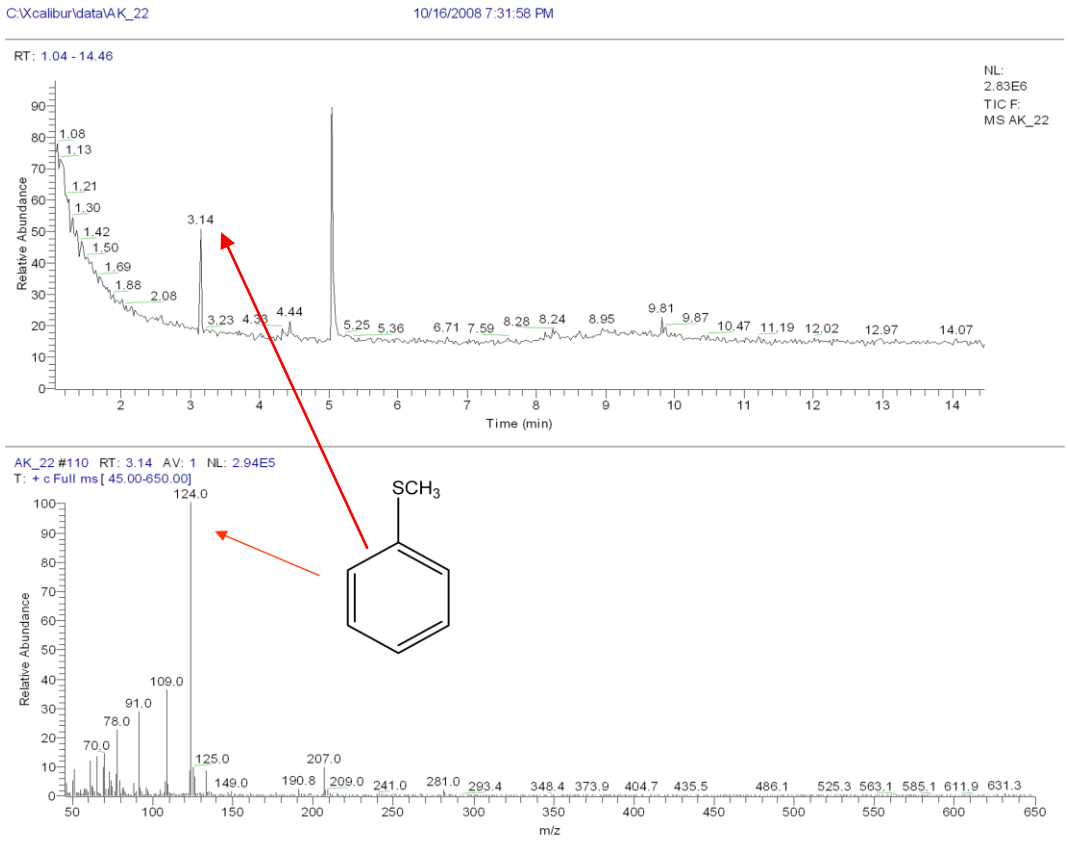


Figure 6.6-2. The GCMS spectra confirming methylation of thiophenol.

6.7 Crystallization of TMT1 an *S*-methyl transferase from *A. thaliana*.

The TMT1 construct described in chapter 6.2 was submitted to the University of St Andrews, Department of Chemistry for crystallisation studies. The construct in pET24a was over-expressed in Rosetta II (DE3) cells with a C-terminal 6-His tag. Initial assays were conducted to confirm enzymatic activity towards a broader range of substrates including Cl^-, Br^-, SCN^-. As a result of this collaboration the first crystal structure of a member of the halomethyl transferases has been revealed. The figure 6.7-1 shows a crystal structure, coloured in a spectrum from N-terminus (blue) to C-terminus (red) (Schmidberger *et al*., 2010).

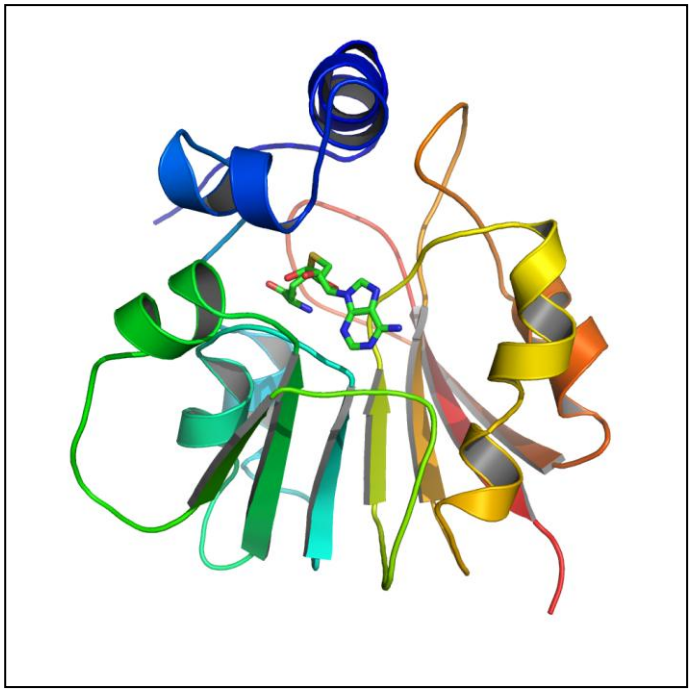


Figure 6.7-1. The crystal structure of *S*-methyl transferase of the *Arabidopsis thaliana* halomethyl transferase (AtHTMT1) with SAH *S*-adenosyl-homocysteine bound to the active site of the enzyme (Schmidberger *et al*,. 2010).

As presented in the publication, the AtHTMT1 enzyme catalyses the reaction most efficiently with thiocyanate and then the halides with an efficiency order of $NCS^- > I^- > Br^- > Cl^-$. More detailed studies of the active site pocket revealed the importance of putative nucleophile binding site as presented in the figure 6.7-2. The recent crystallization of first member of SMT has provided a unique structural basis for architecture, selectivity and catalytic mechanism and confirmed the strict size limitation of the active site pocket. In this case, TMT1 seems to possess very broad substrate specificities, therefore our studies do not permit conclusions about its natural metabolic role *in vivo*.

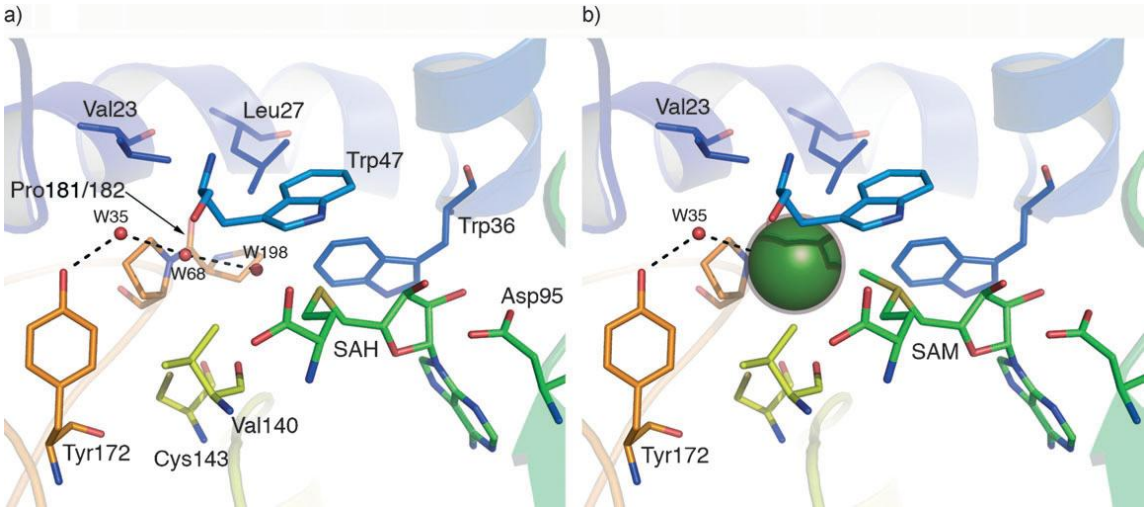


Figure 6.7-2. The active site of *A. thaliana* halomethyl transferase (AtHTMT1).
Picture a) showing the amino acid side groups forming the putative nucleophile binding site with three water molecules (W35, W68, and W198) occupy a channel from the bulk solvent to a proposed nucleophilic binding site occupied by W198.
b) The missing methyl group of (*S*)-SAM is modelled into the structure with space-filling chloride (green sphere, radius) simulate preorganization for reaction (Schmidberger *et al*,. 2010).

6.8 Conclusion

In summary, progress has been made toward identifying plant enzymes capable of methylation of VOSCs. However, additional work is necessary for the unequivocal identification of the methylated F&F compounds formed in this project and a more complete description of the TMT1 activity towards a broader range of chemicals is needed. The role of TMT1 and related enzymes is intriguing and there remains much to learn regarding the relationship between halomethane biosynthesis and thiocyanate/methyl thiocyanate metabolism in plants. The current study has shown the very interesting broader activities of TMT1 toward thiol acceptors and it will be of value to model these substrates into the structural model of TMT1. Despite determining the methylating activity of TMT1 towards some of the F&F compounds *in vitro*, the biotransformations investigated in this chapter could not yield sufficient conversion of free thiols. A

clear limitation of the initial activity studies presented in this chapter is that the small scale of reaction due to the highly expensive methylation group donor *S*-adenosyl-L-[^{3}H-methyl]-L-methionine and *S*-adenosyl-L-methionine. Also more suitable and accurate equipment such a headspace GS-MC which was not available at Durham University, would be recommended for the highly volatile compound detection at low concentrations. Having identified eight different substrates for TMT1, tentative conclusions concerning the broad range of activity were reached. In conclusion, the combined approach using structural and functional studies to understand plant methylation serves as a necessary foundation for the continued discovery of novel SMTs, such as TMT1, that serves an as yet uncharacterised role in plant metabolism.

7 Construction of a pathway to methylate VOSCs in bacteria using a C-S lyase and *S*-methyl transferase

7.1 Introduction

Recent advances in exploring natural product biosynthetic pathways as a route for commercial biotransformation offers a new route to natural F&F production (Berger, 2009). Many natural products of biochemical interest are produced by organisms using dedicated, biosynthetic pathways. Development of a route for production of *S*-methylated flavour compounds by reconstitution of the respective pathways in recombinant bacteria, would be an important accomplishment in applied biotechnology and would provide a proof of concept of linking a two-step enzymatic process. In this chapter, application of biocatalysis for a unique production of methylated free thiols was explored, using the previously tested C-S lyases (CBL, MetC, and TRP) and sulphur methyl transferase (TMT1) combination. The proposed linked reaction catalyzed by two different types of enzyme: lyase and methyl transferase, where cleavage of the carbon-sulphur bond of the starting substrate is followed by transfer of methyl group from *S*-adenosyl methionine (SAM) to free the thiol of the resulting intermediate. This approach was tested *in vitro*, using enzymes that had been previously overexpressed and shown to be active. The possibility of linked bioproduction *in vivo*, in *E. coli* was then investigated with individual enzyme activities monitored by radiochemical and continuous assay. The final system was then tested for activity using GC.

7.2 Single-enzyme activity studies

In the proposed 2-step pathway, an intermediate, obtained after the action of a C-S lyase on a cysteine conjugate would act as a substrate for the second enzyme (*S*-methyl transferase TMT1). In order to establish this 2-step enzymatic reaction, it was first necessary to show each enzyme was functional. It was proposed that, *S*-phenyl-L-cysteine (SPC), could serve as a model substrate for such a two-step reaction with a C-S lyase cleaving the sulphur-carbon bond of SPC, resulting in production of thiophenol, which our studies had shown to be methylated by enzyme (TMT1) in the presence of methyl donor SAM giving the final substrate, methyl phenyl sulphide (MPS). The reaction scheme is shown in figure 7.2-1.

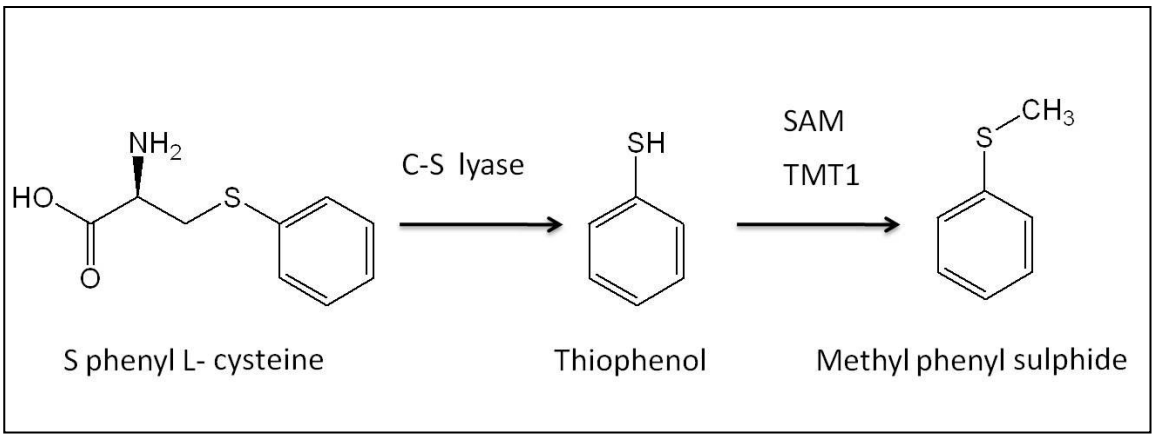


Figure 7.2-1. The proposed scheme of the reconstruction of the C-S lyase / *S*-methyltransferase pathway.

Levels of activity of beta lyase activity were determined by Ellman's assay as previously described in chapter (2.2.5.3). All reactions were carried out in sealed vessels to contain any volatile thiophenol formed and the results are presented in figure 7.2 2. Comparison of beta lyase activity enzymes towards substrate *S*-phenyl-L-cysteine revealed, that cystathionine beta lyase from *E.*

coli (MetC) showed the highest specific activity of (32 nkat/mg) in comparison to the less active TRP, (7.3 nkat/mg) and CBL (1.2 nkat/mg) respectively. The *S*-phenyl-L-cysteine (SPC), used as substrate was highly insoluble in phosphate buffer pH 7.8 buffer; therefore it was dissolved in phosphate buffer pH 3.2 and then the pH was adjusted up to 7.8 prior to enzymatic reaction.

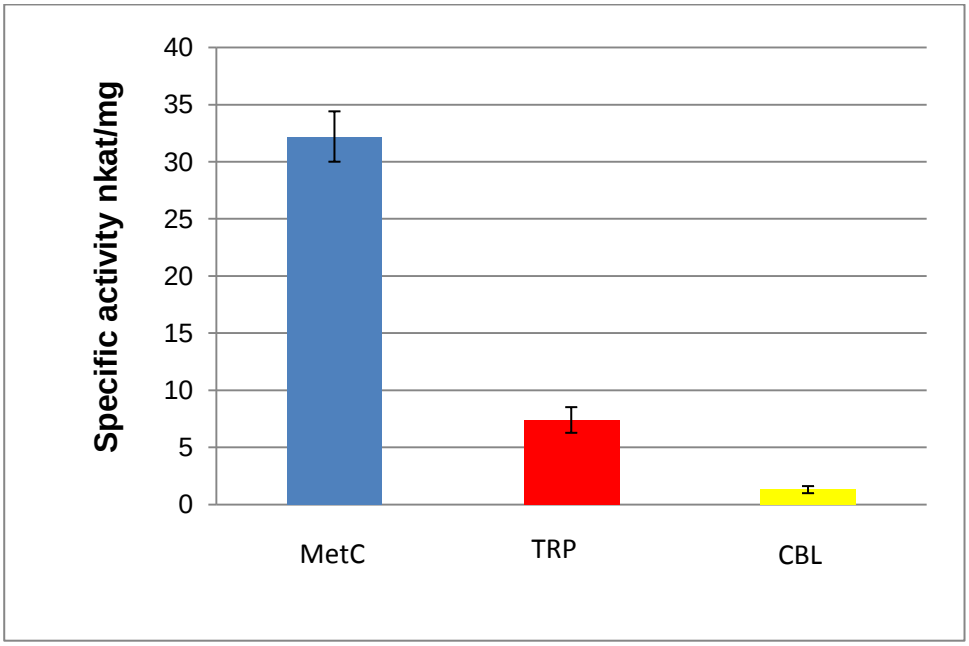


Figure 7.2-2. The comparison of beta lyase specific activity of (TRP, MetC, CBL) towards *S*-phenyl L-cysteine.
(TRP, Tryptophanase beta lyase from *E .coli*; MetC, Cystathionine beta lyase from *E. coli*; CBL, Cystathionine beta lyase from *A. thaliana*).

In order to examine whether previously purified and tested enzymes can conduct two step reaction simultaneously, C-S lyases (CBL, MetC, TRP) and TMT1 were tested in combination *in vitro* for their ability to convert *S*-phenyl-L-cysteine to methyl phenyl sulphide (MPS). Enzymatic activity was measured *via* radiochemical assay as described in section 2.2.5.7. The reaction mixture contained one of the previously purified C-S lyases and TMT1 in the presence

of *S*-phenyl-L-cysteine and *S*-adenosyl-L-[^{3}H-methyl]-L-methionine. All assays were conducted in triplicate and counts were background corrected. The results of the coupled reaction of MetC and TMT1 are presented in figure 7.2-3 and revealed that all coupled enzymes could convert some of the substrate SPC into methyl phenyl sulphide (MPS), with the optimal incubation using MetC and TMT1.

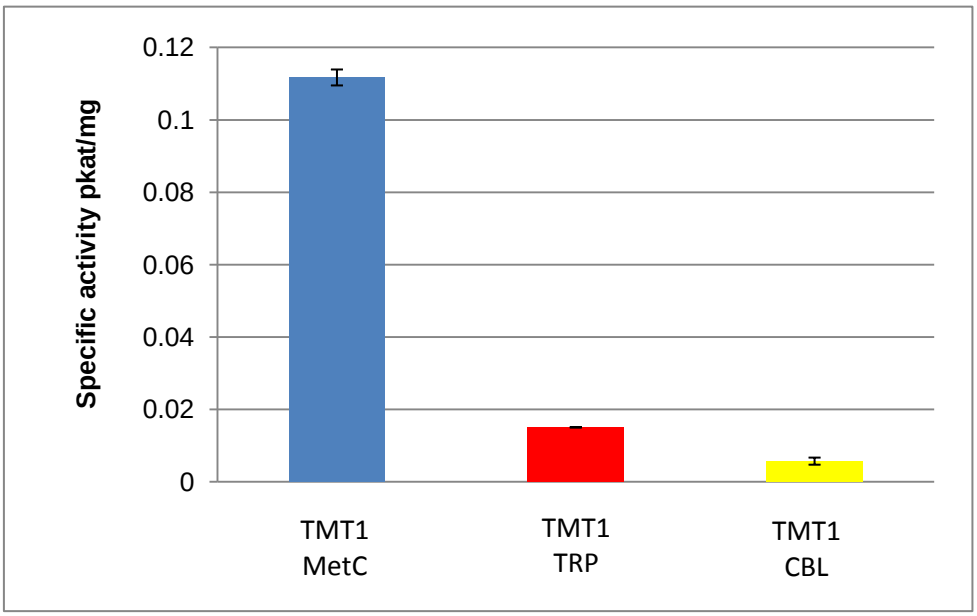
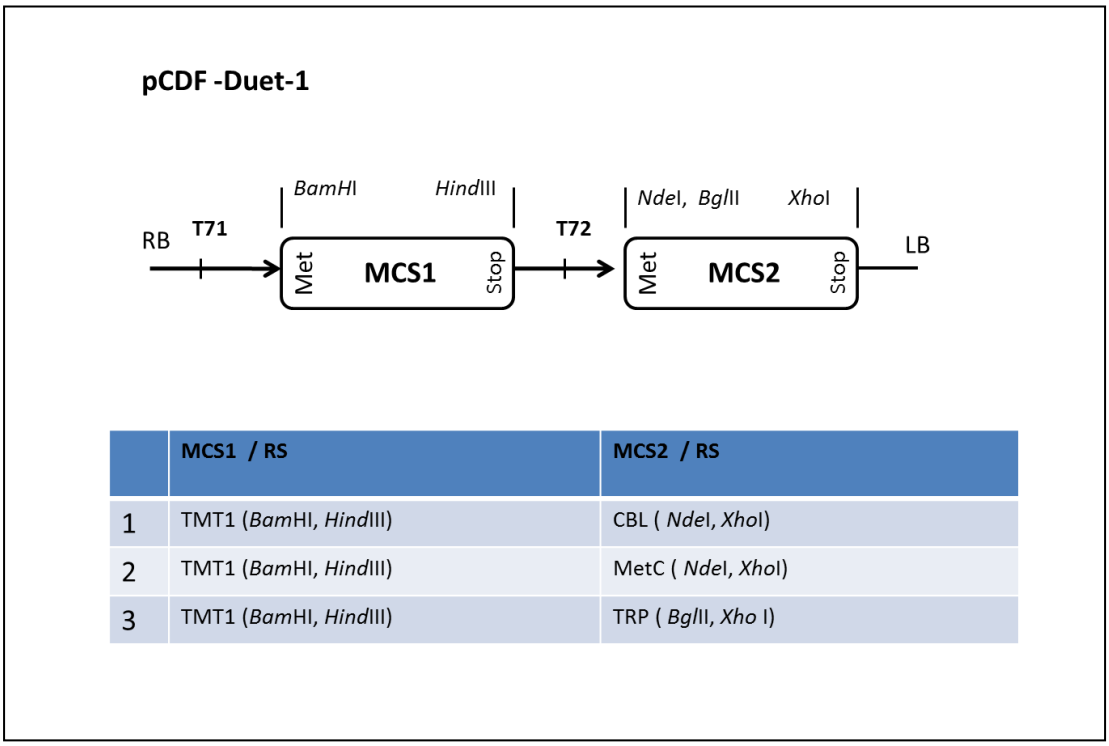


Figure 7.2-3 Comparison of linked enzymatic activity towards *S*-phenyl L-cysteine measured by radiochemical assay.
(TMT1 thiol methyl transferase from *A. thaliana*; MetC, Cystathionine beta lyase from *E.coli*; TRP, Tryptophanase beta lyase from *E.coli*; CBL, Cystathionine beta lyase from *A. thaliana*).

A series of control reactions was performed in the absence of either, methyl donor, C-S lyase or substrate respectively to confirm the specificity of double step conversion. Since all coupled enzymatic reactions were performed successfully, conversion of SPC *in vivo* was attempted.

7.3 Co-expression of enzymes

Development of a route for production of S-methylated compounds by reconstruction of the respective C-S lyase/S-methyltransferase pathway in recombinant bacteria was undertaken using a combination of microbial/plant enzymes. In order to identify co-expressed activity, three different constructs were designed namely: TMT1/CBL, TMT1/MetC and TMT1/TRP respectively. In order to undertake the coexpression of two enzymes, the CDF Duet- 1 vector was chosen. This unique vector was designed for co-expressing multi-protein complexes directly in *E. coli* and possessed streptomycin or spectinomycin resistance, two T7*lac* promoters, two multicloning sites (MCS) regions, and a single T7 terminator. The CDF Duet- 1 vector was already available in-house courtesy of Dr. M. Cann. The plasmid was purified as described in section 2.2.1.9. The general design is presented in figure 7.3-1. Firstly, DNA inserts of TMT1, CBL, TRP, and MetC were amplified by using sets of primers listed in a section 9.4. with a stop codon added to each protein. Cloning was performed by using standard techniques presented in section 2.2.1. The CDF Duet- 1 vector was digested with *Bam*HI, *Hind*III and ligated with TMT1 using T4 DNA ligase. The construct was then purified and its sequence confirmed prior to use. The TMT1 open reading frame construct inserted into first multiple cloning sites (MCS1) was sequenced using the ACYCDuetUP1 Primer (Cat. No. 71178-3) and DuetDOWN1 Primer (Cat. No. 71179-3) as listed in section 9.3.



	MCS1 / RS	MCS2 / RS
1	TMT1 (*Bam*HI, *Hind*III)	CBL (*Nde*I, *Xho*I)
2	TMT1 (*Bam*HI, *Hind*III)	MetC (*Nde*I, *Xho*I)
3	TMT1 (*Bam*HI, *Hind*III)	TRP (*Bgl*II, *Xho* I)

Figure 7.3-1. The co-expression vector for two enzymes: *S*-methyl transferase and cystathionine beta lyase using CDF-Duet-1.
(TMT1, thiol methyl transferase from *A. thaliana*; MetC, Cystathionine beta lyase from *E. coli*; TRP, Tryptophanase beta lyase from *E. coli*; CBL, Cystathionine beta lyase from *A. thaliana*; RB, right border; LB, left border; MCS, multicloning site; RS, restriction site.

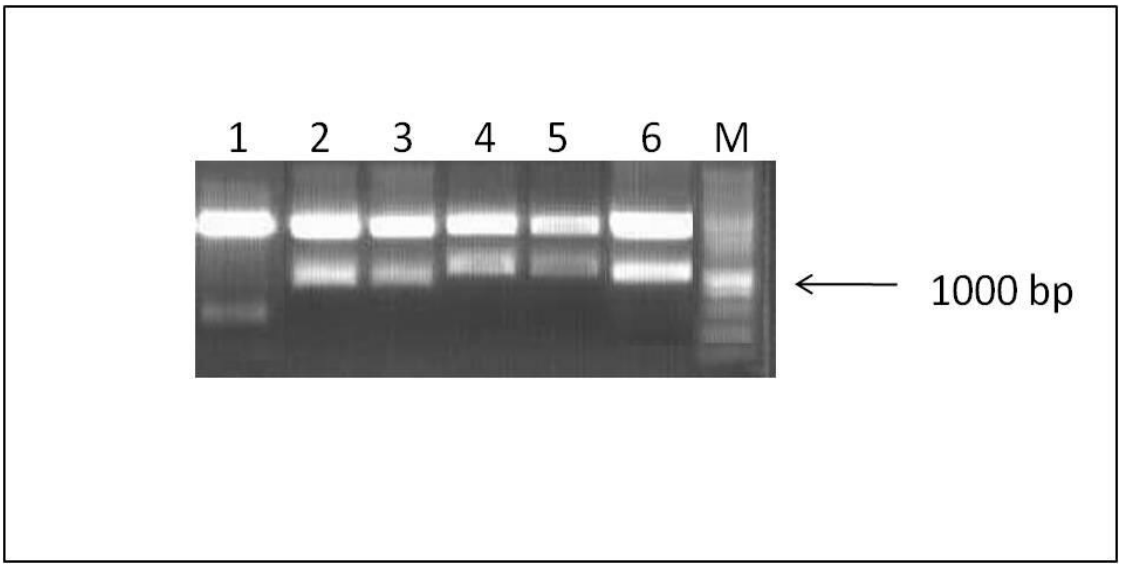


Figure 7.3-2. A restriction digest of coexpression vector pCDF-Duet-1.
Lane 1: construct pCDF-Duet-1-TMT1 digested with *Bam*I and *Hind*III shows TMT1 insert ~ 630bp, Lane 2,3: construct pCDF-Duet-1-TMT1/CBL after digestion with *Nde*I and *Xho*I; Lane 4,5: pCDF-Duet-1-TMT1/TRP after digestion with *Bgl*II and *Xho*I; Lane 6: construct TMT1/MetC; Lane M: Promega 1kb marker.

In the next step, the pCDF-Duet-1-TMT1 construct was digested with *Nde*I and *Xho*I for MetC and CBL. TRP construct was digested with *Bgl*II, and *Xho*I as presented in figure 7.3-1. Each C-S lyase enzyme was then inserted into MCS2 as described in section 2.2.1. Vectors from selected colonies were digested with appropriate restriction enzymes to confirm that the construct was correct and were sequenced to ensure the fidelity of the amplified and cloned DNA fragments. All correct were then were expressed in Rosetta-*gami*^{TM} 2 *E. coli* competent cells.

7.4 Optimisation of construct over-expression

In order to find out the levels of expression of coexpressed enzymes, soluble fractions of *E. coli* bacterial lysate were subjected to SDS-PAGE analysis. As presented in figure7.4-1, both the pCDF-Duet-1-TMT1/TRP construct and pCDF-Duet-1-TMT1/MetC had clearly detectable protein bands. However, no major polypeptide corresponding to the recombinant CBL was apparent in pCDF-Duet-1-TMT1/CBL construct, suggesting that the CBL protein was either poorly expressed or readily degraded. Optimised media (described in section 2.1.1.2) incubated at 28ºC which has shown to improve protein overexpression of MetC and TRP, surprisingly did not improve the production of TMT1. Activity of all three constructs was tested by assaying bacterial lysate in *vitro* using the radiochemical assay as described in section 2.2.5.7, by using 100 µg of crude protein obtained by sonication with the concentration of protein measured using the Bradford assay (2.2.3.9).

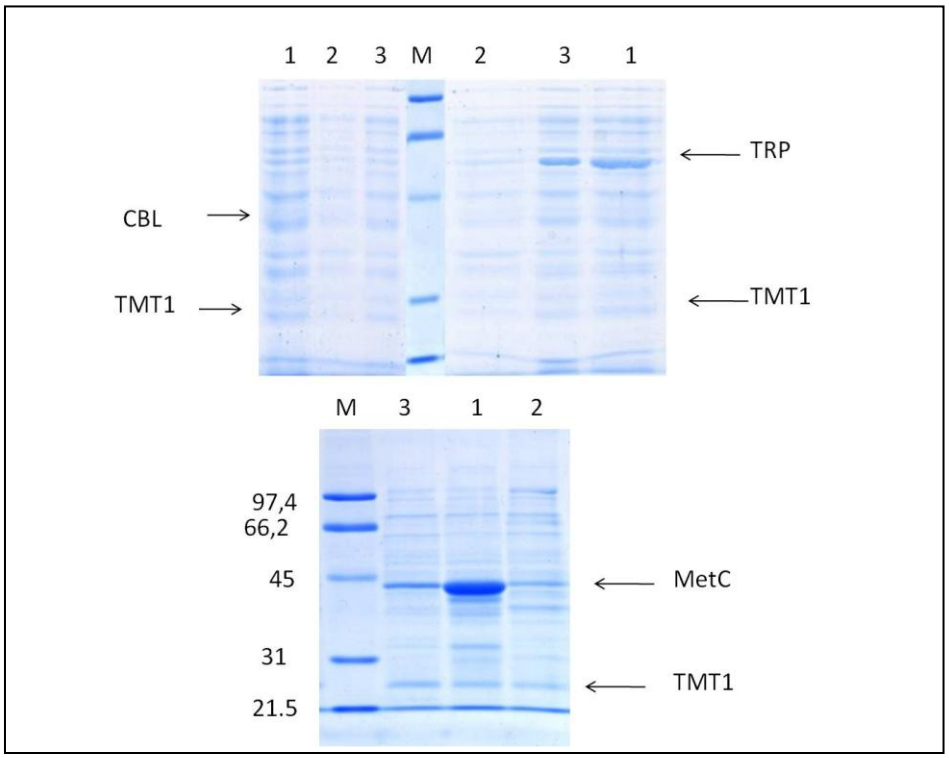


Figure 7.4-1. SDS-PAGE gel stained with Coomassie Blue showing the overexpressed proteins. Lane 1. control; lane 1, optimized medium at 37°C; lane 2, LB medium at 37°C ; lane 3, optimized medium at 28°C; lane M, molecular-weight markers (kDa).

7.5 Comparative study of coupled enzyme constructs

In order to estimate the activity of transformed *E.coli*, two separate experiments were performed. Firstly, the coupled enzymatic activity of TMT1/CBL, TMT1/MetC and TMT1/TRP constructs were tested *in vitro* by radiochemical assay as described in section 2.2.5.7. All constructs tested showed activity towards *S*-phenyl-L-cysteine (SPM) as presented in figure 7.5-1. The highest activity of 0.043 pkat/mg was achieved by the MetC/TMT1 construct. Furthermore, activity was tested in feeding studies performed according to method described in section 2.2.6.2.

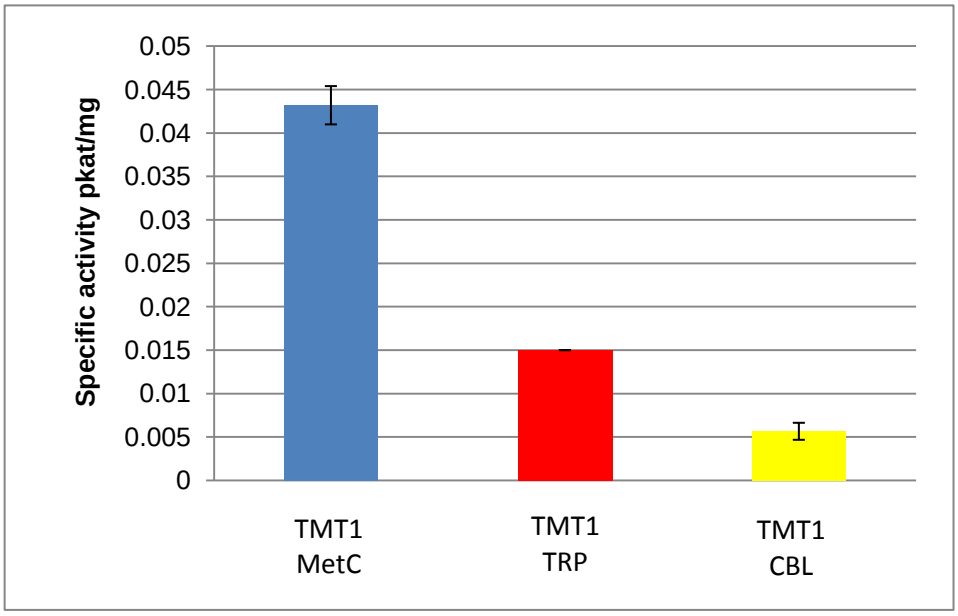


Figure 7.5-1. The results of radiochemical assays performed with coexpressed enzymes. (TMT1, thiol methyl transferase from *A. thaliana*; MetC, Cystathionine beta lyase from *E. coli*; TRP, Tryptophanase beta lyase from *E. coli*; CBL, Cystathionine beta lyase from *A. thaliana*.

The *E. coli* colonies expressing coupled enzyme constructs were supplemented with S-phenyl-L-cysteine up to 0.5 mM concentration and grown overnight in LB medium. The characterisation of the metabolites was carried out after extraction in dichloromethane (DCM) by GC-MS. Unfortunately, the results of the GC-MS analysis were poor and could not been use as a successful determination of product being formed (data not shown). In order to establish accurate and sensitive detection method, further studies were conducted.

7.6 Detection and quantification of volatile products

Due to the equipment limitation at Durham University, it was impossible to use headspace GS-MC analysis, which would be the most suitable for highly volatile compounds detection at small concentration. Therefore, in order to detect and quantify thiophenol and methyl phenyl sulphide (MPS) on a small scale, two available GC methods were developed, using either solvent extraction into ethyl

acetate or dichloromethane (DCM); a solid - phase micro extraction (SPME). The SPME is known as a very sensitive method for solvent free extraction and is regularly used in the flavour and fragrance industry (Cerasek and Pawliszyn, 2006). This SPME technique developed by Supelco, is described as an accurate, inexpensive and a clean method to detect the volatile products from head space (Martendal *et al*., 2007). Although, several successful studies of many volatile compounds have been reported, the analysis of thiophenol and MPS had not been described in the literature.

7.6.1.1 Gas Chromatography using solvent extraction

In order to choose the most suitable organic solvent for gas chromatography, two different solvents were tested to recover the products namely: dichloromethane (DCM) and ethyl acetate. Both solvents were tested to obtain the most efficient extraction and results are shown in 7.6-1. In contrast to ethyl acetate, the DCM extraction results were reliable and efficient. Both standards were run through the GC at varying concentrations (1, 10 and 100 µM) for calibration, illustrating the direct correlation between the GC peak of SPM, MPS and the amount of product formed with retention times of 11.3 and 13.62 minutes respectively as presented in figure 7.6-2. To further confirm the linked enzyme activity, large scale assays with previously purified MetC and TMT1 was performed *in vivo* in the presence of *S*-adenosyl-L-methionine (10 mM). The product of the reaction was then partitioned in DCM and run on the GC according to the method described in section 2.2.6.3.

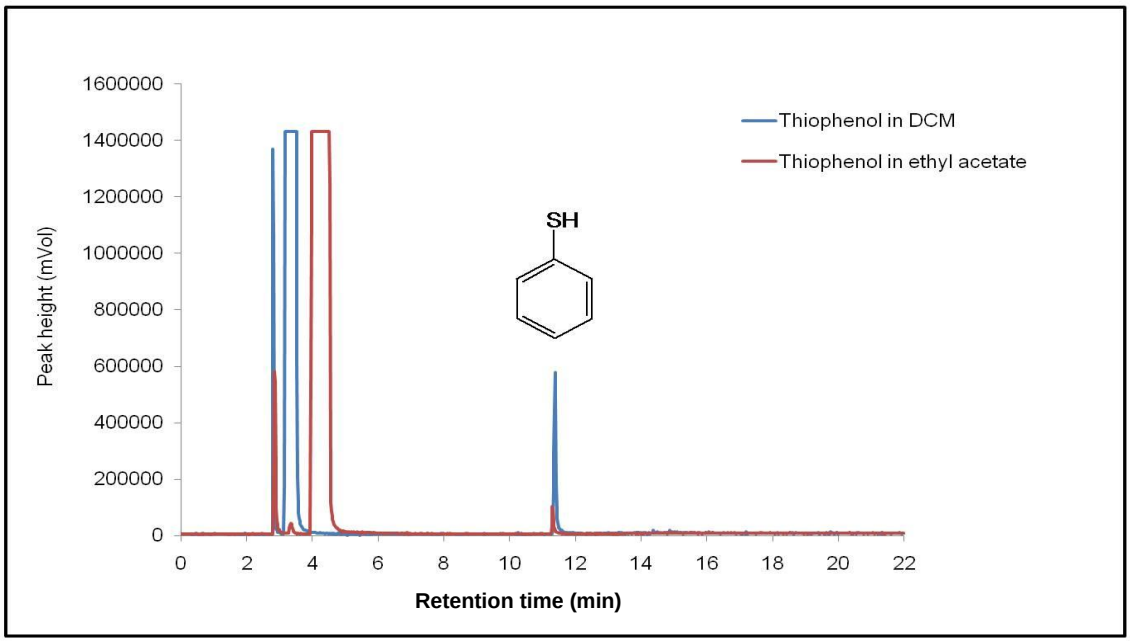


Figure 7.6-1. GC results showing thiophenol (10 µM) standards in DCM and ethyl acetate.

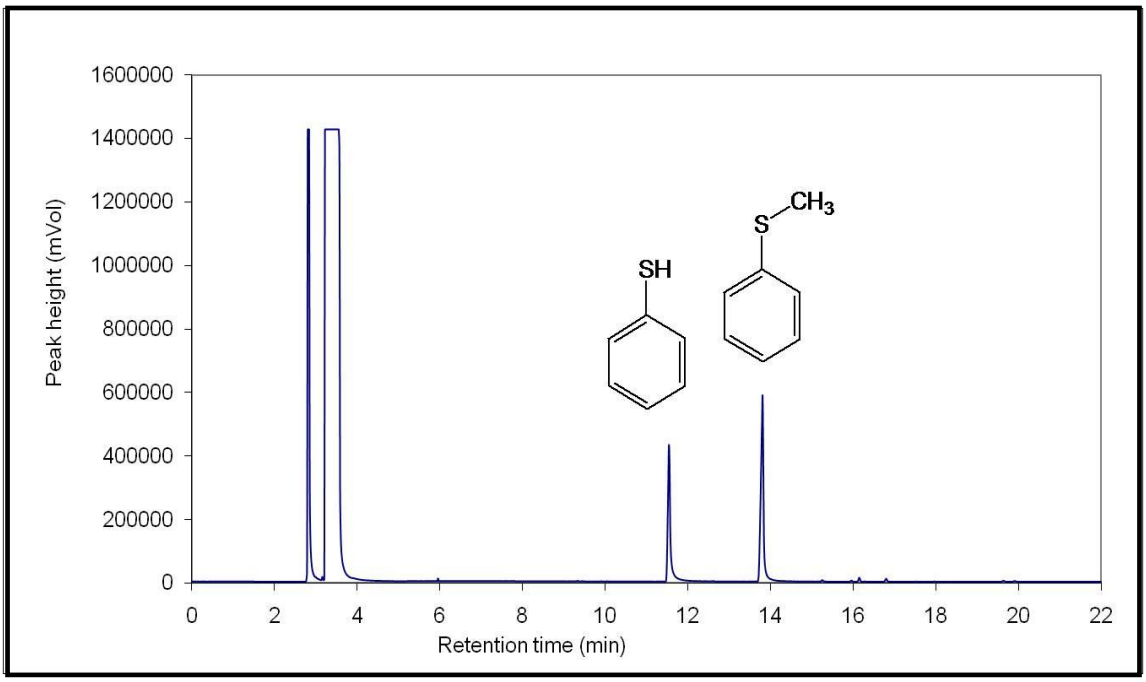


Figure 7.6-2. GC results showing standards thiophenol (10 µM) and methyl phenyl sulphide (10 µM).

The results of this experiment are presented in figure 7.6-3 with the final product of two- step enzymatic reaction - methyl phenyl sulphide (MPS) successfully detected.

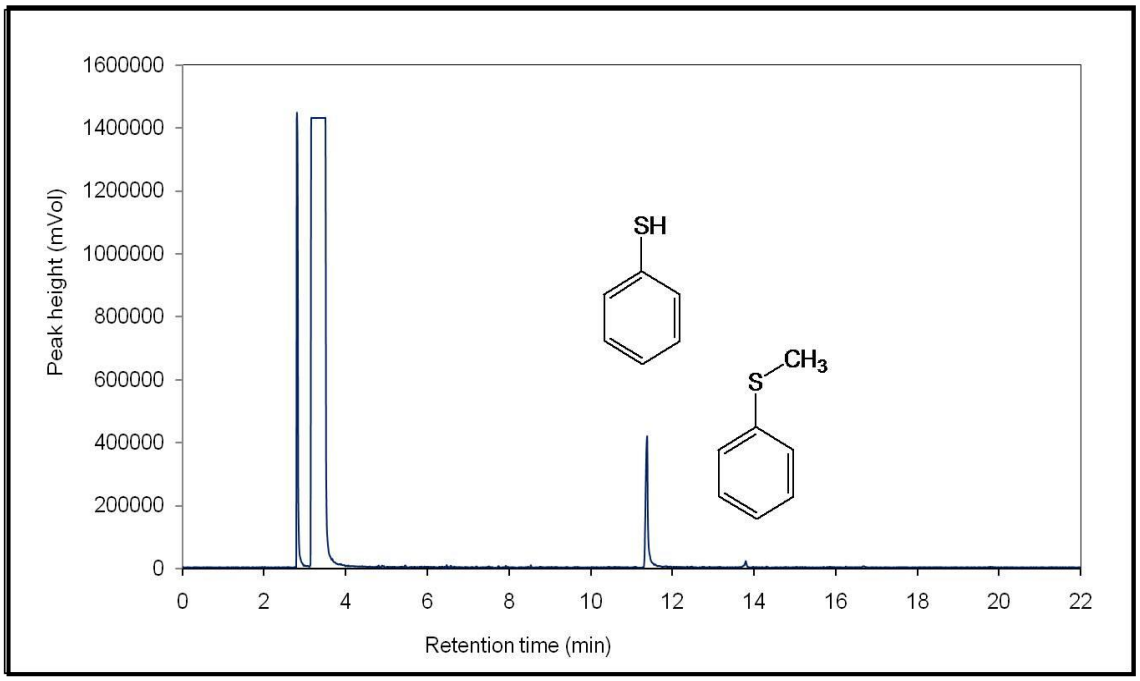


Figure 7.6-3. GC results showing results of MetC and TMT1 of coupled reaction -thiophenol and methyl phenyl sulphide.

7.6.1.2 Solid - phase micro extraction

Direct analysis of the headspace vapours by solid macro phase extraction was performed according to the method described in section 2.2.6.5. using the equipment shown in figure 7.6-4. Various assay conditions and times of incubation were tested in order to establish the most accurate methodologies to monitor the coupled enzyme mediated generation of reaction products as determined by GC.

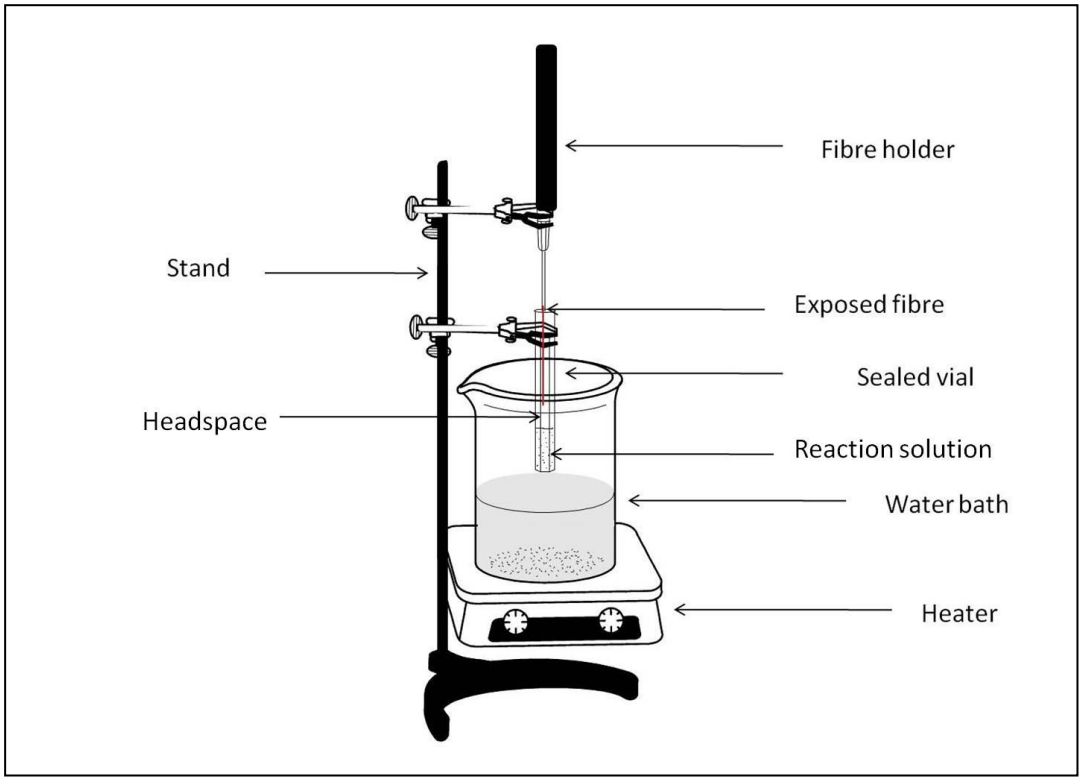


Figure 7.6-4. The scheme showing the apparatus used for SPME experiments.

Both standards of thiophenol and methyl phenyl sulphide (MPS) could be detected, however this method failed in delivering consistent data and failed to detect methyl phenyl sulphide as a product of interest in the two-step reaction of interest.

7.7 Conclusion

The potential to use metabolic engineering to link reactions of interest, and develop a route for the production of *S*-methylated flavour compounds by reconstitution of the respective pathways in recombinant bacteria was investigated. The objective of this work package was to allow the co-expression of a C-S lyase and S-methyltransferase to generate *S*-methylated volatile organosulphur compound methyl phenyl sulphide (MPS) from *S*-cysteinylated precursors *S*-phenyl-L-cysteine (SPC). To derive the most flexible combination

of enzymes, 3 C-S lyases were co-expressed in different combinations with *S*-methyltransferase and tested towards the starting compound. The potential for different enzymes to function together in the pathway could then be tested. In summary the linked 2-step reaction principle was tested and proven possible *in vitro*. Although the process could not operate on a commercial scale the work did prove the concept. A clear disadvantage of the *in vitro* method was the high cost of SAM - the donor of methylation group. To overcome this, the *in vivo* co-expression system was developed. This showed that it was possible to co-express the C-S lyase and TMT. However practical analytical limitation prevented the determination of any reaction products. However, the inability to achieve a product yield greater than 1 % considerably highlighted the problem of converting this interesting biotransformation pathways into a commercially viable process. Direct methylation of flavour precursor with naturally derived methanethiol or hydrogen sulphide, should be considered as a more efficient method of achieving a scalable process to obtain methylated VOSC compounds.

8 Discussion and future work

Significant progress has been achieved at Durham with respect to the commercial production of natural derived F&F compounds. The designed pathway for natural production of F&F organosulphur compounds offers broader scope of compounds and greatly increased application of multistep enzymatic catalysis. In this work, the activity comparison of seven C-S lyases and two *S*-methyl transferases has shown the requirement to select the optimal enzymes within their group. The field of biocatalysis is growing quickly, enzymes developed by nature are powerful tools and their impact is increasingly evident in science and daily life. This work has proven that there is a natural alternative way to obtain chemicals without use of metal catalysts, harmful solvents or insufficient extraction from natural sources. Sustainable technology is a major trend in modern biotechnology (Berger, 2009), unfortunately, the range of natural catalysts is still tiny proportion of the F&F industry. In this work, a newly designed pathway for an enzymatic production of VOSCs has proven to be potentially successful. The fermentation route to the bulk production of active enzymes has been achieved and the biogenic production of high impact volatiles such as methanethiol, hydrogen sulphide thiomenthone and other highly valuable VOSCs from the resulting enriched enzyme preparation confirmed. In future, the search for other useful enzymes in F&F will be formulated by an ever expanding range of genes, being a powerful tool for generating useful molecular diversity in industrial enzymes. The approach requires further extensive research in order to target highly efficient and stable enzymes. Furthermore, the possibility of recycling biocatalysts through immobilization should be investigated as it has not been explored in

this study. There are numerous advantages of immobilized enzymes, it has been shown that a single batch of enzyme can be attached on a solid support, which improved stability, allows re-usage of catalyst and easy separation from reaction environment (Matosevic *et al*., 2011). Furthermore, the added value of extraction of other reaction products like pyruvic acid also known as 2-oxopropanoic acid is an important organic compound used in many drugs, agrochemicals and food additives (Xu *et al*., 2008) could be considered.

Interestingly, the importance of various enzymatic methylation route can be investigated with furfuryl compounds as figure 8.1-1 A, whereas with furfural would generate hemithioacetal figure 8.1-1 (B).

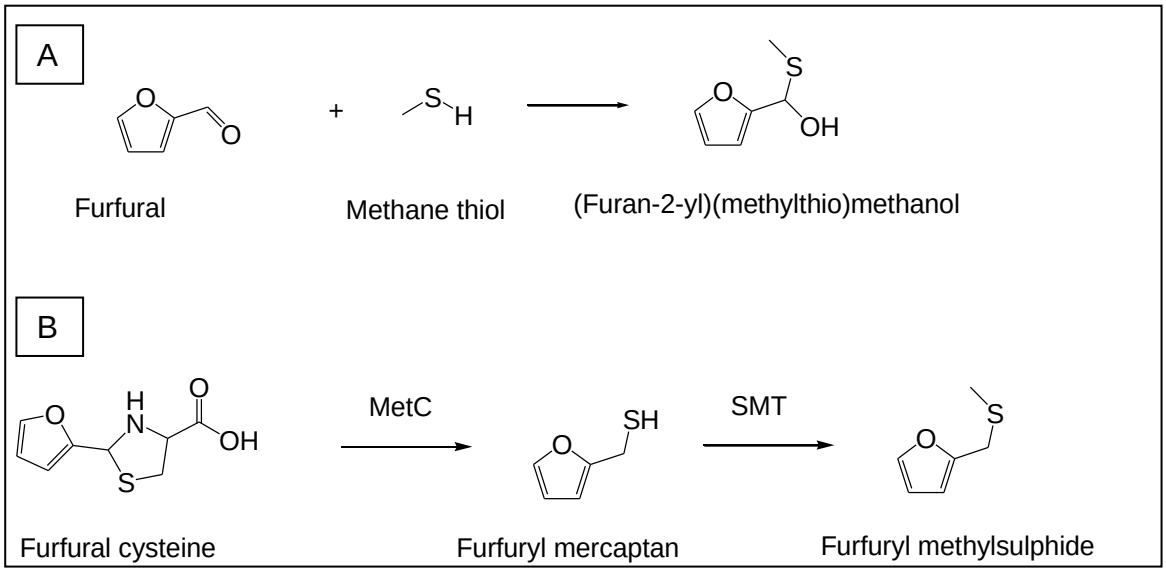


Figure 8.1-1. Proposed biosynthesis pathway for the natural production of methylated organosulphur compounds for the F&F industry.

The coupled co-expression of a C-S lyase and *S*-methyltransferase to directly generate S-methylated volatile organosulphur compounds from *S*-cysteinylated precursors has been developed. The cloning and expression of further novel TMT enzymes with activity toward flavour and fragrance thiols should be investigated. Furthermore, the introductory analysis of two enzymes CBL and

TMT1 and their possible role in fenclorim-cysteine pathway has been presented. As previously deduced, the possible existence of carbon-sulphur lyases and a thiol methyltransferase complex in the model plant *A. thaliana* could not be confirmed. There are several newly discovered and poorly characterised potential members of C-S lyases in *A. thaliana*. For example, cystine lyase recently described as aminotransferase enzyme - (Jones *et al*., 2003) showing carbon-sulphur lyase activity towards cysteine, and C-S lyase SUR1, which plays a role in glucosinolates synthesis (Mikkelsen *et al*., 2004) could potentially play an important role in the fenclorim-cysteine pathway. Due to the volatility of free thiols, accurate quantification of reaction products may be challenging and will depend on effective trapping.

Another important key advantage of using biocatalysts, is their stereo and region-selectivity as described in charter 1.1.5. Some of the desired F&F bioactive compounds are single enantiomers, with others possessing little or even undesirable effects. Hence, from a F&F perspective the different enantiomers of the same compound should be regarded as distinct species. Therefore there is desire to produce enantiomerically pure, single chiral compounds. Enzymes prove to be crucial in creating novel biocatalysts for various reactions. There is every reason to expect that the range and amounts of enzymes used in industry will increase significantly in the near future. The potential of biotransformation promises continued development and broad research of naturally produced chemicals and is the future of highly desirable "green chemistry" of the 21st Century.

9 Supplementary data

9.1 List of Primers

Name of the enzyme	Type of primer	Sequence of primer
γ-yeast (*S. cerevisiae*)	Forward Primer	5'-CTC ATA TGA CTC TAC AAG AAT CTG AT-3'
	Reverse Primer	5'-ATC TCG AGG TTG GTG GCT TGT TTC AAG-3'
β-yeast (*S. cerevisiae*)	Forward Primer	5'-CTC ATA TGA TTG ATC GTA CCG AGT TA-3'
	Reverse Primer	5'-ATC TCG AGG CCA CCC CAT GAA AAT CCC AA-3'
MalY (*E. coli*)	Forward Primer	5'-CTC ATA TGT TCG ATT TTT CAA AGG TC-3'
	Reverse Primer	5'-ATC TCG AGA CGA ACA GCG CGG ATG GCG TT-3'
MetC (*E. coli*)	Forward Primer	5'-CTC ATA TGG CGG ACA AAA AGC TTG AT-3'
	Reverse Primer	5'- ATC TCG AGT ACA ATT CGC GCA AAA CCG GC-3'
CBL (*A. thaliana*)	Forward Primer	5'- TCA TAT GCA GAT CTC AAA TCG GAA CTC-3'
	Reverse Primer	5'-TCT CGA GGA GAG GGA AGG TTT TGA AGG CAA TG-3'
MetCstop *(E. coli)*	Forward Primer	5'-CTC ATA TGG CGG ACA AAA AGC TTG AT-3'
	Reverse Primer	5'-GCC TCG AGT TAT ACA ATT CGC GCA AAA CCG GCG-3'
TMT1 (*A. thaliana*)	Forward Primer	5'-GCG CGC CAT ATG GCT GAA GAA CAA CAA AAC TC-3'
	Reverse Primer	5'-GCG CGC CTC GAT ATT GAT CTT CTT CCA CCT TCC C 3'

CrSMT1*Nde*I *(C. roseus)*	Forward Primer	5'-T CAT ATG ATG AGT TCC CAC GAA GAG AAG AGA AAC-3'
CrSMT1*Xho*I *(C. roseus)*	Reverse Primer	5'-T GCG GCC GCT TAT TTA TAA AAT TCC ATG ACC CAA AAA-3'
CrSMT1*Sal*I Stop *(C. roseus)*	Reverse Primer	5-TGT CGA CTT ATT TAT AAA ATT CCA TGA CCC ACA AA-3'
MGLstop *(P.putida)*	Forward Primer	5'-TCA TAT GAT GCA CGG CTC CAA CAA GCT CC-3'
	Reverse Primer	5'- ATC TCG AGT CAG GCA CTC GCC TTG AGT G-3'

9.2 List of sequencing primers for dual vector pCDFDuet-1

Name of MCS	Name of primer	Sequence
MCS1	ACYCDuetUP1	5'-GGA TCT CGA CGC TCT CCC T-3'
	DuetDOWN1	5'-GAT TAT GCG GCC GTG TAC AA-3'
MCS2	DuetUP2	5'-TTG TAC ACG GCC GCA TAA TC-3'
	T7 Terminator	5'-TTG TAC ACG GCC GCA TAA TC-3'

9.3 List of primers designed for inserts in dual vector pCDFDuet-1

Name of MCS	Name of primer	Sequence
MCS1TMT	Forward Primer	5'-TGG ATC CAT GGC TGA AGA ACA ACA AAA CTC AGA-3'
MCS1TMT	Reverse Primer	5'-AAA GCT TTC AAT TGA TCT TCT TCC ACC TTC CCA-3'
MCS2CBL	Forward Primer	5'-TCA TAT GAT GCA GAT CTC AAA TCG GAA CTC TTT CAA -3'
MCS2CBL	Reverse Primer	5'-ACT CGA GCT AGA GAG GGA AGG TTT TGA AGG CAA-3'
MCS2TRYP	Forward Primer	5'-TAG ATC TAT GGA AAA CTT TAA ACA TCT CCC TGA ACC-3'
MCS2TRYP	Reverse Primer	5'-ACT CGA GTT AAA CTT CTT TCA GTT TTG CGG TGA-3'

9.4 Primers used for controls in qPCR experiment

Gene name	Type of primer	Primer sequence
gapdh	Forward Primer	5'-GGCATTGTTGAGGGACTCAT-3'
	Reverse Primer	5'-TGAGACATCAACGGTTGGAA-3'
ubc	Forward Primer	5'-TTACGAAGGCGGTGTTTTTC-3'
	Reverse Primer	5'-GCTCAGGATGAGCCATCAAT-3'
gctu19	Forward Primer	5'-GGTTCTGGGCTGATTTCATT-3'
	Reverse Primer	5'-CGTATGCTGGAAACCATGTG-3'

9.5 Primers designed for knock out QPCR experiment

Gene name	Type of primer	Primer sequence
At2g43910	Forward Primer	5'-GTTGCTACGTTCCTGCACAA-3'
	Reverse Primer	5'-ATCCAATCCAACAACGAAGC-3'
At2g43920	Forward Primer	5'-TCCATGCATGAACTCCTCAA-3'
	Reverse Primer	5'-ACGAGTGGGAATGGAGTCTG-3'
At2g43940	Forward Primer	5'-CCTGATCGTCATGTTGTTGG-'3
	Reverse Primer	5'-AAGCTCTCCACCAGGTTTCA-'3

9.6 **Amino acid sequences of cloned enzymes.**

Name of the enzyme	Sequence of subcloned enzymes
MGL	MRDSHHNTGF STRAIHHGYD PLSHGGALVP PVYQTATYAF PTVEYGAACF AGEEPGHFYS RISNPTLALL EQRMASLEGG EAGLALASGM GAITSTLWTL LRPGDELIVG RTLYGCTFAF LHHGIGEFGV KVRHVDLNDA KALKAAISSK TRMIYFETPA NPNMQLVDIA AVVEAVRGHD VHVVVDNTYC TPYLQRPLEL GADLVVHSAT KYLSGHGDIT AGLVVGRKVL VDRIRLEGLK DMTGAVLSPH DAALLMRGIK TLALRMDRHC ANAQQVAEFL VRQPQVELIH YPGLPSFAQY ALAQRQMRLP GGMIAFELKG GIEAGRRFMN ALQLFARAVS LGDAESLAQH PASMTHSSYT PQERAHHGIS EGLVRLSVGL EDVEDLLADV EQALQACC
TRP	MENFKHLPEP FRIRVIEPVK RTTRAYREEA IIKSGMNPFL LDSEDVFIDL LTDSGTGAVT QSMQAAMMRG DEAYSGSRSY YALAESVKNI FGYQYTIPTH QGRGAEQIYI PVLIKKREQE KGLDRSKMVA FSNYFFDTTQ GHSQINGCTV RNVYIKEAFD TGVRYDFKGN FDLEGLERGI EEVGPNNVPY IVATITSNSA GGQPVSLANL KAMYSIAKKY DIPVVMDSAR FAENAYFIKQ REAEYKDWTI EQITRETYKY ADMLAMSAKK DAMVPMGGLL CMKDDSFFDV YTECRTLCVV QEGFPTYGGL EGGAMERLAV GLYDGMNLDW LAYRIAQVQY LVDGLEEIGV VCQQAGGHAA FVDAGKLLPH IPADQFPAQA LACELYKVAG IRAVEIGSFL LGRDPKTGKQ LPCPAELLRL TIPRATYTQT HMDFIIEAFK HVKENAANIK GLTFTYEPKV LRHFTAKLKE V
γ-yeast	MTLQESDKFA TKAIHAGEHV DVHGSVIEPI SLSTTFKQSS PANPIGTYEY SRSQNPNREN LERAVAALEN AQYGLAFSSG SATTATILQS LPQGSHAVSI GDVYGGTHRY FTKVANAHGV ETSFTNDLLN DLPQLIKENT KLVWIETPTN PTLKVTDIQK VADLIKKHSA GQDVILVVDN TFLSPYISNP LNFGADIVVH SATKYINGHS DVVLGVLATN NKPLYERLQF LQNAIGAIPS PFDAWLTHRG LKTLHLRVRQ AALSANKIAE FLAADKENVV AVNYPGLKTH PNYDVVLKQH RDALGGGMIS FRIKGGAEAA SKFASSTRLF TLAESLGGIE SLLEVPAVMT HGGIPKEARE ASGVFDDLVR ISVGIEDTDD LLEDIKQALK QATN

β- yeast	MIDRTELSKF GITTQLSVIG RNPDEQSGFV NPPLYKGSTI ILKKLSDLEQ RKGRFYGTAG SPTIDNLENA WTHLTGGAGT VLSASGLGSI SLALLALSKA GDHILMTDSV YVPTRMLCDG LLAKFGVETD YYDPSIGKDI EKLVKPNTTV IFLESPGSGT MEVQDIPALV SVAKKHGIKT ILDNTWATPL FFDAHAHGID ISVEAGTKYL GGHSDLLIGL ASANEECWPL LRSTYDAMAM LPGAEDCQLA LRGMRTLHLR LKEVERKALD LAAWLGNRDE VEKVLHPAFE DCPGHEYWVR DYKGSSGLFS IVLKNGFTRA GLEKMVEGMK VLQLGFSWGG
MalY	MFDFSKVVDR HGTWCTQWDY VADRFGTADL LPFTISDMDF ATAPCIIEAL NQRLMHGVFG YSRWKNDEFL AAIAHWFSTQ HYTAIDSQTV VYGPSVIYMV SELIRQWSET GEGVVIHTPA YDAFYKAIEG NQRTVMPVAL EKQADGWFCD MGKLEAVLAK PECKIMLLCS PQNPTGKVWT CDELEIMADL CERHGVRVIS DEIHMDMVWG EQPHIPWSNV ARGDWALLTS GSKSFNIPAL TGAYGIIENS SSRDAYLSAL KGRDGLSSPS VLALTAHIAA YQQGAPWLDA LRIYLKDNLT YIADKMNAAF PELNWQIPQS TYLAWLDLRP LNIDDNALQK ALIEQEKVAI MPGYTYGEEG RGFVRLNAGC PRSKLEKGVA GLINAIRAVR
MetC	MADKKLDTQL VNAGRSKKYT LGAVNSVIQR ASSLVFESME AKKHATRNRA NGELFYGRRG TLTHFSLQQA MCELEGGAGC ALFPCGAAAV ANSILAFVEQ GDHVLMTNTA YEPSQDFCSK ILSKLGVTTS WFDPLIGADI VKHLQPNTKI VFLESPGSIT MEVHDVPAIV AAVRSVVPDA IIMIDNTWAA GVLFKALDFG IDVSIQAATK YLVGHSDAMI GTAVCNARCW EQLRENAYLM GQMVDADTAY ITSRGLRTLG VRLRQHHESS LKVAEWLAEH PQVARVNHPA LPGSKGHEFW KRDFTGSSGL FSFVLKKKLS NEELANYLDN FSLFSMAYSW GGYESLILAN QPEHIAAIRP QGEIDFSGTL IRLHIGLEDV DDLIADLDAG FARIV

CBL	MEKSVDAMNI KEEASVSTLL VNLDNKFDPF DAMSTPLYQT ATFKQPSAIE NGPYDYTRSG NPTRDALESL LAKLDKADRA FCFTSGMAAL SAVTHLIKNG EEIVAGDDVY GGSDRLLSQV VPRSGVVVKR VNTTKLDEVA AAIGPQTKLV WLESPTNPRQ QISDIRKISE MAHAQGALVL VDNSIMSPVL SRPLELGADI VMHSATKFIA GHSDVMAGVL AVKGEKLAKE VYFLQNSEGS GLAPFDCWLC LRGIKTMALR IEKQQENARK IAMYLSSHPR VKKVYYAGLP DHPGHHLHFS QAKGAGSVFS FITGSVALSK HLVETTKYFS IAVSFGSVKS LISMPCFMSH ASIPAEVREA RGLTEDLVRI SAGIEDVDDL ISDLDIAFKT FPL
CrSMT1	MSSHEEKPSS NKEDDDHSSY ALQLVFSGAL PMVLNAVIKL NVFEIIAKAG PGAKLSPSQI VSQMPTKNPE APVVLDRMLR MLASYSVLTC SVVDFSHGSG QRVYGLSPVS KYFVKNENGG CFGPLLDLLQ DKVLTDIWYE LAPAVLEGGT AFNRAYNMHI FKYTGINQKF NETFNTATIN HAKVIVQEIL KNYKGFENLK TLVDVGGGLG VTLDLITSKY PNLKGINYDL PHVTQNAPTY PGVVHVGGDM FESVPKGDAI FMKWILHDWD DEHCLKLLKN CYKALPENGK VIAVDAILPM NPDNSSSTKH ISQVDLFTLV LYHPGGKERT ENEFLALVAE AGFGGIRKVC VCCDLWVMEF YK
TMT1	MAEEQQNSDQ SNGGNVIPTP EEVATFLHKT VEEGGWEKCW EEEITPWDQG RATPLIVHLV DTSSLPLGRA LVPGCGGGHD VVAMASPERF VVGLDISESA LAKANETYGS SPKAEYFSFV KEDVFTWRPT ELFDLIFDYV FFCAIEPEMR PAWAKSMYEL LKPDGELITL MYPITDHVGG PPYKVDVSTF EEVLVPIGFK AVSVEENPHA IPTRKGKEKL GRWKKIN

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